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Affinity labeling of creatine kinase

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Affinity Labeling of Creatine Kinase

by

Douglas Dominic Buechter

DISSERTATION

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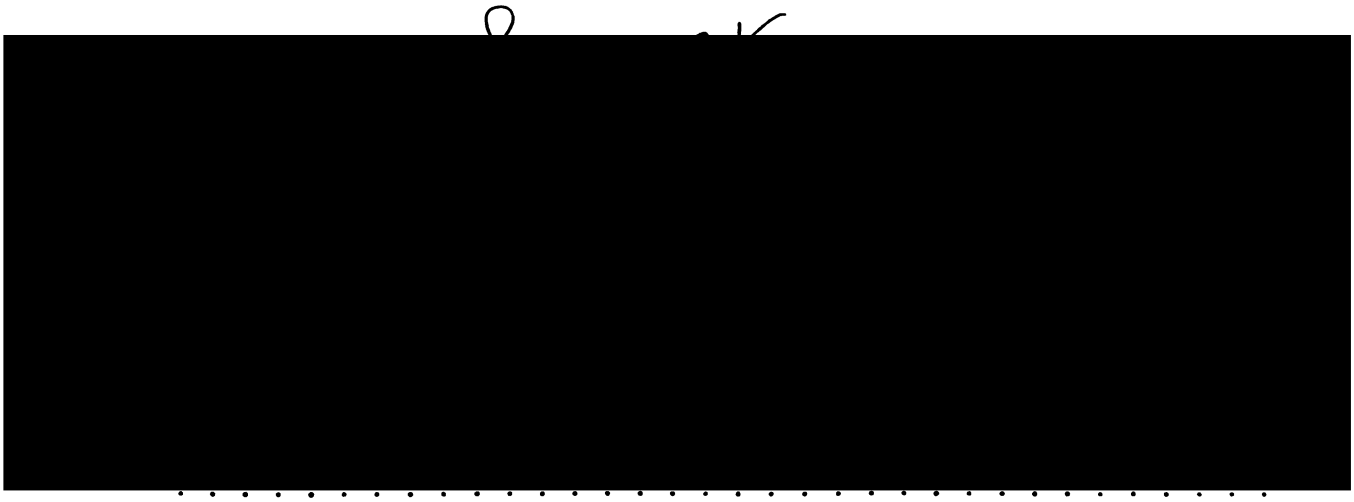

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To my fiancé, Sue.
Her love, support, and encouragement made it all possible.
And to my parents and family,
for their love, support, and understanding throughout the years.

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Affinity Labeling of Creatine Kinase

Douglas Dominic Buechter

Abstract

As part of an ongoing effort to obtain a preparation of creatine kinase (CK) that is suitable for x-ray crystallographic studies, CK has been highly purified from rabbit skeletal muscle. CK so purified is homogeneous by denaturing electrophoresis but displays several bands on native isoelectric focusing gels. Preliminary characterization of these species suggest that they are not the result of either glycosylation or heterogeneous C-termini. This enzyme preparation can be used as a starting point in efforts to characterize fully the multiple species present in purified rabbit muscle creatine kinase.

Cytosolic creatine kinase has also been purified from the electrocyte of *Torpedo californica*. This has provided authentic, tissue-purified enzyme for verification of the identity of the protein expressed from its cDNA in *E. coli*. Benzoylformate decarboxylase expressed in *E. coli* was also purified to confirm that its cDNA encoded for authentic enzyme.

To identify residues of creatine kinase that are potentially involved in catalysis or within the active site, the site of modification of creatine kinase by the affinity label epoxycreatine has been determined. Rabbit muscle creatine kinase was inactivated with [^{14}C]-epoxycreatine and subjected to tryptic digestion. Separation of the resulting peptides gave two fractions that contained radioactivity. Liquid secondary ion mass spectrometry (LSIMS) of the first of these gave a molecular ion consistent with epoxycreatine labeling of the peptide ^{266}AGHPFMWNEHLGYVLTCPSNLGTGLR. Tandem mass spectrometry was used to confirm its sequence and to identify conclusively the site of labeling as the

cysteine residue, Cys-282, in rabbit muscle CK. In a similar manner, the second radioactive fraction was analyzed and found to contain the labeled peptide ^{278}VLTCPSNLGTGLR. This peptide is a chymotryptic fragment of the labeled peptide obtained earlier. Thus, epoxycreatine labels only Cys-282 in rabbit muscle CK.

The results presented here suggest that Cys-282 is very near the creatine binding site. This information is likely to be useful when a crystal structure of CK becomes available. In particular, crystallization of the enzyme with epoxycreatine covalently bound in the active site may give important insights into the binding mode of creatine and the positions of active site residues relative to bound substrates.

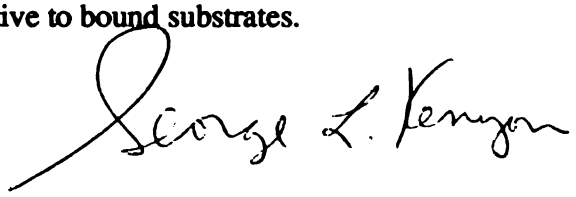


Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMP	Ampicillin
ATP	Adenosine triphosphate
AU	Absorbance unit
BFD	Benzoylformate decarboxylase
BIS	N, N'-Methylene-bis acrylamide
BSA	Bovine serum albumin
CID	Collision-induced dissociation
CK	Creatine kinase
CYS	Cysteine
DPM	Disintegrations per minute
DTT	Dithiothreitol
E-64	N-[N-(L-3-*trans*-Carboxyoxiran-2-carbonyl)-L-leucyl-agmatine
EDTA	Ethylenediaminetetracetic acid
EPR	Electron paramagnetic resonance
FPLC	Fast protein liquid chromatography
GEMSA	Guanidinoethylmercaptosuccinic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
IDP	Inosine diphosphate
IEF	Isoelectric focusing
IPTG	Isopropyl β-D-thiogalactopyranoside
LADH	Liver alcohol dehydrogenase
LB	Luria Bertani
LSIMS	Liquid secondary ion mass spectrometry
MDH	(*S*)-Mandelate dehydrogenase
MES	2-[N-Morpholino]ethanesulfonic acid
MI	Myocardial infarction
MMTS	Methylmethanethiosulfonate
MR	Mandelate racemase

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
PMS	Phenazine methosulfate
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-Tetramethylethylenediamine
TES	N-Tris(hydroxymethyl}methyl-2-aminoethanesulfonic acid
TFA	Trifluoroacetic acid
TLCK	Nα-Tosyl-L-lysine chloromethyl ketone
TPCK	Nα-Tosyl-L-phenylalanine chloromethyl ketone
TPP	Thiamine pyrophosphate
TRIS	Tris(hydroxymethyl) aminomethane

1

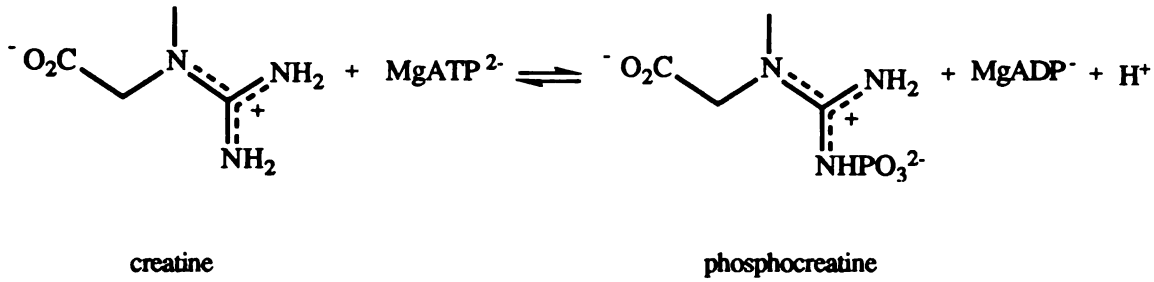
Introduction

I. General Background

A. Physiological Role of Creatine Kinase

In 1927, Fiske and Subbarow (Fiske & Subbarow, 1927) discovered a phosphorylated derivative of creatine in muscle tissue which they termed "phosphagen". Several years later, after the discovery of ATP, Lohmann (Lohmann, 1934) demonstrated that the transfer of a phosphoryl group between phosphagen (phosphocreatine) and ADP was enzyme catalyzed. The role of creatine and phosphocreatine in muscle contraction and other cellular energetic processes was quickly recognized and, in 1954, creatine kinase, the enzyme which catalyzes the phosphoryl transfer, was crystallized from rabbit skeletal muscle (Kuby et al., 1954a).

Creatine kinase (CK, adenosine-5'-triphosphate:creatine phosphotransferase, EC 2.7.3.2) catalyzes the transfer of a phosphoryl group from ATP to creatine, giving rise to phosphocreatine and ADP (Fig. 1.1). Creatine kinase is found in all vertebrates and in some invertebrates. It is present in a wide variety of tissues, including heart, skeletal muscle and brain (Watts, 1973) and in smaller amounts in a variety of other tissues (Jacobus & Lehninger, 1973). CK is a dimeric enzyme of subunit molecular weight approximately 43,000 daltons. There are two known cytosolic CK subunits: brain (B) and muscle (M). These subunits associate to form the muscle (MM), brain (BB), and heterodimer (MB) isozymes. In addition to the cytosolic isozymes there is also an isozyme associated with the inner mitochondrial membrane (Mito-CK).



creatine phosphocreatine

Figure 1.1. The reaction catalyzed by creatine kinase.

CK is important in maintaining levels of "high-energy phosphate" within the cell. Thus the cell, by reversal of the CK reaction (as written in Fig. 1.1), is able to generate ATP under conditions of energy demand or directly at the site of ATP utilization (Saks et al., 1975; Seraydarian & Abbott, 1976). This function is especially important in muscle tissue. For example, isolated, rapidly contracting heart muscle, which would be expected to be quickly depleted of ATP, is actually able to maintain a relatively constant level of ATP for several minutes. However, during this time the level of phosphocreatine drops rapidly, as it is used to replenish the ATP used to maintain muscle contraction (Gudbjarnason et al., 1970; Nassar-Gentina et al., 1978; Neely et al., 1973; Seraydarian & Abbott, 1976).

The creatine kinase reaction has also been postulated to be important in the so-called creatine/phosphocreatine shuttle (Bessman & Geiger, 1981; Jacobus, 1985; Seraydarian & Abbott, 1976). In this hypothesis, the reaction catalyzed by mitochondrial CK is coupled to ATP synthesis, with newly synthesized ATP being utilized to make phosphocreatine. Phosphocreatine then makes its way to ATP-utilizing processes within the cell where the CK associated with these processes catalyzes the local formation of ATP. In this way the energetic potential associated with ATP is "shuttled" within the cell in the form of phosphocreatine. In support of this model it has been found that the MM isozyme of CK is tightly associated with the M-line of the muscle sarcomere (Herasymowych et al., 1980;

Turner et al., 1973) and it has been suggested that the generation of ATP, via the CK reaction, in close proximity to its site of usage, is critical to muscle contraction (Savabi et al., 1983).

In addition to its physiological roles, creatine kinase is important in the diagnosis of myocardial infarction (MI). Serum levels of the isozymes of CK are useful as indicators of the extent and severity of tissue necrosis in MI and are important in its reliable diagnosis (Apple, 1989; Lee & Goldman, 1986; Wu, 1989). In particular, the changes seen following infarction in the levels of the serum "isoforms" of the MM and MB isozymes are important. These isoforms are thought to arise from modification of the enzyme after its release into the blood, and their serum levels reflect the presence and time of onset of tissue damage. The clinical use of serum CK levels in acute myocardial infarction can be complicated by the changes that are also seen in acute skeletal-muscle disease; however, the clinical determination of CK serum levels continues to be a rapid and sensitive component in the diagnosis of acute MI. The isoforms of CK are discussed in more detail in Chapter 2.

Serum CK levels are also of importance in the diagnosis of Duchenne muscular dystrophy. CK levels follow a predictable pattern of an early increase to ~age 5, followed by a rapid decrease as loss of skeletal muscle becomes more severe (Gruemer & Prior, 1987). This pattern can be useful in the differential diagnosis of the several types of muscular dystrophy. An increase in serum CK levels is also of some use in the identification of female carriers of this disease (Rowland, 1988). In obligate female carriers, increased serum CK levels may be the only manifestation of the Duchenne muscular dystrophy gene. This clinical use is likely to become less important as reliable and convenient methods of detecting the muscular dystrophy gene are developed (Rowland, 1988).

B. Creatine Kinase Isozymes

Expression of the CK isozymes is both tissue specific and developmentally regulated. The MM isozyme is found primarily in skeletal and cardiac muscle (Watts, 1973), although there is a report of it having been found in the human brain (Hamburg et al., 1990). The MB isozyme is found predominantly in cardiac muscle (Bessman & Carpenter, 1985), while the BB isozyme has been found in brain, smooth muscle, heart, and placenta (Watts, 1973). In the early stages of development the B subunit is preferentially expressed in embryonic tissue, including skeletal myoblasts. After the terminal differentiation of embryonic skeletal muscle cells, the M-CK gene is transcriptionally activated and expression switches exclusively to the M subunit (Caravatti et al., 1979; Perriard, 1979; Perriard et al., 1987; Watts, 1973). The reasons for the tissue and development specificity are not completely understood but may relate either to as yet unknown functional differences between the isozymes or may reflect differential regulation of expression.

The BB isozyme of CK is the major component of the estrogen-induced protein of the rat uterus, and uterine stimulation by estrogen results in an ~15% increase in CK activity (Reiss & Kaye, 1981). Expression of the BB isozyme has also been found to be stimulated by vitamin D metabolites in the rat (Somjen et al., 1984) and by several other hormones, including gonadotropin-releasing hormone and growth hormone (Koch et al., 1985).

Creatine kinase cDNA from a variety of species has been cloned and sequenced (Babbitt et al., 1986; Benfield et al., 1988; Billadello et al., 1986; Daouk et al., 1988; Mariman et al., 1987; Villarreal-Levy et al., 1987) and both *Torpedo californica* and rabbit muscle CKs have been expressed in and purified from *E. coli.* (Babbitt et al., 1990b; Chen et al., 1990). The entire sequences for the murine M (Jaynes et al., 1988) and rat B CK genes (Benfield et al., 1988) have also recently been determined. The two cytosolic CK

subunits (M and B) are highly homologous; there is ~90% sequence identity between the same subunit across species and ~80% sequence identity between different subunits in the same species (Babbitt et al., 1986). The mitochondrial subunit is considerably different in primary structure from the cytosolic subunits (Haas et al., 1989). It is unable to form dimers with the M and B subunits and can exist as an octamer as well as a dimer (Jacobs et al., 1964; Lipskaya et al., 1989; Lipskaya & Trofimova, 1989). In mitochondria the octamer appears to be the physiologically important species (Quemeneur et al., 1990). The tissue and development specific expression of the CK genes make them attractive candidates for the study of transcriptional and translational regulation, and there is considerable interest in identifying promoter and regulatory elements in the CK genes (Benfield et al., 1988; Jaynes et al., 1988; Johnson et al., 1989; Mariman et al., 1987; Mitchell & Benfield, 1990; Sternberg et al., 1988). Multiple regulatory elements appear to be involved in the regulation of the CK genes, with at least one muscle-specific enhancer having been identified (Jaynes et al., 1988). This element exerts a positive influence on the M-CK gene in differentiated muscle cells but does not appear to be active in embryonic muscle.

Although the complete functional significance of the different CK isozymes is not yet known, there are at least two areas in which a particular isozyme has a unique function. Only the MM isozyme is bound to the M-line of the muscle sarcomere and Mito-CK is the only isozyme found associated with the mitochondrial inner membrane. Presumably these unique functional roles result from specific differences in the primary structures of the isozymes, but these relationships have not yet been delineated.

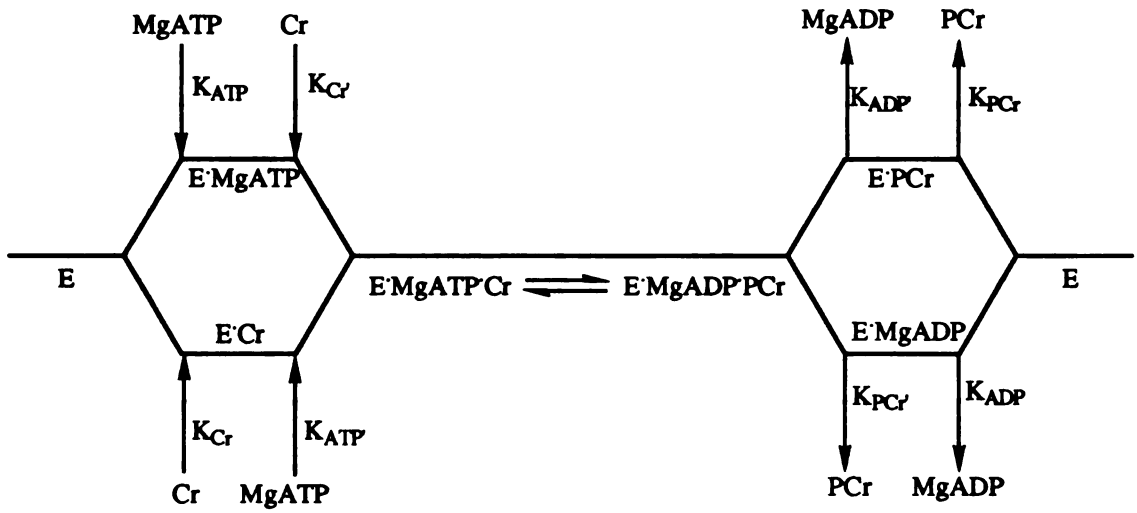


Figure 1.2. Kinetic scheme of creatine kinase. Cr: Creatine. PCr: Phosphocreatine.

II. The Mechanism of Creatine Kinase

A. Introduction

An excellent review of the reaction catalyzed by creatine kinase is available (Kenyon & Reed, 1983); however, because the mechanism of CK and the role of various amino acid residues in catalysis is pertinent to the present work, these topics will be discussed in some detail. At the end of this section an attempt will be made to consolidate the available data and a summary of the events occurring during catalysis will be presented.

B. Kinetic Studies

Creatine kinase catalyzes the transfer of a phosphoryl group between ATP and creatine (Fig. 1.1). Along with creatine and phosphocreatine, the Mg^{2+} complexes of ATP and ADP are substrates for the enzyme (Kuby et al., 1954b; Noda et al., 1960), although other divalent metal ions can be substituted for Mg^{2+} (O'Sullivan & Morrison, 1963;

Table 1.1: Kinetic Constants of the Creatine Kinase Reaction. The Nomenclature is as Shown in Fig. 1.2.

Forward Reaction		Reverse Reaction	
Constant	mM	Constant	mM
K_{ATP}	1.2	K_{PCr}	0.17
$K_{ATP'}$	0.48	$K_{PCr'}$	0.05
K_{Cr}	15.6	K_{ADP}	8.6
$K_{Cr'}$	6.1	$K_{ADP'}$	2.9

Rosenberg & Ennor, 1955). At pH 8, the reaction follows a random bi-bi mechanism (Morrison & James, 1965), as depicted in Fig. 1.2.

All of the enzymatic steps, with the exception of the interconversion of the ternary complexes, are in rapid equilibrium. There are distinct binding sites for the guanidinium and nucleotide substrates (Kuby et al., 1954b; Morrison & James, 1965). Equilibrium isotope exchange studies have confirmed this mechanistic scheme and that it also holds when Mn^{2+} is substituted for Mg^{2+} (Morrison & Cleland, 1966; Morrison & White, 1967). At pH 7, the reaction is essentially random bi-bi, but there is a slight preference for MgATP to bind prior to creatine (Schimerlick & Cleland, 1973). None of the kinetic studies, including quenched-flow cryoenzymic studies (Travers & Barman, 1980) have detected phospho-enzyme or other covalently bound intermediates.

Kinetic parameters of creatine kinase from several species and under several conditions have been determined (Jacobs & Kuby, 1970; Maggio et al., 1977; Morrison & James, 1965; Nihei et al., 1961; Watts, 1973). The values for the enzyme from rabbit muscle at pH 8 and 30 °C (Morrison & James, 1965) are given in Table 1.1.

C. The Active Site of Creatine Kinase

Although there is as yet no x-ray crystal structure available for any creatine kinase, a wealth of information about the binding of substrates and the events occurring during catalysis has been gleaned from kinetic and spectroscopic studies. Much of this work has relied upon three interesting (and fortunate) properties of the enzyme. These are: the ability to form very tightly bound quaternary dead-end complexes; the ability of paramagnetic Mn^{2+} to substitute for Mg^{2+}; and the presence of a highly reactive sulfhydryl residue in or near the active site of the enzyme. These three properties will be briefly discussed before a more detailed description of the active site is given.

Sulfate and orthophosphate anions are competitive inhibitors of CK with respect to MgATP (Nihei et al., 1961). Other small anions, particularly the planar ions nitrate, formate, and thiocyanate, show a more complex inhibition pattern and also markedly enhance the product inhibition seen with MgADP (Milner-White & Watts, 1971). These anions form dead-end quaternary complexes with enzyme, MgADP, and creatine. The planar anion appears to bind at the site on the enzyme which would normally be occupied by the transferring phosphoryl group. This is thought to mimic the transition state of phosphoryl transfer, and the complex has been termed a "transition-state complex" (Milner-White & Watts, 1971). Such transition state complexes have been used extensively in studies of the mechanism of creatine kinase.

The paramagnetic Mn^{2+} ion has been used extensively in spectroscopic studies to probe the active site of CK. The environment of the Mn^{2+} unpaired electron has been investigated by EPR and the effect of the paramagnetic metal on the proton relaxation rate of water molecules in its hydration sphere has been exploited in NMR experiments. These experiments have been particularly helpful in estimating distances in the enzyme substrate complexes.

Finally, creatine kinase possesses one highly reactive sulfhydryl group in each monomer which is in or near the active site (Taylor et al., 1969). It is possible to label this group with a number of sulfhydryl specific reagents, often, but not always, with complete loss of activity. This fact has been used not only to study the possible role of this group in catalysis, but also as a means of introducing EPR active spin labels into the enzyme. This sulfhydryl is discussed in more detail below and in Chapter 4.

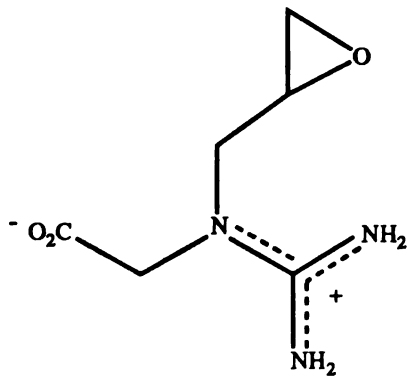
Early experiments of the affect of substrates on the reactivity of the sulfhydryl (O'Sullivan et al., 1966; Watts & Rabin, 1962) and the effect of substrates on the binding of antibodies to the enzyme (Samuels, 1961) suggested that a conformational change occurred upon substrate binding. These observations were confirmed by temperature-jump experiments (Hammes & Hurst, 1969) which showed that the binding of either MgADP or MgATP was accompanied by a conformational change. The binding of creatine to the enzyme-MgATP complex also results in a conformational change (O'Sullivan & Cohn, 1968; O'Sullivan et al., 1966). Interestingly, modification of the reactive sulfhydryl with iodoacetic acid, dinitrofluorobenzene or methylmethanethiosulfonate appears to prevent this change (Markham et al., 1977; O'Sullivan & Cohn, 1968; O'Sullivan et al., 1966; Taylor et al., 1969; Watts & Rabin, 1962). From these observations it has been proposed that the sulfhydryl is involved in a conformational change to the "working" conformation of the enzyme (O'Sullivan & Cohn, 1968; Watts & Rabin, 1962). The exact nature of this change is not known; however, it is thought to result in the "tightening" or closing down of the active site (McLaughlin et al., 1976; Reed & Leyh, 1980). In the transition state complex this is presumably what makes the active site sulfhydryl less susceptible to alkylating agents (Milner-White & Watts, 1971) and restricts the access of solvent to the metal ion (McLaughlin et al., 1976).

The role of the metal ion in catalysis has been extensively investigated by NMR and EPR, with MnATP and MnADP complexes as substrates. The metal ion is coordinated only to the nucleotide substrate and not to the enzyme, and there are water molecules

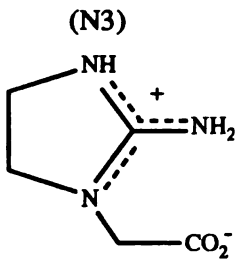
associated with the bound Mn^{2+} (Cohn & Leigh, 1962). EPR was used to obtain approximate distances between the Mn^{2+} and a nitroxide spin label bound at the reactive sulfhydryl group (Cohn et al., 1971; Taylor et al., 1969). The Mn^{2+} is 7-10 Å from the unpaired electron of the nitroxide label in the MnADP enzyme complex and slightly further away in the MnATP complex. The distance between the Mn^{2+} ion and the formate proton in the transition-state complex with formate is approximately 5 Å.

Studies with exchange inert Cr(III) nucleotide complexes (Dunaway-Mariano & Cleland, 1980) have suggested that α, β-coordinated MgADP is the species that binds to the enzyme·phosphocreatine complex. After binding, the conformational change occurs to close down the active site and the metal ion migrates from coordination to the α-phosphate to coordination with the transferring phosphoryl group. Phosphoryl transfer then occurs and β,γ-MgATP and creatine are released as products. The exact timing of the migration of the metal is not known. Reed and Leyh (Reed & Leyh, 1980) have identified all six ligands to the metal in the formate transition-state complex. The metal is liganded to three water molecules, the α and β-phosphates, and the carboxyl of formate. The active site in the transition state is closed and the liganded water molecules are not in exchange with bulk solvent. Additional EPR studies have suggested that in the substrate, product and transition state complexes the metal is liganded to the same atoms (Kenyon & Reed, 1983). This argues against enzyme product or substrate complexes in which the metal is not coordinated to all three phosphates. Ligation of Mg^{2+} to the phosphoryl group of phosphocreatine must then occur prior to phosphoryl transfer and would not be coincident with migration of the metal from the α-phosphate of ADP.

The reaction catalyzed by CK is thought to proceed by an in-line associative transfer of the phosphoryl group. CK does not catalyze the scrambling of ^{18}O in adenosine 5'-[α,β-^{18}O, β-$^{18}O_2$] triphosphate (Lowe & Sproat, 1980) and phosphoryl transfer results in inversion of configuration at phosphorous (Hansen & Knowles, 1981). The observation that the metal ion is liganded to the transferring phosphoryl group (Reed & Leyh, 1980) is



epoxycreatine



cyclocreatine

Figure 1.3. The affinity label, epoxycreatine and the conformationally restricted substrate analogue, cyclocreatine.

also suggestive of an associative process. If the Mg^{2+} acts to stabilize the negative charge on the oxygen of the transfering phosphoryl (and therefore makes the partial charge on the phosphorus atom more positive), this would be expected to increase the electrophilicity of the phosphorus and promote an associative mechanism. However, it should be noted that many of the arguments in support of associative mechanisms are open to other interpretations (Herschlag & Jencks, 1990), and it may not be possible, using presently available methods, to determine definitively whether CK proceeds via an associative or dissociative mechanism.

Several nucleotide analogues have been investigated as substrates of creatine kinase (James & Morrison, 1966). AMP, adenosine, and pyrophosphate are competitive inhibitors of MgADP, while both MgdADP and MgIDP can act as phosphoryl acceptors. In the bound nucleotide, the sugar appears to be in either a C4'-exo or O1'-endo pucker and a high anti-conformation (Rosevear et al., 1987; Rosevear et al., 1981b). The Λ screw sense isomer of bidentate β, γ-MgATP (Dunaway-Mariano & Cleland, 1980) and the Δ isomer of α,β-MgADP (Burgers & Eckstein, 1980) are thought to be substrates. The

phosphates are believed to be in an extended configuration (Dunaway-Mariano & Cleland, 1980). The ligation scheme of the metal in the formate transition state complex has been discussed earlier.

A relatively complete picture has emerged of the structural features of creatine which are important in binding. Glycocyamine is a poorer substrate than creatine (Tanzer & Gilvarg, 1959), and it has been proposed that the N-methyl group is involved in the conformational change which occurs upon substrate binding (McLaughlin et al., 1972). The higher homologs, N-ethyl-N-amidinoglycine (Ennor et al., 1955) and N-propyl-N-amidinoglycine (Rowley et al., 1971), are also substrates. Of importance is the observation that N-propyl-N-amidinoglycine has 1% the V_{max} of creatine and a K_m of 53 mM (McLaughlin et al., 1972). This has led to the design of N-(2,3-epoxypropyl)-N-amidinoglycine (epoxycreatine, Fig. 1.3), the affinity label used in the present work. Cyclocreatine [1-carboxymethyl-2-iminoimidazolidine] (Fig. 1.3) is an excellent substrate for the enzyme, with a V_{max} 90% that of creatine and a K_m of 25 mM (McLaughlin et al., 1972). Cyclocreatine is enzymatically phosphorylated exclusively at the N3 position (Phillips et al., 1979; Struve et al., 1977). This suggests that creatine is phosphorylated on the nitrogen *cis* to the methyl group (and therefore furthest removed from the carboxyl) (Struve et al., 1977). Dietrich et al. (Dietrich et al., 1980), have proposed a model for the steric requirements for creatine binding to CK. There appear to be very tight steric requirements at the creatine binding site with very little tolerance around the guanidinium group, and only enough room for a methyl or methylene group between the α- carbon and the N-methyl group or the N-methyl group and the external guanidinium nitrogen.

D. Active Site Residues

At least partially because of the lack of a crystal structure of CK, it is not possible to identify conclusively residues that are involved in catalysis. Nevertheless, spectroscopic

and chemical modification approaches have implicated several amino acid residues as being in or near the active site. Possible roles in catalysis or binding have been proposed for some of these residues. Only Cys-282, the active site sulfhydryl, has been definitively located in the primary sequence of the enzyme. In this section a brief description is given of the evidence for the involvement of each residue in catalysis.

Chemical modification of CK with diethylpyrocarbonate (Clarke & Price, 1979; Pradel & Kassab, 1968) and pH/rate studies (Cook et al., 1981) have suggested that a histidine acts as an acid/base catalyst, possibly to protonate/deprotonate the guanidinium nitrogen which becomes phosphorylated (Cook et al., 1981). The role of this histidine has been further investigated by NMR (Rosevear et al., 1981a) where it was shown that there are actually three histidines near the active site. Two of these are within 12 Å of the metal ion and one of these has a $pK_a = 7$ by NMR which matches that found in the pH/rate profile. The third histidine is ca. 14 Å from the metal site. The identity of these histidines in the primary sequence of CK is not known.

CK can be inhibited by *p*-nitrophenyl acetate (Clark & Cunningham, 1965) and dansyl chloride (Kassab et al., 1968), apparently as a result of the modification of the ε-amino group of a lysine residue. NMR studies of the transition state complex formed with formate suggest that a lysine interacts with the transfering phosphoryl group and may thereby enhance its electrophilicity (James & Cohn, 1974). A dansylated peptide from chicken muscle CK was isolated and sequenced (Bose & Friedberg, 1971); however, it is not possible to locate definitively this peptide in the sequence of chicken muscle CK. Subsequently, Mahowald (Mahowald, 1969) sequenced a peptide obtained from the crosslinking of CK by 1,5-difluoro-2,4-dinitrobenzene. This reagent reacted with Cys-282 and Lys-196 in rabbit muscle creatine kinase. An unidentified lysine has also been labeled by the 2',3'-dialdehyde derivative of ADP and evidently the enzyme so modified is still able to catalyze the transfer of the phosphoryl group between phosphocreatine and the covalently bound nucleotide analogue (Navinskii et al., 1983).

In addition to the histidine with a pK_a of 7, the effect of pH on the rate and other kinetic parameters of the CK reaction has identified a second group with a pK_a of 6 (Cook et al., 1981). This group is a neutral acid which must be ionized for the binding of either creatine or phosphocreatine. The most obvious candidate for this group is the carboxylic acid of a glutamate or aspartate residue. This acid may interact electrostatically with the tertiary nitrogen of the guanidinium group so as to localize positive charge on this nitrogen and thereby increase the nucleophilicity of the nitrogen which is phosphorylated (Cook et al., 1981). In addition, modification of CK with N-cyclohexyl-N'-β-(4-methylmorpholine)ethylcarbodiimide results in the loss of activity, presumably due to reaction with a carboxyl group (Nevinsky & Gazaryants, 1987). The location of the carboxyl(s) in the primary sequence is not known.

Phenylglyoxal and butanedione (Borders & Riordan, 1975) will each label an arginine residue in CK, and NOE experiments place an arginine in the vicinity of the phosphate groups of the nucleotide substrate (James, 1976). Other spectroscopic studies have implicated a tryptophan residue in the active site (Messmer & Kagi, 1985; Vasak et al., 1979), and a tyrosine residue may also be near the active site, as suggested by modification of CK with iodine (Fattoum et al., 1975).

In addition to the above groups, creatine kinase also contains one highly reactive sulfhydryl group in each subunit. This sulfhydryl can be modified, with complete loss of activity, by a large variety of sulfhydryl specific reagents. These include iodoacetate (Mahowald et al., 1962; Rosenberg & Ennor, 1955), 2,4-dinitrofluorobenzene (Mahowald et al., 1962), and dithiobis(2-nitrobenzoic acid) (O'Sullivan, 1971). Protection against inactivation is afforded by substrates (Mahowald et al., 1962; Rabin & Watts, 1960) and by the transition state complexes (Quiocho & Olson, 1974). Modification of this sulfhydryl with methylmethanethiolsulfonate (MMTS) (Smith & Kenyon, 1974) or with dithiobis(2-nitrobenzoic acid) followed by potassium cyanide (to give the S-cyano derivative) (der Terrossian & Kassab, 1976) apparently resulted in the retention of some

activity: ~20% in the case of the S-SCH_3 and ~73% for the S-CN derivative. From these results it was proposed that modification of the sulfhydryl with bulky or charged groups imposed steric and/or electronic constraints on the enzyme and therefore led to a loss of activity, but that the smaller, uncharged -SCH_3 or -CN groups could be better accommodated by the enzyme and all activity was not lost (Smith & Kenyon, 1974). This proposal has recently been questioned (Zhou & Tsou, 1987) and it may be that the residual activity seen after modification with the cyano or thiomethyl groups is due to the loss of the label, possibly by an intramolecular transfer of the modifying group to a second sulfhydryl (Fawcett et al., 1982). Thomson et al. (Thomson et al., 1964), sequenced a tryptic peptide from CK which had been labeled with ^{14}C-iodoacetate, and Mahowald (Mahowald, 1965) sequenced a similar peptide resulting from digestion of the 2,4-dinitrofluorobenzene-modified enzyme. Following the determination of the primary sequence of rabbit muscle CK (Putney et al., 1984) this sulfhydryl has been identified as Cys-282. This cysteine is invariant in all of the CKs which have been sequenced. Further discussion of the possible role of Cys-282 in catalysis will be presented in Chapter 4.

E. Summary

Creatine kinase is a dimeric enzyme with one active site per subunit. The reaction it catalyzes can be described essentially by a random bi-bi kinetic scheme, although, at pH 7, there is a slight preference for MgATP to bind prior to creatine. Bidentate β, γ-MgATP binds as the Λ screw sense isomer and creatine binds as the zwitterion. Substrate binding induces a conformational change in the enzyme which closes down the active site; Cys-282 may be involved in this process. Arginine and lysine residues interact electrostatically with the phosphates of the nucleotide and may help to anchor it into a favorable conformation for catalysis and/or increase the electrophilicity of the γ-phosphate. A carboxyl group is probably near the active site and may interact with the tertiary nitrogen of the guanidinium

group. A histidine residue probably acts to deprotonate the nitrogen that is phosphorylated. At some point, possibly coincident with the conformational change, the metal becomes cooordinated to all three phosphate groups of the ATP. Phosphoryl transfer then occurs, most likely by an in-line associative mechanism. After transfer, the active site presumably opens back up and the products, phosphocreatine and the Δ screw sense isomer of bidentate MgADP, are released.

This represents a fairly complete picture of the events occurring during catalysis. Unfortunately, most of the evidence for the chemical events of catalysis is indirect and incomplete. In particular, the role played by the active site sulfhydryl is still problematic (see Chapter 4) and the identity of the other groups which participate in catalysis is also questionable. It may not be possible to address adequately these areas until after a crystal structure is available and after the numerous experiments that it may suggest are carried out and fully evaluated.

III. The Goals of the Present Work

A goal of the present work is to identify the residue(s) of creatine kinase which is labeled by the affinity label, epoxycreatine. Epoxycreatine was first demonstrated to be an affinity label of creatine kinase in 1979 (Marletta & Kenyon, 1979); however, the active site residue which it labels has not been determined. It is hoped that the identity of this residue will give important clues to the mechanism of this enzyme and will be an important step toward the complete delineation of the active site of this important enzyme.

In addition, a high-yield and simple purification of the rabbit muscle enzyme is reported. Some preliminary investigations of the microheterogenity problem, which has hampered attempts to obtain high quality crystals of CK, are also reported. These studies

can provide a starting point for additional work in obtaining completely homogeneous enzyme for x-ray work.

Finally, the purification of creatine kinase from *Torpedo californica* and benzoylformate decarboxylase expressed in *E. coli*, are also reported. *Torpedo californica* CK was purified to provide authentic, tissue-purified enzyme for verification of the identity of the protein expressed from cDNA in *E. coli*. Benzoylformate decarboxylase expressed in *E. coli* was also purified to confirm that the cDNA encoded for authentic enzyme.

2

Purification of Creatine Kinase

I. Introduction

Creatine kinase was first purified to apparent homogeneity from rabbit skeletal muscle by Kuby et al (Kuby et al., 1954a). Since then, CK from a number of species and tissues has been purified and characterized (Miller et al., 1982; Quest et al., 1989; Watts, 1973). In the majority of these cases, the purification has been sufficient for most purposes; however, a major problem remains. To date, it has not been possible to obtain crystals of CK of sufficient quality and stability for high resolution x-ray diffraction studies. The overriding reason for this problem appears to be heterogeneity of the enzyme preparations (Hershenson et al., 1986). Highly purified material, homogeneous by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), still consists of a number of species that can be resolved by isoelectric focusing. All of these species evidently have CK activity. Attempts to achieve further purification of these mixtures have met with only limited success (Hershenson et al., 1986). Because it is anticipated that the crystal structure of CK will be a significant step forward in the understanding of this enzyme, a major goal of the Kenyon laboratory is to obtain an enzyme preparation which is pure enough to yield high quality crystals.

The purification of rabbit muscle CK is reported here for two reasons. First, the present purification is a rapid and convenient procedure for obtaining enzyme of very high purity and yield. The major purification techniques employed (ethanol fractionation, Blue Sepharose chromatography, and anion exchange chromatography) have been used separately in previous purifications of CK (Kuby et al., 1954a; Miller et al., 1982; Quest et

al., 1989); however, here they have been combined into a simple and efficient method for obtaining highly purified protein. Secondly, the major hurdle to overcome in determining an x-ray structure of CK is obtaining completely homogeneous enzyme. While the present purification does not completely achieve this goal, and microheterogeneity remains a problem, it is hoped that this purification can be a starting point for attempts to obtain enzyme sufficiently pure for x-ray work. Preliminary experiments which address the microheterogeneity problem are also discussed, and some ideas and suggestions for future approaches to this problem are described.

A purification of creatine kinase from the electrocyte of the electric ray, *Torpedo californica,* is also reported. The electric organ contains a cytosolic CK as well as a membrane-associated form that co-purifies with the acetylcholine receptor (Barrantes et al., 1983; Gysin et al., 1983). The close association of CK with the acetylcholine receptor may have functional significance, and CK possibly acts to replenish the ATP consumed in the energy-requiring processes of transmitter release. Current evidence indicates that both the cytosolic and membrane-associated forms are of the M-type (Gysin et al., 1986; Perryman et al., 1985). CK cDNA from the electric organ of *Torpedo californica* has been cloned, sequenced, and expressed in *E. coli.*(Babbitt et al., 1990b; West et al., 1984). In a collaborative effort with Dr. Patricia Babbitt of the Kenyon lab, the cytosolic CK of the electrocyte has been purified to apparent homogeneity by SDS-PAGE utilizing purification methods developed for rabbit muscle CK. Purified enzyme has been of value in confirming the identity of the enzyme expressed in *E. coli* and as a control in experiments studying the expression of *Torpedo californica* cDNA in *E. coli* (Babbitt et al., 1990b).

II. Experimental

A. Reagents and Materials

ADP (sodium salt), ATP (disodium salt), carboxypeptidase B (porcine pancreas, Type I), creatine, creatine kinase (rabbit muscle, Type I), dithiothreitol, EDTA (disodium salt), Folin and Ciocalteu's phenol reagent, glucose, glycerol, hexokinase (yeast, Type VI), lactate dehydrogenase (rabbit muscle, Type XI), magnesium acetate tetrahydrate, β-mercaptoethanol, MES (potassium salt), MTT, NADH (disodium salt), NADP (sodium salt), phenylmethanesulfonyl fluoride, phosphocreatine, phospho(enol)pyruvate (potassium salt), PMS, potassium acetate, pyruvate kinase (rabbit muscle, Type III), Sigmacote, sulfosalicylic acid, TES (sodium salt), and Tris (free base) were all from Sigma Chemical Co., St. Louis, MO. Acrylamide, ammonium persulfate, Bis, glycine, and TEMED were from BioRad, Richmond, CA. Blue Sepharose CL-6B, Coomassie Blue R250, electrode wicks, IEF ampholytes, and pI standards were from Pharmacia LKB Biotechnology, Piscataway, NJ. GEMSA was from Calbiochem, La Jolla, CA. Trichloroacetic acid was from Aldrich Chemical Co., Milwaukee, WI. Amicon and Centricon ultrafiltration devices were from Amicon Corp., Danvers, MA.

B. Gel Electrophoresis

1. Sodium dodecylsulfate polyacrylamide gel electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli, 1970). Usually, 10% acrylamide/0.3% N,N'-methylene-bis-acrylamide (Bis) resolving gels were used, with 4% acrylamide/0.1% Bis stacking gels. All gels were 6.5 cm x 10 cm x 0.75 mm.

The following recipes were used for four 10% gels. Recipes for all buffers, 30% acrylamide/Bis, and sample dye can be found in Appendix B.

Stacking Gel		Resolving Gel	
stacking gel buffer (4x stock)	2.5 ml	resolving gel buffer (4x stock)	5 ml
30% acrylamide/Bis	1.5 ml	30% acrylamide/Bis	6.6 ml
H_2O	6.0 ml	H_2O	8.4 ml
69% glycerol	0.7 ml	10% ammonium persulfate	100 µl
10% ammonium persulfate	40 µl	TEMED	50 µl
TEMED	12 µl		

The ingredients of the resolving gel, with the exception of the ammonium persulfate and TEMED, were mixed, filtered, and degassed for 10 min. Polymerization was initiated by the addition of the ammonium persulfate and TEMED, and the gels were immediately poured between two glass plates, leaving 2-3 cm at the top for later addition of the stacking gel. After polymerization, all of the ingredients of the stacking gel were mixed and poured on top of the resolving gel, a comb was inserted, and polymerization was allowed to occur. Unused gels were stored moist at 4 °C. Samples were prepared by mixing 2-5 µg of protein (5 µl of a 0.1-1 mg/ml solution) with 5 µl of 2x Laemmli sample buffer (App. B) and 3 µl of sample dye (App. B). Before loading, samples were placed in a boiling water bath for 10 min. Gels were typically run at 15 mA, but no greater than 150 volts. Following electrophoresis, gels were stained for 60 min to overnight in 0.1% Coomassie Blue/25% EtOH/5% AcOH. Gels were destained in 25% EtOH/5% AcOH until the background was clear.

2. Native polyacrylamide gel electrophoresis

Native polyacrylamide gels were run in a manner similar to the procedure given above for SDS-PAGE gels. Buffer recipes can be found in Appendix B. For one 14 x 8.5 cm 10% polyacrylamide gel the following recipes were used:

Resolving Gel		Stacking Gel (4%)	
30% acrylamide/Bis (App. B)	10 ml	30% acrylamide/Bis (App. B)	2.5%
resolving gel buffer	3.75 ml	stacking gel buffer	5 ml
H_2O	13.75 ml	H_2O	10 ml
10% ammonium persulfate	50 µl	10% ammonium persulfate	30 µl
TEMED	15 µl	TEMED	10 µl

Prior to electrophoresis, samples were prepared by adding 5-15 µg of protein (5 µl of a 1-3 mg/ml solution) to 10 µl of running buffer and 17 µl of sample dye (App. B). Electrophoresis was performed at 20 mA until the dye front reached the resolving gel, at which time the current was increased to 25 mA. During electrophoresis, the voltage was not allowed to exceed 200 volts. Staining and destaining procedures were identical to those used with SDS-PAGE gels.

3. Native isoelectric focusing

Native isoelectric focusing (IEF) was performed in 5% polyacrylamide gels with 2.5% of the appropriate pH ampholytes. All gels were 14 cm x 14 cm x 0.75 mm. The following recipes, modified from the manufacturer's specifications (Pharmacia FBE 3000), were followed:

Stock Acrylamide/Bis

acrylamide		19.4 g
Bis		0.6 g
H_2O	qs	200 ml

After stirring for 1 hour with 2.0 g of either Amberlite MB-6 resin or a similar mixed bed resin, the above mixture was filtered and stored in the dark at 4 °C.

For one 14 cm x 14 cm x 0.75 mm gel

stock acrylamide/Bis	9.6 ml
40% ampholytes	1.2 ml
69% glycerol	4.7 ml
H_2O	3.5 ml
10% ammonium persulfate	44 µl
TEMED	15 µl

The above ingredients, with the exception of the ammonium persulfate and TEMED, were mixed together, filtered, and degassed for 5 min. The ammonium persulfate and TEMED were then added and the gel immediately poured between two glass plates, one of which had been siliconized. After polymerization, the gels were overlayed with water and stored at 4 °C. In some instances, gels were poured onto cellophane backing that had been dried onto the non-siliconized plate. Polymerizing the gel on the backing led to greater mechanical stability and did not affect the electrophoretic properties of the gel.

Prior to electrophoresis, the siliconized glass plate was carefully removed, leaving the gel to adhere to the other glass plate or cellophane backing. The plate bearing the gel was placed on the surface of a flat bed electrophoresis apparatus (Pharmacia FBE 3000) with a layer of water between the gel plate and the surface of the apparatus to allow for more efficient and uniform cooling. The apparatus was maintained at 5 °C with a

circulating water bath, but the actual temperature of the gel was not determined. An electrode wick (1.5 x 5 mm) soaked in 0.05 M H_3PO_4 was placed approximately 2 mm in from the edge of the gel nearest the anode. A wick soaked in 1 M NaOH was similarly placed on the cathode edge. In addition, two wicks saturated with 1 M NaOH were placed on the surface of the apparatus, but not in contact with the gel. These wicks were for the purpose of absorbing atmospheric CO_2. The top was placed on the apparatus, the edges sealed with lab tape, and the gel prerun at 15 watts constant power and 1800 volts maximum. The amperage typically fell from 25 mA to 7-8 mA during this period.

Following the prerun, 10-15 µl of a ~1 mg/ml solution of the sample in water (or less than 200 mM buffer) was applied to a small piece of 3M filter paper lying on the surface of the gel. After again sealing the edges with lab tape, electrophoresis was continued for an additional 3-4 hours. After one-half hour, the pieces of 3M paper were removed from the surface of the gel.

Following electrophoresis, if the gel was to be stained with Coomassie Blue, it was fixed for one hour in an aqueous solution of 10% trichloroacetic acid/5% sulfosalicylic acid. The gel was then equilibrated for 2 hours to overnight in 25% MeOH/5% AcOH. After equilibration, the gel was stained for 20 to 30 min with 0.1% Coomassie Blue/25% MeOH/5% AcOH, followed by destaining in 25% MeOH/5% AcOH. In some cases, destaining in 50% MeOH/10% AcOH was necessary to obtain a clear background.

4. Denaturing isoelectric focusing

Isoelectic focusing in the presence of 8 M urea was performed as described for native IEF, except that the following recipe was used for <u>one</u> 14 cm x 14 cm x 0.75 mm gel:

stock acrylamide/Bis (App. B)	9.6 ml
urea	9.1 g
69% glycerol	1.0 ml
40% ampholytes	1.2 ml
10% ammonium persulfate	44 µl
TEMED	15 µl

Samples were prepared as described for native IEF, except that they were dissolved in 8 M urea prior to application to the gel. Focusing and staining were performed as described for native IEF.

C. Activity Assays

1. Spectrophotometric assay

Creatine kinase was assayed by the method of Tanzer and Gilvarg (Tanzer & Gilvarg, 1959). This assay follows the change in absorbance at 340 nm due to the loss of NADH in the following coupled system:

$$\text{creatine} + \text{ATP} \xrightarrow{\text{creatine kinase}} \text{phosphocreatine} + \text{ADP}$$

$$\text{phospho(enol)pyruvate} + \text{ADP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

The following solutions were used:

Glycine Buffer

glycine	200 mM
$Mg(OAc)_2 \cdot 4H_2O$	12 mM
KOAc	200 mM
pH to 9.1 with NaOH (25 °C)	

Assay Mix

ATP (disodium salt)		89 mg
phospho(enol)pyruvate (potassium salt)		10 mg
NADH (disodium salt)		6 mg
pyruvate kinase (500 U/mg)		4 mg
lactate dehydrogenase (940 U/mg)		7 mg
glycine buffer	qs	25 ml

The assay was performed by mixing 350 µl of the assay mix with the appropriate amount of enzyme (usually 0.01-0.02 units) in a 0.5 ml masked cuvette with a one cm pathlength. The absorbance at 340 nm was monitored and the rate of change between 3 and 25 seconds recorded. This value was used for the creatine-independent oxidation of NADH and was only significant with crude tissue extracts. Saturated creatine (150 µl) was then added and a second rate determined in the same manner. The two rates were subtracted to determine the creatine-dependent rate. International units (U), in µmol/min, were calculated from:

$$U = \left(\frac{\Delta AU}{min}\right)\left(\frac{1}{\varepsilon}\right)\left(\frac{1}{l}\right)(v)$$

where:

$\frac{\Delta AU}{min}$ = the rate of change of the absorbance at 340 nm.

ε = the extinction coefficient of NADH (6220 $AU \cdot M^{-1} \cdot cm^{-1}$).

l = the path length of the cuvette in cm.

v = the volume of the assay solution in µl.

For the assay given above this equation can be written as:

$$U = (\text{rate})\left(\frac{1}{6220}\right)(500)$$

All assays were performed at 25 °C on a Hewlett Packard 8452A diode array spectrophotometer.

2. Activity stain

The activity stain couples the CK reaction to the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), with phenazine methosulfate (PMS) acting as an intermediate electron carrier. The coupled reactions are:

$$\text{phosphocreatine} + \text{ADP} \xrightarrow{\text{creatine kinase}} \text{creatine} + \text{ATP}$$

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ATP}$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{6-phosphogluconate} + \text{NADPH}$$

$$\text{PMS (oxidized)} + \text{NADPH} \longrightarrow \text{PMS (reduced)} + \text{NADP}^+$$

$$\text{PMS (reduced)} + \text{MTT (oxidized)} \longrightarrow \text{PMS (oxidized)} + \text{MTT (reduced, colored)}$$

Immediately following electrophoresis, the gel was equilibrated for 20 min in the following solution (usually 100 ml was required for a 14 x 14 cm IEF gel):

In 500 mM Tris·OAc, pH 7.5	
ADP (sodium salt)	2.8 mM
glucose (anhydrous)	370 mM
$MgSO_4$ (anhydrous)	4.1 mM
NADP (sodium salt)	1.2 mM
hexokinase (225 U/mg)	20 µg/ml
glucose-6-phosphate dehydrogenase (85 U/mg)	20 µg/ml

After equilibration, a solution (2 ml) containing 20 mg each of MTT and PMS in 500 mM Tris·OAc, pH 7.5 was added, and the gel was kept in the dark for 15 min. Any bands appearing during this period were considered to be due to a phosphocreatine-independent activity. Following this period, 360 mg of phosphocreatine in 1 ml of the same buffer was added and the gel was again placed in the dark. The dark blue bands indicating CK activity were usually apparent after 15-30 min. After the desired color intensity was achieved, the staining solution was removed, and the gel washed several times with water.

D. Protein Assay

Protein concentrations were determined following the method of Lowry (Lowry et al., 1951). The following solutions were used:

Solution A: 2% Na_2CO_3 in 0.1 N NaOH
Solution B_1: 2% NaK tartrate
Solution B_2: 1% $CuSO_4$
Solution C: 50 ml of soln. A + 0.5 ml of soln. B_1 + 0.5 ml of soln. B_2

To 100 µl of sample containing 2.5 to 30 µg of protein was added 1 ml of soln. C. After vortexing, this solution was allowed to stand at room temperature for 10 min. Folin

and Ciocalteu's phenol reagent (50 µl) was added, the samples were again vortexed and then placed in the dark for 40 min. After this period, the absorbance at 660 nm was determined; a sample containing no protein was used as a blank. Bovine serum albumin (BSA), prepared in the same buffer as the sample, was used as a standard. The A_{660} was linear with the amount of BSA and sample in the range of 2.5 to 40 µg of protein.

E. N-Terminal Sequencing

Protein samples (~100 pmol) were switched into water by ultrafiltration with an Amicon-30. Acetonitrile was added to 20% and the samples were submitted for gas phase Edman degradation to the Biomolecular Resource Center, The University of California, San Francisco.

F. Carboxypeptidase B Digestion

To 25 µg (37 µl of a 0.65 mg/ml solution in 25 mM Tris·HCl, pH 7.6/100 mM NaCl) of purified rabbit muscle CK was added 0.5 µg (2.5 µl of a 0.2 mg/ml solution in the same buffer) of carboxypeptidase B. This was a 1/50 protease/CK ratio. After gentle vortexing, this solution was allowed to sit at room temperature. Aliquots (12 µl) were removed at the desired intervals (30, 90, and 180 min), immediately frozen, and stored at -20 °C until subjected to isoelectric focusing.

G. Purification of Rabbit Muscle Creatine Kinase

1. Tissue extraction

Recipes for purification buffers can be found in Appendix B. Muscle tissue was cut from the rear legs of a 2.5 kg rabbit (male, New Zealand White) and ground twice in a

meat grinder. All subsequent steps were performed at 4 °C unless otherwise indicated. After weighing, this material (207 g) was suspended in 700 ml of Extraction Buffer. The resulting slurry was stirred with an overhead stirrer for 90 min, filtered through two layers of cheesecloth, and centrifuged at 10,000 x g for 45 min. The small pellet was discarded and the supernatant filtered through a 0.2 mm mesh cloth.

2. Ethanol fractionation

To the supernatant (700 ml) was added, over 15 min, 700 ml (1 volume) of absolute ethanol maintained at -20 °C. After addition, the suspension was stirred for 60 min and then centrifuged at 7000 x g for 20 min. The pellet was discarded, and to the supernatant (1360 ml) was added, over 25 min, 2040 ml (1.5 volumes) of absolute ethanol maintained at -20 °C. The resulting suspension was stirred for 60 min and then centrifuged at 7000 x g for 45 min. The supernatant was discarded and the pellet resuspended to 50 ml with MES Buffer. After stirring overnight, the volume was adjusted to 65 ml with MES Buffer and the suspension centrifuged at 100,000 x g for 60 min. The pellet was discarded and the supernatant (62 ml) was carried onto the Blue Sepharose chromatography step.

3. Blue Sepharose chromatography

The creatine kinase-containing supernatant was applied at 1.3 ml/min to 230 ml of Blue Sepharose CL-6B in a 39 x 3 cm column previously equilibrated with MES Buffer. The column was eluted at 1.3 ml/min with MES Buffer until the A_{280} was less than 0.01 AU. This required a total of 1000 ml. At this point elution was switched to TES Buffer, also at 1.3 ml/min. Elution was continued for a total of 864 ml (108 fractions of 8 ml each). The TES fractions that contained creatine kinase activity (#26-108) were combined and concentrated to 22 ml by ultrafiltration with an Amicon YM-30 membrane. The enzyme was stored at 4 °C in this form (TES buffer, approximately 35 mg/ml).

4. Anion exchange chromatography

As needed, the enzyme was purified further by anion exchange chromatography on a Fast Protein Liquid Chromatography (FPLC) system. A portion (1 ml) of the enzyme in TES Buffer was diluted to 2.0 ml with FPLC Buffer A (50 mM Tris·Cl, pH 8.7). This was concentrated to 50 µl with a Centricon-30. After repeating this process one additional time, the volume of the enzyme-containing solution was adjusted to 2.0 ml with FPLC Buffer A, filtered through a 0.45 µm filter, and chromatographed, in 500 µl aliquots, on a Mono Q HR 10/10 anion exchange column. The column was eluted with FPLC Buffer A for 5 minutes, followed by a 0.33%/min increasing gradient of FPLC Buffer B (FPLC Buffer A with 1.0 M NaCl). After 15 minutes of this gradient, the column was washed with 75% FPLC Buffer B for 10 minutes and then re-equilibrated with FPLC Buffer A. Under these conditions, the major CK species eluted at ~2% FPLC Buffer B (20 mM NaCl). CK-containing fractions were combined, concentrated to 2.4 ml by ultrafiltration with a Amicon YM-30 membrane, taken to 1mM DTT, and stored at 4 °C.

H. Purification of Torpedo californica Electric Organ Creatine Kinase

1. Tissue extraction

All of the purification steps were carried out at 4 °C, unless otherwise specified. The buffers were the same as those given for the purification of rabbit muscle CK, unless otherwise indicated. A *Torpedo californica* electric ray was obtained from Pacific Bio-Marine Labs, Venice, CA. The ray was sacrificed and the large honeycomb-like electric organ on the dorsal side excised. The organ (1152 g) was cut into 1-3 cm pieces and homogenized in a Waring blender with one volume of Extraction Buffer. The resulting suspension was filtered through two layers of cheesecloth and centrifuged at 7000 x g for 30 min. The supernatant was set aside and the pellet resuspended in 250 ml of Extraction

Buffer. This suspension was homogenized in 40 ml batches with a teflon homogenizer driven by a power drill at ~250 rpm. The homogenate was filtered through 0.2 mm nylon mesh and added back to the supernatant from the first spin. The combined material (1670 ml) was centrifuged at 16,000 x g for 2 hours. The pellet was discarded and the supernatant (1450 ml) carried on to the ethanol fractionation step.

2. Ethanol fractionation

To the supernatant was added 5800 ml (four volumes) of absolute ethanol maintained at -20 °C. The resulting suspension was stirred for 1.5 hours before filtering through cheesecloth. The large amount of material retained by the cheesecloth was subsequently shown to contain a significant amount of CK (see below) and was saved. The filtrate was centrifuged at 7000 x g for 45 min, the supernatant discarded, and the pellet resuspended in Extraction Buffer. After stirring overnight, this material (800 ml) was centrifuged at 120,000 x g for 45 min. The pellet was discarded and the supernatant (800 ml) treated to remove any remaining nucleic acids by the addition of 1.6 g (80 ml of a 20 mg/ml solution) of protamine sulfate (to give 0.2% w/v). The resulting suspension was stirred for 20 min and then centrifuged at 10,000 x g for 30 min. The supernatant (850 ml) was filtered through a 0.45 μm filter and concentrated to 80 ml by ultrafiltration through an Amicon YM-30 membrane. At this point, it was combined with the concentrated CK-containing supernatant from the ethanol-precipitated material which did not filter through the cheesecloth (see below).

The material from the ethanol precipitation which did not filter through the cheesecloth also contained a significant amount of CK activity. This precipitate was homogenized in ~300 ml of Extraction Buffer with a power drill-driven teflon homogenizer and the final volume brought to 1500 ml with Extraction Buffer. After stirring overnight, this mixture was centrifuged at 9000 x g for 45 min, the pellet discarded, and the

supernatant (1400 ml) centrifuged at 125,000 x g for 45 min. The supernatant from this spin was filtered through a 0.2 μm filter and concentrated to 100 ml by ultrafiltration through an Amicon YM-30 membrane. At this point, the concentrate was combined with the CK-containing material which had filtered through the cheesecloth.

3. Blue Sepharose chromatography

The combined, concentrated material from the ethanol precipitation was filtered through Whatman #1 filter paper, followed by dialysis for 24 hours against two changes of MES Buffer (8 liters). After centrifugation at 13,000 x g for 15 min, the supernatant (250 ml) was applied to 230 ml of Blue Sepharose CL-6B in a 39 x 3 cm column, previously equilibrated with MES Buffer. The column was washed at 1.5 ml/min with MES Buffer until the A_{280} was less than 0.01 AU. This required a total of 420 ml. At this point elution was switched to TES Buffer, also at 1.5 ml/min, and continued for a total of 440 ml (110 fractions of 4 ml each). The TES fractions containing 80% of the total creatine kinase activity (#22-32) were combined and concentrated to 5 ml by ultrafiltration with an Amicon YM-30 membrane.

4. Anion exchange chromatography

The concentrated CK-containing material from the Blue Sepharose column was taken to 50 ml with FPLC Buffer A (50 mM Tris·OAc, pH 8.5), followed by concentration with an Amicon YM-30 to 5 ml. After repeating this two times, the volume of the enzyme-containing solution was adjusted to 35 ml with FPLC Buffer A. As needed, the enzyme was purified in 500 μl aliquots by anion exchange chromatography on a Mono Q HR 5/5 analytical column. The following gradient was used (FPLC Buffer B was FPLC Buffer A with 1 M NaCl.):

Time(min)	% Buffer B
4	0
9	4
19	4
35	10
40	75
45	75

Under these conditions, the major CK-containing peak eluted at 15 min (40 mM NaCl). CK-containing fractions were collected, taken to 1 mM DTT, and stored at 4 °C.

III. Results and Discussion

A. Purification of Rabbit Muscle Creatine Kinase

Given in Table 2.1 is a summary of the purification of creatine kinase from rabbit skeletal muscle. Fig. 2.1 is a photograph of a 10% SDS-PAGE gel of the various steps of the purification.

The purification affords a high yield of enzyme (greater than 500 mg per rabbit) with a 32% yield of the starting units. A 4-fold improvement in specific activity is achieved. The specific activity of the purified enzyme varied slightly with conditions, but was always around 100 U/mg. The enzyme is homogeneous by SDS-PAGE when stained with Coomassie blue. This is the most commonly used measure of protein purity. Even substantially overloaded gels show either a single band or at most only very minor contaminating bands. The enzyme can be stored in TES Buffer at 4 °C for extended periods of time; less than a 10% decrease in the specific activity was seen after 6 months of storage and only very minor bands appeared in the SDS-PAGE of stored material. Because the enzyme was stable in TES Buffer, it was convenient to purify further by anion

Table 2.1: Summary of the Purification of CK from Rabbit Muscle

Purification Step	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (U/mg)
Crude Extract	700	7,666	161,000	21
EtOH and 100,000 x g spin pellet	62	1,658	119,440	72
Blue Sepharose	22	1,130	87,010	77
Mono Q*	--	590	52,955	89

*The values for the FPLC purified material were calculated from the chromatography of a single 1 ml sample of the material from the Blue Sepharose column.

exchange chromatography only the amount needed for a 2-3 week period. This purified enzyme was stored in FPLC Buffer A with 1 mM DTT. No loss of activity was noted over a 3 week period.

A chromatogram of the purification obtained on the Mono Q column is shown in Fig. 2.2. The peak eluting at ~2% FPLC Buffer B contained greater than 95% of the total activity eluting from the column. The other, later eluting peaks, also contained CK activity. In a separate experiment (chromatogram not shown), in which the pH of the initial buffer was 8.5 rather than 8.7 and a Mono Q HR 5/5 column (an analytical column) was used, it was found that the major CK species did not stick to the column and that the other, more basic, species could be resolved into at least five separate peaks. Each of these had CK activity, but none was greater than 1% of the total activity eluting from the column.

Thus, it would appear that the rabbit muscle enzyme is a very heterogeneous population of CK species. These are of very similar molecular weight, and run on SDS-

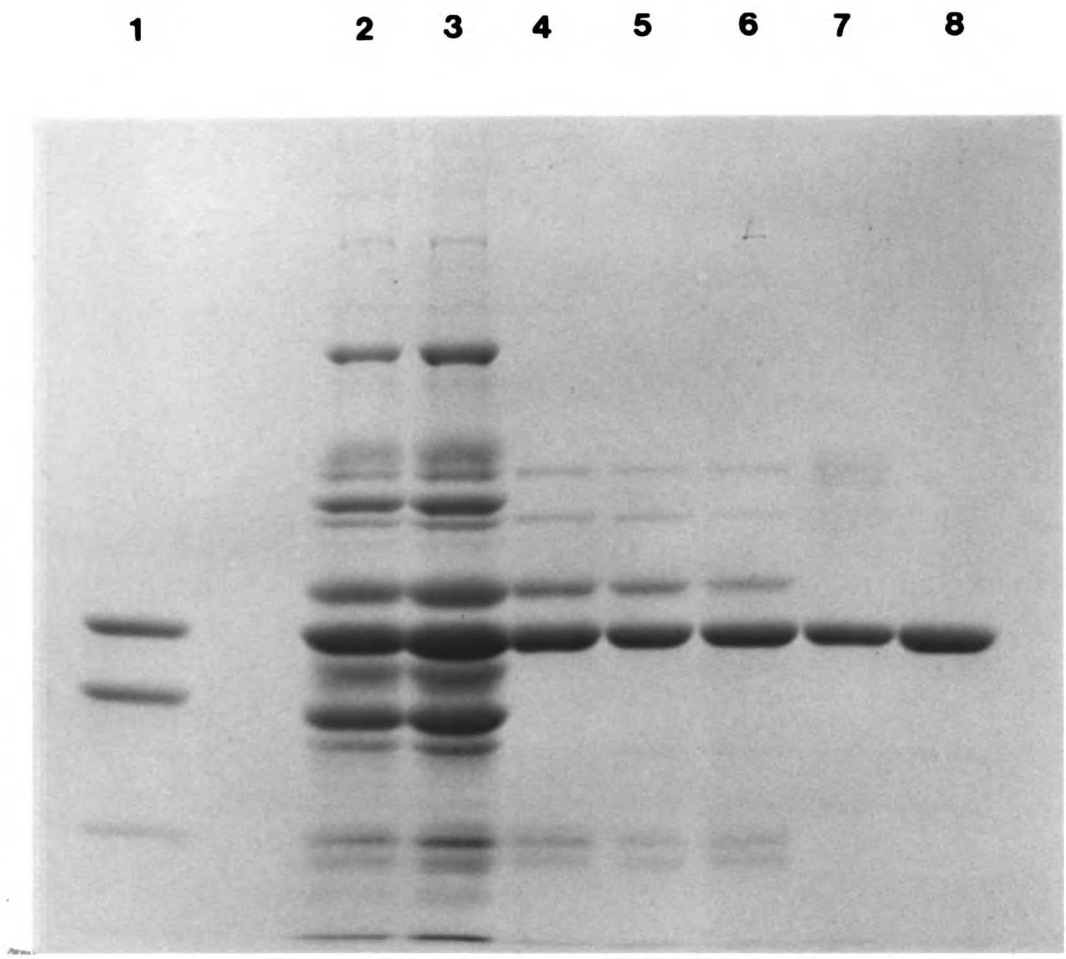


Figure 2.1. Photograph of an SDS-PAGE gel (10%) showing the purification of creatine kinase from rabbit muscle. Lane 1, rabbit muscle CK from Sigma Chemical Co.; lane 2, crude extract; lane 3, supernatant from first 10,000 x g spin; lane 4, supernatant from 50% ethanol fractionation; lane 5, pellet from 80% ethanol fractionation; lane 6, supernatant from 100,000 x g spin; lane 7, after Blue Sepharose chromatography; lane 8, after Mono Q column.

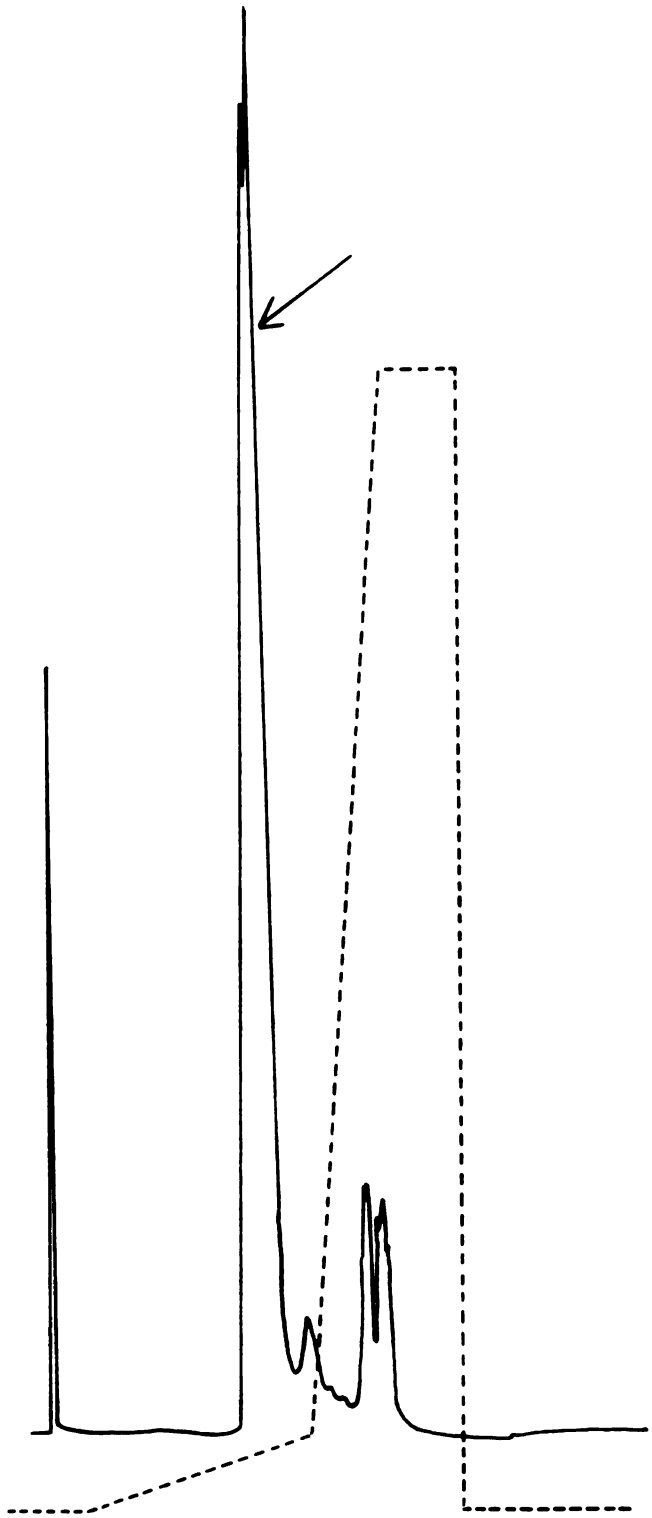


Figure 2.2. Chromatogram of creatine kinase elution from a Mono Q HR 10/10 anion exchange column. Detection was at 280 nm. Other conditions were as outlined in Experimental. The peak containing >95% of the CK activity is indicated by the arrow. (-), Absorbance at 280 nm; (---), percent buffer B.

PAGE as a single band, both before and after FPLC (Fig. 2.1, lanes 7 and 8). Some of these species can be separated readily by FPLC; however, even the FPLC-purified material can be resolved into several bands on an isoelectric focusing gel (Fig. 2.3 and discussion below). The source of this microheterogeneity is not known. A more detailed discussion of this phenomenon is now presented.

B. The "Isoforms" of Creatine Kinase

Fig 2.3 is a photograph of a native isoelectric focusing gel of FPLC-purified rabbit muscle creatine kinase. The gel has been stained for creatine kinase activity and the dark blue/black bands indicate species which possess CK activity. Similar gels, which were stained with Coomassie Blue, did not show any additional bands. Also shown on this gel is a sample of rabbit muscle CK which was expressed in and purified from *E. coli* (Chen et al., 1990). There are at least 9 distinct species in the tissue-purified material, and the major species are also seen in *E. coli*-expressed CK. This multitude of species has been noted before (Hershenson et al., 1986; Panteghini, 1988; Thorstensson et al., 1976) and it is not surprising that this material does not yield high quality crystals.

It is well documented that, at least in plasma, muscle creatine kinase is a substrate for a carboxypeptidase (Billadello et al., 1985; Michelutti et al., 1987; Perryman et al., 1984). Hydrolysis by this protease results in the loss of the C-terminal lysine residue of one or both monomers, thereby generating two possible new dimeric species. Changes in the plasma levels of these isoforms are seen following myocardial infarction. Shown in Fig. 2.4 is a photograph of a pH 3-10 IEF gel of samples taken at various times from a digestion of rabbit muscle CK with carboxypeptidase B, a lysine-specific carboxypeptidase. The resolution of this gel is not so high as that shown in Fig. 2.3 because of the broader pH range; however, the major CK species are clearly evident. Although the major bands disappear during the digestion, they do not coalesce into a

Figure 2.3. Native IEF gel (pH 5-8) of purified rabbit muscle creatine kinase. Lane 1, expressed CK purified from *E. coli*; lane 2, tissue purified CK.

Figure 2.4. Native IEF gel (pH 3-10) of the time course of the digestion of rabbit muscle creatine kinase with carboxypeptidase B. Lane 1, t = 0; lane 2, t = 30 min; lane 3, t = 90 min; lane 4, t = 180 min; lane 5, carboxypeptidase B.

smaller number of bands. This would be expected if they were the result of the presence or absence of the C-terminal lysine. Instead, completely new bands appear to replace those which disappear and the pattern becomes more complex. Conceivably, under the conditions of this experiment, carboxypeptidase B is cleaving more than one residue in from the C-terminus; however, this does not seem likely, given its specificity. These results suggest that the major species present in purified rabbit muscle CK are not the result of the action of a lysine-specific carboxypeptidase.

Brain CK is reportedly a phosphoprotein (Mahadevan et al., 1984). Initial attempts to treat CK with alkaline phosphatase (data not shown) and determine whether this simplifies the patterm of bands seen by IEF, have been inconclusive. The presence of a phosphate group can be detected by treatment with a phosphatase (or by *in vivo* labeling with ^{32}P-labeled phosphate) and also by mass spectrometry. Electrospray mass spectrometry can distinguish the molecular weight of the phosphate group on a protein the size of creatine kinase. Conceivably, isolation and mass spectrometry of the individual bands from IEF gels would be capable of detecting the mass difference due to the phosphate group, or other types of post-translational modifications. A second possible approach could be mass spectrometry of peptides resulting from the proteolytic digestion of creatine kinase. Such peptides may be amenable to tandem mass spectrometry to determine the actual residue(s) bearing the modifying group (Gibson et al., 1987).

Purified rabbit muscle CK gives a positive periodic acid-Schiff reaction (McBride et al., 1990) and the human BB isozyme has been reported to contain sialic acid (McBride & Rodgerson, 1985). These results suggest that some of the multiple forms of the enzyme may result from glycosylation. Unfortunately, the purity of the preparation on which these results were obtained cannot be fully evaluated. The enzyme appears to be homogeneous by SDS-PAGE, but IEF gels were not examined. As a result, it is not possible to determine whether each of the multiple species is glycosylated, or if only one or a small number are. Since *E. coli* reportedly does not carry out glycosylation (Marston, 1987),

Figure 2.5. Native 6% polyacrylamide gel of rabbit muscle creatine kinase. Lane 1, CK-4 μg; lane 2, molecular weight markers.

Figure 2.6. Denaturing IEF gel (pH 3-10) of purified rabbit muscle creatine kinase. Lane 1, 7.5 μg; lane 2, 15 μg; lane 3, pI standards.

the observation that at least the major species are also present in expressed material is a strong argument that glycosylation cannot be the sole explanation for the multiple bands (Chen et al., 1990). An immunologically-based test for sugar residues on proteins (for example, the glycan detection kit marketed by Boehringer Mannheim) may be useful to test for glycosylation. This could be tried on full-length CK, perhaps as a stain for an IEF gel, as well as on separated peptides from the proteolytic digestion of the enzyme. Mass spectrometric characterization of the sugar residues should also be feasible. The types of the glycosidic linkages present and the residues that are modified is not known. This question may also be amenable to investigation by mass specrometry.

There is the possibility that creatine kinase runs on native IEF gels as a population of different multimers, ranging from monomers to octamers, or perhaps higher. Shown in Fig. 2.5 is a photograph of a native 6% polyacrylamide gel of rabbit muscle CK. CK runs as a reasonably sharp band; at least under these conditions CK electrophoreses as a single multimer, presumably the dimer. Shown in Fig. 2.6 is a denaturing IEF gel of rabbit muscle CK. Multiple bands are apparent and the pattern is very similar to that seen with native IEF (compare to Fig. 2.4, lane 1). These results suggest that CK does not run as a number of multimeric species on IEF gels.

There have been reports that certain proteins can electrophorese as complexes with the carrier ampholytes used to establish the pH gradient in IEF gels (Righetti, 1983). These instances are rare but conceivably could account for the multiple bands. No experiments to test for this have been conducted as yet, but such an experiment might involve isolation of the separated bands and refocusing them to determine whether they again run as multiple bands.

It is also possible that the multiple forms arise during the purification. For example: they may result from proteolysis, sulfhydryl oxidation, or deamidation of asparagine or glutamine residues. Although several protease inhibitors (EDTA, PMSF, and GEMSA) are included in the purification buffers, and care is taken to proceed through the purification as

quickly as possible and to perform all steps at 4 °C, this possibility is a serious concern. The specific activity of the enzyme increases throughout each step of the purification and there does not appear to be any step which results in an inordinate loss of activity. Attempts to monitor specifically the number of CK species present at various steps in the purification have not been made. It may be that small changes in specific activity accompanying the production of the multiple bands are obscured by the overall increase in specific activity obtained in the purification. A similar pattern of bands has been noted by others (Cattan et al., 1978; Hershenson et al., 1986) with enzyme that had been purified by different protocols. This would tend to argue against a purification artifact; however, it may be worthwhile to investigate the effect of various steps in the purification on the appearance of the multiple bands.

Translation of brain creatine kinase DNA can be initiated at internal methionines (Soldati et al., 1990). This also might be expected to give rise to multiple CK species. The first internal methionine of rabbit muscle CK is Met-11. Initiation of translation at this site would be expected to give a protein which differs in molecular weight by ~1100 from full length CK. It should be possible to detect this difference by SDS-PAGE, but a second band has never been detected in gels of purified material. Certainly this explanation cannot account for all of the different species.

In conclusion, purified rabbit muscle CK is a mixture of several species of very similar molecular weight, but differing in isoelectric points. The differences between these species are not yet known, although several possibilities can be envisaged and have been discussed. Effective strategies to minimize the production of the multiple forms, or to achieve a better purification of them, may well depend upon identifying the source(s) of the heterogeneity. Once this is accomplished, enzymatic treatment to remove post-translational modifications or adaptations of the purification protocol may be effective approaches. Starting with enzyme from a different source, such as *E. coli*-cloned rabbit brain CK, may also be a possibility. Regardless of the method used, it is of primary importance to obtain

Table 2.2: Summary of the Purification of CK from *Torpedo californica* electric organ

Purification Step	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (U/mg)
Crude Extract	1670	12,692	18,700	1.5
EtOH and 125,000 x g spin pellet	250	n.d.	4000	n.d.
Blue Sepharose	35	157	5775	37
Mono Q*	--	45	1400	32

n.d. Not determined.

*The values for the FPLC purified material were calculated from the chromatography of a single 2.5 ml sample of the material from the Blue Sepharose column.

completely homogeneous enzyme for crystallization and the determination of the x-ray structure of creatine kinase.

C. Purification of Torpedo californica Creatine Kinase

Shown in Table 2.2 is a summary of the purification of creatine kinase from the electric organ of *Torpedo californica*. Fig. 2.7 is a photograph of a 10% SDS-PAGE gel of the various steps of the purification.

The purification is comparable to that of rabbit muscle CK. As shown in both Table 2.2 and Fig. 2.7, the starting tissue contains less CK than rabbit muscle, although CK is still a major protein of the electrocyte. A 7.5% yield of the starting units is obtained, with a 21-fold improvement in specific activity being achieved. There are two points which could be improved upon. First, in an attempt to avoid the problems encountered in this

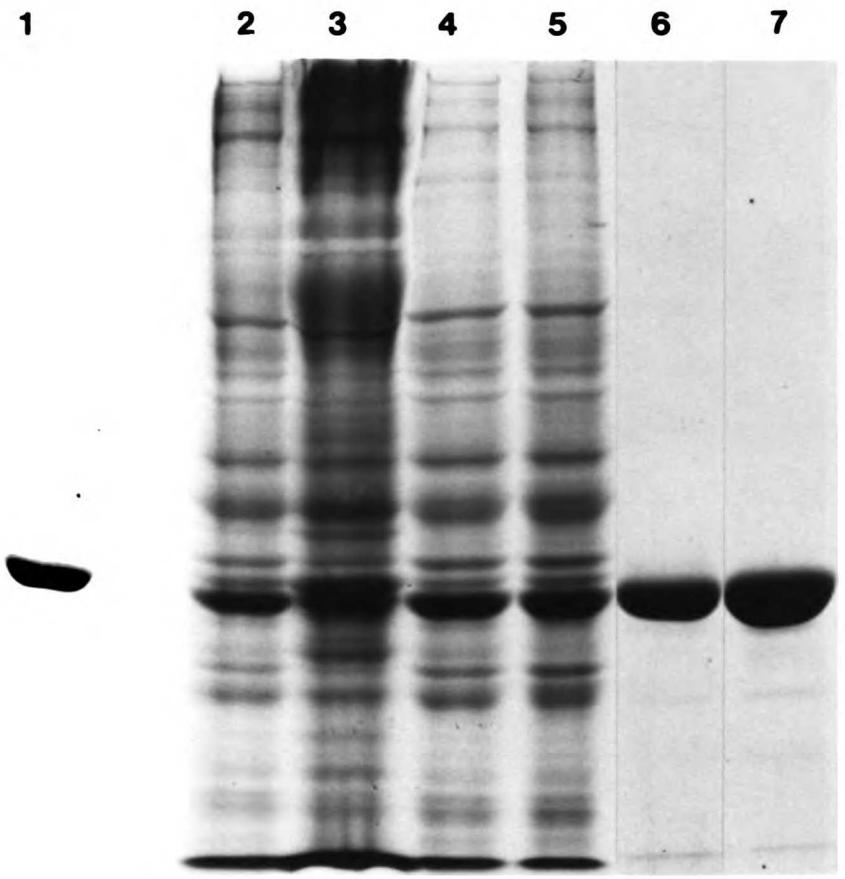


Figure 2.7. Photograph of an SDS-PAGE gel (10%) showing the purification of creatine kinase from *Torpedo californica* electric organ. Lane 1, rabbit muscle CK from Sigma Chemical Co.; lane 2, crude extract; lane 3, pellet from first 16,000 x g spin; lane 4, supernatant from 16,000 x g spin; lane 5, pellet from ethanol fractionation; lane 6, after Blue Sepharose chromatography; lane 7, after Mono Q column.

step, it might be reasonable to investigate a more careful ethanol fractionation. Secondly, the precipitation of nucleic acids with protamine sulfate is probably not necessary and any nucleic acids would likely be removed at later steps and not interfere in the subsequent purification steps. The remainder of the purification proceeded in a straightforward manner, with good yield and purification being achieved at each step. As was the case with rabbit muscle CK, Blue Sepharose and Mono Q chromatography were very effective purification steps. The enzyme is not as stable as rabbit muscle CK, and it is likely that the small drop in specific activity seen following the Mono Q step is the result of the enzyme losing activity over the time period of the purification.

The enzyme is greater than 95% homogeneous by SDS-PAGE (Fig. 2.7). As with the enzyme from rabbit muscle, there were several peaks eluting from the Mono Q column which contained CK activity. The major peak eluting at 4% FPLC Buffer B contained 98% of the activity. Other, later eluting peaks, accounted for the remainder. Because of the relatively lower stability of the FPLC-purified enzyme it was not possible to obtain a reliable IEF gel, and it is therefore unknown whether it also shows multiple bands.

Torpedo californica CK chromatograms on SDS-PAGE as a single band of apparent molecular weight 43,000. The first twenty-one amino acids, determined by Edman degradation, at the N-terminus of the tissue-purified material are:

P-F-G-N-T-H-N-K-W-K-L-N-Y-S-A-^{16}A-E-E-F-P-D

This sequence is exactly that predicted from the nucleotide sequence of the cDNA cloned in *E. coli* (West et al., 1984). It is identical to the previously published sequence (again from Edman degradation) of tissue-purified material (Perryman et al., 1985), with the exception of position 16, which is a lysine in the published sequence. The tissue-purified material has an identical elution pattern from Mono Q as the purified cloned material, and the specific activity of expressed enzyme (20 U/mg) (Babbitt et al., 1990a) is

comparable to that of the tissue-purified. These comparisons are complicated by the fact that the cloned material is obtained as an insoluble aggregate in *E.coli* and must be denatured and refolded before purification (Babbitt et al., 1990b). Nevertheless, it appears that, by the above criteria (N-terminal sequence, Mono-Q elution pattern, and specific activity), the cytosolic CK from *Torpedo californica* is identical to the enzyme that has been expressed in *E. coli* (Babbitt et al., 1990a; West et al., 1984).

3

Affinity Labeling of Creatine Kinase

I. Introduction

Prior to the identification of the active site residue that is modified by epoxycreatine, it was necessary to repeat the characterization of the interaction of the affinity label and enzyme. This chapter describes these experiments and also provides introductory material for the subsequent chapter on the identification of the site of labeling (Chapter 4).

A. Affinity Labeling

Affinity labeling is a valuable and widely used technique in the study of the structure and function of enzymes and proteins. Affinity labeling consists of the covalent modification, in a specific manner, of a group or groups in the active site or binding pocket of a protein. In the most desirable situation, this modification will induce a change in function or structure that can be monitored to gain insight into the role played by the modified residue. Labeling may result in the introduction of bulky or charged groups, or groups that have other desirable chemical properties. Knowing the chemical and physical properties of the label, coupled with the observed change in structure and function caused by its introduction, may allow deductions to be made regarding the role and importance of the modified residue. Such residues may be involved in catalysis, ligand binding, multimer interface contacts, or may be necessary for the maintenance of a particular conformation. The identification of residues that are located in active sites or binding pockets can be important in delineating the geometry and the location of such sites in the 3-dimensional

structure of the protein. Affinity labeling is also used in the study of the interactions between proteins, or between a protein and other cellular structures.

Affinity labels are designed to bind specifically to the region of interest of the protein. As such, they are usually structural analogues of the normal substrate or ligand that have been modified to contain a chemically reactive moiety (usually an electrophilic group). These groups tend to be of only moderate chemical reactivity and are designed to be stable until bound at the active site. Once bound, they may be placed in close proximity to a reactive group of the enzyme (usually a nucleophile), if such a group is suitably positioned. A unimolecular reaction between the reactive group and the label can then occur, leading to the covalent modification of the enzyme residue. In the best of circumstances, reaction will only occur with one active site residue. The covalently modified enzyme can then be separated from excess label, and the alterations in its structure and/or function investigated. An important prerequisite for the complete interpretation of the results of affinity labeling is the identification of the modified residue. It is often possible to make reasonable assumptions regarding the type of modified residue from the known or expected chemistry of the affinity label, or from the properties of the modified enzyme; however, unambiguous identification of the modified residue requires the sequencing of the modified protein. This point is discussed further in Chapter 4.

In order for a compound to be considered an affinity label, it is desirable that it meet the following criteria:

1) Complete inactivation
2) A 1:1 stoichiometry
3) Protection against inactivation by substrates
4) Saturation kinetics
5) Irreversible inactivation

Complete inactivation suggests that the label is bound in or near the active site, and that its presence has a profound effect on normal catalysis. This is evidence that the modified residue plays an important role in catalysis. A 1:1 stoichiometry (moles of affinity label per moles of enzyme active sites) indicates that labeling is not indiscriminate and is likely to be specific for a single residue. If the rate of inactivation is altered by the normal substrates, this implies that labeling occurs near the substrate binding site and does not occur if this site is protected by bound substrate. Saturation kinetics is highly indicative of the formation of an enzyme-inhibitor complex (a Michaelis complex) prior to the inactivation event. This suggests that the inhibitor is binding at the substrate binding site, possibly in a manner similar to the normal substrate. Irreversible inactivation is achieved if the affinity label covalently modifies a residue on the protein. The stability of the label, under the conditions used to investigate the alterations in structure/function that it induces, is critical in the interpretation of the effects of labeling.

If a label meets all of the above criteria, this is taken as strong evidence that it is binding to a residue that is in or near the active site and that modification of this residue has a severe effect on catalysis. In some instances, the labeling of a non-active site residue may meet these criteria; however, if all five requirements are met there is an excellent chance that an active site residue has indeed been modified. It should be noted that, depending upon the role the modified residue plays in catalysis, it may be possible to label it without complete inactivation of the enzyme. This, in and of itself, is an important clue as to the possible role of the residue in catalysis.

A kinetic description of the inactivation of an enzyme by an affinity label has been developed (Kitz & Wilson, 1962; Meloche, 1967). If the reaction obeys saturation kinetics, the inactivation can be described by the scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI_D$$

where E is free enzyme, I is free affinity label, EI is the non-covalent enzyme-inhibitor complex, and EI_D is the covalently modified enzyme. If the concentration of I is saturating, and EI is in rapid equilibrium, the above scheme can be described by the equation:

$$t_{1/2} = \frac{1}{[I]}\left(T_{1/2} K_D\right) + T_{1/2}$$

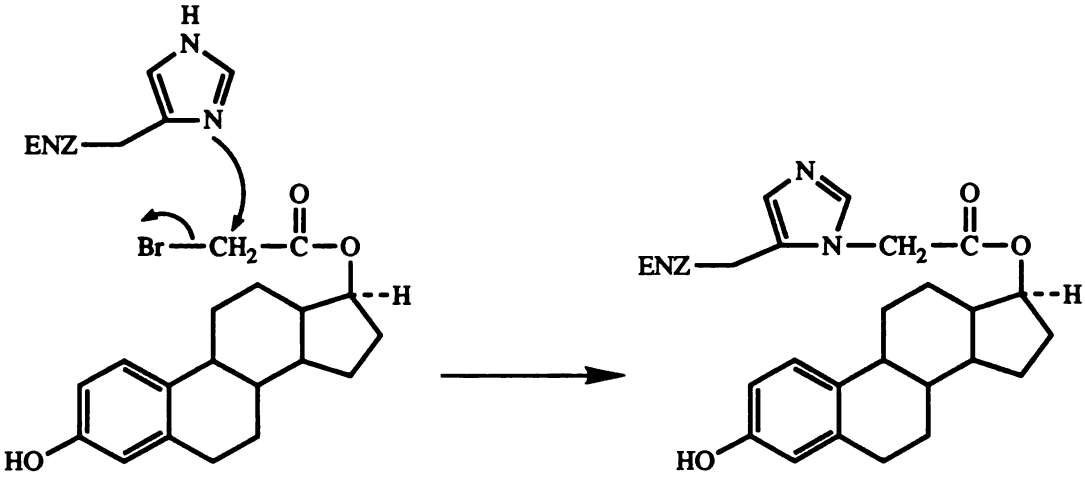
where $t_{1/2}$ is the half-life of inactivation, K_D is $(k_{-1} + k_2)/k_1$, and $T_{1/2}$ is the half-life of inactivation at infinite inhibitor concentration (Meloche, 1967). A plot of log percent activity remaining *vs* time at various inhibitor concentrations will give $t_{1/2}$ values. According to the above equation, a plot of $t_{1/2}$ *vs* 1/[I] will give a straight line with a *y*-intercept of $T_{1/2}$ and a slope of $T_{1/2}K_D$. If saturation kinetics are followed, an enzyme-inhibitor complex must form prior to inactivation, and $T_{1/2}$ will have a finite value. K_D is analogous to the constant, K_M, in Michaelis-Menten kinetics.

B. Types of Affinity Labels

A wide variety of functional groups have been utilized in the design of affinity labels (Cavalla & Neff, 1985; Coleman, 1983; Fersht, 1985; Jakoby & Wilchek, 1977; Plapp, 1982). These include: α-halo carbonyls, aziridines, azido and diazo compounds, aldehydes, fluorosulfonyl groups, and epoxides. Of these, the most common are α-halo carbonyls, the azide and diazo derivatives used in photoaffinity labeling, and epoxides. Brief discussions of the first two will be given to illustrate their applicability, and a more detailed discussion of the use of epoxides will be presented.

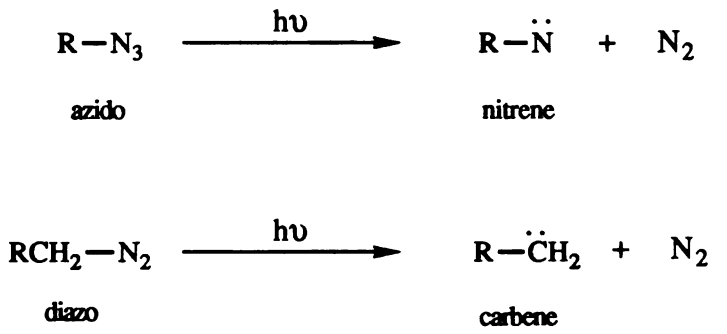
A recent example of the use of an α-halo carbonyl-based affinity label is the irreversible inactivation of 17β-estradiol dehydrogenase by 17α-estradiol-17-

(bromoacetate) (Murdock et al., 1988). This, and similar α-bromoacetoxy steroid derivatives (Murdock et al., 1983), label histidines in the active site of this enzyme:



Interestingly, estrone-3-(bromoacetate), which has the alkylating group attached to the steroid A-ring, alkylates the same histidine as 17α-estradiol-17-(bromoacetate) (Murdock et al., 1983). This histidine is not essential for catalytic activity (Pons et al., 1977). These results suggest that 17α-estradiol-17-(bromoacetate) may be binding in an inverted, "wrong-way", orientation, such that its D-ring lies where the A-ring of the normal substrate would be located (Murdock et al., 1988). Similar bromoacetoxy derivatives of steroids have been used in affinity labeling of other enzymes involved in the oxidation/reduction of steroids (Chen et al., 1989). α-Halo carbonyls will also react with carboxyl, sulfhydryl, and amino groups (Fersht, 1985).

Photoaffinity labeling has also been used extensively as a probe of protein structure and function, particularly of proteins which have no enzymatic activity. Commonly used photoreactive moieties, azido and diazo derivatives, generate highly reactive nitrenes and carbenes, respectively, upon photoactivation.



Photoaffinity labels are chemically unreactive until activated, but once activated tend to show little specificity in the type of group they will modify (Coleman, 1983). Nitrenes and carbenes can undergo a diversity of chemistry, including insertion into carbon-hydrogen bonds (Cavalla & Neff, 1985). This makes possible the labeling of many different types of amino acid residues, particularly those that do not normally have suitably reactive side chains (Chowdhry & Westhimer, 1979). As a result, photoaffinity labeling has been used extensively in the study of proteins that do not carry out catalysis and often have only relatively unreactive groups in their binding pockets. If the lifetime of the reactive species is sufficiently long, non-specific labeling of the protein can result (Ruoho et al., 1973). This is a disadvantage and can make it difficult to draw conclusions about the role of the modified residues. This problem is not necessarily unique to photoaffinity labels and must be considered with any type of affinity label. Several reviews are available on the technique of photoaffinity labeling (Cavalla & Neff, 1985; Chowdhry & Westhimer, 1979; Fedan et al., 1984; Jakoby & Wilchek, 1977).

The use of the epoxide group in affinity labels is of particular importance for the present work. Epoxides are moderate electrophiles and will undergo nucleophilic attack resulting in the opening of the oxirane ring (Fig. 3.1). If water is the attacking nucleophile, the gem diol results; other nucleophiles will give α-substituted alcohols. At neutral and basic pH, nucleophilic attack occurs by an S_n2 mechanism (Parker & Isaacs, 1959; Pocker et al., 1988; Ross, 1950) (Fig. 3.1, A). Attack occurs at the least hindered carbon with

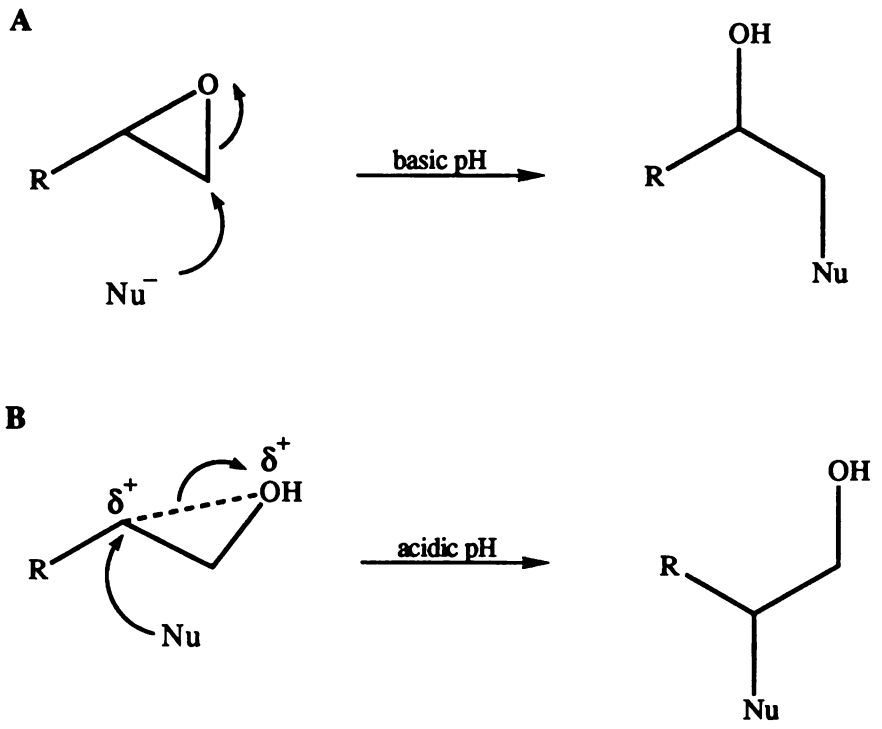


Figure 3.1. The mechanisms of nucleophilic addition to epoxides. (A) Under basic conditions. (B) Under acidic conditions.

inversion of configuration. At low pH, attack on the protonated epoxide also occurs opposite the ring and proceeds with inversion of configuration; in this sense, the mechanism is S_n2 in character (Fig. 3.1, **B**). However, under acidic conditions, the nucleophile often reacts at the carbon that would give the more stable carbocation. This is often the more highly substituted and more hindered position. This would suggest that, at low pH, bond breaking (between the carbon and oxygen) is more pronounced in the transition state and that the carbon at which attack is occurring carries a partial positive charge (Parker & Isaacs, 1959) (Fig. 3.1, **B**).

One of the most widely used epoxide-based affinity labels is conduritol B epoxide (Fig. 3.2). This compound is an active-site directed inhibitor of several enzymes involved in carbohydrate metabolism, including sucrase-isomaltase (Quaroni et al., 1974; Quaroni &

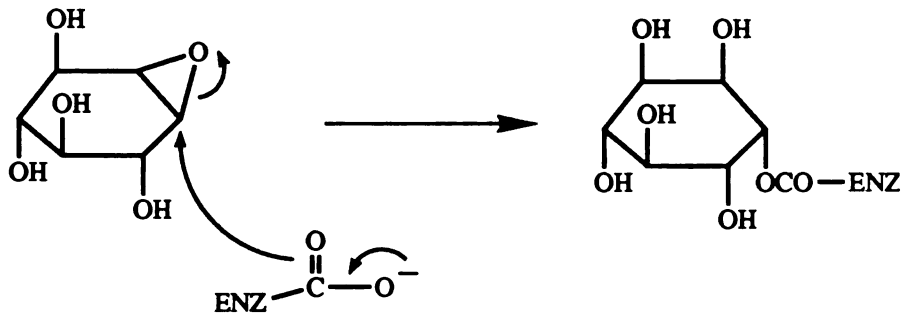


Figure 3.2. The reaction of conduritol B epoxide with an active site carboxylate. The regiochemistry of attack on the epoxide is not known. For convenience, attack at only one carbon of the epoxide is shown.

Semenza, 1976), yeast invertase (Reddy & Maley, 1990), and human acid β-glucosidase (Dinur et al., 1986). In each of these cases, a carboxylic acid group is modified (Fig. 3.2). Affinity labeling with this sugar derivative has been helpful in determining the catalytic mechanisms of these enzymes and in characterizing the differences between the normal acid β-glucosidase and the abnormal enzyme found in patients with Gaucher disease, an inherited lysosomal storage disorder (Grabowski et al., 1985; Grabowski et al., 1986).

Cysteine proteases are irreversibly inhibited by E-64, N-[N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (Fig. 3.3), a natural product isolated from *Aspergillus japonicus* (Hanada et al., 1978). While natural products are not normally classified as affinity labels, this compound exhibits many of the properties of classical affinity labels, including a marked specificity for cysteine *vs.* serine proteases (Shaw, 1990). The epoxide is required for inhibition (Tamai et al., 1981), and the L-isomer is more potent than the D (Barrett et al., 1982). E-64, and related derivatives, alkylate the active site sulfhydryl in cysteine proteases (Fig. 3.3), and alkylation results in the complete loss of activity. NMR (Yabe et al., 1988) and x-ray crystallography studies (Matsumoto et al., 1989; Varughese et al., 1989) of papain inhibited with derivatives of E-64 have

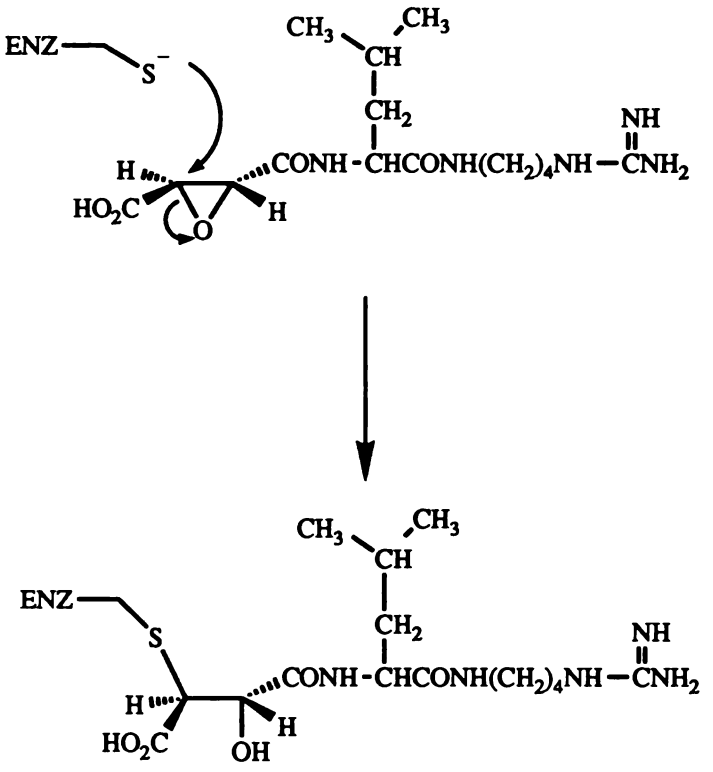


Figure 3.3. Labeling of the active site cysteine of papain by E-64, an epoxide-based affinity label. The regiochemistry of addition of the thiolate is that which has been demonstrated for the reaction of papain and Ep-475, a derivative of E-64 (Yabe et al., 1988).

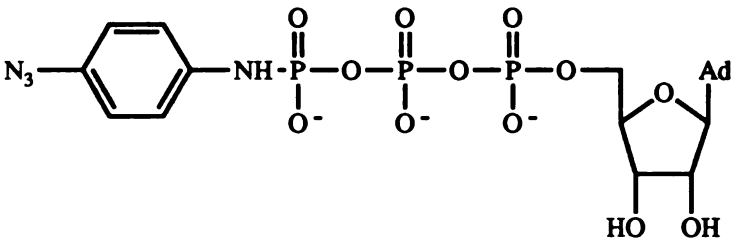
determined that attack occurs in a regiospecific manner, as shown in Fig. 3.3. E-64 has been a valuable tool in the study of the *in vivo* roles of cysteine proteases (Shaw, 1990).

As these selected examples illustrate, epoxide-based affinity labels can be used effectively to label specific residues in the active site of enzymes. There does not appear to be any reason, *a priori*, to assume that the epoxide will react preferentially with any particular type of amino acid residue; rather, as is desirable with an affinity label, the reaction is dependent upon the suitable positioning of the label and active site residue.

C. Affinity Labeling of Creatine Kinase

1. Nucleotide-based affinity labels

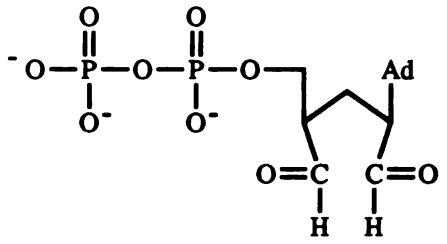
Creatine kinase is irreversibly inhibited upon photolysis of a mixture of the enzyme and ATP γ-*p*-azidoanilide, a nucleotide analogue that has a photolabile azide group attached to the γ-phosphate (Vandest et al., 1980).



ATP γ-*p*-azidoanilide

Partial protection against inactivation is afforded by both ADP and ATP, slight protection by creatine, and complete protection by a mixture of creatine and ADP. Despite the expected non-specific reactivity of the arylnitrene group, inactivation with tritiated label resulted in the irreversible incorporation of only one mole of label per mole of active site. The modified enzyme was found to possess one less free sulfhydryl than unmodified enzyme. Although the modified enzyme was not sequenced, this suggested that a sulfhydryl, possibly Cys-282 (Chapter 1), was the modified group. It was proposed that this sulfhydryl is near the phosphates of the bound nucleotide, although it would appear that the arylnitrene group could easily overlap the creatine binding site.

Rabbit muscle CK can also be inactivated by the dialdehyde derivatives of ATP and ADP (oATP and oADP) (Navinskii et al., 1983).



oADP

Substrates (ATP and ADP) provide protection against inactivation, and one mole of label is incorporated per mole of enzyme active site. Both oADP and oATP are fairly good substrates for CK and have V_{max} values only 4-5 fold less than the natural substrates. The assumption was made that an active site lysine residue was modified, and reduction of the the putative Schiff's base with sodium borotritide resulted in the incorporation of the expected amount of tritium. It was further assumed that the lysine that was labeled is the same one that was shown by NMR experiments to be near the nucleotide phosphate groups (James & Cohn, 1974). No evidence for this assumption was presented.

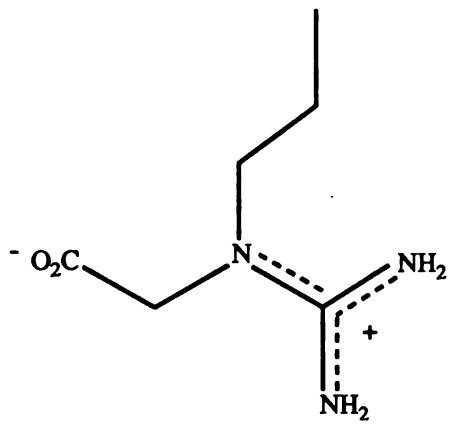
It was reported that the enzyme inactivated by oADP is still able to catalyze the transfer of the phosphoryl group from phosphocreatine to enzyme-bound oADP. This experiment was performed by incubation of [^{14}C]-oADP-inactivated enzyme with phosphocreatine, at pH 8, followed by the removal of any unbound small molecules by gel filtration. Incubation of the resulting enzyme at pH 6 for 5-6 hrs resulted in hydrolysis of the imine linkage and the release of the label from the enzyme. oATP and oADP were then determined by anion exchange chromatography. There appeared to be very little oADP present, but oATP was found. The amounts of each were not quantitated. Unfortunately, the stability of the Schiff's base formed between enzyme and label is not known. Since oADP is a substrate for CK, it is possible that hydrolysis of the imine during the incubation with phosphocreatine gives free oADP that is subsequently enzymatically phosphorylated to oATP. This oATP could then react with the active site lysine. Conceivably, this could occur without the nucleotide ever leaving the active site. Thus, this experiment does not

necessarily address the question of whether the modified residue is essential for catalysis. Perhaps it would have been better if the imine had been reduced prior to this experiment.

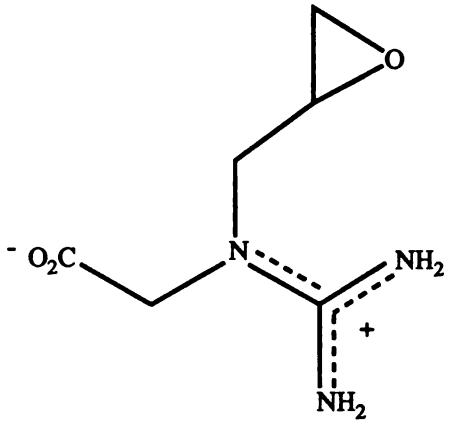
In conclusion, previous affinity labeling studies of CK with nucleotide analogues have been of questionable usefulness. Definitive identification of the labeled residues has not been achieved, and only limited insights have been obtained into the function and structure of the enzyme.

2. Epoxycreatine

Epoxycreatine was designed and synthesized by Marletta and Kenyon (Marletta & Kenyon, 1979) based upon the observation that N-propyl-N-amidinoglycine, although having only 1% the V_{max} of creatine, is a substrate for creatine kinase (Rowley et al., 1971).



N-propyl-N-amidinoglycine



epoxycreatine

Epoxycreatine is an affinity label of CK that fulfills all of the requirements given earlier. The inactivation reportedly follows psuedo-first order kinetics and results in the irreversible incorporation of one mole of label per mole of enzyme active site. The half-life at infinite inhibitor concentration ($T_{1/2}$) is 4.2 min at 0 °C. Saturation kinetics are

followed, although the K_D with the rabbit muscle enzyme is 355 mM, indicating that epoxycreatine binds only very poorly. N-propyl-N-amidinoglycine has a K_M of 53 mM at 1 °C with rabbit muscle CK (McLaughlin et al., 1972), although a much higher value of 395 mM at 30 °C has been reported for the bovine skeletal muscle enzyme (Marletta & Kenyon, 1979). The enzyme·MgADP·nitrate·creatine quaternary complex provides substantial protection against inactivation. Epoxycreatine is also a substrate for CK; inactivation occurs once for every 15 turnovers. There is nothing known about how the putative product of catalysis, phosphoepoxycreatine, interacts with the enzyme. It is also not known whether epoxycreatine covalently bound at the active site can be phosphorylated upon the addition of MgATP to the inactivated enzyme.

Bound epoxycreatine can be removed from the enzyme by relatively mild treatment with alkali (87% of the label is lost by treatment with 0.1 N NaOH for 12 hrs at 25 °C). The rate of loss of the label increases in the presence of hydroxylamine. This was taken as preliminary evidence that a carboxyl group was being modified, presumably the same group suggested from pH/rate studies (Cook et al., 1981). Other than this one hint, it was not determined which residue(s) of the enzyme was modified.

The remainder of this chapter describes some observations made of the interaction of epoxycreatine and creatine kinase. The kinetics of the interaction were determined in a manner similar to that described previously (Marletta & Kenyon, 1979); however, significant differences were initially encountered. These are described, along with possible explanations for them and the steps that were taken to alleviate them. Also included is a description of an attempt to determine whether CK covalently modified by epoxycreatine is catalytically competent.

II. Experimental

A. Reagents and Materials

Rabbit muscle creatine kinase (Type I) and HEPES (free acid) were from Sigma Chemical Co., St. Louis, MO. γ-[^{32}P]-ATP (NH_4^+ salt) was from Amersham, Arlington Heights, IL. All other reagents and materials were obtained as described in Chapter 2.

B. Synthesis of Epoxycreatine

Epoxycreatine was synthesized by the method of Marletta and Kenyon (Marletta & Kenyon, 1979). It was stored desiccated at -20 °C as the crude, slightly yellow colored product from the epoxidation step. As needed, final purification was achieved by chromatography of 25 mg aliquots of the crude product (in some cases treated with rabbit muscle CK, see below) on a C_{18} reverse phase HPLC column (Dynamax 60 Å, 21.4 mm ID x 25 cm L). The column was eluted with water at 5 ml/min. Fractions were collected on ice, lyophilized, and stored desiccated at -20 °C. Only slight hydrolysis (less than 5%) of the epoxide occurred over several months of storage.

C. Reaction of Crude Epoxycreatine with Creatine Kinase

Crude epoxycreatine (300 mg) prepared from the epoxidation of vinylcreatine was dissolved in 3 ml of water. To this was added 20 mg of rabbit muscle creatine kinase (Sigma Chemical Co.) dissolved in 300 µl of water. The resulting solution was allowed to sit at room temperature for 10 min and then centrifuged in a Centricon-30 for 60 min. The filtrate was purified by HPLC in 250 µl aliquots as described above.

D. Effect of Epoxycreatine on the CK Assay Coupling Enzymes

The effect of epoxycreatine on the enzymes (pyruvate kinase and lactic dehydrogenase) used in the coupled creatine kinase assay reaction (see Chapter 2) was determined by following the oxidation of NADH in the coupled system when all of the components were present except creatine kinase and creatine. The following stock solutions, in glycine buffer (see Chapter 2), were prepared:

NADH (disodium salt)	6.6 mM
phospho(enol)pyruvate (potassium salt)	100 mM
ADP (disodium salt)	100 mM
lactic dehydrogenase (940 U/mg)	1.5 mg/ml
pyruvate kinase (500 U/mg)	1.3 mg/ml

In a 500 µl masked cuvette with a 1 cm pathlength were mixed the following amounts of the above stocks:

glycine buffer	450 µl
ADP stock	7.2 µl
NADH stock	16 µl
phospho(enol)pyruvate stock	7.2 µl
lactic dehydrogenase stock	1.5 µl

The resulting concentrations of NADH and phospho(enol)pyruvate are the same as in the normal CK assay (Chapter 2). After mixing, the absorbance at 340 nm was monitored, and the rate of change between 3 and 25 seconds recorded. This value was always less than 0.005 AU/min. Stock pyruvate kinase (2 µl) was added, the absorbance at 340 nm again monitored, and the rate of change between 3 and 25 seconds recorded. This was typically 0.45 AU/min. These two values were subtracted to give the rate of pyruvate kinase-dependent oxidation of NADH.

The K_M of ADP for pyruvate kinase is ~0.2 mM (Ainsworth & MacFarlane, 1973). To determine whether epoxycreatine is an inhibitor of pyruvate kinase with respect to ADP, the concentration of ADP in the assay mix was decreased to 0.15 mM. The assay was repeated with the new concentration of ADP, and the initial velocity recorded. The assay was then repeated once more, except that 2 µl of a 100 mM solution of epoxycreatine in glycine buffer, was added. This is the amount of epoxycreatine that would be present in the assay of 5 µl of an inactivation experiment using 20 mM epoxycreatine (see below).

The effect of epoxycreatine on pyruvate kinase with respect to phospho(enol)pyruvate was determined in an identical manner, except that the concentration of phospho(enol)pyruvate was decreased to 0.08 mM, and the concentration of ADP was left at its original value (1.5 mM). The K_M of phospho(enol)pyruvate with pyruvate kinase is 0.07 mM (Ainsworth & MacFarlane, 1973).

E. Inactivation of Creatine Kinase with Epoxycreatine

Inactivation experiments were performed similar to the procedure of Marletta and Kenyon (Marletta & Kenyon, 1979), except that creatine kinase activity was determined by the pyruvate kinase/lactic dehydrogenase coupled assay (see Chapter 2). To creatine kinase (0.2-2 mg/ml) in 10 mM NaHepes, pH 7.4, was added a sufficient volume of a freshly prepared solution of epoxycreatine (200 mM in 10 mM NaHepes) along with buffer, to give the desired concentration of epoxycreatine, and a final enzyme concentration of 0.1-1.0 mg/ml. Aliquots from the inactivation solutions were withdrawn at the appropriate times, diluted if necessary, and assayed for activity (usually a 1/10 dilution was required). Percent residual activity was calculated from the ratio of the activity of the epoxycreatine-treated enzyme to that of control enzyme that was not treated with epoxycreatine. All inactivations were performed at 0 °C.

F. Attempt to Enzymatically Phosphorylate Enzyme-Bound Epoxycreatine

Creatine kinase (100 μg, 1 mg/ml) was inactivated with 20 mM epoxycreatine as described above. Control enzyme (also 100 μg at 1 mg/ml) was treated in an identical manner, except that it was not inactivated with epoxycreatine. After inactivation at 0 °C for 10 hrs, the enzyme-containing solutions were switched into glycine buffer (see Chapter 2) by ultrafiltration with a Centricon-30 until the concentration of free epoxycreatine in the enzyme-containing solution was expected to be less than 10 nM. The final volumes of the enzyme-containing solutions were then adjusted to 100 μl each with glycine buffer. At this time, the inactivated enzyme had less than 1% of the specific activity of the control enzyme (35 U/mg, assuming full recovery of the enzyme at each step). To both the control and the inactivated enzyme was added 185 μg of Na_2ATP (0.33 μmol, 185 μg/5 μl) in glycine buffer, along with 1 x 10^{-5} μmol of γ-[^{32}P]-ATP (NH_4^+ salt, 5 μl, 50 μCi, 5000 Ci/mmol). This amount of radiolabeled ATP is negligible compared to the amount of cold ATP, so that the final ATP concentration was 3.3 mM. The final specific activity was 0.17 Ci/mmol. Both solutions were allowed to sit at room temperature for 4 hours. At this time, the epoxycreatine-treated enzyme had no detectable activity and control enzyme had 56% of the starting units. Aliquots (50 μl) were withdrawn from each, and non-enzyme bound radioactivity was removed by repeated ultrafiltration through a Centricon-30. Prior experience with ultrafiltration using Centricons had given no indication that creatine kinase binds to any significant degree to the Centricon membrane. After ultrafiltration was repeated six times, the counts in the filtrates were reasonably low (less than 3000 cpm). At this time, the enzyme-containing solution was removed from the Centricon and counted for radioactivity. The counts remaining in the final filtrate were also determined.

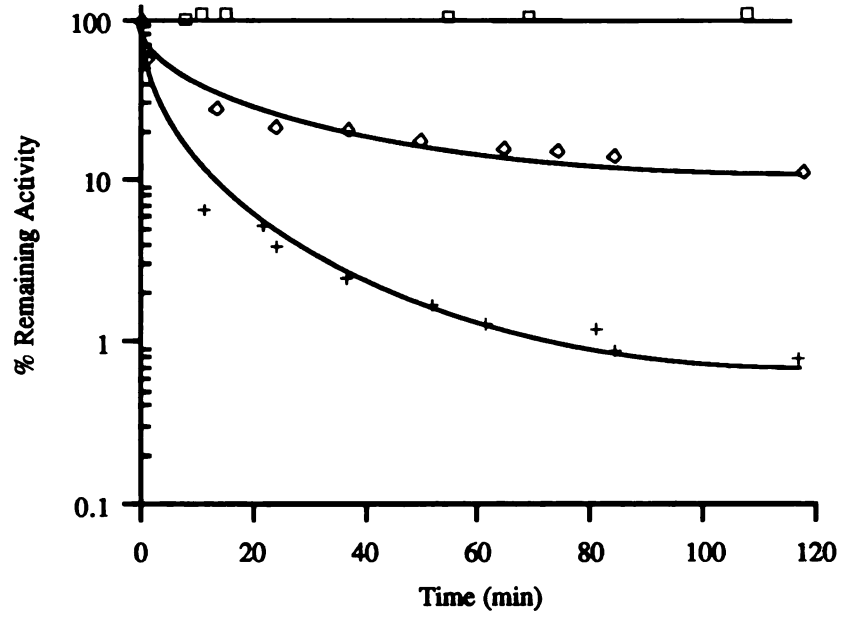


Figure 3.4. Time course of inactivation of creatine kinase by epoxycreatine. (□) Control, no epoxycreatine. (◊) 12 mM epoxycreatine. (+) 20 mM epoxycreatine. All inactivations were done at a creatine kinase concentration of 0.1 mg/ml. Curves are drawn through the points to highlight the non-linearity and are not fits to any particular equation.

III. Results and Discussion

A. The Reaction of Creatine Kinase and Epoxycreatine

The time courses of inactivation at two epoxycreatine concentrations are shown in Fig. 3.4. These experiments were performed at a CK concentration of 0.1 mg/ml and epoxycreatine concentrations of 12 and 20 mM, respectively. It is immediately evident that the inactivation is biphasic and does not follow simple psuedo-first order kinetics. There is a rapid drop in activity that lasts for several minutes, followed by a slower decrease that eventually goes to complete inactivation. Several other epoxycreatine concentrations gave similar results (data not shown). This is in contrast to the results reported previously

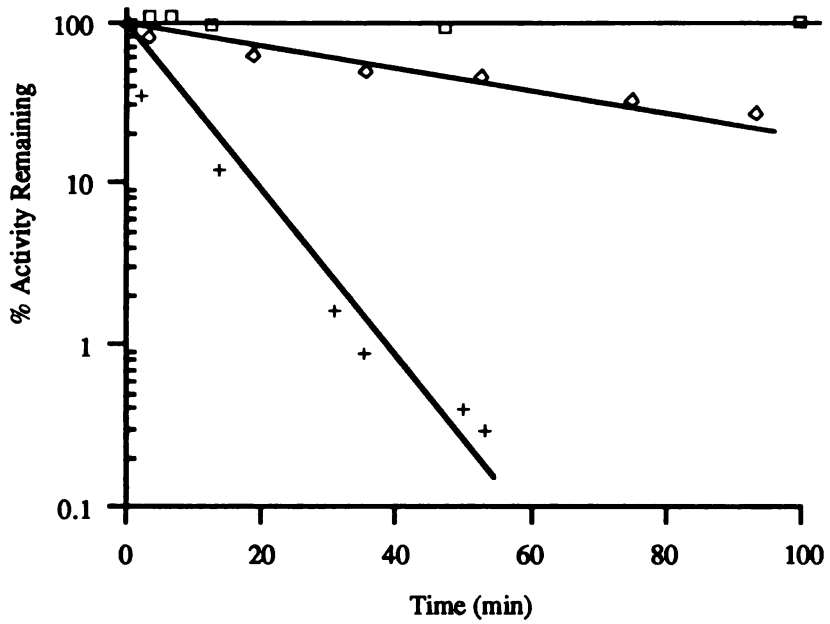


Figure 3.5. Time course of inactivation of creatine kinase by epoxycreatine. (□) control, no epoxycreatine. (◊) 20 mM epoxycreatine. (+) 100 mM epoxycreatine. All inactivations were done at a creatine kinase concentration of 1.0 mg/ml. The deviation from linearity is most noticeable at 100 mM epoxycreatine.

(Marletta & Kenyon, 1979), where psuedo-first order kinetics were seen. Several possibilities for this discrepancy are discussed below.

Previously, the activity of creatine kinase was determined by following the production of the proton in the CK reaction with a pH-stat (Marletta & Kenyon, 1979). In the present work, CK activity was determined with an assay that couples the CK-catalyzed formation of ADP to the oxidation of NADH *via* pyruvate kinase and lactic dehydrogenase (see Chapter 2). There was the possibility that a partial explanation for the biphasic kinetics was an effect of epoxycreatine on one of these coupling enzymes. This possibility was ruled out by determining that epoxycreatine, at the concentrations added to the assay mixture when assaying a typical inactivation experiment, had no effect on the activity of either pyruvate kinase or lactic dehydrogenase. This was the case when either ADP or

phospho(enol)pyruvate were present at concentrations close to their K_M values. Since pyruvate kinase is assayed by coupling to lactic dehydrogenase, any effect on lactic dehydrogenase should also have been detected in these experiments. In all cases, however, identical initial velocities were obtained in both the presence and absence of epoxycreatine.

The reagents used in the epoxidation of vinylcreatine to epoxycreatine are hydrogen peroxide and sodium tungstate (Na_2WO_4). The actual oxidizing species is sodium pertungstate (Na_2WO_5). Sodium pertungstate is a potent inhibitor of creatine kinase (Nyugen, 1990). The yellow color resulting from this species is evident immediately upon addition of sodium tungstate to aqueous hydrogen peroxide, and the crude product resulting from the work-up of the epoxidation reaction (see Experimental) retains a slight yellow color. The HPLC purification of epoxycreatine should remove any sodium pertungstate (or sodium tungstate) since these charged compounds should not bind to a reverse phase C_{18} column. HPLC-purified epoxycreatine does not have a noticeable yellow color. However, as a simple means to ensure that no pertungstate remained in the purified epoxycreatine, the crude mixture of epoxycreatine and vinylcreatine was reacted with rabbit muscle creatine kinase for several minutes prior to HPLC purification. Addition of the enzyme to the clear, yellow solution of the crude reaction product resulted in the immediate disappearance of the yellow color. After separation of the enzyme and unreacted epoxycreatine by ultrafiltration, the epoxycreatine was purified by HPLC, in the usual manner. Only a slight decrease in yield was noted after this procedure. The kinetics of the inactivation of CK with purified affinity label treated in this manner were no different than those seen with untreated affinity label, and identical inactivation curves were obtained (data not shown). This suggested that a species such as pertungstate was not the problem. However, to ensure the complete removal of tungstate and pertungstate, the crude epoxidation mixture was routinely treated in this manner prior to the final HPLC purification step.

An additional possibility for the biphasic kinetics was that there is more than one species present in the enzyme preparation. As noted earlier, the initial inactivation experiments were performed at an enzyme concentration of 0.1 mg/ml. In re-examining the previous experiments of Marletta and Kenyon (Marletta & Kenyon, 1979), it was noted that an enzyme concentration of 1 mg/ml had been used. As shown in Fig. 3.5, repeating the inactivation at a CK concentration of 1 mg/ml gave curves which approximate first-order kinetics much more closely. These curves are very similar to those which were obtained previously (Marletta, 1978). There is, however, still an initial fast phase of inactivation, although it is much less pronounced.

These results demonstrate that the rate of modification of CK by epoxycreatine depends upon the enzyme concentration. The most reasonable explanation for this behavior is that the monomer and dimer (or other multimeric species) react with epoxycreatine at different rates. Perhaps the monomer reacts at a much faster rate than the dimer. It would be expected that the equilibrium between monomer and dimer would shift to the dimer as the concentration is increased. The difference in reactivity could result from the active site being more fully "formed" in the dimer, and the binding of epoxycreatine into the active site in the dimer may be more demanding than the binding to the monomer. In other words, the monomer is possibly a less specific, more "floppy" enzyme, than the dimer. There is some evidence that the two subunits of the dimer are arranged asymmetrically (Degani & Degani, 1979; Price & Hunter, 1976), although it appears that the monomer alone is active (Wang et al., 1990). The remaining portion of the fast inactivation phase apparent in Fig. 3.5 may be the result of the presence of a small, but finite, amount of monomer in the preparation. This phase may disappear if the enzyme concentration were raised even further, although this experiment was not tried.

Other possible explanations (Fee et al., 1974; Knorre & Chimitova, 1981; Meloche, 1967; Ray & Koshland, 1961; Tsou, 1962) for the non-first order kinetics in these

experiments were also considered. Those that are the most applicable are briefly discussed below.

Epoxycreatine modifies CK with a 1:1 stoichiometry (Marletta & Kenyon, 1979). One mole of label is incorporated per mole of active site. This suggests, but does not prove, that only one group on the enzyme is being labeled. The best approach to determine if more than one group is labeled is to identify the site of labeling. As described in Chapter 4, it has been established in the course of this work that only one group on the enzyme is labeled. A second possibility consistent with the specificity of labeling is that only one group in each monomer is modified, but that reaction at one monomer alters the rate of modification of the reactive group of the other. Biphasic kinetics could be seen if reaction of epoxycreatine at the active site of one subunit decreased the rate of reaction at the other subunit. This is consistent with the previously noted possibility of asymmetrically associated subunits.

The epoxycreatine used in these experiments is a mixture of enantiomers. Given the high enantioselectivity usually exhibited by enzymes, it would not be surprising if the enantiomers reacted at different rates with the enzyme. One enantiomer could bind to (but not react with) the enzyme and prevent inactivation by the other. An argument against this explanation is provided by the high epoxycreatine/CK ratios (25 mM epoxycreatine with 0.1 mg/ml CK is a 3000:1 molar ratio) used in these studies. At these ratios, the relative proportions of the two enantiomers should not change significantly throughout the course of the inactivation, even if only one enantiomer is reactive.

An additional possibility is that the gem diol resulting from the non-enzymatic hydrolysis of epoxycreatine may inhibit the inactivation. This possibility cannot be ruled out, although epoxycreatine is reported to be stable under the conditions of these experiments (Marletta & Kenyon, 1979). Epoxycreatine is also a substrate for creatine kinase. The ability of phosphoepoxycreatine to inhibit the enzyme has not been investigated. As epoxycreatine is turned over, the concentration of phosphoepoxycreatine

will increase. If it does not inhibit the enzyme, or does so at a rate significantly different than epoxycreatine, biphasic kinetics could also result.

By using a CK concentration of 1 mg/ml in the inactivation experiments, it was possible to repeat the results obtained by Marletta and Kenyon (Marletta & Kenyon, 1979). The stoichiometry of the inactivation was not independently verified; however, given the similarity to previous results when an enzyme concentration of 1 mg/ml was used, it is reasonable to assume that the inactivation was proceeding as previously described. On the basis of these results, it was decided to use these inactivation conditions (1 mg/ml enzyme) in subsequent experiments to determine the site of labeling (see Chapter 4).

B. Is Epoxycreatine-Modified Creatine Kinase Catalytically Competent?

Epoxycreatine-modified creatine kinase is not able to catalyze the phosphorylation of creatine. This is not surprising, and as discussed earlier, is evidence that epoxycreatine is bound in the enzyme's active site. Creatine kinase is able to catalyze the transfer of a phosphoryl group from MgATP to epoxycreatine. This raises the question of whether the epoxycreatine-modified enzyme is capable of catalyzing the transfer of the phosphoryl group from MgATP to enzyme-bound epoxycreatine. The answer to this question has important implications for the mechanism of the enzyme and, if positive, directly addresses whether the residue to which epoxycreatine is bound is involved in the chemical steps of catalysis. To approach this question, an experiment was designed to determine if addition of γ-[^{32}P]-ATP to the modified enzyme resulted in the incorporation of covalently-bound radioactivity on the enzyme. Presumably, any bound ^{32}P (over and above that seen with unmodified, control enzyme) would be the result of the transfer of the γ-phosphate to the bound affinity label.

Table 3.1: Free and Enzyme-bound Radioactivity (dpm) from the Incubation of Epoxycreatine-modified Creatine Kinase with γ-[^{32}P]-ATP. Free dpm is the number of counts in the final ultrafiltration filtrate.

Epoxycreatine-modified Creatine Kinase		Control, unmodified Creatine Kinase	
Enzyme-bound dpm	Free dpm	Enzyme-bound dpm	Free dpm
2754	3054	4568	1697

As shown in Table 3.1, after incubation of modified enzyme with γ-[^{32}P]-ATP for 4 hrs in glycine buffer at room temperature, there was no indication that the modified enzyme contained any more bound counts than unmodified, control enzyme. This experiment was so designed that, if 100% of the bound epoxycreatine were phosphorylated, 865,000 dpm of ^{32}P should have remained bound to the enzyme. The concentration of ATP, at 3.3 mM, was several times greater than the K_M of MgATP (0.48 mM, see Table 1.1), although the K_M of MgATP with the epoxycreatine-modified enzyme is not known. Since the standard coupled assay for creatine kinase (see Chapter 2) is performed in glycine buffer, it would be expected that this would be a suitable buffer for this experiment. Epoxycreatine remained bound to the enzyme during the course of this experiment since the modified enzyme did not possess any activity at the end of the 4 hr incubation period. Control enzyme retained over 50% of the starting activity. This experiment provided no evidence that the modified enzyme is able to catalyze the transfer of a phosphoryl group from ATP to the bound label.

There are several possible reasons why the modified enzyme is not able to catalyze the phosphorylation of the bound label. Chief among these is that the modified residue is absolutely essential for catalysis. These results would suggest that it is. Alternatively, it could be that epoxycreatine is not bound in a productive manner, and phosphoryl transfer does not occur, not because of the essentiality of the modified residue, but because the

substrates are not positioned properly for transfer. In addition, the position of equilibrium for the phosphorylation of bound label is not known, but conceivably could lie far in the direction of free ATP. The lability of the phosphorylated label to hydrolysis is also not known.

Phospho(enol)pyruvate carboxykinase that has been modified with the dialdehyde derivative of GDP (oGDP) is not able to catalyze the transfer of the phosphate group from phospho(enol)pyruvate to the enzyme-bound oGDP (Guidinger, 1990). As discussed earlier, there is a report that creatine kinase modified by the dialdehyde derivative of ADP is capable of transfering the phosphoryl group from phosphocreatine to the enzyme-bound label. As far as is known, this is the only example where this type of experiment has been successful with a kinase; however, the problems with this work have been discussed earlier.

It is not possible to distinguish among the explanations given above for the negative result obtained in this experiment. As discussed more fully in Chapter 4, it is possible that the modified residue is essential for catalysis. On the other hand, there is precedent for not assuming that this is the case without further information. For example, it is possible to label a reactive cysteine residue in bacterial luciferase with a variety of reagents, all of which lead to the complete inactivation of the enzyme. However, when this residue is changed to alanine by site-specific mutagenesis, the mutant enzyme retains activity (Baldwin et al., 1989). This example highlights the possible pitfalls in attempting to assign roles in catalysis for specific residues based solely upon chemical modification studies.

4

Site of Modification of Creatine Kinase by Epoxycreatine

I. Introduction

A. Purpose of Study

The residue(s) of creatine kinase that is modified by epoxycreatine has not been previously identified. As mentioned in Chapter 3, it is imperative to do this if a complete interpretation of the results of the affinity labeling studies is to be made. In addition, since a crystal structure of creatine kinase is not yet available, the identification of active site residues, particularly the location of them with respect to bound substrates, is an important goal in mechanistic studies of CK and will also be important in determining the location and delineating the boundaries of the active site when an x-ray structure becomes available. In this chapter is reported the identification of the active site residue of rabbit muscle creatine kinase that is labeled by epoxycreatine.

B. Experimental Approach

1. Introduction

The approach taken in determining the labeled residue can be summarized as follows:

1) Proteolytic digestion of [^{14}C]-epoxycreatine-labeled CK
2) Separation by HPLC of the resulting peptides
3) Mass spectrometric identification of the [^{14}C]-labeled peptides
4) Confirmation of the site of labeling by mass spectrometry

Each of these steps is discussed below.

2. Proteolytic digestion of creatine kinase

The amino acid sequence (predicted from the cDNA sequence) of rabbit muscle creatine kinase is known (Putney et al., 1984). As a result, it is possible to predict the sequences and molecular weights of the peptides that will result from the digestion of CK with a protease of known specificity. After digestion, these peptides can be separated either by HPLC or by other types of chromatography. The sequence, and position in the enzyme sequence, of each of the separated peptides can be deduced from knowledge of the molecular weights of each of them. This approach assumes that the sequence of each peptide is unique in the protein and that every peptide has a unique molecular weight. With smaller peptides this may not be the case, and there may be more than one peptide with a given sequence or with the same molecular weight. Peptides with the same amino acid composition, or that contain amino acids that are of the same residue weight, will also have the same molecular weight. Additional information, such as direct sequence determination, is required to distinguish among these peptides. Statistically, a tetrapeptide of a given sequence will occur only once in every 160,000 residues in a random protein sequence. Thus, in most cases, if the amino acid sequence of the protein is known, it should be possible to "map" the majority of the protein sequence onto the chromatogram of the separated peptides.

A requirement of the proteolytic digestion is that it generate peptides that can be easily separated by the chosen chromatographic method and that will be amenable to the

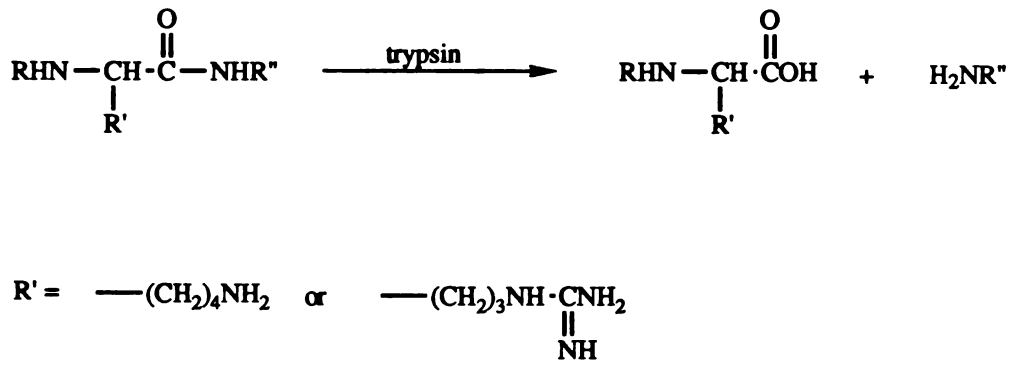


Figure 4.1. Cleavage of a polypeptide by the serine protease trypsin. R and R" represent the remaining portions of the polypeptide.

method chosen for molecular weight or sequence determination. The serine protease trypsin was selected for the present work because it most nearly fulfills these prerequisites. Trypsin cleaves peptide bonds in polypeptides on the C-terminal side of lysine and arginine residues (Walsh, 1970) (Fig. 4.1). It exhibits good specificity and activity and usually gives reproducible cleavage patterns. As discussed more fully in Results, digestion of CK with trypsin is expected to give a range of peptides that are suitable for HPLC separation and mass spectrometric analysis. Prior to digestion, it is common practice to reduce any disulfides in the protein and to alkylate the free sulfhydryls with iodoacetic acid, or a similar reagent. This insures that disulfides do not form between the peptides during the course of the digestion and usually gives more reproducible results.

Proteolytic digestion of unlabeled enzyme can be used to determine the optimum time of digestion, and the HPLC chromatogram of the unlabeled peptides can help to identify potential sites of modification (see below). Digestion of the affinity labeled enzyme is performed by inactivation with epoxycreatine, removal of excess label by dialysis or ultrafiltration, reduction, carboxymethylation, and digestion in the same manner as unmodified enzyme.

3. HPLC separation of proteolytic peptides

After a suitable protease is chosen and digestion is performed, it is necessary to develop an appropriate method to separate the resulting peptides. There are several chromatographic methods available (Allen, 1981); however, reverse phase HPLC is the current method of choice. Small and medium sized peptides generally are separated easily on C_{18} columns. Larger molecular weight peptides (> 5000) can often be separated by chromatography on either a C_8 or C_4 column. Resins made specifically for the separation of peptides (such as that in the Vydac C_{18} Protein/Peptide column used here) are available. These have larger pore sizes than the comparable C_{18} resins that are used for the separation of smaller molecules. Such resins give excellent resolution of peptides and proteins. Typical solvent systems consist of water-trifluoroacetic acid (TFA) mixtures, with gradients of an increasing concentration of an organic modifier. TFA and similar acids will usually increase the solubility of the peptides and enhance resolution. Several types of organic solvents are used, including methanol and isopropanol, although acetonitrile often gives the best results. Many examples of the proteolytic digestion of proteins and the HPLC separation of the resulting peptides are available (DiBussolo, 1984; Freiser & Gooding, 1987; Harlow & Switzer, 1990; Mahoney & Hermodson, 1980; Powell & Thorpe, 1988; Wilson et al., 1982).

4. Liquid secondary ion mass spectrometry of the separated peptides

At this point, it was expected to have an HPLC chromatogram of the digest of both [^{14}C]-epoxycreatine-modified and unmodified CK. These chromatograms would consist of a number of peaks, possibly the number expected, although it was more likely that there would be some overlap in the separation and a certain amount of either over- or underdigestion of the protein. The next step was to determine the sequences of the

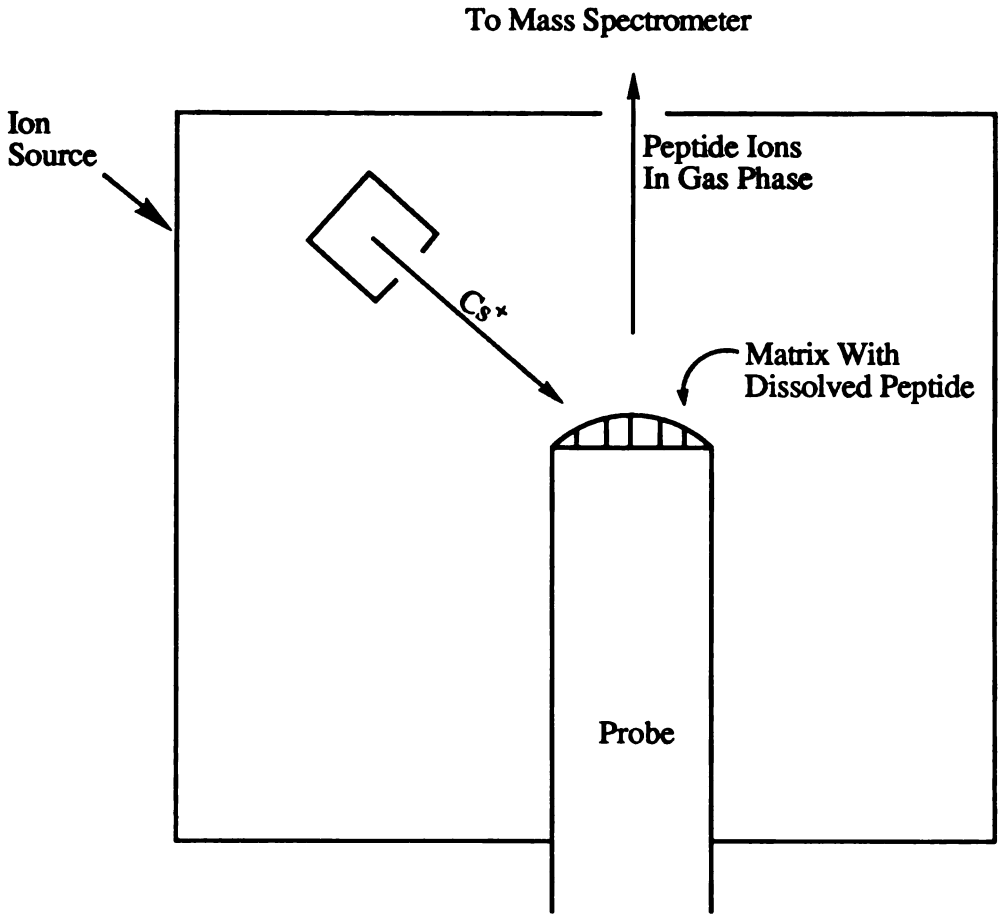


Figure 4.2. Diagram of the source block of a liquid secondary ion mass spectrometer.

[^{14}C]-labeled peptides in the digest of the [^{14}C]-epoxycreatine-labeled enzyme. Mass spectrometry was chosen as the means of achieving this goal.

Liquid secondary ion mass spectrometry (LSIMS) (Fig. 4.2) is a technique that makes possible the determination of the molecular weight of molecules that would not normally be amenable to electron impact methods (Aberth et al., 1982). This is usually the case with highly polar or very large molecules that are not volatile under normal conditions. LSIMS can be used to produce gas-phase ions of many of these types of molecules, including peptides.

LSIMS is performed by first dissolving the peptide in a nonvolatile matrix; glycerol/thioglycerol mixtures are often used. This mixture is placed on the mass spectrometer probe tip, and the probe is inserted into the instrument. The matrix is then bombarded with cesium ions. These ions penetrate the surface layer (~50 Å) of the liquid matrix and impart their kinetic energy to both matrix and peptide molecules at the surface. This is sufficient to "sputter" off any peptides present into the gas phase. For this process to occur, the peptide must possess a certain degree of surface activity. Most of the resulting, gas-phase peptide molecules will be neutral, but some will bear either positive or negative charges. These charged peptides are accelerated into the mass spectrometer where they are separated by their mass-to-charge ratio, as in conventional electron impact mass spectrometry. Because both negatively and positively charged species are obtained, detection in LSIMS can be in either the positive or negative mode. Positively charged species resulting from the protonation of the peptide will be detected at the molecular weight plus one; negatively charged species are detected at the molecular weight less one.

LSIMS is a "soft-ionization" technique. Gas phase ions produced by LSIMS do not generally possess sufficient energy for significant fragmentation, and thus primarily only molecular ions are seen. Extensive structural information, inherent in the fragmentation patterns, is therefore not available in a typical LSIMS experiment. However, as discussed earlier, for peptides from the proteolytic digestion of an enzyme of known sequence, the molecular weight is usually sufficient to identify the sequence of a given peptide.

In routine use of the LSIMS instrument at UCSF, it is possible to detect peptides in picomole amounts. The mass range, again in routine use, is typically from 300 to 3500 mass units. Hexylation of the carboxyl groups of small, hydrophilic peptides can be employed to enhance their surface activity, often providing a substantial increase in the abundance of their molecular ions (Falick & Maltby, 1989). Peptides larger than 3000-3500 mass units may require subdigestion with a second protease to produce peptides more

amenable to LSIMS. Several proteases, possessing a variety of cleavage specificities, are available for this purpose (Allen, 1981).

There are two mass spectrometric-based strategies available for the identification of covalently-modified peptides in a proteolytic digest of an enzyme. First, each fraction from the HPLC separation of a digest of the unlabeled enzyme can be subjected to LSIMS. In most cases, this should make it possible to identify peptides in the chromatogram that cover the entire sequence of the enzyme. "Mapping" the sequence of the enzyme in this manner may also be useful for other purposes. Following this same procedure with the peptides resulting from the digestion of the modified enzyme should again identify peptides representing the entire sequence, except that one or more of them will be shifted in mass by the incremental molecular weight of the modifying group. If necessary, the labeled peptides can be further analyzed to confirm the sequence and the site of modification. This method suffers from the disadvantage of not necessarily being able to identify peptides resulting from low levels of labeling. In addition, it is not always possible to identify peptides representing the entire sequence of the enzyme.

In the second strategy, modified peptides can be identified by inactivating the enzyme with radiolabeled affinity label. Fractions from the HPLC separation that contain radioactivity will presumably contain labeled peptides. LSIMS can then be used to identify these peptides and the site of labeling confirmed by sequencing. This method requires the synthesis of radioactive affinity label but can then be used to detect and isolate minor sites of labeling. Following this procedure does not preclude the subsequent identification of all of the peptides in the digest. For these reasons, it is the superior method and has been used in the present work.

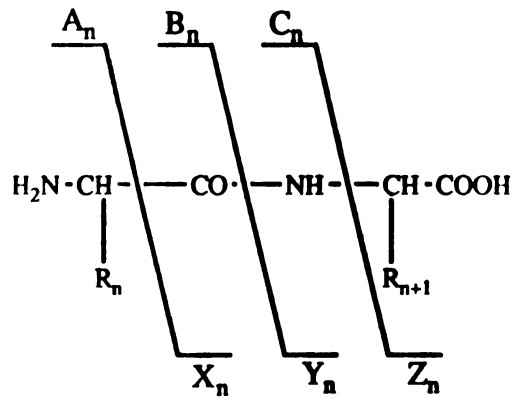
5. Tandem mass spectrometry of the labeled peptides

Once the labeled peptides have been identified, it is necessary to confirm the sequence of them and conclusively pinpoint the labeled residue. Traditionally, peptide sequencing has been done by Edman degradation (Allen, 1981). The labeled residue can usually be identified by determining at which cycle radioactivity is encountered. Alternatively, it may not be possible to sequence through the labeled residue. If the sequence of the peptide is already known, this is usually sufficient to identify the labeled residue. If the sequence is not known, it may be necessary to perform an amino acid analysis of the peptide or additional analyses to suggest possibilities for the labeled residue.

Edman degradation is not always able to determine unambiguously the site of modification. This may be especially true if the peptide of interest is a minor component in a mixture of peptides. As an alternative to Edman degradation, tandem mass spectrometry is a rapid and sensitive method for the sequencing of peptides. Tandem mass spectrometry, as the name implies, is performed by connecting together two mass spectrometers. The first is a typical LSIMS instrument in which peptide ions can be separated on the basis of their mass-to-charge ratio. At the end of this instrument, instead of a detector, is a slit where only the ion of interest is focused. This slit is followed by a gas collision cell where the ion is vibronically activated upon collision with helium atoms. The ion undergoes unimolecular dissociation reactions, the product ions of which enter the second mass spectometer. This fragmentation is termed collision-induced dissociation (CID). In effect, this process makes it possible to obtain the complete fragmentation pattern of the desired ion free of any contaminating components (ions) originating from the sample.

In the second mass spectrometer, the mass spectrum of only the selected ion is recorded. Many of the fragment ions obtained from peptides in this process retain the side chains of the individual amino acids. Thus, these fragments contain sequence information,

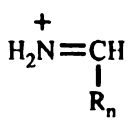
A



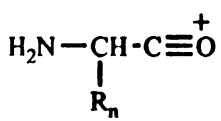
B

N-Terminal Fragments **C-Terminal Fragments**

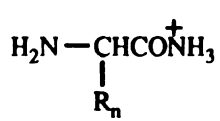
A series



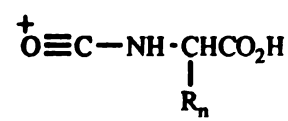
B series



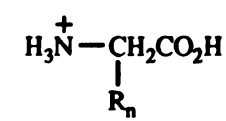
C series



X series



Y series



Z series

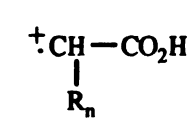


Figure 4.3. Fragmentation patterns commonly observed in collision-induced dissociation spectra of peptides. (A) Sites of bond cleavage of the peptide backbone. A, B, and C ions result from charge retention on the N-terminus. X, Y, and Z ions result from charge retention on the C-terminus. Nomenclature according to Roepstorff and Fohlman (Roepstorff & Fohlman, 1984), as modified by Biemann (Biemann, 1988) (B) The structure of the ions in each series.

and tandem mass spectrometry is a means of gaining back the structural information that is not available in LSIMS.

Peptide fragmentation patterns in tandem mass spectrometry follow what are now well established rules (Biemann & Scoble, 1987; Johnson & Biemann, 1987; Johnson et al., 1988) (Fig. 4.3). Major fragmentation occurs along the polypeptide backbone, giving rise to successively larger ions that are increased in weight by an incremental amount that is characteristic of each amino acid in the sequence. Ions resulting from cleavage at successive positions along the polypeptide chain are collectively referred to as an "ion series". By identifying the ions in a given series, it is possible to "walk" along the peptide and determine the amino acid at each position. There are six major ion series that are observed: three with charge retention on the C-terminus and three with retention on the N-terminus (Fig. 4.3). These make it possible to sequence the peptide from both the N-and C-termini, and each ion series provides confirmatory evidence for the sequence. In addition, certain ion series result from side chain fragmentation processes (**v**, **w**, and **d** series). These do not provide sequence information for the amino acid at which fragmentation occurs but do contain the side chains for the sequence up to the point of fragmentation. Such processes also permit identification of the isomeric amino acids, leucine and isoleucine. It typically takes 3-5 min to record the CID spectrum of a medium sized peptide (~2000 molecular weight), although identification of the ion series and interpretation of the spectrum can be more demanding. Nevertheless, with experience, spectra can be interpreted in a reasonably short period of time. Tandem mass spectrometry is subject to the same mass range and sensitivity limitations as LSIMS.

Tandem mass spectrometry can be used to identify covalently-modified residues in peptides because of the shift in molecular weight seen in those ions that contain the modifying group (Biemann & Scoble, 1987; Janes et al., 1990; Kaur et al., 1989). Ions in a given series will be consistent with the sequence of the peptide, up to the modified residue. Ions containing the modified residue will be increased incrementally in mass by

the weight of the modifying group. From this information, it is possible to obtain the sequence of the peptide and to identify conclusively the site of labeling.

C. Summary

The combination of steps outlined above constitute a simple and effective method to identify the site of modification of an affinity-labeled enzyme or protein, or to determine the location of post-translational modifications. LSIMS and tandem mass spectrometry are both rapid and sensitive techniques, and they proved to be effective means of identifying the site of modification of creatine kinase by epoxycreatine. In particular, as described below, tandem mass spectrometry was invaluable in confirming the sequence of labeled peptides and in unambiguously determining the site of labeling.

II. Experimental

A. Reagents and Materials

[^{14}C]-Cyanamide (57 mCi/mmol) was from American Radiolabeled Chemicals, St. Louis, MO. Hydrogen peroxide (50%) was from Fisher, Fair Lawn, NJ. Cyanamide was from Aldrich Chemical Co., Milwaukee, WI. Sodium tungstate dihydrate was from Alfa Products, Beverly, MA. Sequencing grade endoproteinase Glu-C was from Boehringer-Mannheim, Indianapolis, IN. TPCK-treated bovine pancreatic trypsin, TLCK, and iodoacetic acid were from Sigma Chemical Co., St. Louis, MO. All other reagents and materials were obtained as described in Chapters 2 and 3. Iodoacetic acid was recrystallized from benzene prior to use.

B. [^{14}C]-N-Amidino-N-(2-propenyl)glycine

[^{14}C]-N-amidino-N-(2-propenyl)glycine was synthesized as described (Marletta & Kenyon, 1979), except that the label was introduced as [^{14}C]-cyanamide. This results in the carbon of the guanidinium group being labeled. Freshly diethyl ether-extracted cyanamide (32 mg, 0.75 mmol) was added to a small glass conical vial. [^{14}C]-Cyanamide (1.48 mg, 0.035 mmol, 57 mCi/mmol) was added to this with the aid of 60 µl of water. To this solution was added 6.5 µl of 58% ammonium hydroxide, followed by 77 mg (0.67 mmol) of N-(2-propenyl)glycine (Marletta & Kenyon, 1979) as a solid. Following gentle vortexing, the vial was allowed to sit at room temperature. A solid began to crystallize after 8 hours. After 5 days, the solid was ground to a fine powder with the closed end of a melting point capillary. Acetone (1 ml) was added, the suspension was stirred, and the solid was allowed to settle for 5 min. The supernatant was decanted, and the solid was washed twice with additional 1 ml volumes of acetone. The crude product was dried *in vacuo* on a Speed Vac Concentrator (Savant). The dried material was suspended in 1 ml of hot ethanol and hot water added dropwise until all of the solid went into solution. This solution was placed at 5 °C overnight, the liquid decanted, and the crystals washed one time with ice-cold ethanol. The recrystallized product was dried *in vacuo* to give 70 mg (67% yield) of [^{14}C]-N-amidino-N-(2-propenyl)glycine.

C. [^{14}C]-Epoxycreatine

[^{14}C]-Epoxycreatine was synthesized by the method of Marletta and Kenyon (Marletta & Kenyon, 1979). [^{14}C]-N-amidino-N-(2-propenyl)glycine (70 mg, 0.44 mmol, 0.24 mCi/mmol) was dissolved in 0.4 ml of 50% hydrogen peroxide. While stirring this solution with a small magnetic flea, 6 mg of sodium tungstate dihydrate was added as a solid. The resultant clear, light green solution was stirred at room temperature. After 90 min, the solution was transferred to a glass scintillation vial and the crude product

precipitated by the addition of 5 ml of ice-cold acetone. This suspension was allowed to sit on ice for 10 min, the acetone decanted, and the solid washed twice with additional 5 ml volumes of ice-cold acetone. After drying *in vacuo*, the crude product was purified by HPLC as described in Chapter 3. The final yield of pure [^{14}C]-epoxycreatine was 4.5 mg (7%).

D. Specific Activity of [^{14}C]-N-Amidino-N-(2-propenyl)glycine

The concentration of [^{14}C]-N-amidino-N-(2-propenyl)glycine in a solution of unknown concentration was determined by HPLC. To prepare a standard curve, aliquots (1, 2, 4, 6, and 10 μg) of a solution of N-amidino-N-(2-propenyl)glycine (0.1 mg/ml) were chromatographed on a C_{18} HPLC column (Vydac, 25 cm x 4.6 mm) with water as solvent at 1 ml/min and detection at 220 nm. A single peak with a retention time of 5 min was observed in each injection. To generate the standard curve, the area under this peak for each injection was plotted *vs.* the amount injected. This curve was linear (r = 0.999) over the range of 1-10 μg.

A small portion of [^{14}C]-N-amidino-N-(2-propenyl)glycine was dissolved in 2.5 ml of water. Two separate aliquots (30 and 60 μl) of this solution were chromatographed on the above HPLC column. A single peak at 5 min was obtained in each case. The area under this peak was determined for each injection and the amount of material injected determined from the standard curve described above. For the 30 μl injection, a concentration of 0.054 mg/ml was obtained; the 60 μl injection gave a concentration of 0.055 mg/ml. These give an average concentration of 3.5×10^{-7} mmol/μl. An additional portion (50 μl) of the above solution was counted for radioactivity on a Beckman LS5000 TA scintillation counter. Quenched standards supplied by Beckman were used to construct a quench curve. Counting the sample, and using this quench curve, gave 9544 dpm in the 50 μl sample. There are 2.22×10^{9} dpm/mCi, so this sample contained 4.3×10^{-6} mCi, or

8.6×10^{-8} mCi/µl. If the concentration was 3.5×10^{-7} mmol/µl, this gives a specific activity of 0.24 mCi/mmol.

E. Tryptic Digestion of Rabbit Muscle Creatine Kinase

Creatine kinase (both epoxycreatine-modified and unmodified) was dissolved at 10 mg/ml in alkylation buffer (6 M guanidine·HCl/100 mM Tris·Cl/1 mM EDTA, pH 8.3) containing 2 mM freshly added DTT. After this solution sat at room temperature for 60 min, sufficient 50 mM iodoacetic acid in water was added to give a 1.2x molar excess over total thiols. This solution was allowed to sit in the dark at room temperature for 1.5 hrs. The alkylation reaction was quenched by the addition of β-mercaptoethanol to 1% (v/v) and the quenched solution was switched into digestion buffer (100 mM NH_4HCO_3/0.1 mM $CaCl_2$, pH 8.0) by repeated ultrafiltration through an Amicon YM-30 membrane or a Centricon-30. The volume of the filtered solution was adjusted with digestion buffer to give an enzyme concentration of 5 mg/ml and TPCK-treated trypsin (5 mg/ml in 0.1 mM HCl) was added to give a CK/trypsin ratio of 50/1. Digestion was allowed to progress at room temperature (usually for 5 hours). The reaction was quenched by the addition of TLCK (18 µg/ml in water) to give a 1.1x molar excess over trypsin. Digests were lyophilized and stored at -70 °C.

F. Separation of Tryptic Digests

Samples from the tryptic digestion of CK were dissolved in water containing 0.1% TFA and chromatographed on a Vydac Protein/Peptide C_{18} column (25 cm x 4.6 mm). A linear gradient from 100% solvent A to 70% solvent B at a rate of 0.7%/min was used, where solvent A was 0.1% TFA in water and solvent B was 0.08% TFA in acetonitrile. The flow rate was 1 ml/min and detection was at 215 nm. Collected fractions were taken to

dryness on a Speed Vac Concentrator (Savant) and stored at -70 °C. Fractions were counted for radioactivity on a Beckman LS5000 TA scintillation counter.

G. Endoproteinase Glu-C Digestion

The peptide (~10 µg) to be digested was dissolved in 10 µl of 75 mM sodium phosphate buffer, pH 7.8. Endoproteinase Glu-C (2.4 µl of a 0.5 mg/ml solution in the same buffer) was added, the solution gently vortexed and allowed to sit at room temperature. After 5 hours, the digest solution was taken to dryness *in vacuo* and stored at -70 °C. Peptides were purified by HPLC as described above for the tryptic digest, except that the column was eluted with 0.1% TFA in water for 5 min prior to starting the gradient. Collected peptides were taken to dryness and stored at -70 °C.

H. Hexylation of Peptides

The hexylation reagent was prepared by the addition of 0.4 ml of acetyl chloride to 3 ml of ice-cold n-hexanol that had been dried over K_2CO_3 and freshly distilled (Falick & Maltby, 1989). This was stored at -20 °C. Hexylation was performed by dissolving the peptide at ~1 µg/µl in the hexylation reagent. After sitting at 45 °C for one hour, this solution was taken to dryness *in vacuo*. Hexylated peptides were stored at -70 °C.

I. Liquid Secondary Ion Mass Spectrometry

Liquid secondary ion mass spectrometry (LSIMS) analyses were carried out on a Kratos MS50S double focusing mass spectrometer equipped with a high field magnet and a cesium ion source (Falick et al., 1986). Samples from the HPLC separation of the tryptic peptides were dissolved in 5 µl of 0.1% TFA in water. A portion of this solution (1-5 µl) was added to 1 µl of glycerol:thioglycerol (1:1)/1% TFA. This mixture was placed on the

probe tip, the probe inserted into the instrument, and the mass spectrum recorded. The observed masses of the peptides were corrected to the true masses by adding or subtracting a correction factor (usually between 0.4-4 mass units) determined by calibrating the instrument with a reference sample of a mixture of compounds of known molecular weight (Ultramark 1621, PCR Research Chemicals, Gainesville, FL).

J. Tandem Mass Spectrometry

Collision-induced dissociation (CID) analyses were performed on a Kratos Concept IIHH four sector mass spectrometer equipped with a cesium ion source and an electro-optical multichannel array detector able to record 4% of the mass range simultaneously (Walls et al., 1990). The pressure of the helium collision gas was so adjusted that the intensity of the selected precursor ion was decreased to 30% of its initial value (Walls et al., 1990). The collision cell was floated at 4 keV.

III. Results

A. Synthesis of [^{14}C]-Epoxycreatine

The specific activity of the [^{14}C]-epoxycreatine used in this work was 0.24 mCi/mmol. This was determined by measuring the specific activity of [^{14}C]-N-amidino-N-(2-propenyl)glycine, the precursor to [^{14}C]-epoxycreatine. If the [^{14}C]-epoxycreatine synthesized from this compound is pure, it should have an identical specific activity. The HPLC purification does result in epoxycreatine that is pure by HPLC and by ^{1}H-NMR. The specific activity of the [^{14}C]-cyanamide obtained from American Radiolabeled Chemicals was stated to be 57 mCi/mmol. In the synthesis of [^{14}C]-N-amidino-N-(2-propenyl)glycine, this material was diluted 22-fold. This should have given a product with

POSITION	SEQUENCE	PROTONATED MOLECULAR WEIGHT
(1 - 8)	PFGNTHNK	914.45
(9 - 10)	YK	310.18
(11 - 14)	LNYK	537.30
(15 - 24)	SEEEYPDLSK	1196.53
(25 - 31)	HNNHMAK	851.39
(32 - 39)	VLTPDLYK	948.54
(40 - 40)	K	147.11
(41 - 42)	LR	288.20
(43 - 44)	DK	262.14
(45 - 85)	ETPSGFTLDDVIQTGVDNPGHPFIMTVGCVAGDEESYTVFK	4373.03
(86 - 95)	DLFDPIIQDR	1231.63
(96 - 100)	HGGFK	545.28
(101 - 104)	PTDK	460.24
(105 - 106)	HK	284.17
(107 - 115)	TDLNHENLK	1083.54
(116 - 129)	GGDDLDPHYVLSSR	1530.72
(130 - 131)	VR	274.19
(132 - 134)	TGR	333.19
(135 - 137)	SIK	347.23
(138 - 147)	GYTLPPHCSR	1130.54
(148 - 150)	GER	361.18
(151 - 151)	R	175.12
(152 - 155)	AVEK	446.26
(156 - 169)	LSVEALNSLTGEFK	1507.80
(170 - 171)	GK	204.13
(172 - 176)	YYPLK	683.38
(177 - 195)	SMTEQEQQQLIDDHFLFDK	2352.08
(196 - 208)	PVSPLLLASGMAR	1311.75
(209 - 214)	DWPDAR	759.34
(215 - 222)	GIWHNDNK	983.47
(223 - 235)	SFLVWVNEEDHLR	1643.82
(236 - 241)	VISMEK	706.38
(242 - 246)	GGNMK	506.24
(247 - 250)	EVFR	550.30
(251 - 251)	R	175.12
(252 - 258)	FCVGLQK	794.42
(259 - 264)	IEEIFK	778.43
(265 - 265)	K	147.11
(266 - 291)	AGHPFMWNEHLGYVLTCPSNLGTGLR	2870.39
(292 - 297)	GGVHVK	596.35
(298 - 303)	LAHLSK	668.41
(304 - 306)	HPK	381.22
(307 - 313)	FEEILTR	907.49
(314 - 315)	LR	288.20
(316 - 318)	LQK	388.26
(319 - 319)	R	175.12
(320 - 340)	GTGGVDTAAVGSVFDISNADR	2008.96
(341 - 357)	LGSSEVEQVQLVVDGVK	1785.96
(358 - 364)	LMVEMEK	879.43
(365 - 365)	K	147.11
(366 - 368)	LEK	389.24
(369 - 380)	GQSIDDMIPAQK	1302.64

Figure 4.4. The sequences and molecular weights of the peptides expected from the tryptic digestion of rabbit muscle creatine kinase.

a specific activity of ~2.6 mCi/mmol. Why the actual specific activity was much lower is not known but possibly results from the starting [^{14}C]-cyanamide being impure. The [^{14}C]-cyanamide was purchased as a solid packaged in a small glass vial. The solid had a slight green color to it but was not further purified. When the specific activity of the final product was determined to be much lower than expected, American Radiolabeled Chemicals was contacted. Upon checking, they determined that the [^{14}C]-cyanamide was impure and, after purifying it further, they supplied a new sample. The reaction was repeated with this material; however, the results were even worse and a specific activity of 0.15 mCi/mmol was obtained. At this point, it was decided to go ahead and use the product with a specific activity of 0.24 mCi/mmol in the inactivation experiments and hope it would be sufficient.

B. Tryptic Digestion of Unmodified Creatine Kinase

Shown in Fig. 4.4 are the sequences and protonated molecular weights of the primary peptides expected from the digestion of rabbit muscle creatine kinase with trypsin. Because protonated molecular ions (M + 1) are obtained from LSIMS, all molecular weights in the following discussion are also given as the protonated molecular weights. There are 34 lysines and 18 arginines in rabbit muscle creatine kinase, and there are fifty-two expected tryptic peptides (one of the lysines is at the C-terminus). There are six single amino acids and seven dipeptides expected. The peptides range in molecular weight from 147 to 4373 mass units and in size from one to forty-one amino acids. With the exception of the peptide of molecular weight 4373, all of the peptides are less than 3000 molecular weight. Thus, excluding the forty-one amino acid peptide, the majority of the sequence of CK is contained in peptides that are of an appropriate size to be separated by HPLC and should also be amenable to LSIMS. In addition to the primary peptides shown in Fig. 4.4, there are 124 possible partial peptides that arise from missing less than 4 cuts and are less

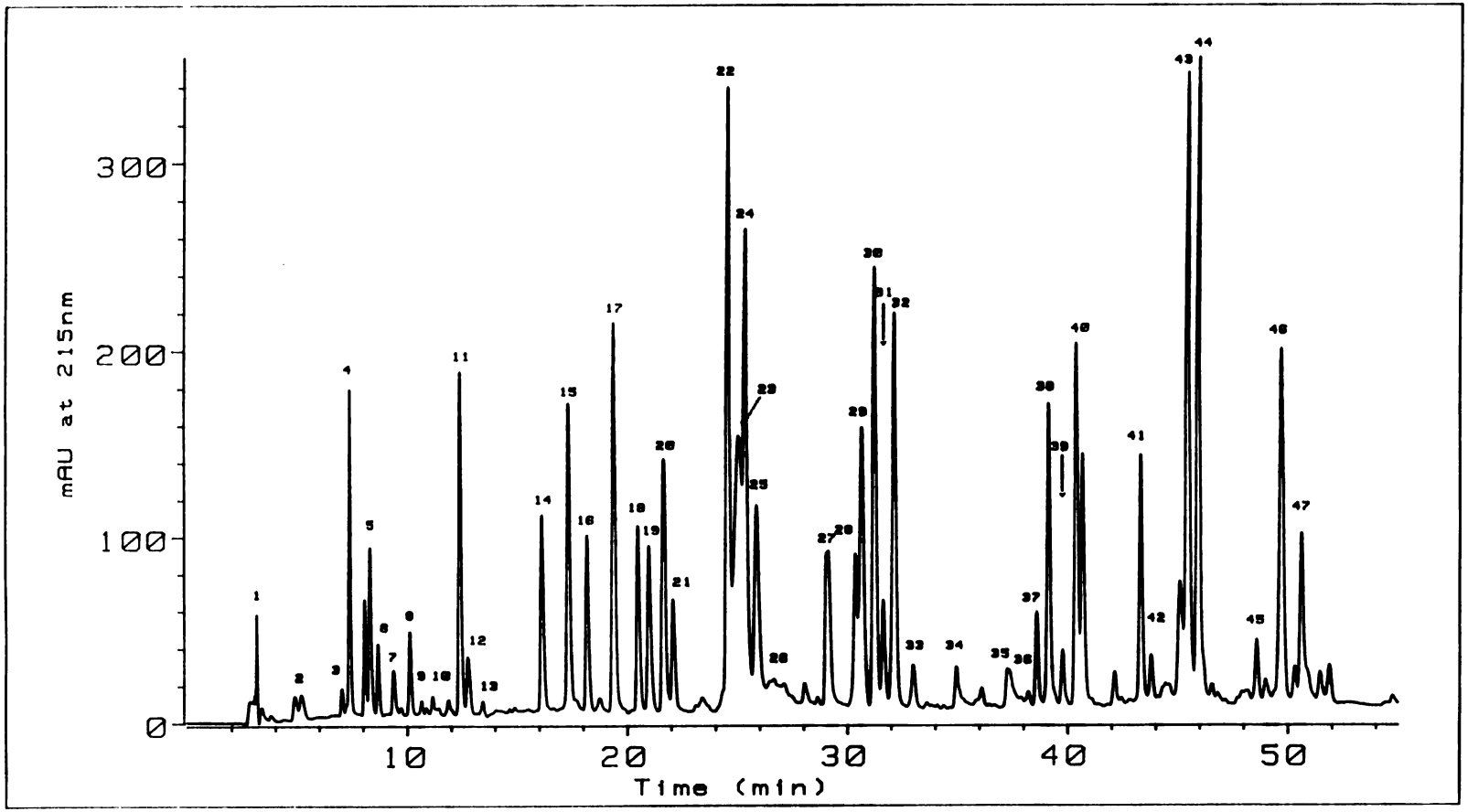


Figure 4.5 HPLC chromatogram of the separation of the peptides from a five hour digestion of 325 µg of rabbit muscle creatine kinase with TPCK-treated trypsin. Fractions that were collected and analyzed by mass spectrometry are numbered.

than 4000 molecular weight. Most of these partial peptides would be expected to be amenable to LSIMS.

There are four free cysteines in each subunit of rabbit muscle CK. In the digest of the unlabeled enzyme these should each bear a carboxymethyl group, -CH_2COOH, because the enzyme is alkylated with iodoacetic acid prior to digestion. The tryptic peptides that contain these cysteines, peptides 45-85, 138-147, 252-258, and 266-291 in Fig. 4.4, should be shifted in molecular weight (plus 58 mass units) by the presence of this group. The same should hold true for the epoxycreatine-modified enzyme, assuming it has the same number of free sulfhydryls. Although rabbit muscle CK does not contain any disulfides, the enzyme was routinely reduced with DTT prior to alkylation. This was added insurance that all of the thiols would be free.

A digestion time of five hours was typically used in this work. Chromatograms (not shown) of the separated peptides from digests quenched prior to five hours showed consistent changes as the time of digestion increased. Allowing digestion to proceed longer than five hours (up to 24 hrs) resulted in little or no change in the chromatograms. The digest at five hours was perfectly reproducible. Five hours was also chosen because it was felt that it represented a good compromise between the need to have complete and reproducible digestion and the possibility of the enzyme-bound epoxycreatine being labile under the digestion conditions. Tryptic digestion is usually performed at a pH greater than 8.5; a pH of 8.0 was chosen for the present work to avoid loss of the label if it were bound *via* an ester linkage, as suggested from previous work (Marletta & Kenyon, 1979).

A chromatogram of the HPLC separation of a five-hour tryptic digest of reduced and carboxymethylated rabbit muscle creatine kinase is shown in Fig. 4.5. Fig. 4.6 lists the peptides which were found in the HPLC fractions numbered in Fig. 4.5. Over 47 peptides are well separated, although several of the peaks clearly contain more than one peptide. Each of these fractions was collected, dried on a Speed Vac Concentrator (Savant) and subjected to LSIMS. As an example, Fig. 4.7 shows a typical LSIMS spectrum

Fraction	Peptide	Position
1	DK	43
2	GER	148
3	—	—
4	HPK	304
5	GGNMK	242
6	AVEK	152
7	SIK	135
8	LQK	316
9	—	—
10	—	—
11	HNNHMAK	25
12	KLEK* or GERR*	365/148
13	—	—
14	GGVHVK	292
15	PFGNTHNK	1
16	LNYK	11
17	HGGFKPTDK*	96
18	LAHLSK	298
19	EVFR	247
20	HKTDLNHENLK* and TDLNHENLK	105/107
21	VISMEK or EVFRR*	236/247
22	GIWHNDNK	215
23	GYTLPPHCSR and DWPDAR	138/209
24	SEEEYPDLSK and GYTLPPHCSR	15/138
25	GYTLPPHCSR and YYPLK	138/172
26	—	—
27	LMVEMEK	358
28	GQSIDDMIPAQK and FCVGLQK	369/252
29	RFCVGLQKIEEIFKK* and RFCVGLQK* and GQSIDDMIPAQK	251/251/369
30	VLTPDLYK and FEEILTR	32/307
31	VLTPDLYK and IEEIFK	32/259
32	GGDDLDPHYVLSSR	116
33	—	—
34	—	—
35	—	—
36	—	—
37	RGTGGVDTAAVGSVFDISNADR*	319
38	GTGGVDTAAVGSVFDISNADR	320
39	YKLNYKSEEEYPDLSK*	9
40	LSVEALNSLTGEFK and DLFDPIIQDR	156/86
41	LSVEALNSLTGEFK	156
42	—	—
43	SFLVWVNEEDHLR	223
44	AGHPFMWNEHLGYVLTCPSNLGTGLR	266
45	—	—
46	LNYKSEEEYPDLSKHNNHMAK* and SMTEQEQQQLIDDHFLFDKPVSPLLLASGMAR* and PVSPLLLASGMAR	11/177/196
47	LGSSEVEQVQLVVDGVKLMVEMEKKLEK*	341

Figure 4.6 Peptides identified by mass spectrometry in the numbered fractions in Fig. 4.5. Peptides resulting from incomplete proteolytic cleavage are indicated by an asterisk.

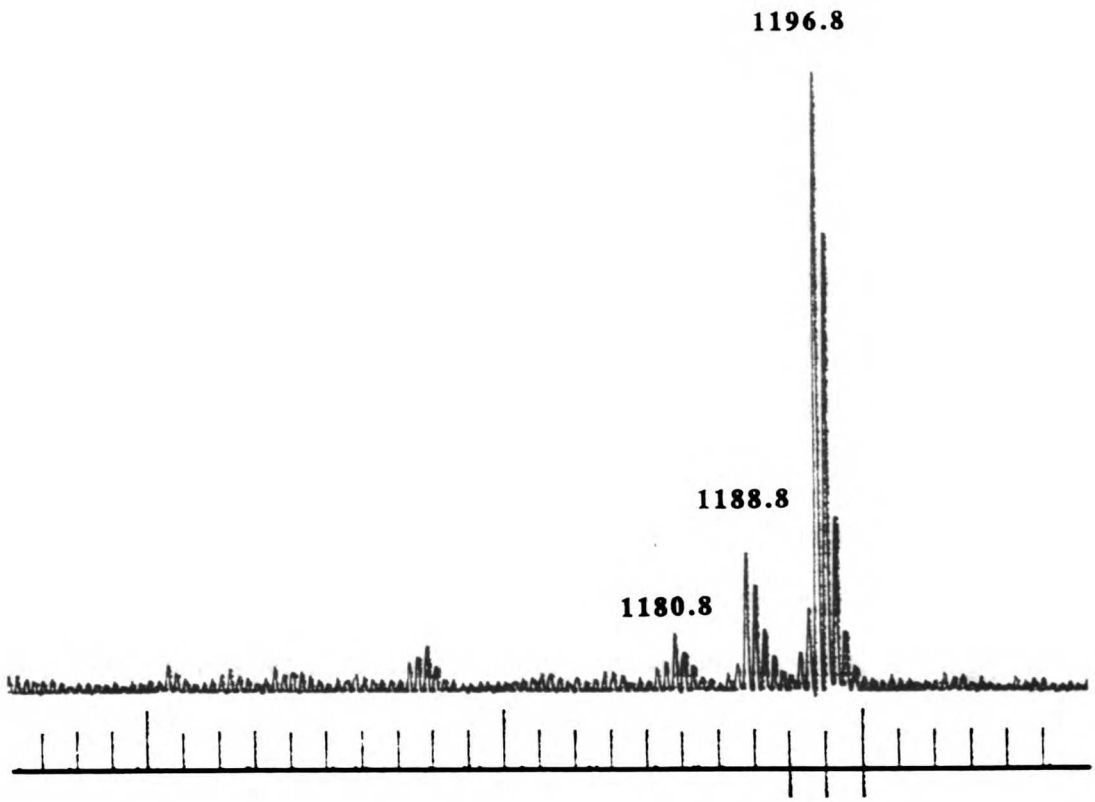


Figure 4.7 A portion of the liquid secondary ion mass spectrometry spectrum of fraction #24 in Fig. 4.5.

obtained from one of these fractions, in this case fraction #24 in Fig. 4.5. This fraction contained two peptides. The protonated molecular weight of the first of these, at m/z 1196.8, was within 0.5 mass units of the protonated molecular weight expected for peptide 15-24 in Fig. 4.4. The second ion, at m/z 1188.8, was within 0.5 mass units of the protonated molecular weight of peptide 138-147 in Fig. 4.4, if this peptide, which has one cysteine, is modified by one carboxymethyl group (plus 58 mass units). The ion at 1180.8

is minus 16 mass units from 1196.8. Such ions are commonly observed in LSIMS spectra and probably result from loss of an -NH_2 group. In all cases, a molecular weight had to be within 0.5 mass units of the expected molecular weight before it was assigned to a peptide. In some instances partial peptides, due to incomplete proteolytic cleavage, were found. These are indicated in Fig. 4.6.

Several of the fractions from the digest did not give ions by LSIMS and the peptides contained in them had to be hexylated before yielding abundant molecular ions. These included fractions #1, 2, 4, 5, 6, 7, 8, 12, and 15 (Fig. 4.5). Hexylation results in an increase in mass of 84 for the esterification of the C-terminal carboxyl and additional increases of 84 for any side chain carboxyls. The hexylation reaction proceeded smoothly, in good yield, and with no apparent side products.

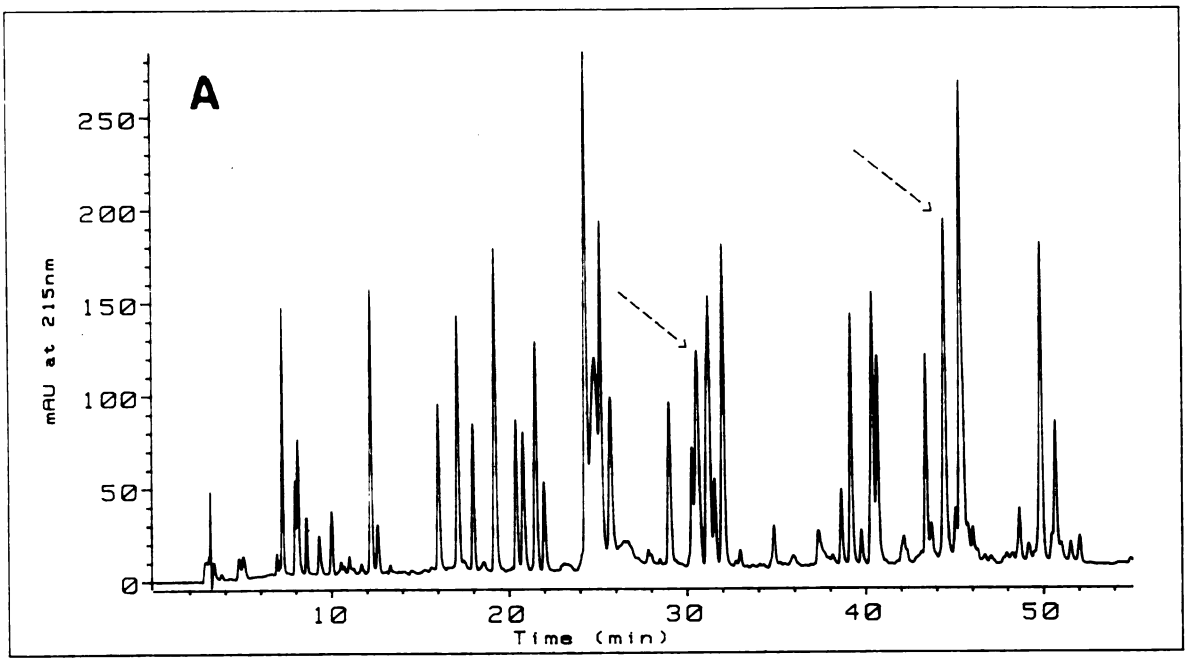
Peptide 45-85 in Fig. 4.4 has a protonated molecular weight of 4373.03. This is above the limit that can be observed by LSIMS, as performed in the present work. In an attempt to locate this peptide in the chromatogram, several of the later eluting peaks were subjected to subdigestion with endoproteinase Glu-C. This protease cuts on the C-terminal side of glutamates in ammonium carbonate or ammonium acetate buffer and on the C-terminal side of both glutamates and aspartates in sodium phosphate buffer. Peptide 45-85 contains three glutamates and four aspartates. Digestion in phosphate buffer was chosen because these conditions would be expected to give peptides of appropriate size. Subdigestion of fraction #45 was not successful; only undigested material was seen upon HPLC of the subdigestion reaction mixture. Subdigestion of fractions #46 and 47 did result in the production of additional, smaller peptides. None of these peptides was consistent with peptide 45-85, but they did help to confirm the identity of the peptides in these fractions. The subdigestion peptides found in fraction #47 are the same as some of those seen in the subdigestion of fraction #46; inspection of the chromatogram of the tryptic digest (Fig. 4.5) suggests that these two fractions are not completely resolved and that #47 is likely "contaminated" with #46. Thus, it was not possible to locate peptide 45-85 in the

PFGNTHNKYK$_{10}$LNYKSEEEYP$_{20}$DLSKHNNHMA$_{30}$KVLTPDLYK K$_{40}$LR
DK ETPSGF$_{50}$TLDDVIQTGV$_{60}$DNPGHPFIMT$_{70}$VGCVAGDEES$_{80}$YTVF
K DLFDP$_{90}$IIQDRHGGFK$_{100}$PTDKHKTDLN$_{110}$HENLKGGDDL$_{120}$DPHYVL
SSR V$_{130}$RTGR SIKGYT$_{140}$LPPHCSRGER$_{150}$RAVEKLSVEA$_{160}$LNSLT
GEFK G$_{170}$K YYPLKSMTE$_{180}$QEQQQLIDDH$_{190}$FLFDKPVSPL$_{200}$LLASG
MARDW$_{210}$PDARGIWHND$_{220}$NKSFLVWVNE$_{230}$EDHLRVISME$_{240}$KGGN
MKEVFR$_{250}$RFCVGLQKIE$_{260}$EIFKKAGHPF$_{270}$MWNEHLGYVL$_{280}$TCPSN
LGTGL$_{290}$RGGVHVKLAH$_{300}$LSKHPKFEEI$_{310}$LTR LR LQKRG$_{320}$TGGVD
TAAVG$_{330}$SVFDISNADR$_{340}$LGSSEVEQVQ$_{350}$LVVDGVKLMV$_{360}$EMEK
KLEKGQ$_{370}$SIDDMIPAQK$_{380}$

Figure 4.8 The amino acid sequence of rabbit muscle creatine kinase. Individual amino acids are represented by one letter codes. Boxed portions of the sequence are those that were identified in the tryptic peptides.

chromatogram. Conceivably, it is in one of the later eluting fractions and is resistant to digestion with endoproteinase Glu-C. Further attempts to locate it were not undertaken. This was not expected to present a problem in determining the site of modification by [^{14}C]-epoxycreatine, as long as all of the enzyme-bound radioactivity was accounted for.

Shown in Fig. 4.8 is the sequence of rabbit muscle creatine kinase. Residues that are boxed are those portions of the sequence that were located in the tryptic map. It was possible to identify 88% of the sequence of the enzyme in the tryptic peptides. The portion of the sequence that was not found is accounted for mainly by the forty-one amino acid peptide discussed above. Many of the fractions from the separation of the tryptic peptides contained additional, unidentified peptides, usually as minor components. The molecular weights of these peptides did not match any of the expected primary or secondary tryptic



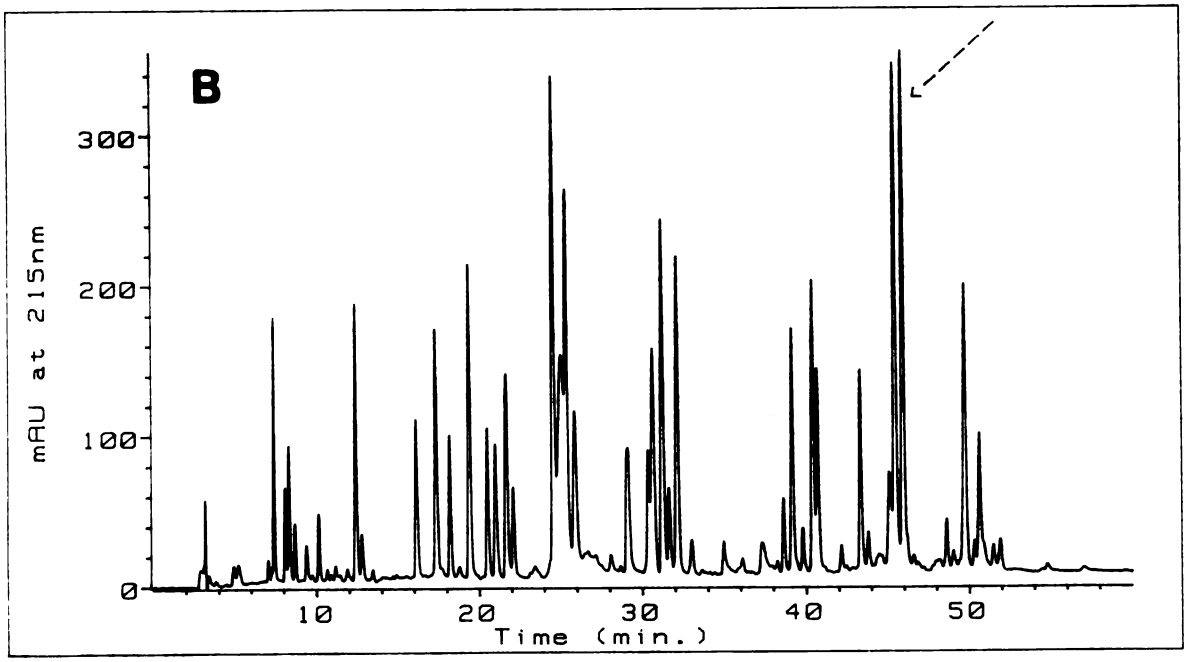


Figure 4.9 (A) HPLC chromatogram of the separation of the peptides from a five hour digestion of 250 µg of [^{14}C]-epoxycreatine-modified rabbit muscle creatine kinase with TPCK-treated trypsin. Arrows show the fractions which contained radioactivity. (B) HPLC chromatogram of the separation of unmodified creatine kinase shown in Fig. 4.5.

peptides. It is possible they arise from overdigestion, a low level of a non-creatine kinase protein, or from the multitude of species present in the enzyme preparation (see Chapter 2). The majority of the peptides, however, are accounted for in the expected sequence of rabbit muscle CK.

C. Tryptic Digestion of Epoxycreatine-modified Creatine Kinase

Shown in Fig. 4.9A is a chromatogram from the HPLC separation of a five hour tryptic digest of [^{14}C]-epoxycreatine-modified creatine kinase. For comparison purposes, Fig. 4.9B shows the separation of the digest of unmodified CK previously shown in Fig. 4.5. The chromatogram shown in Fig. 4.9A was obtained from 250 μg of CK, at 1mg/ml, that had been inactivated with 20 mM epoxycreatine. The inactivation was allowed to proceed for ~10 half-lives, 10 hrs in this case. At this time, the inactivated enzyme had less than 1% of the activity of control enzyme that was not treated with epoxycreatine. Excess [^{14}C]-epoxycreatine was removed by repeated ultrafiltration through a Centricon-30 ultrafiltration device. The final filtrate from this process contained 90 cpm. After removal of the excess [^{14}C]-epoxycreatine, the enzyme activity was not checked again. There were no apparent differences in the behavior of the modified and unmodified enzymes either during the course of the digestion or subsequent chromatographic separation of the peptides.

Visual inspection of the chromatograms in Fig. 4.9 reveals two points of difference between them. In the chromatogram of the digest of [^{14}C]-epoxycreatine-modified CK (Fig. 4.9A) there is a peak at 44.5 min (indicated by an arrow in Fig. 4.9A) that is not seen in the chromatogram of unmodified enzyme (Fig. 4.9B). In addition, the chromatogram of unmodified enzyme contains a large peak at 46 min (fraction #44 in Fig. 4.5 and indicated by an arrow in Fig. 4.9B) that is not seen in the chromatogram of the [^{14}C]-epoxycreatine-modified enzyme. No other differences are apparent in these chromatograms.

Fractions containing obvious peaks in the chromatogram were collected as well as fractions covering the areas between the peaks. Fractions were collected out to 110 min (90% acetonitrile). Upon scintillation counting of the collected fractions from the separation of the digest of [^{14}C]-epoxycreatine-modified CK, it was found that the the fraction at 44.5 min (Fig. 4.9A) contained 900 dpm and that at 31.5 min (Fig. 4.9A) contained 305 dpm. Background counts were ~25 dpm. No other fractions contained more than 50 dpm, except a fraction collected between 41.3 and 43 min that contained 100 dpm. This area of the chromatogram does not contain any species which absorbs significantly at 215 nm. No radioactivity was found in the void volume of the column and no additional counts were found at a high percentage of acetonitrile.

If the specific activity of the [^{14}C]-epoxycreatine used in this experiment was 0.24 mCi/mmol, a total of 3000 dpm should have been bound to the enzyme. This assumes that labeling was 100% and that no enzyme (or label) was lost in the inactivation, reduction and carboxymethylation, and chromatography steps. The two fractions indicated by the arrows in Fig. 4.8A, therefore, account for ~40% of the expected bound radioactivity. There are several possible explanations for the less than quantitative recovery of radioactivity. The most obvious is the loss of a portion of the enzyme during the digestion procedure. From the inactivation of the enzyme with [^{14}C]-epoxycreatine until the final HPLC purification, there are numerous points at which some enzyme may have been lost, particularly two ultrafiltration steps. It would not be unreasonable to expect that these losses might be 20-30%. It is possible that label is lost during these procedures as well, and given the reported lability of the label this is not unreasonable. The small peak remaining at 46 min in the chromatogram of labeled enzyme may be peptide that lost the label prior to the carboxymethylation or may be from the small amount of enzyme that was not labeled by epoxycreatine. The possibility that an additional site of labeling, perhaps one more labile than those observed here and therefore lost during the digestion, is present cannot be completely ruled out. However, there is no evidence for multiple sites of labeling under

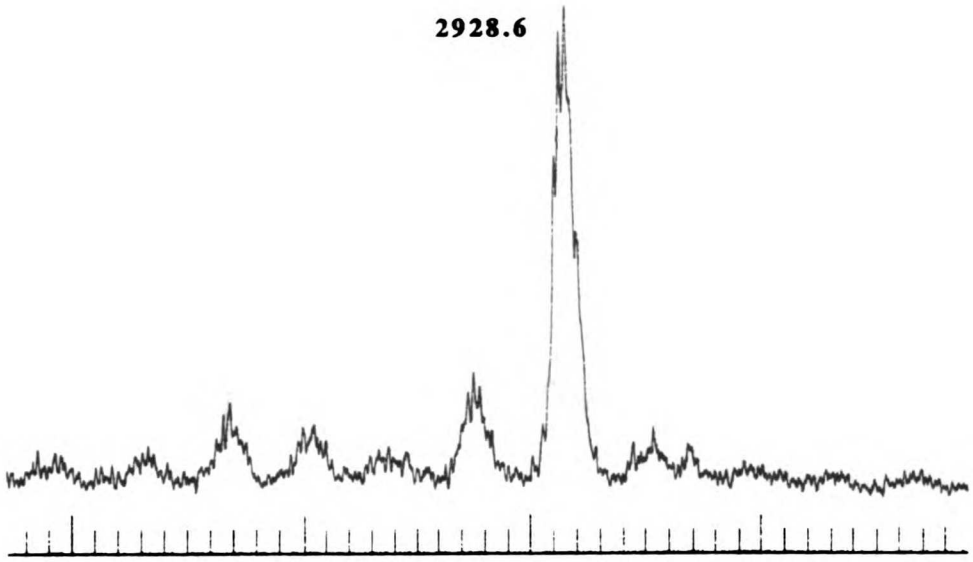


Figure 4.10 Liquid secondary ion mass spectrometry spectrum of the fraction at 46 min (Fig. 4.8B) in the chromatogram of the HPLC separation of the tryptic digest of creatine kinase.

these conditions. In fact, the finding by Marletta and Kenyon (Marletta & Kenyon, 1979) that a 1:1 stoichiometry of moles of affinity label to moles of enzyme active sites is observed under identical conditions is a strong argument that labeling is occurring at only one site. The present observation that two fractions in the digest contain radioactivity is discussed below.

These results suggest that two fractions from the HPLC separation of the tryptic digest contained peptides that were labeled with epoxycreatine. One of these was the fraction at 44.5 min in the chromatogram of the epoxycreatine-modified enzyme. The second was the fraction at 31.5 min in the chromatogram of modified enzyme. This chromatographic peak shape of this fraction was not visually different from that of the corresponding fraction in the chromatogram of unmodified CK. Attention was focused on each of these fractions separately in an attempt to identify the labeled peptide(s) in each.

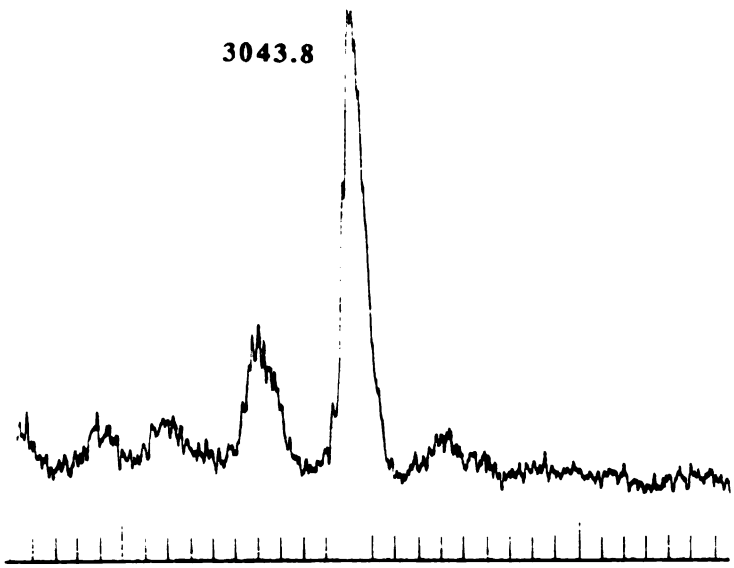
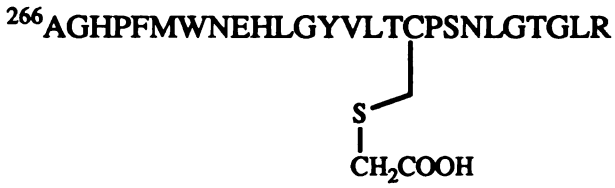


Figure 4.11 Liquid secondary ion mass spectrometry spectrum of the fraction at 44.5 min (Fig. 4.8A) in the chromatogram of the HPLC separation of the tryptic digest of epoxycreatine-modified creatine kinase.

CK that was labeled with non-radioactive epoxycreatine was used in this portion of the work.

D. The Labeled Peptide in the Fraction at 44.5 min.

Since it was expected that the labeled peptide in the fraction at 44.5 min in the digest of modified CK resulted from labeling of the peptide in the fraction at 46 min in the digest of unmodified enzyme, attention was intially focused on identification of the peptide in the fraction at 46 min in the digest of unmodified CK. Shown in Fig. 4.10 is the LSIMS spectrum of the fraction at 46 min in the chromatogram of *unmodified* creatine kinase. The protonated molecular weight of the peptide in this fraction is at m/z 2928.6. This molecular weight corresponds to peptide 266-291 in Fig. 4.4, if there is a carboxymethyl group present on the single cysteine in this peptide:



The expected molecular weight for this peptide with the carboxymethyl group is 2928.4. Since this peak was no longer present in the chromatogram of labeled enzyme, it was suspected that it had been labeled with epoxycreatine and had changed in retention time.

The most likely candidate for this labeled peptide was the new peak at 44.5 min in the chromatogram of labeled enzyme (Fig. 4.9A). The LSIMS spectrum of this fraction is shown in Fig. 4.11. The protonated molecular weight of the peptide in this fraction is at m/z 3043.8. If 173 mass units are subtracted for the expected molecular weight of bound epoxycreatine, this leaves 2870.8. This is within 0.4 mass units of the protonated molecular weight of peptide 266-291, *without the carboxymethyl group*. Thus, it would appeared that this peptide is labeled with epoxycreatine, but is not carboxymethylated. A reasonable explanation for this result is that epoxycreatine is bound at or near the cysteine and that its presence prevents the subsequent carboxymethylation of this residue. The tentative conclusion was made that epoxycreatine was labeling this cysteine. This residue is Cys-282 in the sequence of rabbit muscle creatine kinase.

To identify conclusively the labeled residue it was necessary to sequence this peptide. Tandem mass spectrometry was chosen for this purpose. However, the molecular weight of this peptide, 3043.8, is somewhat above the size of a peptide that could be expected to yield a CID spectrum containing complete sequence information. Inspection of the sequence of the peptide showed it to contain one glutamic acid residue. Digestion with endoproteinase Glu-C should give two peptides:

v						1347				785	671	558		400			
w		1778				1346		968	871	784	670	557				229	
x				1693	1530	1431		1217	940	843	756	642	529	472	371		
y			1724					1191	914	817	730	616	503		345		175
z			1708	1650	1487	1389	1275	1173		800	713		486	429		272	159
	His	Leu	Gly	Tyr	Val	Leu	Thr	Cys*	Pro	Ser	Asn	Leu	Gly	Thr	Gly	Leu	Arg
a	110	223		443	542	655		1032	1130	1217	1331	1444				1772	
b	138	251					784	1060	1158		1359	1472			1687		
c								1077		1262	1376	1489	1546				
d										1201	1288	1402		1585		1730	

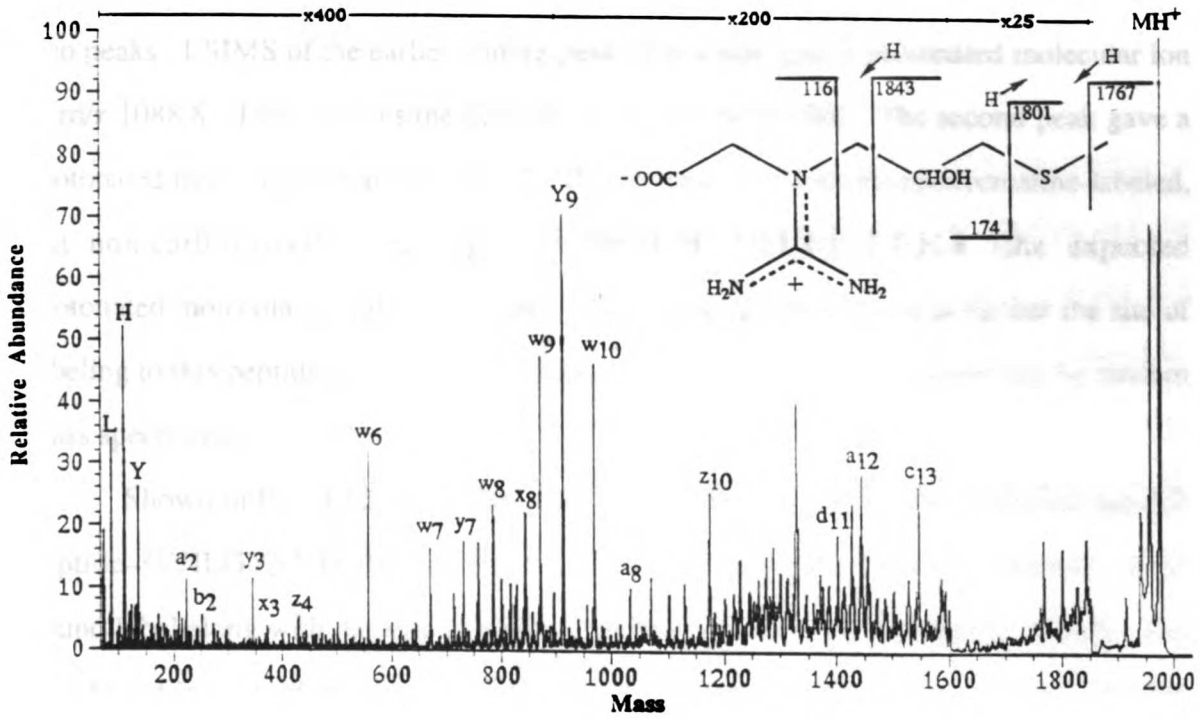


Figure 4.12 CID spectrum of the peptide ^{275}HLGYVLT^{282}CPSNLGTGLR. Observed fragment ions are listed above the spectrum. Ions belonging to the same series are listed in rows. The insert shows the proposed structure for the label bound at Cys-282, as well as the ions expected from the fragmentation of the label.

^{266}AGHPFMWNEHLGYVLTCPSNLGTGLR

↓ Endoproteinase Glu C

^{266}AGHPFMWNE and ^{275}HLGYVLTCPSNLGTGLR

The smaller of these two would have a protonated molecular weight of 1088.47, while the larger would have a protonated molecular weight of 1800.96. Each of these should be amenable to tandem mass spectrometry.

HPLC separation of the products from endoproteinase Glu-C digestion of the peptide in the fraction at 44.5 min in the chromatogram of epoxycreatine-labeled CK gave two peaks. LSIMS of the earlier eluting peak of this pair gave a protonated molecular ion at m/z 1088.8. This matches the peptide–^{266}AGHPFMWNWE. The second peak gave a protonated molecular ion at m/z 1974.2. This is consistent with the epoxycreatine-labeled, but non-carboxymethylated peptide–^{275}HLGYVLTCPSNLGTGLR (the expected protonated molecular weight is 1973.96). This result helped to localize further the site of labeling to this peptide and also gave a peptide that was suitable for sequencing by tandem mass spectrometry.

Shown in Fig. 4.12 is the high energy CID spectrum of the epoxycreatine-labeled peptide–^{275}HLGYVLTCPSNLGTGLR . The inset shows the proposed structure of the bound label along with the ions expected from fragmentation of the labeling moiety. The ions found in each of the ion series are listed above the spectrum. Abundant C-terminal sequence ions were observed as is usually the case for peptides containing arginine at the C-terminus (Johnson et al., 1988). All of the observed ions in the ion series are consistent with the sequence shown for this peptide up to the cysteine residue. A 173 mass unit shift in the sequence ions is observed beginning at this position. For example, the expected z_{10} ion for this sequence, if Cys-282 were not modified, would be at m/z 1000; however, the

observed z_{10} ion, with epoxycreatine bound at Cys-282, is at m/z 1173 (Fig 4.12), exactly the expected increase. Ions in the other series completely confirm the sequence and the site of labeling. Side chain fragmentation ions are observed in the N-terminal sequence series, d_{10}, d_{11}, d_{12}, d_{14}, and d_{16}. This is consistent with the introduction of the basic guanidinium group of epoxycreatine at Cys-282. The occurrence of **d**-ions is often indicative of the presence of a basic amino acid on the N-terminal side of the site of cleavage (Johnson et al., 1988). Additional side chain fragmentation ions (the **v** and **w** series) are also observed, again completely consistent with the proposed sequence. These results confirmed the sequence of this peptide and conclusively identified the site of labeling as Cys-282.

E. The Labeled Peptide in the Fraction at 31.5 min.

Having determined the major site of labeling, attention was turned to the fraction at 31.5 min in Fig. 4.9A that contained ~25% of the radioactivity. This fraction did not appear to be visually different in the chromatograms of the digests of the labeled and unlabeled enzyme (compare Fig. 4.9A to 4.9B). Inspection of these chromatograms indicated that this fraction, in each digest, contained several peptides. The LSIMS spectra from these fractions contained the same major ions; four molecular ions were seen in each fraction. Three of these are listed in Fig. 4.6 for fraction #29, the other, at m/z 852.4, was also found in fraction #28 (Fig. 4.6) and results from incomplete separation of these two fractions. In addition to these major ions, the fraction from the labeled digest contained a low abundance protonated molecular ion at m/z 1503.8 that was not present in the digest of unlabeled enzyme. This was the only difference apparent between these two fractions.

The protonated molecular ion at m/z 1503.8 is consistent with the epoxycreatine-labeled, but non-carboxymethylated peptide:

v			1246			785	671	558		400		
w		1346		968	871		670	557		413		229
x		1431		1217		843		642	529	472	371	314	201
y						817	730	616	503	446	345	288	175
z		1389	1275			801	714	599	487	430	329	272	159

Val-Leu-Thr-Cys*-Pro-Ser-Asn-Leu-Gly-Thr-Gly-Leu-Arg

a	72	185	286	562	659	746	860	973	1030	1132	1189	1302
b	100	213	314	590	687	774	888		1058	1160		1330
c					704	791	905		1076		1234	
d						730	817	931		1116		1260

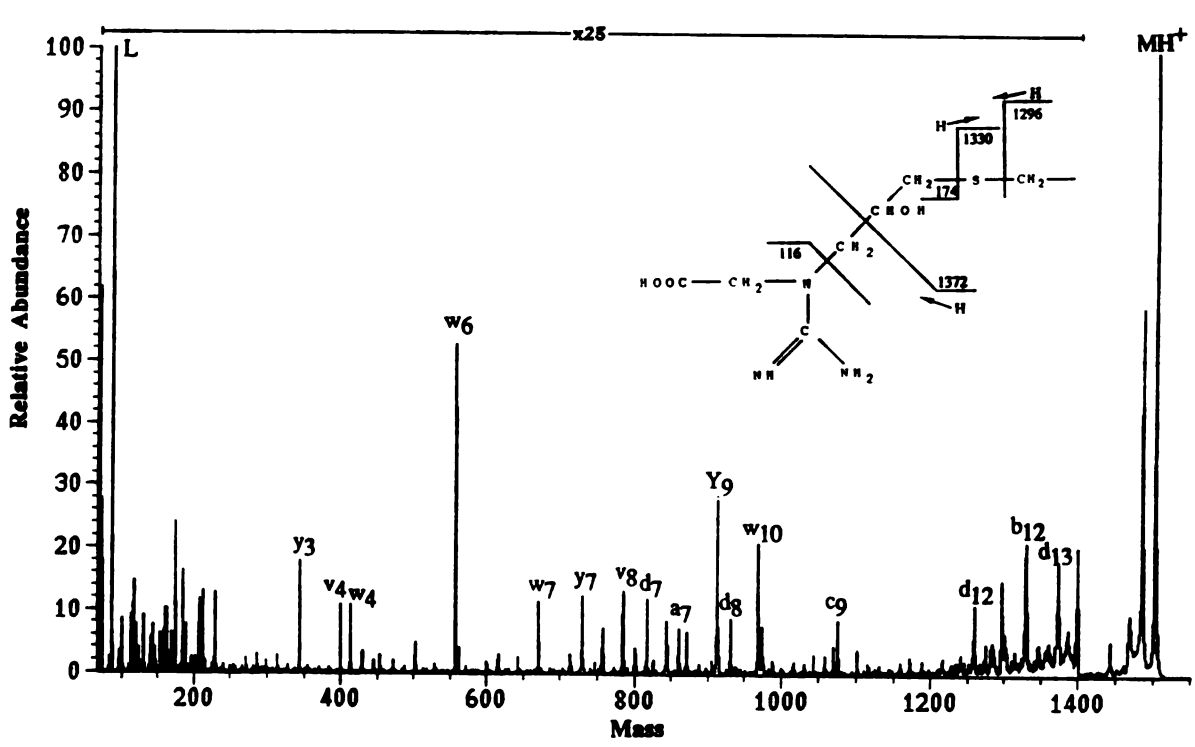


Figure 4.13 CID spectrum of the peptide ^{279}VLTCPSNLGTGLR. Observed fragment ions are listed above the spectrum. Ions belonging to the same series are listed in rows. The insert shows the proposed structure for the label bound at Cys-282, as well as the ions expected from the fragmentation of the label.

^{279}VLTCPSNLGTGLR

The expected protonated molecular weight, if this peptide were labeled with epoxycreatine, is 1503.8. Labeling again appears to be at the cysteine, since the peptide is not carboxymethylated. This peptide is evidently a chymotryptic fragment of the larger peptide described above (peptide 266-291); it most likely arises from chymotryptic activity associated with the trypsin, or, less likely, from the nearby basic guanidinium moiety of epoxycreatine being recognized as a substrate by trypsin.

Several HPLC conditions were investigated in an attempt to separate this peptide from the other peptides in this fraction. Gradients of increasing acetonitrile as low as 0.1%/min were used. These gave only marginal improvements in the separation. After several attempts, a fraction that was enriched in the desired peptide was obtained. This fraction still contained at least one other peptide, but the ion at m/z 1503.8 was considerably improved in abundance and, most importantly, was of sufficient abundance for CID analysis.

The high-energy CID spectrum of this peptide (Fig. 4.13) again showed abundant C-terminal fragmentation ions, as well as several N-terminal fragmentation ions. Ions consistent with the fragmentation of bound epoxycreatine are present, as diagrammed in the inset of Fig. 4.13. The presence of epoxycreatine at Cys-282 is clearly evident from several of the ion series. Again, as an example, the expected a_4 ion for this sequence, if Cys-282 were not modified, would be at m/z 389. Instead, the a_4 ion is at m/z 562, exactly the shift expected for bound epoxycreatine. The modification is at Cys-282; ions representing the sequence up to Cys-282 are consistent with the sequence shown, with no additional modified residues.

IV. Discussion

The developments of the past decade in protein chemistry and molecular biology require rapid, sensitive and reliable methods for protein analysis. Mass spectrometry is one of the fastest developing fields in this area and is now commonly used in the analysis of molecules of biological significance (Burlingame et al., 1990). In particular, soft ionization techniques, such as liquid secondary ion mass spectrometry, are valuable methods for determining the weights of the molecular ions of peptides obtained from the proteolytic digests of proteins of known sequence. Peptide mapping by mass spectrometry is a rapid and reliable method for amino acid sequence confirmation (Burlingame et al., 1990) and a useful tool for determining post-translational (Settineri et al., 1990) and other types of covalent modifications of proteins and peptides (DeWolf et al., 1988; Kaur et al., 1989). Tandem mass spectrometry complements soft ionization techniques by allowing the fragmentation pattern of selected ions to be obtained. This method enables the complete sequence of a peptide to be deduced and makes possible the sequencing of peptides and proteins of previously unknown structure (Biemann & Scoble, 1987; Burlingame et al., 1990). Tandem mass spectrometry is the method of choice for determining the exact site and nature of covalent modification in proteins and peptides.

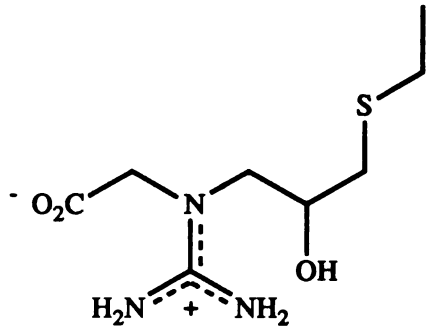
In this work, a combination of LSIMS and tandem mass spectrometry has been used to determine the specific amino acid residue of creatine kinase that is covalently modified by the affinity label, epoxycreatine. These two techniques nicely complemented each other in analyzing the peptides from the tryptic digestion of creatine kinase. The majority of the primary sequence could be mapped by LSIMS of the HPLC-separated peptides (Fig. 4.8). Several HPLC fractions obviously contained more than one peptide; however, this presented no problem in obtaining a molecular weight for each of them. Eighty-eight percent of the amino acid sequence of rabbit muscle creatine kinase, as deduced from the cDNA sequence (Putney et al., 1984), has been confirmed by this work.

Several peptides were found in the tryptic digest that could not be accounted for by expected tryptic peptides. The source of these peptides is not known, but further mass spectrometric analysis of them would likely be an excellent approach to the elucidation of the structures of the multiple species seen in purified rabbit muscle CK by isoelectric focusing (see Chapter 2).

The shift to a shorter retention time of the single peak in the chromatogram of the digest of epoxycreatine-labeled CK suggested that the peptide in this fraction was labeled with epoxycreatine. LSIMS of this fraction supported this conclusion. The observation that the labeled peptide did not appear to be carboxymethylated provided the first evidence that modification of Cys-282 had occurred. This was confirmed from the CID spectrum (Fig. 4.12). Because no additional changes were seen in the chromatogram, it appeared that only this peptide was labeled. This would not be unexpected since epoxycreatine labels CK with a 1:1 stoichiometry (Smith & Kenyon, 1974). It was a surprise, therefore, to find that a second HPLC fraction contained a significant amount of radioactivity. Attempts to separate further the peptides in this fraction were only marginally successful. However, this peptide was amenable to tandem mass spectrometry and gave an excellent CID spectrum (Fig. 4.13). This allowed the determination of an unambiguous sequence for it and also conclusively identified the site of labeling as Cys-282. The use of tandem mass spectrometry was especially critical in this case; the sequencing of peptide mixtures by conventional methods, such as Edman degradation, often gives ambiguous results, especially if the peptide of interest is a minor component.

Epoxycreatine labels cysteine-282. Only this residue is modified; no other significant amounts of radioactivity were detected in the fractions from the HPLC separation of the peptides resulting from the tryptic digestion of [^{14}C]-epoxycreatine labeled CK. The proposed structure for the labeled peptide is:

^{266}AGHPFMWNEHLGYVLTCPSNLGTGLR



This structure is that resulting from nucleophilic attack of the thiol of Cys-282 on the terminal carbon of the epoxide. This is likely to be the favored mode of opening of the oxirane ring, at least if the reaction were occurring free in solution (see Chapter 3). However, the regiochemistry of attack by Cys-282, within the confines of the active site, could be dictated, not only by the steric properties of epoxycreatine, but also by the constraints imposed upon the system by the enzyme. This could result in attack at the other, methine, carbon of the epoxide. The actual structure of the bound label is not known.

Contrary to the tentative conclusion from previous work (Marletta & Kenyon, 1979), epoxycreatine does not label a carboxyl group in the active site of creatine kinase. The suggestion that it does was made based upon the observation that the bound label was removed from the enzyme under relatively mild basic conditions and that loss of the label was enhanced by hydroxylamine. Removal of the label from Cys-282 by base can be explained by a simple reversal of the alkylation reaction (Fig. 4.14). This reaction would lead to the free thiolate at Cys-282 and free epoxycreatine. Under basic conditions, epoxycreatine would be expected to be hydrolyzed rapidly to the diol. The diol was detected upon treatment of the inactivated enzyme with base (Marletta & Kenyon, 1979). This is consistent with hydrolysis of an ester linkage as proposed by Marletta and Kenyon (Marletta & Kenyon, 1979), but is also consistent with the mechanism of base catalyzed loss of the thioether-linked label proposed here. The mechanism by which hydroxylamine

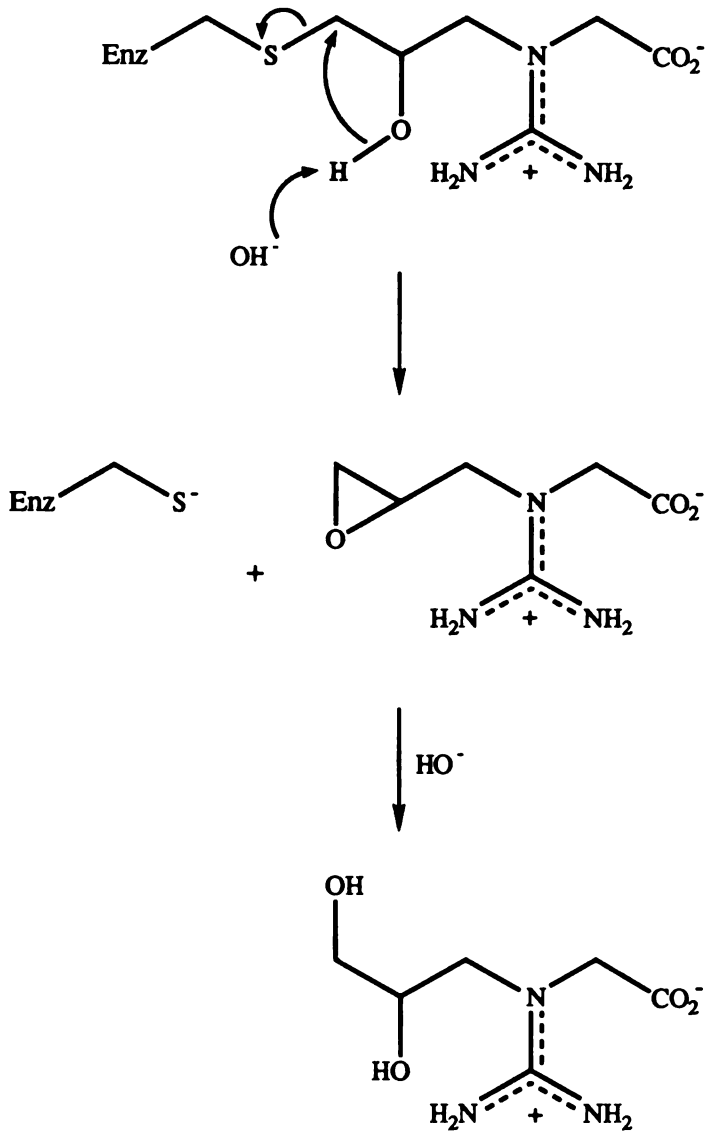


Figure 4.14 Proposed mechanism for the removal of creatine kinase-bound epoxycreatine under basic conditions.

enhances loss of the label is not known, but may be the result of a unique environment within the enzyme active site.

The role that Cys-282 plays in catalysis continues to be controversial. A mechanism for CK that has Cys-282 playing an integral part in the chemical steps of phosphoryl transfer has been proposed (Rabin & Watts, 1960; Watts & Rabin, 1962).

There has been little experimental evidence to support this mechanism. As discussed in Chapter 1, formation of the -$SSCH_3$ or -SCN derivatives at Cys-282 apparently results in the retention of some activity. This has led to the suggestion that Cys-282 is involved in maintaining the correct conformation at the active site (Hooton, 1968) or that its integrity is necessary for the conformational change to the "working" enzyme that occurs upon substrate binding (Markham et al., 1977; O'Sullivan & Cohn, 1968; Reed & Cohn, 1972; Taylor et al., 1969). Site-directed mutagenesis of Cys-282 in rabbit muscle CK to alanine and serine has been carried out (Chen et al., 1990). Preliminary data shows that the alanine mutant has ~1/5000 the activity of native enzyme and that the serine mutant has ~1/200. The ability of the hydroxyl group of serine to serve in the same capacity in the enzyme mechanism as the thiol of cysteine cannot be ruled out at present. Further characterization of these and other Cys-282 mutants is ongoing. A more definitive resolution of this controversy may be possible when a crystal structure of CK is obtained.

The location of Cys-282 with respect to the substrate binding sites is equally controversial. Some studies have indicated that modification of the sulfhydryl has little effect on nucleotide binding (O'Sullivan & Cohn, 1968; O'Sullivan et al., 1966; Roustan et al., 1970) and that it is likely to be near the creatine binding site. Alternative proposals suggest that Cys-282 has a significant effect on nucleotide binding (Maggio et al., 1977) (Vandest et al., 1980) and is not involved in creatine binding (Wang et al., 1988). Epoxycreatine fulfills all of the requirements of a classical affinity label and is also turned over by the enzyme fifteen times for every inactivation event (Marletta & Kenyon, 1979). This strongly suggests that it binds to the creatine binding site, in a manner similar to creatine itself, and therefore that Cys-282 lies near the creatine binding site. The epoxypropyl group is considerably longer than the N-methyl group of creatine (~4-4.5 Å *vs* ~1.4 Å for total C-C and C-N bond lengths). It is conceivable that this group has a certain amount of conformational flexibility and may be able to partially overlap the nucleotide binding site. If this is the case, Cys-282 may lie more at the interface of the two

sites, perhaps in an appropriate position to mediate the conformational change and "closing down" of the active site that occurs upon substrate binding (McLaughlin et al., 1976; Reed & Leyh, 1980). This may help to explain some of the ambiguous results found in previous attempts to localize this residue. In either case, the thiol group of Cys-282 is suitably positioned with respect to enzyme-bound epoxycreatine to be within "striking" distance of the epoxide ring.

Despite the many ambiguities, the role played by Cys-282 in phosphoryl transfer is likely to be crucial for efficient catalysis, even if it is not directly involved in the chemical steps. The results presented here suggest that Cys-282 is very near the creatine binding site. The importance of such information is aptly illustrated in the case of adenylate kinase. A crystal structure of this enzyme has been available for a number of years (Schulz et al., 1974; Egner et al., 1987) and extensive NMR studies have been carried out to investigate the binding of substrates (Fry et al., 1985; Honggao et al., 1990; Mildvan & Fry, 1987). Despite this wealth of information, there is still considerable controversy regarding the exact location of the substrate binding sites (Shyy et al., 1987; Fry et al., 1988; Vetter et al., 1990) and the roles which individual amino acid residues play in catalysis (Honggao et al., 1990; Diederichs & Schulz, 1990). One reason for this is that it has been difficult to co-crystallize adenylate kinase with substrates. Much of the crystallographic work has been done with the enzyme complexed with Ap5A, a bisubstrate inhibitor of the enzyme. This can be a general problem with many enzymes; substrates are often turned over many times during the time course of experiments to measure their binding. An "average" of the enzyme·substrate and enzyme·product complexes is then obtained. If the equilibrium constant between these species is close to one, as is often the case (Knowles, 1980), this can make interpretation of such experiments difficult. For this reason, substrate analogues that are not catalytically competent, such as Ap5A, are often used to investigate the binding of substrates. This is a valuable technique, but it cannot be guaranteed that such analogues will bind in the same manner as substrates. Results obtained with such inhibitors must be

viewed with caution and confirmed by additional investigations, perhaps with other inhibitors.

The above discussion highlights the importance of substrate analogues, such as epoxycreatine, in the evaluation of the binding of substrates from crystal structures. The results presented here are likely to be useful when a crystal structure of CK becomes available. In particular, crystallization of the enzyme with epoxycreatine covalently bound in the active site may give important insights into the binding mode of creatine and the positions of active site residues relative to bound substrates. The full elucidation of the role of Cys-282, and other active site residues, in catalysis by creatine kinase may have to wait until such a structure is available and the numerous experiments which it may suggest are performed and fully evaluated.

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Appendix A

Purification of Benzoylformate Decarboxylase Expressed in *Escherichia coli*

I. Introduction

The mandelate pathway of *Pseudomonas putida* (ATCC 12633) (Fig. A1) enables growth of this microorganism on either (*R*)- or (*S*)-mandelate as sole carbon source (Gunsalus et al., 1953). The first enzyme in this pathway, mandelate racemase (MR), catalyzes the interconversion of the two enantiomers of mandelic acid. (*S*)-Mandelate, but not the *R* isomer, is oxidized to benzoylformate by the next enzyme in the pathway, an (*S*)-specific mandelate dehydrogenase (MDH). Benzoylformate is subsequently decarboxylated to benzaldehyde by benzoylformate decarboxylase (BFD). Benzoic acid, obtained from the oxidation of benzaldehyde by either an $NADP^+$- or an NAD^+-dependent benzaldehyde dehydrogenase, is then converted ultimately to acetyl-CoA and succinyl-CoA *via* the β-ketoadipate pathway (Ornston & Stanier, 1966).

BFD was first purified to homogeneity from *Pseudomonas putida* by Hegeman (Hegeman, 1970). The enzyme requires thiamine pyrophosphate as a cofactor. No other cofactors appear to be necessary. The specific activity of the purified enzyme was reported to be 193 U/mg (Hegeman, 1970). Later purifications (Reynolds et al., 1988) resulted in homogeneous enzyme with a specific activity of only 34 U/mg. This discrepancy is briefly discussed later. The enzyme electrophoreses in denaturing polyacrylamide gels at an apparent molecular weight of 57,500 (Reynolds et al., 1988). It is not known whether the enzyme is a multimer, although it was originally reported (Hegeman, 1970) that the enzyme

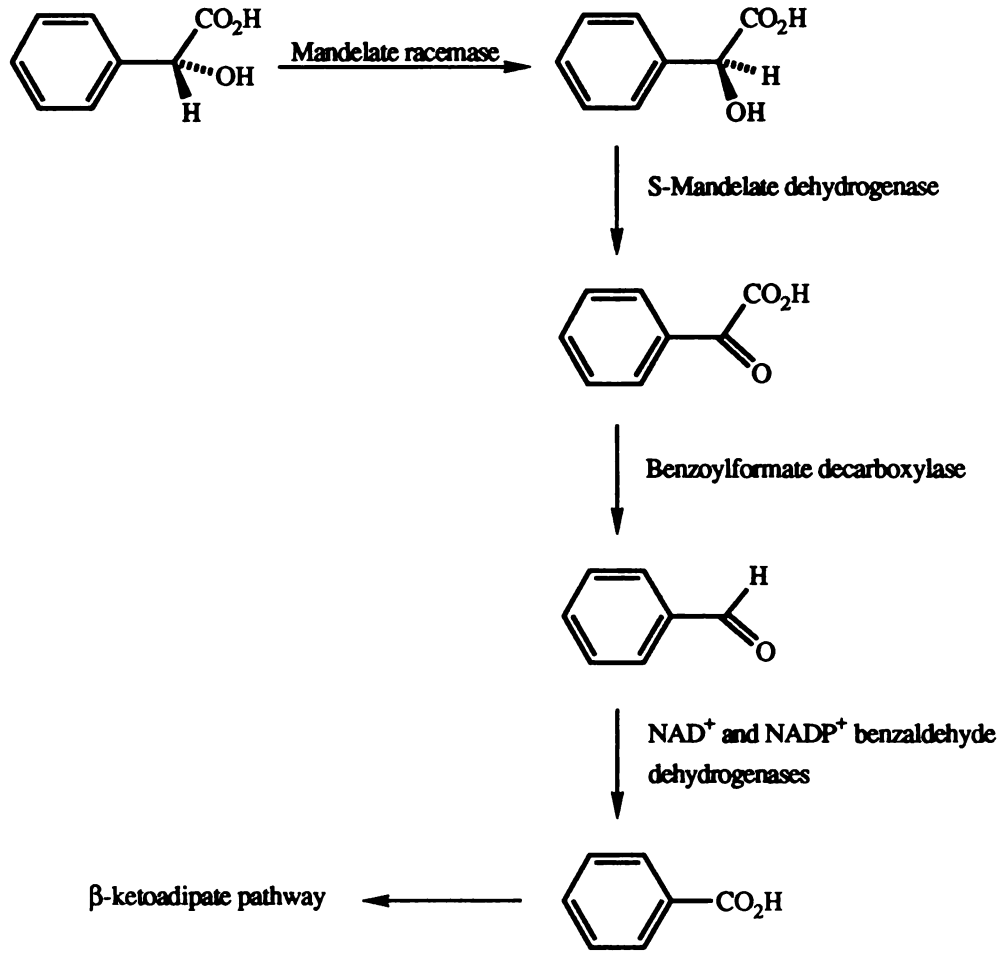

Figure A1. The mandelate pathway of *P. putida* ATCC 12633.

runs on Sephadex columns at an apparent molecular weight of 80,000. The molecular weight predicted from the cDNA sequence is 53,600 (Tsou et al., 1990). Thus, the enzyme is evidently running on these columns as the dimer.

The mandelate pathway is inducible by either racemic mandelic acid or benzoylformic acid (Hegeman, 1970), and the genes for all of the enzymes are coordinately regulated (Hegeman, 1966). A 10.5-kb restriction fragment of *P. putida* chromosomal DNA that encodes the genes of the pathway has been cloned (Tsou et al., 1990). The first three enzymes of the mandelate pathway (MR, MDH, and BFD) are encoded on an operon.

At least one of the genes for the benzaldehyde dehydrogenases is close by but is independently transcribed. The location of the gene for the second benzaldehyde dehydrogenase is not yet known.

As part of an ongoing project to characterize fully each of the enzymes and genes in the mandelate pathway, a subfragment of the 10.5-kb restriction fragment that contains the gene for BFD has been subcloned into the expression vector pKK233-2 (Tsou et al., 1990). This vector utilizes the inducible *trc* promoter and contains an ampicillin resistance gene. The present work describes the expression and purification of BFD from *E. coli* transformed with the pKK233-2 vector with the BFD gene insert (pBFDtrc). Comparison of the enzyme so purified to that purified from *P. putida* has confirmed that pBFDtrc encodes for authentic benzoylformate decarboxylase.

II. Experimental

A. Reagents and Materials

IPTG was from Boehringer Mannheim, Indianapolis, IN. Horse liver alcohol dehydrogenase, thiamine pyrophosphate chloride, and lysozyme were from Sigma Chemical Co., St. Louis, MO. S-200 Sephacryl and DEAE-Sephacel were from Pharmacia LKB Biotechnology, Piscataway, NJ. Benzoylformic acid was from Aldrich Chemical Co., Milwaukee, WI. All other reagents were obtained as described in previous chapters.

B. Purification of Benzoylformate Decarboxylase from E. coli

A single colony of *E. coli* JM105 transformed with pBFDtrc growing on LB agar with 50 mg/ml ampicillin was used to inoculate 1 ml of LB medium containing 50 mg/ml

ampicillin (LB/amp medium). After overnight growth at 37 °C, two 15 µl LB/amp medium cultures were each inoculated with 500 µl of this starter. After growth at 37 °C for 4 hours, each 15 ml culture was used to inoculate 1500 ml of LB/amp medium in 2800 ml Fernbach flasks. Broths were grown at 37 °C until the OD_{590} reached 1.0, at which time they were induced by the addition of IPTG to 0.2 mM. Growth was continued for an additional 2 hours and the cells harvested by centrifugation at 5000 x g for 20 min. Wet cells were weighed and stored overnight at -20 °C. All of the following steps were performed at 4 °C unless otherwise indicated. All of the buffers used in the purification contained 0.1 mM PMSF, except those in the FPLC step. The cells (12.4 g) from 3 liters of culture were thawed and suspended in 75 ml of 50 mM sodium phosphate buffer, pH 7.0. To the suspended cells was added 1.24 ml of a 10 mg/ml solution of lysozyme in 50 mM sodium phosphate buffer, pH 7.0. After stirring for 2 hours, the resulting suspension was sonicated for 8 cycles of 3 min each. The sonicated mixture was taken to 130 ml total volume with 50 mM sodium phosphate buffer, pH 7.0, and centrifuged at 100,000 x g for 60 min. The supernatant was decanted and the pellet discarded.

Solid ammonium sulfate sufficient to give 50% saturation (39.5 g) was added to the supernatant (126 ml) over a 10 min period. After stirring for 60 min, the suspension was centrifuged at 10,000 x g for 20 min. An additional 24.6 g of solid ammonium sulfate (to give 75% saturation) was added, over 15 min, to the supernatant (140 ml). This mixture was stirred for 90 min, followed by centrifugation at 10,000 x g for 20 min. The pellet was resuspended in 28 ml of 100 mM sodium phosphate buffer, pH 6.0, and heated in a water bath at 55 °C for 10 min. This mixture was cooled in an ice bath and centrifuged at 15,000 x g for 15 min. The pellet was discarded and the supernatant dialyzed overnight against two changes of 2 liters each of 50 mM sodium phosphate buffer, pH 6.0.

The dialyzed material (34 ml) was clarified by centrifugation at 15,000 x g for 15 min and then loaded onto a column of 185 ml of DEAE-Sephacel, previously equilibrated in 50 mM sodium phosphate buffer, pH 6.0. The column was eluted at 1 ml/min with a

linear gradient from 0 to 0.5 M NaCl in 50 mM sodium phosphate buffer, pH 6.0. Fractions (7 ml) were assayed for BFD activity and those containing greater than 3 U/ml were combined and concentrated to 2.1 ml by ultrafiltration using an Amicon XM-50 membrane. The concentrated BFD-containing solution was loaded onto a column of 465 ml of S-200 Sephacryl and eluted at 14 ml/hour with 50 mM sodium phosphate buffer, pH 6.0. Fractions (4.5 ml) containing greater than 1.5 U/ml of BFD activity were combined and concentrated to 3.5 ml by ultrafiltration (XM-50 membrane).

The enzyme was further purified by FPLC using a Mono Q (Pharmacia) column. Following filtration to remove debris, the enzyme-containing solution was chromatographed using a 110 min linear gradient of 150-400 mM NaCl in 50 mM sodium phosphate buffer, pH 6.0. Fractions containing BFD activity were analyzed by SDS-PAGE, and those that were the most electrophoretically homogeneous were combined and concentrated by ultrafiltration to ~1 mg/ml (XM-50 membrane). The enzyme was stored at 4 °C.

C. N-terminal Sequence Analysis

FPLC-purified BFD (200 pmol) was prepared as described in Chapter 2 and submitted for gas phase N-terminal sequencing to the Biomolecular Resource Center, University of California, San Francisco.

D. Assay of Benzoylformate Decarboxylase

Benzoylformate decarboxylase was assayed by the horse liver alcohol dehydrogenase (LADH) coupled assay as described previously (Weiss et al., 1988). This assay follows the change in absorbance at 340 nm due to the loss of NADH in the following coupled system:

$$\text{benzoylformate} \xrightarrow{\text{benzoylformate decarboxylase}} \text{benzaldehyde}$$

$$\text{benzaldehyde} + \text{NADH} \xrightarrow{\text{horse liver alcohol dehydrogenase}} \text{benzyl alcohol} + \text{NAD}^+$$

Assay mix was prepared by adding to 25 ml of 100 mM sodium phosphate buffer, pH 7.0, the following components:

NADH (disodium salt)	5.4 mg
TPP chloride	11.7 mg
LADH (horse liver, 1.6 U/mg)	32 mg

A solution (1.5 mg/ml) of benzoylformic acid in water was also prepared.

The assay was performed by mixing 490 µl of the assay mix with the appropriate amount of BFD (usually ~0.05-0.10 units) in a 0.5 ml masked cuvette with a 1 cm pathlength. The absorbance at 340 nm was monitored and the rate of change between 3 and 25 seconds recorded. This value was used for the non-benzoylformate dependent oxidation of NADH and was only significant with crude cell extracts. Benzoylformic acid solution (10 µl) was added and a second rate determined in the same manner. The two rates were subtracted to determine the benzoylformic acid-dependent rate. International units (U), in µmol/min, were calculated as in the spectrophotometric assay of creatine kinase described in Chapter 2. All assays were performed at 25 °C on a Hewlett Packard 8452A diode array spectrophotometer.

E. Electrophoresis and Protein Assays

SDS-PAGE and Lowry protein determinations were performed as described in Chapter 2 and Appendix B.

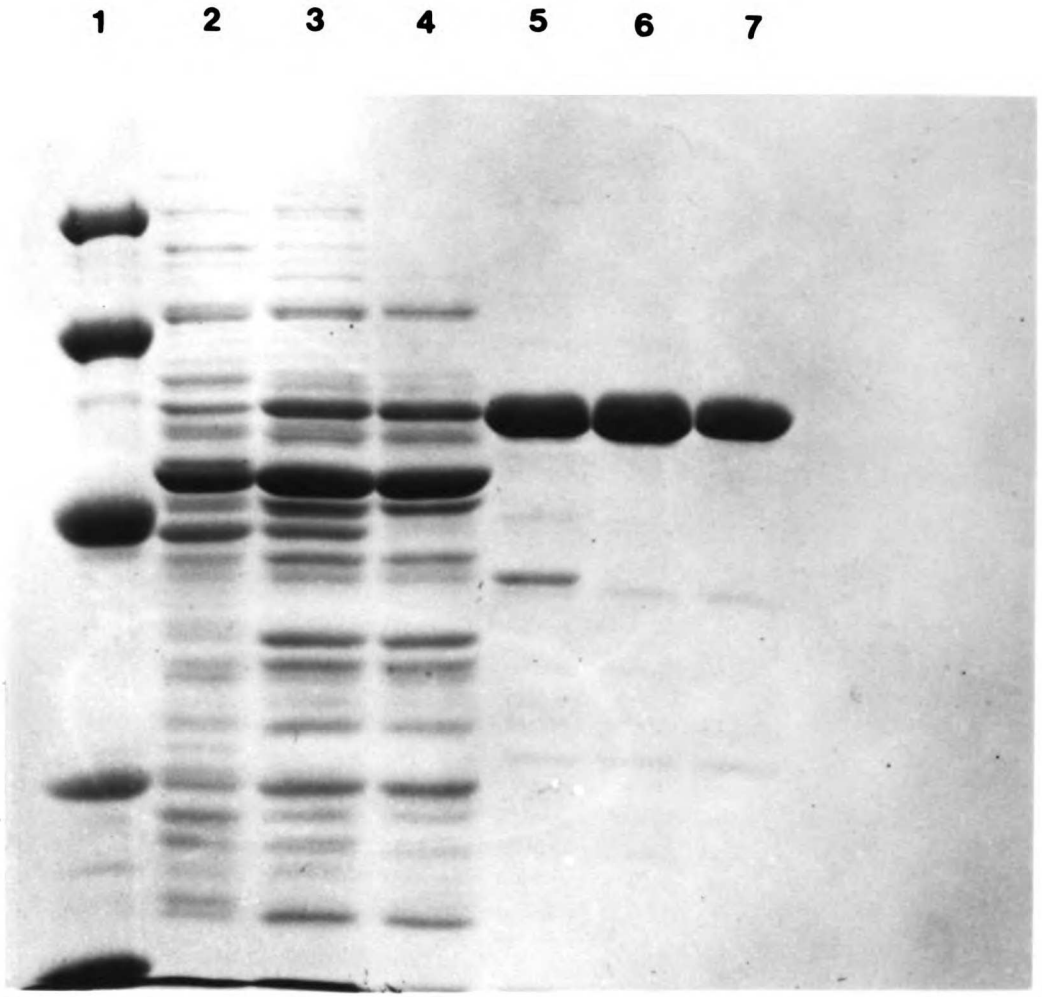

Figure A2. Photograph of a 10% SDS-PAGE gel showing the purification of benzoylformate decarboxylase from *E. coli* transformed with pBFDtrc. Lane 1, Molecular weight standards; lane 2, crude extract; lane 3, 75% $(NH_4)_2SO_4$ pellet; lane 4, heat treatment supernatant; lane 5, after DEAE-Sephacel; lane 6, after Sephacryl S-200; lane 7, after FPLC.

Table A1: Summary of the Purification of BFD from pBFDtrc Transformed *E. coli*

Purification Step	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (U/mg)
Crude Extract	130	756	4662	6
$(NH_4)_2SO_4$ (75% sat.) pellet	28	280	4200	15
heat treatment supernatant	34	240	4200	17
DEAE-Sephacel	2.1	12	2262	189
Sephacryl S-200	3.5	10	1074	107
Mono Q*	--	3.2	148	46

*Calculated from the chromatography of a single 38 µl aliquot of the Sephacryl S-200 purified enzyme.

III. Results and Discussion

Given in Table A1 is a summary of the purification of benzoylformate decarboxylase from *E. coli* transformed with pBFDtrc. Fig. A2 is a photograph of a 10% SDS-PAGE gel of the various steps of the purification. The purification followed the purification from *Pseudomonas putida* described previously (Hegeman, 1970). In this case, however, it was found that treatment of the crude extract with manganese chloride to remove nucleic acids abolished all activity. This step was subsequently omitted without any detrimental effect. A final FPLC step was also included in the purification. This was particularly effective in removing a small amount of contaminant which migrated at a very similar molecular weight to BFD on SDS-PAGE.

The enzyme purified from pBFDtrc transformed *E. coli* is greater than 95% homogeneous as judged by SDS-PAGE (Fig A2). The molecular weight estimated from SDS-PAGE is 57,000. This agrees with that found by SDS-PAGE for the enzyme purified

from *P. putida* (Reynolds et al., 1988), although the molecular weight predicted from the DNA sequence is 53,600 (Tsou et al., 1990). The first eight N-terminal residues (Ala-Ser-Val-His-Gly-Thr-Thr-Tyr) are those predicted from the sequence of the gene encoded by pBFDtrc, if the N-terminal methionine is removed by *E. coli*, and exactly match those previously determined for the pure enzyme from *P. putida* (Garcia, 1987). The FPLC-purified enzyme has a specific activity of 46 U/mg. This is comparable to the 34 U/mg found for pure enzyme from *P. putida* (Reynolds et al., 1988). As shown in Table A1, the specific activity of the enzyme after the anion exchange step is 189 U/mg. This is considerably higher than that of the final FPLC-purified enzyme and is similar to that originally found by Hegeman (Hegeman, 1970). It is not known at this time why the specific activity drops during the purification; however, it is known (Weiss et al., 1988) that the enzyme rapidly loses activity upon dilution. Possibly, this is the result of the dissociation of a dimer to a less active monomer. Although enzyme-containing fractions were concentrated as quickly as possible, it is possible that the enzyme is diluted and loses activity while being chromatographed. Attempts to prevent this loss of activity and obtain enzyme sufficiently pure for x-ray crystallography are underway. It should be noted that the preparation following the DEAE-Sephacel purification step is sufficiently pure for most purposes and retains the highest specific activity (189 U/mg).

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Appendix B

Recipes of Buffers and Solutions

I. Gel Electrophoresis

A. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

Stacking Gel Buffer (4x stock)	
Tris (free base)	0.5 M
SDS	0.4%
pH to 6.8 with AcOH (25 °C)	

Resolving Gel Buffer (4x stock)	
Tris (free base)	1.5 M
SDS	0.4%
pH to 8.7 with AcOH (25 °C)	

Running Buffer (1x)	
Glycine	0.19 M
Tris (free base)	0.025 M
SDS	0.1%
pH to 8.3 with AcOH (25 °C)	

2x Laemmli Sample Buffer	
Tris (pH 6.8)	0.0625 M
SDS	2.3%
β-mercaptoethanol	5%
Glycerol	10%

30% Acrylamide/Bis	
Acrylamide	30%
Bis	0.8%
Filter and store in the dark at 4 °C	

Sample Dye (1.0 ml)		
Sucrose		0.5 g
1% Bromphenol Blue		100 µl
H_2O	qs	1.0 ml

B. Native Polyacrylamide Gel Electrophoresis

Stacking Gel Buffer	
Tris (free base)	500 mM
pH to 6.8 with HCl (25 °C)	

Resolving Gel Buffer	
Tris (free base)	3 M
pH to 8.8 with HCl (25 °C)	

Running Buffer

Tris (free base)	25 mM
Glycine	192 mM

pH should be ~8.4 (25 °C)

II. Purification Buffers

Extraction Buffer

Tris (free base)	50 mM
β-mercaptoethanol	5 mM
EDTA (disodium salt)	5 mM
PMSF	0.1 mM
GEMSA	0.2 mM

pH to 7.4 with AcOH (4 °C)

MES Buffer

MES (potassium salt)	10 mM
KCl	40 mM
DTT	1 mM
EDTA (disodium salt)	5 mM
PMSF	0.1 mM
GEMSA	0.2 mM

pH to 6.0 with HCl (4 °C)

TES Buffer

TES (sodium salt)	10 mM
KCl	40 mM
DTT	1 mM
EDTA (disodium salt)	5 mM
PMSF	0.1 mM
GEMSA	0.2 mM

pH to 8.0 with HCl (4 °C)