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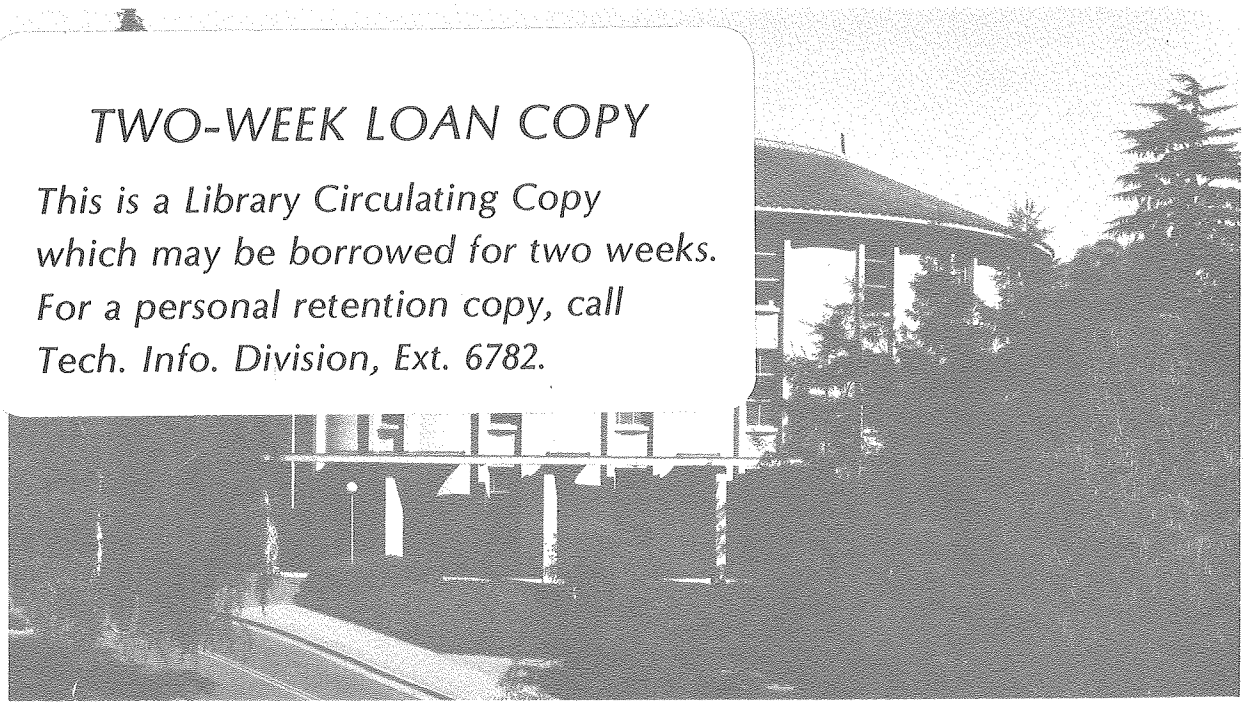

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INVESTIGATION OF MOLYBDENUM-SUBSTRATE INTERACTIONS WITH X-RAY ABSORPTION SPECTROSCOPY.

Joseph P. Smith, Jon A. Kirby, Melvin P. Klein*,
Alan Robertson, and Albert C. Thompson.

Laboratory of Chemical Biodynamics,
Lawrence Berkeley Laboratory,
University of California, Berkeley, CA. 94720

Running Head: X-Ray Absorption Studies of Nitrogenase

INTRODUCTION

Molybdenum is always found in active preparations of nitrogenase. This fact, along with the results of studies of inorganic models for nitrogenase, such as the molybdate cysteine complex of Schrauzer[1] suggest that molybdenum plays an important role in the enzyme mechanism. In light of the many molybdenum-dinitrogen complexes that have been prepared, it is tempting to speculate that the molybdenum is somehow involved with the binding of substrates, prior to reduction by the enzyme. To fully understand the function of molybdenum in nitrogenase, it is important to know whether, and under what conditions, molybdenum-substrate interactions will occur. We have been using x-ray absorption spectroscopy to investigate this problem and in this paper, we will describe our approach and the results we have obtained to date.

The intense, tunable x-ray source that is occasionally available at the Stanford Synchrotron Radiation Laboratory has made it practical to study samples with the low concentrations of the absorber of interest typical of metalloprotein systems. Several studies of metalloprotein systems with the synchrotron radiation source have been recently reported.[2,3]

The theory and interpretation of x-ray spectra have been discussed in published reports[4,5] and only a brief synopsis will be presented here. For purposes of discussion, the x-ray absorption spectrum may be divided into two parts, viz, the edge region, located within $\pm$ 50eV of the sharp increase in adsorption at the adsorption edge; and the extended x-ray absorption fine structure or EXAFS, which extends from 50-1000eV above an absorption edge.

In the edge region, we observe transitions from core levels (1s, in the present case) to low lying unoccupied molecular orbitals. Edge structure is determined by the metal site symmetry, the identity of the surrounding ligands and the strength of the metal-ligand interaction.

At higher energies, a continuous distribution of final states is available for occupation by the ejected photoelectron. The absorption coefficient in the energy range of 50-1000eV above the edge is modulated due to backscattering of the ejected photoelectron from atoms in the vicinity of the absorbing atom. These modulations are the EXAFS, which may be thought of as an interferogram whose structure is sensitive to the distances between the absorber and nearby scatters, the nature of the scattering atom, and the degree of local order around the absorbing atom. The EXAFS is written as a normalized modulation of the absorption cross section in photoelectron wave vector (k) space. Where $k = \frac{1}{\hbar} 2m(E-E_o)^{1/2}$ (1)

$$\chi(k) = \frac{\mu(k) - \mu_o(k)}{\mu_o(k)}$$

In equation 1, $\mu_0(k)$ is the absorption cross section in the absence of any modulation. The dependence of EXAFS on local structure is given [4] by equation 2.

$$\chi(k) = -\sum_j \frac{N_j}{k R_j^2} |f(\pi,k)| e^{-\sigma_j^2 k^2} e^{-R_j/\lambda_j} \sin(2kR_j + \alpha_j(k)) \quad (2)$$

Here (k) has been written as a sum of damped sinusoids. Each term in the sum represents the contribution of a shell of N_j identical atoms located at a distance Rj from the absorber. The backscattering amplitude function $f_j(\pi,k)$ describes the interaction between photoelectron and scatterer. The effects of thermal and structural disorder are represented by $e^{\sigma^2 k^2}$ factor. The effect of interations potentials on the photoelectron phase is given by the total phase shift $\alpha j(k)$. Finally, a mean free path factor ($e^{-Rj\lambda}$) accounts for inelastic decay of the photoelectron. In equation 2, the backscattering amplitude is characteristic of the type of scattering atom, while the total phase shift is a combined characteristic of the absorber-scatterer pair.

The above considerations indicate that the x-ray absorption spectrum is sensitive to the arrangement and nature of the ligands around a metal atom

In principle, if dinitrogen were to bind directly to molybdenum, or bind to some other enzyme site causing an indirect perturbation of the coordination environment of molybdenum, the x-ray absorption spectrum would be altered.

Our experimental approach to the use of x-ray absorption sepctroscopy can be summarized as follows. Solutions of the molybdenum-iron component of nitrogenase are prepared and equilibrated with either argon or dinitrogen. The molybdenum absorption edge spectra and EXAFS of these samples are measured and compared, along with the spectra of selected model compounds.

EXPERIMENTAL

Ezympe Samples. The Mo-Fe component of Azotobacter vinelandii was prepared according to the procedure of Shah and Brill.[6] In one experiment, we used Clostridium Pasteurianum Mo-Fe component prepared by M. Henzl of the University of Wisconsin at Madison. The average specific activities of pooled Azotobacter preparations used in this work ranged from 1500 to 1650 nanomoles of ethylene produced per minute per millgram of protein. For samples run as frozen solutions, recovery of activity after X-ray experiments was 90% or better. For the experiment where spectra were taken on solutions held at 4°C, the recovery of the argon equilibrated sample was 75% and that of the dinitrogen equilibrated sample 88%.

Inorganic Model Compounds. Tris (3-4 dithiotoluene) Mo (VI) was prepared in a benzene solution by the method of Gilbert and Sandell.[7] Other molybdenum compounds were reagent grade commercially available chemicals.

X-ray spectra. X-ray spectra were recorded at the Stanford Synchrotron Radiation Laboratory. This facility has been described elsewhere.[8] Model compound spectra were recorded in the absorption mode using ion chamber detectors. Enzyme spectra were recorded in both the absorption and the fluorescence mode.[9] Due to severe absorption background problems, only fluorescence EXAFS data are presented here.

RESULTS

The absorption edge spectra of several molybdenum compounds are shown in figure 1. In figure 2, the absorption spectra of two nitrogenase samples at 4°C are presented.

In all of the edge spectra, the absorption of the Mo K-shell was isolated by fitting a first or second order polynominal to the pre-edge region of the data followed by extrapolation and subtraction of the fitted function form the entire data set.

Two types of EXAFS experiments were performed on nitrogenase samples. Three separate data collections were made on nitogenase samples held at -90°C. Each data collection included samples equilibrated with argon and dinitrogen. A single large data collection was performed on samples held at +4°C. The average EXAFS of nitrogenase samples at -90°C are shown in figure 3. In figure 4, we show the EXAFS of nitrogenase samples at 4°C.

In an effort to measure the effects of dinitrogen coordination on molybdenum EXAFS, Timothy Walker of our laboratory prepared samples of trans-bis dinitrogen [1,2,bis diphenylphosphinoethane]Mo(0) and its tetrahydride analog. In figure 5, we present the EXAFS of these compounds.

DISCUSSION

Edge Spectra. The spectra of model compounds (fig. 1) illustrate the sensitivity of the edge region structure to the environment of the molybdenum. Progressing from the octahedral $K_3Mo\ Cl_6$ to more distorted 6-coordinate structures (Tris 3,4 dithiotoluene)Mo(VI), MoO_2 and MoO_3) the absorption edge becomes broader and more complex. The spectrum of sodium molybdate dihydrate shows the edge shape characteristic of the tetrahedrally coordinated species with formally d^0 configurations.[10]

The absorption edge of nitrogenase is broad and featureless on the low energy side of the first absorption maximum. On the high energy side of the first absorption maximum, there is a distinct shoulder. For six coordinate first row transition metal ions, shoulders on the high energy side of the first absorption maxima have been attributed to splitting of the excited state due to asymmetric coordination.[11] Comparison of the two enzyme spectra shows little difference in the low energy side of the absorption edge. The energy of the infleciton point in the absorption edge has been used as a qualitative indicator of molybdenum "coordination charge". There is no change (within an error of leV) in the inflection point energy due to the presence of dinitorgen.

When dinitrogen is added, the shoulder on the high energy side of the first absorption maximum becomes somewhat broader. Schemes for the interpretation of this feature have been proposed,[13] but in the absence of a detailed model of the molybdenum site in nitrogenase, a quantitative interpretation of the changes observed is premature.

In summary, the presence of dinitrogen causes only minor changes in the molybdenum edge structure of nitrogenase. These results show that no drastic change in the coordination environment of molybdenum occurs upon the addition of dinitrogen. Without a detailed model of the structure of the molybdenum site, it would be premature to attempt a more quantitative explanation of the changes observed. On a qualitiative level, extension of these experiments to nitrogenase samples equilibrated with other substrates or inhibitors might permit a more detailed interpretation to be formulated.

EXAFS STUDIES

The EXAFS of the nitrogenase samples have been analyzed by Fourier transformation.[4] In figure 6, the Fourier transforms of the -90°C nitrogenase EXAFS are presented. In addition to the transforms of the EXAFS of samples

equilibrated with N_2 and argon, we show the Fourier transform of the difference between the Mo-Fe+N_2 and the Mo-Fe+Ar EXAFS. The difference EXAFS was obtained by subtracting the normalized Mo-Fe+Ar (k) from that of Mo-Fe+N_2. The difference transform should highlight any features of the Fourier transforms due to the presence of dinitrogen.

Examination of the transforms in figure 6 shows that both samples display a major periodicity at an apparent radius of 1.72A, with shoulder at 2.5Å. There are no significant differences in the transforms.

The results of the Fourier transform method depend on the weighting of the data in k space prior to computation of the Fourier transform. By weighting different parts of the k space domain more heavily than others, information can be obtained about the dependence of EXAFS amplitude on k. When the high k part of the data is given greater weight in computing the transform, peaks due to scatterers with higher atomic numbers and less thermal and structural disorder tend to grow with respect to peaks due to scatterers of lower atomic number and greater disorder. In figure 7 we show the Fourier transforms of the -90°C data weigted by k^3. The growth of the peak at 2.5 Å component suggests that the 2.5 A component is due to scatterer(s) with higher atomic numbers than the scatterer(s) responsible for the 1.72A component.

In figure 8, we show the transforms of nitrogenase data taken on samples at 4°C. There is a major periodicity at 1.72 Å, as was observed in the -90° data. However, there are additional peaks at 3.62 A and 5.32 Å (N_2) and 4.06 Å and 4.82 Å (Ar) which are not present in the -90° data. There appears to be a significant difference in the N_2 and Ar transforms. The interpretation of these results presents several problems. The first is that of the peaks at distances of greater than 4 Å.

Experience with the analysis of EXAFS data from systems of known structure indicates that unless there are multiple scatterers, detection of peaks beyond 4 A is not possible. It could be proposed, knowing that a number of iron atoms are present in the molybdenum-iron cofactor, that the molybdenum is present in a novel cluster structure with multiple molybdenum-iron distances. This explanation does not explain the apparent alterations in structure that occur on freezing. To formulate a consistent explanation for both -90°C and 4°C data, it would have to be assumed that a) a significant structural change occurs during freezing; b) upon thawing, the molybdenum site assumes a highly unusual (by EXAFS standards) structure; and c) Mo-substrate interactions which occur at 4°C are blocked at -90°C. An alternative to the above speculations is to assume that the 4°C data contains some pathological artifact which gives rise to the peaks at large r. Under usual circumstances, these discrepancies would be resolved by simple repetition of the experiment at 4°C. However, the present situation with respect to instrument time at Stanford make it unlikely that these experiments will be repeated soon.

In summary, the EXAFS results show that the presence of nitrogen produces no change in the nearest neighbors of molybdenum in nitrogenase. Both -90°C data and 4°C data are in agreement on this point. In the -90°C data a shoulder on the main peak in the Fourier transform appears to be due to a different type of scatterer than that responsible for the 1.72 A peak. The data taken at 4°C show peaks extending out to 5.3 A. These peaks are altered in the presence of N_2. Due to the large differences between the 4°C and 90°C data, and the unusually large radii of peaks in the 4°C data, the apparent differences due to the presence of N_2 are open to question.

Finally, we will consider the EXAFS of the molybdenum-dinitrogen complexes (fig. 5). In these compounds molybdenum is surrounded by 4 phosphorous atoms (at 2.45 Å) and in one case, two nitrogen atoms as well (2.01 Å). The beating

of two slightly different frequencies is apparent in the low k region of the data for the dinitrogen complex. The tetrahydride EXAFS is composed of a single major frequency.

The results of this preliminary study show that in these compounds coordination of molybdenum by dinitrogen is detectable under some circumstances. It is clear, however, that the EXAFS is dominated by the scattering of 4 phosphorous atoms. This has implications for the study of the molybdenum-substrate interactions in nitrogenase. If N_2 entered the coordination sphere of molybdenum, its presence could be hidden by the presence of a larger number of strong scatters such as sulfur or iron. This is particularly true if the Mo-N distance was close to the Mo-X distance, where X represents the other nearest neighbors in nitrogenase. At the present time, we are continuing our EXAFS studies of molybdenum dinitrogen complexes in an effort to better define the conditions under which Mo-N coordination could be detected by EXAFS.

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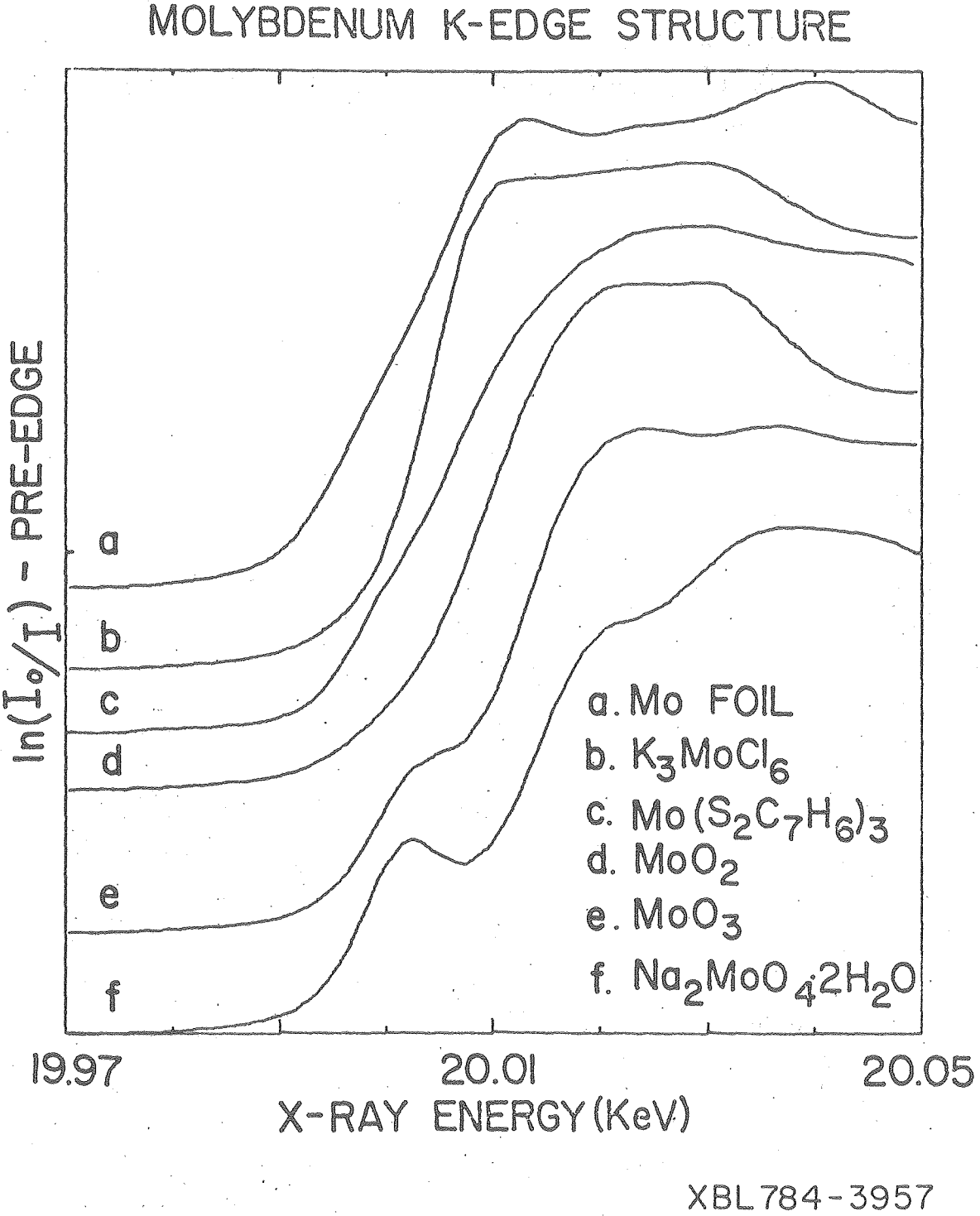

Figure 1. Edge Spectra of Molybdenum Compounds. Data points taken every 2.5eV.

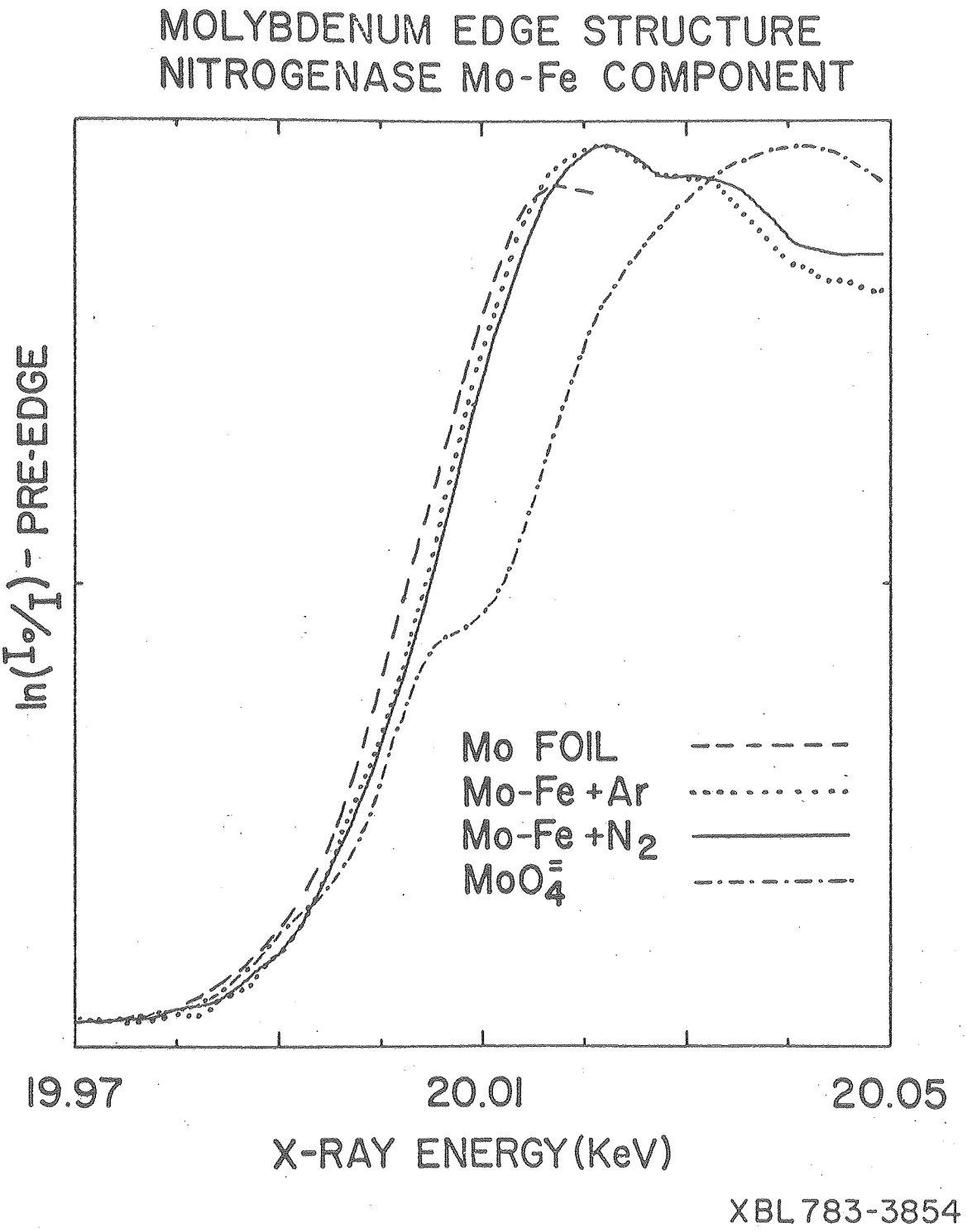

Fig. 2. Edge Spectra of nitrogenase samples at 4°C. Spectrum of molybdenum foil shown as an energy reference. Data points taken every 1.6 eV.

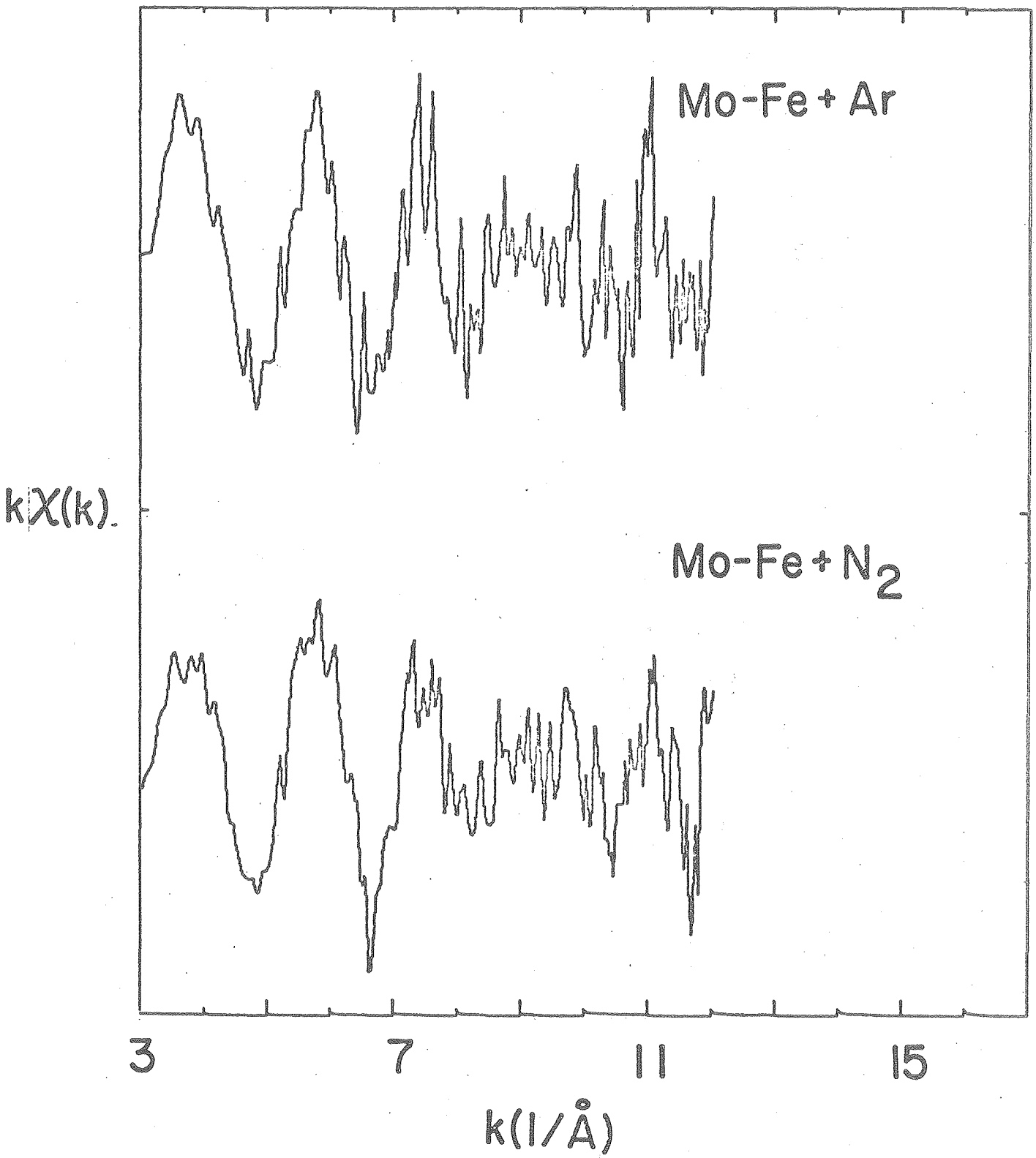

Fig. 3. Averaged Mo EXAFS of nitrogenase at -90°C

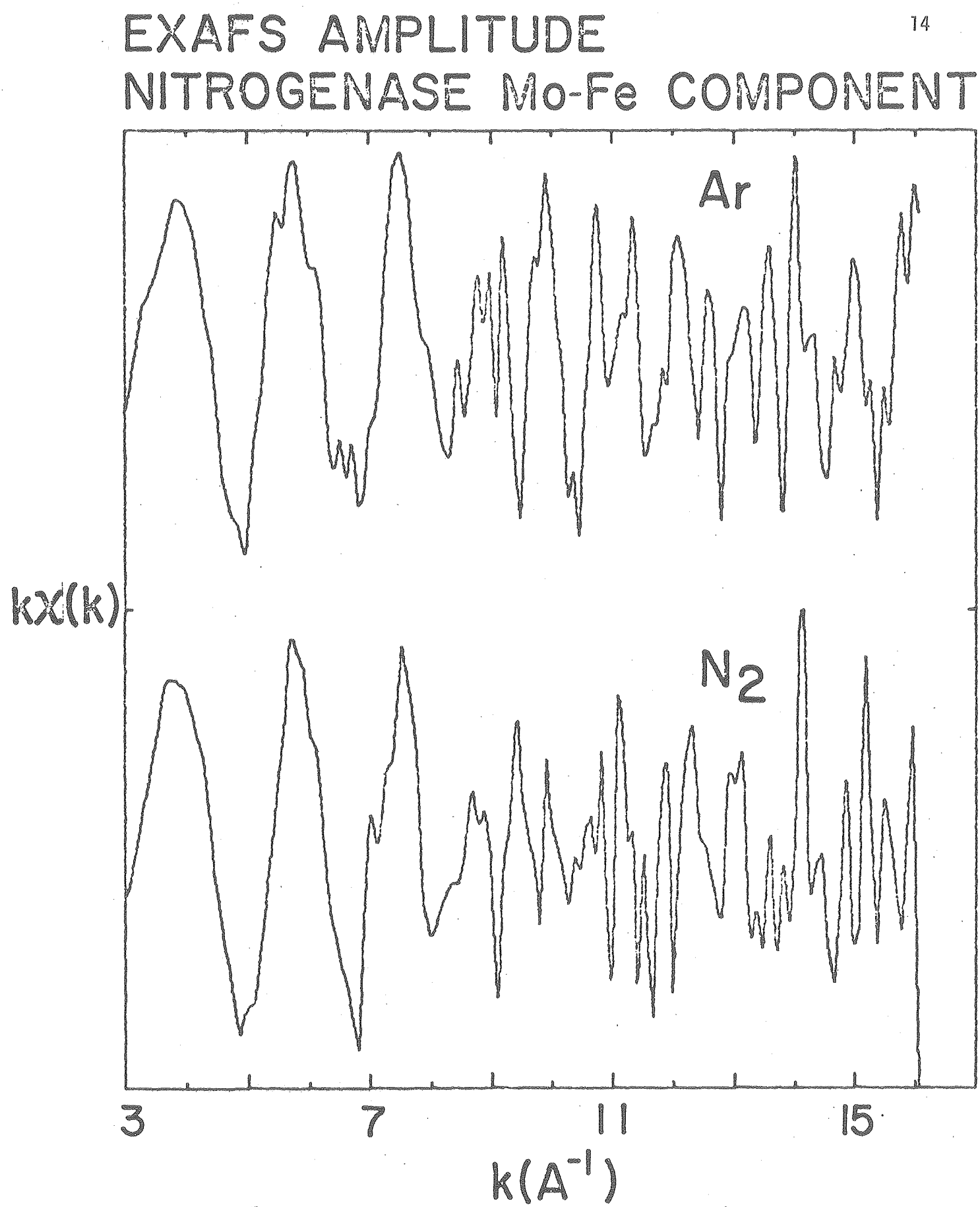

Figure 4. Average Mo EXAFS of nitrogenase at +4°C

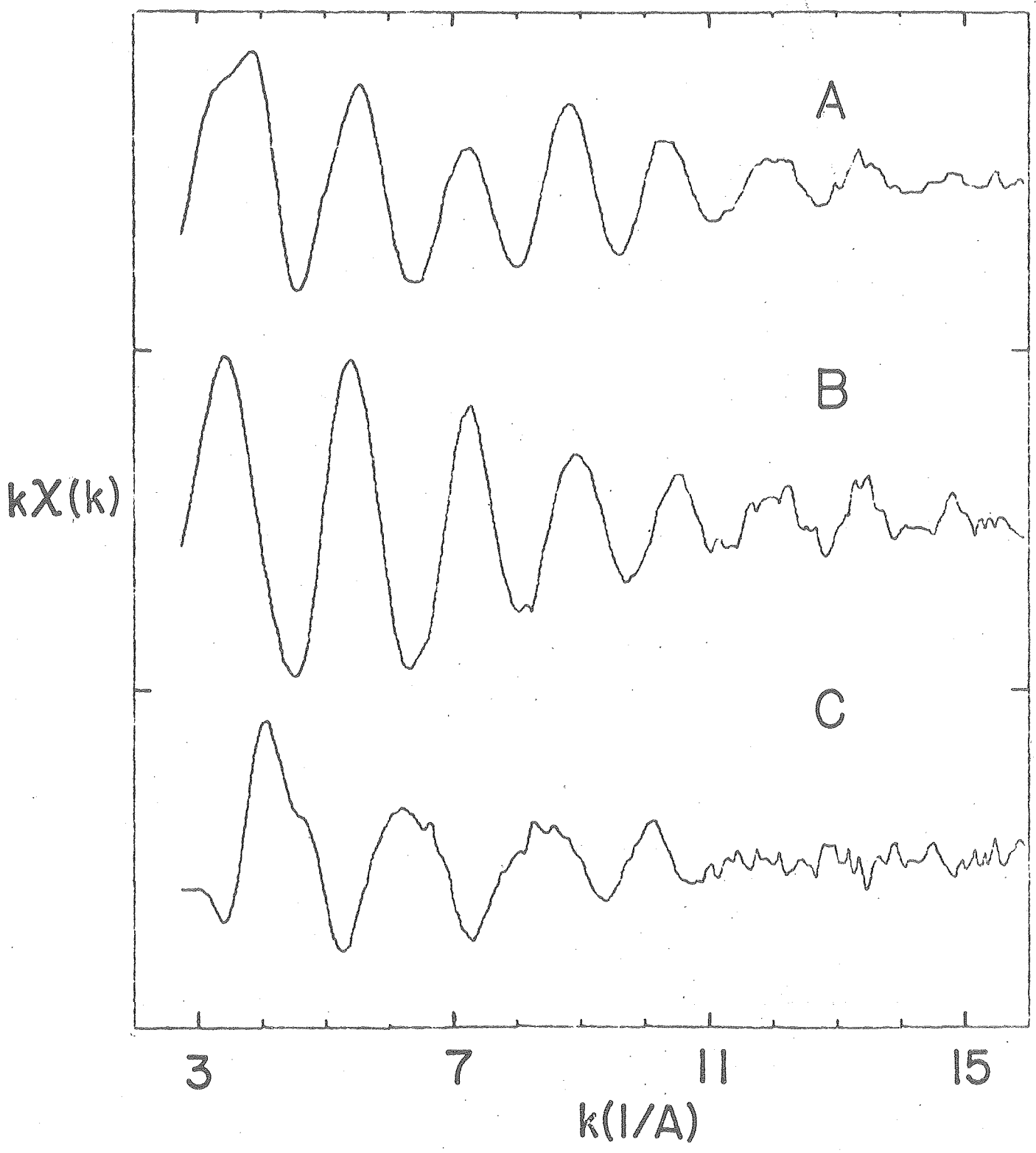

Figure 5. EXAFS of A) trans bis dinitrogen [1,2 bis (diphenylphosphinoethane)]Mo(o)
B. [1,2 bis (diphynl phosphinoethane)] molybdenum tetrahydride
C. Difference EXAFS A-B.

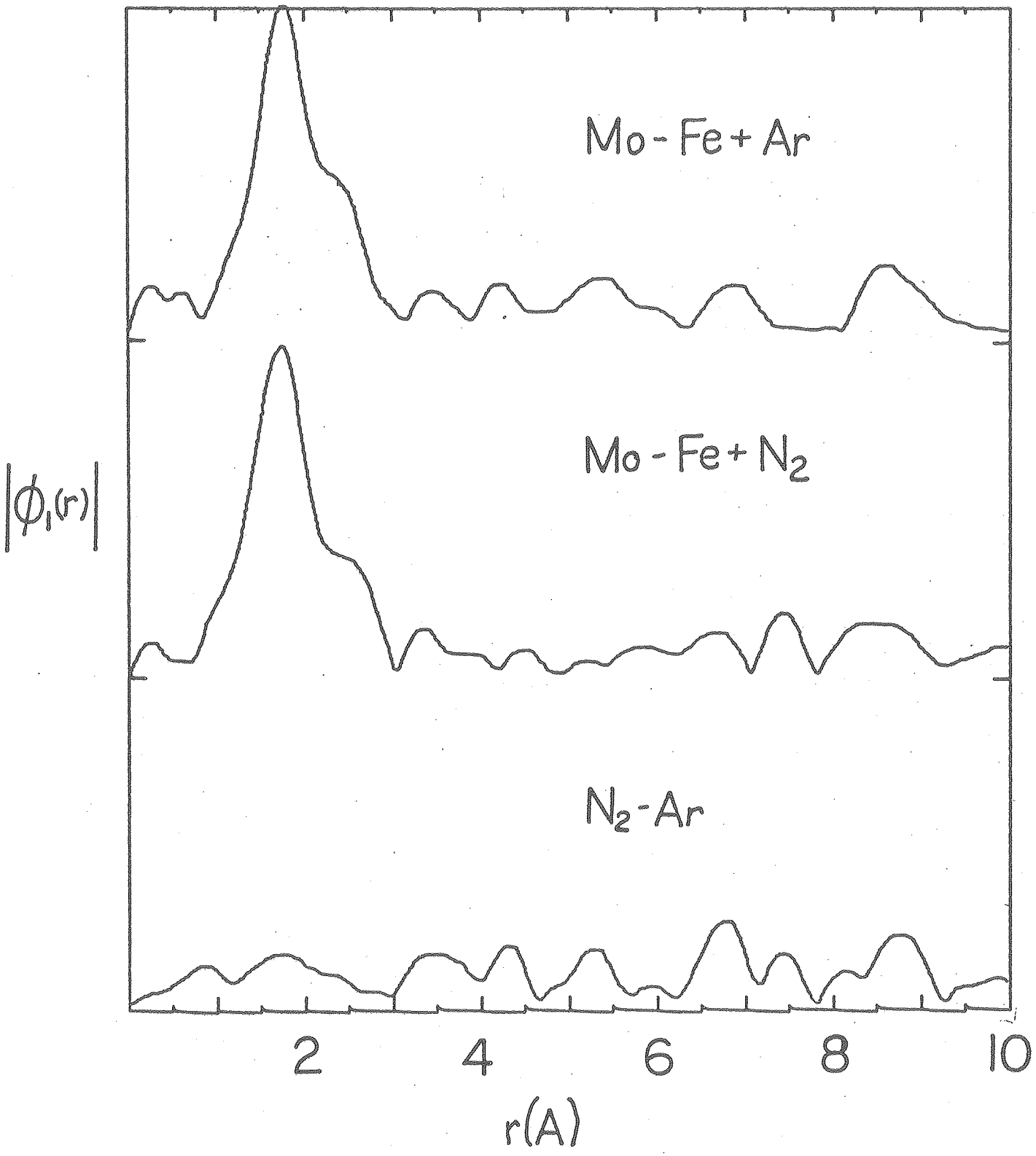

Figure 6 Fourier transform of -90°C nitrogenase EXAFS. Data used from 3-12 1/A. k' weighting.

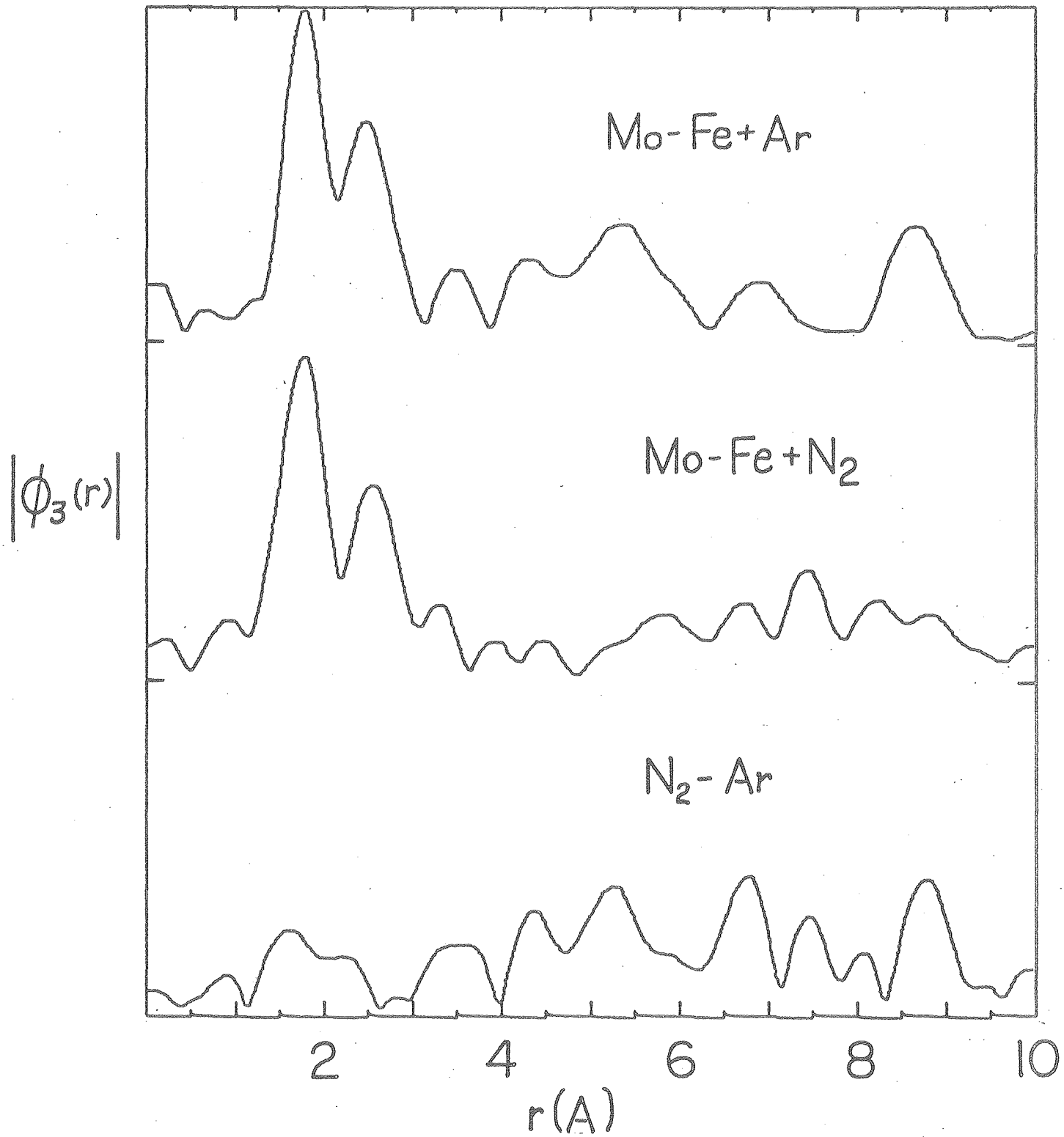

Figure 7. Fourier transforms as in Figure 6, but with k^3 weighting.

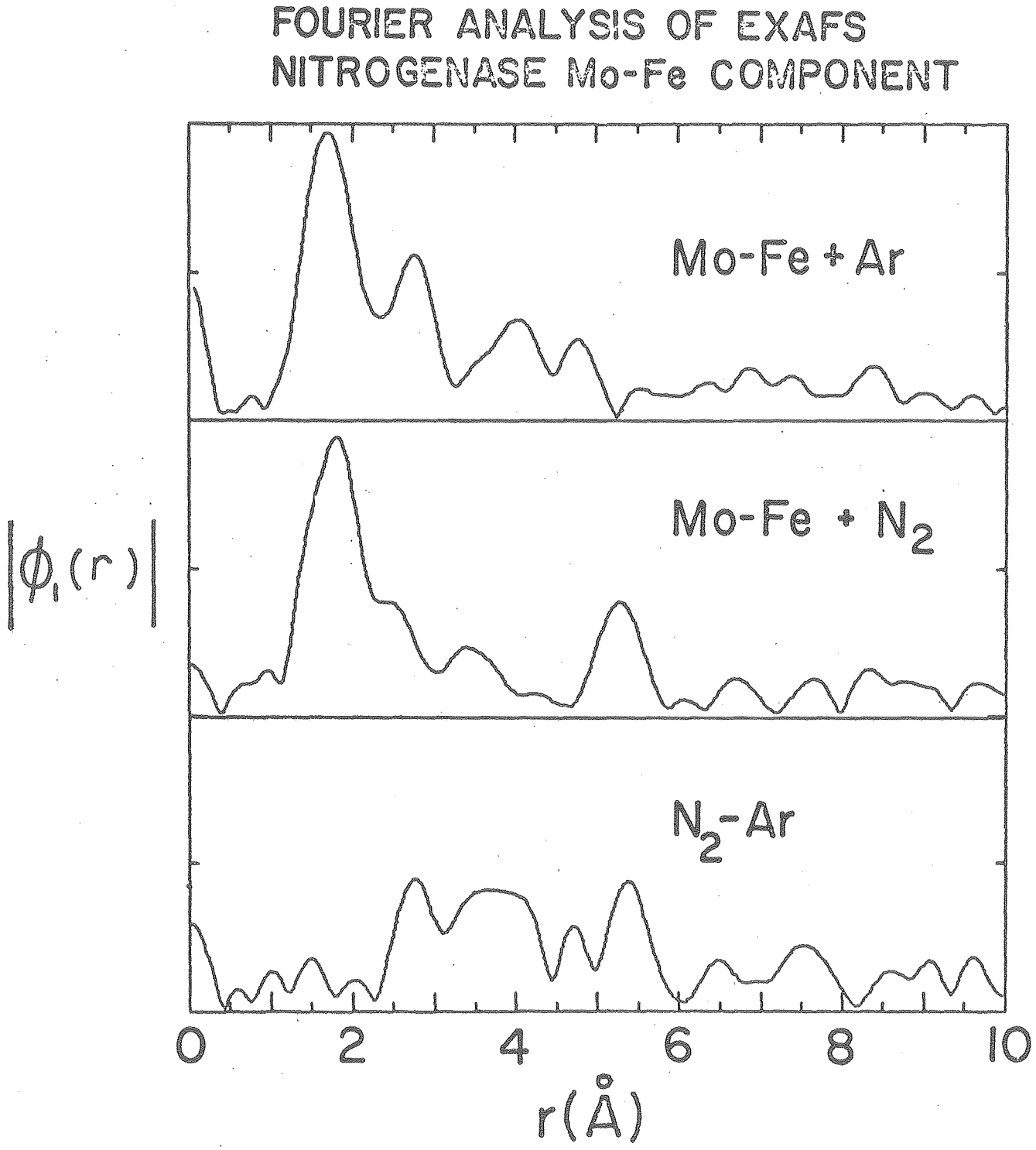

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Figure 8 Fourier transform of nitrogenase EXAFS. (4°C). k' weighting, 3-12 1/A k space domain.