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# Evidence from $^{13}C$ NMR for Polarization of the Carbonyl of Oxaloacetate in the Active Site of Citrate Synthase†

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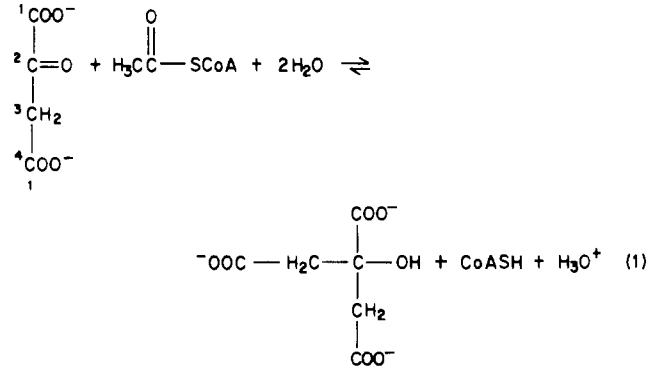
ABSTRACT: The carbon-13 NMR spectrum of oxaloacetate bound in the active site of citrate synthase has been obtained at 90.56 MHz. In the binary complex with enzyme, the positions of the resonances of oxaloacetate are shifted relative to those of the free ligand as follows: C-1 (carboxylate), −2.5 ppm; C-2 (carbonyl), +4.3 ppm; C-3 (methylene), −0.6 ppm; C-4 (carboxylate), +1.3 ppm. The change observed in the carbonyl chemical shift is successively increased in ternary complexes with the product [coenzyme A (CoA)], a substrate analogue (*S*-acetonyl-CoA), and an acetyl-CoA enolate analogue (carboxymethyl-CoA), reaching a value of +6.8 ppm from the free carbonyl resonance. Binary complexes are in intermediate to fast exchange on the NMR time scale with free oxaloacetate; ternary complexes are in slow exchange. Line widths of the methylene resonance in the ternary complexes suggest complete immobilization of oxaloacetate in the active site. Analysis of line widths in the binary complex suggests the existence of a dynamic equilibrium between two or more forms of bound oxaloacetate, primarily involving C-4. The changes in chemical shifts of the carbonyl carbon indicate strong polarization of the carbonyl bond or protonation of the carbonyl oxygen. Some of this carbonyl polarization occurs even in the binary complex. Development of positive charge on the carbonyl carbon enhances reactivity toward condensation with the carbanion/enolate of acetyl-CoA in the mechanism which has been postulated for this enzyme. The very large change in the chemical shift of the reacting carbonyl in the presence of an analogue of the enolate of acetyl-CoA supports this interpretation.

Citrate synthase (EC 4.1.3.7) catalyzes the condensation of oxaloacetate (OAA) with acetyl coenzyme A (acetyl-CoA) to form citrate (eq 1). The enzyme has been the subject of intense scrutiny for many years. Sufficient structural and kinetic data are available to suggest what catalytic strategies are used by this enzyme. The chemical mechanism is thought to involve generation of the carbanion (enolate) of acetyl-CoA which condenses with the carbonyl of OAA to form *S*-citryl-CoA as an intermediate (Eggerer, 1965; Weidman & Drysdale, 1979; Eggerer & Remberger, 1963; Bayer et al., 1981). It has been proposed that the carbonyl of OAA could




(1)

interact with an electrophilic residue, resulting in polarization of the C=O bond with substantial positive charge development at the carbonyl carbon (Srere, 1966). Polarization of the

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carbonyl would enhance its reactivity toward carbanion addition. Evidence for such polarization, however, has previously been limited to the observation from crystallographic studies (Wiegand et al., 1984; Remington et al., 1982) that the environment of the carbonyl is extremely polar with several potentially or obligatorily positively charged residues (Arg-329, His-320, and His-238) in close proximity to the carbonyl oxygen. His-320 is in a position to donate a proton. We have sought evidence for carbonyl polarization from $^{13}C$ NMR and have attempted to estimate its importance as a catalytic strategy for this enzyme.

## Materials and Methods

Crystalline citrate synthase was a product of Sigma Chemical Co., St. Louis, MO. Enzyme crystals were dissolved in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.50, and dialyzed extensively to remove $(NH_4)_2SO_4$. Enzyme solutions were concentrated to ~100 mg/mL by using a CX-30 immersible ultrafiltration concentrator (Millipore Corp.).

The concentrations of OAA, acetyl-CoA, and citrate synthase specific activity were all determined by using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method (Srere, 1969).

*S*-Acetonyl-CoA was prepared according to Rubenstein & Dryer (1980) and carboxymethyl-CoA according to Bayer et al. (1981). CoA and acetyl-CoA were obtained from Sigma Chemical Co.

*Specifically Labeled [$^{13}C$]Oxaloacetates.* Labeled (90% $^{13}C$) aspartates were the products of KOR Isotopes (2-$^{13}C$ label) and Merck Isotopes (1,3- and 4-$^{13}C$ labels). Aspartate aminotransferase (EC 2.6.1.1) (0.1 mg) was used to catalyze the transamination between the labeled aspartate (0.1 M) and $\alpha$-ketoglutarate (0.1 M) to yield glutamate and OAA (0.4 mL, 50 mM phosphate buffer, pH 7.5). After equilibration, the pH of the reaction mixture was rapidly lowered to pH 1 with concentrated HCl (~6 $\mu$L). The solution was then passed over a column (0.9 × 4 cm) of the free-acid form of AG-50W-X8 (Bio-Rad) and washed through with 0.1 M HCl. The amino acids were retained on the resin. The fractions containing OAA (contaminated with $\alpha$-ketoglutarate) were frozen and stored at −20 °C. While OAA is most stable in the free-acid form, about 10% loss occurred upon storage of frozen solutions for 24 h. Oxaloacetate readily decarboxylates to yield pyruvate and bicarbonate (particularly if metal cations are present). At the beginning of the NMR experiment, ethylenediaminetetraacetic acid (EDTA) was added to give a final concentration of $10^{-4}$ M in the NMR sample, and the OAA samples were rapidly neutralized to pH 7.50 by using 1 M Tris base and reassayed with citrate synthase (Srere, 1969).

Under our conditions, free OAA has a half-life of a few hours. Fortunately, the stability of OAA is greatly increased when bound to citrate synthase. When added to the enzyme in substoichiometric amounts, less than 10% decomposition occurs in 24 h at 15 °C. Oxaloacetate was assayed before and after each NMR experiment.

Small-molecule contaminants arising from the decomposition of OAA during preparation were removed as follows. OAA was added to an enzyme solution at a concentration ~10% over the active site concentration to form the binary complex. The sample, ~1 mL, was then applied to the top of a 2.8-mL column of preswollen G-25 and centrifuged briefly to recover the protein. As a result of the very tight binding of OAA to the enzyme, OAA eluted with the protein while the contaminants were left behind in the gel. Approximately 80% of the OAA was recovered with the enzyme free of decomposition products and other contaminants (see Results).

*Nuclear Magnetic Resonance Spectroscopy.* Carbon-13 spectra were obtained at 90.56 MHz by using a Bruker WH-360 spectrometer equipped with a 10-mm multinuclear probe. Proton-decoupled spectra were obtained by using broad-band noise decoupling centered at the frequency of water protons. With a decoupler power of 2.1 W, and the variable temperature controller set at 3 °C, the sample temperature was maintained at 15 °C. A sweep width of 26 000 Hz was used, and 32K data points were collected. The pulse width and recycle time were varied in an attempt to optimize parameters for each experiment (Becker et al., 1979). The final sample composition included 20% $D_2O$ (for internal lock), ~0.1 M acetonitrile (as internal chemical shift reference), $10^{-4}$ M EDTA, and a final enzyme concentration of 48–96 mg/mL (1–2 mM active sites). A typical spectrum of bound $^{13}C$-labeled OAA required 16 h of data collection. Chemical shifts were internally referenced to the acetonitrile cyano resonance which was given a value of 118.90 ppm relative to tetramethylsilane ($Me_4Si$). The IUPAC-recommended convention was followed (IUPAC, 1976).

A spin–echo experiment using the Carr–Purcell–Meiboom–Gill (Meiboom & Gill, 1958) sequence was performed for the [4-$^{13}C$]OAA binary complex. Even echoes were collected at 2, 8, 16, and 30 ms after the initial $\pi/2$ pulse.

## Results

*Chemical Shifts of Oxaloacetate Bound to Citrate Synthase.* Oxaloacetate binds unusually tightly to the enzyme in the binary complex ($0.6 < K_{diss} < 5$ $\mu$M in 4 M urea; Srere, 1966; Johansson et al., 1973). In the presence of a slight excess of enzyme (at an active site concentration of 1 mM), stable binary complexes can be studied by NMR techniques without possible ambiguities arising from the presence of unbound ligand. Since the OAA binding affinity is increased in ternary complexes (Johansson & Pettersson, 1974; Weidman et al., 1973; Bayer et al., 1981), this holds for them as well. The chemical shift changes, from those of free OAA, for each specifically labeled carbon of OAA bound to citrate synthase in binary and selected ternary complexes (obtained in the presence of saturating concentrations of the second ligand) are shown in Table I. The effect of binding on the chemical shift of the carbonyl carbon is unusually large. Spectra are shown in Figure 1 for [2-$^{13}C$]OAA free in solution (A) and bound to citrate synthase in the binary complex (B) and ternary complexes with acetonyl-CoA (C) and carboxymethyl-CoA (D). The bottom spectrum (E) shows the result of adding an excess of OAA (over the concentration of active sites) to the solution in experiment C. Neither the resonance positions nor line widths of free and bound OAA were affected by this addition. The resonance found at 204.7 ppm in the free OAA spectrum (A) and in the experiment with excess OAA (E) arises from [2-$^{13}C$]pyruvate, resulting from decomposition of free OAA. Note the absence of any pyruvate resonance in the spectra (B–D) in which the binary complex had been separated from unbound contaminants by the gel filtration procedure described under Materials and Methods.

*Line Widths ($T_2$) for Each Carbon of OAA.* In all cases, magnetic field inhomogeneity limited the resolution to ~2 Hz as monitored by the line width of the acetonitrile (internal reference) methyl resonance. All the resonances of OAA bound to enzyme exceeded this by a factor of 5–100. The line widths for bound resonances, obtained by Lorentzian line-shape analysis, are given in Table I. The precision of these measurements, determined by multiple experiments for some samples, was ±10–15%. The greater uncertainty in some values (indicated as approximate) results from an inadequate

Table I: Chemical Shift Changes[a] and Line Widths[b] of Oxaloacetate Bound to Citrate Synthase in Binary and Ternary Complexes

| | | | ternary complexes | | |
|---|---|---|---|---|---|
| | | binary complex | CoA | S-acetonyl-CoA | carboxymethyl-CoA |
| C-1 (carboxyl) | $\Delta\delta$ | −2.5 | −3.2 | −3.6 | −3.6 |
| | $\Delta\nu_{1/2}$ (Hz) | 27 | 24 | 26 | 24 |
| C-2 (carbonyl) | $\Delta\delta$ | +4.3 | +4.8 | +5.9 | +6.8 |
| | $\Delta\nu_{1/2}$ (Hz) | 57 | 50 | 43 | 39 |
| C-3 (methylene) | $\Delta\delta$ | −0.6 | | −0.6 | −0.3 |
| | $\Delta\nu_{1/2}$ (Hz) | ~170 | | ~150 | ~145 |
| C-4 (carboxyl) | $\Delta\delta$ | +1.2 | +1.6 | +0.7 | +3.8 |
| | $\Delta\nu_{1/2}$ (Hz) | 33 | | | 13 |

[a] The precision of chemical shift values is ±0.1 ppm. Chemical shifts for free OAA are the following: C-1, 168.1 ppm; C-2, 199.8 ppm; C-3, 49.1 ppm; C-4, 174.8 ppm. [b] Line widths were determined by Lorentzian line-shape analysis. Independent experiments for some samples indicate a precision of ±10–15% for these measurements except where the values are indicated as approximate.

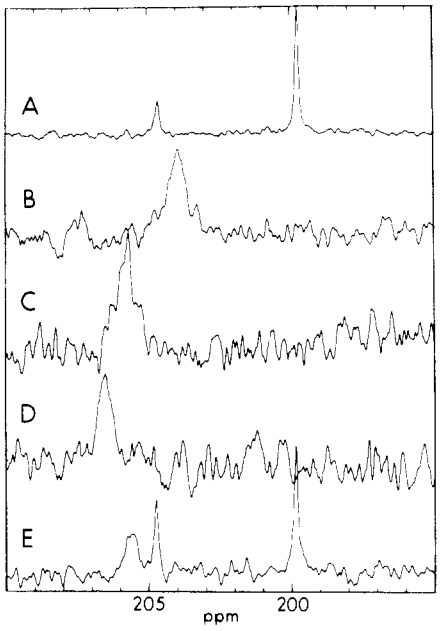


FIGURE 1: $^{13}C$ NMR spectra of [2-$^{13}C$]oxaloacetate [pulse width = 6.4 μs (21°), recycle time = 1 s, exponential filter = 10 Hz; all samples in 50 mM Tris buffer, pH 7.50, and 0.1 mM EDTA, 10 °C]. (A) 1.86 mM OAA (smaller resonance at 204.7 ppm is the decarboxylation product [2-$^{13}C$]pyruvate), 17 561 transients. (B) Binary complex with citrate synthase; 0.94 mM citrate synthase sites and 0.85 mM OAA, 58 614 transients. (C) Ternary complex with acetonyl-CoA; 0.83 mM citrate synthase sites, 0.71 mM OAA, and 0.88 mM acetonyl-CoA, 54 429 transients. (D) Ternary complex with carboxymethyl-CoA; 0.51 mM citrate synthase sites, 0.39 mM OAA, and 0.40 mM carboxymethyl-CoA, 82 431 transients. (E) Ternary complex with acetonyl-CoA and excess OAA; 0.68 mM citrate synthase sites, 0.72 mM acetonyl-CoA, 1.48 mM OAA (before experiment), and 1.23 mM OAA (after experiment), 32 662 transients.

signal/noise ratio when the bound resonance is very broad (especially C-3).

The line widths at half-height [$\Delta\nu_{1/2} = (\pi T_2^*)^{-1}$ where $T_2^*$ is the apparent first-order time constant for decay of $xy$ magnetization] may reflect the true transverse $T_2$ relaxation time for each carbon in the absence of chemical shift dispersion. This was tested by a spin–echo experiment for the C-4 binary complex. The line width for this carbon in the binary complex is 33 Hz ($T_2^* \sim 10$ ms) while in ternary complexes it is 13 Hz ($T_2^* \sim 20$ ms). With echo times of 2 and 8 ms (43 000 and 82 000 transients, respectively), the signal from this carbon was observed, although much diminished in intensity, 50–75%, in the 8-ms experiment. No C-4 resonance could be observed with echo times of 16 and 30 ms. For the 16-ms experiment, signals could still be detected from protein carbons expected to have relatively short correlation times (side-chain carboxylates, ~181 ppm, methylene carbons of lysine, ~39 ppm, etc.). Because of a limited signal/noise ratio, we could not have observed the signal after ~2 $T_2$; therefore, this result is compatible with a single resonance of line width 33 Hz where $T_2 = T_2^*$ and not with the presence of chemical shift dispersion of several resonances with line widths of 13 Hz or less.

For carbons 2 and 4 (for which reasonably accurate line widths could be obtained for both binary and ternary complexes), the line widths in the binary complex are substantially larger than those in ternary complexes (Table I). In contrast, the C-1 line width shows very little dependence upon the nature of the complex.

*Chemical Exchange between Free OAA and OAA Bound in Binary and Ternary Complexes.* We can make qualitative statements about the chemical exchange regime for each resonance. A quantitative determination of exchange rates by NMR would require measurements of line widths and chemical shifts at a series of known concentrations of excess ligand. Because of the instability of OAA, this was not possible. By the time a sufficient number of transients had been recorded for adequate signal/noise ratios, substantial amounts of the OAA in excess of the number of binding sites had decomposed. Furthermore, the resonance in the binary complex may be affected by additional chemical exchange processes between more than one form of bound OAA, vide infra.

In general, binary complexes were found to be in fast to intermediate exchange with free OAA. As more and more excess OAA was added to the solution (at the beginning of the experiment), the resonance was found to move toward that of the free OAA and to first broaden before narrowing again as more excess OAA was added. The frequency difference between the free OAA and that bound in binary complexes ranges from 54 Hz (C-3) to 390 Hz (C-2) at 90.56 MHz. The observation of fast to intermediate NMR exchange behavior for the binary complex is roughly compatible with the 330-$s^{-1}$ off-rate constant for OAA from the binary complex estimated from kinetic data (Johansson & Pettersson, 1974).

Ternary complexes with 1,2- and 4-$^{13}C$-labeled OAA were found to be in slow exchange as exemplified by the ternary complex spectrum with acetonyl-CoA in the presence of excess [2-$^{13}C$]OAA shown in scan E of Figure 1. In all cases, signals for both free and bound OAA could be observed simultaneously. The line widths for both resonances were independent of the OAA concentration. Line widths for OAA in these ternary complexes were found not to depend on the nature of the second ligand regardless of the effect of the second ligand on the OAA chemical shift.

The dissociation rate constant for OAA from ternary complexes is very small. For conditions in which populations in the two sites are equal (approximated in our experiments, the uncertainty arising from the instability of the free OAA), then

$$\pi\Delta\nu_{1/2,\text{obsd}} = 1/T_{2F} + k_{-1} \quad (2)$$

where $\Delta\nu_{1/2,\text{obsd}}$ is the line width of the free ligand observed in the presence of exchange, $1/T_{2F} = \pi\Delta\nu_{1/2}$ for the free ligand in the absence of exchange (enzyme), and $k_{-1}$ is the rate constant for dissociation of the ligand from the complex. Since we observe no effect at all on the free OAA line width resulting from the presence of the complex, then the second term on the right of eq 2 must be very much smaller than the first. Thus, $k_{-1}$ must be $<<2\pi$ Hz = 6.3 $s^{-1}$.

## Discussion

*Interpretation of the Chemical Shift Changes.* (*A*) *C-2* (*Carbonyl*). The large shift to higher frequency (4–7 ppm) induced by the enzyme is consistent with extensive polarization of the carbonyl of OAA. In strong acid solutions of simple aliphatic ketones, complete protonation of the carbonyl results in a shift to higher frequency of 20–30 ppm (Maciel & Ruben, 1963; Maciel & Natterstad, 1965; Tiffon & Dubois, 1978). Similarly, in solutions of strong Lewis acids such as $BF_3$, shifts of ~20 ppm are observed (Fratiello et al., 1976). These shifts in ketone resonances are understood to result from the change in $\pi$-bond polarity which occurs upon protonation or interaction with a strong Lewis acid. This interpretation is strengthened by the reasonably successful theoretical calculations of trends in carbonyl $^{13}C$ chemical shifts by de Jeu (1970) and Tiffon & Dubois (1978) which were made on this basis and by the linear correlation observed between the solvent effects on the IR carbonyl stretching frequency and on the $^{13}C$ chemical shift for several ketones (Ueji & Nakamura, 1976). Thus, there is strong support in model systems for interpretation of changes in carbonyl $^{13}C$ chemical shifts in terms of changes in C=O bond order (Tiffon & Dubois, 1978). The chemical shift change we observed would then correspond to a reduction of the C=O bond order from 2 to 1.7 (20–30% protonation of the carbonyl).

These results illustrate the special utility of spectroscopic probes in mechanistic studies. A change in bond order from 2 to 1.7 is easily detectable by NMR. However, it corresponds (using Pauling's rule) to an increased C=O bond length of only 0.05 Å, a change which is undetectable by present crystallographic techniques. Yet this change in bond order could mean a catalytic enhancement of $>10^{+9}$ (vide infra).

A persuasive argument in favor of our interpretation is that the chemical shift change already apparent in the binary complex is successively increased in ternary complexes as the second ligand better approximates the structure of the enolate of acetyl-CoA. Consistent with the expectation for an intermediate lying along the reaction pathway, polarization of the carbonyl (as monitored by its chemical shift change) is maximally increased in the ternary complex with the acetyl-CoA enolate analogue carboxymethyl-CoA (Bayer et al., 1981).

Other explanations of the chemical shift change can be excluded. We have tentatively assigned a chemical shift of 98.2 ppm to C-2 of the OAA enol. Since the observed shift changes in the opposite direction on binding to the enzyme, enolization of OAA in the active site is not occurring to any significant degree. This is not surprising since the keto form of OAA has been shown to be the true substrate (Gruber et al., 1956) and enolization would decrease the reactivity of the carbonyl toward addition of the carbanion of acetyl-CoA. The change in the C-2 chemical shift also cannot arise from ring current effects. On the basis of the crystal coordinates (Wiegand et al., 1984), the ring current shift generated at C-2 by aromatic residues is less than 1 ppm (Perkins & Dwek, 1980).

(*B*) *C-3* (*Methylene*). The very small change observed in the chemical shift of the methylene carbon is compatible with partial protonation of the carbonyl. Shifts of $\leq$1 ppm are observed in the $\alpha$ $sp^3$ carbon upon carbonyl protonation (de Jeu, 1970; McClelland & Reynolds, 1976) or coordination to a strong Lewis acid (Fratiello et al., 1976).

(*C*) *C-1* (*Carboxylate*). The substantial increased shielding of the $\alpha$-carboxylate may result from polarization of the adjacent carbonyl. Protonation of the carbonyl in $\alpha$–$\beta$-unsaturated ketones such as 3-ketobutene results in a increased shielding of the $\alpha$-methine carbon of ~+3 ppm while the $\beta$-carbon experiences significant deshielding (Olah et al., 1972). This has been explained to result from a redistribution of charge over the unsaturated carbon skeleton upon carbonyl protonation. In the more polarizable carboxylate, a redistribution of negative charge density away from the carboxylate oxygens closer to the carboxylate carbon as the adjacent C=O bond is made more polar (by protonation) seems entirely reasonable.

(*D*) *C-4* (*Carboxylate*). We are unsure of the proper interpretation of the chemical shift changes for this carbon which result from binding to the enzyme. The key may lie in the differences between the OAA structure and/or protein interactions present in the acetonyl-CoA and carboxymethyl-CoA complexes. The direction of the chemical shift change upon binding observed in the binary complex and further increased in the carboxymethyl-CoA ternary complex is partially reversed in the acetonyl-CoA complex. The 1.2 ppm shift change observed for this carboxylate (increasing to 3.8 ppm in the carboxymethyl-CoA ternary complex) is larger than would be expected to result from a local environmental effect (usually <1 ppm; Palmer et al., 1982) and larger than that experienced by the carboxylate-containing ligands bound to other proteins by apparently similar salt bridges (Palmer et al., 1982).

*Complexities in the Interpretation of $^{13}C$ Chemical Shift Changes.* To understand in detail all the chemical shift changes which we have observed is not possible at present. It seems most useful to us to rationalize the effects primarily by analogy with the well-known pronounced chemical shift changes which accompany polarization of carbonyl carbons. However, it is by no means certain, given the complex behavior of $^{13}C$ NMR shifts [see p 30ff of Jardetzky & Roberts (1981)] and of enzyme–ligand interactions, that this is the full explanation for the shifts we have observed even for the carbonyl carbon. In particular, OAA appears to be immobilized in a fixed conformation in the active site (vide infra), and we have no information from model systems to allow us to estimate the contribution of this fixed geometry to the chemical shift change.

*Immobilization of Bound OAA.* Since C-3 contains two covalently bonded protons, the dipolar contribution should dominate the relaxation for this carbon. The line width of the C-3 OAA (methylene) resonance in ternary complexes suggests a rotational correlation time appropriate for the enzyme molecule as a whole. A line width of 150–200 Hz reflecting dipolar relaxation from two protons at a distance of 1.07 Å under conditions of broad-band decoupling corresponds to a correlation time of $(5–7) \times 10^{-8}$ s (Dwek, 1973). For a globular protein the size of citrate synthase, the calculated rotational correlation time is $8.1 \times 10^{-8}$ s [see Lakowicz et al., (1983) and references cited therein; Singh et al., 1970; Srere, 1975]. Thus, if C-3 were experiencing no other relaxation processes, this result implies that the bound OAA is immobilized on the enzyme and is constrained to rotate with it.

Dipolar relaxation from the methylene protons is not sufficient to explain the line widths of the other carbons. Other mechanisms including dipolar relaxation from other protons and relaxation arising from chemical shift anisotropy may contribute to the line widths.

*Evidence for Chemical Exchange within the Binary Complex.* The line widths for some carbons in the binary complex are larger than those in ternary complexes. Explanations for

this observation include chemical shift dispersion, magnetic effects, chemical exchange with free OAA, and chemical exchange within the binary complex. We present arguments against the first three of these and suggest that the excess line width of binary complexes is the consequence of chemical exchange processes *within* that complex.

For chemical shift dispersion to contribute to the line width, a number of environments (in slow exchange) with slightly different chemical shifts is required. The results of the spin–echo experiment for the C-4 binary complex are entirely compatible with the observed line width being a true reflection of irreversible $T_2$ processes (such as chemical exchange). It seems unlikely that magnetic effects of the local protein environment (dipolar relaxation from protons of nearby active site residues) on the line width are greater in the binary complex than in ternary complexes since the strength of all other interactions of OAA and protein clearly increases upon formation of ternary complexes. It also does not seem likely that line-broadening effects arising from chemical shift anisotropy would be greater in binary than in ternary complexes since the rotational correlation time in the tighter ternary complexes (vide supra) is already close to that of the enzyme itself. The additional broadening found in binary complexes over that in ternary complexes cannot reflect a chemical exchange process with free ligand because the amount of free OAA (~0.5%) in these experiments is too low to affect the line width. We have confirmed this conclusion by simulating spectra for carbon 4 using the general line-shape equation for a two-site system (Gutowsky & Holm, 1956; Feeney et al., 1979).[1]

If the binary complex were to consist of several significantly populated forms with different OAA chemical shifts which interconvert with one another, then the "excess" line width in the binary complex can be explained by an exchange process between these forms. Since only a single OAA resonance is observed for the ternary complexes differing in chemical shift but having about the same line width, it seems reasonable that when the various ternary complexes are produced, a single state is stabilized to the exclusion of others.

With this interpretation, the different carbons of OAA show different sensitivities to the presumed exchange processes in the binary complex. The line width for C-1 is essentially constant for both binary and ternary complexes, while that for C-4 changes by over a factor of 2 between binary and ternary complexes. It is tempting to speculate that C-2 and C-4 protein interactions are involved in the exchange processes in the binary complex while C-1 is not. It is as if the C-4 end of the molecule experiences two or more different environments in the binary complex with a rate of exchange between them that is comparable to the chemical shift frequency differences. On the other hand, while the C-1 environment (as reflected by its chemical shift) differs in the various complexes, no exchange process between these environments can be observed in the binary complex.

There is crystallographic evidence (Remington et al., 1982; Wiegand et al., 1984) for two substantially different conformations corresponding to an open and a closed form of this enzyme. Our line-width data suggest the existence in solution of a conformational equilibrium between two forms which may correspond to open and closed forms of the binary complex.

[1] Using dissociation constants of 1–5 $\mu$M and dissociation rate constants from 15 to 1500 $s^{-1}$ and assuming a true binary complex line width of 30 Hz shifted 1.6 ppm from the free ligand which was assumed to have a line width of 2 Hz, we theoretically obtained only very small perturbations which would be experimentally undetectable on either the line width or the chemical shift of the bound resonance.

*Consequences of Carbonyl Polarization for Catalytic Enhancement.* The most convincing demonstrations of substrate destabilization by carbonyl polarization (protonation) are the FTIR studies of dihydroxyacetone phosphate bound to triosephosphate isomerase and glyceraldehyde 3-phosphate bound to aldolase (Belasco & Knowles, 1980, 1983). The effect of polarization on the C=O stretching frequency is interpreted by comparison to the IR frequency shift obtained upon protonation of acetone in concentrated sulfuric acid solutions (the same model we are using for interpreting $^{13}C$ NMR shifts) and indicates 20% and 25% protonation in the active sites of these two enzymes, respectively.

Roberts et al. (1976) examined the chemical shift change (0.8 ppm) of the amide carbonyl carbon in a transition-state analogue inhibitor which is induced when it binds to aspartate transcarbamylase. They interpreted this shift as evidence for protonation of the carbonyl oxygen and suggested that it implies about 20% protonation in the transition state for the reaction.

The shift change we have observed in the OAA carbonyl resonance is greater than that observed upon transfer of acetone from water to trifluoroacetic acid solution (Maciel & Ruben, 1963) and for OAA in the carboxymethyl-CoA ternary complex may correspond to 30% protonation. Simple aliphatic ketones have $pK_a$'s near −2.5 for their conjugate acids. If one assumes a near-linear correlation of chemical shift changes with carbonyl bond polarization (as has been established for carbonyl protonation; McClelland & Reynolds, 1976), a ~7 ppm change corresponds to a shift in an unfavorable equilibrium by a factor of $>10^9$ (at pH 7 only $10^{-9.5}$ of a base whose conjugate acid has a $pK_a$ equal to −2.5 is protonated).[2] Whether or not this calculation is quantitatively correct, our results clearly support substantial substrate destabilization by ketone polarization of OAA in the active site of citrate synthase.

It has been proposed (Jencks, 1975) that part of the free energy released upon substrate binding might provide a driving force that results in a local destabilization at the reacting site of the substrate and a consequent reduction in the height of the activation barrier. We have presented evidence here for an example of substrate destabilization: polarization of the reacting carbonyl of oxaloacetate which occurs upon binding to the active site of citrate synthase. Carbonyl polarization of OAA upon binding to citrate synthase presents a paradox. The strong interactions of the enzyme with OAA are indicated by the unusually tight binding ($K_{diss} < 1$ $\mu$M). Of the overall standard free energy change for the citrate synthase reaction at pH 7, −17 kcal $mol^{-1}$, the binding of OAA accounts for −10

[2] Consideration should be given to the question as to whether the $pK_a$ of the conjugate acid of a simple ketone (e.g., acetone) should be close to that of the protonated ketone dicarboxylate of OAA. Good models are difficult to find since such a species could exist in an appreciable concentration only in an enzyme active site. We have examined several models in an attempt to estimate the effects of the 1- and 4-carboxylates on the $pK_a$ of a ketone conjugate acid. Compared to a simple amine ($pK_a$ for *sec*-butylamine = 10.6), the amino of glycine has a $pK_a$ of 9.9 (as a model for the effect of the 1-carboxyl) while aspartate with both a 1- and a 4-carboxyl has a $pK_a$ of 10.00 for a total change of ~−0.6 $pK_a$ unit. On the other hand, compared to acetic acid ($pK_a$ = 4.76), oxalate (as a model for the effect of the 1-carboxyl) has a $pK_a$ (symmetry corrected) of 3.89 while malonic acid (as a model for the effect of the 4-carboxyl) has a $pK_a$ (symmetry corrected) of 5.39 for an overall effect of ~−0.2 $pK_a$ unit. The difference in these models may result from the effects of hydrogen bonding in malonic acid with smaller additional contributions from geometrical differences. Since neither model exactly corresponds to the protonated ketone in oxaloacetate, we see no reason to prefer one over the other. In either case, these differences are small compared to the effect of the enzyme.

kcal $mol^{-1}$. The presence of a carbonyl is absolutely required for tight binding; succinate and other dicarboxy acids without a carbonyl have weak or no affinity for the enzyme (Srere, 1966). From where does the free energy come to shift an unfavorable equilibrium (carbonyl polarization) by a factor of $10^9$? The *observed* affinity of the enzyme for OAA is already very high, and the *intrinsic* affinity (if carbonyl polarization did not occur) must be even greater. Therefore, there must be some highly exergonic process associated with OAA binding which does not take place upon binding other dicarboxylic acids.

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