Biochemical Characterization of Fibrinolysin-Cleaved Ovine Pituitary Prolactin

by

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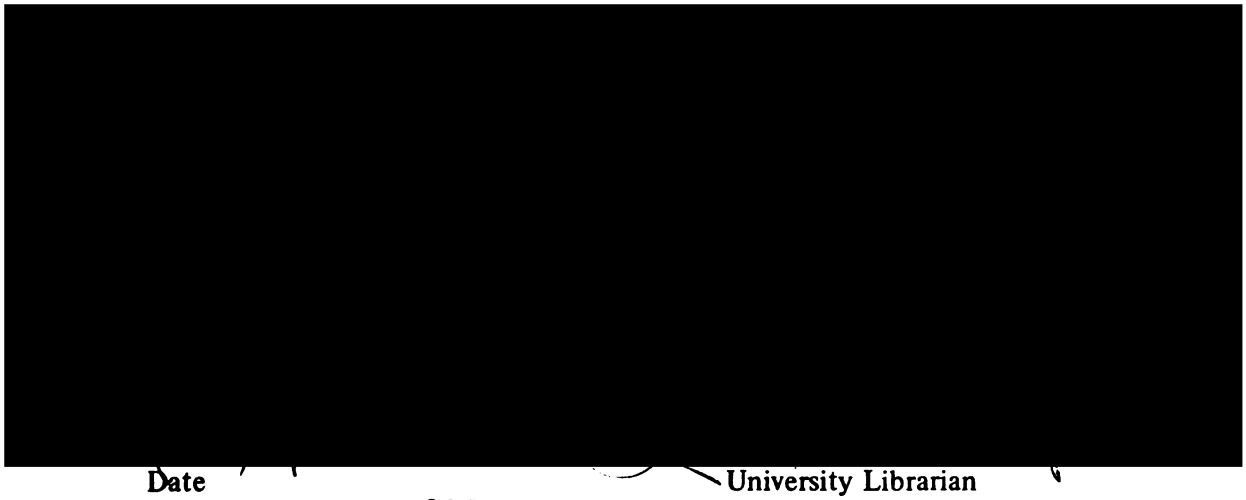

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BIOCHEMICAL CHARACTERIZATION OF FIBRINOLYSIN-CLEAVED OVINE PITUITARY PROLACTIN

Michael Dell Jibson

Ovine prolactin has been found to undergo specific cleavage of the peptide bond between Met-53 and Ala-54 when subjected to limited digestion by bacterial fibrinolysin (EC 3.4.21.7). The fragments created, oPRL-(1-53) and oPRL-(54-199), were purified by exclusion and ion exchange chromatography, and characterized by molecular weight determination, amino acid and end-group analysis. oPRL-(54-199) was found to exist as a dimer in solution. Although each fragment was found by circular dichroism to have a well-defined conformation, neither fragment had biological, immunological, or receptor-binding activity. Recombination of the fragments restored much of the native prolactin structure, and the recombinant had full immunological, and 13% receptor-binding activity. Fluorescein-isothiocyanate was used to label oPRL-(1-53), and a dissociation constant of 0.144 μM was measured at 30°C for the recombined fragments by fluorescence polarization. Frontal analysis was used to measure the dissociation constants at 2°C and 23°C. In addition, frontal analysis was used to measure the monomer-dimer equilibrium constants at 2°C and 23°C. Determination of equilibrium constants at these temperatures allowed the calculation of ΔH and $T\Delta S$

for both the dimerization and recombination reactions. At 23°C ΔH was 5.78 kcal/mol for oPRL-(54-199) monomer formation, and -0.06 kcal/mol for the recombination. Under the same conditions, the values of $T\Delta S$ were -2.68 kcal/mol and 13.42 kcal/mol. It was apparent from these measurements that the recombination reaction was driven by the entropic forces of hydrophobic bonds and charge-charge interactions, while the major contribution to dimer formation was from hydrogen bonds and vanderwaals forces. The kinetics of fragment recombination were followed by fluorescence polarization and intensity. Changes in molecular weight followed second-order kinetics, with a rate constant of 2.80×10^5 M^{-1}-sec^{-1}. Fluorescence intensity changes showed equivocal kinetic plots, with rates 10 x lower than for the recombination. These findings suggest that the rate-limiting step in the overall recombination reaction is binding of the two fragments, rather than oPRL-(54-199) monomer formation, or a conformational change in the recombining peptides.

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ABBREVIATIONS

Ac	acetate
BSA	bovine serum albumin
ch	chapter
CD	circular dichoism, circular dichroic
DEAE	diethylaminoethane
DTT	dithiothreitol
FITC	fluorescein isothiocyanate (isomer I)
FTC-	fluorescein isothiocarbamyl-
GH	growth hormone, pituitary somatotropin
HCS	human chorionic somatomammotropin
HGH	human growth hormone
β-LPH	β-lipotropin
MRW	mean residue weight
$NaDodSO_4$	sodium dodecyl sulfate
oPRL	ovine pituitary prolactin
ORD	optical rotatory dispersion
PCS	phase combining system
PRL	pituitary prolactin
RCAM	reduced carbamidomethylated
rec	recombined, recombinant
RIA	radioimmunoassy
RRA	radioreceptorassay
Tris	tris-(hydroxymethyl)aminomethane
UV	ultraviolet

CHAPTER 1

PROLACTIN STRUCTURE AND FUNCTION

A. THE CHEMISTRY OF PROLACTIN

The existence of a pituitary factor with lactogenic activity was first demonstrated by Stricker & Grueter (1928), who used bovine pituitary extracts to stimulate milk production in the pseudopregnant rabbit. This finding was confirmed by Corner (1930) using sheep pituitary extracts on similarly treated animals. The widespread distribution of this factor was hinted by the work of Riddle *et al*. (1932), who found that a fraction of bovine pituitary extracts could stimulate the growth of the pigeon crop sac. The determination that this response was both specific and dose-dependent (Riddle *et al*., 1933) suggested that a similar factor was present in avian as well as mammalian species. Since that time pituitary extracts from all vertebrate classes have been shown to possess activity when tested in the crop sac (Bern, 1975). The name prolactin, suggested by Riddle *et al*. (1932), has gained almost universal acceptance for this factor, although the terms mammotropin (Lyons & Catchpole, 1933), galactin (Gardner & Turner, 1933), lactogen (Reece & Turner, 1937), and leutropin (Astwood, 1941) have also been proposed. More recently, the name paralactin has

been suggested for the fish hormone, following the observation that the crop sac response to fish PRL was qualitatively different from that of rat or toad PRL (Chadwick, 1970).

ISOLATION. Highly purified preparations of PRL were first isolated from ovine pituitaries in "crystalline" form (White et al., 1937), while Lyons (1937) outlined a procedure involving precipitation of the hormone from either ovine or bovine pituitary extracts. These techniques were subsequently modified (Li et al., 1942; Cole & Li, 1955; Li et al., 1970) in order to increase the purity and specific activity of the final product.

Since the initial work involving pituitaries from sheep and cattle, prolactin has been isolated, in varying degrees of purity, from the pig (Epstein, 1964), rat (Ellis et al., 1969), human (Lewis et al., 1971), chicken (Scanes et al., 1975), dog (Papkoff, 1976), fish (Farmer et al., 1975; Farmer et al., 1977), chinchilla (Jibson & Talamantes, 1978), turkey (Burke & Papkoff, 1980), salmon (Idler et al., 1978), fin whale (Kawauchi & Tubokawa, 1979), kangaroo, wallaby, oppossum (Farmer et al., 1981), mouse (Kohmoto, 1975; Shoer et al., 1978), amphibians (Hayashida et al., 1973), monkey (Guyda & Friesen, 1971), and goat (McNeilly & Andrews, 1974).

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named for its lactogenic activity, prolactin has been shown to possess a wide variety of functions among vertebrates (Riddle, 1963; Bern & Nicoll, 1968; Ball, 1969; Nicoll & Bern, 1972; Ensor, 1978). The specialization of PRL function appears to be characteristic of individual vertebrate classes, and in some cases species, without any apparent underlying theme (Nicoll & Bern, 1972).

Among fishes, most known functions involve osmoregulation (Ensor, 1978), although this generalization does not take into account the variations in the specific osmoregulatory role of PRL among different teleost species (Bern, 1975). In addition, PRL has been implicated in the control of fat metabolism, pigmentation, thyroid function, and parental behavior (Ensor, 1978).

PRL action among amphibians parallels, to a large extent, that noted for fishes, however, additional effects have been reported on metamorphosis and growth. Ensor (1978) has suggested that an increase in the number of PRL functions has occurred through evolution, with additional activities developing in higher species. Although this line of reasoning implies that "lower" species evolve more slowly than "higher", the pattern does appear consistent with observations to this point.

Studies on PRL function in reptiles indicate the importance of somatotropic and metabolic activities, as well as the established role in osmoregulation. In

addition, an anti-gonadotropic activity has been noted.

The first indication of a lactogenic activity for PRL occurred among birds, with the stimulation of crop "milk" in some species. Additional functions related to reproduction in these species include formation of the brood-patch, parental behavior, and a synergistic effect, with estrogens, on the ovary. Migratory functions have also been noted, such as fat deposition, feather growth, and behavioral changes.

Mammary growth and development and stimulation of lactation are the best-known functions of prolactin among mammals. In addition, effects have been noted on the male reproductive system, on lipid metabolism, osmoregulation, and parental behavior. PRL has been shown to be involved in the reproductive cycle of some species, with luteotropic and luteolytic activity noted, depending on the presence of other hormones.

CRITERIA OF PURITY. Although Li et al. (1957) presented sedimentation data indicating that their preparation of oPRL was monodisperse, evidence of molecular heterogeneity subsequently came to light. Using countercurrent distribution, Cole & Li (1958) found that in some solvent systems three active components of oPRL could be isolated. These same investigators (Cole & Li, 1959) also used electrophoresis to isolate several fractions of oPRL, all of

which were active in the pigeon crop sac and had identical end-groups.

One source of heterogeneity was investigated by Squire et al. (1963), who found conditions under which oPRL underwent either reversible or irreversible aggregation, and by Andrews (1966), who noted a concentration-dependent aggregation of the hormone. It was subsequently reported that the degree of aggregation in these studies had no effect on crop sac stimulating activity (Li & Flux, 1964), and Wallis et al. (1980) found rat PRL dimers and aggregates with full immunoreactivity. Similar size differences were reported in preparations of human PRL (Suh & Frantz, 1974) accompanied by data suggesting reduced biological activity for the larger forms and evidence of secretion in vivo (Garnier et al., 1978). Other investigators, however, felt that these "big" and "big-big" human PRL's were artifacts of isolation (Fang et al., 1978). In each of these studies the large-molecular-weight variants were found to be dimers or aggregates rather than prehormone forms.

Several electrophoretic components of oPRL have been reported on starch gels (Samuelsson & Li, 1964) and by isoelectric focusing (Houghton & Li, 1978). Studies of the ovine (Lewis et al., 1970) and bovine (Graf et al., 1970) hormones indicated that these forms may be due to progressive deamidation of the molecules.

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PHYSICAL PROPERTIES. Ovine and bovine PRL could not be separated by free-boundary electrophoresis, and both molecules were characterized by an isoelectric point of 5.73 (Li et al., 1940a). Porcine PRL was shown to differ slightly, with an isoelectric pH of 5.85 (Bewley & Li, 1975a), and the salmon hormone had pI=6.0 (Idler et al., 1978).

The sedimentation behavior of ovine (Squire et al., 1963) and porcine (Bewley & Li, 1975a) PRL has been examined, and in each case a sedimentation coefficient of 2.18 S was determined. When combined with partial specific volumes of 0.721 cm^3/gm for oPRL (Li, 1942) and 0.732 cm^3/gm for the porcine hormone (Bewley & Li, 1975a), respective molecular weights of 23,300 and 22,400 were established. The Stokes radius of each molecule has been measured by gel filtration and in each case was found to be 24.9 A at pH 8.2 (Bewley & Li, 1975a).

The solubilities of ovine and bovine PRL were investigated by Li et al. (1941a) using NaCl solutions. Both molecules behaved as pure substances, but showed somewhat different solubility behavior as ionic strength was varied. A similar comparison of ovine and porcine PRL in ammonium sulfate solutions likewise showed differences in the properties of the two hormones (Bewley & Li, 1975a). These findings suggested that, in spite of similarities in size and amino acid composition, each of these molecules

has a characteristic surface structure, reflected in solubility properties.

AMINO ACID SEQUENCE. The complete amino acid sequence of oPRL is shown in Fig. 1 (Li et al., 1970; Li, 1976). The molecular weight of the molecule, based on the sequence, is 22,674 at its isoelectric point. Primary structures have also been determined for porcine (Li, 1976), bovine (Wallis, 1974), and human PRL (Shome & Parlow, 1977), and a partial sequence of PRL from the fin whale has been proposed (Kawauchi & Tubokawa, 1979). In addition, gene sequences for rat (Cooke et al., 1980; Chien & Thompson, 1980; Gubbins et al., 1980) and ox (Miller et al., 1981) PRL have been presented.

A number of comparisons have been made involving the sequences of oPRL, HGH, and HCS (Bewley & Li, 1970; Bewley & Li, 1971a; Niall et al., 1971; Dayhoff, 1972) and ovine GH (Bewley et al., 1972), as well as comparisons of ovine and porcine PRL (Li, 1976). Each of these studies has indicated that similarities in PRL and GH primary structures suggest their evolution from a common precursor, but attempts to locate residues crucial to biological activity have not been successful. In addition, very detailed evolutionary schemes for PRL and GH have not been consistent with one another (Miller et al., 1981).

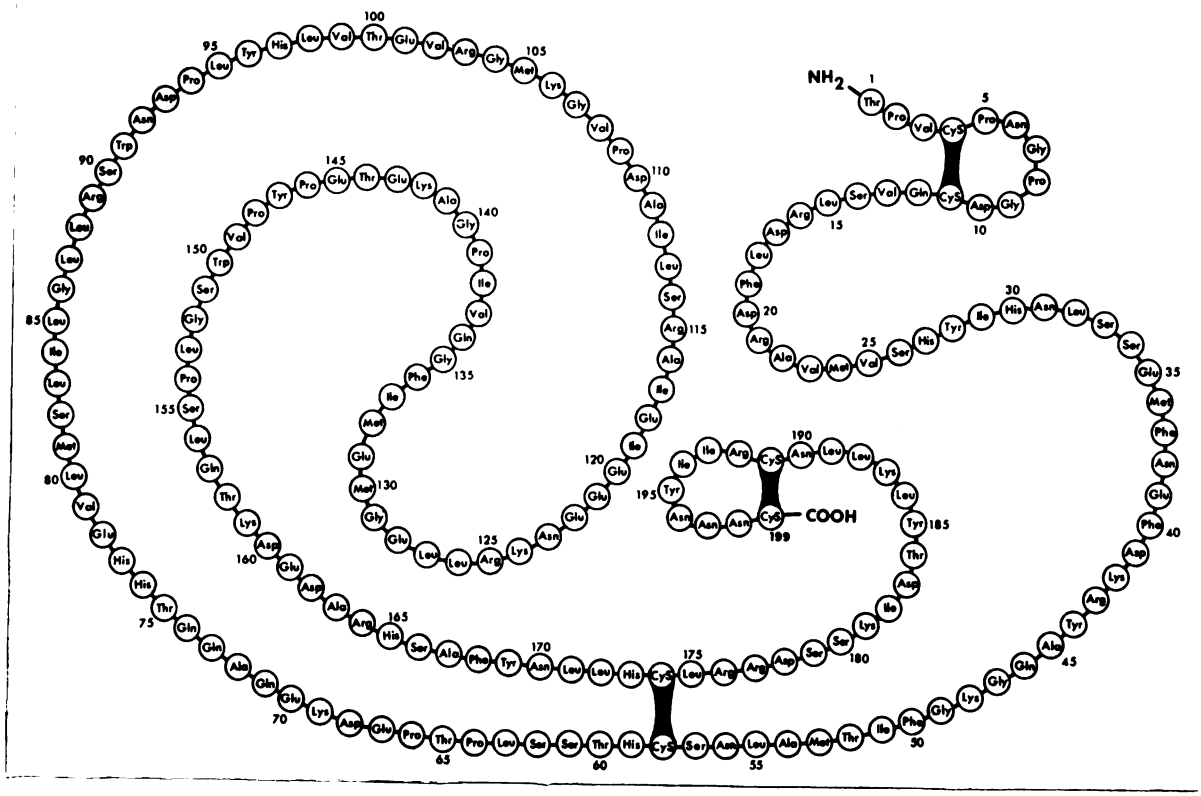

Fig. 1. The amino acid sequence of oPRL.

POST-TRANSLATIONAL PROCESSING. The sequence of nucleotides constituting the oPRL structural gene includes codons for 26 amino acids not found with the isolated hormone, located at the amino terminus of the protein (Cooke et al., 1980; Chien & Thompson, 1980; Gubbins et al., 1980). Mauren & Mckean (1978) also found an extended PRL chain synthesized by rat pituitary cells in culture following a 3-minute labeling procedure. After 10 minutes, however, only the shorter sequence was present. Using a cell-free system these same investigators were able to induce synthesis of the longer chain, but found that it was selectively cleaved to the shortened form following addition of the membrane fraction of dog pancreas. These findings give evidence for the synthesis of a pre-PRL chain in the rat, which is then enzymatically converted to PRL. The gene sequence found for bovine PRL similarly codes for a pre-hormone region of at least 10 amino acids, but the length of the complete sequence was not determined (Miller et al., 1981).

In addition to the conversion of pre-PRL to PRL, Farmer et al. (1976) found evidence for conformational differences between the stored and secreted forms of the rat hormone. Although no discrepancies were noted in amino acid composition or end-group between the two forms, both biological and immunological activities were somewhat higher in the stored material, and the tryptophan region

of the CD spectra showed a marked difference. Comparisons of biologic and immunologic activities between stored and secreted rat PRL (Asawaroengchai & Nicoll, 1977) and among the electrophoretic components of stored and secreted rat PRL have also been shown to differ significantly (Asawaroengchai et al., 1978), and to change in response to sex and age of donor, and period of incubation.

The synthesis and secretion of a cleaved form of PRL by rat pituitary cells was reported by Mittra (1980a). This material, consisting of the 16000 dalton N-terminal and 8000 dalton C-terminal regions of PRL held together by a single disulfide bond, was found both in fresh pituitary glands and secreted by the gland in vitro. The relative concentrations of cleaved and intact PRL appeared to be correlated to physiological and pharmacological conditions. In a companion study (Mittra, 1980b), the isolated N-terminal fragment was found to induce a significant increase in the rates of DNA synthesis and cell division of mammary epithelial cells, while intact rat PRL was ineffective. In consequence of these findings, Mittra (1980b) has proposed that cleaved and intact PRL may have separate effects on the mammary gland, the intact material being exclusively lactogenic and the cleaved hormone mitogenic.

SPECTROSCOPY. The importance of the ultraviolet absorp-

tion spectrum of proteins as a means of studying chromophoric residues and determining concentration has been discussed by Bewley (1977) in relation to HCS. The absorptivity ($E^{0.1\%}$) of oPRL was determined to be 9.09 at 277 nm (Li et al., 1970) on the basis of amino acid analysis, and the same value has been used for the porcine (Bewley & Li, 1975a) and whale hormones (Kawauchi & Tubokawa, 1979). In contrast, an absorptivity value of 5.54 at 277 nm was reported for tilapia PRL (Farmer et al., 1977), the lower value being attributable to the presence of a single Trp residue rather than the two found for most PRL's.

Difference absorption spectra of both ovine (Kawauchi et al., 1973) and porcine (Bewley & Li, 1975a) PRL at pH 8.2 vs. pH 4 showed a weak blue-shift minimum at 290 nm with shoulders at 298 nm and 280 nm, and spectra taken in 50% acetic acid showed larger peaks at the same wavelengths. Similar blue-shifts were also observed for porcine PRL in 6 M guanidine-HCl at pH 8.2 (Bewley & Li, 1975a).

Spectroscopic titrations of oPRL at 292 nm (Aloj & Edelhoch, 1970) and 295 nm (Ma et al., 1970) indicated that 6 of 7 Tyr residues were ionized, with pK=11.2-11.3. A similar titration of porcine PRL gave a pK of 11.15 and a total of 6 Tyr residues ionized, with pK=10.2-10.3 (Ma et al., 1970).

The fluorescence spectrum of oPRL has been examined in neutral, acidic (Aloj & Edelhoch, 1970; Bewley & Li, 1975a), and alkaline conditions (Kawauchi et al., 1976). At neutral pH, the excitation maximum occurred at 292 nm and was attributed primarily to Trp. The emission maximum was found to be 336.5 nm. Transfer of the protein to acidic conditions resulted in an emission peak shift to 340 nm, and an increase of ~50% in quantum yield. At pH 12, oPRL lost 50% of its quantum yield as compared to neutral pH. The porcine hormone was found to exhibit similar properties when examined at neutral and acid pH, with an emission maximum at 337.9 nm (Bewley & Li, 1975a).

The circular dichroism spectrum of oPRL under neutral conditions indicated an α-helix content of 50-60% (Aloj & Edelhoch, 1970; Bewley & Li, 1972). In the region of side-chain absorption, a single positive band occurs at 297-298 nm and a negative plateau consisting of poorly resolved bands appeared between 260nm and 280 nm. In acidic medium, Bewley & Li (1972) reported a 15-25% decrease in intensity below 250 nm, and the α-helix content was found to drop to 40% (Aloj & Edelhoch, 1970). Little change in the spectrum was noted up to pH 11. In 5 M guanidine-HCl, most of the spectrum was lost in both near and far UV regions.

The CD spectrum of porcine PRL, at pH 8.2, was similar to the ovine hormone below 250 nm and above 290 nm

(Bewley & Li, 1975a). The porcine molecule, however, shows a rapidly increasing region of negative ellipticity from 290 nm to 250 nm. Under acidic conditions and in the presence of 6 M guanidine-HCl, porcine PRL behaves similarly to oPRL. The CD spectrum of tilapia PRL at pH 8.2 shows a near UV spectrum similar to that of porcine PRL, and an α-helix content of 65 ± 5% (Farmer et al., 1977). Fin whale PRL also yields a near UV CD spectrum similar to that of the porcine hormone, and is approximately 50% helical (Kawauchi & Tubokawa, 1979).

These spectral properties have been used in combination to examine the titration of oPRL with acid, alkali, and guanidine-HCl (Aloj & Edelhoch, 1970; Kawauchi et al., 1976). Both fluorescence and absorption spectra indicated a transition between pH 5 and pH 3 (Aloj & Edelhoch, 1970), while fluorescence and CD spectra indicated a conformational shift between pH 11 and pH 12 (Aloj & Edelhoch, 1970; Kawauchi et al., 1976). A discrepancy was noted, however, in the alkaline titration, between the fluorescence quantum yield and mean residue ellipticty at 297, 260, and 222 nm, with only the fluorescence data indicating a transition between pH 8 and pH 11 (Kawauchi et al., 1976). Titration of oPRL with guanidine-HCl, followed by absorption, fluorescence, and CD spectra, indicated a transition between 6 and 10 M at pH 8, and between 2 and 8 M at pH 5.2 (Aloj & Edelhoch, 1970).

CHEMICAL MODIFICATIONS. Early attempts to reduce the three disulfide bonds of oPRL involved complete denaturation of the protein with 10 M urea (Li & Cummins, 1958). The product of this reduction was found to be inactive in the pigeon crop sac. Bewley & Li (1969) used dithiothreitol to completely reduce oPRL at pH 8.1 in the absence of denaturants. This reaction was highly dependent on concentration of DTT and on the pH of the reaction mixture, leading to the suggestion that partial reduction of the molecule might be possible. The conditions required for partial oPRL reduction were reported by Doneen et al. (1979), who were able to selectively reduce one, two, or three of the disulfides. Cys-4-11 was most susceptible to reduction, followed by Cys-191-199. Loss of these two covalent bonds had no effect on biological activity when tested in the pigeon crop sac or mouse mammary gland, but completely abolished activity in the Gillychthys urinary bladder. This finding contrasted with an increase in fish bladder activity when only Cys-4-11 was reduced and alkylated, which could be related to the absence of an N-terminal disulfide in teleost PRL (Farmer et al., 1977).

Chymotrypsin has been used to examine the susceptibility of oPRL to loss of biological activity through enzymatic digestion. It was found that a 25% digestion of oPRL resulted in no detectable change in crop sac (Li,

1962) or immunological activity (Trenkle et al., 1963). Continuing the digestion to 50% brought about a 50% loss of biological potency and was accompanied by the appearance of fragments with molecular weights of approximately 14,000 (Sluyser & Li, 1963) and 20,700 (Sluyser & Li, 1964). These findings were interpreted as evidence for the existence of an "active core" in the PRL chain, to which the remainder of the molecule was largely extraneous. In spite of a recommendation by Niall et al. (1973), however, that studies of conformation be abandoned in favor of a search for the active core, this line of reasoning has not gained general acceptance. An alternative explanation for these findings was provided by studies on cleaved forms of rat PRL (Mittra, 1980b; Mittra, 1980), bovine GH (Forsham et al., 1958; Sonenberg et al., 1965; 1968; Nadler et al., 1967) and HGH (Yadley & Chrambach, 1973; Yadley et al., 1973; Lewis et al., 1975) that showed altered or increased biological activities, suggesting the possibility that activity of these hormones is regulated in part by in vivo proteolysis (Mittra, 1980b; Mittra, 1980).

Selective modification of Trp-150 with o-nitrophenylsulfenyl chloride (NPS-Cl) brought about large changes in the side-chain CD spectrum of oPRL, and was accompanied by a 75% loss of crop-sac activity (Kawauchi et al., 1973). With the additional modification of TRP-91,

changes were noted in both the side-chain and amide bond regions of the CD spectrum, and crop sac activity was completely abolished. Exclusion chromatography of the 2-NPS derivative indicated that this molecule had formed a dimer. Oxidation of the two Trp residues with 2-(2-nitrophenylsulfenyl)-3-methyl-3'-bromoindolemine abolished crop sac activity and was accompanied by a 50% loss of α-helix content, although immunological activity was retained (Houghton & Li, 1978). Cleavage of the oPRL molecule at the two Trp residues, and removal of the 59-residue fragment lying between them caused no further change in helix content, but destroyed immunological activity.

Partial oxidation of oPRL methionines with hydrogen peroxide modified four of the seven residues, at positions 24, 53, 105, and 130 (Houghton & Li, 1976). This derivative was indistinguishable from native oPRL when examined by rate of tryptic digestion, CD in the side-chain region, or pigeon crop sac, but showed a 10% reduction in α-helix content, and a 25% reduction in proportion of monomer. Complete oxidation of Met brought about additional changes, including loss of crop sac activity, 20% reduction in α-helix content, alteration of side-chain CD spectrum, and a 2.5-3.0 times increase in rate of tryptic digestion. Alkylation of the 7 Met's with iodoacetic acid brought about changes equivalent to those noted for

complete oxidation. It was suggested that the four readily-modified Met's are exposed to solvent in the native molecule, while the remaining three residues can only react after a major conformational change. Reduction of the seven oxidized Met residues could be accomplished with mercaptoacetic acid, but only with complete reduction of disulfide bonds (Houghton & Li, 1979).

The role of charged groups in oPRL was investigated in several early studies involving oxidation, acetylation, guanidination, and esterification. In initial experiments, crop sac activity was abolished by destruction of primary amines with nitrous acid (Li et al., 1939a). A somewhat less drastic treatment involved acetylation of oPRL, also accompanied by inactivation of the hormone (Li & Kalman, 1946; Li et al., 1939b). In order to differentiate between the α- and ε-amino groups, Geschwind & Li (1957) guanidinated oPRL and determined that the derivative molecule retained biological activity. Subsequent acetylation did not inactivate the hormone, suggesting that the positively-charged Lys side-chains, rather than the presence of amino groups, are required for biological activity. The importance of oPRL carboxyl groups was demonstrated by esterification with methanol, which showed a negative correlation between degree of modification and crop sac activity (Li & Fraenkel-Conrat, 1947).

Iodination of oPRL has been carried out under a

variety of conditions, both as a structural probe and also in preparation for the development of a RIA. Early studies indicated that only Tyr residues were involved in the iodination reaction, and that, following reaction with all phenolic side-chains, the molecule was devoid of biological activity (Li et al., 1940b; Li et al., 1941b). The isoelectric point of the iodinated material was shifted from pH 5.8 to pH 4.7. The preparation of a biologically active iodinated oPRL involved a less drastically modified molecule (Cox, 1951). A more systematic study of oPRL iodination was carried out by Kawauchi et al. (1977), who examined the relative reactivities of the seven Tyr residues. Tyr-44 was found to be the most readily iodinated of the residues, and the diiodinated derivative of oPRL modified at this residue was fully active in the pigeon crop sac. Tyr-195 was also considered highly reactive, Tyr-147, Tyr-169, and Tyr-185 were partially active, and Tyr-28 and Tyr-96 were unreactive under nondenaturing conditions. An iodinated derivative of bovine PRL was somewhat less active than the native hormone in the crop sac and mammary gland, but showed increased RIA activity (Bullough & Wallis, 1977).

The Tyr residues of oPRL have also been modified by reaction with tetranitromethane (Ma et al., 1970; Kawauchi et al., 1977). The nitrated hormone retained biological activity, but appeared to undergo some conformational

change, as evidenced by ionization properties (Ma et al., 1970), CD spectra, exclusion chromatography, and tryptic digestion (Kawauchi et al., 1977).

IMMUNOCHEMISTRY. Studies using antibodies to PRL's or GH's have proven useful both in examining structural similarities among hormones from various species and in making quantitative determinations of PRL levels in serum, crude pituitary extracts, or purified preparations. Guinea pig antiserum to porcine PRL was found to react with ovine and human PRL, but rat PRL and tilapia pituitary extracts did not cross-react (Clarke & Li, 1974).

A homologous RIA for oPRL was first developed by Aria & Lee (1967), who found cross-reactivity with bovine PRL, but not with human PRL or HGH. Jacobs et al. (1972), however, found antibodies to oPRL able to cross-react with both the human and porcine hormones, and caprine PRL was found to be indistinguishable from oPRL in the RIA (McNeilly & Andrews, 1974).

Similar results were obtained in a RIA using antibodies raised to bovine PRL, which cross-reacted with the porcine hormone (Rand & Odell, 1971). In contrast, antibodies raised to kangaroo PRL did not cross-react with the hormone from the sheep, rat, or dog (Farmer et al., 1981). Homologous RIA's have also been developed for rat PRL (Kwa & Verhofstad, 1967; Niswinder et al., 1969; Neill &

Reichert, 1971) and mouse PRL (Sinha et al., 1972).

Among primates, antibodies to monkey PRL were cross-reactive with human PRL, but not HGH, giving early evidence for the existence of the human hormone (Hwang et al., 1971). Isolation of human PRL allowed development of a homologous RIA, which showed cross-reactivity with bovine and ovine PRL, a slight reaction with mouse PRL, but no response with the rat hormone (Sinha et al., 1973).

RIA data using antibodies to two bird PRL's are currently available. The homologous chicken PRL RIA did not show activity with PRL from the sheep, ox, or rat, nor with pituitary extracts of the pig or mouse, but gave a positive response with pituitary extracts from the guinea fowl, pheasant, pigeon, and quail (Yamamoto et al., 1980). Antibodies to turkey PRL did not cross-react with sheep or rat PRL, but no other bird hormones were tested (Burke & Papkoff, 1980).

Tilapia PRL has been used for the development of a homologous RIA (Farmer et al., 1977) that did not cross-react with PRL or pituitary extracts from the shark, perch, or sturgeon. In contrast, tilapia PRL was cross-reactive with antisera to rat and turtle GH. A number of amphibian PRL's have also been found to cross-react with rat GH antisera (Hayashida et al., 1973).

Although these studies indicate considerable variation among antisera raised to a single hormone and the

behavior of hormones from closely related species, some generalizations regarding protein structure are also suggested. It is clear that at least some immunological determinants of mammalian hormones are shared, not only among various mammals, but also among fish and amphibians. As might be expected, however, closely related species showed greater cross-reactivity than distant species. Cross-reactivity also appeared to be unidirectional with respect to evolutionary development, with antisera to mammalian hormones binding non-mammalian PRL's, while the converse relationship, binding of non-mammalian hormones to mammalian hormone antibodies has not been observed.

B. SECONDARY STRUCTURE PREDICTION OF PROLACTINS AND SOMATOTROPINS[1]

Structure-function relationships of pituitary hormones and HCS have been the subject of numerous studies and several recent reviews (Sherwood, et al., 1972; Li, 1974, 1975; Bewley & Li, 1977; Bewley, 1977). These studies report chemical modification, immunochemical, CD, and fluorescence data, as well as speculation on the significance of differences in amino acid sequence among homologous molecules. This information has led to considerable insight into the involvement of some aspects of protein

[1]This section is taken from Jibson and Li (1979).

structure in determining the nature and action of these hormones. Nevertheless, the exact relationship between the function of peptide hormones and their primary structure and conformation remains somewhat obscure.

At present, X-ray diffraction studies on the tertiary structure of pituitary and chorionic hormones are not available in the literature. In the absence of this information, prediction of protein structure has been suggested as a means of locating structural homologies among proteins (Argos, 1976, 1977), and of determining some aspects of the relationship between secondary structure and function (Chou & Fasman, 1975, 1978; Eterovic & Ferchmin, 1977). It was therefore of interest to undertake the prediction of secondary structure of these hormones, and to compare structural homology with that of sequence.

METHODS. The prediction method of Chou and Fasman (1974a, b) was used, with parameters based on the structures of 29 proteins (Fasman et al., 1976; Chou & Fasman, 1977). Ambiguous regions, having potential for more than one type of ordered secondary structure, were predicted to have the structure of highest potential in that region. All predicted conformations were examined to determine the relative location of disulfide loops and regions of ordered structure, but in no case was it necessary to alter the predicted structures.

Sequences used in these predictions were those of HGH (Li et al., 1966, 1973b; Li & Dixon, 1971; Li 1972), HCS (Li et al., 1971, 1973b), oPRL (Li et al., 1970; Li, 1976), and porcine PRL (Li, 1976).

Determination of structural homology between HGH and HCS, and between ovine and porcine PRL was based on direct comparison of the sequences, without aligning them for maximum homology. Comparisons of oPRL with HGH and HCS were made using the alignment of Bewley and Li (1971).

RESULTS. The predicted structures of HGH and HCS are shown in Table 1. HGH contains 37 residues in regions having both α-helix and β-sheet potential, while HCS has 7 residues in such regions. Each of the five α-helix regions predicted for HGH is found at an analogous position in the HCS sequence. HCS is also predicted to contain an additional segment of helix at residues 84-88. Predicted β-sheet regions in the two molecules are somewhat less homologous, with 6 of 9 segments in HGH also found in HCS. Each of the 16 β-turns predicted for HGH is found at a similar position in HCS. However, HCS shows 4 additional turns, not predicted for HGH. Comparison of the two predictions shows 70% of the residues in the same type of secondary structure. With residues at boundaries between α-helix and β-sheet potential also considered to be homologous, 82% of the residues are predicted to be in

the same type of secondary structure. The predicted secondary structure of HGH is shown graphically in Fig. 2.

Structures predicted for ovine and porcine PRL are shown in Table 2. The predictions show 20 residues in oPRL having both α-helix and β-sheet potential, and 28 such residues in porcine PRL. Both molecules are predicted to contain 6 helical segments, 5 of which are found in homologous positions, Each of the 7 β-sheet regions predicted for oPRL is also found in the predicted porcine PRL structure, while porcine PRL contains 2 additional β-sheet segments. Both molecules are predicted to contain 18 β-turns, with 15 in homologous positions. Comparison of the predicted structures of these two molecules shows 74% of the residues in homologous regions. With boundary residues and residues in ambiguous regions included, the homology increases to 78%.

Comparison of HGH and oPRL shows 48% of the aligned residues to be in regions of the same predicted structure. HCS compared with oPRL shows 44% of the residues in homologous positions. The predicted secondary structure of oPRL is shown graphically in Fig. 3.

Table 1

Predicted secondary structures of HGH and HCS

α-Helix		β-Sheet		β-Turn		Random coil	
HGH	HCS	HGH	HCS	HGH	HCS	HGH	HCS
11– 25	9– 25	6– 10	1– 8	26– 29	26– 29	1– 5	–
30– 36	30– 36	–	50– 53	–	37– 40	37– 39	–
65– 69	64– 68	74– 87	75– 83	40– 43	39– 42	44– 46	43– 45
–	84– 88	90– 96	90– 94	47– 50	46– 49	51– 59	58– 59
115–120	112–120	101–104	–	–	54– 57	64	–
169–174	167–174	111–114	–	60– 63	60– 63	–	73– 74
		121–124	121–125	70– 73	69– 72	88– 89	89
20%	27%	136–143	–	97–100	97–100	–	95– 96
		163–167	163–166	105–108	105–108	–	101–104
		175–180	175–181	107–110	108–111	125–128	126–127
				129–132	128–131	–	138–140
		30%	22%	132–135	132–135	144–146	145–146
				–	134–137	155–156	153–156
				–	141–144	168	–
				147–150	147–150	185–186	186
				151–154	149–152	191	191
				157–160	157–160		
				159–162	159–162	19%	14%
				181–184	182–185		
				187–190	187–190		
				31%	37%		

*Percent of residues predicted to be in each type of secondary structure is shown at the bottom of each column.

Table 2

Predicted secondary structures of ovine and porcine PRL*

α-Helix		β-Sheet		β-Turn		Random coil	
Ovine	Porcine	Ovine	Porcine	Ovine	Porcine	Ovine	Porcine
16– 22	16– 22	11– 15	8– 15	4– 7	4– 7	1– 3	1– 3
33– 40	33– 40	23– 32	23– 32	7– 10	–	45– 46	45– 46
69– 77	69– 78	–	51– 55	41– 44	41– 44	51– 55	–
–	101–107	79– 91	79– 91	47– 50	47– 50	63– 64	63– 64
112–122	112–122	97–102	97–100	56– 59	56– 59	78	–
127–132	127–132	133–137	133–137	59– 62	59– 62	92	92
157–167	–	–	146–149	65– 68	65– 68	103	–
		168–175	166–175	93– 96	93– 96	142–144	142–145
26%	25%	182–195	186–195	104–107	–	149	–
				108–111	108–111	–	157–159
		31%	35%	123–126	123–126		
				138–141	138–141	9%	7%
				145–148	–		
				150–153	150–153		
				153–156	153–156		
				–	160–163		
				–	162–165		
				176–179	176–179		
				178–181	178–181		
				–	182–185		
				196–199	196–199		
				34%	33%		

*Percent of residues predicted to be in each type of secondary structure is shown at the bottom of each column.

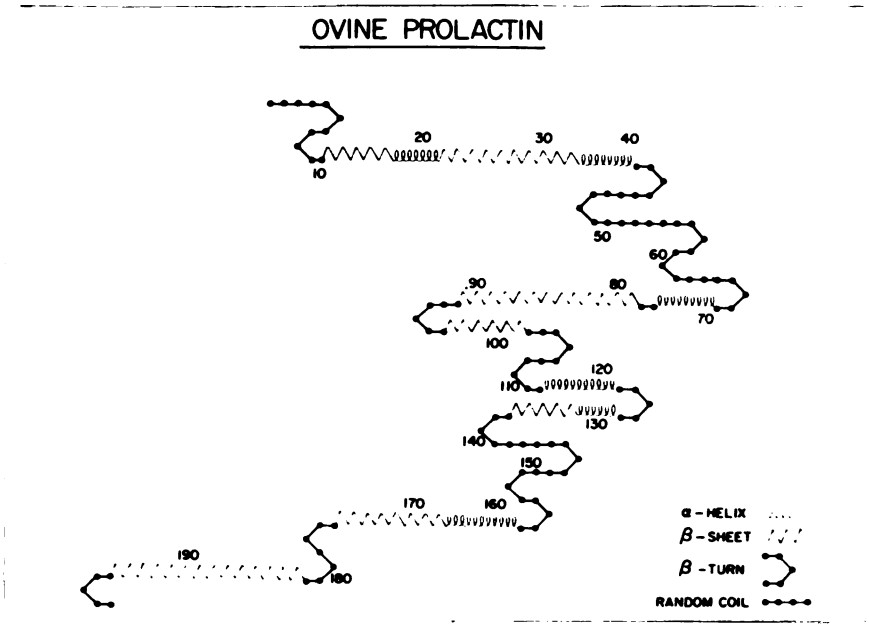

Fig. 3. Predicted secondary structure of oPRL

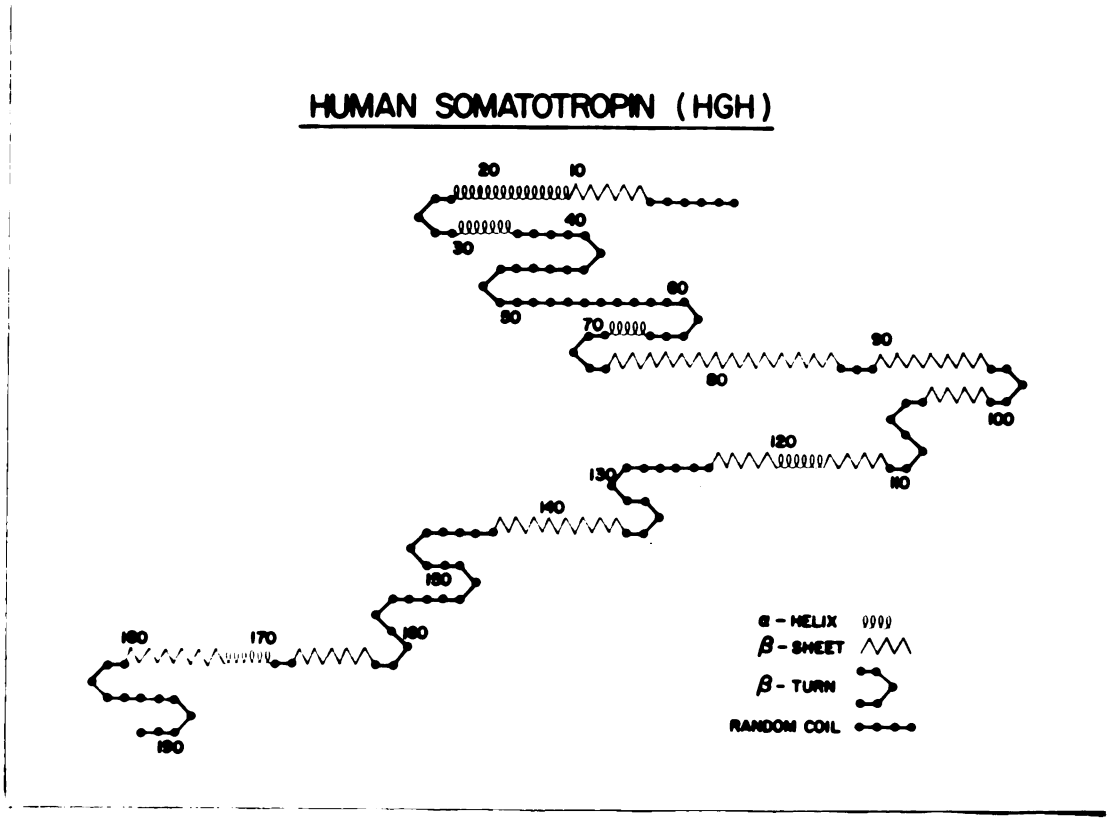

Fig. 2. Predicted secondary structure of HGH

DISCUSSION. Since the early work of Holzwarth (1964) and Holzwarth and Doty (1965), CD has been considered the method of choice for evaluation of protein secondary structure. Numerous CD studies of HGH have reported α-helix contents of 45-55% (Bewley & Li, 1967, 1972; Bewley et al., 1969; Aloj & Edelhoch, 1972; Holladay et al., 1974), which do not compare favorably with the predicted helix content of 20% reported here. Similarly, the α-helix content of HCS has been determined by CD and ORD to be 44-51% (Aloj & Edelhoch, 1971; Bewley & Li, 1971b; Aloj et al., 1972), while predicted to contain 27% α-helix in this study. CD of both oPRL (Aloj & Edelhoch, 1970; Bewley & Li, 1972; Farmer et al., 1976) and porcine PRL (Bewley & Li, 1975a) has shown 45-60% α-helix, as opposed to 26% and 25% predicted here for ovine and porcine PRL, respectively.

In order to account for the discrepancy observed between experimental and predicted values of α-helix and β-sheet content, it is important to note that both CD and the Chou-Fasman predictions have certain limitations. Several methods of determining α-helix content from CD data have been published, based on analysis of synthetic peptides (Bewley et al., 1969; Greenfield & Fasman, 1969), and of X-ray structures (Chen & Yang, 1971). While most estimates are based on studies of synthetic peptides, it has been found that these measurements are not above

question (Chen et al., 1972, 1974), and determination of α-helix must be considered an estimate, with an uncertainty of approximately 10% (Bewley & Li, 1972).

Estimation of β-sheet and β-turn content by CD involves several problems, such as dependence of the spectrum on the length of β-sheet segments (Chen et al., 1972, 1974), and dependence of the spectrum on the conformation of neighboring strands (Woody, 1969). Furthermore, the CD spectra of the β-sheet and β-turn are difficult to distinguish from one another, they are of weak intensity compared to the α-helix spectrum, and they may overlap CD bands due to Tyr and Trp side-chains. Thus it is difficult to make unequivocal estimates of these secondary structures (Chen et al., 1977).

Chou and Fasman (1974b) have reported 70-80% accuracy in predicting secondary structure. This technique is most effective for small, thermostable, monomeric proteins (Sternberg & Thornton, 1978), but has not been effective in predicting the structure of short, non-rigid peptides in aqueous solution (St.-Pierre et al., 1976; Yang et al., 1977; Wu & Yang, 1978). The method was effective in locating 80-90% of α-helix regions when applied to adenyl kinase (Schulz et al., 1974), but ends of helices were not predicted accurately, and β-sheet and β-turn segments showed only 60-70% agreement with the X-ray structure.

The Chou-Fasman prediction rules are also known to

involve ambiguities, the resolution of which may not be consistent among different users (Yang et al., 1977). This fact has been demonstrated for the secondary structure of ovine β-LPH, which has been predicted in three studies (St.-Pierre et al., 1976; Yang et al., 1977; Jibson & Li, 1979). In these accounts, predictions ranged from 45% to 65% α-helix, 5% to 16% β-sheet, and 19% to 31% β-turn. While the general features of these predictions are in agreement, it is apparent that caution must be exercised in producing very detailed predictions.

In spite of the limitations of secondary structure predictions, it has been suggested that structural homologies between proteins might best be seen by this method (Argos, 1976, 1977). Comparison of the sequences of HGH and HCS show 85% of the residues to be identical, and 96% to be homologous (Bewley & Li, 1971a; Bewley et al., 1972). These values are somewhat higher than the predicted 82% homology between the secondary structures of the two molecules. Attention has also been drawn to the fact that over 50% of the amino acid differences between HGH and HCS are found in residues 1-75, while only 15% of the changes are found in residues 126-191 (Li et al., 1971, 1973b; Sherwood et al., 1972). The division of these sequences into more and less variable regions is much less dramatic in these predictions, because the differences in predicted structure between the two

molecules are evenly distributed throughout the chains.

Sequence comparison of ovine and porcine PRL has shown 94% homology in their primary structures (Li, 1976), as opposed to the 78% homology in secondary structure predicted in this study. Similarly, sequence homology between HGH and oPRL has been reported to be approximately 60% (Bewley & Li, 1971a; Bewley et al., 1972), while homology in secondary structure is predicted here to be 44%.

In view of these predictions, the possibility should be considered that apparently minor changes in the sequence of these proteins may result in relatively large alterations in secondary structure. This possibility may, of course, be extended to tertiary structure as well. It may thus be unwise to attempt correlation of a single, or a few, amino acid changes with broad differences in activity, without consideration of possible changes in secondary or tertiary structure.

C. AMINO ACID SEQUENCE HOMOLOGIES AMONG PROLACTINS AND SOMATOTROPINS

The comparison of amino acid sequences of proteins having similar biological activities but originating from a variety of species, has been useful both in the study of evolutionary patterns of development and in the examination of structure- function relationships (Dayhoff, 1969). It has been suggested that invariant amino acids, or highly conserved regions of the peptide chain, are more crucial to the conformation and, therefore, function, of related proteins than are those showing greater diversity among species. This proposal has led to the consideration of amino acid sequence as a potential probe into higher order structure and its relationship to biological activity.

The determination of the complete primary structures of prolactins and somatotropins from several species allowed a comparison of these molecules and location of identical amino acids, conservative replacements, and non-conservative changes (Bewley & Li, 1970; 1971; Niall, 1971; Bewley et al., 1972; Dayhoff, 1972; Li, 1976). Although these studies presented convincing evidence for a common precursor to prolactins and somatotropins, the limited number of sequences available at that time made it impossible to evaluate the significance of specific amino

acid replacements.

The recent publication of several additional prolactin and somatotropin sequences has prompted us to undertake a broader comparison of species differences. In order to increase the effectiveness of this comparison, we have developed a simple means of evaluating primary structure changes. Although not strictly quantitative, this technique makes it possible to identify patterns in the distribution of both highly conserved and highly variable amino acids, and can easily incorporate additional sequences as they become available.

METHODS. The prolactin sequences used in this study were those of the human (Shome & Parlow, 1977), sheep (Li, 1976), ox (Wallis, 1974), pig (Li, 1976), and rat (Cooke et al., 1980). The primary structure of rat prolactin was based on the gene nucleic acid sequence; all others were determined by amino acid sequencing. The somatotropin sequences used were those of the human (Roskam & Rogeon, 1979; Martial et al., 1979), sheep (Li et al., 1973a), ox (Graf & Li, 1974), horse (Zakin et al., 1976), and rat (Seeburg et al., 1977). The human and rat structures were taken from gene sequences. Human chorionic somatomammotropin (Li et al.,1973b; Bewley, 1977) was included with the growth hormones on the basis of primary structure.

The sequences used in each comparison were aligned

for maximum homology. HGH and HCS were aligned with the prolactins as described by Bewley and Li (1970), with extensions as needed to include all residues of each sequence.

In order to evaluate amino acid differences between molecules, each sequence was compared individually with all other sequences within its group. The same amino acid replacement occurring at at given position between several pairs of sequences was counted only once. Values shown in the histograms were based on the designations of Dayhoff (1969): replacements with acceptance rates at least 40 times that predicted by chance were given a value of 1, those with rates from 21 to 39 a value of 2, 7-20 a value of 3, 1-6 a value of 4, and those not observed a value of 5. Gaps were assigned a value of 3 on the basis of their average mutability with all amino acids. Equivocal asparagine- aspartic acid and glutamine-glutamic acid assignments were taken as the more conserved residue at that position.

The probability of occurrence of conserved or variable amino acids within any given segment of a sequence was calculated from the binomial formula:

$$P(x) = \frac{n!}{x!(n-x)!} p^x q^{(n-x)}$$

where P(x) is the probability of x occurrences of conserved or variable residues, n is the length of the

segment being examined, p is the probability of occurrence of conserved or variable residues for the total sequence (taken as the fraction of such residues actually observed for the sequence - Table 3), and q=1-p. For each group of molecules, the minimum number of consecutive occurrences of conserved or variable residues required to generate $P(x) \leq .01$ was determined, and every possible segment ranging in length from that minimum number up to 40 residues was examined. The number of segments expected to have $P(x) < .01$ for a random distribution of conserved or variable amino acids was also calculated from the above equation and the total number of segments examined. The chi-square test was then used to compare observed and expected numbers of segments with $P(x) \leq .01$. Conserved and variable segments were identified in separate searches.

RESULTS AND DISCUSSION. The primary structures of the prolactins used in this study are shown in Fig. 4, with the degree of variability at each amino acid position indicated by the histogram. The number of amino acids differing from the majority of sequences for each of the prolactins used is given in Table 4. Nearly half of the total deviations from the majority occur in the rat hormone. Removal of this species from the analysis leaves 71% of the residues identical among the remaining sequences and 84% conserved (variability ≤ 1).

The number of identical residues among the prolactins, and those classified as conserved (including identical), intermediate, and variable are given in Table 3. The total number of segments examined, and the number of those segments having $P \leq .01$ are shown in Table 5. The lengths of the segments examined were determined by the minimum number of consecutive conserved or variable amino acids required to generate $P \leq .01$.

The distribution of conserved amino acids among the prolactins is not significantly different from that expected for a random arrangement of conserved residues. In contrast, the variable residues in these sequences appear to be grouped into two highly variable regions, as indicated by the values given in Table 5. Segments with the lowest P-values are given in Table 6.

The somatotropin sequences compared in this study are shown in Fig. 5, and the classification of residues is summarized in Table 3. Although the somatotropin comparison includes 6 sequences, instead of the 5 used for prolactin, the percentage of identical and conserved residues is slightly higher than was found for the prolactins, and the proportion of variable residues is only half as great as that previously noted. The highly conserved nature of the somatotropin primary structures is further illustrated by the observation that 75% of the variations from the majority of sequences are found in HGH or HCS (Table 7).

Among the remaining four species 84 % of the residues are identical and 94% conserved.

The lengths and total numbers of somatotropin segments examined are given in Table 5. The numbers of segments with $P \leq .01$ for either conserved or variable residues were lower than expected for a random sequence. The significance of numbers below predicted levels is questionable, however, because the observed occurrences of conserved or variable residues converge with expected values as segment size increases. This was, of course, not a problem for cases in which observed values were higher than predicted. It may be concluded, therefore, only that the arrangement of conserved and variable amino acids in the somatotropins is at least as uniform as expected for a random distribution.

In order to investigate the role of sequence in structure- function relationships, a comparison was made of the prolactins with the lactogenic somatotropins HGH and HCS (Ferguson & Wallace, 1961; Josmovich & MacLaren, 1962; Doneen, 1976). This study was of interest because it involved consideration of molecules more divergent than just prolactins from related species, or growth hormones of pituitary and placental origin, while allowing comparison of molecules with at least one similarity in biological activity.

The variability shown among these proteins is

presented in Fig. 6, with the classification of residues given in Table 3. As expected for this diverse group of molecules, the proportion of conserved residues is much lower than for prolactins or somatotropins alone.

Table 5 gives the results of the searches for conserved and variable segments in these lactogenic sequences. The most striking features of the analysis are the sharp increase in highly conserved segments and the decrease in variable regions when compared to the prolactins alone. The most notable of these regions, representing local minima of P-values, are indicated in Table 6. It appears from these results that the distribution of conserved amino acids in these sequences is not random, but rather arranged so as to retain the primary structure of two particular regions.

Although the technique described here involves several arbitrary choices, such as the designations of conserved and variable amino acids, the nonrandom distribution of these classes strongly suggests a correlation between amino acid variability, as here defined, and some factor determining the function of these proteins. It should be kept in mind, however, that examination of homology from such a limited number of primary structures, and several of those from closely related species, may lead to skewed patterns of amino acid changes. Nevertheless, the value of sequence comparisons should be greatly

increased by the availability of proteins from a wider variety of species. As additional sequences are determined, the presence of more pronounced patterns in conserved and variable residues may develop, allowing correlation of these chemical properties with biological activity.

Table 3

Numbers of residues from PRL's and somatotropins classified as identical, conserved (variability ≤ 1), intermediate ($1 <$ variability < 6), and variable (variability ≥ 6).

	Identical		Conserved		Intermediate		Variable		Total
	No.	%	No.	%	No.	%	No.	%	
Prolactins	99	49.7	136	68.3	40	20.1	23	11.6	199
Somatotropins	101	52.3	141	73.1	39	20.2	13	6.7	193
Lactogenic sequences	30	12.9	62	26.7	86	37.1	84	36.2	232

Table 4

Number of amino acids within each of the PRL sequences differing from the majority of sequences at a given position.

	Number of variations	Percent of total variations
Sheep	18	9.4
Ox	16	8.3
Pig	21	10.9
Human	51	26.6
Rat	86	44.8
Total	192	

Table 5

Comparison of observed and expected numbers of segments having significantly (P .01) more conserved or variable amino acids than expected for a random distribution.

Prolactins	conserved	13-40	4858	25.4	16
	variable	3-40	6783	35.2	251*
Somatotropins					
Lactogenic sequences	conserved	4.40	7807	42.5	491*
	variable	5.50	7578	43.2	22

*Significantly greater than expected value (P .001) in chi-square test

Table 6

Location and probability of occurrence of the most conserved and most variable segments in the PRL and lactogenic sequences. The number of such segments among the somatotropins was not significant.

	Conserved		Variable	
	Segment	P	Segment	P
Prolactins			75-87	.00013
			131-168	.00186
Lactogenic sequences	64-75	.00067		
	207-219	.00007		

Table 7

Number of amino acids within each of the somatotropin sequences differing from the majority of sequences at a given position.

	Number of variations	Percent of total variations
Sheep	12	7.4
Ox	10	6.2
Horse	8	4.9
Rat	11	6.8
Human	55	34.0
HCS	66	40.7
Total	162	

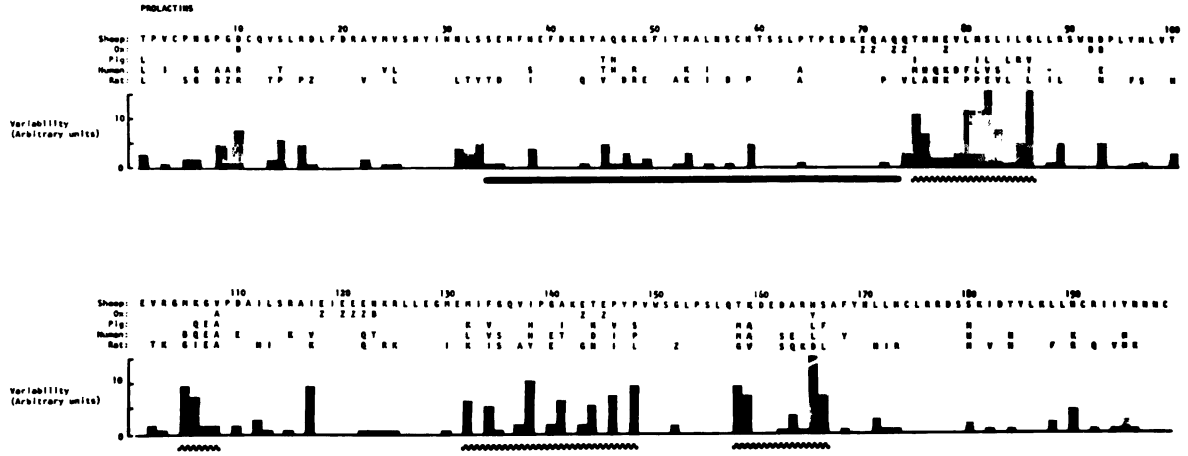

Fig. 4. Primary structures of PRL's. Only residues differing from the uppermost sequence are given. Dashes represent gaps introduced to maximize homology. The histogram gives variability at each position in the sequence.

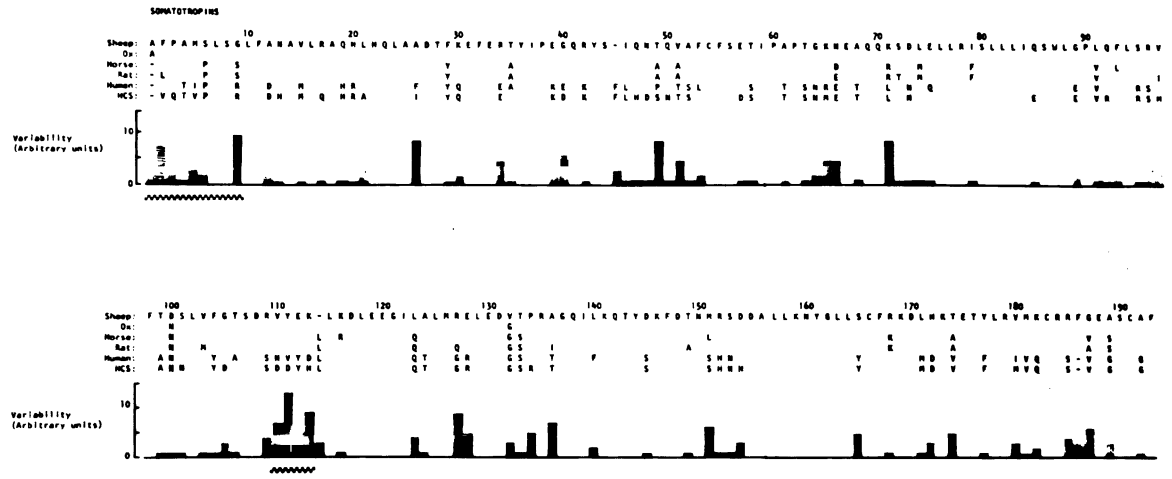

Fig. 5. Primary structures of GH and HCS. Only residues differing from the uppermost sequence are given. Dashes represent gaps introduced to maximize homology. The histogram gives variability at each position in the sequence.

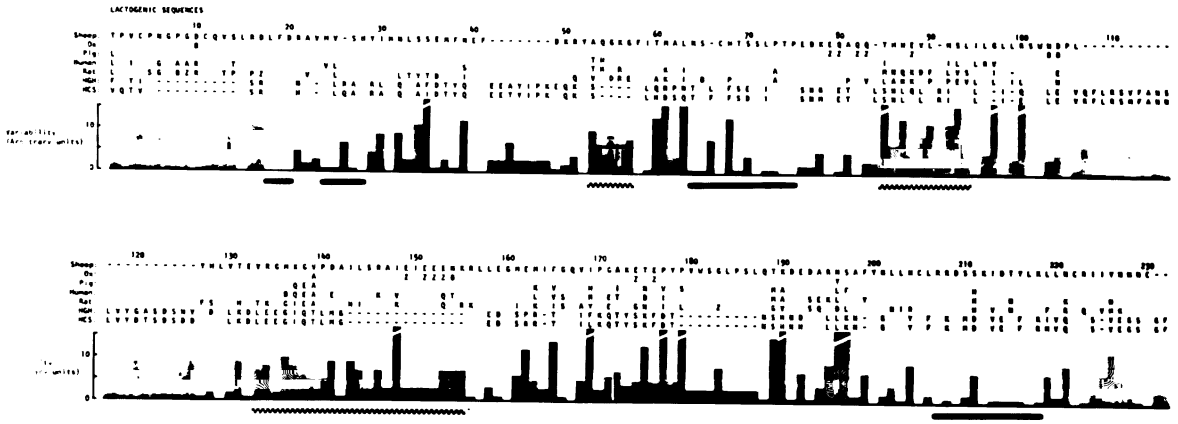

Fig. 6. Primary structures of the lactogenic sequences: PRL's, HGH, and HCS. Only residues differing from the uppermost sequence are given. Dashes represent gaps introduced to maximize homology. The histogram gives variability at each position in the sequence.

CHAPTER 2

DIGESTION OF PROLACTIN WITH FIBRINOLYSIN

PROTEIN COMPLEMENTATION. Complementation has been defined as "the restoration of a biological activity by noncovalent interaction of different proteins or polypeptides" (Zabin & Villarejo, 1975). Most often, enzyme subunits have been studied as examples of complementing systems, with independently synthesized peptide chains interacting to form an active complex (Schlesinger & Levinthal, 1965; Schlesinger, 1970; Zabin & Villarejo, 1975). The subunit proteins constituting these enzymes have been characterized as typically having stable, globular conformations in the unbound state, allowing recognition among binding components. Upon complementation, the subunits undergo conformational changes which manifest themselves in the appearance of biological activity (Zabin & Villarejo, 1975).

A somewhat less extensively studied form of protein complementation involves the interaction of protein fragments <u>in vitro</u> (Anfinsen & Scheraga, 1975). Studies in this area have been directed toward the determination of the minimum requirements within a peptide chain for maintenance of the native conformation. An excellent example of this type of study is the complementation of bovine pancreatic ribonuclease fragments (Richards &

Vithayathil, 1959; Kato & Anfinsen, 1969). It was determined in these studies that, with the enzymatic cleavage and removal of the first 20 residues of the peptide chain (ribonuclease S-peptide), the remaining 104-residue chain (ribonuclease S-protein) could be inactivated by enzymatic scrambling of its four disulfide bonds. This instability toward reduction of disulfide suggested that the S-protein lacks the structural information sufficient to form a single conformation. Addition of the S-peptide, however, prevented disulfide bond rearrangement, and, when added to the scrambled S-protein, provided sufficient information to allow reformation of the correct cysteine pairing.

Cleavage of ribonuclease to remove residues 119-124 from the C-terminus caused complete inactivation of the enzyme (Lin et al., 1970; Gutte et al., 1972). This activity was not restored by complementation with a synthetic peptide corresponding to ribonuclease-(117-124). Recombination with ribonuclease-(116-124), however, restored 60% of the enzyme activity, and 98% was restored by addition of residues 111-124.

Perhaps the most remarkable behavior of the ribonuclease fragments was the formation of an active ternary complex involving the S-peptide, des-(119-124)-S-protein, and residues 111-124 (Lin et al., 1970). None of these components was active when tested alone, and neither of the terminal fragments alone was able to regenerate the

activity of ribonuclease-(21-118). When each of the fragments was present, however, 30% of the activity of the native enzyme was restored.

Recombination of protein fragments does not appear to be a general phenomenon, however, as numerous examples of noncomplementing systems have been reported (Anfinsen & Scheraga, 1975). Nevertheless, for those cases in which complemention does occur, interaction of fragments may be useful in identifying regions of the peptide chain critical for proper tertiary structure and may provide both qualitative and quantitative information on the intramolecular forces involved in maintenance of protein conformation.

COMPLEMENTATION OF HGH AND HCS FRAGMENTS. Partial digestion of bovine GH with trypsin or chymotrypsin has been reported to increase the nitrogen-retention activity of the hormone in humans (Forsham et al., 1958; Sonenberg et al., 1965; Kato & Anfinsen, 1969; Sonenberg et al., 1968), and decrease the antigenicity of the molecule (Moudgal, 1962), while maintaining full tibia activity (Li et al., 1959). In addition, limited trypsin and pepsin digests of bovine GH showed immunological cross-reactivity with the human hormone (Laron et al., 1964; Sonenberg et al., 1968).

Similar studies on HGH indicated that partial plasmin

digestion resulted in greater crop sac and tibia activity (Yadley & Chrambach, 1973; Yadley et al., 1973), accompanied by a slight decrease in RIA activity (Clarke et al., 1974). The possibility that in vivo proteolytic cleavage might potentiate HGH activity was raised by the isolation from human pituitary extracts of several cleaved forms of the hormone, immunologically indistinguishable from native HGH, but with increased lactogenic and growth promoting activities (Singh et al., 1974). These biological and immunological properties were also found for fibrinolysin-treated HGH, from which residues 138-147 had been removed (Lewis et al.,1975). Reduction and alkylation of disulfide bonds in plasmin- modified HGH allowed isolation of two peptide chains, HGH-(1-134) and HGH-(141-191) (Reagan et al., 1973; Li & Graf, 1974). These fragments were reported to exhibit both growth- promoting and lactogenic activities, although with potencies considerably less than that of the native hormone (Li & Graf, 1974). Subsequent studies, however, reported that only the N-terminal fragment had significant biological activity (Reagan et al., 1975; Rao et al., 1976). Both fragments gave RIA inhibition curves parallel to that of HGH-(1-134), showing 25% the immunological activity of the native hormone, and HGH-(141-191) showing 1-2% (Clarke et al., 1974).

The conformations of the HGH fragments have been

examined by circular dichroism and exclusion chromatography (Li & Bewley, 1976; Bewley & Li, 1978). The secondary structure of HGH-(1-134) appeared similar to that of the native hormone, but the tertiary structure, as evidenced by CD spectra, differed markedly. Conformational changes in the C-terminal fragment appeared to result in the formation of a random coil. The apparent molecular weight of HGH-(1-134) was 47,000, corresponding to a trimer of the fragment. There was no evidence for self-association of HGH-(141-191).

The kinetics and thermodynamics of dissociation of the reduced and alkylated plasmin fragments of HGH have been studied by CD, fluorescence, and exclusion chromatography (Bewley & Li, 1978). The reaction proceeded in a slow, exergonic fashion, with an equilibrium constant of 1.77 M and a free energy of dissociation of -340 cal/mol at 25^{o}C. The proximity of the dissociation constant to unity suggests that the existence of a trimeric N-terminal and monomeric C-terminal fragment is nearly as favorable energetically as the conformation of the reduced and alkylated hormone.

Recombination of the HGH fragments has been reported to completely restore biological activity (Li & Bewley, 1976; Li et al., 1976). In addition, RIA data indicated that the recombinant molecule is nearly identical immunologically to the native hormone. Both exclusion

chromatography and CD studies showed a complete recovery of native conformation upon fragment recombination.

The role of HGH-(141-191) in maintaining the biological activity of the recombinant molecule was investigated by the use of several synthetic analogs to the fragments in the association reaction (Li et al., 1977; Li et al., 1978; Blake & Li, 1978; Li et al., 1981). Each of the analogs ranging in length from 47 to 57 amino acids was found to bind the natural N-terminal fragment. All of these recombinant molecules showed nearly full recovery of biological and immunological activity as compared to native HGH. In contrast, a synthetic analog to HGH-(140-182) showed no evidence of recombination with natural HGH-(1-134) (Li et al., 1981). These findings suggest that the minimum requirement for recombination lies somewhere between residues 182 and 187 of the C-terminal fragment.

Investigation of the action of plasmin on HCS led to the isolation of fragments analogous to those of HGH, but missing residue 134 from the N-terminal fragment (Li & Houghton, 1978). Neither of these fragments had biological or immunological activity. The complementation of HCS fragments with those of HGH resulted in recovery of 50% growth-promoting activity when the HGH N-terminal was combined with the HCS C-terminus. In contrast, the biological activity of HCS-(1-133) with HGH-(141-191) was much

lower (Li, 1978). Examination of these mixed recombinants for immunological and membrane- binding properties indicated that determinants for both activities are found predominantly within the N-terminal fragment (Burstein et al., 1978). These findings are not surprising considering that there are only two amino acid differences between the HCS and HGH C-terminal fragments, as compared to 25 differences in their N-terminal fragments (Bewley & Li, 1971).

An examination of this evidence indicates that the C-terminal fragments of HGH and HCS are not able to fold into their native conformations, but that their sequences alone contain sufficient information to allow specific binding in complementation reactions. In contrast, the N-terminal regions of both HGH and HCS appear to retain at least some of their native secondary and tertiary structures, but require the smaller fragment to maintain their complete native conformations.

A. FIBRINOLYSIN CLEAVAGE OF OPRL. Birk and Li (1978) reported the partial digestion of oPRL by fibrinolysin (EC 3.4.21.7). Disc gel electrophoresis showed the formation of two major components following 9 h digestion at room temperature with a 1:100 enzyme to substrate ratio (w:w). These components were isolated by exclusion chromatography on Sephadex G-100 in 0.01 M NH_4HCO_3.

The larger fragment was found to have the N-terminal sequence Ala-Leu-Asx-, but no C-terminal residues were liberated by the action of carboxypeptidase A. A molecular weight of 17,500 was found by $NaDodSO_4$ gel electrophoresis, while column chromatography indicated 37,000, suggesting the formation of a dimer in nondenaturing solutions. These findings led to the conclusion that this fragment consists of residues 54-199, which was confirmed by amino acid analysis.

Sequence analysis of the smaller fragment revealed Thr-Pro-Val- at the amino terminus and Ile-Thr-Met- at the carboxy-terminus. The molecular weight of the peptide was found to be >7,000 by $NaDodSO_4$ gel electrophoresis and >8,000 by exclusion chromatography. These findings, together with amino acid analysis, indicated that the smaller fragment was oPRL-(1-53).

CD spectra of the two fragments showed major changes in the Trp and Phe regions of the spectra. In the far UV region, both peptides showed evidence of α-helical structure, but the helix contents were considerably below that of the native molecule.

The fragments were found to be devoid of biological activity when tested in the pigeon crop sac. Immunological activity with antiserum to oPRL from either the guinea pig or rabbit was also absent when examined by gel double-diffusion on Ouchterlony plates or complement

fixation.

Recombination of the fragments was observed when 1.6 mg (0.1 μmol) oPRL-(1-53) were combined with 1.2 mg (0.2 μmol) oPRL-(54-199) in 1 ml 0.1 M Tris-HCl buffer, pH 8.2. The chromatographic behavior of the recombined fragments was identical to that of native oPRL. CD spectra of the recombinant showed a complete restoration of α-helix and a partial recovery of tertiary structure, the latter most notable in the spectrum of Trp. Small differences remained, however, between the CD spectra of native and recombinant oPRL throughout the region of side-chain absorption.

The recombined oPRL fragments behaved almost indistinguishably from native oPRL both on Ouchterlony plates and in complement fixation, indicating a nearly complete recovery of immunological activity. Biological activity, however, was not restored by recombination, and the potency of the recombinant was estimated to be only 2% that of the native hormone in the crop sac.

B. FIBRINOLYSIN ACTIVITY

Although bacterial fibrinolysin has been commercially available for several years, the molecule has not been sufficiently well characterized for the Commission on Biochemical Nomenclature of the International Union of Biochemistry (1978) to distinguish it from plasmin.

Physicochemical studies on the enzyme are currently being carried out by Y. Birk (personal communication), therefore this work was limited to an examination of the effect of fibrinolysin on oPRL, to determine which residues (if any) constitute the preferred substrate for proteolysis.

MATERIALS AND METHODS. Purified fibrinolysin was generously provided by Y. Birk. The highly purified enzyme was prepared by chromatography of commercially-available fibrinolysin on DEAE-cellulose, eluted with a continuous salt gradient. The enzymatically active fraction of the elution contained 1% of the starting material; this was dialyzed against 0.01 M NH_4HCO_3, pH 8.4, then concentrated by dialysis against glycerol. The glycerol-concentrated solution (1 mg/ml) was stable at room temperature for several months; however, lyophilization of the purified enzyme resulted in loss of activity (Y. Birk, personal communication).

oPRL, prepared as described by Li et al. (1970), was supplied by C. H. Li. Before digestion with fibrinolysin, this material was subjected to the additional purification steps of Sluyser & Li (1964), followed by column chromatography on Sephadex G-100 (62 x 3.5 cm) in 0.01 M NH_4HCO_3, pH 8.4. The monomer fraction was lyophilized and used in subsequent procedures.

Five mg of the oPRL monomer were dissolved in 2 ml

0.05 M NH_4Ac, pH 8.2 for the enzymatic digestion. Twenty µl of the fibrinolysin solution (20 µg) were added, the mixture was kept at room temperature 17 h, and the solution was lyophilized. The hygroscopic product was partially dissolved in 0.5 ml 0.1 M HOAc, and was completely solubilized by addition of 2 drops of glacial HOAc.

The dissolved digestion product was submitted to peptide mapping (Katz et al., 1959; Sairam & Li, 1975), and the most intense ninhydrin-positive spots were cut out and eluted into 1 ml 0.1 M NH_4OH. Aliquots were removed from the eluates and used for end-group analysis (Gray, 1967; Woods & Wong, 1967) or amino acid analysis (Spackman et al., 1958).

RESULTS. The peptide map of the complete fibrinolysin digest of oPRL is shown in Fig. 7. One hundred three ninhydrin-positive spots were identified in the map, but most of these spots overlapped one another to some degree, limiting the number of peptides that could be identified by end-group and amino acid analysis.

Of the spots labeled, numbers 1-37 and 103 were used for end-group analysis; 22 of these had single end-groups, while the remainder showed evidence of as many as 4 end-groups. The number of times each amino acid was found at the N-terminus of the fibrinolysin-cleaved oPRL peptides is shown in Table 8. By far the most common amino acids

found were Leu, Lys, and Val, accounting for 60% of all end-groups identified. Several other amino acids did, however, occur at the amino- terminus of at least one of the peptides examined.

In order to examine the possibility that the end-groups observed were created by completely random cleavage, Table 8 also shows the expected number of end-group occurrences for this case, calculated on the basis of oPRL amino acid composition, and the results of the chi-square test of differences between observed and expected values. The assumptions inherent in the chi-square test require that it only be used in cases with expected values of at least 5. This constraint restricted the test to the values of Leu, Glu, and Asp. Of these, only Leu was detected significantly more often than expected ($P<.005$). It is apparent, however, that both Lys and Val also occurred with more than random frequency, although the chi-square test could not be used in those cases. Amino acid analysis was performed on 19 of the eluted peptides. Of these, most showed compositions that appeared to originate from more than a single peptide, or which corresponded to more than one fragment of the oPRL sequence. It was possible, however, to make tentative assignments for the positions of 4 peptides, as shown in Table 9. Examination of the 8 cleavages required to generate these fragments reveals Leu once on the N-terminal

side and twice on the C-terminal side of the cleaved bond. The ninhydrin spot labeled #1 on the peptide map was found to consist almost exclusively of Leu (Table 9), suggesting the production of large amounts of free Leu during the digestion.

Table 8. Number of times each amino acid was found at the N-terminus of fibrinolysin-oPRL fragments, and the number of occurrences expected for random cleavage of oPRL. The chi-square test was used to determine significance of differences between observed and expected values with expected values 5.

Amino Acid	Observed Occurrences	Expected Occurrences
Leu	16	7.1[a]
Lys	12	2.8
Val	9	3.1
Asp	4	6.7
Tyr	4	2.1
Glu	3	6.7[b]
Ile	3	3.4
Ala	2	2.8
Arg	2	3.4
Gly	1	3.4
His	1	2.4
Met	1	2.1
Phe	1	1.8
Ser	1	4.6
Thr	1	2.8
Cys	0	1.8
Pro	0	3.4
Trp	0	0.6

[a]Chi-square value significant at .005 level.
[b]Chi-square value not significant at .05 level.

Table 9. Amino acid composition of several peptides isolated from fibrinolysin cleavage of oPRL. Peptide numbers are from Fig. A. Peptide analyses are compared to oPRL segments indicated. Peptide bond cleavages required to generate each fragment are also shown.

Amino Acid	Peptide 26 Obs	176-185	Peptide 27 Obs	62-75	Peptide 32 Obs	160-166	Peptide 33 Obs	157-166	Peptide 1 Obs
Lys	1.0	1	0.9	1	0.1	0	0.8	1	
His					1.0	1	1.0	1	1.0
Arg	1.5	2			1.0	1	0.7	1	
Asx	2.4	2	1.2	1	1.8	2	2.5	2	0.9
Thr	0.9	1	1.7	2			1.0	1	
Ser	1.9	2	1.3	1	1.0	1	1.1	1	0.3
Glx	0.3	0	4.8	5	1.2	1	1.9	2	0.3
Pro			2.1	2					
Gly	0.2	0	0.2	0	0.1	0			0.9
Ala			1.1	1	1.1	1	0.9	1	
Val	0.1	0	0.1	0					1.3
Ile	1.0	1							2.8
Leu	0.1	0	1.0	1					51.8
Tyr	0.9	1	0.1	0					
Phe									0.7
Cleavage Required	Leu-Arg Tyr-Leu		Ser-Ser Thr-His		Lys-Asp Ser-Ala		Leu-Gln Ser-Ala		

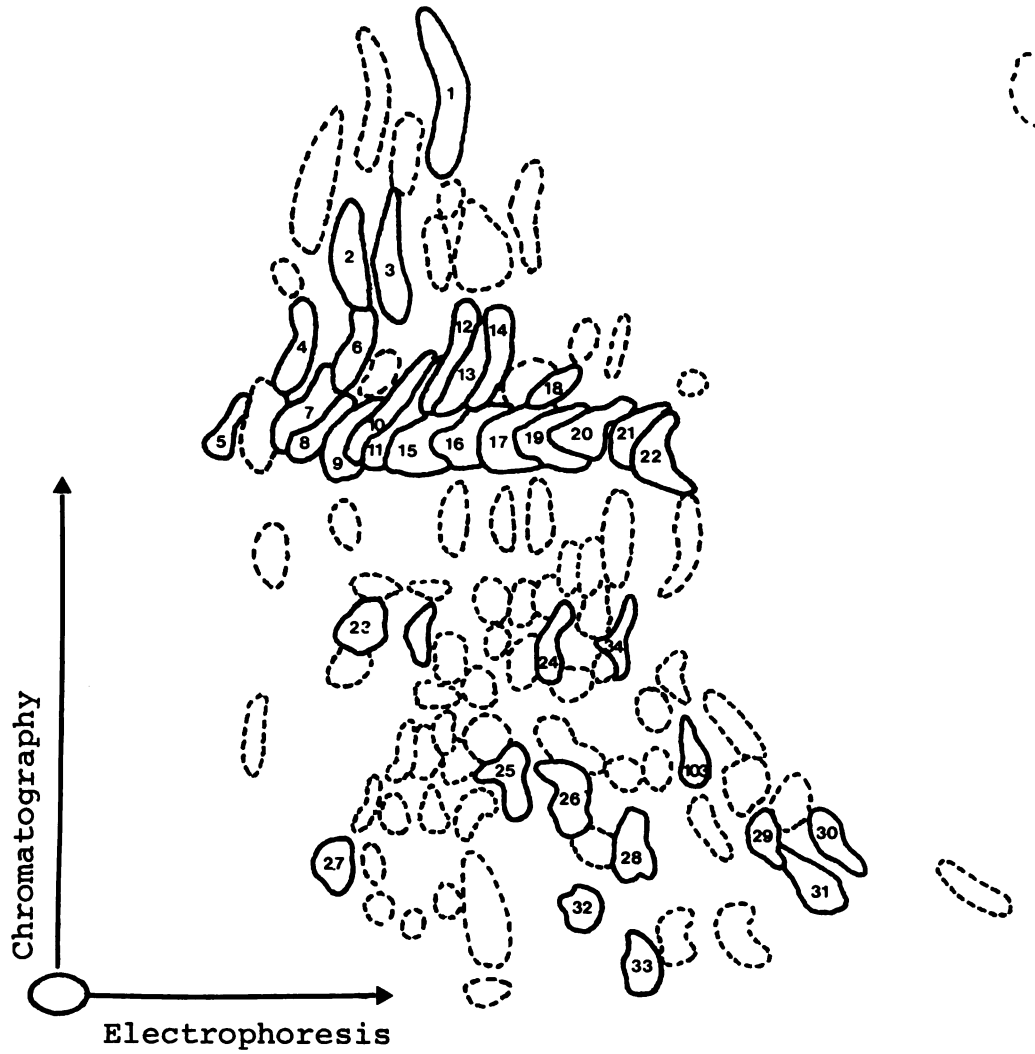

Fig. 7. Peptide map of complete fibrinolysin digest of oPRL. Numbered spots were eluted from the paper.

DISCUSSION. The variety of amino acids involved in peptide bond cleavage by fibrinolysin demonstrates the low specificity of the enzyme toward primary structure. Especially noteworthy in this regard is the prevalence at cleavage points of amino acid side-chains as chemically divergent as Leu and Lys. This may be an indication of some specificity involving conformation as well as amino acid side-chains. The higher rate of cleavage at Lys as compared to Arg may be an artifact resulting from the proximity of dansyl-Arg and ε-dansyl-Lys in the solvent systems used here for end-group analysis, leading to false-negative findings for N- terminal Arg.

The large number of ninhydrin spots present after peptide mapping suggests that fibrinolysin is active against a wide variety of residues, as previously suggested by the results of end-group analysis. Of greater significance in this regard, however, was the identification of several peptides ranging in length from 7 to 14 residues. Since only one of the spots analyzed showed a single amino acid, it is reasonable to assume that the 103 spots on the peptide map arose from peptides overlapping in the oPRL sequence, formed by incomplete digestion at most peptide bonds. Consistent with this conclusion was the isolation of both oPRL-(157-166) and oPRL-(160-166), demonstrating only partial digestion of the 159-160 bond.

The detection of a relatively high concentration of

free Leu indicates some preference for cleavage at that residue, consistent with the results of end-group analysis. The presence of free Leu, considered together with the observation of Leu at the C-terminus of 2 of the 8 cleavage points identified, may suggest a preference for this side-chain on either side of the hydrolyzed bond. In addition, the possibility of exopeptidase activity cannot be ruled out.

Although the precise nature of fibrinolysin specificity was not determined by this study, it is apparent that the Met-Ala bond of oPRL selectively cleaved by limited digestion is not specified entirely by the amino acids involved. It appears, instead, that other factors, such as conformation, play a role in determining the principle site of cleavage.

C. KINETICS OF FIBRINOLYSIN DIGESTION

It is clear from the results of complete fibrinolysin digestion of oPRL that a large number of sites are available for proteolytic cleavage. In order to determine the optimum conditions for preferential splitting of the 53-54 peptide bond, restricting as much as possible hydrolysis of other bonds, a study of the kinetics of digestion was undertaken. The purpose of this study was to separate the rate of cleavage between residues 53 and 54 from the overall rate of proteolysis, and to determine what

criteria could be used to monitor the digestion, so as to terminate it when the maximal concentration of oPRL-(1-53) and oPRL-(54-199) was reached. In addition, two lots of fibrinolysin were compared, to determine their relative rates of digestion.

MATERIALS AND METHODS. Crude preparations of bacterial fibrinolysin were obtained from Calbiochem, lots 400857 (A) and 602339 (B). The digestion of oPRL and subsequent chromatography were carried out as described by Birk and Li (1978), with the exception that the length of digestion was varied between 6 h and 21 h.

Changes in the peak wavelength of the oPRL fluorescence emission spectrum during the digestion were followed with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The concentration of oPRL monomer, prepared as described above, was 1 mg/ml in 0.01 M NH_4HCO_3, pH 8.4, and purified fibrinolysin was present at 1 µg/ml. Aliquots were removed from the digest periodically for $NaDodSO_4$ gel electrophoresis as described by Laemmli (1970).

The kinetics of peptide bond cleavage were followed by alkali uptake with a Radiometer Titrator 11 pH-stat. A 1 mg/ml oPRL solution was prepared in dilute NaOH (pH 8.0), then adjusted to pH 8.10 .01 with 0.01 M or 0.005 M NaOH by the titrator. Purifed fibrinolysin was added to

an enzyme:substrate ratio of 1:1000 for initial rate studies and 1:180 for complete digestion. The digestion was carried out under a nitrogen atmosphere, and was monitored until no further addition of alkali was observed.

CD spectra were taken with a Cary 60 spectropolarimeter equipped with a model 6002 circular dichroism attachment. Pathlengths were 0.1 or 1.0 cm, sample concentration was 1 mg/ml in 0.01 M NH_4HCO_3, pH 8.4, and the dynode voltage did not exceed 600 V. The molecular weight of 22,674 and mean residue weight of 113.9 were determined from the sequence of oPRL. Spectra were reported in terms of molar ellipticity in the near UV region (above 250 nm) and mean residue ellipticity in the far UV region (below 250 nm). α-Helix content was estimated as described by Bewley et al. (1969).

Protein concentration was determined by absorption with a Beckman DK-2A spectrophotometer. A correction for light scattering was made from optical density between 360 nm and 320nm (Leach & Scheraga, 1960). An absorptivity ($E^{0.1\%}$) at 280 nm of 0.909 for oPRL was taken from Bewley and Li (1972).

RESULTS. $NaDodSO_4$ gels of aliquots removed at various times during the digestion are shown in Fig. 8. Although the intensity of stained bands was not measured quantitatively, a progressive decrease in oPRL, and a concomitant

increase in lower molecular weight material, may be noted.

The elution profiles of fibrinolysin digests for different time periods and for the two enzyme preparations are shown in Fig. 9. The positions of the several peaks reported by Birk and Li (1978) were consistent through the entire range of digestion times, with both fibrinolysin lots. In contrast, the relative concentrations of these components showed considerable variation. The most notable changes were found for the α-peak, which was more prominent in the digests involving preparation B than A, and which became less intense as the period of digestion was increased for either preparation.

In addition to the α, β, γ, and C-components originally reported, a series of smaller-molecular-weight peaks (ε_1, ε_2, ε_3) appeared, and increased in intensity as the time of digestion progressed. This material was found to consist of hygroscopic, low- molecular-weight peptides whose composition was not further investigated.

The uptake of alkali during the early stages of digestion is shown in Fig. 10, together with the changes in Trp fluorescence of oPRL over the same time period. The fluorescence changes during this time appeared linear. At 4 h, an average of 7 peptide bonds per molecule had been cleaved. A complete digestion resulted in an average cleavage of 22 bonds per molecule.

The CD spectrum of the 4 h digest is shown in Fig.

11, together with the spectrum of native oPRL. The α-helix content of the digested material was 35 ± 5%.

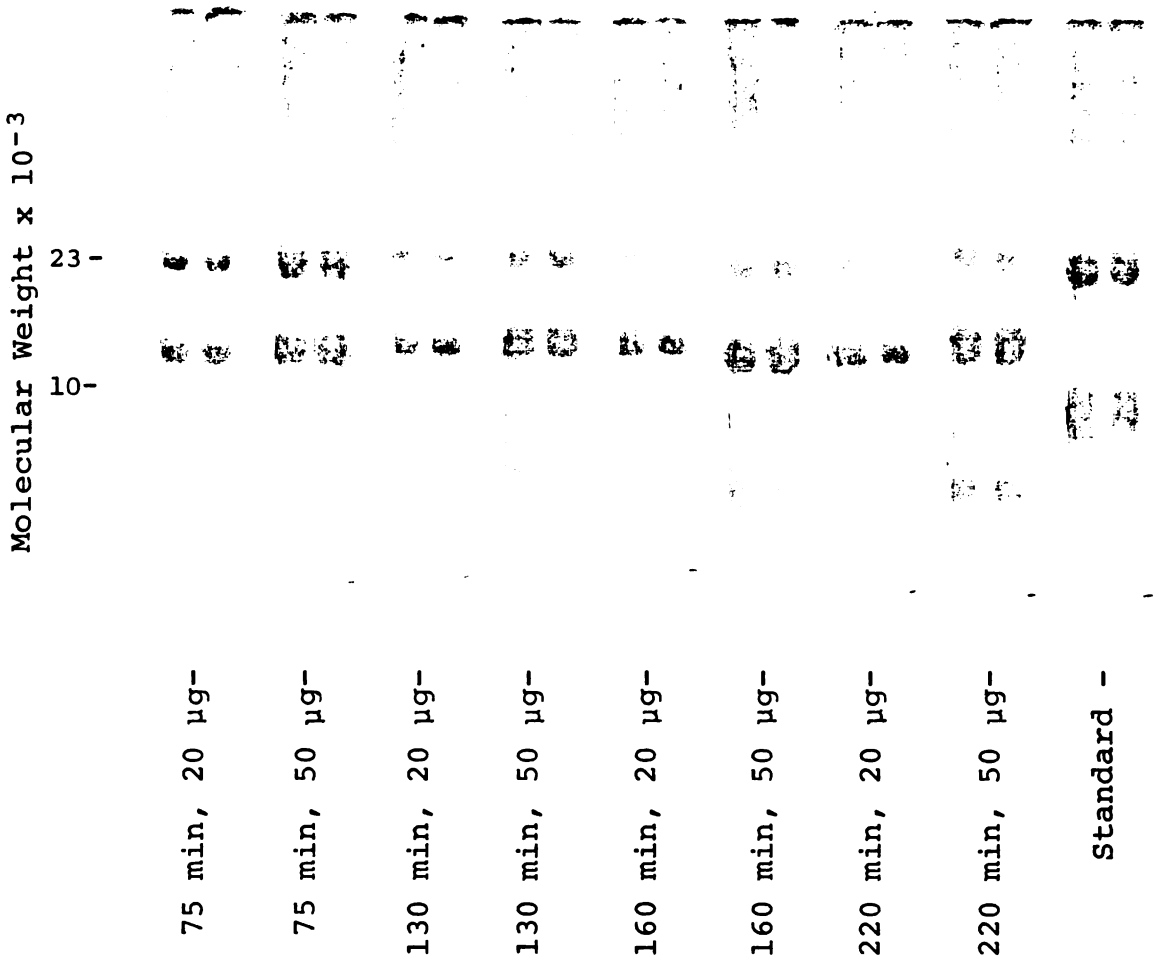

Fig. 8. $NaDodSO_4$ gel electrophoresis of oPRL digestion with fibrinolysin. Time of digestion and amount of material on each gel is shown. Molecular weight standards were oPRL and β-LPH.

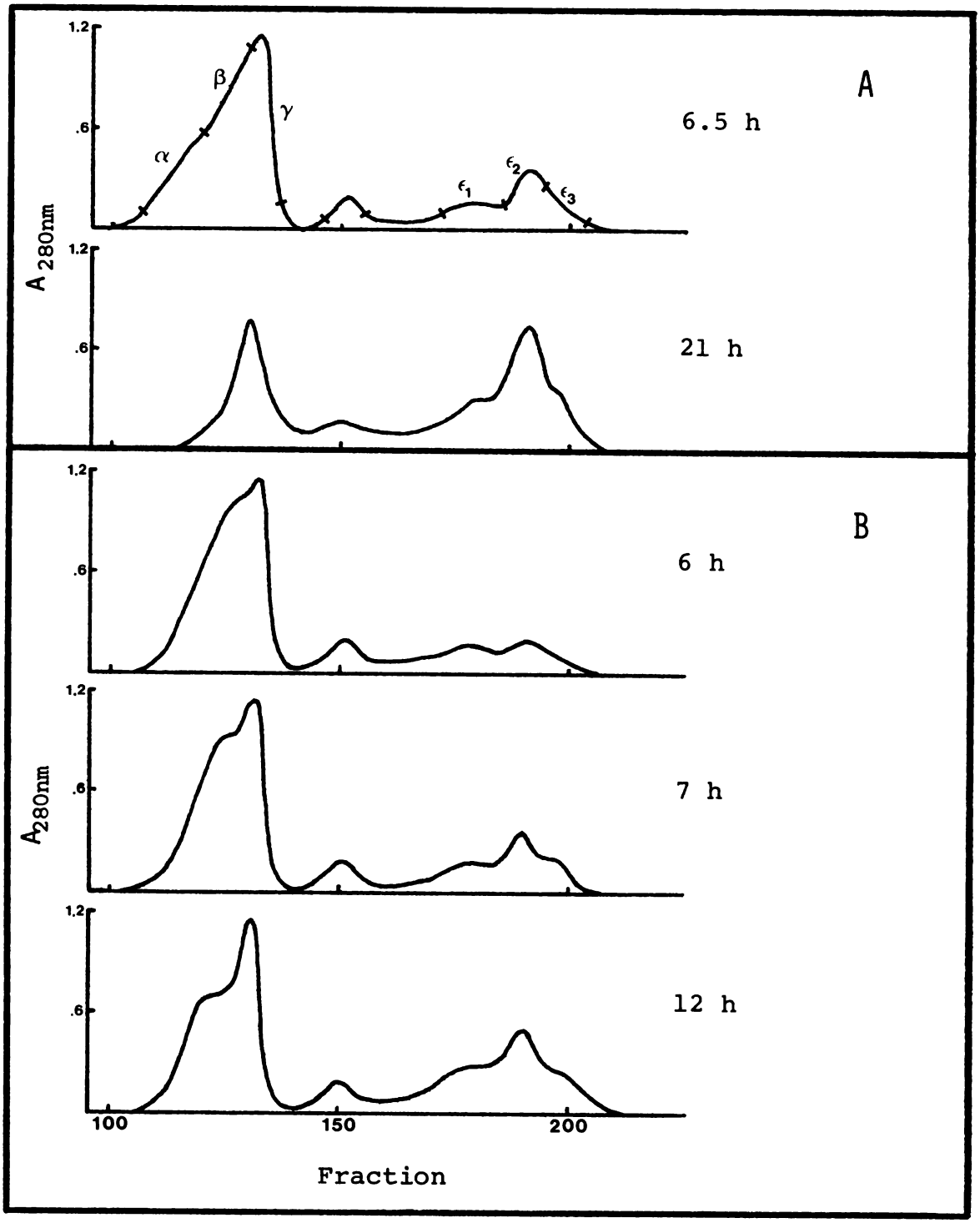

Fig. 9. Elution profiles of fibrinolysin-treated oPRL, following digestion times indicated, on Sephadex G-75 in 20% HOAc. Diagrams A and B represent two different lots of fibrinolysin. Fractions α, β, and γ were those of Birk and Li (1978).

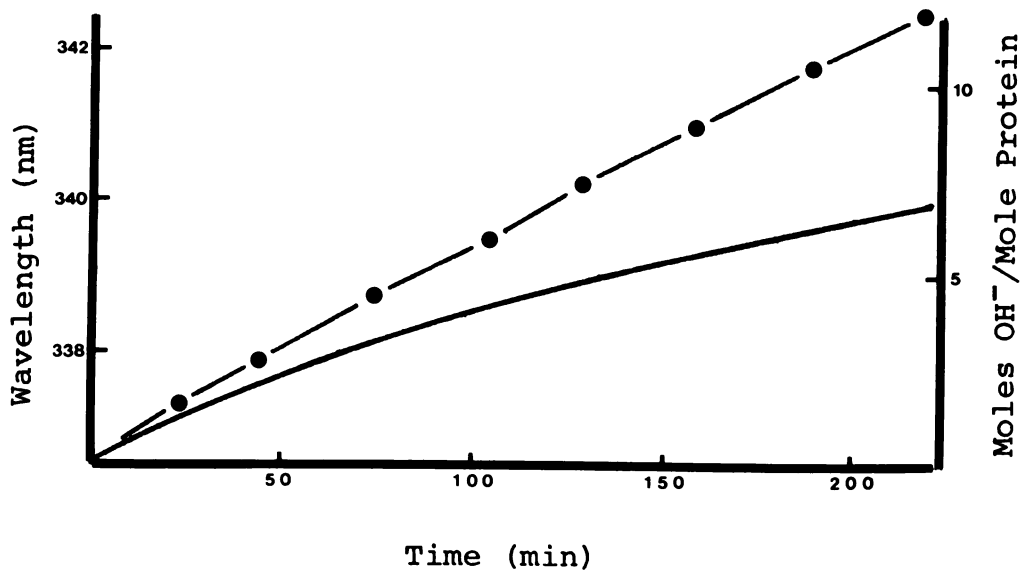

Fig. 10. Time course of peak fluorescence emission wavelength (-·-) and alkali uptake (——) of oPRL during fibrinolysin digestion. The excitation wavelength was 295 nm.

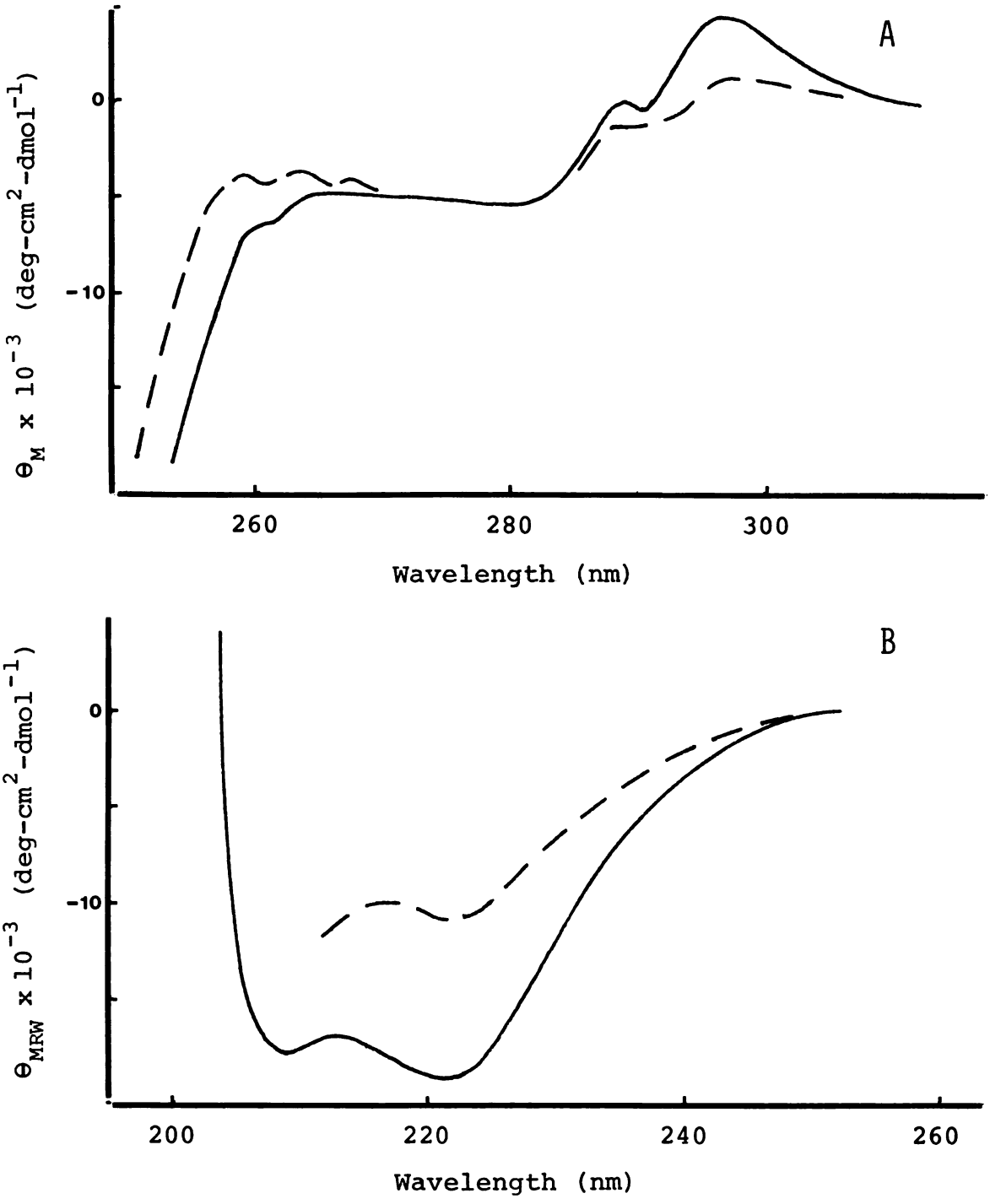

Fig. 11. CD spectra of native oPRL (——) and a 4 h fibrinolysin digest of oPRL (----) at pH 8.4, in the region of side-chain absorption (A) and amide bond absorption (B).

DISCUSSION. The apparent differences between the two lots of fibrinolysin compared in this study may be accounted for by assuming different levels of enzyme activity. There were no apparent differences between these preparations in the peak positions or changes in relative concentrations as time of digestion increased.

Fraction-α was reported by Birk and Li (1978) to consist of undigested oPRL. It was not surprising, therefore, to find a progressive decline in the concentration of this material as the digestion progressed. Fractions- and -C, however, showed a long period of stable concentration. The steady increase in the intensities of components ε_1, ε_2, ε_3, gave evidence for extensive cleavage throughout the digestion.

A similar ambiguity may be noted between alkali uptake and $NaDodSO_4$ gel electrophoresis patterns. At 220 min an average of 7 bonds per molecule had been broken, but the two major cleavage products remained evident on the gels. CD spectra also gave evidence for the retention of most of the native oPRL secondary and tertiary structure at 240 min.

The apparent linearity of oPRL fluorescence changes during the digestion was in marked contrast to the decrease in rate of alkali uptake over the same period. Apparently, substrate availability limited the rate of proteolysis, but cleavage of peptide bonds was not the

rate-determining factor for fluorescence changes.

Among the possible explanations for these observations are the following. First, a steady state condition may be established, in which the rate of major fragment formation equals its rate of degradation. Under these conditions the levels of oPRL-(1-53) and oPRL-(54-199) would remain relatively constant through much of the course of digestion, while the intact molecule would be degraded, and formation of low-molecular- weight material would be apparent.

A second explanation proposes that hydrolysis of the 53-54 peptide bond is rapidly catalyzed by the enzyme, but without facilitation of subsequent cleavage as a result of this initial nick. In this case, it might be expected that only after the relatively slow hydrolysis of two or more bonds would the rigidity of the native oPRL structure be lost, and the molecule become susceptible to more rapid attack by fibrinolysin, allowing the enzyme to thoroughly degrade a small proportion of the substrate, while leaving most of the oPRL molecules with a single cleavage.

Finally, interpretation of these data may involve the possibility of fibrinolysin exopeptidase activity. It is possible that the large number of peptide bonds cleaved without loss of three-dimensional structure or disappearance of the two primary cleavage products on gels resulted from selective removal of a few terminal residues of the

major fragments.

D. IDENTIFICATION OF PARTIAL DIGESTION PRODUCTS

The isolation procedures of Birk and Li (1978) were examined and compared to several alternative techniques for fragment purification. Of special interest was the separation of intact oPRL from oPRL-(54-199) in the β-region of the elution profile. In addition, a more quantitative evaluation of fragment membrane-binding properties than was previously presented was undertaken.

MATERIALS AND METHODS. The isolation procedures of Birk and Li (1978) were used for the initial preparation of the fragments. The purity of the peptides was then examined by slab gel electrophoresis in the presence of $NaDodSO_4$ (Ames, 1974), with 20% acrylamide. Molecular weight standards used were BSA (69,000), ovalbumin (45,000), HGH (22,000), myoglobin (16,900), α-lactalbumin (14,500), and β-LPH (10,000).

End-groups were determined by the dansyl-chloride method (Gray, 1967; Woods & Wang, 1967), and the positions of the fluorescent spots were compared to dansyl-amino acid standards. Amino acid analyses were performed in triplicate, with a Beckman 119C amino acid analyzer, according to the method of Spackman et al. (1958). Amino acid compositions were compared to those expected for

oPRL, oPRL-(1-53), and oPRL-(54-199). The chi-square test was used to determine the goodness of fit between the analyses and expected compositions.

A tryptic digest of fraction-C was obtained from 0.6 mg of the material with 12 µg trypsin (Worthington Biochemical Co., 217 u/mg) in 0.2 M NH_4Ac, pH 8.1 for 20 h at room temperature. The digest was used for peptide mapping (Kato et al., 1959; Sairam & Li, 1975), and ninhydrin-positive spots were cut from the paper, eluted into 0.1 M NH_4OH, and submitted to amino acid analysis and end-group analysis.

The radioreceptorassay[1] was performed with late pregnant rabbit mammary gland membranes (Shiu et al., 1973) and ^{125}I-oPRL prepared by the lactoperoxidase method (Thorell & Johansson, 1971; Garnier et al., 1978). Modifications of the method were subsequently described by Wong et al. (1981).

Molecular weights of the two fragments under nondenaturing conditions were determined by exclusion chromatography according to the method of Laurent and Killander (1964). Standards used were BSA (stokes radius=37.0 Å) and myoglobin (stokes radius=18.9 Å).

A portion of the β-fraction shown in Fig. 9 was lyophilized and rechromatographed on Sephedex G-75 in 20%

[1]This assay was performed by K. Hines.

HOAc as described above. Each of the major peaks was then lyophilized and passed through a Sephadex G-100 upward-flow column (57 x 1.4 cm) in 0.01 M NH_4HCO_3, pH 8.4.

A second portion of the β-fraction, containing 55 mg of lyophilized material, was used for exclusion chromatography on Sephadex G-100 (63 x 3.5 cm) in 0.01 M NH_4HCO_3, pH 8.4. Each of the major peaks was lyophilized and examined by $NaDodSO_4$ gel electrophoresis as described above.

The remaining 153 mg of the lyophilized β-fraction was dissolved in 0.01 M NH_4HCO_3, pH 8.4, then dialyzed against the same buffer to remove any residual HOAc from the previous procedures. The solution was loaded on a DEAE-cellulose column (33 x 1.2 cm) equilibrated in the same buffer, then eluted with a continuous NaCl gradient. Both the absorbance at 280 nm and the salt concentration were determined for the fractions collected. Salt concentration was measured as electrical resistance, which was then compared with a standard regression line of log(resistance) vs. log[NaCl] determined for NaCl in the same buffer. The fractions of interest were examined by $NaDodSO_4$ gel electrophoresis.

RESULTS. The $NaDodSO_4$ gel electrophoresis pattern of oPRL-(1-53) shows a single major band corresponding to a molecular weight of 6,000 (Fig. 12). In addition, a faint band with a molecular weight of 9,000 was visible. Two

bands near the top of the gel appear to have been the result of contamination with the standards used.

oPRL-(54-199) showed a major band with a molecular weight of 17,000 (Fig. 12). A considerable amount of lower-molecular-weight material was also present, however, as well as a faint band of molecular weight 23,000. When determined by exclusion chromatography, the molecular weight of the major band was 33,900 ± 600.

End-group analysis of fraction C showed a single spot corresponding to dansyl-Thr. The results of the amino acid analysis are shown in Table 10, calculated according to expectations for the fragment. Chi-square values for treatment of the amino acid analysis as oPRL or oPRL-(54-199) each indicated a probability of occurrence less than 0.005.

End-group analysis of peak-Y indicated the presence of Ala at the N-terminus, and gave no evidence of major contaminants. The amino acid analysis of the material is given in Table 10. Calculation of raw data as oPRL and oPRL-(1-53) gave chi- square values corresponding to $0.05<P<0.975$ for the entire sequence and $P<.005$ for residues 1-53.

The displacement of ^{125}I-oPRL from rabbit mammary membranes by native oPRL, oPRL-(1-53), and oPRL-(54-199) is shown in Fig. 13. No membrane binding activity was detected for the small fragment even at a concentration

1000-fold higher than that at which the native hormone was active. Displacement by oPRL-(54-199) yielded a curve parallel to that of oPRL, but with 1% the activity of the native material.

The peptide map obtained for the trypsin-digest of fraction-C contained 6 major spots (Fig. 14), as expected for oPRL-(1-53). Amino acid and end-group analyses, however, showed that only 4 of these peptides corresponded to the N-terminal region of oPRL (Table 11). One of the additional peptides was found to have the composition and end-group of oPRL-(178-181). The end-group of peptide #1 was that of oPRL-(49-53), but the amino acid analysis was equivocal, and this material was not assigned. None of the major spots contained oPRL-(1-16), and each of the spots eluted from the map showed a background amino acid concentration of approximately 10%.

Chromatography of the β-fraction on Sephadex G-75 in 20% HOAc resulted in the elution of a single asymmetric peak (Fig. 15). This was divided into two fractions as shown, each of which was passed through a calibrated column for molecular weight determination. The two major components had molecular weights of 48,000 and 37,000. A number of minor peaks emerged later in the elution, apparently consisting of lower-molecular-weight material.

Passage of the β-Fraction through Sephadex G-100 at pH 8.4 resolved two major components (Fig. 16). Analysis

of these peaks and the starting material by gel electrophoresis (Fig. 17) revealed that peak B consisted of a single major band with a molecular weight of 23,000, and a diffusely-stained region extending from the major band to a molecular weight of 6,000. Peak A, in contrast, showed four discrete bands, with molecular weights in the same range.

Ion exchange chromatography of the β-fraction separated the material into several distinguishable components (Fig. 18). Aliquots from these fractions were examined by gel electrophoresis as shown in Fig. 19. Fractions A and C, eluted at NaCl concentrations between 6 mM and 9 mM, appeared to consist almost exclusively of undigested oPRL, with a slight trace of a 17,000 molecular weight component in C. A high concentration of the 17,000 molecular weight species was apparent in the remaining fractions. In addition, lower-molecular-weight peptides began forming discrete bands at Fraction G, in 13 mM salt.

Table 10. Amino acid analyses of oPRL-(1-53) and oPRL-(54-199). Correspondence between expected and observed values is shown by the chi-square test.

Amino Acid	oPRL-(1-53)		oPRL-(54-199)	
	Expected	Observed	Expected	Observed
Ala	2	2.5	7	6.8
Arg	3	2.6	8	8.7
Asp	7	6.2	15	13.7
Cys	2	2.7	4	3.4
Glx	4	6.6	18	19.7
Gly	4	4.2	7	7.9
His	2	2.0	6	6.8
Ile	2	1.6	9	7.6
Leu	3	4.0	20	20.6
Lys	2	1.9	7	7.2
Met	3	1.5	4	4.4
Phe	4	3.2	2	2.4
Pro	3	3.6	8	9.7
Ser	4	3.6	11	9.5
Thr	2	1.8	7	7.3
Val	4	3.0	6	5.2
	χ^2=11.7		χ^2=3.55	
	P = 0.9		P =0.995	

Table 11. Amino acid and end-group analyses of trypsin-oPRL-(1-53) fragments. Peptide analyses are compared to oPRL segments indicated.

Amino Acid	Peptide 1		Peptide 2		Peptide 3		Peptide 4		Peptide 5		Peptide 6	
	Obs	49-53	Obs	17-21	Obs	22-43	Obs	44-48	Obs	178-181	Obs	43
Asp	1.5	0	43.3	2	22.8	3	3.6	0	3.1	1		
Thr	0.3	1					1.2	0	0.4	0		
Ser	0.9	0			16.1	3	1.9	0	7.7	2		
Glx	0.4	0			10.8	2	28.2	1	0.5	0		
Gly	5.9	1	2.1	0	1.4	0	24.5	1			0.7	0
Ala					5.6	1	24.7	1				
Val					16.6	2	0.6	0				
Met					7.3	2						
Ile					4.3	1	1.3	0				
Leu			25.7	1	6.4	1	2.2	0				
Tyr					2.4	1	11.4	1				
Phe	11.6	1	24.3	1	10.3	2						
His					12.2	2	1.4	0				
Lys					4.9	1	29.7	1	3.8	1		
Arg			22.5	1	4.5	1	0.8	0			2.7	1
End-Group:	Gly	Gly	Asp	Asp	Ala	Ala	Tyr	Tyr	Asp	Asp	Arg	Arg

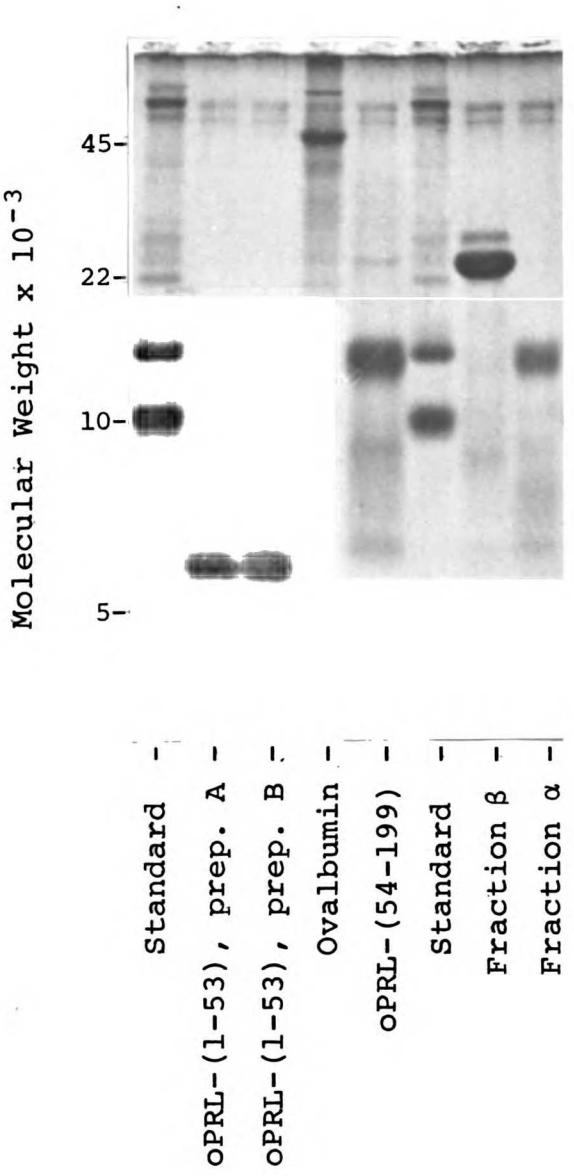

Fig. 12. $NaDodSO_4$ gel electrophoresis of isolated oPRL fragments, following digestion with fibrinolysin. Molecular weight standards were BSA, ovalbumin, HGH, myoglobin, α-lactalbumin, and β-LPH.

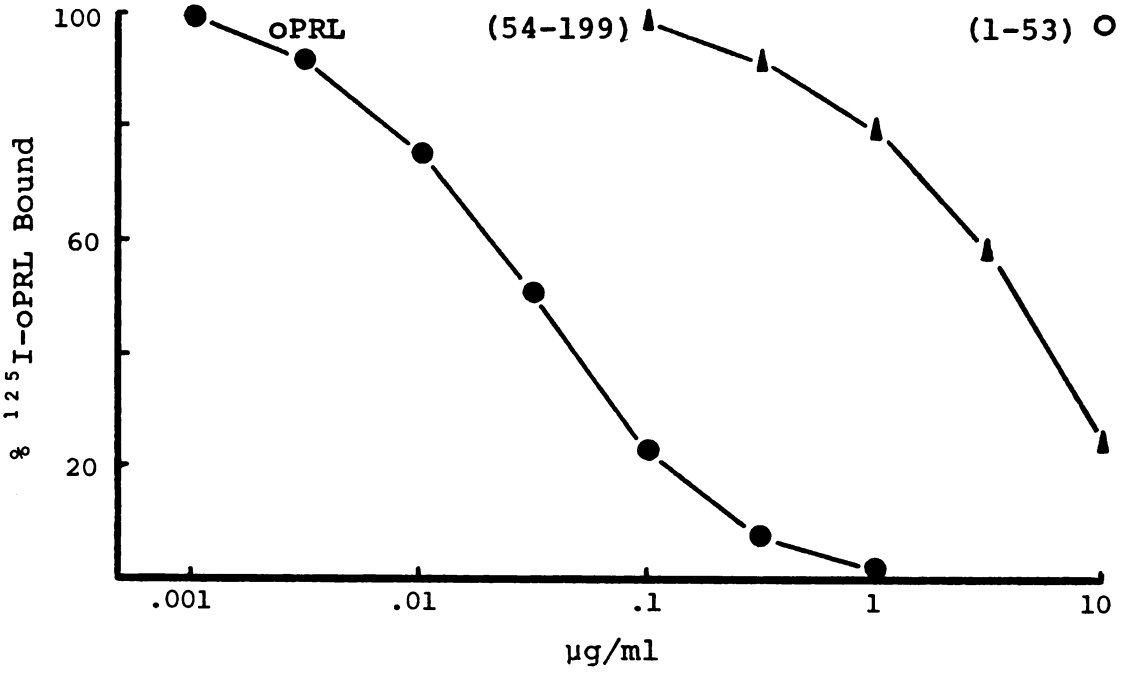

Fig. 13. Displacement of ^{125}I-oPRL by oPRL, oPRL-1-53, and oPRL-(54-199) from pregnant rabbit mammary tissue.

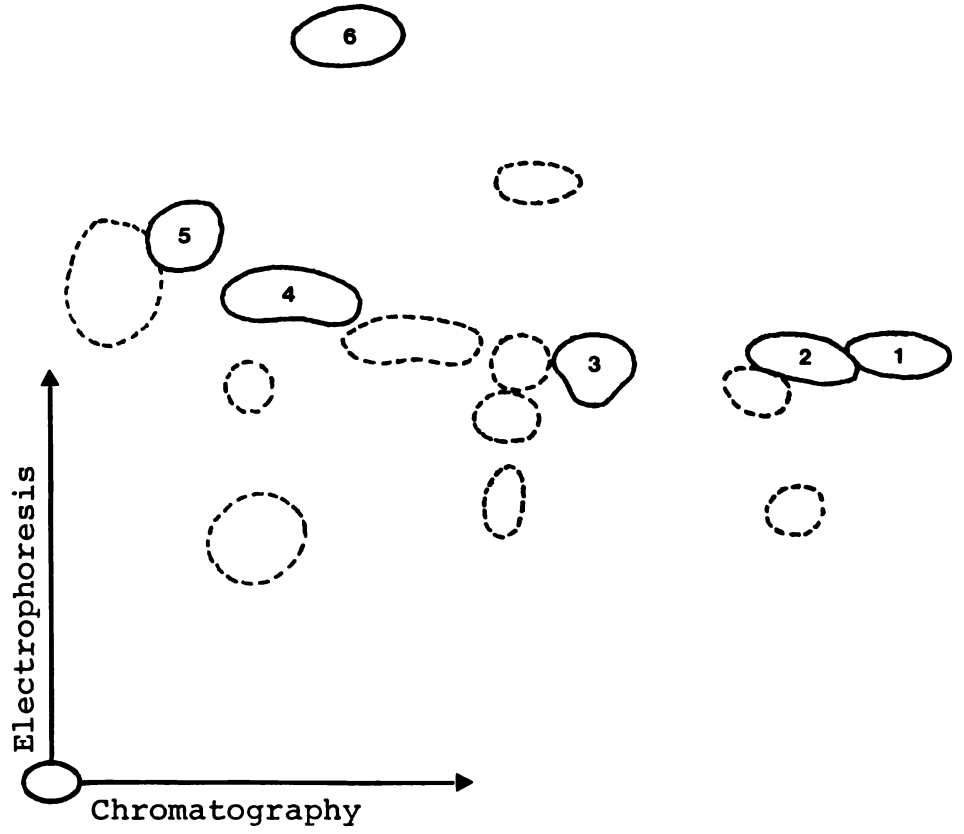

Fig. 14. Peptide map of oPRL-(1-53) trypsin digest. Numbered spots wee eluted from the paper.

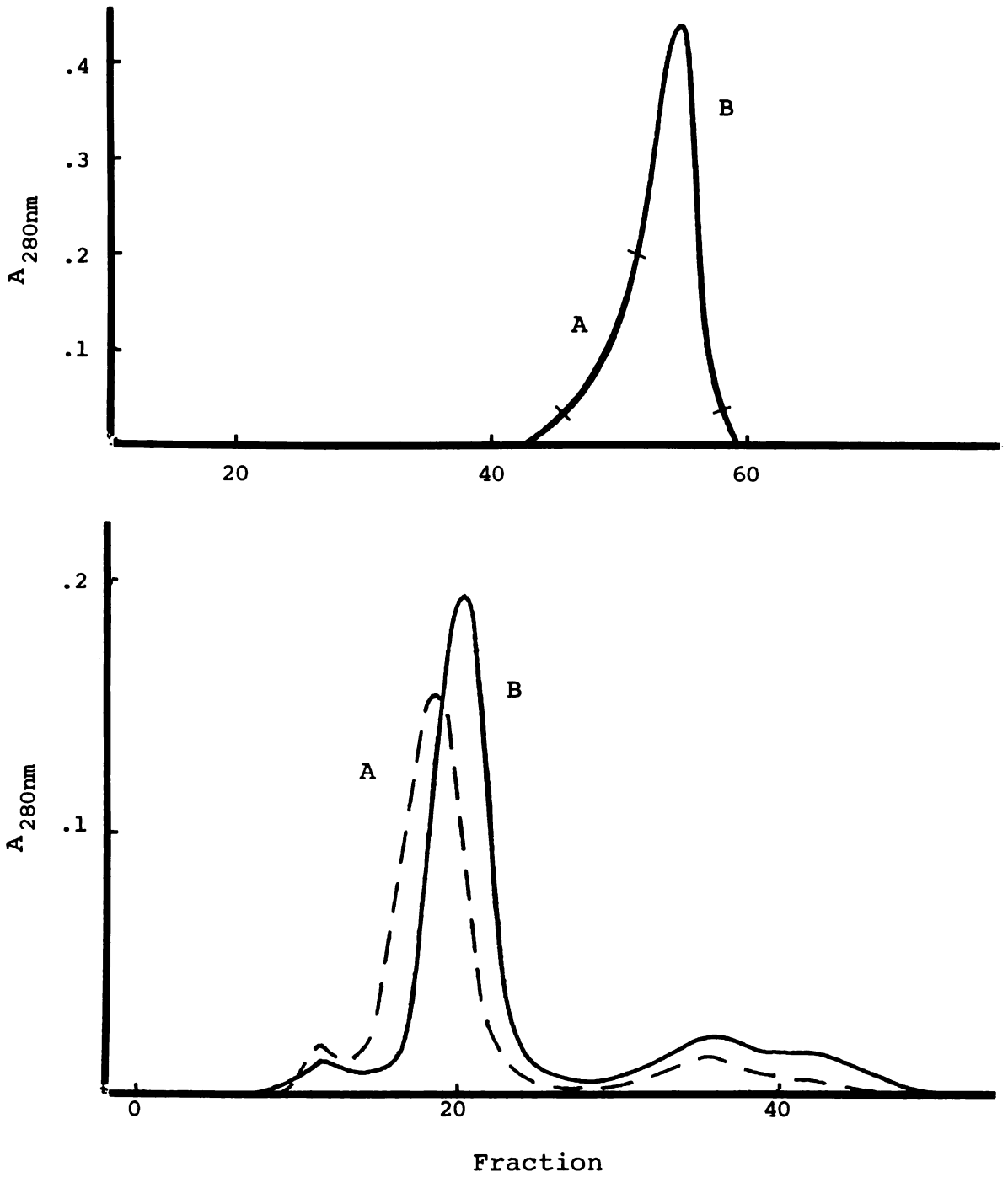

Fig. 15. Upper panel: Elution profile of peak β on Sephadex-G-75 in 20% HOAc. Lower panel: Elution of A and B fractions on Sephadex G-100 in 0.01 M NH_4HCO_3, pH 8.4

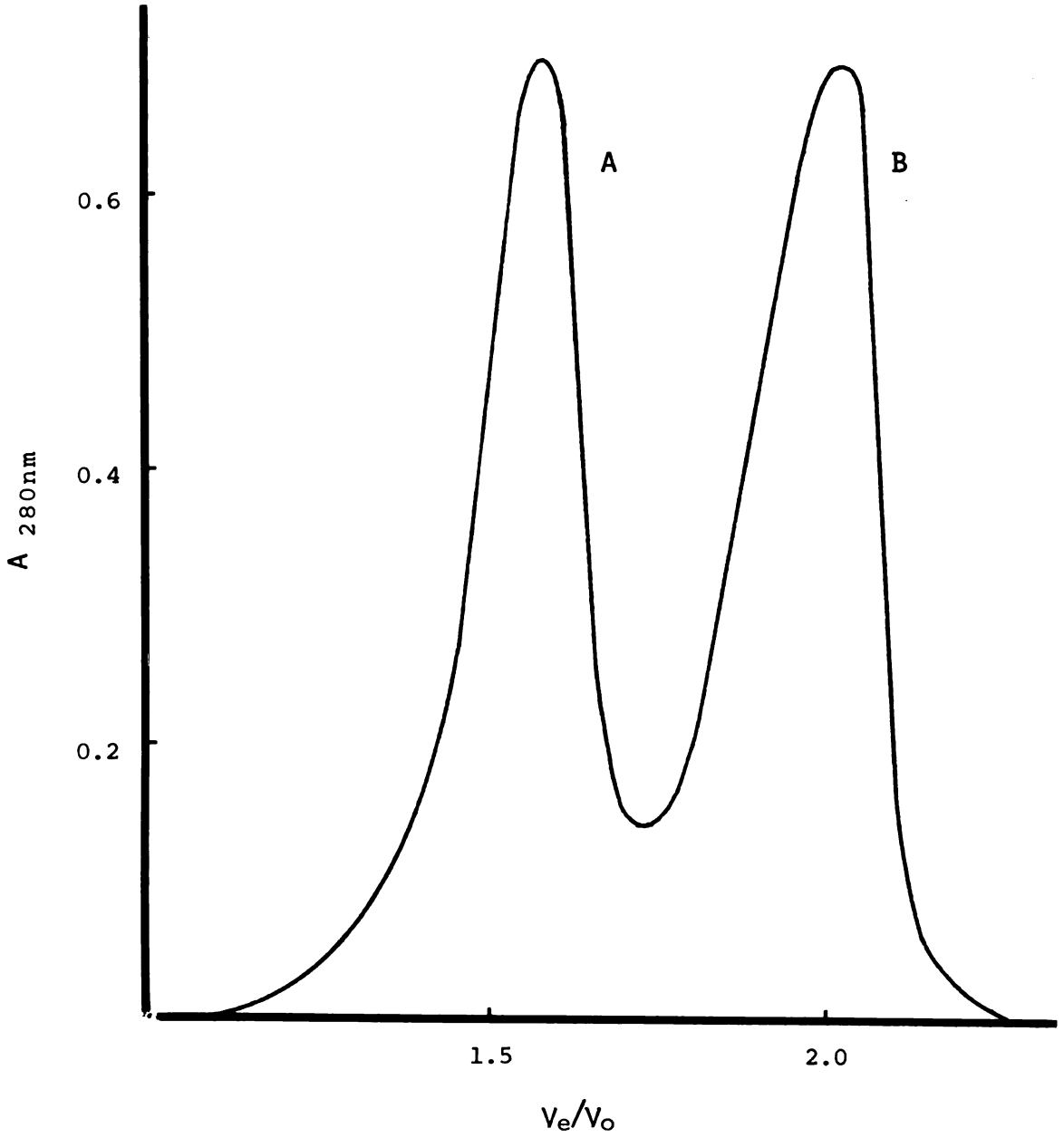

Fig. 16. Elution Profile of fraction β from Sephadex G-100 in 0.01 M NH_4HCO_3, pH 8.4.

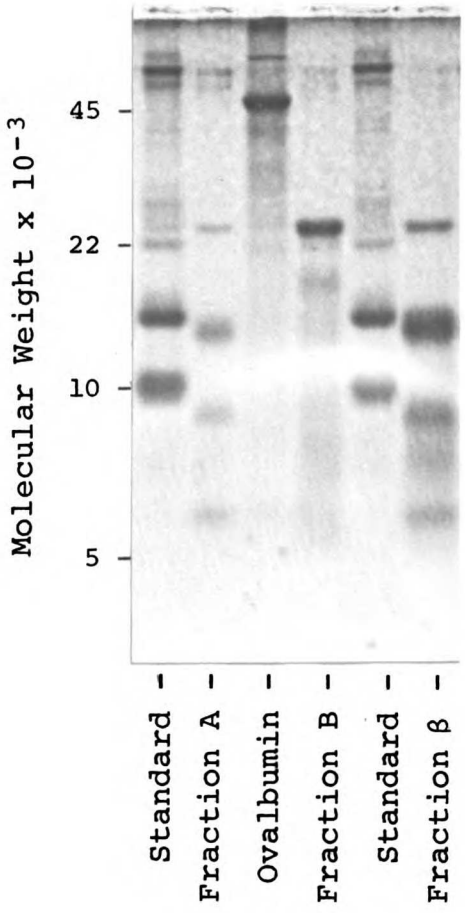

Fig. 17. $NaDodSO_4$ gel electrophoresis of fraction β following elution from Sephadex G-100 (Fig. 16). Molecular weight standards were BSA, ovalbumin, HGH, myoglobin, β-lactalbumin, and α-LPH

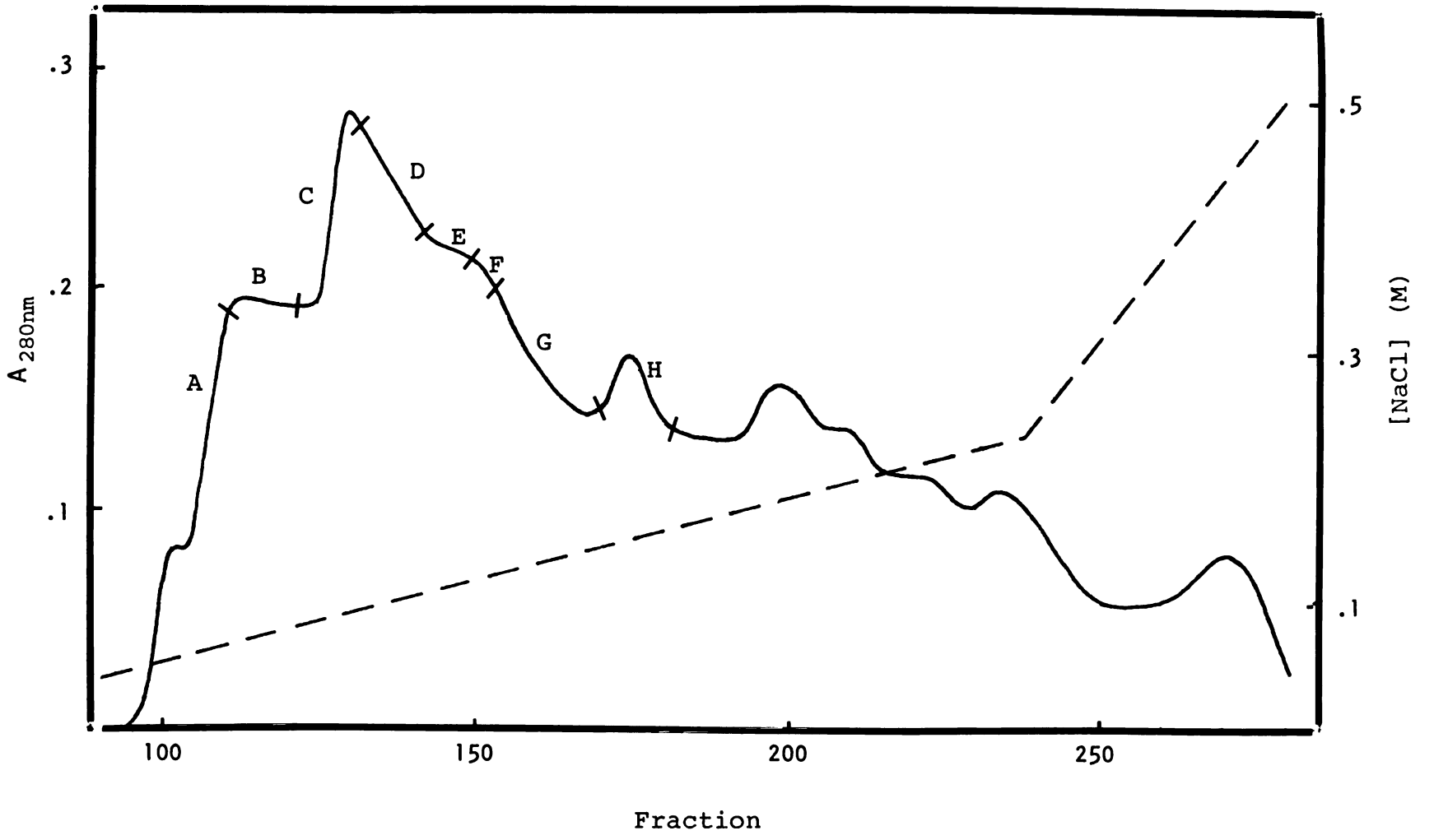

Fig. 18. Elution profile of fraction β from DEAE-cellulose (——) and concentration of NaCl gradient used in the elution (----).

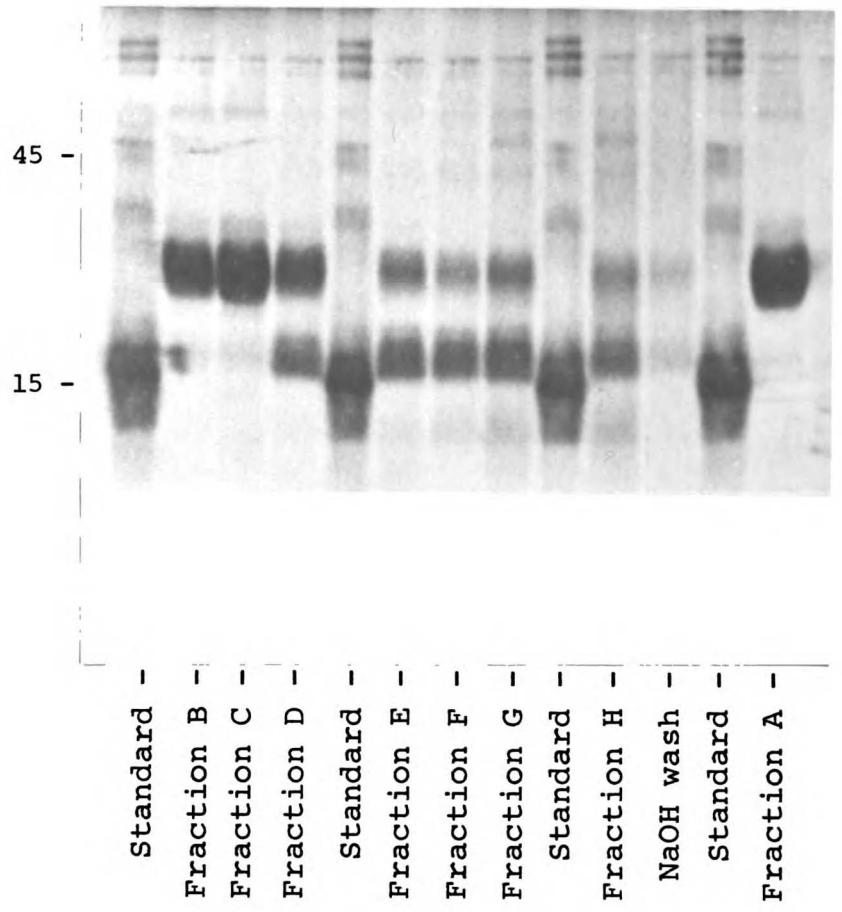

Fig. 19. $NaDodSO_4$ gel electrophoresis of fraction β following elution from DEAE-cellulose (Fig. 18). Molecular weight standards were ovalbumin, myoglobin, β-LPH, and α-lactalbumin.

DISCUSSION. Although a single end-group was determined for the oPRL-(1-53) preparation, both gel electrophoresis and the peptide map indicated that a small amount of contaminating material was present in the preparation. It is not clear why a peptide apparently consisting of residues 178-181 was associated with the fragment, but it may have resulted from some noncovalent interaction. Although this peptide and oPRL-(1-53) are close to the 58-174 disulfide bond, there was no evidence to suggest a covalent link, as no trace of Cys was found in these analyses, and no major contaminating band was found on the $NaDodSO_4$ gels.

The absence of residues 1-16 and 49-53 in the peptide map may have been due to poor reaction of those peptides, which are devoid of Lys residues, with ninhydrin. In this case, the several minor spots noted would be expected to contain the missing fragments.

The RRA data indicated that oPRL-(1-53) lacked any significant membrane binding activity. This conclusion was later confirmed by Wong *et al*. (1981) for both RRA and RIA. The lack of ability to displace labeled oPRL in the RRA also demonstrated that the fragment preparation was not contaminated with undigested material.

The presence of contaminating material in the preparation of oPRL-(54-199) was evident on the $NaDodSO_4$ gel. A faint band running close to oPRL-(1-53) suggested that a small amount of this fragment was present. Other

bands corresponded to undigested oPRL and a molecular weight of 9,000-10,000.

The molecular weight of the fragment under nondenaturing conditions was consistent with the suggestion of Birk and Li (1978) that the material exists as a dimer in aqueous solution. The molecular weight of 33,900 was very close to that of 33,330, calculated from the sequence, as expected for globular proteins that do not interact with Sephadex (Laurent & Killander, 1964). The inability of exclusion chromatography to remove the contaminants from this preparation may be a consequence of noncovalent interaction among the various components in the solution.

The detection of 1% membrane binding activity may be the result of oPRL contamination. Similar levels of activity were reported, however, by Wong et al. (1981), for both RRA and RIA, but this may also have been due to a small amount of undigested material.

Repeated passage of the β-fraction through G-75 in 20% HOAc gave very poor resolution of oPRL and oPRL-(54-199). The eluted components had molecular weights apparently corresponding to dimers of undigested oPRL and oPRL-(54-199). Although the fragment was originally reported to be a dimer (Birk & Li, 1978), nothing was said concerning the state of aggregation of the intact material. The elution pattern of the original digest did, however, show oPRL as the first protein to emerge from the

column, suggesting that either both oPRL and oPRL-(54-199) behaved as dimers or both behaved as monomers in 20% HOAc. If the latter was the case, then dimerization of oPRL may have been the result of repeated lyophilization, or of lyophilization from 20% HOAc. The resolution of material passed through Sephadex G-100 at pH 8.4 was considerably better than that obtained in acid. The content of the isolated peaks, however, showed a much greater purification of undigested oPRL than oPRL-(54-199). The inability to separate the large fragment from intact material and smaller components may have been the result of noncovalent interaction between these peptides and oPRL, with binding to the latter being the least stable. The earlier elution of the multicomponent peak was consistent with oPRL-(54-199) dimerization, and suggests that the smaller components are either noncovalently bound to the fragment, or represent nicking within the disulfide loops of the peptide, which results in separation of the chains under the reducing conditions used for electrophoresis.

The best separation of components appears to have been achieved with DEAE-cellulose. The use of a rather shallow salt gradient resulted first in the elution of undigested material, followed by oPRL-(54-199) with minimal contamination by smaller peptides. Intact oPRL, however, appeared over a wide range of salt concentrations, suggesting that components with several isoelectric

points were present, possibly differing in degree of deamidation (Lewis et al., 1970).

The low-molecular-weight material required the highest salt concentration for elution. Although it was possible to selectively remove oPRL-(54-199) from the DEAE, this does not rule out the possibility that the smaller peptides originated from nicks in the large fragment, as nicked forms might interact more strongly with the ion exchanger.

The final method of oPRL-(54-199) isolation, based on these findings, first involved removal of undigested oPRL by gel filtration in 20% HOAc, then at pH 8.4. RRA data indicated that these steps removed at least 99% of the intact hormone. Smaller peptides or nicked material could then be removed by ion exchange chromatography, leaving a homogeneous preparation of the intact fragment.

CHAPTER 3

STRUCTURE OF THE OPRL FRAGMENTS AND RECOMBINANT

Although oPRL-(1-53) and oPRL-(54-199) are devoid of biological activity (Birk & Li, 1978), each fragment has sufficient structural integrity to allow recognition of, and binding to, the complementary peptide. While this structural requirement could be restricted to sequence, evidence of secondary and tertiary structure for both fragments raises the possibility that the conformation of the molecules is involved in the recombination process. In order to investigate this possibility, a study of the spectroscopic properties of the fragments was undertaken.

A. ULTRAVIOLET ABSORPTION SPECTRA

The principal use of the UV absorption spectra of these molecules was concentration determination. In order to make these measurements, it was necessary to establish the absorptivity of the fragments. In addition, some information on chromophore environment can be obtained from absorption properties (Wetlaufer, 1962), allowing use of these spectra in estimating the degree of folding of the fragments.

MATERIALS AND METHODS. UV absorption spectra were taken on a Perkin-Elmer 552 spectrophotometer, equipped with a temperature control apparatus to maintain sample and

reference temperatures at 25°C. The pathlength in each case was 1 cm and the band-pass was 1 nm. Spectra were scanned from 360 nm to 240 nm. Absorption values taken at 5 nm intervals between 360 nm and 320 nm were used to correct for light scattering according to the method of Leach and Scheraga (1960).

Absorptivity values were first estimated, as suggested by Wetlaufer (1962), on the basis of amino acid composition. Amino acid analysis (ch. 2) was also used to determine the concentration of the fragments. These values were then compared with the absorption spectra of the same samples. Finally, absorptivity values were measured according to the method of Bewley (unpublished), which involved comparison of the absorption spectra before and after sample digestion with thermolysin. By this means it was possible to eliminate the conformational contribution to the absorptivity of the protein (Wetlaufer, 1962).

This technique involved determination of the absorption spectrum at neutral pH, followed by addition of a 1:1000 enzyme:substrate ratio (w:w) of thermolysin. The digestion was carried out for 20 h at 30°C. The exact positions of absorption peaks and shoulders were determined from a second derivative spectrum. The sample was adjusted to pH 2 with 10 µl concentrated HCl and the second derivative was again scanned, to insure that no

further conformational changes had occurred. The solution was then divided between the sample and reference cells, and the sample was adjusted to pH 13 with 10 µl concentrated NaOH. The molar extinction coefficient of Tyr under these conditions is 1,745 M^{-1} at 287 nm (Bewley, unpublished), allowing measurement of the protein concentration.

The possibility that a Tyr modification was catalyzed by some contaminant in the crude fibrinolysin was tested by digesting duplicate 1 mg samples of oPRL with 1 µg purified fibrinolysin, and duplicate 1 mg samples with 55 µg crude fibrinolysin. Both reactions were carried out at 37°C for 20 h. Absorption and pH 13 difference spectra were compared.

The action of crude and purified fibrinolysin on N-acetyl-L-tyrosinamide at pH 7.8, 8.7, and 9.5 was also examined. The Tyr solution was adjusted to an absorbance of ~0.3, corresponding to the Tyr content of ~0.6 mg/ml oPRL. 50 µg crude fibrinolysin and 1 µg pure fibrinolysin were used in separate treatments, at each pH. The ratios of neutral to pH 13 difference absorbances were measured for each solution at 7 h and 40 h.

RESULTS. The UV absorption spectrum of oPRL-(54-199) is shown in Fig. 20. The broad peak is centered at 278 nm and is somewhat less flat than the same region of the

native oPRL spectrum. The shoulder at 290 nm is similar in both spectra. The absorptivity of oPRL-(54-199) at 280 nm was calculated from its sequence to be 1.08 ml/mg. From amino acid analysis, the value was determined to be 1.04 (mean of two analyses), and, on the basis of the thermolysin digest, 1.059 ± .016 (mean ± S. E. of 4 analyses).

Also shown in Fig. 20 is the spectrum of oPRL-(1-53). This spectrum is characterized by a well-defined maximum at 276 nm, fine structure between 255 nm and 270 nm, and the absence of a shoulder at 290 nm. In addition, the spectrum is considerably less intense than that of the other molecules. The absorptivity of the fragment was estimated from the sequence to be 0.461 ml/mg at 276 nm. Determination of absorptivity by thermolysin digestion resulted in a value of 0.944 ml/mg.

Examination of the ratio of Tyr absorbance at neutral pH to the difference absorption of ionized Tyr for the thermolysin-digested fragment gave a value of 1.01 (Fig. 21), compared to the value of 0.60 ± .03 (6 determinations) for N-acetyl-L-tyrosinamide. It appears from these numbers that only 60% of the absorbing Tyr of oPRL-(1-53) ionize at pH 13.[1]

The possibility that a conformational effect was

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[1] I am indebted to T. A. Bewley for this observation.

responsible for the incomplete ionization was tested by repeating the spectra in 5 M guanidine-HCl. The results in this case were unchanged by the presence of the denaturant.

The absorptivity of oPRL-(1-53) was again determined by the difference spectrum method described above, but with a 20 h acid hydrolysis in place of the thermolysin digest. The absorptivity in this case was found to be 0.860 ml/mg, and the ratio of ionizing to absorbing Tyr was 0.92, corresponding to 55% ionization.

A comparison of theoretical and observed contents of Tyr in amino acid analyses of oPRL-(1-53) and oPRL-(54-199) revealed an observed to expected ratio of 1.00 ± .03 (3 determinations) for the large fragment, and 0.84 ± .05 (4 determinations) for the small. This difference in ratios was significant at the 0.005 level.

In order to determine which of the two Tyr residues of oPRL-(1-53) was responsible for these findings, a tryptic digest and peptide map of the fragment were done as in ch. 2. Amino acid analysis of the two Tyr-containing peptides, oPRL-(22-43) and oPRL-(44-48), revealed Tyr contents 44% and 42% as great as expected on the basis of other residues present (Table 11). Optical examination of the tryptic fragments was not possible because of excessive light scattering.

The comparison of Tyr ionization for oPRL samples

digested with crude and pure fibrinolysin revealed a 5% decrease in ionization with the crude enzyme (Fig. 22). If complete ionization for the pure fibrinolysin preparation is assumed, this corresponds to ionization of 6.7 Tyr of oPRL digested with crude fibrinolysin. No differences appeared among any of the N-acetyl-L- tyrosinamide solutions with both crude and purified enzyme.

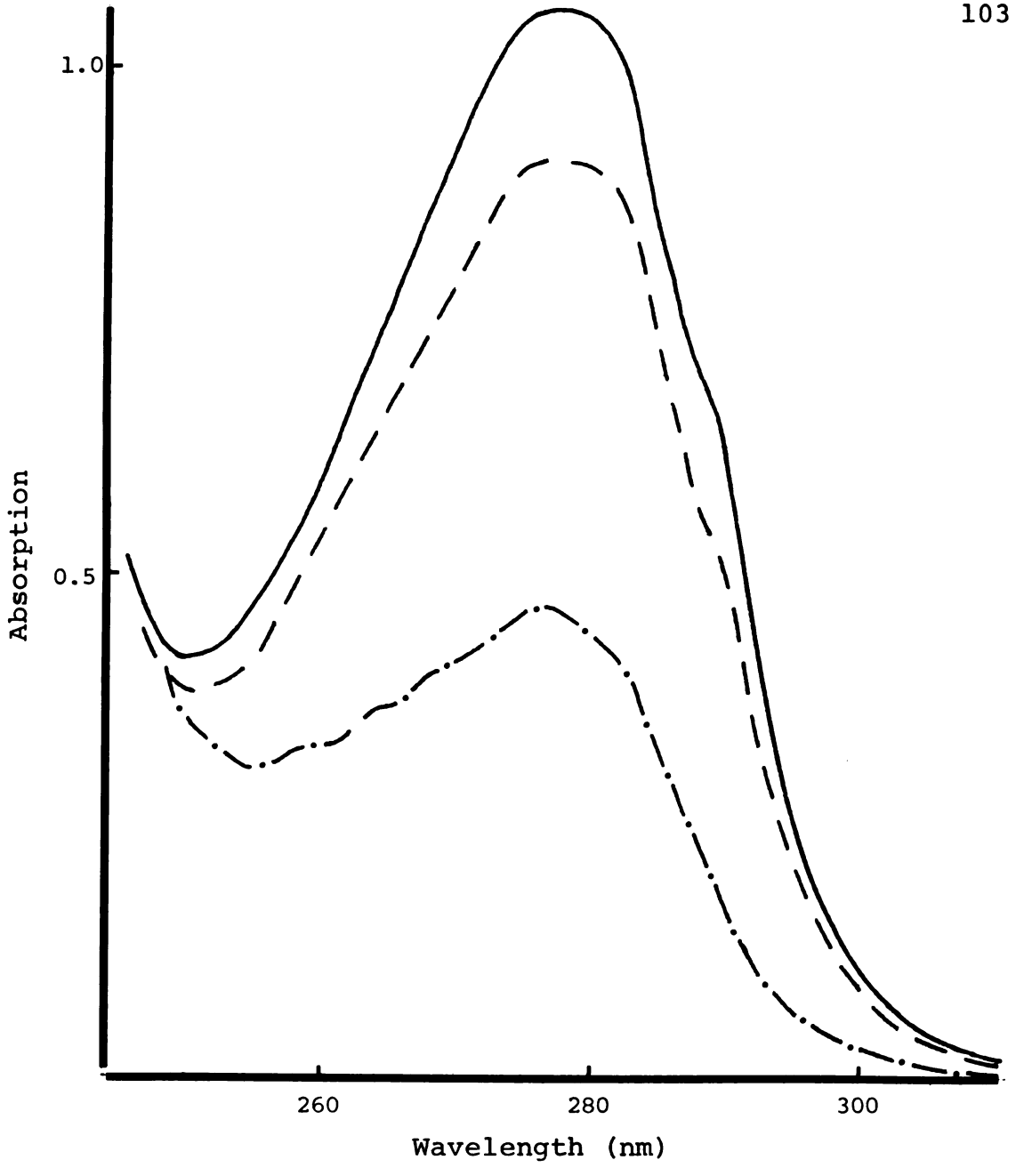

Fig. 20
UV absorption spectra of oPRL (---), oPRL-(1-53) (- · -), and oPRL-(54-199) (——) at 1 mg/ml, pH 8.4.

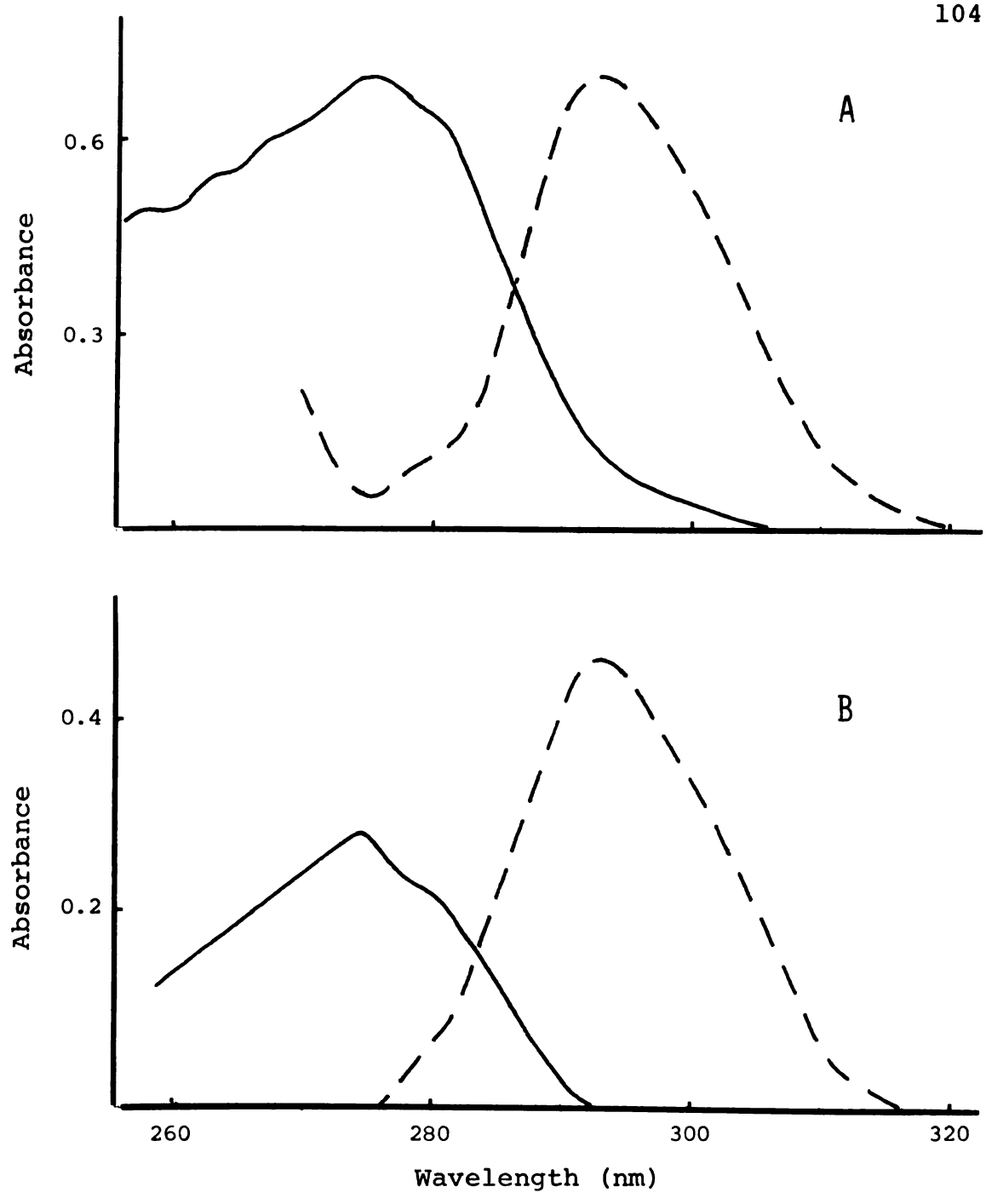

Fig. 21

A. UV absorption spectra of thermolysin-digested oPRL-(1-53) at pH 8.2 (——), and the pH 2 vs pH 13 difference spectrum of the same material (---).

B. UV spectra of N-acetyl-L-tyrosinamide under the same conditions as above.

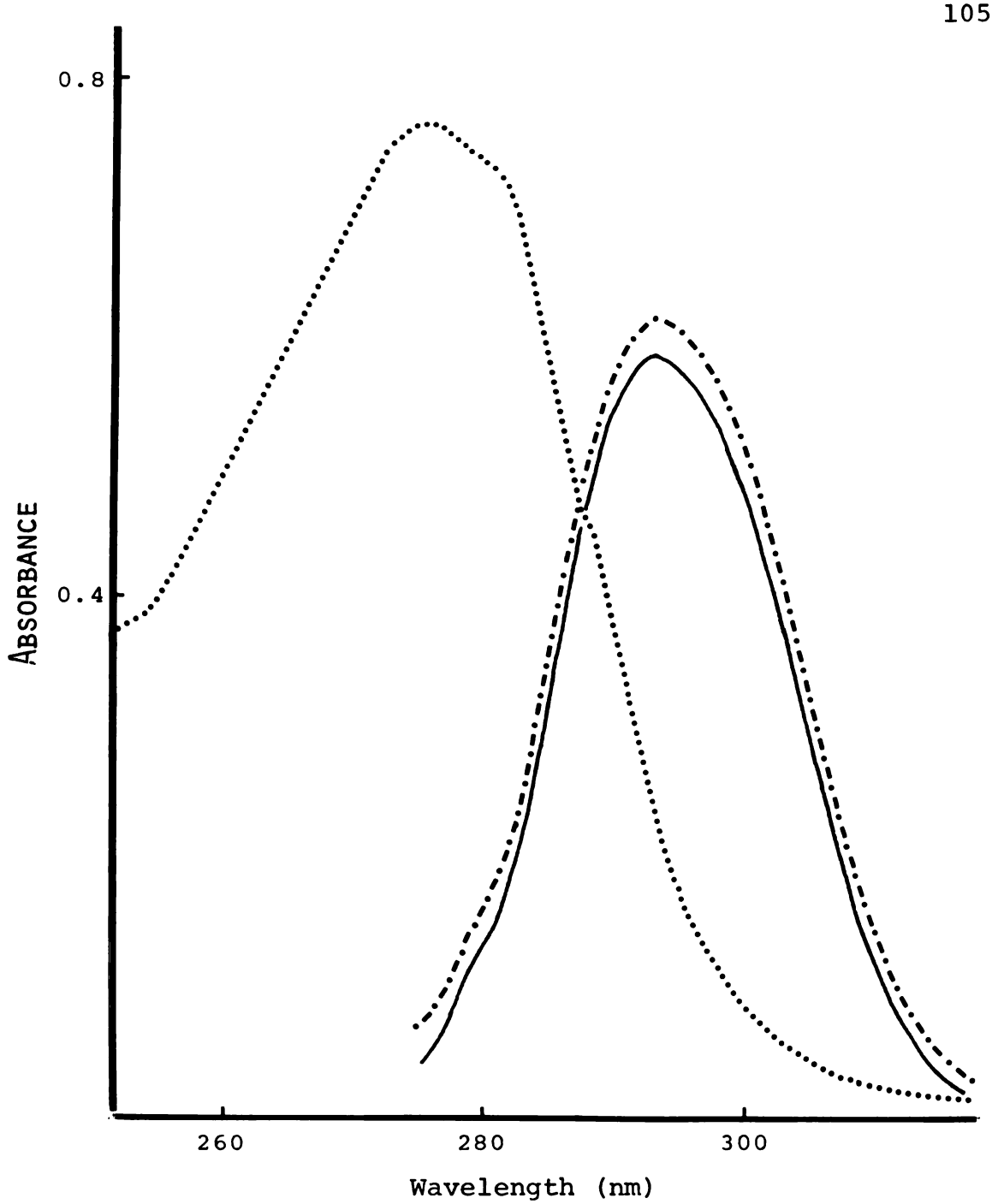

Fig. 22. UV absorption spectrum of oPRL digested with crude or purified fibrinolysin (···), and the pH 2 vs pH 13 difference spectra of oPRL after digestion with crude (——) and pure (-·-) enzyme.

DISCUSSION. The absorptivity of oPRL-(54-199), as determined by three different methods, was 10% greater than that of oPRL. The value of 1.06 ml/mg was used in subsequent procedures, since the thermolysin-digest method of absorptivity determination has been found to give results within 1-2% of those obtained by nitrogen analysis (Bewley, unpublished). The absorptivity of 1.08 ml/mg, calculated on the basis of sequence, was closer to the experimental value than is the case for most proteins (Wetlaufer, 1962). This, however, is also true of native oPRL, which has calculated and experimental values of 0.916 and 0.909 (Li et al., 1970).

The absorptivity determination for oPRL-(1-53) led to the interesting discovery of incomplete Tyr ionization for the fragment. This effect was not due to the conformation of the peptide, since neither treatment with guanidine-HCl nor titration to pH 13 reversed it, while both of these conditions permit ionization of all 7 Tyr residues of oPRL (Ma et al., 1970). In addition, the absorbance of Tyr at pH 2 was below 275 nm, suggesting complete immersion in aqueous medium (Herskovitz & Sorenson, 1968).

In the absence of a conformational effect, Tyr modification is a reasonable explanation for the observations. Amino acid analysis consistently showed a Tyr content lower than expected for oPRL-(1-53), but not for oPRL-(54-199). The amino acid analyses of the oPRL-(1-53)

tryptic digest did not, however, show any anomolous peak unique to the Tyr- containing peptides. Amino acid analysis of oPRL-(1-53) also showed only a 15% loss of Tyr, compared to the 40% loss seen for the ionization. It is possible that the modification was partially reversed by acid hydrolysis.

Attempts to locate a modified Tyr in the oPRL-(1-53) sequence also proved equivocal, as both Tyr-containing peptides had less than half the expected concentration of Tyr. This could be an indication of modification at both residues, or it may have been an artifact, not detected because this procedure was performed only once.

The use of crude fibrinolysin as a modification catalyst appears to have been partially successful with oPRL, but not with N-acetyl-L-tyrosinamide. The difference between ionization of 6.7 and 7.0 Tyr residues was small, but reproducible, and, therefore, probably real. The failure of the enzyme to modify a model compound was not altogether surprising, since a conformationally-dependent modification might not occur with the free residue.

Although most modified forms of Tyr do not show an absorption peak near 275 nm (Strickland, 1974), one notable exception is O-methyltyrosine (Wetlaufer et al., 1958), which shows an absorption maximum at 274 nm at neutral pH. The pH 11.5 difference spectrum of O-

methyltyrosine, however, is shifted to 284 nm, as compared to 293 nm for unmodified Tyr. The possibility that one or both of the oPRL-(1-53) Tyr residues was converted to O-methyltyrosine is also attractive from the chemical standpoint, as this ether would be expected to be cleaved by HCl rather slowly (Morrison & Boyd, 1973), possibly accounting for the decrease in modified Tyr seen by amino acid hydrolysis as compared to the spectrophotometric measurements.

Several enzymes are known to catalyze the formation of O-methyl compounds, among them are phenyl O-methyltransferase (Axelrod & Daly, 1968) and protein O-methyltransferase (Kim & Paik, 1970), both found in mammalian tissue. Neither of these enzymes appears to modify Tyr, however. Nevertheless, the existence of an enzyme with the function of forming O-methyltyrosine was suggested by the isolation of puromycin, which contains the Tyr ether, from Streptomyces alboniger (Waller et al., 1953). It is possible that some contaminant in the fibrinolysin preparations used here serves a similar function in the bacteria from which the enzyme was isolated. Unfortunately, this hypothesis could not be confirmed for the fragment, because of insufficient material.

B. FLUORESCENCE SPECTRA

Fluorescence spectra provide both a means of

detecting peptides at low concentration, and also a sensitive probe into structural changes at the chromophores. The characterization of fluorescence emission spectra reported here were undertaken primarily as a prelude to recombination studies in which the fluorescent properties of the fragments would be followed during the reaction.

MATERIALS AND METHODS. Fluorescence emission spectra were taken with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation wavelength was 290 nm, with a 10 nm band-pass; emission spectra were recorded, also with a band-pass of 10 nm. The pathlength along both the excitation and emission axes was 1 cm. The maximum concentration at which a linear relationship should be observed between concentration and fluorescence intensity was estimated for each peptide according to the method of Hercules (1966), based on molar absorptivity at the excitation wavelength, determined by UV absorption spectra. All spectra were taken at sample concentrations within this linear region. In order to facilitate comparison of fluorescence intensity among the peptides used in this study, all spectra were adjusted to a uniform molar concentration, and a correction was made for the Raman scattering peak centered at 324 nm (Parker & Rees, 1960).

RESULTS. The maximum oPRL concentration at which the relationship between fluorescence intensity and protein

concentration showed good linearity was calculated to be 0.11 mg/ml (4.9 μM). The limiting value for oPRL-(1-53) was 0.25 mg/ml (41.5 μM), for oPRL-(54-199) was 0.07 mg/ml (4.2 μM), and for recombinant oPRL was 0.09 mg/ml (3.9 μM).

The fluorescence emission spectra of isolated and recombined oPRL-(54-199) are compared with that of the native hormone in Fig. 23. The oPRL emission maximum at 337 nm was red-shifted to 340 nm in oPRL-(54-199), and remained at the higher wavelength even after fragment recombination. In addition, isolation of oPRL-(54-199) brought about a 60% increase in relative fluorescence that was reversed by recombination of the fragments. oPRL-(1-53) showed less than 1% the fluorescence intensity of the other molecules, with a maximum at 350 nm.

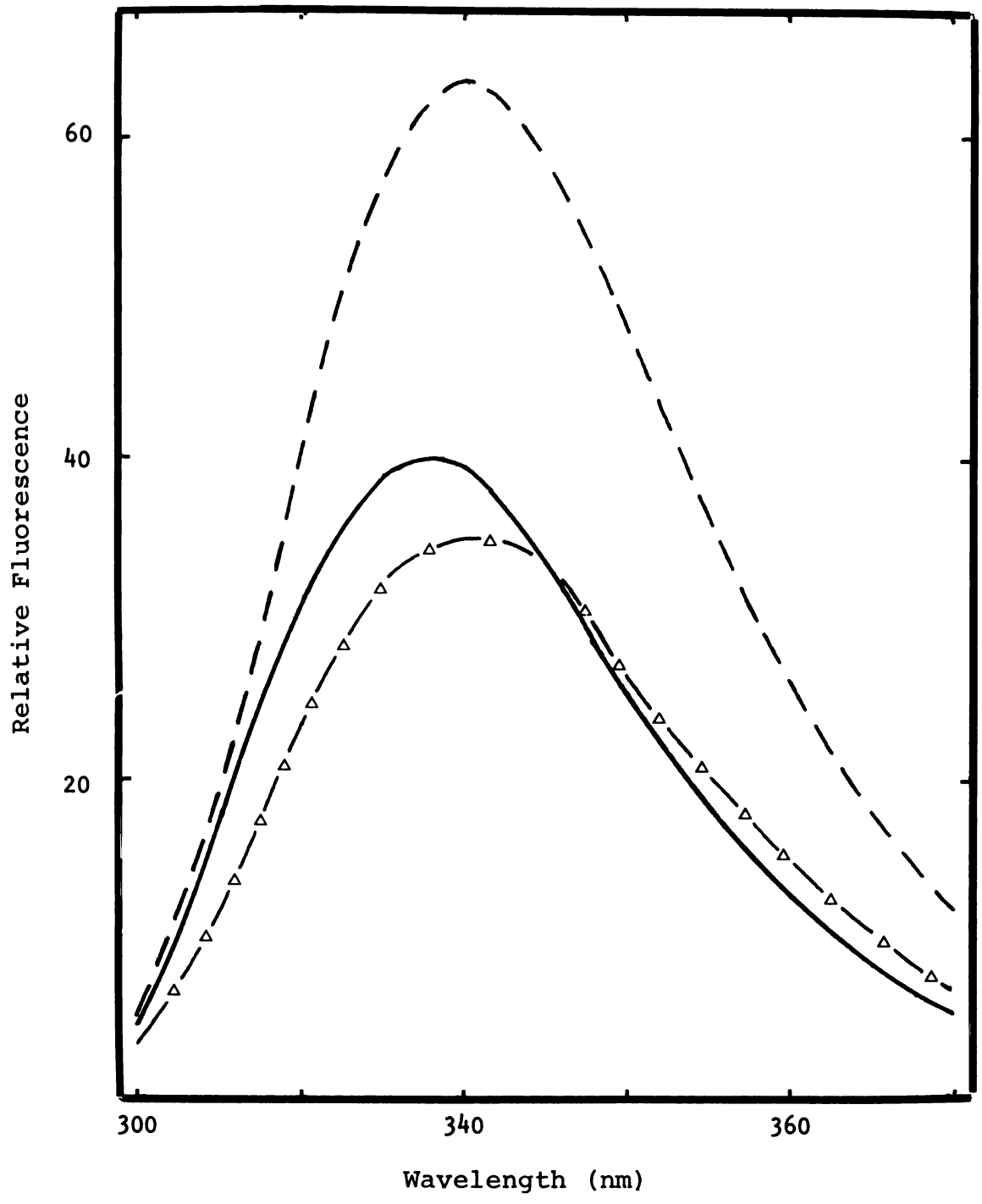

Fig. 23.
Fluorescence emission spectra of oPRL (——), oPRL-(54-199) (---), and recombinant oPRL (△). The excitation wavelength was 295 nm. All spectra were adjusted to equivalent molar concentrations.

DISCUSSION. A direct comparison of the fluorescence intensities of oPRL and its fragments, adjusted to uniform concentration, allowed determination of the relative fluorescence quantum yields, even though absolute quantum yields were not measured. In order for these comparisons to be valid, it was necessary to insure that the assumption of a linear relationship between concentration and fluorescence intensity not be violated. According to the equation of Hercules (1966), the deviation from linearity at the levels of protein used here should be less than 2%.

The shifts in emission peak wavelength of oPRL-(54-199) and recombinant oPRL as compared to the native hormone were consistent with an increased exposure of Trp side-chains to the aqueous medium. The wavelength position of indole emission has been found to range from below 320 nm, in a very hydrophobic environment, to 350 nm when totally exposed to water (Cowgill, 1969; Edelhoch & Chen, 1980). A shift of 3 nm, therefore, may reflect a relatively minor change in the local environment, and suggests that the Trp residues of the fragment were not completely exposed to the aqueous solvent. The failure of this peak to return to its native position after fragment recombination indicated that the repair of oPRL structure was not complete.

In contrast to these shifts in peak position were the changes in relative peak intensities. The sharp increase

in relative fluorescence of oPRL-(54-199) as compared to the native molecule may reflect removal of one or both Trp residues from the proximity of a quenching group, such as a peptide carbonyl, disulfide bond, or basic side-chain (Cowgill, 1969). It also appears unlikely on the basis of fluorescence intensity that the indole rings of the fragment were completely exposed to the solvent, as water is an effective quenching agent (Edelhoch & Chen, 1980). The return of the emission peak to approximately the intensity of the native hormone upon fragment recombination suggests that at least part of the native Trp environment was restored.

The failure of oPRL-(1-53) to show significant fluorescence emission when excited at 295 nm is expected for a peptide devoid of Trp (Cowgill, 1969; Edelhoch & Chen, 1980), since the peak Tyr excitation wavelength is typically between 275 nm and 280 nm (Cowgill, 1976).

C. CIRCULAR DICHROISM STUDIES

Circular dichroism provides a sensitive probe into the local environments of amino acid chromophores (Strickland, 1974). The principal advantage of this technique over other optical methods is the ability to resolve the contributions of Trp, Tyr, Phe, and disulfide into characteristic bands, allowing these residues to be examined individually. In addition, the far UV region of the CD

spectrum shows the overall secondary structure of proteins.

MATERIALS AND METHODS. CD spectra were obtained as described in chapter 2, except that sample concentrations ranged from 0.4 mg/ml to 1.0 mg/ml. The mean residue weight of oPRL-(1-53) was taken as 113.7, and that of oPRL-(54-199) was 114.2. These values were calculated from the sequence of the fragments, and they differ slightly from those used by Birk & Li (1978).

The sum of fragment spectra in the near UV region was determined by adding the molar ellipticities of the two species at each wavelength. In the far UV region the sum was obtained from the weighted mean of fragment mean residue ellipticities, with the weighting factor taken as the proportion of residues contributed by each fragment, as shown:

$$[\theta]_{A\ B,\lambda} = \frac{\frac{C_A}{MRW_A}\ [\theta]_{A,\lambda} \quad \frac{C_B}{MRW_B}\ [\theta]_{B,\lambda}}{\frac{C_A}{MRW_A} \quad \frac{C_B}{MRW_B}}$$

where [θ] is the mean residue ellipticity at a given wavelength, λ = wavelength, A and B are the two fragments used, C is concentration in mg/ml, and MRW = mean residue weight.

A sample of oPRL was passed through a Sephadex G-75 column (132 x 2.5 cm) in 20% HOAc, lyophilized, and rechromatographed on Sephadex G-100 in 0.01 M NH_4HCO_3, pH 8.4. Dimers and higher-molecular-weight aggregates were

discarded after each separation.

Recombined oPRL was prepared by mixing 0.69 mg (115 nmol) oPRL-(1-53) with 0.975 mg (58 nmol) oPRL-(54-199) in 1 ml buffer. The solution stood at room temperature 6 h, followed by lyophilization. Dried material was dissolved in 1 ml 0.01 M NH_4HCO_3, pH 8.4 and the solution was passed through a Sephadex G-100 column (55 x 1.4 cm) equilibrated in the same buffer. Eluted material was lyophilized.

RESULTS. The CD spectra of the isolated oPRL fragments are shown in Fig. 24, together with the spectra of the native and HOAc-treated hormones. The native and acid-treated material were indistinguishable throughout the near and far UV regions of the spectra.

oPRL-(1-53) shows a broad negative ellipticity below 310 nm, with a sharp increase in intensity below 290 nm (see Fig. 23 for an expanded scale). Fine structure bands appear as a negative maximum and shoulder at 278 nm and 284 nm, and as positive maxima at 255 nm, 264 nm, and 270 nm. Negative bands at 209 nm and 220 nm are approximately half as intense as those of the native molecule, and correspond to an α-helix content of 25 ± 5%.

oPRL-(54-199) also shows a broad negative CD envelope below 310 nm. A positive maximum is apparent at 292 nm, as well as fine structure bands between 260 nm and 287 nm. In the region of amide bond absorption, the negative max-

imum at 209 nm appears identical to that of oPRL. In contrast, the band centered at 220 nm is approximately 20% less intense than that of the native hormone. Taken together, these bands suggest an α-helix content of 45± 5%.

Fig. 25 shows the CD spectra of recombinant oPRL compared with the native hormone, the sum of the individual fragment spectra, and fibrinolysin-treated oPRL (from Fig. 11). In the near UV region, the recombined molecule shows positive bands at 288 nm and 297 nm, similar in position to, but less intense than, those of intact oPRL. Fine structure bands may also be seen between 260 nm and 285 nm in the recombinant, but were not apparent in the spectrum of the native protein. The negative maxima in the far UV region are approximately 25% less intense for the recombinant than for the native hormone, corresponding to an α-helix content of 35-40%.

The sum of the two fragment spectra show major differences from the recombined oPRL in three areas of the spectrum. At 257 nm the positive maximum of the fragment sum is considerably more intense than that of the recombinant. The positive shoulder of recombined oPRL at 288 nm is red-shifted to 291 nm for the individual fragments, and the recombinant positive band at 297 nm is not apparent for the fragments. The far UV bands of the recombinant are indistinguishable from those of the

individual fragments.

Partial digestion of oPRL with fibrinolysin resulted in a loss of positive dichroism at 290 nm and 297 nm, and in the appearance of fine structure bands between 257 nm and 275 nm. CD bands at 209 nm and 220 nm show a major reduction of intensity, and the fibrinolysin-treated material is estimated to have an α-helix content of 35± 5%.

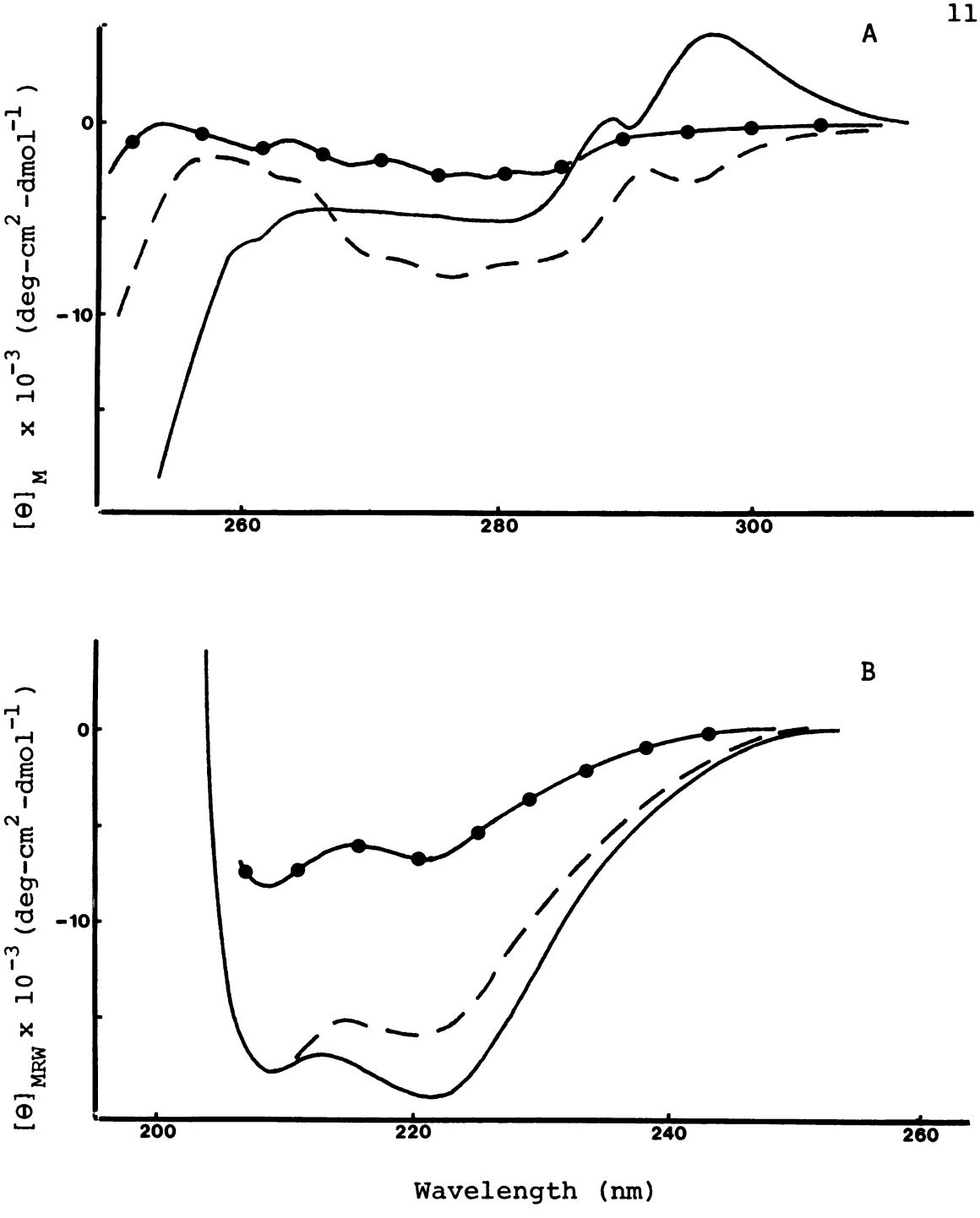

Fig. 24. CD spectra of native and HOAc-treated oPRL (——), oPRL-(1-53) (—●—), and oPRL-(54-199) (---) in the regions of side-chain (A) and amide bond (B) absorption.

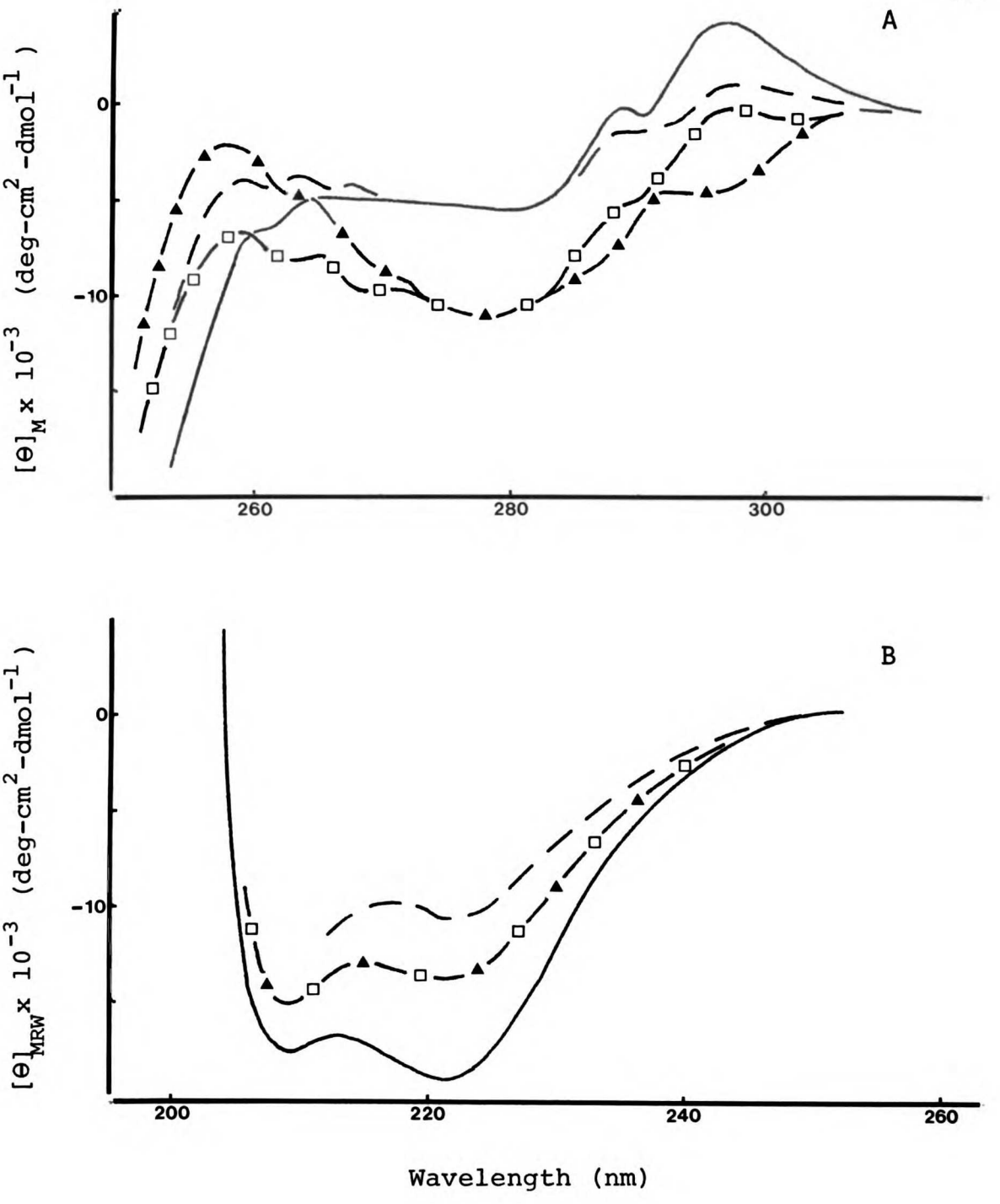

Fig. 25.
CD spectra of oPRL (——), fibrinolysin-treated oPRL (---), unrecombined fragments (▲), and the recombinant (□) in the regions of side-chain (A) and amide bond (B) absorption.

DISCUSSION. Among the side-chain chromophores present in oPRL-(1-53), only the disulfide bond is known to show ellipticity above 295 nm (Strickland, 1974). The CD of disulfide bonds is dependent on both rigidity (Woody, 1973) and proximity of peptide bonds or side-chains (Strickland et al., 1974). It appears that these conditions are met in the fragment.

Fine structure at 278 nm and 284 nm may be attributed to the [0 + 800 cm^{-1}] and [0 - 0] transitions of Tyr, and suggest that the phenol group is at least partially exposed to a hydrophilic environment (Strickland, 1974). Additional bands below 275 nm may be assigned to Phe, however the large number of possible Phe transitions in this region precludes the assignment of individual bands (Strickland et al., 1974).

The presence of far UV bands corresponding to 20 ± 5% α-helix indicates that 8-13 residues of the fragment form a helical structure. This evidence of secondary structure, together with the rigidity of the disulfide and only partial exposure of Tyr suggest that oPRL-(1-53) has a well-defined conformation, in contrast to the small fragment of plasmin-HGH (Bewley & Li, 1978).

Although both Trp and disulfide bonds of oPRL-(1-53) show dichroic bands above 300 nm (Strickland, 1974), both native oPRL and oPRL-(54-199) have only positive indole bands. It appears, therefore, that the broad negative

envelope of the fragment is due, in part, to the presence of rigid disulfide. The positive maximum at 292 nm is typical of the [0 - 0] transition of the Trp ^1L_b band in a hydrophilic environment (Strickland, 1974). Fine structure bands correspond closely in wavelength position in this fragment to the bands of oPRL-(1-53), and may be assigned as for the smaller fragment.

An α-helix content of 45 ± 5% suggests that 60-70 residues of the fragment are in a helical conformation. The presence of a large amount of α-helix, and the intensity of bands throughout the near UV region indicate a rigid conformation for this fragment.

It is apparent from the spectrum of partially digested oPRL that treatment with fibrinolysin brought about significant structural changes. The most striking shift in the near UV region is the loss of intensity of the 297 nm band assigned to Trp in a hydrophilic environment. Accompanying this decrease is the loss of intensity of the shoulder at 289 nm, attributable to the [0 + 850 cm^{-1}] transition of the Trp ^1L_b band (Strickland, 1974). The possibility that this decrease results from loss of an underlying disulfide band does not seem likely in view of the unchanged region below 285 nm, where disulfide transitions are typically centered (Woody, 1973). It appears, therefore, that a change in the local environment of one or both Trp residues occurs as a result of fibrinolysin

cleavage.

The loss of native oPRL structure is also apparent in the far UV region. The drop from 50% α-helix to 35% represents a loss of about 30 helical residues. It is unlikely that these spectral changes resulted from complete digestion of a portion of the oPRL molecules, as this would have been reflected in the Phe and Tyr regions of the spectrum. It appears, instead, that partial digestion brought about significant changes in the native oPRL conformation. In light of this determination, it seems reasonable to compare the recombinant molecule not only to the native, but also to the fibrinolysin-cleaved hormone.

Comparison of the sum of the two fragment spectra with native and partially-digested oPRL allowed investigation of the structural elements altered by separation of the fragments. In the near UV region the Trp bands at 297 nm and 289 nm are replaced by a single band at 292 nm, suggesting a substantial increase in the freedom of indole rotation, as well as a decrease in the hydrophobicity of the local environment (Strickland, 1974).

An additional change may be noted in the Phe spectrum, between 250 nm and 270 nm. Although fibrinolysin treatment of oPRL brought about some change in this region, the large positive shift centered at 257 nm was not apparent until after isolation of the fragments. The intensity of Phe bands has been shown to be highly

dependent on conformation, while the wavelength of the bands was found to shift very little, even in a wide variety of environments (Strickland, 1974). These findings suggest that the conformational changes involved in fragment isolation affect both Phe and Trp residues.

The effect of isolation on Tyr residues is more equivocal, as the Trp bands overlap this region, and loss of indole dichroism could account for the decrease at 280 nm. In addition, fine structure bands in the fibrinolysin-treated material are not sufficiently distinct to allow comparison with the sum of fragment spectra.

The far UV bands of the fibrinolysin-treated hormone appear somewhat less intense than those of the isolated fragments. The difference in α-helix content between these bands is 5%, suggesting that the differences in intensity may not be real. The possibility cannot be excluded, however, that the individual fragments have more helical structure than the partially digested material.

The CD spectrum of recombinant oPRL shows the restoration of Trp bands at 297 nm and 289 nm. Although not as intense as the similar bands in the native and fibrinolysin-treated material, the wavelength positions are the same, and represent a major change in the Trp environment upon recombination.

The Phe bands are similarly shifted toward the form

of the native and cleaved hormone, although they remain distinct. In addition, a 1 nm red-shift may be noted in the Phe peaks, possibly reflecting a change from hydrophobic to hydrophilic environment.

The α-helix bands of the recombined fragments appear indistinguishable from the sum of the individual fragment spectra, and remain significantly less intense than those of the native hormone.

These spectral studies indicate the both fragments retain considerable secondary and tertiary structure, although differences between fragment and native oPRL conformation remain. Some elements of the native conformation appear to be restored upon recombination of the fragments, however, the restoration of α-helix structure reported by Birk & Li (1978) was not confirmed by this study. Possibly their determination of sample concentrations was not accurate , as the absorptivity values of the fragments and recombinant were not established at that time. The differences in side-chain chromophores between the native and recombinant molecules may be partially accounted for on the basis of conformational changes occurring upon fibrinolysin digestion, and are not entirely products of the recombination itself. It appears, therefore, that the native oPRL conformation requires the integrity of the 53-54 peptide bond, as well as the presence of both fragments.

CHAPTER 4

EQUILIBRIUM AND KINETIC STUDIES ON THE RECOMBINATION OF OVINE PROLACTIN FRAGMENTS

A. FLUORESCENCE POLARIZATION STUDIES OF THE RECOMBINATION EQUILIBRIUM[1]

Circular Dichroism studies have shown that oPRL-(1-53) and oPRL-(54-199) recombine rapidly on mixing to give a spectrum very similar to that of the native hormone (Birk & Li, 1978). The recombinant was shown to exhibit full immunoreactivity as revealed by microcomplement fixation and immunodiffusion. However, the pigeon crop-sac stimulating activity of the recombinant was minimal. In order to investigate this apparent dichotomy, an examination of the interaction of the fibrinolysin fragments by fluorescence polarization (Weber, 1953) was undertaken. Although this method has most commonly been applied to antigen-antibody binding (Dandliker, 1977; Dandliker et al., 1978), the principles involved can be generalized to study any reaction producing a sufficiently large molecular weight change in the fluorescent species (Weber, 1953; Dandliker, 1977; Dandliker et al., 1978). The usefulness of this technique has been greatly expanded in recent

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[1] This section was taken, with some modifications, from Jibson et al. (1981).

years by the development of appropriate instrumentation (Kelly et al., 1976) and by the characterization of fluorescein isothiocyanate as a fluorescent label (Maeda et al., 1969). We report here studies on the equilibrium behavior of the fluorescein thiocarbamyl (FTC) derivative of oPRL-(1-53) and oPRL-(54-199).

MATERIALS AND METHODS. PRL was isolated from sheep pituitary glands by the method of Li et al., (1970). Fragments were produced by partial digestion of oPRL with bacterial fibrinolysin as described (Birk & Li, 1978; ch. 2). The concentration of oPRL-(1-53) was determined based on an absorptivity at 276 nm of 0.46 ; the concentration of oPRL-(54-199) was determined based on an absorptivity at 280 nm of 1.06 (ch.3).

Fluorescein isothiocyanate (isomer I), synthesized as described (Kawauchi et al., 1969), was provided by K. Muramoto. FTC-oPRL-(1-53) was obtained by allowing peptide to react 4 h with a 1.5 M excess of the fluorescein isothiocyanate isomer in 0.1 M NH_4Ac (pH 8.2) as described (Maeda et al., 1969). Residual fluorescein isothiocyanate was removed by chromatography on a Sephadex G-25 column (1.4 x 18 cm) in 0.10 phosphate buffer (pH 7.4). The peptide peak (~100 mg, 2% peptide) was lyophilized and redissolved in 10 ml of 0.01 M NH_4HCO_3 (pH 8.4) as a stock solution. Polarization experiments were carried out with

5-20 µl of the stock solution plus 2 ml of the NH_4HCO_3 buffer. All labeling and storage procedures were carried out in darkness.

The concentration of the labeled peptide solution was determined by duplicate amino acid analyses with norleucine as a concentration standard. Amino acid analyses were carried out as described above (ch. 2). The extent of labeling was determined by the absorbance at 490 nm, with a molar extinction coefficient of 61,000 assumed (Maeda et al., 1969). The molar ratio of label to peptide was 0.37.

Fluorescence polarization was measured with a MAC-2 polarization spectropolarimeter (Japan Immunoresearch, Takasaki, Japan; distributed in the United States by Meloy Laboratories, Springfield, VA), equipped with a circulating water bath for temperature control. Instrument specifications were described by Maeda (1979) and Kinoshita et al., (1980). Polarization was measured in terms of P-values (Maeda et al., 1969; Perrin, 1926), given in arbitrary units. Competitive binding data are reported as percentage change in polarization (% ΔP) of FTC-oPRL-(1-53) initially in the totally unbound state. Other binding data are shown as Scatchard plots after analysis of polarization as described (Dandliker et al., 1964; Dandliker, 1977; Dandliker et al., 1978). All data were collected at 30±0.5°C.

Fluorescence intensity changes accompanying binding were determined with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation wavelength was 492 nm (7.3 nm band width), and the emission wavelength was 515 nm (8.5 nm band width). The ratio of bound to free fluorescence intensities for FTC-oPRL-(1-53) was determined to be 1.04. This value was used in preparation of data for Scatchard analysis.

RESULTS. Competitive binding studies were performed by measuring polarization of solutions containing oPRL-(1-53) and FTC-oPRL-(1-53) in several molar ratios with a total concentration of 0.25 μM. Polarization was again determined after addition of 0.50 μM oPRL-(54-199). Changes in P-values observed for three different molar ratios of labeled and unlabeled oPRL-(1-53) are shown in Table 12. Use of as much as a 14-fold excess of unlabeled over labeled peptide yielded the same change in polarization upon recombination as did the 2-fold excess.

Binding data obtained from polarization measurements with several concentrations of FTC-oPRL-(1-53) and oPRL-(54-199) are in Table 13. Scatchard plots of these data are shown in Fig. 26. Numerical information from these analyses is given in Table 14. The mean dissociation constant from these data was 0.144 μM. The B-intercept (intercept in Fig. 26 with the abscissa labeled bound

FTC-oPRL-(1-53), μM) of each line should correspond to the concentration of oPRL-(54-199) used. Normalization of all values to [oPRL-(54-199)]=1.00 μM allowed calculation of a mean intercept of 1.01 μM (see Table 14), in excellent agreement with the expected value. The free energy of dissociation for recombinant oPRL at 30^{o}C was found to be 9.50 kcal/mol.

Table 12

Competitive binding of FTC-labeled and unlabeled oPRL-(1-53)[a] to oPRL-(54-199)[b].

oPRL-(1-53)/FTC-oPRL-(1-53)	%ΔP[c] ± SEM
2/1	9.57 ± 0.59
5/1	9.50 ± 0.17
14/1	9.93 ± 1.21

[a]The total concentration of labeled and unlabeled oPRL-(1-53) was 0.25 μM.

[b]The concentration of oPRL-(54-199) was 0.50 μM.

[c]Each value of percent change in polarization was the mean of three determinations.

Table 13

Binding data derived from fluorescence polarization measurements

oPRL-(54-199) μM	FTC-oPRL-(1-53) μM		Bound/Free mol ratio
	Total	Bound	
0.25	0.50	0.215	0.75
	0.60	0.232	0.63
	0.75	0.251	0.51
	1.00	0.281	0.39
0.50	0.35	0.230	1.92
	0.50	0.301	1.51
	0.60	0.334	1.26
	1.00	0.399	0.66
0.75	0.35	0.257	2.78
	0.50	0.357	2.50
	0.60	0.424	2.41
	1.00	0.517	1.07
1.00	0.35	0.271	3.46
	0.50	0.385	3.35
	0.60	0.446	2.91
	1.00	0.556	1.25

Table 14

Analysis of Scatchard plots* of binding of FTC-oPRL-(1-53) and oPRL-(54-199)

oPRL-(54-199) (μM)	K_d (μM)	B-intercept* (μM)	$\frac{\text{B-intercept (μM)}}{\text{oPRL-(54-199) (μM)}}$	G_d (kcal/mol)
0.25	0.179	0.346	1.38	9.36
0.50	0.134	0.496	0.99	9.53
0.75	0.144	0.704	0.94	9.49
1.00	0.117	0.734	0.73	9.62
Mean±SEM	0.144±.013		1.01±0.14	9.50±.05

* See Fig. 26.

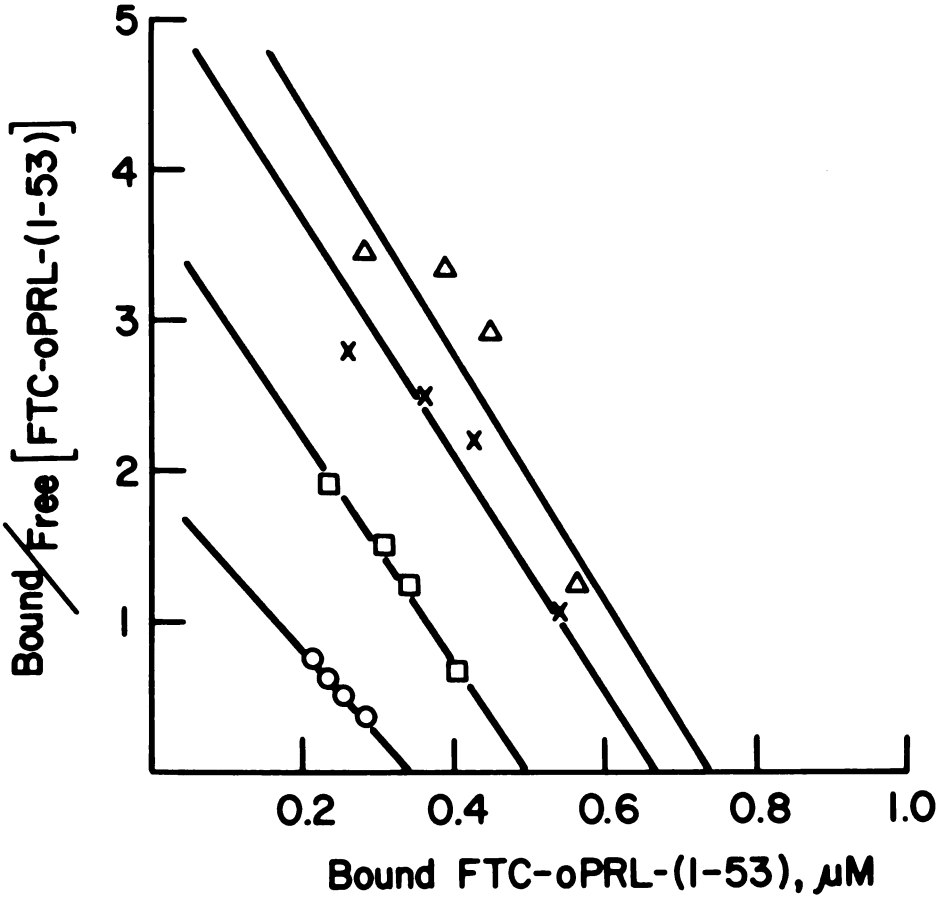

Fig. 26.
Scatchard plot of binding data (Table 13) of FTC-oPRL-(1-53) and oPRL-(54-199) at 30°C. Each line represents a single concentration of oPRL-(54-199): 0.25 M (○), 0.50 M (□), 0.75 M (x), and 1.00 M (△). The dissociation constant, K_d is obtained from the Scatchard equation

$$\frac{[\text{Bound}]}{[\text{Free}]} = -K_d + [\text{Bound}]_{max} \quad .$$

DISCUSSION. oPRL-(1-53) appears to have been satisfactorily labeled by fluorescein isothiocyanate. Because the reaction proceeded well at pH 8.2, labeling of the N-terminal amino group should predominate over reaction with Lys residues (Maeda et al., 1969). The fluorescein/oPRL-(1-53) mol binding ratio of 0.37:1 is consistent with this conclusion. This level of binding also indicates that in all cases unlabeled oPRL-(1-53) was present with the FTC fragment. This fact was dealt with explicitly in the competitive binding studies, but was not considered significant in the determination of the dissociation constant.

The presence of a fluorescein label on oPRL-(1-53) does not appear to affect binding of the fragment to oPRL-(54-199). Competitive binding with labeled and unlabeled oPRL-(1-53) showed the same change in polarization upon binding oPRL-(54-199) in each case tested. P-values in this study were a function of the proportion of bound to free FTC-oPRL-(1-53). Therefore, it is clear that this ratio remains unchanged regardless of the relative concentrations of labeled and unlabeled material.

Scatchard analysis of the binding data permitted a determination not only of the dissociation constant for the recombination, but also the number of binding sites available to FTC-oPRL-(1-53). The close correspondence between the B-intercepts of the Scatchard plots and the concentrations of oPRL-(54-199) used is consistent with

the one-to-one binding expected for a specific interaction between the two fragments.

The apparent divergence between physicochemical and biological properties of recombinant oPRL may be explained in part by a dissociation constant of 0.144 μM. At the fragment concentrations used by Birk and Li (1978) for CD studies, nearly 99% of the oPRL-(54-199) and 50% of the oPRL-(1-53) were in the recombined form. Thus, the assumption of complete recombination appears to have been valid for these measurements. In contrast, under the conditions used for the pigeon crop-sac assay, less than 50% of the fragments would be expected to be in the bound state even at the highest concentration used. Therefore, it is unlikely that this test yielded meaningful results.

When the equilibrium properties of the recombination are taken into account, a much closer correspondence may be noted between physical and biological properties (Wong et al., 1981). Even as the recombination approaches 100%, however, only 13% of the radioreceptor-binding activity is restored. Thus, the equilibrium characteristics of the recombinant account only in part for the change in biological properties of the recombined molecule.

B. EQUILIBRIUM STUDIES BY FRONTAL ANALYSIS

The measurement of an equilibrium constant for interaction of oPRL-(1-53) and oPRL-(54-199) at 30°C

helped determine the biological and immunological activities of the recombinant (Wong et al., 1981), but left unresolved several other questions regarding the chemical nature of the recombination reaction. The fluorescence polarization measurements were based on molecular weight changes of the FITC- labeled oPRL-(1-53) and did not take into account the self-association of oPRL-(54-199) leading to formation of a stable dimer (Birk & Li, 1978; ch. 2). In order for a one-to-one association to occur, the recombination process must involve at least two steps: dissociation of the oPRL-(54-199) dimer and noncovalent binding of the two oPRL fragments. In order to separate the equilibrium constants of these two reactions, it was necessary to examine the monomer-dimer equilibrium of oPRL-(54-199).

Although fluorescence polarization has been used in the study of self-associating systems (Malencik & Anderson, 1972), the monomer-dimer reaction, involving a molecular weight change of only a factor of 2, was not a favorable system for study by this technique (Dandliker, 1977; Dandliker et al., 1978). Frontal analysis has been used for the study of equilibrium parameters both in self-associating (Ackers & Thompson, 1965; Ackers, 1967) and 3-component systems (Nichol & Winzor, 1964). This method requires the assumptions that the solute molecule does not interact noncovalently with the gel matrix, and that the

equilibrium reactions are sufficiently rapid that a stable equilibrium condition is approximated throughout the elution. Both of these conditions appear to be met in this case, as molecular weight measurements of oPRL-(54-199) by exclusion chromatography gave no evidence of interaction with the gel (Birk & Li, 1978; ch. 2), and the recombination reaction proceeds rapidly, with accompanying loss of fragment dimer (Birk & Li, 1978; ch. 3). Frontal analysis also permitted determination of the monomer-dimer equilibrium constant without labeling of the fragment, since the elution profile could be followed by intrinsic Trp fluorescence.

In addition to examining the monomer-dimer equilibrium of oPRL-(54-199), it was also of interest to determine the free energy of association for the complete reaction at more than one temperature, in order to estimate the contributions of entropy and enthalpy to binding (Tanford, 1961). Frontal analysis also proved to be of value in this regard, by allowing equilibrium measurements both of the fragment self-association and recombination reactions at low temperature.

Frontal analysis of the recombination reaction required a system in which the elution of oPRL-(1-53) could be monitored, and in which the recombinant migrated at the same rate as the unlabeled fragment (Nichol & Winzor, 1964). The small fragment was labeled by reducing

and alkylating, with ^{14}C-iodoacetamide, the single disulfide bond. This label was chosen because it was not located at the N-terminus, as FITC had been, and also because the disulfide bond involved did not appear crucial to maintaining the conformation of the intact hormone (Doneen et al., 1979). The second condition was met by the use of Sephadex G-50, which completely excluded both the recombinant and oPRL-(54-199).

MATERIALS AND METHODS. Cys-(4,11) of oPRL-(1-53) was reduced by addition of a 1.4 M excess of DTT (Calbiochem) over peptide sulfhydryl. The CD spectrum of the solution was taken as described (ch. 3) after a 30 min incubation period. An additional 1.4 M excess of DTT was added and the CD spectrum was again scanned. (1-^{14}C)-Iodoacetamide was obtained in ethanol solution from New England Nuclear (NEC-221, lot # 1337- 041; 19.1 mCi/mmol), was dried by rotary evaporation, and mixed with a 3-fold excess of unlabeled iodoacetamide (Calbiochem, lot #502369). A 30 M excess of iodoacetamide over DTT and peptide sulfhydryl was allowed to react 10 min, followed by removal of excess material by chromatography on Sephadex G-25. The labeled peptide was lyophilized, and the monomer fraction was isolated on Sephadex G-50, followed by amino acid analysis as described previously (ch. 2).

Frontal analysis involves the exclusion chromatogra-

phy of a large sample volume, typically at least as great as the total colume volume. Unlike the more common zonal analysis, in which small volumes are eluted as narrow bands, elution profiles in frontal analysis consist of plateaus of sample concentration, characterized by a specific height and positions of leading and trailing fronts. Height in this case corresponds to the concentration of the sample loaded on the column and fronts are functions of the sample elution properties, as in zonal analysis. In samples with more than one constituent, each separable molecular species forms an independent plateau and fronts. In self-associating systems, the position of the leading front is determined by the number average molecular weight of the sample.

Frontal analysis of the recombination was performed at both 23°C and 2°C with ^{14}C-(RCAM)-oPRL-(1-53) at concentrations from 0.15 μM to 0.25 μM and oPRL-(54-199) from 0.15 μM to 0.19 μM. Concentrations of both species were determined as described (ch. 3). The two components were allowed to stand at their final temperature at least 15 min, followed by pumping onto a Sephadex G-50 upward-flow column (30 x 0.9 cm) at 7.5 ml/hr; 4 min fractions were collected. Aliquots of 0.5 ml were removed, combined with 4.5 ml PCS and counted on a Packard BPL scintillation spectrophotometer. Plateaus representing bound and free labeled material were used in the equation $K_{AS}=\frac{(C^{\alpha}-C^{\beta})}{C_B^{\beta}(C_A^{\alpha}-C_B^{\alpha}+C_B^{\beta})}$

where C represents initial concentration, is plateau of bound material, A is oPRL-(54-199), B is ^{14}C-(RCAM)-oPRL-(1-53), and C is the recombinant. Analysis of the monomer-dimer equilibrium was performed at 23°C and 2°C with oPRL-(54-199) concentrations between 0.50 μM and 1.20 μM. Concentration determination and column chromatography were performed as above. The column was calibrated as described (ch. 2). Fractions were read for fluorescence with an excitation wavelength of 286 nm and an emission wavelength of 340 nm. The centroid position of the leading front was taken as $\bar{\nu}$ in the equation

$$\alpha=(\bar{\nu}-\sigma_p)/(\sigma_m-\sigma_p)$$

where α is weight fraction of monomer, M refers to monomer, P to polymer, and σ is defined by

$$\sigma=(V_e-V_o)/(V_t-V_o)$$

with V_e being the elution volume of the sample, V_o the column void volume, and V_t the total column volume (Ackers & Thompson, 1965). The value of σ_m was estimated from the calibration proteins, and σ_p, which was measured at high concentration. The centroid of the leading front was determined from the position of the least squares line for the linear region of the front at the half- height of the plateau.

The weight fraction of monomer, α, can be substituted into the equilibrium equation for the self-association:

$$K = \frac{[An]}{[A]^n} = \frac{(1-\alpha)}{n\alpha^n(C_o)^{n-1}}$$

where $(1-\alpha)$ = weight fraction of n-mer, C_o = initial concentration of the monomeric unit, (αC_o) = molar concentration of monomer, and $(1-\alpha)C_o/n$ = molar concentration of the n-mer.

Free energies of dissociation (ΔG_d) were calculated from the dissociation constants (K_d), according to the equation $\Delta G = -RT\ln K_d$, where T = absolute temperature, and R is the gas constant (1.987 cal/deg-mol). The entropies and enthalpies of dissociation (ΔS_d and ΔH_d) were calculated from the relation $\Delta G = H - T\Delta S$, after determining ΔG_d at more than one temperature.

RESULTS. The CD spectra of oPRL-(1-53) during reduction and alkylation are shown in Fig. 27. A broad shift in the spectrum was observed in conjunction with the first addition of DTT. The second addition of DTT caused no further change. The amino acid analysis of ^{14}C-(RCAM)-oPRL-(1-53) showed no trace of unlabeled Cys (Table 15). A calibrated G-50 column allowed isolation of the monomer fraction of the labeled fragment (Fig. 28).

A typical elution pattern for the recombination reaction at 23°C is shown in Fig. 29, with plateaus indicated. The mean K_d values of three determinations each, at 23°C and 2°C are given in Table 16, together with the free energy of dissociation at each temperature, and values at 30°C obtained by fluorescence polarization. A plot of ΔG

vs. T is shown in Fig. 30. The correlation coefficient of the 3 points is 0.997, the slope of the line, corresponding to $-\Delta S^{\circ}$, is 40.8 cal/deg-mol, and the ΔG-intercept, equal to ΔH°, is -2.91 kcal/mol. At 23°C, $T\Delta S^{\circ}$ for the dissociation reaction is -12.08 kcal/mol.

The elution profiles of two analyses of oPRL-(54-199) are shown in Fig. 31, with the front centroids indicated. Both leading and trailing fronts showed excellent linearity, and in no case was the coefficient of correlation less than 0.98. The results of chromatography with several concentrations at 2°C and 23°C are shown in Table 17. Determination of the change in dissociation enthalpy and entropy for the reaction gave ΔH°= 5.78 kcal/mol, and ΔS°= -9.52 cal/deg-mol. At 23°C the $T\Delta S$ term for dissociation of the dimer was -2.82 kcal/mol.

Table 15

Amino acid analysis of ^{14}C-[RCAM]-oPRL-(1-53)

Amino Acid	oPRL-(1-53)	Observed
Asx	7	7.3
Thr	2	2.5
Ser	4	3.8
Glx	4	6.7
Pro	3	1.8
Gly	4	5.5
Ala	2	3.7
Val	4	4.2
Ile	2	1.6
Leu	3	3.3
Tyr	2	1.8
Phe	4	3.0
His	2	1.7
Lys	2	2.1
Arg	3	0.5
Cys	2	0
Met	3	1.1

Table 16

Thermodynamic values calculated from equilibrium measurements of the oPRL fragment recombination at the temperatures indicated. The values of K_d are given in μM, those of ΔG°, ΔH°, and $T\Delta S^\circ$ in kcal/mol.

Temp. (°C)	K_d ± SE	ΔG_d° ± SE	$T\Delta S^\circ$
2	.226±.023	8.33 ±.06	-11.2
23	.175±.026	9.12 ±.08	-12.1
30	.144±.013	9.50 ±.05	-12.4

ΔS°= -0.041
ΔH°= -2.91

Table 17

Thermodynamic values calculated from equilibrium measurements of the oPRL-(54-199) monomer-dimer reaction at the temperatures indicated. Values of K_d are given in μM, those of ΔG°, ΔH°, and $T\Delta S^\circ$ in kcal/mol.

Temp. (°C)	K_d ± SE	ΔG°_d ± SE	$T\Delta S^\circ$
2	.198±.021	8.44 ±.06	-2.49
23	.425±.032	8.63 ±.04	-2.68

ΔS°= -0.0091
ΔH°= 5.95

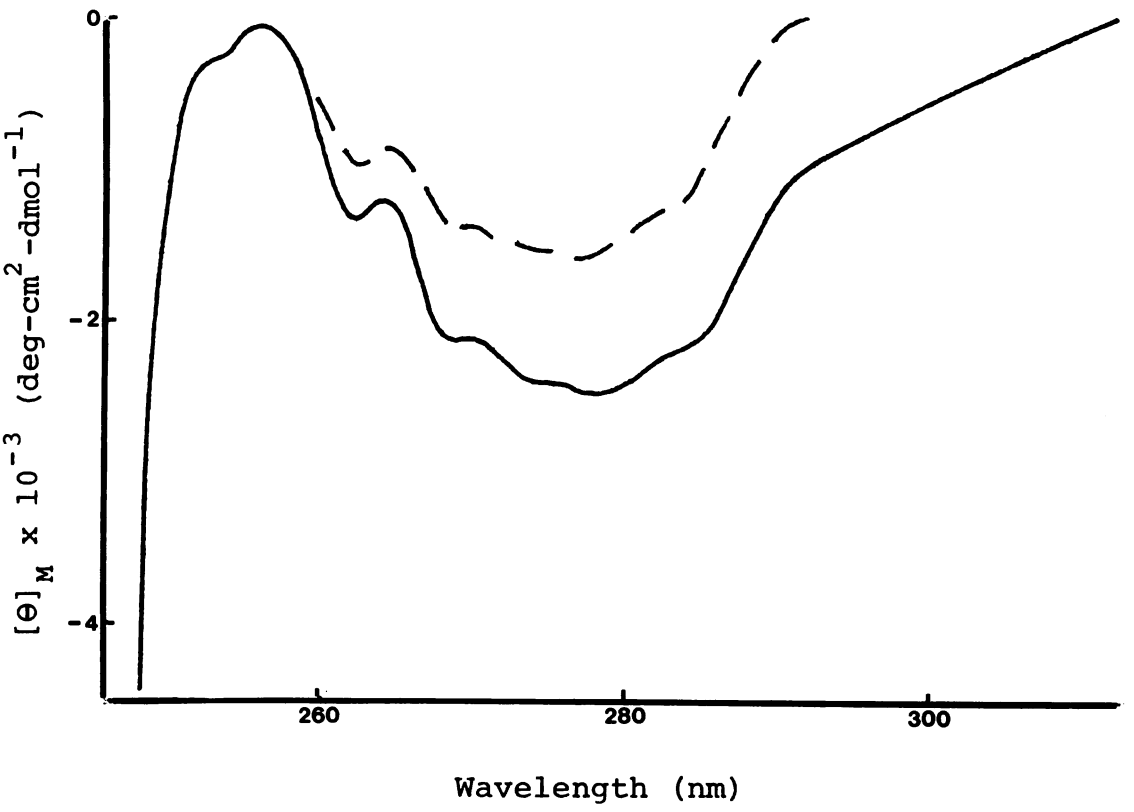

Fig. 27.
CD spectra of oPRL-(1-53) in the region of side-chain absorption before (——) and after (---) reduction and alkylation.

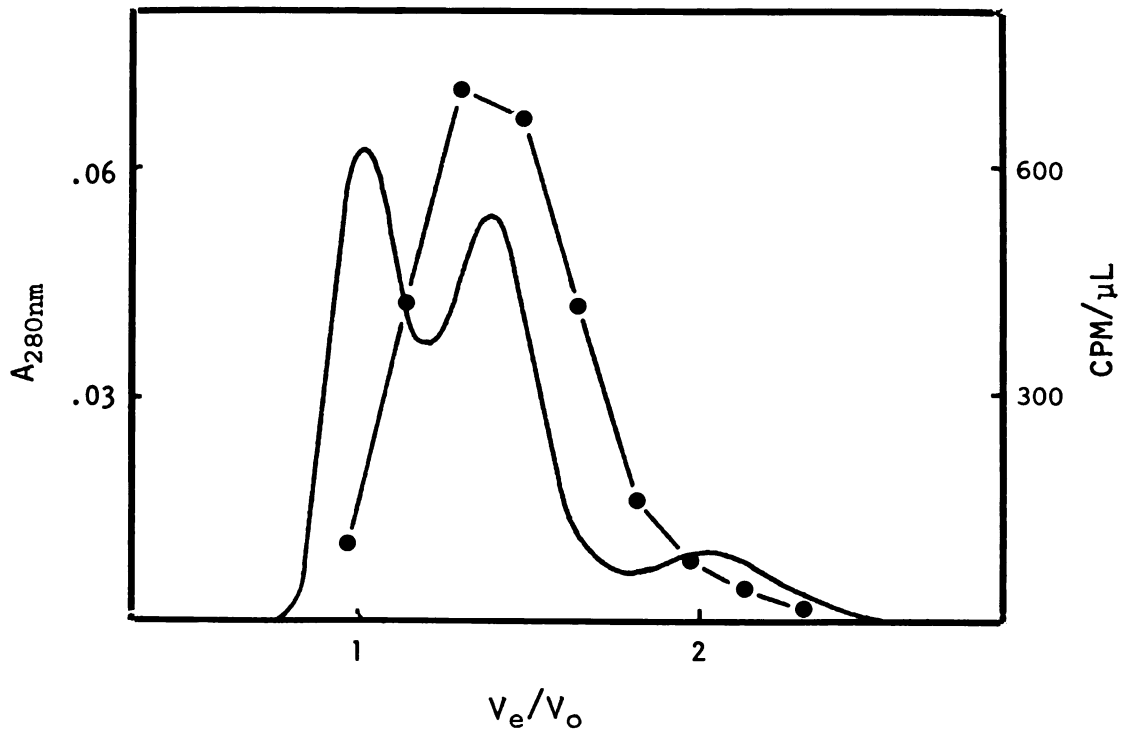

Fig. 28.
Elution profile of ^{14}C-[RCAM]-oPRL-(1-53) from Sephadex G-50, monitored by UV absorbance (——) and radioactivity (---).

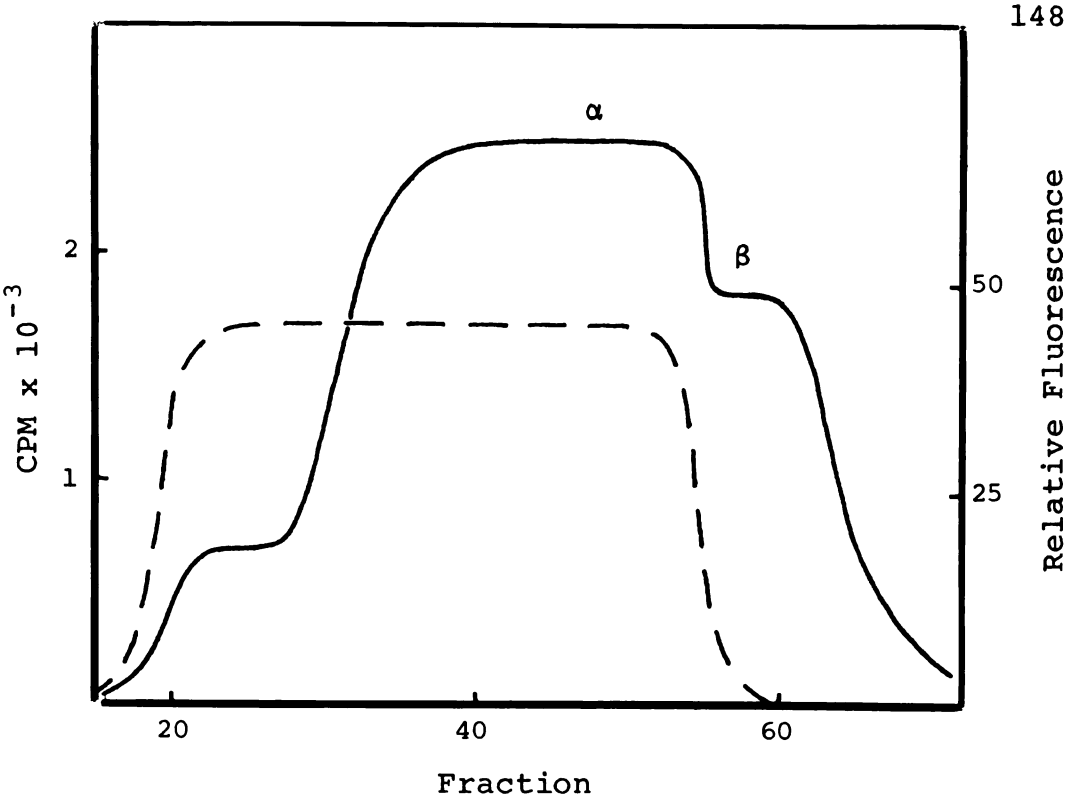

Fig. 29.
Elution profile of recombinant oPRL formed with ^{14}C-[RCAM]-oPRL-(1-53) and oPRL-(54-199), followed by fluorescence (λ_{ex}=295 nm, λ_{em}=340 nm) (---) and radioactivity (——). Plateaus used in equilibrium calculations are labeled α and β.

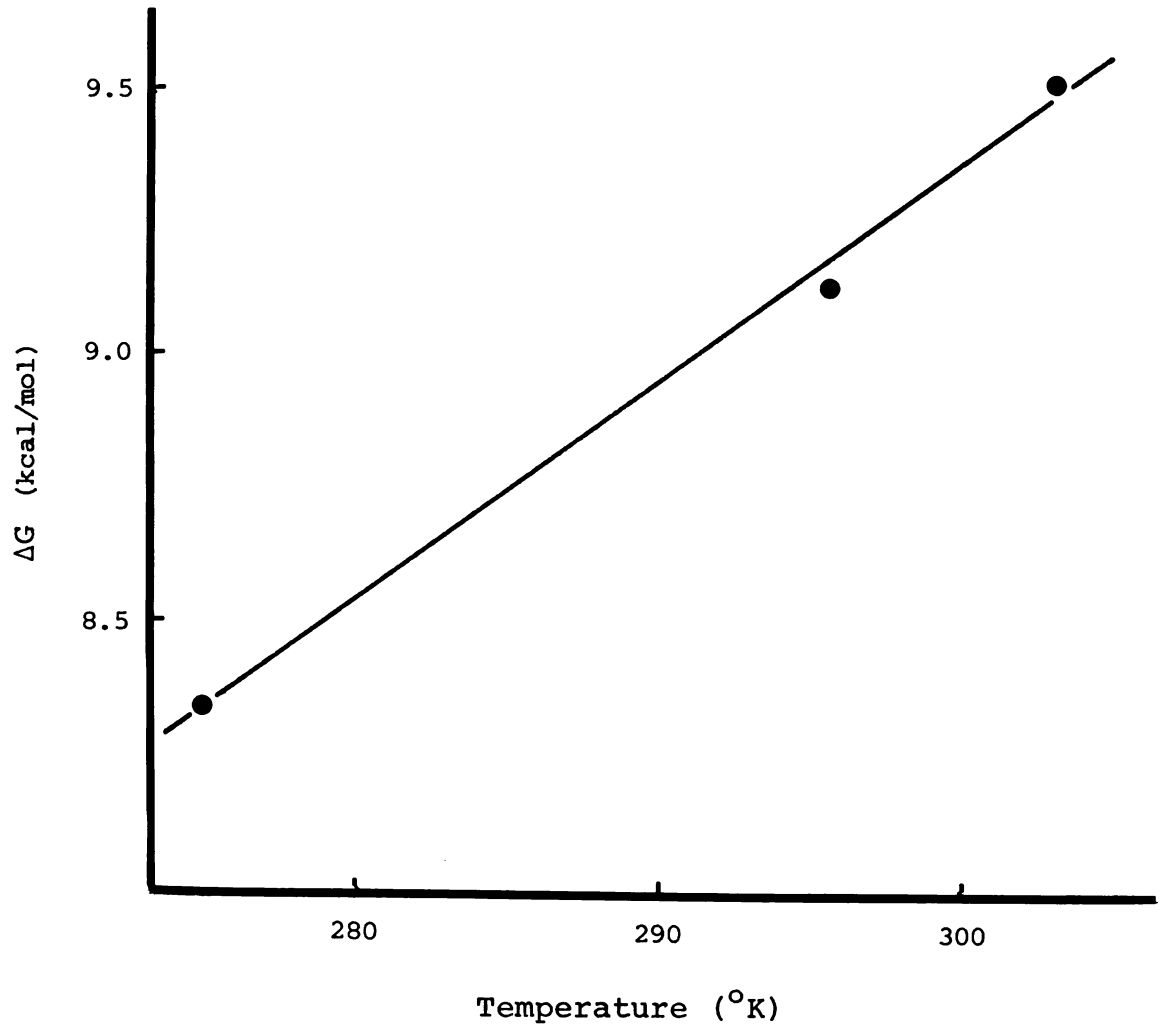

Fig. 30.
Plot of free energy of dissociation (ΔG_d) as a function of absolute temperature for recombinant oPRL. Points at 275°K and 296°K were obtained by frontal analysis, that at 303°K by fluorescence polarization. The least squares line for the points is shown.

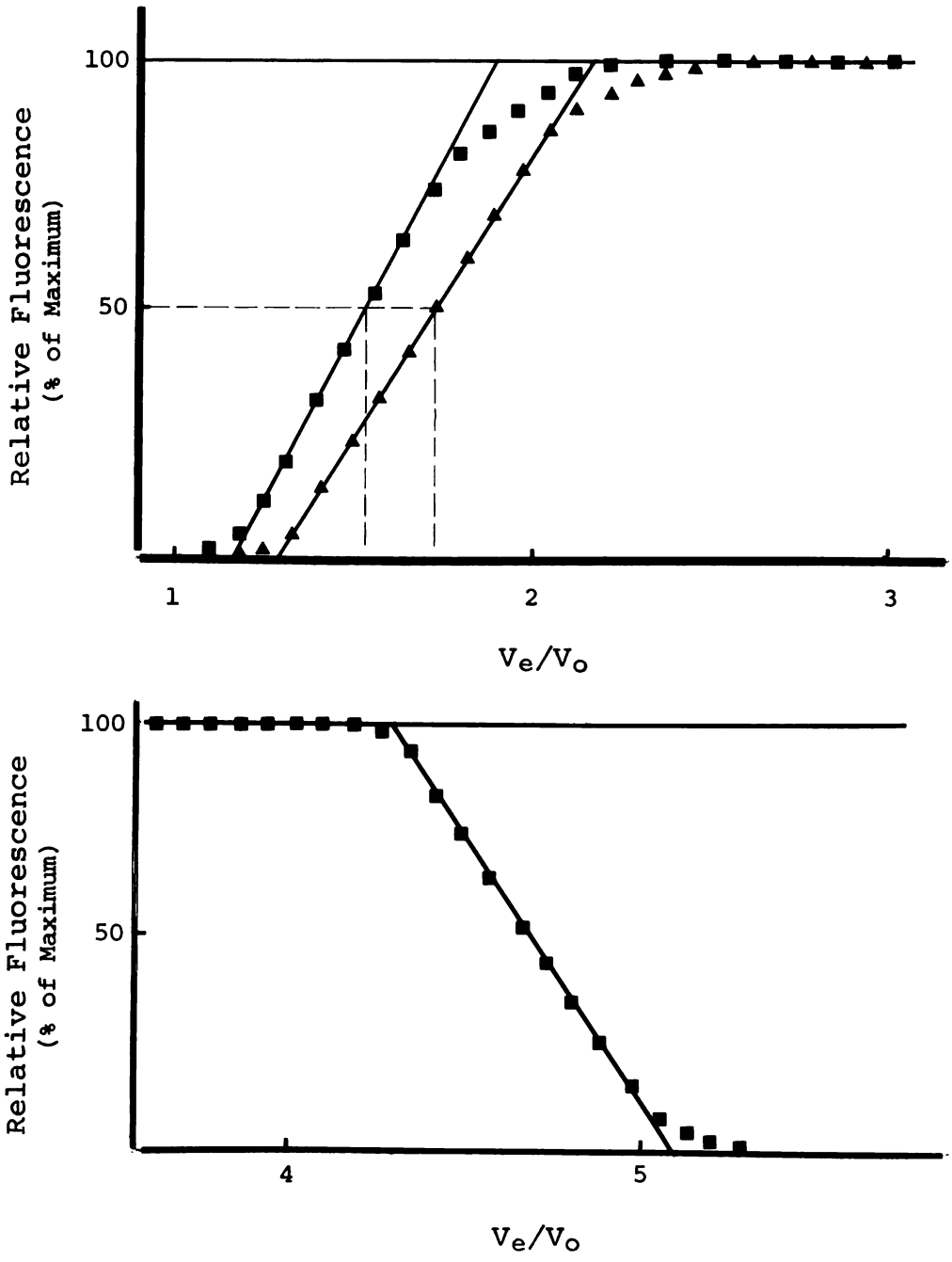

Fig. 31.
Elution profiles of the leading and trailing fronts of the oPRL-(54-199) monomer-dimer reaction at 12.8 μM (■) and 0.53 μM (▲). Least squares lines are shown for the fronts and plateau (—), and the positions of the leading front centroids are indicated (--).

DISCUSSION. The labeling of oPRL-(1-53) with [1-^{14}C]-iodoacetamide appears to have been complete, as shown both by the CD spectrum, which exhibited complete loss of peptide disulfide, and also by amino acid analysis, which was devoid of Cys. The binding properties of the ^{14}C-labeled fragment were indistinguishable from those of FTC-oPRL-(1-53), as shown by the comparison of binding energies in Fig. 30. Competitive binding of labeled and unlabeled oPRL-(1-53) was not performed in this case because of limited availability of the fragment.

The use of frontal analysis in measuring the dissociation constant of recombinant oPRL was superior to fluorescence polarization in nearly every regard. The chromatographic method required no special equipment, was simple to perform, involved the same amount of time as zonal exclusion chromatography, could be performed over a wide temperature range, was based on few underlying assumptions, and did not involve complex mathematical manipulations. In addition, frontal analysis provided a simple means of measuring the self-association of oPRL-(54-199) without requiring the use of an extrinsic label. In contrast, fluorescence polarization could only be performed with a specially-designed instrument, required 6-8 h of work to obtain a single equilibrium measurement, frequently gave erratic and inconsistent results, involved a number of assumptions regarding the behavior of the

fluorescent label, required a long series of mathematical approximations, was effective over a very small temperature range ($\sim$10^{o}C), and was not capable of examining a monomer-dimer reaction. Both techniques used very small amounts of material, typically on the order of 5 nmol of each fragment for one determination.

The self-association of oPRL-(54-199) appeared from molecular weight measurements to be a dimerization (Birk & Li, 1978; ch. 2). That conclusion was confirmed by the elution pattern of the trailing front, the first derivative of which reflects the degree of polymerization (Ackers & Thompson, 1965). The linear fronts obtained for the fragment gave evidence for the presence of only two molecular-weight species.

The determination of a free energy of dissociation of 0.425 μM at 23^{o}C for the oPRL-(54-199) dimer was consistent with observations that the fragment existed as a dimer in solutions with concentrations as low as 0.1 mg/ml (Birk & Li, 1978; ch. 2). A comparison of the enthalpy and entropy values of the association at 23^{o}C indicate that both terms contribute to the dimerization, with the enthalpy providing about twice as much energy to the process as the entropy. The enthalpy of protein-protein binding apparently arises from hydrogen bonds and vanderWaals interactions, while hydrophobic forces and salt bridges give rise to the entropy contribution (Kauzmann,

1959). It is possible that each of these factors is involved in the formation of oPRL-(54-199) dimers, with hydrogen bonds and vanderWaals forces predominating.

The free energy of dissociation for the recombinant includes at least two reactions, one involving both fragments, and one only the dimerization. Since free energy, enthalpy, and entropy are, by definition, state functions, the pathway by which a recombinant oPRL molecule becomes a free oPRL-(1-53) and dimer oPRL-(54-199) is of no consequence with regard to these thermodynamic properties. In order to separate the recombination process from the dimerization, the model shown in Fig. 32 was used. The dissociation of dimer was based on a single dimer molecule dissociating into 2 monomers, and the sign is opposite to the dissociation of the recombinant. Therefore, as shown in Table 18, the overall dissociation for the process $AB \rightleftharpoons A+B$ alone is 13.42 kcal/mol. Similar analysis at $2^{\circ}C$ yields ΔG_d = 12.53 kcal/mol. From ΔG values at these temperatures, ΔH was determined to be 875 cal/mol at $23^{\circ}C$, and $T\Delta S$ was -12.55 kcal/mol. It is apparent from these numbers that the entropic forces of hydrophobic and ionic bonds dominate the recombination, in contrast to the oPRL-(54-199) dimer formation. A comparison of the energy of recombination with the conformational energies of GH from various species (Holladay et al., 1974) shows that it is within the range of 8-14 kcal/mol established for the

rat, ox, and sheep hormones. This similarity may indicate that the total conformational entropy of oPRL is somewhat higher than that of GH.

Table 18

Thermodynamic values calculated for the recombination of oPRL fragments in the absence of the oPRL-(54-199) monomer-dimer equilibrium. All values are given in kcal/mol.

		$A \rightleftarrows \frac{1}{2}AA$	$AB \rightleftarrows \frac{1}{2}AA+B$	$AB \rightleftarrows A+B$
2°C	ΔG°	-4.22	8.33	12.55*
	ΔH°	-2.97	-2.91	0.06
	$T\Delta S^\circ$	1.24	-11.22	-12.46
23°C	ΔG°	-4.31	9.12	13.43*
	ΔH°	-2.97	-2.91	0.06
	$T\Delta S^\circ$	1.34	-12.08	-13.42

*The discrepancy between ΔG° and $H^\circ T\Delta S^\circ$ is derived entirely from the estimation of these values shown in Fig. 30.

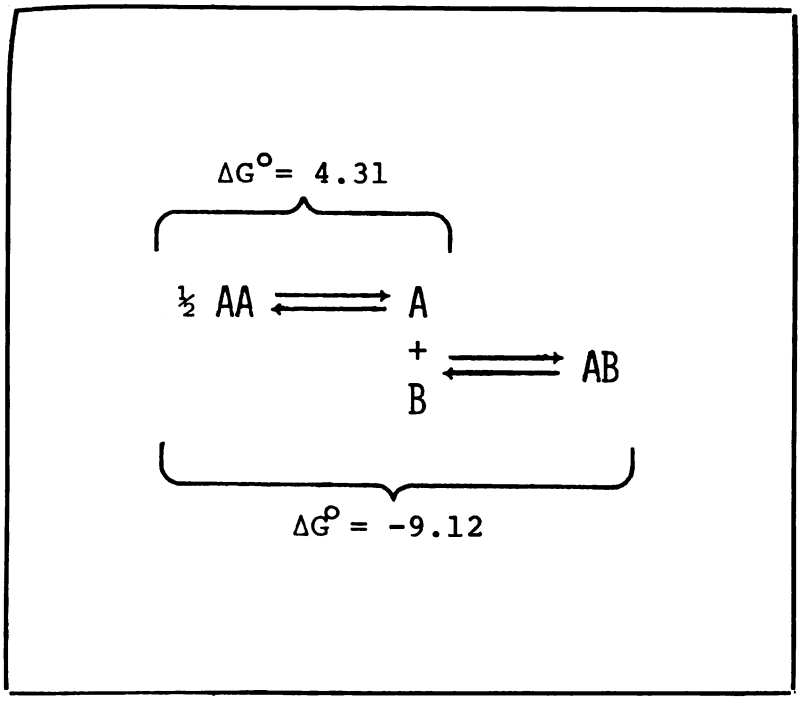

Fig. 32.
Thermodynamics of the recombination of oPRL-(1-53) with the dimer of oPRL-(54-199). This model was used to separate the thermodynamic states of the reactions and does not represent the sequence of steps. ΔG° values are given in kcal/mol.

C. KINETICS OF FRAGMENT RECOMBINATION

Following the determination of the recombination equilibrium constant, it was of interest to examine the kinetics of the same process, to establish the reaction order and rate. The association of the two fragments has already been shown to involve at least two steps: formation of oPRL-(54-199) monomer and formation of a one-to-one complex of the fragments. Whatever the sequence of these two steps, dissociation of the oPRL-(54-199) dimer is a first-order reaction and binding of the two fragments is a second- order process. Determination of the apparent kinetic order of the recombination would establish which of these steps is rate-limiting in the reaction. Since changes in fluorescence polarization in this case are a function only of changes in molecular weight, this technique was used as a probe for recombination.

In addition to these association-dissociation steps, the recombination also involves changes in the fluorescence and CD spectra (Birk & Li, 1978; ch. 3). Although these spectral shifts could simply represent changes in chromophore environment brought about by the binding of the two fragments, they instead appear to be reflective of conformational differences between the free and recombined peptides, as indicated by the biological and immunological properties of the fragments and recombinant. The possi-

bility of a rate-determining conformational change was investigated by following the kinetics of fluorescence changes during the reaction.

MATERIALS AND METHODS. Fluorescence polarization measurements were made as described above, with oPRL-(1-53) prepared previously (ch. 4). A solution of fluorescent-labeled peptide was placed in the 2 ml sample cell to obtain a baseline polarization measurement. The unlabeled fragment was then added in a 20 µl volume and rapidly mixed. Polarization was recorded as a continuous chart reading, rather than the time-averaged digital output used for equilibrium measurements. Concentrations of FTC-oPRL-(1-53) ranged from 0.125 µM to 0.25 µM, and those of oPRL-(54-199) ranged from 0.0625 µM to 0.125 µM.

The analysis of kinetics given by Dandliker (1977; Dandliker et al., 1978) requires the use of high reactant concentrations to determine the limit of bound polarization. In order to avoid this problem, the following analysis was used. Number average molecular weight is defined by the equation

$$M = \frac{\Sigma N_i M_i}{\Sigma N_i}$$

where N = molar concentration and M = molecular weight. In this case only the fluorescent-labeled material is considered, therefore $M_n = \frac{[F]M_F + [R]M_R}{F_o}$

where F = FTC-oPRL-(1-53), R = FTC-rec-oPRL, and F_o = initial concentration of F. Substituting

$$F_o-[F] = [R]$$

and solving for [F] gives

$$[F] = \frac{(M_n-M_R)F_o}{(M_F-M_R)}$$

There is a linear relationship between M_n and change in polarization (ΔP) (Dandliker et al., 1964, 1978; Dandliker, 1977), therefore M_n can be replaced by

$$M_n = a\Delta P+b$$

giving

$$[F] = \frac{F\ (a\Delta P+b-M_R)}{M_F-M_R}$$

In order to evaluate a and b, endpoints were determined for both ΔP and M_n. Before the start of the reaction, $\Delta P=0$ and M_n was the molecular weight of FTC-oPRL-(1-53) (6,300). The constant b, therefore, is simply the molecular weight of the labeled fragment. At the completion of the recombination, ΔP reaches its maximum value and M_n can be calculated from the initial concentrations of reactants used and the equilibrium constant determined above (ch. 4). The concentration of the recombinant can also be determined directly from ΔP values, by substituting equation (3) into (6) and solving for [R], giving

$$[R] = \frac{F_o a\Delta P}{(M_R-M_F)}$$

First order kinetics were examined by plotting [F] vs. time; second order kinetics were evaluated by plotting

$$\frac{1}{[F_o-N_o]} \ln \frac{[N_o][F_o-R]}{[F_o][N_o-R]}$$

(where N is oPRL-(54-199)) as a function of time. In both cases the rate constant, k, was given by the slope of the plot.

Fluorescence measurements were made as described above (ch. 3), with an excitation wavelength of 290 nm and an emission wavelength of 340 nm. Changes in fluorescence intensity were followed during the reaction. The concentration of oPRL-(1-53) was either 1.96 μM or 19.6 μM, that of oPRL-(54-199) was 1.21 μM or 12.1 μM. The two fragment solutions were injected simultaneously into the sample cell from syringes. The injection time was ~2 s, and the machine response time after injection was 2-3 s. Fragment and recombinant levels were used in first- and second-order kinetic plots as above.

RESULTS. Fluorescence polarization measurements of the fragment recombination were used to determine the first- and second-order plots shown in Fig. 33. Curvature in the first-order plots is evident from the beginning of the reaction. The curvature of the second-order plots appears to increase sharply after approximately 2 min. The rate

constant calculated from the initial segments of the three curves was 2.80 ±.36 x 10^5 M^{-1}-sec^{-1}.

The first-order curves obtained from changes in Trp fluorescence were rather equivocal (Fig. 34). The plots of fragment levels during recombination without an excess of either fragment could both be interpreted as being linear. As expected, the plot of oPRL-(54-199) levels in the presence of a 16 x molar excess of oPRL-(1-53) also appeared linear. In contrast, the levels of oPRL-(1-53), when reacted with a 6 x molar excess of oPRL-(54-199), clearly did not follow a first-order decay.

Consideration of the fluorescence intensity data as second order showed good linearity for the recombination when similar fragment concentrations were used (Fig. 34). The second-order rate constant determined for this case was 6.35 x 10^3 M^{-1}-sec^{-1}. The second-order curves obtained with an excess of either fragment were not linear.

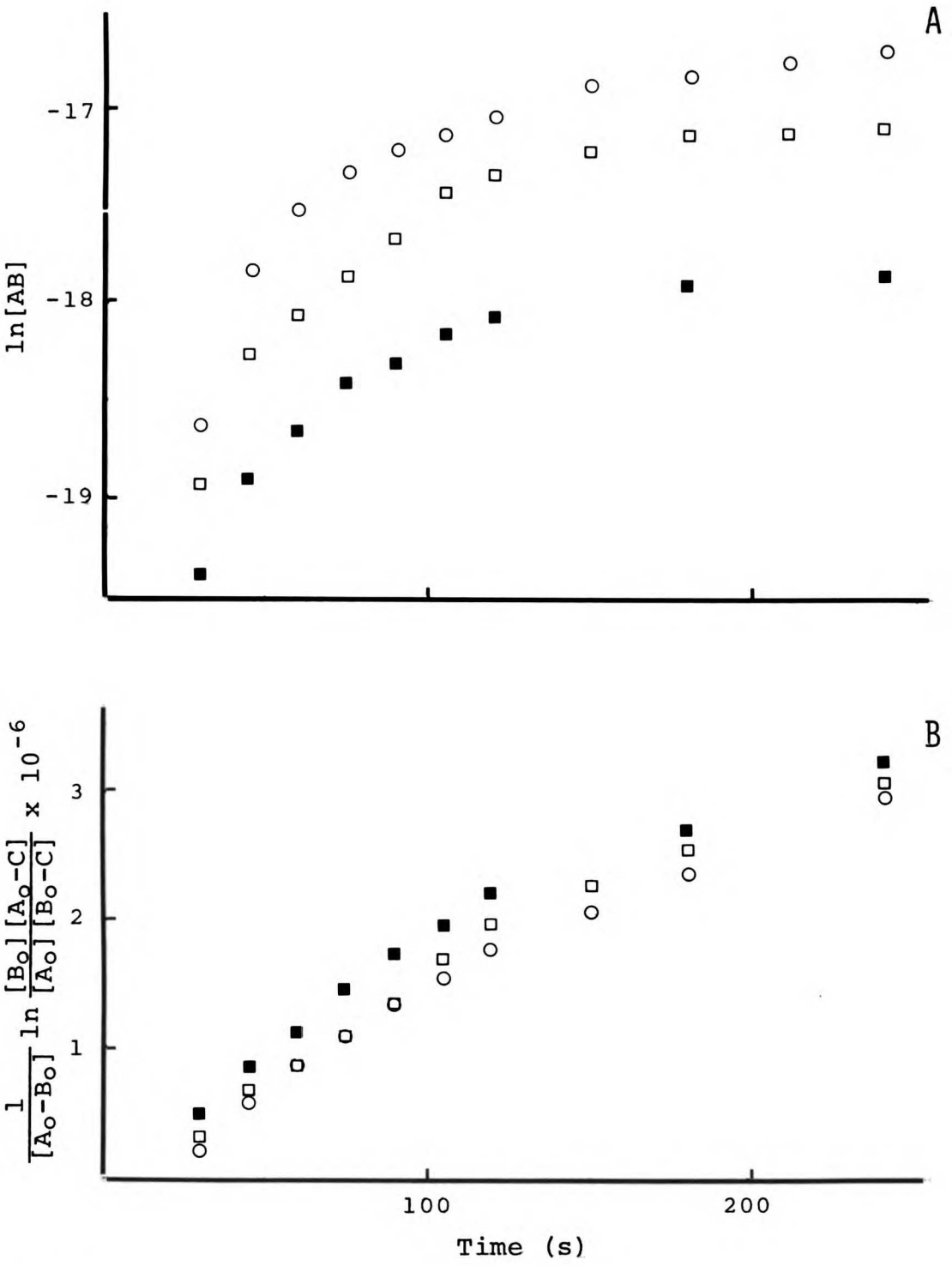

Fig. 33.
Recombination of oPRL fragments followed by fluorescence polarization at 0.125 (■), 0.20 (□), and 0.25 μM (○) FTC-oPRL-(1-53) considered as a first (A) and second(B) order reaction. A is FTC-oPRL-(1-53), B is oPRL-(54-199), and C or AB is the recombinant. In each case [A]/[B] = 2.

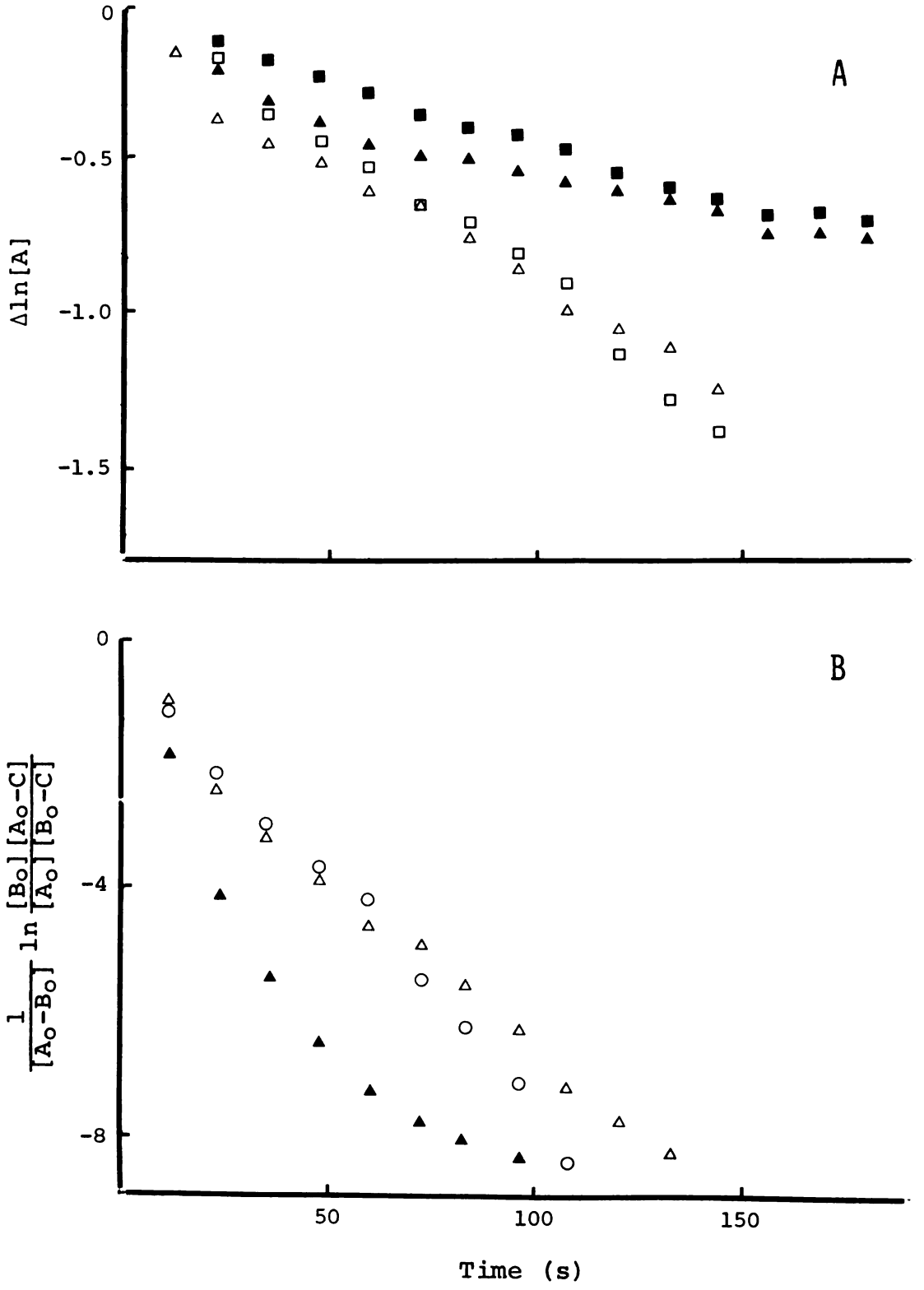

Fig. 34. A: [oPRL-(1-53)] from fluorescence intensity, with initial concentration of 1.96 M and 1.21μM (■) or 12.1μM (▲) oPRL-(54-199); [oPRL-(54-199)] initially at 1.21μM and 1.96 μM (○) or 19.6μM (△) oPRL-(1-53).
B: Second-order plot with concentrations as above.

DISCUSSION. It appears from the non-linearity of the first-order polarization plots that the association of the fragments is not rate-limited by dissociation of the oPRL-(54-199) dimer, or by a conformational change preceding the recombination. It is not clear why the second-order plots were non-linear after the initial stages of the reaction. Because the dissociation constant at 23°C was less than 0.175 μM, the backward rate constant could be neglected in the calculations. It is possible that the rates of monomer formation and recombination are similar to one another, in which case the reaction would not appear as clearly first or second order.

The linearity of the second-order plot of fluorescence intensity changes also indicates that the conformational change reflected in Trp fluorescence is not a limiting step in fragment recombination. The possibility that fluorescence changes are caused exclusively by burial of residues, rather than by a conformational change in the fragments, can also be rejected, because of the difference in rates of fluorescence intensity and polarization changes. The rate of molecular weight restoration in the recombination reaction was 44 x higher than the rate of conformational repair, suggesting that the recombination precedes the slower shift in tertiary structure.

CHAPTER 5

CONCLUSIONS

The isolation and recombination of two oPRL fragments has proven to be a useful probe into the structure of the hormone, allowing investigation of both the covalent and noncovalent bonds required to maintain its native conformation. A review of oPRL structure-function studies shows that chemical modification of the hormone has long been used in the determination of structural requirements for biological activity, but no attempt had been made previously to measure quantitatively the forces involved in maintaining the conformation of this protein. The importance of the integrity of the peptide chain was shown by preferential cleavage of the Met-Ala bond between residues 53 and 54. This modification in primary structure brought about significant changes in the secondary and tertiary structures of the molecule. The loss of 30% of the total helical content of oPRL upon hydrolysis of a single peptide bond suggests the importance of this region of the molecule to its overall conformation. This is especially noteworthy in light of the minor conformational shifts in the protein accompanying the loss of other covalent bonds, such as the terminal disulfides (Doneen et al., 1979). The prediction of oPRL secondary structure reported here also indicates that residues 53-54 lie in a region of low

helix-forming potential, suggesting that tertiary interactions contribute to the stability of at least part of the hormone secondary structure.

A shift in tertiary structure upon hydrolysis of the 53-54 bond is also apparent from the changes in the local environment at one or both of the Trp residues. Neither of these residues, located at positions 91 and 150, is close to the site of cleavage in the sequence. Therefore, these changes must represent folding of the molecule such that a Trp lies close to the bond, or a structural shift upon fibrinolysin treatment that extends over a large distance across the molecule.

In contrast to the loss of some structural elements with cleavage of a single bond, much of the oPRL structure appeared stable enough to withstand both treatment with 20% HOAc and isolation of the fragments. This was true of 70% of the original α-helix content and much of the local environment of the Tyr residues. Apparently, these structures are maintained by more local interactions and do not require the overall integrity of the molecule. A third group of interactions did not require the 53-54 bond, and could be regenerated after recombination of the fragments. These contacts were shown by restoration of much of the native side-chain CD spectrum.

The stability of oPRL-(54-199) in aqueous solution was increased by self-association to form a dimer.

Measurement of the monomer-dimer equilibrium constants at two temperatures allowed estimation of the free energy, enthalpy, and entropy of the self-association. These quantities are of special interest because ΔH reflects energies derived from hydrogen bonds and vanderWaals forces, while $T\Delta S$ arises from hydrophobic bonds and charge-charge interactions (Kauzmann, 1959). Both enthalpic and entropic forces appear to make a significant contribution to dimer formation, with enthalpy accounting for 70% of the total binding energy at room temperature.

A similar analysis of the reaction of oPRL-(1-53) with the oPRL-(54-199) monomer-dimer equilibrium revealed that the recombination is not only much more dependent on entropic forces than is the dimer formation, but also that the enthalpy is unfavorable to the reaction.

A combination of thermodynamic measurements for oPRL-(54-199) dimer formation and the overall recombination reaction allowed determination of the forces involved in the interaction of the individual fragments. These calculations showed that the binding of the two fragments is driven by the entropy of the reaction, with a negligible contribution from changes in enthalpy. This finding indicates that the two fragments are held together almost exclusively by hydrophobic forces and salt bridges, with little contribution from hydrogen bonds or vanderWaals interactions. In addition, it appears that dimerization

does not reproduce the interactions of the native or recombined molecules, but instead represents formation of a different set of stabilizing contacts.

It is possible that some aspects of the oPRL conformation, such as helical regions, are maintained by hydrogen bonding and vanderWaals forces, but these structures do not appear to influence, or be influenced by, the recombination of fragments. The failure of the recombination to restore the complete α-helix of the native molecule is consistent with this conclusion. Although the total conformational energy of oPRL has not been measured, Holladay et al., 1974) have examined these values for rat, ox, and sheep GH, and have reported energies of 8-14 kcal/mol. The free energy for recombination of the oPRL fragments is -13.4 kcal/mol, possibly indicating that a large proportion of the total conformational energy of native oPRL is lost upon isolation of the fragments.

Studies of recombination rates indicate that fragment interaction follows second-order kinetics. This finding rules out the possibilities that oPRL-(54-199) monomer formation or some conformational change constitute rate-limiting steps in the recombination reaction. In addition, the rates of changes in fluorescence intensity occur much more slowly than the recombination itself, indicating that the fragments must interact before the conformational shift affecting the fluorescence intensity can occur.

Thus, the restoration of some aspects of the native oPRL conformation appears to be dependent on, and not to limit, the recombination of the fragments.

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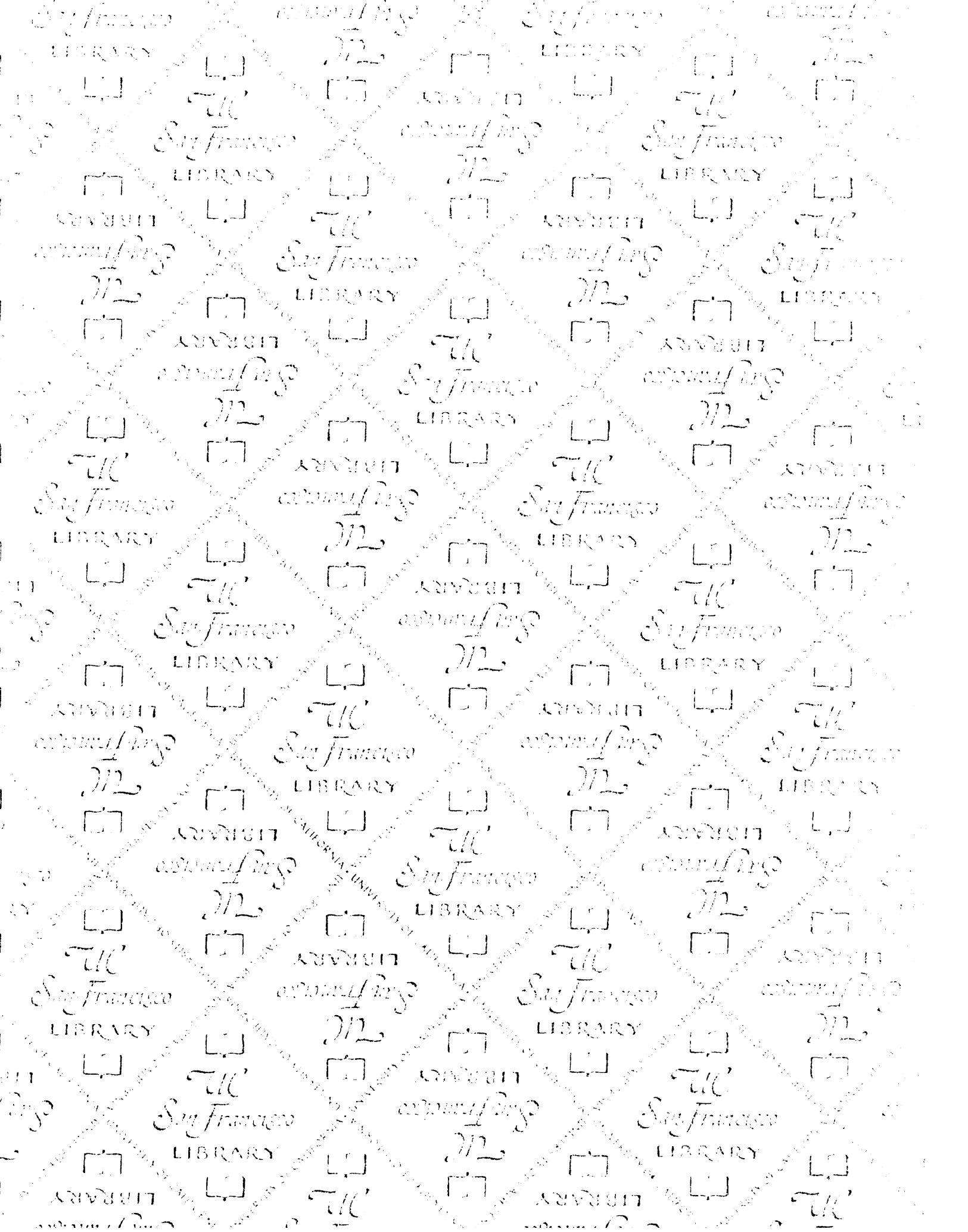
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