Aspartate Transcarbamylase of Escherichia coli

HETEROGENEITY OF BINDING SITES FOR CARBAMYL PHOSPHATE AND FLUORINATED ANALOGS OF CARBAMYL PHOSPHATE

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JOHN A. RIDGE, MARY FEDARKO ROBERTS, MARTIN H. SCHAFFER,§ AND GEORGE R. STARK§

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Some preparations of both native aspartate transcarbamylase from Escherichia coli and catalytic subunit have fewer tight binding sites per oligomer for carbamyl-P than the number of catalytic peptide chains. In contrast, the number of sites for the tight-binding inhibitor N-(phosphonacetyl)-L-aspartate does equal the number of catalytic chains in each case. Binding of the labile carbamyl-P was determined using rapid gel filtration, with conversion to stable carbamyl-L-aspartate during collection. Native enzyme (six catalytic chains) obtained from cells grown under the conditions of J. C. Gerhart and H. Holoubek (J. Biol. Chem. (1967) 242, 2886-2892) has 5.4 tight sites for carbamyl-P at pH 8.0 (Kₐ = 9.9 μM), whereas native enzyme from cells grown with higher concentrations of glucose, uracil, and histidine (to yield more enzyme per unit volume of culture) has only 1.9 tight sites at pH 8.0 (Kₐ = 4.6 μM) and only 2.3 tight sites at pH 7.0 (Kₐ = 2.6 μM). At pH 8.0, catalytic subunit (three catalytic chains) obtained from the former native enzyme has 2.2 tight sites for carbamyl-P (Kₐ = 2.4 μM) and the number of sites is 2.3 in the presence of 35 mM succinate, whereas catalytic subunit obtained from the latter native enzyme has 1.8 tight sites (Kₐ = 3.6 μM) in the absence of succinate and 2.3 tight sites in its presence.

The number of tight binding sites is also less than the number of subunit peptide chains in ¹⁹F nuclear magnetic resonance experiments performed with catalytic subunit and two fluorinated analogs of carbamyl-P at comparable concentrations of analogs and active sites. A model is proposed in which incomplete removal of formylmethionine from the NH₂ termini of the enzyme under conditions of extreme derepression affects affinity for ligands.

Application of NMR spectroscopy to problems of biological interest has increased rapidly with the advent of Fourier transform methodology and with the availability of instruments capable of observing nuclei other than ¹H. ¹⁹F is an attractive NMR probe for several reasons, and there are now several studies in which substrate analogs labeled with this nucleus have been used (1-4) or in which the protein has been modified covalently with a fluorine-containing probe (5, 6). ¹⁹F NMR is almost as sensitive as ¹H NMR, but the range of chemical shifts is much larger and the shifts are extremely sensitive to changes in environment. Since the substrate analog usually contains the only ¹⁹F in the system, backgrounds are low. Thus, spectra can be obtained at comparable molar concentrations of protein and ¹⁹F. In contrast, experiments with ¹H probes are usually done with ligand concentrations (for examples with aspartate transcarbamylase, see Refs. 7 and 8). A possible disadvantage of working with ¹⁹F is that its electronegativity is much greater than that of ¹H, for which ¹⁹F is usually substituted. To the extent that this difference is important, substrate analogs labeled with ¹⁹F may behave very differently from substrates upon binding to enzymes.

In initial experiments, we found that FLAP, a trifluoro analog of carbamyl-P, binds tightly to substantially fewer than three sites per catalytic subunit trimer, even though the transition state analog PALA binds tightly to all three sites. Although partial saturation of C₃R₄ by carbamyl-P was observed by Rosenbusch and Griffin (9) using equilibrium dialysis, these workers also reported that C₃ has 2.9 tight binding sites for carbamyl-P. However, there was a good deal of scatter in the experiments with C₃, especially at low ligand concentrations, due at least in part to extensive decomposition of carbamyl-P during the dialysis. The apparent disagreement between our results with FLAP and the results of Rosenbusch and Griffin with carbamyl-P led us to study the binding of

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* The abbreviations used are: FLAP, 3,3,3-trifluoro-2,2-dihydroxypropyl 1-phosphonate; C₃R₆, native aspartate transcarbamylase from Escherichia coli, comprising three dimeric regulatory subunits and two trimeric catalytic subunits; C₃, the catalytic subunit; PALA, N-(phosphonacetyl)-L-aspartate.
Binding of Carbamyl Phosphate to Aspartate Transcarbamylase

Materials and Methods

Growth of Cells

Most experiments with carbamyl-P were performed with enzyme preparations freshly made from a batch of cells grown at the New England Enzyme Center in 1969 (Batch A). The cells were stored at -20°C and preparations of enzyme made periodically from them were used for all the work reported from this laboratory after 1969. Enzyme from cell Batch A was also used for all the 14C NMR experiments reported in this paper. Enzyme from a different batch of cells (Batch B) was kindly provided by Dr. Howard Schachman, University of California, Berkeley. As shown in detail below, the properties of C,R, and C,R, prepared from Batch A and Batch B differed. Cell Batch B was grown according to Gerhart and Holoubek (12), in medium containing 4.0 g/liter of n-glucose, 50 mg/liter each of L-histidine and L-leucine, and 4 mg/liter of uracil. For cell Batch A these quantities were changed, in order to obtain a higher yield of protein/liter of growth because the yields of cells are higher.

Enzyme

Native aspartate transcarbamylase prepared as described by Gerhart and Holoubek (12) was stored at -20°C as an ammonium sulfate precipitate. The enzyme was dissolved in 40 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM 2-mercaptoethanol before use. Catalytic subunit was prepared as described by Gerhart and Holoubek (12). Protein concentrations were determined by quantitative amino acid analysis, with standards run concurrently. There was no significant change from the amino acid compositions determined previously.

Materials

[14C]Carbamyl-P for binding studies (New England Nuclear Corp.) was precipitated from cold ethanol with unlabeled carrier in order to remove radiochemical impurities. Less than 0.2% of the radioactivity remained after acidification and heating. Specific radioactivity was determined by colorimetric assay (15) after complete enzymatic conversion to carbamyl aspartate. Radioactivity of the same sample was determined after acidification and evaporation, to remove any volatile radio-active impurity. [14C]Carbamyl-P for use in assays was synthesized according to Davies et al. (16), L-[14C] aspartate was obtained from the New England Nuclear Corp., and succinate was recrystallized from H2O. All other compounds were reagent grade and were not purified further. All buffers were prepared using quartz-distilled H2O.

Binding Experiments

The binding of carbamyl-P to enzyme was measured at 28°C according to Hummel and Dreyer (11), using Sephadex G-25. Columns were equilibrated with buffer and [14C]carbamyl-P and each experiment was begun by placing a known amount of enzyme in the same solution onto the column. Concentrations of enzyme in stock solutions were determined by amino acid analysis except for the experiments of Fig. 3, where the concentration was determined from the absorbancy at 280 nm and checked by titrating the active sites with PALA as described by Collins and Stark (13). Fractions from the columns were collected directly into scintillation vials. To convert carbamyl-P in the fractions rapidly into carbamyl aspartate, a chemically stable form, each vial contained 4 μg of C,R, and 20 mM L-aspartate in buffer. Following collection, each fraction was acidified with acetic acid and the contents were evaporated to dryness in an oven. [14C]Carbamyl-L-aspartate is stable to this procedure, but [14C]cyanoate and CO2 decomposition products of carbamyl-P, are removed. Thus, the radioactivity remaining after heating in acid reflects accurately the amount of carbamyl-P present in each fraction as it is collected, whereas decomposition of carbamyl-P during the run is seen as a sloping baseline, and can be determined quantitatively in each experiment. Scatchard plots (17) were constructed from data obtained in a series of experiments with different concentrations of carbamyl-P. Since only one class of binding sites was detected, the data were fit by a straightforward least-squares regression.

Syntheses of FLAP and Reduced FLAP

Structures and synthetic routes are shown in Fig. 1.

TABLE 1

| Structure | Calculated | Found |
|-----------|------------|-------|
| FLAP      | C 34.15, F 23.15, H 5.73, N 11.38, P 12.58 | C 34.30, F 22.89, H 5.16, N 11.01, P 12.63 |
| Reduced FLAP | C 33.50, F 22.89, H 5.16, N 11.01, P 12.63 | C 33.50, F 22.89, H 5.16, N 11.01, P 12.63 |

FLAP—A solution of 100 mg (0.41 mmol) of the phosphonamide in 10 ml of water was acidified to pH 1.0 and allowed to stand for 2 h. A proton NMR spectrum showed that the N-phosphonomonoephosphate doublet had collapsed to a singlet, indicating that hydrolysis was complete. FLAP was purified by elution from Dowex 1-X8 (formate) with a linear gradient from 3 M formic acid to 3 M HCl. The structure of FLAP was confirmed by NMR analysis. The proton NMR spectrum of FLAP had a doublet centered 2.00 ppm from external tetramethyl silane, JNH-HPO = 16 Hz; the 19F spectrum had a doublet centered 7.37 ppm (ppm) in trocaric acid, JNH,19F = 2.0 Hz; the 31P spectrum (with proton noise decoupling) had a single broad peak 25.14 ppm.

FIG. 1. Routes for the syntheses of FLAP and reduced FLAP.
Binding of Carbamyl Phosphate to Aspartate Transcarbamylase

RESULTS

Binding of Carbamyl-P to C₄ and C₄R₄—A typical elution profile of carbamyl-P from Sephadex G-25 in the presence of enzyme (Fig. 2) has a sloping baseline (solid line) due to decomposition of carbamyl-P on the column, and a peak of bound carbamyl-P that emerges with the enzyme at the excluded volume. As illustrated in Fig. 2, we define each limit of the peak by an increase of more than 1 standard deviation (dashed line) above the mean number of counts per min in a least squares fit of the sloping baseline. Behind the peak is a trough (not shown in Fig. 2) that extends to the included volume of the column, representing a region where the amount of carbamyl-P has been depleted by the enzyme. The areas of the peak and the trough should be identical but, since the peak is broad and shallow, it is more difficult to define and we have not calculated its area. However, we have used separation of the peak and the trough by a flat baseline as a criterion for equilibration on the column.

Most experiments were performed at 28°C, pH 8.0, in 90 mM Tris-acetate buffer, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA. One study of C₄R₄ was at 28°C, pH 7.0, in 40 mM imidazole acetate buffer, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, the conditions used by Rosenbusch and Griffin (9). Preparations of C₄ and C₄R₄ were derived from two different batches of cells grown under different conditions. Enzymes prepared from cell Batch A have a number of tight binding sites for carbamyl-P smaller than the number of catalytic peptide chains and, for C₄, the number of sites increases significantly in the presence of succinate, an analog of L-aspartate (Fig. 3). Enzymes prepared from cell Batch B have a number of tight binding sites for carbamyl-P more nearly equal to the number of catalytic chains and, for C₄, the number increases only very slightly when a high concentration of succinate is added (Fig. 4). Scatchard plots for the binding of carbamyl-P to C₄R₄ are shown in Figs. 5 and 6. C₄R₄ from cell Batch A binds 2 to 3 molecules of carbamyl-P per oligomer, whereas C₄R₄ from Batch B binds nearly 6. All the results at pH 8 are summarized in Table I.

Although fewer than three tight binding sites for carbamyl-P were observed with C₄ from both batches of cells, the concen-
FIG. 3. Scatchard plots for the binding of carbamyl-P to C₄ from cell Batch A at pH 8.0 and 28° in 90 mM Tris-acetate buffer. (○) no succinate; (△) 3.5 mM succinate; (□) 35 mM succinate. The data were calculated as if succinate were able to bind only to the C₄-carbamyl-P complex and not to C₄; see Table I for details.

FIG. 4. Scatchard plots for the binding of carbamyl-P to C₄ from cell Batch B at pH 8.0 and 28° in 90 mM Tris-acetate buffer. (○) no succinate; (□) 35 mM succinate. The data were calculated as if succinate were able to bind only to the C₄-carbamyl-P complex and not to C₄; see Table I for details.

FIG. 5. Scatchard plots for the binding of carbamyl-P to C₄R₄ from cell Batch A at 28°. (○) pH 8.0 in 90 mM Tris-acetate buffer; (△) pH 7.0 in 40 mM imidazole acetate buffer.

FIG. 6. A Scatchard plot for the binding of carbamyl-P to C₄R₄ from cell Batch B at pH 8.0 and 28° in 90 mM Tris-acetate buffer.

The concentration range of carbamyl-P that could be investigated was limited, so that a third binding site with an affinity 10 times smaller would not have been detected. We have shown previously that C₄ and C₄R₄ from cell Batch A have three or six sites, respectively, for the tightly bound inhibitor PALA (13, 21) and this stoichiometry of PALA binding was confirmed with the preparation of C₄ used in the experiment of Fig. 3. Rosenbusch and Griffin (9) reported previously that the number of binding sites for carbamyl-P to C₄R₄ increased at pH 7 from three to six in the presence of succinate; we used succinate with C₄ to test whether the third potential binding site for carbamyl-P would be revealed in the presence of this aspartate analog. Such an experiment can be performed...
because, fortuitously under the conditions we have used, the constants for dissociation of succinate from the C₄:succinate binary complex and the C₄:carbamyl-P:succinate ternary complex are both 3.5 mM (21). Hence, succinate does not perturb the equilibrium C₄ + carbamyl-P ⇌ C₄:carbamyl-P because it binds equally well to C₄ and to C₄:carbamyl-P. As shown in Fig. 3 and Table I, 3.5 mM succinate does increase the number of sites for carbamyl-P and a further increase is observed with 35 mM succinate.

Binding of carbamyl-P to C₄R₄ was difficult to observe in the presence of succinate at pH 8, because succinate competes more effectively with carbamyl-P for C₄R₄ than it does for C₄ at this pH. At 0.5 μM carbamyl-P, increasing concentrations of succinate cause a decrease in the amount of carbamyl-P bound to the enzyme, with half-decrease at 4 mM succinate (data not shown). At appreciably higher concentrations of succinate, no binding at all is observed. Using preparations of C₄R₄ from cells grown under conditions similar to the ones we have used (see below) Rosenbusch and Griffin (9) did observe that, at pH 7, 5 mM succinate increased the number of tight binding sites for carbamyl-P from 3.2 ± 0.5 to 5.94 ± 1.35; the carbamyl-P concentrations used in the presence of succinate were 10 to 100 μM. They also observed that Kₛ for carbamyl-P increased from 14 μM without succinate to 27 μM with succinate, an apparent paradox now explained by the binding of succinate to free C₄R₄ (21). Jacobson and Stark (21) also observed that Kₛ for the dissociation of succinate from C₄:succinate is only slightly lower at pH 7 than at pH 8, whereas Kₛ for the dissociation of succinate from C₄:carbamyl-P:succinate decreases much more. Thus the affinities of succinate for C₄R₄ and C₄R₄:carbamyl-P may be nearly equal at pH 7.

**Inhibition of C₄ by FLAP**—FLAP is competitive with carbamyl-P (Fig. 7) and noncompetitive with L-aspartate (Fig. 8) at pH 7.8. A replot of the slopes of Fig. 7 versus FLAP concentration is a parabola. At the low concentrations of carbamyl-P and L-aspartate employed, the activity observed in the experiment of Fig. 7 is almost exclusively due to binding of carbamyl-P to the tight sites only. The parabolic competitive inhibition observed with FLAP is very reminiscent of the pattern observed with phosphate, interpreted by Porter et al. (22) as evidence for a complex with 2 phosphates per active site. By analogy, it may be possible to form E:FLAP and E:(FLAP)₂ complexes at each active site. By fitting the parabola, Kₛ values of about 0.04 and 0.1 mM can be calculated for the 1st and 2nd molecules. Other possibilities for parabolic competitive inhibition involve interaction among the active sites of C₄: the binding of FLAP to a low affinity site of a complex with stoichiometry C₄:FLAP, may strengthen the binding of FLAP to the high affinity site already occupied, or the binding of FLAP to one active site of C₄ may decrease the affinity of the unoccupied site or sites for carbamyl-P. Under the conditions of Fig. 7, there can be no significant binding of carbamyl-P and FLAP to the same active site, since noncompetitive rather than competitive inhibition would then be observed.

**NMR Studies with FLAP and C₄**—All experiments were done with C₄ from cell Batch A. ¹³C NMR signals for free FLAP (1046.8 Hz upfield of internal trifluoroacetate) and 3.6 mM FLAP in the presence of 1.5 mM C₄ (0.2 mM Tris-acetate buffer, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 8.0) are shown in Fig. 9. Scatchard plots of the data (Fig. 10) show that FLAP binds to C₄ with at least two affinities. At pH 7.0, the data are described best by a model in which 1 molecule of FLAP binds very tightly (Kₛ₁ = 0.05 mM), while 2 others bind much more weakly (Kₛ₂ = 1.77 mM). At pH 8.0, the relative stoichiometry

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**Table I**

| Cell batch | Enzyme species | [Succinate] | Number of sites | Kₛ observed⁴⁺ | Kₛ calculated |
|------------|----------------|-------------|----------------|---------------|---------------|
| A          | C₄             | 0           | 1.78           | 3.6           |               |
| A          | C₄             | 3.5         | 2.20           | 4.7           | 7.2           |
| A          | C₄R₄           | 35          | 2.34           | 5.7           | 0.52          |
| A          | C₄R₄           | 0           | 1.90           | 4.6           |               |
| B          | C₄             | 0           | 2.21           | 2.4           |               |
| B          | C₄R₄           | 35          | 2.29           | 2.2           | 0.20          |
| B          | C₄R₄           | 0           | 5.39           | 9.9           |               |

**Fig. 7.** Inhibition of C₄ by FLAP, varying carbamyl-P at constant aspartate (1 μM), 28°C. The concentration of C₄ was 2.5 μg/ml; velocity is in mol/min/g of C₄. FLAP concentrations ranged from 0 to 0.59 mM, as shown in the figure. The buffer was 10 mM Tris-acetate, 0.1 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7.8. The assay of Davies et al. (16) with [¹⁴C]carbamyl-P was used.

**Fig. 8.** Inhibition of C₄ by FLAP, varying L-aspartate at constant carbamyl-P (0.5 mM), 28°C. FLAP concentration ranged from 0 to 16 mM, as shown in the figure. All other conditions were as for Fig. 1, except that the assay of Porter et al. (22) with [¹⁴C]L-aspartate was used.
Binding of Carboxylic Phosphate to Aspartate Transcarbamylase

FIG. 9. A, \(^{19}F\) NMR spectrum of FLAP at pH 8.0. \(\delta_F\) is given as Hz upfield from internal trifluoroacetate. The coupling is due to phosphorus, \(J_{19F19P} = 1.8\) Hz. B, \(^{13}P\) NMR spectrum of FLAP (3.6 mM) and C\(_5\) (1.5 mM) at pH 8.0.

Fig. 10. Scatchard plots for the binding of FLAP to C\(_5\) (○) 50 mM imidazole acetate, 10 mM 2-mercaptoethanol, 1 mM EDTA (pH 7.0); (■) 0.2 M Tris-acetate, 10 mM 2-mercaptoethanol, 1 mM EDTA (pH 8.0). The solid lines represent best fits to the model with one tight binding site: at pH 7.0, \(K_{LO} = 0.05\) mM and \(K_{HI} = 1.77\) mM; at pH 8.0, \(K_{LO} = 0.05\) mM and \(K_{HI} = 0.27\) mM. The dotted line represents the best fit to the model with two tight binding sites: at pH 8.0, \(K_{LO} = 0.08\) mM and \(K_{HI} = 0.80\) mM.

is less clear and the data can be described by models with one tight site \((K_{LO} = 0.05\) mM\) and two weak sites \((K_{HI} = 0.27\) mM\) or two tight sites \((K_{LO} = 0.08\) mM\) and one weak site \((K_{HI} = 0.8\) mM\). The error in measuring \(\delta_F\) \((\pm 0.4\) Hz\) causes a constant error in \(\psi(I)\) of \(\pm 0.10\); for 8 degrees of freedom \(\chi^2\) is 12.9 for the model with one tight site and 9.9 for the one with two tight sites. From the maximum likelihood ratio for the two models, there is a slight (2 to 1) preference for two tight sites at pH 8.0.

Approximately two tight sites are also seen in the binding of carbamyl-P to C\(_5\) at pH 8.0. At pH 7.5, the data are fit best by assuming that there is one tight site and at pH 8.5, a model with two tight sites gives the best fit (Table II). At each pH, both \(K_{HI}\) and \(K_{LO}\) decrease as the ionic strength decreases. At pH 7.0 and pH 8.0, there is a two-fold decrease in \(K_{HI}\) as the buffer concentration is lowered from 200 mM to 10 mM and \(K_{LO}\) also decreases by at least a factor of 2. The binding of carbamyl-P is affected similarly by ionic strength, although the effect is much greater: \(K_D = 14\) mM in 200 mM buffer and 1 mM in 10 mM buffer at pH 8.0 (21).

The chemical shift observed upon binding of FLAP to C\(_5\), \(\Delta\), is also pH-dependent (Fig. 11): at pH 7.0, \(\Delta\) is 19 Hz, and at pH 8.0 it is 10.4 Hz. Although \(K_D\) is affected by ionic strength, \(\Delta\) is not. The pH dependence for the binding of FLAP, quite different from that for the binding of carbamyl-P, can be interpreted in two ways: either the structure of FLAP changes with pH and the high pH form binds more tightly, or a group on the enzyme with \(pK_a = 7.6\) must be unprotonated for FLAP to bind with high affinity to a second site. To investigate the first possibility, the \(^{19}F\), \(^{1}H\), and \(^{31}P\) NMR spectra of FLAP

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The inactive 5-thio-2-nitrobenzamide derivative of C\(_5\) binds 1 molecule of carbamyl-P with normal affinity \((K_D = 14\) mM at pH 8.0\) while the others bind much more weakly (23). FLAP binds to this derivative with enhancement of the difference in affinities. At pH 8.0, there is clearly only one tight site; \(K_{LO} (0.05\) mM\) is similar to the values calculated for the binding of FLAP to unmodified C\(_5\) but \(K_{HI}\) is increased to 2.5 mM.
TABLE II

| pH    | Buffer concentration | Technique | \(K_{\text{M}}\) | \(K_{\text{H}}\) |
|-------|----------------------|-----------|------------------|----------------|
|       |                      | nM       |                  |                  |
|       |                      | 1        | 0.10             | 2.0             |
| 7.0   | 200                  | NMR      | 1                | 0.05            |
| 50    | NMR                  | 1        | 1.77             |                 |
| 10    | NMR                  | 1        | 1.0              |                 |
| 5     | Difference spectroscopy |        |                  | 1.2             |
| 7.5   | 200                  | NMR      | 1                | 0.05            |
| 8.0   | 200                  | NMR      | 1                | 0.05            |
| 10    | NMR                  | 1        | 0.27             |                 |
| 5     | Difference spectroscopy |        |                  | 0.04            |
| 8.5   | 200                  | NMR      | 1                | 0.05            |
| 10    | NMR                  | 1        | 0.43             |                 |

*Imidazole acetate buffers at pH 7.0, Tris-acetate buffers at other pH values. All NMR experiments in D$_2$O, difference spectroscopy and kinetics in H$_2$O.

\(n_{\text{Ho}} = 3 - n_{\text{Lo}}\)

Not enough data were obtained for low concentrations of FLAP in these experiments to calculate \(K_{\text{M}}\). For calculation of \(K_{\text{M}}\), the \(K_{\text{M}}\) values were assumed to be in the range 0.05 to 0.10 mM and a best fit was then determined.

These data can be fit by models with either one or two tight binding sites (see the text). Dissociation constants are calculated for each case.

From parabolic competitive inhibition versus carbamyl-P, data of Fig. 7.

were observed in D$_2$O as a function of pH, and the pK\(_a\) values found were compared with pK\(_a\) values from titration of FLAP with KOH. As shown in Table III, the phosphate protons have pK\(_a\) values of 1.8 and 6.3 and the hydroxyl of the hydrated carbonyl group begins to titrate at pH 12. There is no uptake of KOH by FLAP between pH 7.0 and pH 8.0 but there is a marked upfield movement of the chemical shift of the methylene protons and a small movement of the fluorine shift, perhaps indicating that there is a pH-dependent conformational change of FLAP without an ionization. Since the change in 19F shift with pK\(_a\) 8 for free FLAP is small (1.5 Hz) compared to the change in shift for FLAP bound to the enzyme between pH 7 and pH 8 (8.6 Hz), and since the titration of the shift is much sharper than predicted for titration of a single group (Fig. 11), the pH dependence must be due at least in part to titration of C\(_3\) and not solely to a conformational change of FLAP.

Competition between FLAP and Other Anions—In addition to the general effect of ionic strength on the binding of FLAP, there is a more specific competition between FLAP and carbamyl-P analogs or dicarboxylic acids. For example, the NMR signal for unbound FLAP reappears upon addition of phosphate to a solution of FLAP and C\(_3\). The transition state analog PALA also competes. Dissociation constants derived from competition experiments are shown in Table IV. Succinate, which dissociates from the active site of C\(_3\) succinate with \(K_{\text{a}} = 3.5\) mM (21), displaces FLAP with \(K_{\text{a}} = 2.6\) mM. The data indicate that ternary complexes with C\(_3\), FLAP, and acids do not form and that FLAP is displaced instead.

Difference Spectra—Spectra were recorded as described previously (25) using a Cary 15 double beam spectrophotometer and cells with a path length of 0.44 cm. FLAP (12.1 mM)
and C₉ (1.8 mg/ml) in low ionic strength buffer (5 mM imidazole acetate, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7.0) give a difference spectrum with maxima at 281.4 nm and 289.2 nm and a magnitude 78% of that obtained with carbamyl-P under these conditions. The Kᵦ for FLAP at pH 7.0 obtained from a spectral titration is 1.2 mM, assuming three equivalent sites. A Scatchard plot of the data is linear. The value 1.2 mM is much greater than the value of Kᵦ₉ determined in the NMR experiments. Adding 10 mM succinate results in a new difference spectrum just like the one induced by succinate alone (25), indicating that succinate displaces those molecules of bound FLAP responsible for the difference spectrum. The spectral magnitude decreases as the ionic strength of the buffer increases. In 200 mM imidazole acetate with 10 mM FLAP and 2 mg/ml of C₉, no difference spectrum at all is observed, even though FLAP still binds tightly to C₉ as judged by ¹⁹F NMR studies. Both this effect and the large difference between Kᵦ₀ from NMR experiments and the Kᵦ derived from the difference spectrum titration indicate that tight binding of the first molecule of FLAP to C₉ is not accompanied by a change in the near ultraviolet absorption spectrum of the enzyme. If it is assumed that the tight site is saturated without formation of a difference spectrum, spectrophotometric titration of the weak sites leads to an estimate of 0.6 mM for Kᵦ₉ in 5 mM buffer, a value to be compared with 1.0 mM in 10 mM buffer determined by NMR.

Interaction of Reduced FLAP with C₉—The binding of FLAP and reduced FLAP to C₉ were compared to help evaluate the role of the hydrated carbonyl group. The phosphonate protons of reduced FLAP have pKₐ values of 2.7 and 7.3, by titration with KOH. Both ionic forms are assumed to bind to C₉ equally well (19). With low concentrations of buffer (10 mM imidazole acetate, pH 7.0, and 10 mM Tris-acetate, pH 8.0) Δₓ = 16 Hz and Kₓₙ = 1.8 mM at pH 7 and Δₓ = 10 Hz and Kₓₙ = 0.55 mM at pH 8. The change in chemical shifts upon binding and Kₓₙ values are similar to those of FLAP. The data were not adequate to determine accurate values for Kᵦ₀ but two classes of binding sites were definitely observed. Succinate and L-aspartate displace reduced FLAP from C₉.

FLAP and Cₓ₉—Accurate dissociation constants or estimates of cooperativity could not be derived in this case because the bound chemical shift is about 2 Hz at pH 7.0 and only about 1 Hz at pH 8.0, so that changes are extremely small and difficult to measure accurately. The decreased magnitude of the bound shifts indicates that the electronic environment of FLAP is more like its environment in water when it is bound to Cₓ₉ than when it is bound to C₉. The bound linewidth for FLAP at pH 7.0 is larger with Cₓ₉ (26 Hz) than with C₉ (12 Hz), as expected from the different sizes of the two enzyme species.

**DISCUSSION**

Relation of Conditions for Cell Growth to the Number of Tight Binding Sites for Carbamyl-P—Using equilibrium dialysis, Rosenbusch and Griffin (9) found 3.2 ± 0.5 tight binding sites for carbamyl-P to Cₓ₉ at pH 7.0, Kᵦ = 14 μM, compared with 2.3 tight sites and Kᵦ = 2.6 μM from our experiment done under essentially the same conditions (Fig. 5). The cells used by Rosenbusch and Griffin were grown under conditions identical with those for cell Batch A, except that 50 μg/liter of L-leucine was also present. Their yield of enzyme was about twice that obtained when the cells were grown under the conditions of Gerhart and Holoubek (12). Therefore, Cₓ₉ from two independent batches of cells grown in the richer medium have substantially fewer than six tight sites for carbamyl-P. Furthermore, C₉ from the cells grown in Basel has about two tight binding sites for carbamyl-P at pH 7 or pH 8 without succinate and about three with eucineinate, roughly in agreement with our values at pH 8.

The New England Enzyme Center has grown mutant cells for the isolation of aspartate transcarbamylase under the conditions used for cell Batch A since 1969, and enzyme from such cells has been used by a number of investigators. Both Cₓ₉ and C₉ from cell Batch A have one binding site for PALA per catalytic chain, and Hammes et al. (26) have shown that C₉ also prepared from cell Batch A, has three binding sites for carbamyl-P in the presence of succinate at high ligand concentrations. Since the maximum velocities for most preparations of enzymes are quite close to the values given by Gerhart and Holoubek (12) when the assays are performed at high concentrations of carbamyl-P, we conclude that the sites with low affinity for carbamyl-P can be filled eventually and that these sites are then fully active. Furthermore, Rosenbusch* has found that the allosteric properties of Cₓ₉ prepared from cells grown in the richer medium are reproducible and very similar to the properties of enzymes from cells grown according to Gerhart and Holoubek (12). Again, such measurements are usually done at high concentrations of carbamyl-P.

**A Possible Cause for Weak Carbamyl-P Binding**—Both Weber (27) and Hervé and Stark (28) found about two-thirds of the number of free NH₃ termini expected from the number of peptide chains for both C₉ and regulatory subunit prepared from cells grown according to Gerhart and Holoubek (12). Weber (29) also reported that highly variable amounts of methionine and threonine were found as the NH₃ termini in different preparations of regulatory subunit. These results imply that, under conditions of derepression, when a large amount of a single protein is being synthesized, the enzymes which ordinarily remove formylmethionine from nascent chains (30) may be overwhelmed, so that in part formylmethionine or methionine remains instead of the NH₃ termini present in normal cells. Perhaps incomplete removal of formylmethionine is even less efficient in the richer medium, where the yield of enzyme is greater. If C₉ chains with formylmethionyl or methionyl termini were to bind carbamyl-P less well than chains with NH₃-terminal alanine, the low number of tight binding sites for carbamyl-P in both preparations of C₉ studied here would be explained. In the case of Cₓ₉, from cells of Batch A, there are only about two tight binding sites for carbamyl-P, whereas six is the number predicted from the sum of the two C₉ subunits. Therefore, the nature of the NH₃ termini of the regulatory chains in Cₓ₉ may also affect carbamyl-P binding. A correlation between the nature of NH₃ termini and the properties of Cₓ₉ might also provide an explanation for the formation and properties of the unusual enzyme produced in the presence of 2-thiouracil and studied by Kerbiriou and Hervé (31, 32). In this enzyme, homotropic cooperativity has been lost. Since 2-thiouracil is converted to 2-thiouridine phosphate in vivo (see Ref. 33 for a recent discussion and references) and since 2-thio-UMP has been reported to be a strong allosteric inhibitor of Cₓ₉ (34), it may be that 2-thio-

*J. P. Rosenbusch, Biozentrum, Basel, Switzerland, personal communication.

*H. Blair, New England Enzyme Center, personal communication.
Binding of Carbamyl Phosphate to Aspartate Transcarbamylase

UMP or 2-thio-UTP in high concentration in the cells stabilizes a structure of nascent R chains in which correct processing of the NH₂ termini is impeded.

Obviously, careful further work will be required to substantiate or disprove the suggestions made above. However, regardless of the detailed explanation, it is important now to determine the number of tight binding sites for carbamyl-P in preparations of enzyme to be used for binding studies of any kind and for some other kinds of experiments as well. As we have discussed, the capacity of the enzyme to bind 1 molecule of PALA per catalytic chain and to exhibit normal maximum velocity and allosteric properties at high carbamyl-P concentrations does not necessarily mean that the enzyme is chemically homogeneous. With new appreciation for the possible effects of heterogeneity, we must view with new caution our work (23, 35) and the work of others (for example, Ref. 36) with derivatives of C₄ and C₅R₄ in which the number of tight binding sites for carbamyl-P or PALA has been altered through chemical modification. We suggest also that studies of nucleoside triphosphate binding (37-40) and temperature jump studies with nucleoside triphosphates (41-43) may be complicated by enzyme heterogeneity.

Experiments with FLAP and Reduced FLAP—With C₄, the changes in chemical shifts upon binding (Δδ) for FLAP and reduced FLAP indicate shielding of the trifluoromethyl group. However, Δδ is relatively small (10 to 20 Hz) compared with the values 65 to 100 Hz for the CF₃ groups of substrate analogs bound to chymotrypsin, where there is a hydrogen bond between the imidazole of histidine 57 and the carbonyl oxygen of the analogs (1, 2). The small value of Δδ for the binding of FLAP to C₄R₄ indicates that the presence of the regulatory subunit alters the environment of the CF₃ group in the active site. Although counterbalancing effects are possible, it is likely that there is little difference in polarity between the environment in C₄ and the one in solution.

Although both FLAP and carbamyl-P bind tightly to about two sites on C₄ at pH 8, the number of tight sites for FLAP decreases to one at pH 7. In contrast, Rosenbusch finds two tight sites for the binding of carbamyl-P to C₄ at pH 7 or pH 8. It can be seen from Fig. 5 that the number of tight sites for binding of carbamyl-P to C₄R₄ increases slightly with decreasing pH. The pH dependence of Δδ and K₄ is not caused by titration of the ligands since FLAP and reduced FLAP have very different titration curves in water but the same dependence of Δδ and K₄ on pH. The anomalous pH dependence for FLAP may indicate that the highly electronegative CF₃ group interacts with the enzyme in a manner that distorts the active site. Such an interaction is also indicated by the properties of an inactive derivative of C₄ in which the single sulphydryl group in the active site (23) has been modified with CF₃—C(OH)₂CH₃—. This derivative binds carbamyl-P but not succinate in the presence of carbamyl-P (44), just like the inactive thionitrobenzoate derivative, also modified on the sulphydryl group (23). In both cases, electronegative parts of the modifying groups may be near enough to positively charged groups of the enzyme to impede their binding to a carboxylate group of succinate.

Pre-existing Asymmetry or Negative Cooperativity in C₄—Although C₄R₄ is a classic example of an allosteric enzyme, cooperative binding of ligands to unmodified C₄ has not been described previously, except for the kinetic experiments of Heyde (45) with ITP as an inhibitor. Jacobson and Stark (23) had shown that only 1 molecule of PALA binds tightly to C₄ in which the sulphydryl group in each active site has been modified with a thionitrobenzoate group. Although the possibility of intrinsic asymmetry was considered, a model in which a conformational change and negative cooperativity were involved was favored. Kempe and Stark (46) have shown that C₄ modified in the active sites with one or two molecules of pyridoxamine-P produces a large increase in fluorescence upon the binding of PALA, but that the fully modified derivative, with 3 molecules of pyridoxamine-P per trimer, does not, even though it can be shown that PALA still binds to this species. A model involving interaction among the three active sites of the partially modified species was used to explain these findings.

At pH 7, unmodified C₄ has one tight binding site for FLAP and, from Rosenbusch's data, two tight binding sites for carbamyl-P. If these properties are to be explained by intrinsic asymmetry, three different kinds of sites are required. Negative cooperativity, in which conformational changes upon partial saturation result in decreased affinity at unoccupied sites, is an attractive alternative, although at present we do not have evidence to choose definitively between these models.

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J A Ridge, F Roberts, M H Schaffer and G R Stark

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