Evidence from $^{13}$C NMR for Protonation of Carbamyl-P and N-(Phosphonacetyl)-L-aspartate in the Active Site of Aspartate Transcarbamylase*  

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Nuclear magnetic resonance has been used to study the binding of $[^{13}$C]carbamyl-P (90% enriched) to the catalytic subunit of Escherichia coli aspartate transcarbamylase. Upon forming a binary complex, there is a small change in the chemical shift of the carbonyl carbon resonance, 2 Hz upfield at pH 7.0, indicating that the environments of the carbonyl group in the active site and in water are similar. When succinate, an analog of L-aspartate, is added to form a ternary complex, there is a large downfield change in the chemical shift for carbamyl-P, consistent with interaction between the carbonyl group and a proton donor of the enzyme. The change might also be caused by a ring current from a nearby aromatic amino acid residue. From the pH dependence of this downfield change and from the effects of L-aspartate analogs other than succinate, the form of the enzyme involved is proposed to be an isomerized ternary complex, previously observed in temperature jump and proton NMR studies. The downfield change in chemical shift for carbamyl-P bound to the isomerized complex is 17.7 ± 1.0 Hz. Using this value, the relative ability of other four-carbon dicarboxylic acids to form isomerized ternary complexes with the enzyme and carbamyl-P has been evaluated quantitatively.

The $^{13}$C peak for the transition state analog N-(phosphonacetyl)-L-aspartate (PALA), 90% enriched specifically at the amide carbonyl group, is shifted 20 Hz downfield of the peak for free PALA upon binding to the catalytic subunit at pH 7.0. In contrast, the peak for $[^{1}$C]phosphonacetamide shifts upfield by about 6 Hz upon binding. Since PALA induces isomerization of the enzyme and phosphonacetamide does not, these data provide further evidence consistent with protonation of the carbonyl group only upon isomerization. The degrees of protonation in strong acids of the carbonyl groups of PALA, phosphonacetamide and urethan (a model for the labile carbamyl-P) have been determined, as have the chemical shifts for these compounds upon full protonation. From these data it is calculated that the amide carbonyl groups of carbamyl-P and PALA might be protonated to a maximum of about 20% in the isomerized complexes at pH 7.0. The change in conformation of the enzyme carbamyl-P complex upon binding L-aspartate, previously proposed to aid catalysis by compressing the two substrates together in the active site, may be accompanied by polarization of the C=O bond, making this ordinarily unreactive group a much better electrophile.

A keto analog of PALA, 4,5-dicarboxy-2-ketopentyl phosphonate, also binds tightly to the catalytic subunit and induces a very similar conformational change, whereas an alcohol analog, 4,5-dicarboxy-2-hydroxypentyl phosphonate, does not bind tightly, indicating the critical importance of an unhindered carbonyl group with trigonal geometry.

We have proposed previously that the carbonyl oxygen of carbamyl-P may interact with a general acid in the active site of aspartate transcarbamylase during catalysis, activating it for nucleophilic attack by the amino group of L-aspartate (1). The idea that the carbonyl oxygen rather than the amide nitrogen of carbamyl-P would be a likely site for protonation is supported by NMR studies of model compounds. Amides, ureas, and alkyl carbamates are protonated by strong acids solely on the carbonyl oxygen atoms regardless of the nature of their N- or O-substituents (2). When succinate, an analog of L-aspartate, binds to the catalytic subunit-carbamyl-P complex, a large conformational change is detected by several methods and it has been proposed that this change aids
catalysis by compressing the amino group of aspartate and the carbonyl group of carbamyl-P together in the active site (1, 3). Studies by Beard and Schmidt (4) of the binding of succinate to the subunit-carbamyl-P complex by 1H NMR indicate that between pH 7 and pH 10, two protonated groups are involved in the conformational change. One, with pK about 8, participates directly in the binding of succinate and another, with pK about 7, is thought to be at a site distinct from the succinate binding site. The latter group may interact with the carbonyl group of carbamyl-P, and may be involved in the conformational change.

There are several recent reports in which 13C NMR of carbonyl carbon atoms has been used to provide information about enzyme mechanisms. These include the binding of bicarbonate to the proteins ribulose 1,5-diphosphate carboxylase (5), carbonic anhydrase (6), myoglobin (7), and transferrin (8). 13C NMR provides the possibility to examine a crucial detail of the catalytic mechanism of aspartate transcarbamylase, the postulated protonation of the carbonyl group of carbamyl-P, using this substrate directly rather than an analog which might interact with the enzyme somewhat differently.

The 13C chemical shift of carbonyl carbon atoms is determined largely by the local paramagnetic term in the Karplus and Pople theory (9, 10). This theory has been used successfully to interpret 13C NMR studies with model compounds, and we use it now to interpret the effects observed with aspartate transcarbamylase. With model compounds, the dependence upon solvent indicates that 13C chemical shifts for carbonyl carbons are determined largely by the local paramagnetic term in the Karplus and Pople theory (11). 13C NMR studies with model compounds and with aspartate transcarbamylase, using this substrate directly rather than an analog which might interact with the enzyme somewhat differently.

A large downfield change in the chemical shift of [13C]carbamyl P is observed in the isomerized ternary complex of aspartate transcarbamylase with carbamyl-P and succinate, and also in the isomerized binary complex with [13C]PALA, a transition state analog. Because of the established relationship between protonation of model compounds in hydrogen bonding systems and the direction of change in chemical shift, we can interpret our results with the enzyme in terms of protonation of the carbonyl groups in the active site. Alternative interpretations are also considered.

**Materials and Methods**

[13C]Carbamyl-P-KN13CO was prepared by fusing [15C]urea, 90% enriched, (Merck, Sharp and Dohme of Canada, Ltd.), with KNO3. Enzyme was used to interpret the effects observed with aspartate transcarbamylase, the postulated protonation of the carbonyl group of carbamyl-P, using this substrate directly rather than an analog which might interact with the enzyme somewhat differently.

The abbreviations used are: PALA, N-(phosphonacetyl)-L-aspartate; DIKEP, 4,5-dicarboxy-2-ketopenylphosphonate; DIHOP, 4,5-dicarboxy-2-hydroxypropylphosphonate.

1. The chemical shifts of urethan, a model for carbamyl-P, are virtually identical in solvents which cannot donate protons and which differ markedly in polarity (dimethylformamide, dioxane, dimethylsulfoxide, benzene, ethyl acetate, tetrahydrofuran, acetanilide, pyridine). Furthermore, the shifts in all these solvents are upfield of the shift in water (12).

2. The abbreviations used are: PALA, N-(phosphonacetyl)-L-aspartate; DIKEP, 4,5-dicarboxy-2-ketopenylphosphonate; DIHOP, 4,5-dicarboxy-2-hydroxypropylphosphonate.
**Determination of Inhibition Constants**—Constants for the binding of dicarboxylic acids to free C₄ were determined at pH 7.0 and 28° by the method of Jacobson and Stark (27) using ¹³C aspartate. The concentrations in the assays were: catalytic subunit, 1 µg/ml; t-aspartate, 0.8 µM; imidazole acetate, 100 mM; 2-mercaptoethanol, 2 mM; EDTA, 0.2 mM. All inhibitors were 20 mM except succinate, which was 1.8 mM. Carbamyl-P concentrations were varied between 1 and 20 µM. Inhibition constants for DIHOP and DIKEP were determined as described by Davies et al. (28). The concentrations in the assays were: catalytic subunit, 1.8 µg/ml; t-aspartate, 40 mM; Tris acetate (pH 8.0), 0.2 M; 2-mercaptoethanol, 2 mM; EDTA, 0.2 mM; [¹³C]carbamyl-P, 2.5 mM. The concentrations of DIKEP and DIHOP were varied, up to 5 mM.

**NMR Spectroscopy**—Spectra with proton noise decoupling were obtained for [¹³C]carbamyl-P and [¹³C]PALA at 25.2 MHz using a Varian XL-100 spectrometer equipped with a Nicolet Technology Corp. data system and pulse unit. Spectra for [¹³C]phosphonacetamide were obtained at 20 MHz using a Varian CFT-20 spectrometer. To facilitate comparison of the phosphonacetamide data with the PALA data, phosphonacetamide shifts are reported as though they had been obtained at 25.2 MHz. Because of the long T₂ of the carbamyl-P (carbonyl) group (35 ± 10) a small flip angle (10–15°) was used for the radiofrequency pulse with a recycle time of 1.0 s. The free induction decay was multiplied by a negative exponential corresponding to 1 to 2 Hz line broadening prior to Fourier transformation. Difference spectra were calculated with Nicolet software. [¹³C]carbamyl-P (1.7 ppm) and [¹³C]PALA (14.4 ppm) were used as an internal standard. The chemical shift of its carbonyl group was found to be invariant as a function of pH in phosphate buffer by comparison with external benzene or dioxane. Samples in D₂O buffers were contained in 12-mm tubes. For experiments in H₂O, a 10-mm tube containing the sample was inserted into a 12-mm tube containing D₂O for the lock signal. An aliquot of 200 mM carbamyl-P was added to the enzyme solution and the resulting mixture was kept cold until placed in the spectrometer. Since the total time required for an experiment with carbamyl-P was 2 to 12 h, decomposition of carbamyl-P at the normal probe temperature of 37° would have been a major problem. The problem was circumvented by lowering the probe temperature to 23° and by using 40 mM phosphate buffer. Rosenbusch and Griffin (25) have found the half-life of carbamyl-P to be 20 h under these conditions.

Spectra for [¹³C]carbamyl-P were obtained at 40.5 MHz using a Varian XL-100 spectrometer. The sweep width was 1000 Hz, the flip angle was 30°, and the acquisition time 0.6 s. Inorganic phosphate of the buffer (40 mM) was used as an internal standard. Since phosphate is known to have a pH-dependent chemical shift and since it binds to the enzyme, external H₂PO₄ was used to be sure that the phosphate shift was invariant at pH 7.0 with 1.0 mM enzyme, with and without the substrate.

**Calculation of Bound Shifts**—The quantitative criterion for fast exchange is that 1/r, the reciprocal of the lifetime in the bound state, must be large compared to the chemical shift between the two states: 1/r > > 2πΔν. For protonation of amide and urethane carbonyls the exchange is that 1/r, the reciprocal of the lifetime in the bound state, must be large compared to the chemical shift between the two states:

where δ₀ and δ₁ are the chemical shifts of bound hydrogens and free carbamyl-P respectively, and (EC)/(C₀) and (C)/(C₀) are the fractions of those species. The shifts from the free carbamyl-P peak are expressed as

\[ \Lambda_{\text{free}} = \delta_{\text{free}} - \delta_{0} = \sum \frac{EC}{C} \Lambda_{\text{EC}} \]

where \( \Lambda_{\text{EC}} \) is defined as the chemical shift for bound carbamyl-P. Kleppe (30) and Porter et al. (31) have shown that phosphate is a competitive inhibitor for carbamyl-P. At 40 mM phosphate and 2 mM carbamyl-P, approximately 15% of the active sites are occupied by phosphate and 84% by carbamyl-P. Calculated from the coupled equilibrium equations with the assumption that the concentration of free enzyme is negligible compared to those of the enzyme complexes.

Dicarboxylic acids bind not only to the catalytic subunit-carbamyl-P binary complex, but also to free catalytic subunit (27). Enzyme (E) is distributed among five species: E/carbamyl-P, E-phosphate, E-carbamyl-P-dicarboxylate, and E-dicarboxylate. To calculate the relative amount of each, the constants for dissociation of dicarboxylic acids from the ternary complex (Kₛ) and binary complex (Kₛ⁺) are needed. The various equilibria yield eight equations and eight unknowns which can be reduced to a quadratic equation under the reasonable assumptions that the concentration of free catalytic subunit is negligible and that unbound phosphate and succinate are approximately equal to total phosphate and succinate. As an example, with 2 mM carbamyl-P, 1 mM active sites, 40 mM potassium phosphate, 10 mM succinate in D₂O at apparent pH 7.0, with Kₛ⁺ (carbamyl-P) = 7 μM (31), Kₛ (phosphate) = 1 mM (31), Kₛ⁺ (succinate) = 1.7 mM and Kₛ (succinate) = 0.25 mM, 97% of the enzyme is in the ternary complex, 2% is in the binary complex with carbamyl-P, 1% is in the binary complex with phosphate, and less than 0.1% is in the binary complex with succinate. For a dicarboxylic acid such as L-malate, where the interaction of the acid with free catalytic subunit (Kₛ) = 1.53 mM is stronger than the interaction with the subunit-carbamyl-P complex (Kₛ⁺ = 10.4 mM), the fraction of subunit-L-malate rises to 8% under our experimental conditions.

**RESULTS**

**Dissociation Constants for Dicarboxylic Acids**—Heyde et al. (32) and Jacobson and Stark (27) have shown that dicarboxylic acids bind to free catalytic subunit in competition with carbamyl-P in H₂O at pH 8.0. In order to calculate concentrations of the various complexes in NMR experiments, we determined constants for dissociation of dicarboxylic acids from catalytic subunit alone and from the subunit-carbamyl-P complex in D₂O at apparent pH 7.0 (Table I). The constant for dissociation of succinate from the ternary complex with carbamyl-P (Kₛ⁺) is substantially smaller than the constant for dissociation of succinate from the binary complex (Kₛ⁺). For D-malate, these two constants are nearly equal. All other dicarboxylic acids tested bind more tightly to the free subunit than to the complex with carbamyl-P.

**TABLE I**

| Dicarboxylic Acid | Kₛ from subunit | Kₛ from subunit-carbamyl-P |
|------------------|-----------------|---------------------------|
| L-Malate         | 1.5             | 10.4                      |
| D-Malate         | 3.2             | 2.5                       |
| Maleate          | 0.54            | 0.59                      |
| Maleate          | 1.4             | 1.4                       |
| MesoMalate       | 1.7             | 0.25                      |
| Succinate        | 2.3             | 0.45                      |

*The values 0.17 mM at pH 8.0 (27) was multiplied by 0.64, the factor by which Kₛ for succinate decreases between pH 8 and pH 7, and by 0.76, the factor for succinate in going from a buffer in H₂O at pH 7.0, to a buffer in D₂O at the same apparent pH.

*The value 4.3 mM at pH 7.9 (31) was multiplied by 0.25, the factor by which Kₛ for succinate decreases between pH 7.9 and pH 7, and by 0.54, the factor for succinate in going from a buffer in H₂O at pH 7.0, to a buffer in D₂O at the same apparent pH.

*In 100 mM imidazole acetate buffer, H₂O, pH 7.0.
$^{13}$C NMR and Aspartate Transcarbamylase

![NMR Spectra](Fig. 1)

**FIG. 1.** NMR spectra of $^{[13}C$carbamyl-P in 40 mM potassium phosphate buffer, 5 mM 2-mercaptoethanol, 0.2 mM EDTA, D$_2$O, pH 7.0. Each spectrum represents 7000 to 12,000 transients, acquisition time, 1.0 s; sweep width, 2000 Hz; flip angle, 10 to 15°. $a$, carbamyl-P alone (1.7 mM); $b$, carbamyl-P (1.7 mM) plus catalytic subunit (1.0 mM in active sites); $c$, sample $b$ plus succinate (10 mM); $d$, carbamyl-P (1.7 mM) catalytic subunit (0.92 mM in active sites) and L-malate (10 mM).

$^{13}$C$[^{13}C]$Carbamyl-P and Catalytic Subunit—The chemical shift for free carbamyl-P in phosphate buffer in D$_2$O at apparent pH 7.0 is 156.3 ± 0.5 Hz upfield of internal urea or 160.8 ppm downfield of external tetramethylsilane (Fig. 1a). This signal is a doublet with carbon coupled to $^{31}$P by 4.5 Hz. Carbamyl-P is fully ionized above pH 7 and the chemical shift does not vary between pH 7.0 and 9.5. When catalytic subunit is added, there is a small but reproducible upfield change in the shift of 0.5 to 1.0 Hz (Fig. 1b), corresponding to a change of 2 Hz (0.1 ppm) for the bound species. This small upfield change indicates that the environment of the carbonyl group of carbamyl-P in the active site is slightly different than in water.

$^{13}$C$[^{13}C]$Carbamyl-P, Catalytic Subunit and Succinate—When succinate is added, a downfield change in the shift is observed for carbamyl-P (Fig. 1c). The magnitude of the change depends on the concentration of succinate (Table II). The large change for carbamyl-P bound in the ternary complex at apparent pH 7.0, 15 to 16 Hz, may indicate that the binding of succinate causes the carbonyl group of carbamyl-P to interact with a proton donor of the enzyme, as discussed below. The change in shift for carbamyl-P in the ternary complex decreases with increasing pH, suggesting that the complex is being titrated (Table III). Beard and Schmidt (4) have observed a pH-dependent relaxation of succinate protons upon the binding of succinate to the catalytic subunit carbamyl-P complex and suggest that two protonated groups which affect succinate binding are titrated between pH 7 and pH 10 and that there is an isomerization of a ternary complex. As shown in Fig. 2, there are two binary complexes of catalytic subunit and carbamyl-P in fast exchange with succinate and a conformational isomer of the ternary complex EHJ in which the ligands are tightly bound, (EHJ)$^\ddagger$. Beard and Schmidt propose that a group on the enzyme with pK 8.2 is directly involved in the binding of succinate, and that a group with pK 6.9 is involved in the conformational change. We have fit our $^{13}$C data to this

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$\text{pK}_a$ values approximately cancel the glass electrode correction, so that extents of ionization in D$_2$O and in H$_2$O are comparable at the same pH meter readings. Constants for dissociation of succinate from binary and ternary complexes are somewhat lower at apparent pH 7.0 in D$_2$O compared to pH 7.0 in H$_2$O: $K_a$ in D$_2$O is 0.76 times the value in H$_2$O and $K_a$ in H$_2$O is 0.54 times the value in H$_2$O (Table I). Using these values to calculate the distribution of enzyme species, the chemical shifts for bound carbamyl-P are the same in D$_2$O and in H$_2$O. The data in Table II are for H$_2$O. For the two highest concentrations of succinate, the calculated change in bound shift is 15.6 Hz at pH 7.0. For the same experiment in D$_2$O, the change for the bound species is 15.9 Hz at apparent pH 7.0 (Table III). It has been observed previously that D$_2$O has no significant effect on the maximum velocity of the catalytic subunit, and that the velocity in H$_2$O at any pH is about the same as the velocity in D$_2$O at an apparent pH 0.4 unit higher (35).
Changes in chemical shifts for $^{13}$C carboxyl-P in ternary complexes with succinate as a function of apparent pH in D$_2$O at 23°C

| Apparent pH | H$_2$O | D$_2$O* |
|-------------|---------|---------|
| 7.0         | 0.45    | 0.25    |
| 7.3         | 0.97    | 0.87    |
| 7.6         | 1.7     | 0.80    |
| 8.0         | 3.2     | 0.65    |
| 8.3         | 5.3     | 0.47    |

*Calculated using formulae and constants from Beard and Schmidt (4) for 23°C.

**Calculated by assuming that all species with carboxyl-P bound except for the isomerized ternary complex (EH$_2$J)' have changes in chemical shift equivalent to that for catalytic subunit carboxyl-P (2 Hz).

Model by assuming that the change in shift for bound carboxyl-P in EHI and EH$_2$I is 2 Hz, the value for the change in the binary complexes, i.e., the isomerized complex (EH$_2$J)' is the only species in which bound carboxyl-P experiences a large downfield change in chemical shift. The fraction of ternary complex isomerized can be evaluated as a function of pH at 23°C from the distribution equations and optimized constants of Beard and Schmidt (4). From the relative amount of (EH$_2$J)' at apparent pH 7.0, the change in shift for carboxyl-P in the isomerized complex is calculated to be 17.7 ± 1.0 Hz (Table III). This value is not significantly different up to apparent pH 8.3, i.e., the observed change stays proportional to the amount of (EH$_2$J)'. The fit is good at pH 7.3 and 7.8: at pH 8.0 and 8.3 the errors are greater because the observed changes are smaller.

$^{13}$C Carboxyl-P, Catalytic Subunit, and Other Dicarboxylic Acids—Other dicarboxylic acids also cause downfield changes of the chemical shift for $^{13}$C carboxyl-P in ternary complexes, but none are as large as with succinate (Table IV). The fraction of enzyme present as an isomerized ternary complex (EH$_2$J)' can be calculated from the shifts observed if it is assumed that the change for each isomerized complex is the same, 17.7 Hz. These calculations are compared with other measures of the degree of conformational change under “Discussion.”

$^{13}$C PALA and Catalytic Subunit—The chemical shift for the amide carboxyl group of free PALA is extremely pH-dependent because it is sensitive to ionization of the phosphonate group (Fig. 3). The second phosphonate proton dissociates with pK$_a$ about 7.5; the signal for PALA trianion is 187.8 Hz and that for PALA tetra-anion 235.8 Hz downfield of internal ura. At pH 7.0, the chemical shift for PALA is about 200 Hz, indicating that about 25% of free PALA is in the tetra-anion form at this pH. Like the carboxyl-P signal, the signal for PALA is a doublet. The coupling with $^{31}$P is slightly pH-dependent (4 Hz at pH 7.0, 3.5 Hz at pH 8.4).

Since PALA binds very tightly to the enzyme ($K_1 = 2.7 \times 10^{-4}$ M at pH 7 and 28°C (20)), free and bound PALA should be in slow exchange. At pH 7.0, with about 2 eq of PALA per active site, two peaks with roughly comparable linewidths and intensities are observed, consistent with slow exchange (Fig. 4A). The upfield peak is at the position of free PALA at pH 7.0 and it increases in intensity with increasing concentration of free PALA. The other peak, 20 Hz downfield, is due to bound PALA. Since the peak for bound PALA is partially obscured by the envelope of signals from the enzyme carbonyl groups (Fig.
TABLE IV

Changes in chemical shifts for bound carbamyl-P in ternary complexes
with different dicarboxylic acids in D2O at apparent pH 7.0

| Dicarboxylic acid | Calculated change for bound carbamyl-P |
|------------------|---------------------------------------|
| Succinate        | 15.9 ± 0.9                            |
| L-Malate         | 7.2 ± 2.5                             |
| Mesotartrate     | 4.8 ± 1.4                             |
| Maleate          | 6.6 ± 1.1                             |
| d-Malate         | 1.4 ± 1.2                             |

FIG. 3. Chemical shifts for the amide carbonyl of [13C]PALA (b)).
The solid line is a theoretical titration curve for pK = 7.5 and a total shift of 48 Hz.

4B), the contribution of the enzyme was subtracted to yield a difference spectrum (Fig. 4C).

[13C]Phosphonacetamide and Catalytic Subunit—The carboxyl group of free phosphonacetamide has a chemical shift which is also extremely pH-dependent due to phosphonate ionization. The pKₐ for dissociation of the second phosphonate proton is 7.1 and the chemical shift of the dianion is 85 Hz downfield of the shift for the monoanion. At pH 6.9, the coupling constant with 31P is 4.7 Hz. Phosphonacetamide competes with carbamyl-P, with Kᵢ = 0.66 mM at pH 8.0 (31); bound and free phosphonacetamide are in fast exchange when enzyme is present (40). A single resonance was observed, consistent with fast exchange, in two experiments with phosphonacetamide (4.3 and 1.5 mM) in the presence of catalytic subunit (0.8 mM) at apparent pH 7.0 in D2O, where 41% of free phosphonacetamide is in the dianion form. The observed change in shift was small, and the change for bound phosphonacetamide was calculated to be 5 to 7 Hz upfield of that for free phosphonacetamide at the same pH (299.6 Hz downfield of internal urea). Because the observed shift is very sensitive to small variations in pH in this case, the pH of the enzyme solution was checked carefully before and after each experiment and found to be constant within 0.01 unit.

[31P] Spectra with Carbamyl-P and Catalytic Subunit—The binding of carbamyl-P (15 mM) to catalytic subunit (1.3 mM in active sites) at pH 7 in the presence of 40 mM phosphate causes an observed upfield change in shift of 6.3 Hz. The calculated change for bound carbamyl-P is 78 Hz upfield. A downfield change would be expected if proximity of a positively charged group of the enzyme were the dominant factor; the opposite direction of the observed change may indicate the influence of an aromatic ring in the vicinity of the phosphate group of bound carbamyl-P. Adding succinate to the binary complex causes a smaller further change in the 31P chemical shift, 2 Hz downfield. This result with 31P is in contrast to the much larger effect at the carbonyl group with [13C]carbamyl-P.

Interaction with DIKEP and DIHOP—The contribution of the amide carbonyl group of PALA to affinity for catalytic subunit was investigated with the analogs DIKEP, in which the carbonyl group is that of a ketone, and DIHOP, in which the ketone has been reduced to an alcohol. The Kᵢ values at pH 8 are 6.5 × 10⁻⁷ M for DIKEP and 4 × 10⁻⁸ M for DIHOP, each value based on the total concentration of all the optical isomers. The Kᵢ for DIHOP may be even higher than 4 × 10⁻⁸ M, since contamination by 1 to 2% of DIKEP, which would account for all the inhibition observed, cannot be ruled out. Catalytic subunit was titrated with DIKEP by ultraviolet difference spectroscopy according to the procedure of Collins and Stark (20) in 40 mM glycylglycine buffer at pH 7.0. The magnitude of the difference spectrum at saturation was the
same as the magnitudes of those obtained with PALA or carbamyl-P plus succinate, with a major peak at 288.8 nm and a minor peak at 281.1 nm. If only L-DIKEP binds tightly, indication that there are likely to be aromatic rings in the active site, and Schmidt et al. (40) proposed that the downfield change in chemical shift observed for the protons of methyl phosphate upon binding to the catalytic subunit, much larger than the change for the protons of acetyl phosphate, might be due to the proximity of an aromatic ring edge-on to the methyl group. If the edge of such a ring were to approach the carbonyl group of carbamyl-P as a result of the conformational change induced by the binding of succinate, it might well cause the effect we observe. It is difficult to choose definitively between ring current and protonation on the basis of the current NMR evidence alone, but an aromatic ring near the carbonyl group of carbamyl-P would not aid catalysis, whereas protonation of the carbonyl oxygen atom is a most attractive mechanistic possibility; therefore we favor it. With L-DIKEP, protonation is much less likely because of the lower pKₐ of the ketone carbonyl, and yet the conformational change with the catalytic subunit is indistinguishable from the one obtained with PALA. Therefore, ¹³C NMR experiments with L-DIKEP should be very helpful in choosing between protonation of the carbonyl group and other mechanisms.

In the catalytic mechanism we have proposed previously (1, 3) a substantial change in conformation occurs when dicarboxylic acids are bound, and substituents in the position of the amino group of L-aspartate interfere both with affinity for the enzyme and with the extent of conformational change upon saturation. Support for a large conformational change upon the binding of succinate has been obtained by difference spectroscopy (1), change in sedimentation coefficient (45), changes in circular dichroism (43) and optical rotatory dispersion (44), temperature jump measurements (29), and proton NMR (4). The present results with [¹³C]karbamyl-P indicate that this conformational change may be accompanied by significant protonation of the carbonyl group. The variation of the downfield change in chemical shift with pH fits very well a model in which the isomerized complex (EH₃D⁺) is the only species in which this protonation occurs.

When other dicarboxylic acids are substituted for succinate, smaller downfield shifts are observed (Table IV). By assuming that the downfield shift for [¹³C]karbamyl-P occurs only in the isomerized complex (EH₃D⁺) and that the magnitude of this shift is independent of the nature of the dicarboxylic acid, we can calculate the fraction of ternary complex isomerized in each case (Table V). For L-malate, the fraction isomerized is in reasonably good agreement with estimates from temperature-jump and sedimentation experiments, each of which have been carried out under somewhat different conditions (Table V). For L-malate, the degree of conformational change estimated from difference spectroscopy is much lower than estimates obtained by the other methods and, for D-malate, difference spectra indicate extensive conformational change but the ¹³C NMR experiment shows little effect on bound carbamyl-P. Perhaps the perturbation of tryptophan accompanying binding of a dicarboxylic acid to the ternary complex reflects not only conformational change but also changes in the polarity of the environment of this chromophore caused by nearby ion pairs. This possibility has been suggested previously by Griffin et al. (45) on the basis of circular dichroism data. Another possible explanation is that the hydroxyl group of D-malate is near enough to the carbonyl group of carbamyl-P to provide some shielding.

Jacobson and Stark (27) have shown recently that both
TABLE V
Comparison of various measures of conformational change in ternary complexes of catalytic subunit and carbamyl-P with several dicarboxylic acids

| Dicarboxylic acid | \( ^{13}C \text{NMR} \) at pH 7.0 | Difference spectra (room temp.) | \( T_{\text{jump}} \) (28°, pH 7.4) | \( \Delta S^0 \) (20 to 22°) |
|-------------------|-------------------------------|--------------------------------|---------------------------------|-----------------------------|
| Succinate         | 0.91                          | 0.91                           | 0.88                            | 0.93                        |
| l-Malate          | 0.47                          | 0.15                           | 0.40                            | 0.70                        |
| d-Malate          | 0.17                          | 0.66                           | ND                              | ND                          |
| Mesotartrate      | 0.35                          | ND                             | ND                              | ND                          |
| Malate            | 0.44                          | ND                             | ND                              | ND                          |

*The fraction isomerized for succinate is from Beard and Schmidt (4) at pH 7.0 and 23°. All other values are relative to this, from the data of Table IV, assuming that the large downfield shift for carbamyl-P occurs only in the isomerized complex (EHJ) and that the magnitude of the shift in (EHJ) is independent of the nature of the dicarboxylic acid.

Collins and Stark (1) found the following magnitudes for the difference spectra, relative to the one given by subunit plus carbamyl-P 1.0: succinate = 2.8, L-malate = 1.3, d-malate = 2.3. Taking the fraction isomerized for succinate to be 0.91 (4), the fraction isomerized for L-malate = (0.3 x 0.91/1.8) = 0.15 and that for d-malate = (1.3 x 0.91/1.8) = 0.66.

From Hammes et al. (29). The fraction isomerized for succinate at pH 7.4 and 28° from Beard and Schmidt (4) is 0.75.

Kirschner and Schachman (44) determined a corrected value of 1.60 for succinate and 0.77 for L-malate at ligand concentrations of 20 mM. The corrected value for saturating L-malate, using \( K_r = 12.1 \text{mM} \), is 0.77/0.62 = 1.24, and for succinate is 1.67. From the data of Beard and Schmidt (4), the fraction isomerized for succinate is 0.91 at pH 7.0 and 28°.

Not determined.

L-malate and mesotartrate are capable of triggering an overall conformational change of native aspartate transcarbamylase in the presence of carbamyl-P in a manner similar to the effect of succinate previously described by Gerhart and Pardee (46). From the data of Table V it is apparent that these two analogs of L-aspartate also cause appreciable fractions of the ternary complex with catalytic subunit to become isomerized.

PALA and Phosphonacetamide—Interpretation of the 20-Hz downfield shift observed upon the binding of PALA to catalytic subunit is complicated by the necessity of doing the experiment at a pH near 7.0, since the pK\(_a\) for dissociation of the second phosphate proton is 7.5 and ionization is accompanied by a downfield change in chemical shift of 48 Hz. If the pK\(_a\) for the phosphate portion of PALA were to change to a value below about 6 upon binding, so that the only bound form of PALA at pH 7 were the phosphon dianion, the chemical shift for PALA would be expected to move about 36 Hz downfield upon binding from this effect alone. Since a downfield change of 20 Hz is actually observed, a compensating change of 16 Hz upfield from some interaction at the carbonyl group of PALA is required, a result greatly different from the 17.5-Hz downfield shift observed for carbamyl-P in the presence of succinate. Alternatively, if the pK\(_a\) for the phosphate of bound PALA were changed to a value above about 8.5, the chemical shift for PALA would move 12 Hz upfield upon binding from this cause alone, and the observed change of 20 Hz downfield would imply a change of 39 Hz downfield from the interaction of the carbonyl group.

In order to distinguish among these two extreme possibilities and the intermediate cases in which the pK\(_a\) for the phosphate of bound PALA lies between 6 and 8.5, we examined the binding of phosphonacetamide to the enzyme, since only small effects would be expected from any interaction with the carbonyl group of this compound, by analogy with the case of carbamyl-P in the absence of succinate. At apparent pH 6.94, the chemical shift for phosphonacetamide (pK\(_a\) 7.1 in D\(_2\)O) moves only about 6 Hz upfield upon binding, a small effect comparable to the 2-Hz upfield change seen with carbamyl-P (which is completely ionized at pH 7, so that there is no possible complication from a change in ionization state). The results with phosphonacetamide indicate strongly that the pK\(_a\) of this compound is affected very little upon binding to the enzyme, i.e. the enzyme has about the same affinity for the mono- and di-anion forms. If the enzyme also has approximately equal affinities for the trianion and tetra-anion forms of PALA, the downfield change of 20 Hz observed upon binding would reflect primarily a difference in the environment of the carbonyl group, similar to the change observed with carbamyl-P plus succinate. Of course, the conformations of the enzyme with PALA or phosphonacetamide bound are quite different. However, the \( ^{31}P \text{NMR} \) data with carbamyl-P alone and in the presence of succinate indicate that the environment of this phosphate di-anion changes much more when the binary complex is formed than it does when succinate adds to form the isomerized ternary complex.

Relative Basicities of Carbonyl Groups and Changes in Chemical Shifts upon Protonation—Chemical shifts relative to urea are given in Table VI for carbamyl-P, urethan, PALA, and phosphonacetamide. In order to interpret data for interactions of carbamyl-P and PALA with the catalytic subunit in terms of protonation of the carbonyl groups, we need to know something about their relative basicities and about how much their chemical shifts change upon full protonation. Both carbamyl-P and PALA are too labile in strong acid to determine these quantities directly, so values have to be inferred from the properties of model compounds such as urethan, butyramide, and phosphonacetamide, as described in Table VII. The downfield change in chemical shift of 17.7 Hz for carbamyl-P in the isomerized ternary complex (EHJ) represents 20% of the change obtained with urethan and phosphonacetamide upon full protonation. If the pK\(_a\) of the phosphate group of PALA is unaltered upon binding to the enzyme, the 20-Hz downfield change in chemical shift observed upon binding of PALA to catalytic subunit corresponds to about the same change with carbamyl-P and succinate. Since the pK\(_a\) values for the carbonyl groups of PALA and carbamyl-P are about the same.

| Compound          | Chemical shift observed minus chemical shift for internal urea |
|-------------------|--------------------------------------------------------------|
| Urethan           | +179                                                         |
| Carbamyl-P        | -156.3                                                      |
| PALA (acid)       | +124                                                        |
| PALA (trianion)   | +188                                                        |
|PALA (tetraniion)  | +236                                                        |
|Phosphonacetamide (acid) | +179                                                   |
|Phosphonacetamide (monooanion) | +265                                      |
|Phosphonacetamide (dianion) | +350                                                   |
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served in concentrated H2SO4. The energy to drive such change in pK, required to achieve protonation 20% of the time is about 2 units lower than in the corresponding amino acid (48). As a rough guide, the units. Comparable inductive effects are seen for substitutions at the site of the enzyme, where specific acid catalysis is possible.

Nevertheless, the inductive effects of -OPOs2- and -OPO3- on the pK, of the carbonyl group are bound to be quite different and should be taken into account, even if only approximately. From the comparison of butyramide with phosphonacetamide (48) it can be seen that substitution of an oxygen for an amide methylene (-0.8 pK unit) to the pK, for phosphonacetamide; (b) add the effect of the phosphonate group in going from butyramide to phosphonacetamide (-1.7 pK units) to the pK, of urethan. (Table VII) it seems quite reasonable for these groups to experience about the same change in the isomerized complexes.

We would like to know pKa values for the amide carbonyl groups of carbamyl-P and PALA when they are bound to the enzyme. Fully protonated enzyme-bound carbamyl-P would be NH4+CH2CH2COOH, and Table VII gives an estimate of the pKa for the species NH4+CH2CH2COO-. Of course, the first species can exist in appreciable concentration only in the active site of the enzyme, where specific acid catalysis is possible. Nevertheless, the inductive effects of -OPO42- and -OPO3- on the pKa of the carbonyl group are bound to be quite different and should be taken into account, even if only approximately. From the comparison of butyramide with phosphonacetamide in Table VII it can be seen that substitution of -CH2PO2H2 for -CH2CH2CH2 decreases the pKa by 1.7 units. Comparable inductive effects are seen for substitutions of -CH2CO2H for -CH2CH2CH2 (48). As a rough guide, the pKa of the NH+ group in a peptide NH2CH2CHR(CO)ONHR is about 2 units lower than in the corresponding amino acid NH2CHR(CO)2-. Therefore, we estimate that the pKa for the species NH2CH2CHR(CO)2- is about -4 or -3 and that the change in pKa required to achieve protonation 20% of the time at pH 7 is 9 or 10 units. An alternative model is that partial protonation occurs all the time, with an effect on the chemical shift 20% of that for full protonation. The energy to drive such a process need not be 20% of the energy to drive full protonation, so the change in pKa estimated above can be regarded as an upper limit.

Conclusions—a modified stepwise scenario can be proposed for the action of aspartate transcarbamylase by combining the present results with previous evidence summarized with references by Jacobson and Stark (3). The binding of the substrates is ordered. Carbamyl-P, the first substrate to bind, interacts first with a readily accessible site on the enzyme through its phosphate group, followed by a conformational change in which a hydrogen bond is formed between a proton donor of the enzyme and the carbonyl group. Close proximity of the enzyme to the NH2 group helps to hold the bound carbamyl-P rigidly. L-Aspartate then binds, causing a second conformational change in which the NH2 group is pushed toward the carbonyl group of carbamyl-P. The aspartate is held rigidly by both carboxylates and also by close steric interactions with the enzyme, so that compression of the two substrates takes place only in a productive direction, along the reaction coordinate. During the conformational change, the hydrogen bond to the carbonyl group of carbamyl-P becomes stronger. Protonation or perhaps deformation of the amide activates this carbonyl group, which is otherwise very unreactive toward nucleophiles. This activated form of carbamyl-P must be well protected from solvent in isomerized complexes, since the catalytic subunit does not stimulate the decomposition of carbamyl-P in the presence of succinate.6 The pKa values of the carbonyl groups of PALA and carbamyl-P are similar, so that each may be protonated to about the same extent on the enzyme. In the case of DIKEP, shortening of a hydrogen bond to the carbonyl groups should also take place upon compression, but to the extent that protonation is important we would expect the effect on the 13C-labeled chemical shift of this ketone to be much less, since a ketone is a much weaker base than the amides of PALA or carbamyl-P. Experiments with 13C-enriched acetyl-P in the presence of succinate might help to reveal whether distortion of the amide bond of carbamyl-P plays a role in activating the carbonyl group. Fourier transform infrared measurements with the 13C-labeled compounds we have used would also be quite informative.

Energy to drive both the conformational change of the enzyme and the proposed highly unfavorable shortening of the hydrogen bond (protonation of the carbonyl group) must of course be derived from unrealized potential binding energy of the substrates. Although the estimate is very rough, the pKa values of the carbonyl groups of carbamyl-P and PALA may be increased by as much as 9 or 10 units in the isomerized complexes. Since the observed affinity of the enzyme for PALA is about 106 M-1 at pH 7, PALA could bind with an affinity of 1017 M-1 or so in the absence of conformational change and carbonyl interaction, corresponding to a binding energy of about 23 kcal/mol at 25°. Although this energy seems very large, it is comparable to recent estimates of some actual binding energies (the binding of avidin to biotin has a free energy of -20 kcal/mol) and to calculations of potential binding energies in the absence of conformational change and carbonyl interaction, corresponding to a binding energy of about 23 kcal/mol at 25°.

a *O. Modebe and G. R. Stark, unpublished results.
could contribute 40 entropy units, or about ~12 kcal/mol to the free energy of binding.

The nature of the postulated proton donor is unknown, but it should be noted that Beard and Schmidt (4) provide evidence that a group with pKₐ 7 is involved in isomerization of the ternary complex, and Greenwell et al. (51) have implicated the presence of 2 histidine residues in the active site through a study of the photoxidation of subunit-pyridoxal-P complexes. Decisive information on the nature of the catalytic residues and on the possible role of ring currents in the effects we have observed with ¹³C-labeled compounds should come eventually from crystallographic maps of aspartate transcarbamylase (52), perhaps in the presence of PALA.

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