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3-^{13}C-METHIONINE-LABELLED E. COLI ALKALINE PHOSPHATASE

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SUMMARY:- 3-^{13}C-methionine has been biosynthetically incorporated into E. coli alkaline phosphatase using strain CW3747 which is auxotrophic for Met. ^{13}C NMR of the dimeric native enzyme labelled at the eight methionine residues of the primary structure shows a dispersion of resonance signals permitting resolution of at least five methione environments, none of which coincide with the chemical shift position of free methionine. At acid pH, ^{13}C signal intensity is shifted to a chemical shift consistent with solvent exposure. However, three discrete resonances are observed, suggesting a retention of defined structure. The labelled protein thus can serve as a probe of conformational alterations of the enzyme.

The three dimensional structure of proteins is not static but can adopt a range of conformations. The control of the range of structures accessible can, in some cases, be assigned to the association of protein ligands or apparently local modifications of covalent protein structure. Defining the extent to which structural information is communicated through the polypeptide chain remains a difficult and incompletely resolved problem. An example of such a system is the E. coli enzyme alkaline phosphatase. This dimeric Zn(II) enzyme undergoes substantial structural changes as a consequence of substrate association, changes in the medium, binding of the essential metal ions and limited proteolytic cleavage (1-3). Evidence for the presence of conformational alterations has been previously obtained on the basis of, inter alia, circular dichroic measurement of the peptide bond region, hydrogen exchange studies, magnetic resonance methods and differential scanning calorimetry (1-3).

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In principle, magnetic resonance methods afford a means of directly assessing the extent of structural alterations. The chemical shifts of, for example, fluorine and carbon atoms have been previously shown to be influenced by their immediate environment within a protein structure (4,5). Moreover, changes in chemical shift as well as other magnetic resonance parameters (e.g. relaxation times, magnitude of the Nuclear Overhauser Effect) have been shown to provide a measure of alterations in structure. To initiate investigations of this type in the alkaline phosphatase system, selective ^{13}C enrichment of methionine residues labelled at the 3 carbon was performed. The use of ^{13}C-labelled methionine has previously been employed in studies of myoglobin, cytochrome C and dihydrofolate reductase (7-9). There are eight methionine residues per subunit of the enzyme located at sequence positions 4, 53, 134, 264, 302, 398, 400 and 442 (10). Based on the most recent information available on the three-dimensional structure of the enzyme, (11) these sequence positions sample a variety of environments differing in their location with respect to the protein surface, enzyme active center (and metal binding locus) and the region of subunit contact. Methionine residues are thus limited in number, simplifying the interpretation of data obtained, and yet sample a variety of structural loci. In addition, the bacterial cell strain CW3747 which has been routinely employed in the induced biosynthesis of alkaline phosphatase is a methionine auxotroph.

MATERIALS AND METHODS: 3-^{13}C-L-methionine (73% isotopically enriched) and 3-^{14}C-L-methionine (99% isotopically enriched) were obtained from ICN-KOR Isotopes and New England Nuclear respectively. Spectroscopically pure $MnSO_4$ was obtained from Johnson-Matthey, Inc. All other chemicals were reagent grade and used without further purification. E. coli strain CW3747 (ATCC 27527) were grown to late log phase ($OD_{550} \sim 1.0$) on a 10 L scale using a New Brunswick bench top fermentor. A minimal Tris-glucose medium (4) supplemented with 10 µg/ml thiamine, 20 µg/ml methione and 30 mM KH_2PO_4 was employed. Cells were harvested by centrifugation and resuspended in a fresh minimal media supplemented with 10 µg/ml isotopically labelled methionine and 6 x 10^{-5} M KH_2PO_4. The resuspended cells were maintained in aerated culture at 37° for 18-24 hr to maximize the yield of alkaline phosphatase. Cells were then harvested and the alkaline phosphatase isolated as previously described (11). Chromatographic purification of the enzyme was performed on a 1.6 cm x 34 cm DEAE cellulose column using 0.01 M Tris, pH 8.0 as the buffer with 1 x 10^{-5} M Zn(II) 1 x 10^{-2} M Mg(II) also present. NMR spectra were recorded on and IBM AF 270 spectrometer at 28°, using a 10 mm frequency-tunable probe and irradiating at 67.9 MHZ and 109.3 MHZ for detection of ^{13}C and ^{31}P respectively. Samples of 1.6 ml to 2.0 ml were made 20% in D_2O to provide a field-frequency lock. All carbon spectra were obtained using a sweep width of 15151 Hz (223 ppm) and a 60° - 70° flip angle sampling 32K or 64K data points. Broad-band proton decoupling was used

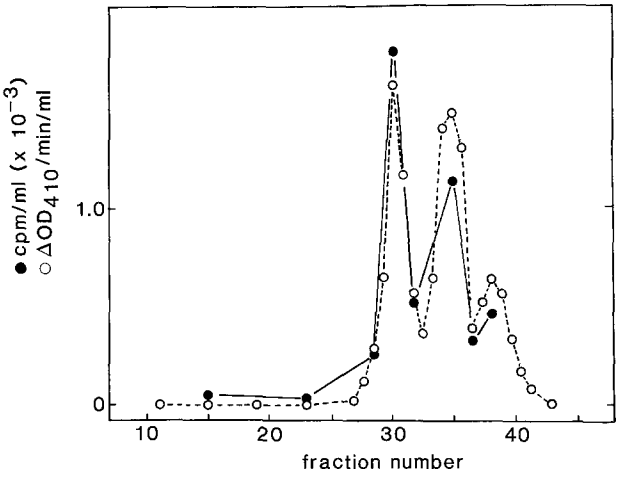


Figure 1. Elution profile of Anion Exchange Chromatography of Alkaline Phosphatase. Chromatography of alkaline phosphatase grown with ^{14}C-labelled methionine introduced during the induced biosynthesis of the enzyme was performed as described in Materials and Methods. Fractions of 5 mL were collected employing 500 mL of elution buffer with a linear salt gradient of 0 - 0.15 M NaCl. Enzyme activity (o) was monitored as para-nitrophenyphosphate hydrolase activity (12); Radiolabel incorporation (●) was determined on the basis of ^{14}C counts determined from aliquots taken from the indicated fractions.

for all spectra acquisition. Chemical shifts are reported relative to the chemical shift of tetramethyl silane. Circular dichroic spectra were recorded on a Jasco 500-C spectropolarimeter using 0.1 cm path-length cells.

Assessment of the extent of incorporation was performed by the addition of a small amount of ^{14}C-labelled methionine to the cell culture mixed with the ^{13}C-labelled amino acid. Induced protein synthesis was carried out and the crude enzyme product isolated. Shown in Figure 1 is a portion of the elution profile obtained on ion exchange chromatography of the protein. Radioactivity and phosphohydrolase activity comigrate as three resolved peaks. The peaks correspond to the "isozymes" normally observed in preparations of the enzyme. These species arise from deletion of the amino terminal Arg residue from none, one or both of the subunits of the dimeric enzyme. No significant kinetic or structural difference has been reported for these enzyme variants and mixtures of these species were employed for the magnetic resonance experiments. Quantitation of the amounts of radioactivity incorporated indicated that the isolated enzyme was enriched >70% in methionine added in the second phase of the biosynthetic protocol. For the synthesis reported methionine isotopically enriched

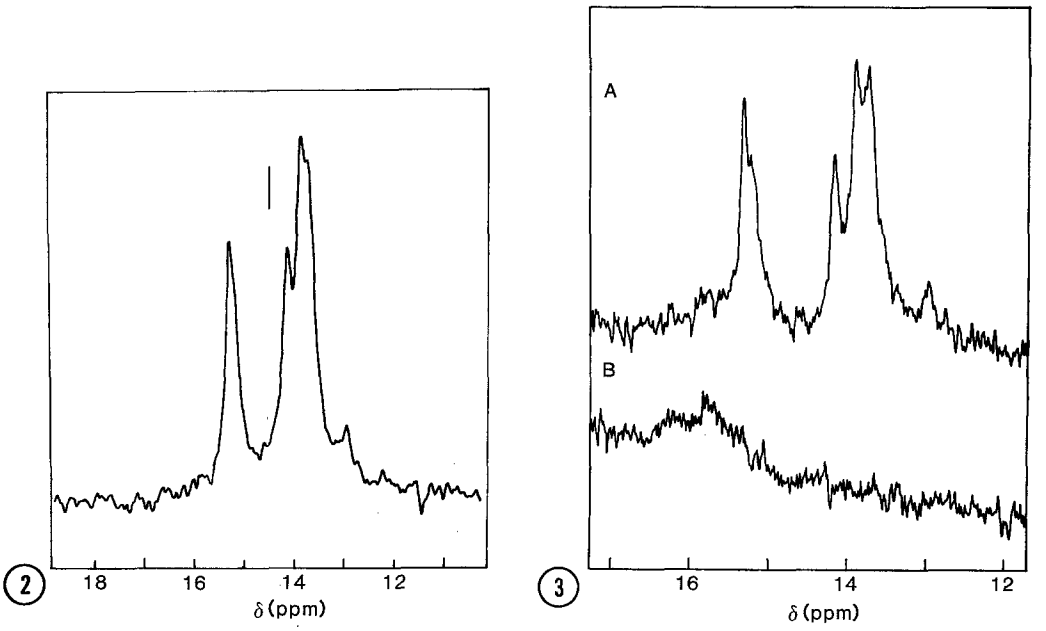


Figure 2. Difference Spectrum of ^{13}C-Met-Labelled and Unlabelled Alkaline Phosphatase. Conditions: 0.33 M alkaline phosphatase, 0.01 M Tris, 1 x 10^{-3} M Mg(II), 1 x 10^{-5} M Zn(II) pH 8.0. Data were obtained sampling 32K data points with a 60° flip angle collecting 30K (unlabelled) and 100K (labelled) transients with an acquisition time of 0.54 sec. Data are displayed with a line broadening of 5 Hz.

Figure 3. ^{13}C NMR Spectra of Unlabelled and 3-^{13}C-Methione Labelled Alkaline Phosphatase. Conditions: 0.33 x 10^{-3} M alkaline phosphatase, 0.01 M Tris, 1 x 10^{-3} M Mg(II), 1 x 10^{-5} M Zn(II) pH 8.0. Data were obtained sampling 32K data points using a flip angle of 60° with an acquisition time of 0.54 sec; (A) 3-^{13}C-methionine alkaline phosphatase (100,000 transients), (B) alkaline phosphatase (30,000 transients). Spectra are displayed with a line-broadening of 1 Hz.

~70% with ^{13}C at the 3-carbon was employed yielding an enzyme product which was ~50% ^{13}C-enriched.

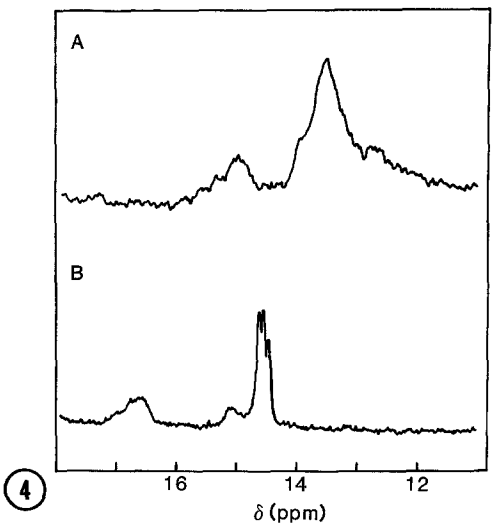
Shown in Figure 2 is a difference spectrum of labelled and unlabelled enzyme displaying the chemical shift range of 10-18 ppm. With the exception of resonances assigned to the carbon atoms of the Tris buffer, all other regions of the ^{13}C difference spectrum from -10 ppm to 120 ppm showed no observable resonance intensity. Shown in Figure 3 is a comparison of comparable ^{13}C spectra of the labelled and unlabelled protein. All features of the difference spectrum are reproduced in the unsubtracted spectrum of the labelled enzyme. The dispersion of chemical shifts of the methyl methionine resonances of the protein fortunately occur at the upfield extrema of the envelope of resonances due to

other alkyl carbons present at natural abundance. The extent of ^{13}C incorporation and the chemical shift position of the resonance of the labelled protein obviate the requirement of performing difference spectroscopy to monitor spectral changes in future experiments.

The symmetric dimer of alkaline phosphatase contains eight methionine residues per subunit (9). Both crystallographic and prior magnetic resonance studies indicate that when the four Zn^{+2} and two Mg^{+2} binding sites of the dimer are saturated with metal ions, the environments of corresponding residues of the two subunits occupy identical environments (1,10,12). The ^{13}C resonance intensity observed thus represents eight methionine environments in the enzyme. Since phosphate binding to the enzyme has been shown to produce structural alterations in the protein (1,2), a ^{31}P NMR spectrum of the enzyme sample was obtained to determine if bound phosphate was present. No resonance intensity was observed under sampling conditions which would have readily detected the presence of as little as a 5% level of bound phosphate.

The chemical shift of the 3-carbon of methionine under the experimental conditions of Figures 2 and 3 occurs at 14.54 ppm. Thus no resonance intensity in the enzyme structure is observed at a chemical shift corresponding to a completely solvent exposed environment. Based on studies with model peptides, there is no evidence to indicate that the dispersion of resonance intensity can be assigned to the influence of flanking residues per se (13). Thus distinct environments within the protein structure are apparently reflected in the discrete resonances observed.

The sample was then made 6 μM in Mn^{+2} ion to determine if a distinction between accessible residues, lying at or near the protein surface, and those lying in a more buried state could be made on the basis of magnitude of the influence of the paramagnetic ion. As shown in Figure 4A, resonances centered at ~15 ppm appear to be selectively broadened suggesting that the resonance intensity at this position corresponds to residues lying near the protein surface. In view of the influence of the presence of the paramagnetic metal ion on the entire spectrum, however, such conclusions must remain tentative.



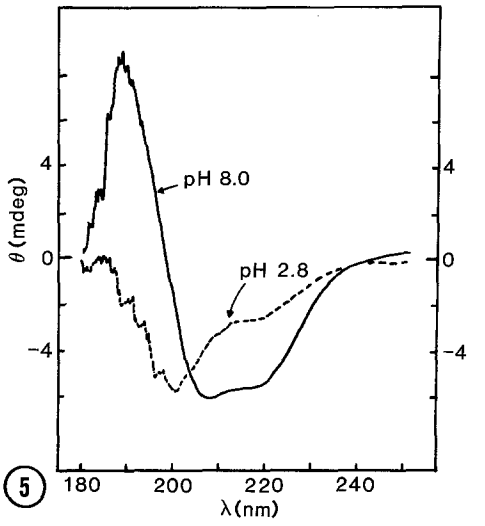


Figure 4. Influence of Mn(II) and Low pH. Conditions: (A) 0.15 x 10^{-3} M alkaline phosphatase, 0.01 M Tris, 1 x 10^{-3} M Mg(II), 1 x 10^{-5} M Zn(II), 6 x 10^{-6} M Mn(II), pH 8.0. (B) as (A), sample pH adjusted to pH 2.6 with acetic acid and sample made 0.5 x 10^{-3} M in EDTA. Data were obtained sampling 64K data points using a flip angle of 70° with an acquisition time of 1.049 sec, collecting 30,000 transients. Spectra are displayed with a line broadening of 2 Hz (A) and 1 Hz (B).

Figure 5. Circular Dichroism of Alkaline Phosphatase. Dilutions of enzyme employed in the magnetic resonance experiments shown in Figure 3A (solid line, protein concentration 0.3 mg/mL^{-1}) and Figure 4B (dashed line, protein concentration 0.3 mg/mL^{-1}) were monitored from 180 nm to 250 nm. Medium conditions are as described for Figures 3A and 4B.

The pH of the sample was then adjusted to pH 2.6 and EDTA was added to limit the line-broadening influence of the Mn^{+2} ion present. At pH values below pH 4 the enzyme has been shown to release the firmly bound metal ions with a concomitant loss of activity and undergo substantial conformational changes resulting is dissociation of subunits and a loss of secondary structure (15). Shown in Figure 5 are CD spectra in the region of peptide bond absorbance illustrating the magnitude of these changes for aliquots of the samples used in the NMR studies.

The ^{13}C NMR spectrum of the sample at pH 2.6 is shown in Figure 4B. The marked structural changes are reflected in an attenuation of the dispersion of the ^{13}C resonances which now appear as three resolved peaks at 14.60 ppm, 14.53 ppm and 14.46 ppm. These chemical shifts do not correspond to the resonance positions observed for the methionine residues of the native structure. Indeed the chemical shift region from 14.3 ppm to 14.7 ppm is devoid of resonance

intensity in the native protein spectrum. The chemical shift of the 3-carbon of free methione at pH 2.6 occurs at 14.48 ppm suggesting that the collapse of structure results in exposure of all methionine residues to solvent. However, while structural features of the native enzyme are largely lost on acidification, the observation of several distinct resonances at pH 2.6 indicates that the protein structure is neither completely unfolded (which would be anticipated to result in a single resonance for the solvent exposed methionines) nor randomized. On restoration of the enzyme medium to pH 8.0 following exposure to acidic conditions, reconstitution of protein structure and function has been demonstrated (1). The retention of a degree of ordered structure, as indicated by the low pH NMR spectrum, may permit the elucidation of aspects of the refolding pathway of the enzyme. Studies to assign the resonances observed to specific amino acid residues in the sequence and to employ the labelled enzyme as a probe of more subtle conformational alterations are currently in progress.

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