SELECTIVELY ENRICHED [γ-^{13}C]-HISTIDINE AS A NUCLEAR MAGNETIC RESONANCE PROBE OF ENZYME STRUCTURE AND FUNCTION. SYNTHESIS AND INCORPORATION INTO *E. coli* ALKALINE PHOSPHATASE.

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SUMMARY: The ^{13}C nmr spectrum of *E. coli* alkaline phosphatase in which the γ positions of the histidine residues are enriched in ^{13}C is reported. The labelled enzyme samples were prepared by *in vivo* incorporation of [γ-^{13}C]-histidine or [γ-^{13}C]-β,β-dideuteriohistidine. Eight clearly resolved ^{13}C nmr signals were observed, corresponding to the eight or nine histidine residues per subunit. The observed narrow linewidths and sensitivity to environment of histidine C_γ resonances in alkaline phosphatase suggest that ^{13}C enrichment at this quaternary carbon position will prove useful in determining details of enzyme structure and mechanism by means of ^{13}C nmr measurements.

Carbon-13 nuclear magnetic resonance has to a great extent failed to live up to its promise of providing a technique for investigating individual amino acid residues in proteins. In this communication, we demonstrate that this failure may be overcome, at least in the case of histidine residues, by preparing proteins which are enriched in ^{13}C at quaternary carbon positions.

Previous difficulties in applying ^{13}C nmr techniques to studies of protein structure are the result of problems with sensitivity, overlap of signals, and assignment of signals. The inherently low sensitivity of the ^{13}C nmr technique has in general allowed useful natural abundance spectra to be obtained only for relatively small proteins (M.W.$\lesssim$20,000) (1) which can be

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obtained in comparatively large amounts. The sensitivity problem can be alleviated in some cases by preparation of samples uniformly enriched in ^{13}C(2,3) or by advances in instrument sensitivity. However, the problems of overlap and assignment of signals still remain severe, particularly in the case of larger proteins.

To alleviate these difficulties, several workers have employed incorporation of specifically ^{13}C enriched amino acids into proteins. For example, [δ_2-^{13}C]-L-histidine has been incorporated into several enzymes for this purpose. In α-lytic protease(4) and semisynthetic ribonuclease-S'(5), where only one histidine residue was labeled, useful information was derived from the properties of the single, broad resonance observed. Unfortunately, the large linewidth of these signals (∿30 Hz), leads to resolution problems when more than one histidine in the protein is labeled. Browne et al.(6,7) were unable to resolve the overlapping signals from the four labeled histidine residues in tryptophan synthetase α-subunit, even though the C^{δ_2} hydrogens had been replaced by deuterium to reduce the linewidths of individual resonances.

Quaternary (nonprotonated) ^{13}C nuclei generally have nmr signals which are narrower than those of ^{13}C nuclei having directly attached hydrogens (1). In addition, the chemical shifts of signals from various quaternary carbons have been observed to be relatively sensitive to perturbations in environment(8). Preparation of proteins enriched in ^{13}C at quaternary carbon locations thus could alleviate the overlap problem encountered in previous studies. In this communication, we report the synthesis of an amino acid enriched in ^{13}C at a quaternary position, [γ-^{13}C]-DL-histidine, and its deuterium analog [γ-^{13}C]-β,β-dideu-

terio-Dl-histidine,* and the ^{13}C nmr spectra resulting from their incorporation into E. coli alkaline phosphatase.

MATERIALS AND METHODS

The synthesis of [γ-^{13}C]-DL-histidine involved initial conversion of barium carbonate (90% enriched in ^{13}C) to ethyl [β-^{13}C]-glycinate hydrochloride(9-13). The ethyl glycinate was transformed to ethyl [4-^{13}C]-imidazole-4-carboxylate (14). Reduction with lithium aluminum hydride (15) or lithium aluminum deuteride yielded [4-^{13}C]-4-hydroxymethylimidazole or its dideuterated counterpart, which were then converted to singly or doubly labeled DL-histidine (15,16).

Incorporation of both [γ-^{13}C]-histidine and [γ-^{13}C]-β,β-dideuteriohistidine into alkaline phosphatase was achieved using a histidine auxotroph of E. coli (17). Enzyme production was induced to coincide with the introduction of 10 mg/l of labeled DL-histidine into the growth medium, and the enzyme was purified as previously described (17).

RESULTS AND DISCUSSION

E. coli alkaline phosphatase (M.W. 86,000) was chosen for study in order to test the predicted utility of [γ-^{13}C]-histidines as structural probes in relatively large proteins. The enzyme is a zinc metalloprotein consisting of two identical subunits, each containing 8 or 9 histidine residues (18). Although none of these histidines has been shown to have a direct role in catalysis, there is indirect evidence for their participation as zinc ligands at the two active sites (19-21).

The ^{13}C nmr spectrum of the enzyme containing [γ-^{13}C]-β,β-dideuterio-histidine (Figure 1)** shows eight resonances corresponding to the eight or nine residues per subunit. The peaks numbered 1,2,5 and 8 clearly correspond to resonances from single histidine side chains. Peaks 6 and 7, which occur in a portion of the spectrum where there is a substantial background signal from ^{13}C nuclei present at natural abundance, also

*Replacement of the β-hydrogens by deuterium is expected to provide further enhancement of resolution (cf. references 7 and 8).

**The spectrum of the enzyme containing [γ-^{13}C]-histidine is very similar to Figure 1 except that several pairs of resonances are not as well resolved.

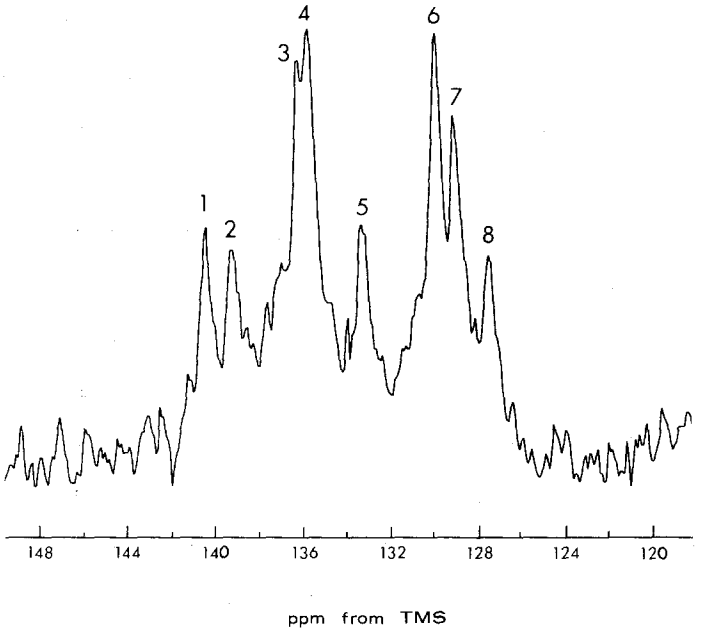

Figure 1. Fully proton-decoupled ^{13}C nmr spectrum of 1.6mM [γ-^{13}C]-β,β-dideuteriohistidine alkaline phosphatase in 0.1M Tris·HCl (D_2O), 0.1M NaCl, pH 7.8. The spectrum was recorded at 25.14 MHz using a 0.4 sec. acquisition time, a 1.1 sec pulse delay, 4.0 Hz digital broadening, and 24,000 acquisitions. The enzyme sample had been rendered phosphate-free by dialysis against EDTA followed by reconstitution by dialysis against Zn^{+2} and Mg^{+2}.

appear to be signals from individual histidines, but the possibility that signals from three residues occur in this region cannot be excluded at present. The barely resolved resonances 3 and 4 come from two or possibly three histidines.

The most notable feature of the spectrum, other than the large number of resolved resonances, is the large chemical shift range (about 14 ppm) exhibited by the individual histidine residues in the native enzyme. This is about double the range reported for the signals assigned to histidine C_γ carbons in natural abundance spectra obtained for a number of smaller diamagnetic proteins (1). The likelihood that several

histidines are liganded directly to Zn^{+2} could partially explain the large chemical shift range. However, resonances are found both upfield and downfield from those previously reported.

The observed narrow linewidths and sensitivity to environment of histidine C_γ resonances in alkaline phosphatase and also in E. coli aspartate carbamylase (22) suggest that ^{13}C enrichment at the histidine quaternary position will prove to be valuable in the study not only of the gross conformations of enzymes but also of local effects. For example, if, as suspected, there are several histidine residues at the active site of alkaline phosphatase, ^{13}C spectra of the labeled enzyme may well reflect local perturbations and thus help to resolve such questions as stoichiometry of metal binding, details of phosphate binding, pH effects, and the nature of subunit interactions. These and related topics are currently under investigation.

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