Phosphorus-31 Nuclear Magnetic Resonance Study of the Active Site Phosphohistidine and Regulatory Phosphoserine Residues of Rat Liver ATP-Citrate Lyase†

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ABSTRACT: ^{31}P NMR has been used to investigate the nature of the two chemically distinct phosphorylation sites of ATP-citrate lyase from rat liver. The "regulatory" or "structural" phosphorylation site is acid stable and known to be phosphoserine. The "catalytic" site is very acid labile and has been suggested by different workers to contain either phosphohistidine or an acyl phosphate group. We have demonstrated the presence of both endogenous phosphoserine and phosphoserine introduced after treatment of the lyase with the catalytic subunit of cAMP-dependent protein kinase. This structural phosphate group could be titrated and was readily removed by alkaline phosphatase; these facts, together with the narrow line width of the ^{31}P NMR signal, suggest that it is relatively mobile and located near the surface of the protein. ^{31}P NMR spectra of ATP-citrate lyase that had previously been exposed to fairly high concentrations of potassium chloride (1.5 M), or that had been denatured in detergent and 2-mercaptoethanol, clearly identified phosphohistidine as the catalytic phosphate group. That phosphohistidine is indeed a catalytic intermediate was demonstrated by the disappearance of the resonance in the presence of the substrates citrate and coenzyme A. The line width of the phosphohistidine resonance indicated that the catalytic phosphohistidine residue has negligible residual mobility on the protein. These results are consistent with the pattern of earlier observations on the chemical environments of phospho groups that serve a regulatory or structural role as opposed to a catalytic function in proteins.

The enzyme ATP-citrate lyase catalyzes the formation of acetyl coenzyme A (acetyl-CoA) from citrate in the cytoplasm to be used for the synthesis of fatty acids and cholesterol:

$$\text{citrate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + P_i$$

The enzyme is a tetramer (molecular weight 440 000) of four apparently identical subunits (Singh et al., 1976). Catalysis by this enzyme involves the participation of a phosphoenzyme intermediate (Inoue et al., 1966a), resulting from phosphorylation of the enzyme at the "catalytic" site by the substrate MgATP in the first step of the overall reaction [for a review, see Spector (1972)]. This acid-labile catalytic site is phosphorylated to a maximum of two phosphate groups per enzyme tetramer and has been shown to contain phosphohistidine (Cottam & Srere, 1969). However, this identification has been considered inconsistent with the lability of this residue to neutral hydroxylamine, a characteristic typical of acyl phosphates; treatment with hydroxylamine followed by Lossen rearrangement of the hydroxamate gave the product expected for γ-glutamyl phosphate (Inoue et al., 1966b). We have also encountered instability of this residue to hydroxylamine, an observation that prompted us to investigate the precise nature of this phosphorylation site.

In addition, the lyase can be phosphorylated at a chemically distinct "structural" or "regulatory" site, to a limit of two phosphoserines per enzyme tetramer. Phosphorylation at this site has been observed in vitro in response to both cAMP-dependent (Guy et al., 1980; Pierce et al., 1981) and -independent protein kinases (Ramakrishna & Benjamin, 1981), in vivo (Linn & Srere, 1979), and in response to insulin (Alexander et al., 1979; Pierce et al., 1982) and glucagon (Pierce et al., 1981). However, this structural phosphorylation does not lead to any detectable change in the kinetic properties of the enzyme (Ranganathan et al., 1980; Avruch et al., 1981), and its physiological function remains unknown.

The occurrence of two such chemically and functionally distinct phosphorylation sites thus makes ATP-citrate lyase an ideal candidate for analysis using phosphorus-31 nuclear magnetic resonance (^{31}P NMR).[1] This technique has recently proven extremely useful in determining the nature of phosphate covalently attached to proteins [for a recent review, see Matheis & Whitaker (1984)] and in studying the functional role of such phosphate groups in proteins (Sykes, 1983; Vogel & Bridger, 1983a; Brauer & Sykes, 1984).

In this paper, we report on our use of ^{31}P NMR to study phosphorylation at the structural site of ATP-citrate lyase and to confirm the identity of the catalytic phosphorylated residue as phosphohistidine. The NMR signals of the two types of phosphorylated residues have vastly different line widths, which we believe reflect their strongly contrasting environments within the enzyme.

Materials and Methods

Chemicals. [γ-^{32}P]ATP was purchased from New England Nuclear. *Escherichia coli* alkaline phosphatase, type III, was

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[1] Abbreviations: NMR, nuclear magnetic resonance; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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from Sigma. Affi-Gel Blue (100–200 mesh) was obtained from Bio-Rad. All other chemicals were of the highest purity available.

Purification of Enzymes. ATP-citrate lyase was purified to homogeneity from the livers of male Sprague-Dawley rats that had been starved for 2 days and then refed for 3 days on a high carbohydrate diet immediately prior to sacrifice (Osterlund & Bridger, 1977). The purification procedure was essentially that described earlier (Vogel & Bridger, 1981), modified in later cases to replace the final gel filtration step by affinity chromatography. Here, the enzyme was dialyzed against 20 mM potassium phosphate, 1 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM DTT, pH 7.5, containing 0.2 M KCl. The enzyme was then applied to a column (1 × 40 cm) of Affi-Gel Blue, and the column was washed with 250 mL of buffer containing 0.2 M KCl. High-activity ATP-citrate lyase was eluted with a linear gradient (500 mL) of 0.2–2 M KCl, both in the same phosphate buffer. This KCl-treated lyase had a specific activity of 8.7 units/mg or greater and ran as one band on polyacrylamide gel electrophoresis in the presence or absence of SDS. Lyase activity was measured by the coupled assay (Srere, 1959) as described earlier (Osterlund & Bridger, 1977).

The catalytic subunit of cAMP-dependent protein kinase was purified from rabbit thigh muscle according to method II of Bechtel et al. (1977) with an additional hydroxylapatite step. Protein kinase activity was detected by the filter paper assay as described by Bechtel et al. (1977). One unit was defined as that catalyzing the transfer of 1 μmol of phoshate from [γ-^{32}P]ATP to histone per minute at 30 °C.

Phosphorylation of ATP-Citrate Lyase. Lyase containing ^{32}P at the catalytic site only was prepared by incubation of the purified enzyme (final concentration 1–2 mg/mL) in 50 mM Tris-HCl, pH 8.4, with 3 mM $MgCl_2$ 10 mM DTT, and 0.5 mM [γ-^{32}P]ATP (sp act. 50–100 dpm/pmol) for 10 min at 37 °C. The reaction was stopped by the addition of EDTA to 20 mM. ^{32}P-labeled lyase was isolated by gel filtration on Sephadex G-100, at 4 °C. ^{32}P incorporation was measured by Cerenkov radiation.

ATP-citrate lyase was phosphorylated at its structural sites by incubation of lyase (1–2 mg/mL) in 50 mM Tris-HCl, pH 7.5, with 3 mM $MgCl_2$, 10 mM DTT, and 0.5 mM [γ-^{32}P]-ATP together with 5 units of the catalytic subunit of cAMP-dependent protein kinase for 90 min at 30 °C. The reaction was stopped and ^{32}P-labeled lyase recovered as described above.

ATP-citrate lyase was analyzed by SDS–polyacrylamide gel electrophoresis with the method of Weber & Osborn (1969). Enzyme containing ^{32}P at the catalytic site could be detected by covering unstained gel directly with parafilm and placing it at −20 °C for autoradiography. Incorporation of acid-stable structural phosphate was determined by autoradiography after staining of the gel.

Dephosphorylation of ATP-Citrate Lyase. The enzyme containing structural phosphates (either endogenous or those added in vitro by protein kinase) was incubated in 50 mM Tris-HCl and 1 mM DTT, pH 8.4, with 0.75 unit of *Escherichia coli* alkaline phosphatase for 6 h at 30 °C. Dephosphorylated lyase was recovered by gel filtration on Sephadex G-100.

^{31}P NMR Spectroscopy. Phosphorylation with unlabeled ATP was as described for the preparation of ^{32}P-labeled enzyme, except that the protein concentration was 12 mg/mL. The degree of phosphorylation was determined by parallel treatment of a sample labeled with [γ-^{32}P]ATP under otherwise identical conditions, and the incorporated ^{32}P was measured as described above. The phosphorylated enzyme was concentrated with a Millipore CX-30,000 immersible membrane filtration system with continuous vibrating agitation. SDS and 2-mercaptoethanol, where required, were added to 1% (w/v) and 1% (v/v), respectively, and the mixtures were incubated for 2 h at 30 °C. All samples were clarified by centrifugation prior to NMR analysis, D_2O was added to 14% (v/v), and the pH (meter) was checked and adjusted where necessary. Titration of the protein was carried out in 50 mM Tris-HCl, 20 mM HEPES, and 10 mM KCl; the pH was adjusted with concentrated solutions of Tris, HEPES, or MES.

Model compounds were obtained from Sigma except for 3-phosphohistidine, which was synthesized from calcium imidazole diphosphate and histidine by the method of Rosenberg (1964). Standards were dissolved in 50 mM Tris-HCl, 1 mM DTT, and 15% (v/v) D_2O.

^{31}P NMR of protein samples was carried out on a Nicolet NT300WB spectrometer at 121.5 MHz. Samples were of 3.5–4 mL in 12 mm o.d. flat-bottom NMR tubes. Pulses of 70° were used, and the delay time between free induction decays was 1 s. The spectral width was ±4000 Hz. Protein spectra were not proton decoupled and were recorded with a line broadening of 20 Hz. Chemical shifts are referenced to 85% H_3PO_4.

Results and Discussion

^{32}P-Labeled ATP-Citrate Lyase. Phosphorylation of ATP-citrate lyase at its catalytic site resulted in the incorporation of no more than two phosphates per tetramer, indicating half of the sites reactivity. This class of phosphoryl group was acid labile and was removable by incubation with 0.05 M hydroxylamine, pH 8.0, for 20 min at 30 °C as observed earlier by Inoue et al. (1968). Incorporation of total phoshate (i.e., structural plus catalytic) could reach a maximum of about three per tetramer with previously dephosphorylated enzyme but only two with our preparations of native enzyme; the precise degree of phosphorylation varied from batch to batch of prepared enzyme.

^{31}P NMR of Model Compounds. The chemical shifts of various model compounds have been reported by Vogel & Bridger (1983b). These values were found to be unaffected by the presence of 1% SDS and 1% 2-mercaptoethanol.

^{31}P NMR of the Structural Phosphate Groups of ATP-Citrate Lyase. Native lyase that had been phosphorylated at its catalytic site only gave a single peak with a chemical shift of 4.9 ppm at pH 7.4 (Figure 1A). This value corresponds well with the chemical shift expected for phosphoserine [for a review of the chemical shifts of other phosphoproteins see Matheis & Whitaker (1984)] and represents endogenous structural phosphoserine. The size of this peak was greatly increased when the lyase was pretreated in vitro with the catalytic subunit of cAMP-dependent protein kinase (Figure 1B). This phosphoserine residue was readily hydrolyzed by treatment of the enzyme with *E. coli* alkaline phosphatase (Figure 2). Titration of the endogenous phosphoserine residue was only possible over the narrow range of pH 8.4–6.2 due to problems with protein precipitation at lower pH values. However, as shown in Figure 3, over this pH range it follows very closely the titration curve obtained for SerP-68 of ovalbumin (Vogel & Bridger, 1982a). This titratable behavior suggests that the residue is exposed to the solvent, which together with its narrow line width (20 ± 5 Hz) and ready removal by alkaline phoshatase is consistent with the phosphoserine residue being reltively mobile and located on the surface of the enzyme.

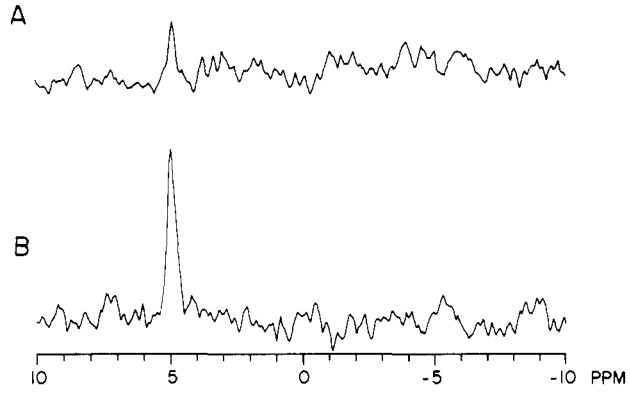

FIGURE 1: ^{31}P NMR spectra of the structural phosphate groups of ATP-citrate lyase: (A) endogenous phosphate in enzyme as isolated, 16 mg/mL, in 50 mM Tris-HCl, 1 mM DTT, and 14% D_2O, pH 7.4 (45 000 scans); (B) enzyme after phosphorylation with the catalytic subunit of cAMP-dependent protein kinase, 19 mg/ml, in 50 mM Tris, 1 mM DTT, and 14% D_2O, pH 7.4 (7000 scans). Enzymes were purified without KCl treatment. For other conditions, see text.

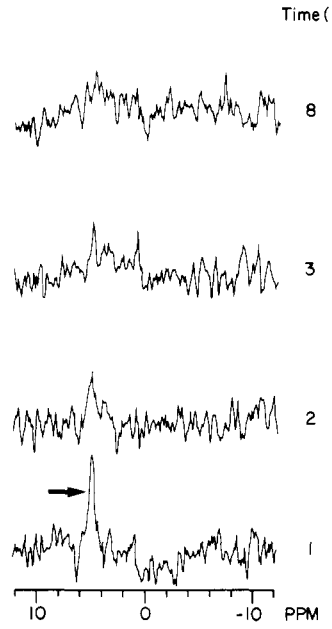

FIGURE 2: ^{31}P NMR spectra showing dephosphorylation of ATP-citrate lyase by *E. coli* alkaline phosphatase. Lyase (19 mg/mL) was in 50 mM Tris-HCl, 1 mM DTT, and 14% D_2O, pH 7.4, plus 0.6 unit of *E. coli* alkaline phosphatase. Incubations were at 22 °C in an NMR sample tube, 3000 spectra being acquired per 1-h incubation. Other conditions are as described in the text. Endogenous phoshoserine is indicated by the arrow; inorganic phosphate released during incubation was visualized after addition of EDTA (data not shown).

ATP-citrate lyase can also be phosphorylated in a cAMP-independent manner by ATP-citrate lyase kinase (Ramakrishna & Benjamin, 1981). Phosphorylation has been shown to produce both phosphoserine and phosphothreonine in ATP-citrate lyase from adipose tissue (Ramakrishna et al., 1981). In neither native nor SDS-denatured ATP-citrate lyase from rat liver did we observe a ^{31}P NMR signal that could be attributed to endogenous phosphothreonine, for which a chemical shift of approximately 4 ppm might be expected (Vogel & Bridger, 1983b).

^{31}P NMR of Catalytic Phosphate of ATP-Citrate Lyase. During early experiments using lyase that had not been subjected to high concentrations of KCl during the purification procedure, we were unable to detect the catalytic phosphate group using ^{31}P NMR. However, when this protein was denatured in SDS, the catalytic group was clearly visible (Figure

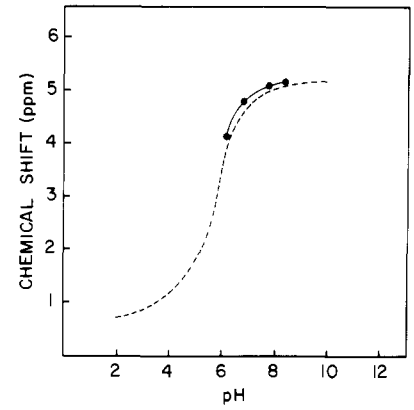

FIGURE 3: Partial ^{31}P NMR titration of endogenous structural phosphate of ATP-citrate lyase (●). For comparison, the titration of the SerP-68 of ovalbumin is shown by the dashed line [from Vogel & Bridger (1982a), reproduced with permission]. Enzyme was isolated by a KCl purification step; other conditions were as described in text.

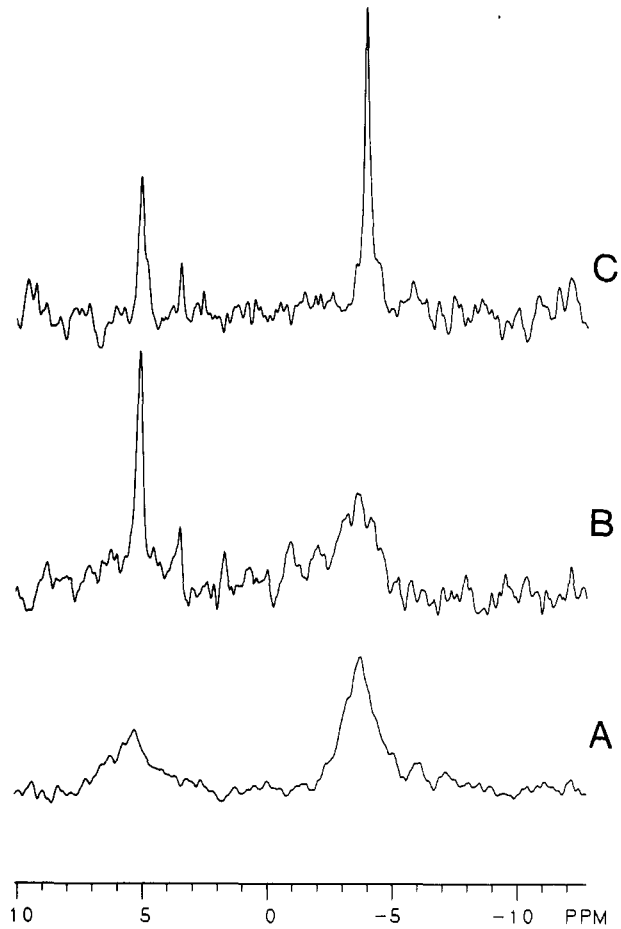

FIGURE 4: ^{31}P NMR spectra of ATP-citrate lyase: (A) non KCl treated enzyme, 32 mg/mL, in 50 mM Tris-HCl, 1% SDS, 1% 2-mercaptoethanol, and 14% D_2O, pH 8.1 (50 000 scans); (B) KCl-treated enzyme, 30 mg/mL, in 50 mM Tris-HCl, 50 mM KCl, 10 mM DTT, and 14% D_2O, pH 8.4 (40 000 scans); (C) KCl-treated enzyme, 25 mg/mL in 50 mM Tris-HCl, 1% SDS, 1% 2-mercaptoethanol, and 14% D_2O, pH 8.4 (30 000 scans).

4A). The peak at 5.4 ppm corresponds to endogenous phoshoserine, while the resonance at −3.7 ppm was assigned to 3-phosphohistidine. The chemical shift of this phosphohistidine residue is unusual in that it appears downfield from values quoted for both free and enzyme-bound 3-phosphohistidine, which resonate at −4.7 ppm at pH 8.0 (Gassner et al., 1977) and −4.8 ppm at pH 7.2 (Vogel & Bridger, 1982b), respectively. In no case did we observe a signal indicative of

an acyl phosphate group for which a chemical shift of approximately 1.5 ppm would be expected (Vogel & Bridger, 1983b).

Although this confirms the presence of phosphohistidine as the catalytic phosphorylated residue, any demonstration and study of its involvement as a catalytic intermediate would be precluded by the strongly denaturing conditions used. Accordingly, we sought to find conditions where the enzyme retained its activity but where the phosphohistidine signal might still be detectable. It was therefore gratifying to discover that the enzyme that had been purified by affinity chromatography in the presence of relatively high concentration of KCl was fully active and yielded a distinct signal corresponding to phosphohistidine at −3.7 ppm (Figure 4B). Endogenous phosphoserine can be seen at 5.1 ppm, and a small amount of inorganic phosphate is detectable (3.7 ppm). The line widths of the two phosphoryl groups are vastly different, 16 and 200 Hz for phosphoserine and phosphohistidine, respectively, thus reflecting the different environments of these phosphoryl groups within the enzyme. Denaturation of KCl-treated enzyme in SDS (Figure 4C) resulted in a marked reduction in the line widths (12 Hz) of both phosphoryl groups, to values consistent with their being relatively mobile residues. If the tertiary structure of the enzyme were responsible for the downfield shift of phospho groups with respect to standard compounds, one would expect denaturation to diminish such interactions. However, the chemical shifts of these resonances were unaffected by SDS. Similar behavior has been observed for other phospho proteins, such as the chemical shift and titration behavior of the phosphoserine residues of ovalbumin that are almost unaffected by denaturation in 8 M urea and 25 mM DTT (Vogel & Bridger, 1982a).

In order to confirm that the observed phosphohistidine resonance was in fact that of the catalytic phosphorylated intermediate, it was necessary to determine if it reacted with the other enzyme substrates. As shown in Figure 5, addition of citrate plus CoA resulted in the disappearance of the phosphohistidine signal, with a marked release of the P_i resonance. The broad resonance at −10 ppm is due to CoA and suggests binding to the enzyme (CoA also contains a resonance at −4.7 ppm; this signal is masked by the phosphoserine resonance).

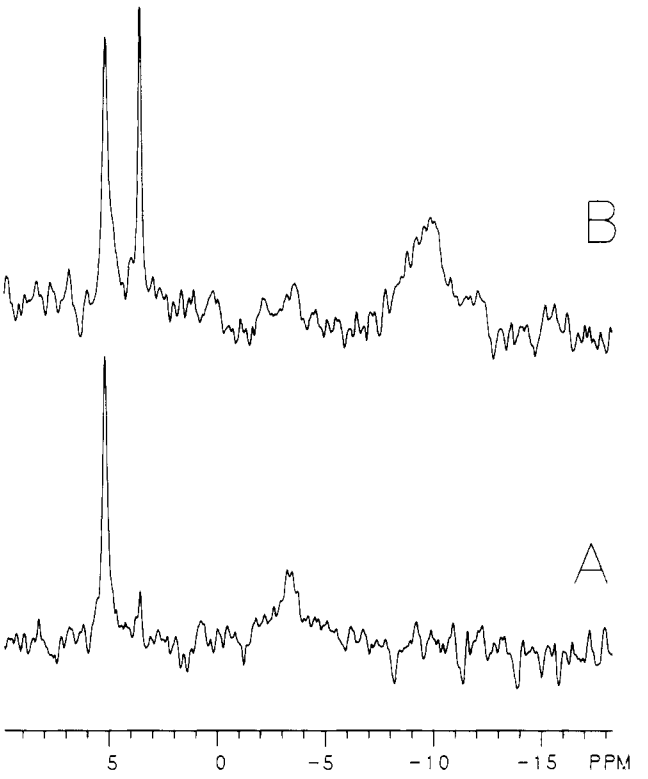

FIGURE 5: ^{31}P NMR spectra showing reaction of the catalytic phosphate group of KCl-treated ATP-citrate lyase with substrates citrate and CoA: (A) 30 mg/mL in 50 mM Tris-HCl, 50 mM KCl, 10 mM DTT, and 14% D_2O, pH 8.4 (40 000 scans); (B) plus 5 mM potassium citrate and 0.2 mM CoA (40 000 scans).

Conclusions

The experiments reported herein demonstrate the utility of ^{31}P NMR to identify and determine the local environment of phosphorylated residues on protein molecules, even those as large as ATP-citrate lyase. This enzyme, with its two classes of phosphorylated groups, provides further confirmation for suggestions (Vogel & Bridger, 1982a,b; Vogel et al., 1982; Vogel, 1984) that the structural or regulatory phosphate groups would generally be found on the surface of enzymes in environments where their mobility would be unrestricted, while phosphate groups that served as catalytic intermediates would be sequestered in buried environments within the active site, with essentially no residual mobility and with greatly reduced access to solvent. Moreover, these results confirm the site of phosphorylation at the active center as a histidine residue, inconsistent with previous suggestions (Inoue et al., 1966b) of the presence of acyl phosphate. We believe, however, that the unusual environment of this residue, which gives broad ^{31}P NMR signals under most conditions, could account for the uncharacteristic chemical reactivity of this phosphorylated amino acid side chain.

Using the calculations and theory outlined by Vogel et al. (1982), developed for the enzyme-bound and relatively immobile 3-phosphohistidine residue of *E. coli* succinyl-CoA synthetase, we have estimated the line width expected for 3-phosphohistidine bound to an enzyme of molecular weight 440 000. Since the correlation time, τ_c, is proportional to molecular weight, for ATP-citrate lyase τ_c would be approximately 132 ns. With this value and an anisotropy term, $\Delta\sigma(1 + \eta^2/3)^{1/2}$, of 236 ppm, as determined for succinyl-CoA synthetase, 3-phosphohistine bound to ATP-citrate lyase would be expected to have a line width of approximately 115 Hz. For KCl-treated lyase, we observed line widths in the region of 150–200 Hz. The phosphohistidine signal of non KCl treated enzyme in the absence of SDS appears to have been excessively broad and thus not discernible above the base line. A possible explanation for this would be that, at the high concentrtions (>30 mg/mL) used for these experiments, the lyase had undergone some degree of aggregation. Also, our studies have indicated the presence of two conformations of the phosphohistidine resonance (unpublished observation); thus exchange broadening could also contribute to increased line-width values.

ATP-citrate lyase catalyzes a trisubstrate reaction and is believed to proceed in a three-step mechanism via an enzyme–phosphate intermediate, shown here to be phosphohistidine. The second step in the reaction mechanism has been suggested to involve the formation of citryl phoshate (Walsh & Spector, 1969). Thus, this mechanism may be similar to that proposed for *E. coli* succinyl-CoA synthetase, which is also believed to involve two covalent intermediates, namely, a 3-phosphohistidine residue and enzyme-bound succinyl phosphate (Bridger, 1974; Vogel & Bridger, 1982b). Using fully active KCl-treated ATP-citrate lyase, we are now in a position to use ^{31}P NMR to study its mechanism, with approaches similar to those previously exploited for *E. coli* succinyl-CoA synthetase.

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