Electrostatics are central to the function and regulation of Escherichia coli aspartate transcarbamylase, and modeling has suggested that long range electrostatic effects are likely to be important (Glackin, M. P., McCarthy, M. P., Mallikarachchi, D., Matthews, J. B., and Allewell, N. M. (1989) Proteins Struct. Funct. Genet. 5, 66–77; Oberg, H., Trikha, J., Yuan, X., and Allewell, N. M. (1993) Proteins Struct. Funct. Genet., in press). To investigate this possibility from an experimental standpoint, we have examined the effects both of assembly and of removing ionizable and polar side chains outside the active site (Glu-50, Tyr-165, and Tyr-240) on the pH dependence of the kinetic parameters of aspartate transcarbamylase. The holoenzyme (c3r6) assembles from three regulatory dimers (r2) and two catalytically active trimers (c3). pH dependences of the enzyme kinetic parameters suggest that the mechanisms of productive binding of L-Asp to the binary complexes of the catalytic subunit (c4) and holoenzyme (c4r2) with carbamyl phosphate are different. In contrast, the Michaelis complex appears similar for both c3 and c4r2 except for pH shifts of 1 pH unit. Results also indicate that the catastrophic mechanism of the holoenzyme does not involve reverse protonation, as has recently been proposed for the catalytic trimer (Turnbull, J. L., Waldrop, G. L., and Schachman, H. K. (1992) Biochemistry 31, 6562–6569). The tyrosines at positions 165 and 240 are part of a cluster of interactions that links the catalytic subunits in the T state (the c1:c4 interface) and which is disrupted in the T → R transition. The effects of mutating the two Tyr residues are quite different: Y240F has higher than wild-type activity and affinity over the entire pH range, while Y165F has activity and affinity an order of magnitude lower than wild-type. Removal of the regulatory subunits from Y165F increases activity and affinity and restores the pH dependence of the wild-type catalytic subunit. Like Y165F, E50A has low activity and affinity over the entire pH range. Linkage analysis indicates that there is long range energetic coupling among the active site, the c:r subunit interfaces, and residue Y165. The substantial quantitative difference between Y165F and Y240F, both of which are at the c1:c4 interface about 14–16 Å from the closest active site, demonstrates specific path dependence, as opposed to general distance dependence, of interactions between this interface and the active site.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) was one of the first systems exploited to study intramolecular signal transduction (Yates and Pardee, 1956) and remains an important system for analyzing molecular mechanisms of recognition, communication, and regulation. This enzyme catalyzes the first committed step in pyrimidine biosynthesis, formation of N-carbamyl-L-Asp from carbamyl phosphate and L-aspartate. Aspartate transcarbamylase binds L-Asp cooperatively, and its enzymatic activity is allosterically regulated by nucleotide triphosphates (Yates and Pardee, 1956; Gerhart and Pardee, 1962, 1963; Wild et al., 1989). Recent reviews include Allewell (1989), Hervé (1989), Kantrowitz and Lipscomb (1990), Wild and Wales (1990), and Lipscomb (1992, 1994).

The protein is a dodecamer consisting of six catalytic (c) chains and six regulatory (r) chains organized as two catalytic trimers (c3) and three regulatory dimers (r2). Binding of substrates and substrate analogs induces a T → R transition in which the holoenzyme expands by 12 Å along its 3-fold axis. The switch to the high affinity R structure eliminates contacts between c3 subunits (the C1:C4 interface), one set of c3:r2 contacts and domain interactions in the r chain but simultaneously strengthens a second set of c3:r2 contacts, interchain interactions in c3 and interdomain interactions in c chains. Critical features of this transition include large movements of the 80s and 240s loops in the c chains and closure of the c chain domains (see Fig. 1).

Because both the substrates of aspartate transcarbamylase and its regulatory nucleotides have several negative charges, electrostatic effects might be expected to figure heavily in both catalysis and the allosteric mechanism. A number of studies have substantiated this expectation. Gerhart and Pardee (1964) showed that the pH optimum for catalytic activity shifts from 7 at low aspartate concentrations to 8.3 at high aspartate concentrations. These pH optima are assigned to the T and R structural states of the enzyme. In the first systematic study of pH effects, Pastra-Landis et al. (1978) demonstrated differences in the pH dependence of the holoenzyme and catalytic subunit. Thiry and Hervé (1978) and Tauc et al. (1982) showed that the effects of ATP and CTP on enzymatic activity vary markedly with pH. Mutants for which the pH dependence of catalytic activity is different from the wild-type have been found (Ladjimi and Kantrowitz, 1987; Xi et al., 1990; Waldrop...
Electrostatics and pH Dependence of Aspartate Transcarbamylase

In this study we have examined the pH dependence of the kinetics of the holoenzyme and a set of single site mutants in which ionizable or polar side chains outside the active site have been replaced by nonionizable, nonpolar side chains. Two of the mutations (Y165F and Y240F) occur in a cluster of interactions at the c1:c4 interface that forms a critical link between catalytic subunits in the T structural state and is disrupted in the R state (Newton and Kantrowitz, 1990). Because the interactions between the mutated residues and ionizable groups involved in the catalytic mechanism?

MATERIALS AND METHODS

Proteins—Wild-type and mutant holoenzymes were isolated from E. coli strain EK1104 transformed with the appropriate plasmid as described previously (Nowlan and Kantrowitz, 1985). EK1104 and plasmids containing the wild-type pyrB gene (pEK2), and Y240F (pEK33) were provided by Dr. E. R. Kantrowitz (Department of Chemistry, Boston College, Chestnut Hill, MA 02167). Plasmid containing the mutant gene for Y165F was obtained from Dr. J. R. Wild (Department of Biochemistry and Biophysics, Texas A & M University, College Station, TX). E50A was obtained as the purified protein from Dr. Kantrowitz.

c1 and c3 subunits were prepared following Yang et al. (1978) with the modifications introduced by Burz and Allewell (1982). Proteins were stored as precipitates in 3.6 M (NH4)2SO4, 0.1 M Tris-HCl, 0.2 mM dithiothreitol, 0.2 mM EDTA, and 0.2 mM dithiothreitol, pH 8.3, at 4°C. The purity of c1 and c3 assayed by non-denaturing PAGE (Davis, 1964) at pH 8.3, was found to be greater than 95%, with only c1 and c3 aggregates being detectable as contaminants.

Protein concentrations for wild-type and E50A were determined spectrophotometrically at 280 nm using extinction coefficients of 0.59 ml/(mg-cm) for c1, 0.72 ml/(mg-cm) for c3 (Gerhart and Holoubek, 1967). Extinction coefficients for Y165F and Y240F were determined using a colorimetric protein concentration assay (Bio-Rad Protein assay, Bio-Rad); the values obtained were 0.56 and 0.52 ml/(mg-cm), respectively.

Chemicals—Carbamyl phosphate, L-Asp, carbamyl L-Asp, Tris, bis-Tris, and Caps were purchased from Sigma, while the bisubstrate analog, assayed as >80% pure and used without further purification, was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892. Neohydrol (1-[3-(chlorormercuri-2-methoxypropyl)urea] was a gift from Marion Merrell Dow Pharmaceutical Inc. (Cincinnati, OH) and was recrystallized from absolute ethanol before use.

Assay—The colorimetric assay, developed by Prescott and J ones (1969) and modified by Pastra-Landis et al. (1978), was used to monitor formation of carbamyl-L-Asp. The buffer system used was 0.02 M Tris-Cl, 0.02 mM bis-Tris, and 0.02 mM Caps, 0.2 mM dithiothreitol, 0.2 mM disodium ethylenediaminetetraacetate (EDTA), and 0.2 mM Tris, pH 8.3. A 1 ml sample was incubated at 25°C with a 3 ml aliquot. The reaction was stopped by adding 1 ml of ice-cold 25% ethanol and 2 ml of ice-cold 4 M absolute ethanol. The mixture was vortexed and centrifuged for 5 minutes at 12000 × g. The supernatant was removed and the precipitate was washed with 1 ml of ice-cold 4 M absolute ethanol. The precipitate was washed twice with 1 ml of ice-cold 4 M absolute ethanol and was then redissolved in 2 ml of 4 M absolute ethanol. The absorbance at 500 nm was measured.
EDTA (Pastra-Landis et al., 1978). Assays were carried out at 25°C, and assay tubes were equilibrated in a water bath for at least a half of an hour before each assay. The amount of enzyme used in each experiment, which varied with pH and the mutant being studied, was chosen to produce 0.02–0.2 μmol of carbamyl L-Asp in 5 min. Separate carbamyl-L-Asp standard curves were generated for each individual titration curve, since this assay is highly sensitive to room temperature, ambient light, and the batch of color reagent. Stock carbamyl phosphate solutions (48 mM) were prepared fresh in buffer immediately before each titration and kept on ice to keep decomposition to a minimum. Data Analysis—The nonlinear least squares program NONLIN (Johnson and Frasier, 1985) was used throughout. We initially attempted to resolve the kinetic parameters using the algorithms of Pastra-Landis et al. (1978). In the absence of substrate inhibition, saturation curves were fit to the Hill equation

$$V = \frac{V_{\text{max}}[S]^{n}}{K_{s}^{n} + [S]^{n}}$$

(Eq. 1)

where $K_s$ is the dissociation constant for inhibitory substrate binding and $V_s$ is the limiting velocity when substrate inhibition is maximal (Pastra-Landis et al., 1978). However, values of the fitted parameters obtained with this equation were highly correlated and the fitted values of $V_{\text{max}}$ and $K_{s}^{n}$ were much larger than appeared reasonable in light of the raw data, as Pastra-Landis et al. (1978) reported. We find that the equation

$$V = \frac{V_{\text{max}} + V[S]K_{s}}{I + K_{s}[S]^{n} + [S]K_{s}}$$

(Eq. 2)

where $K_s$ is the dissociation constant for inhibitory substrate binding and $V_s$ is the limiting velocity when substrate inhibition is maximal eliminates these problems. This equation describes cooperative substrate binding, incorporated as a Hill coefficient, and partial uncompetitive substrate inhibition.

Plots of log $V_{\text{max}}$ and log $V_{\text{max}}/K_{s}^{n}$ versus pH were fit to equations in which these parameters are partitioned into contributions from molecules in different states of ionization and with different catalytic efficiencies and/or substrate affinities (Cleland, 1977; Turnbull et al., 1992). With the exception of Y165F, all of the data could be fit by models that assume that both $V_{\text{max}}$ and $K_{s}^{n}$ are modulated by the ionization states of one or two independent groups

$$K_{s}^{n} = K_{s}^{a}E+P$$

$$K_{s}^{b}pK_{s}^{b}$$

$$pK_{s}^{b}$$

$$pK_{s}^{b}$$

$$pK_{s}^{b}$$

Scheme I.

where E is the free enzyme, E-CP is the binary complex of aspartate transcarbamylase with carbamyl phosphate, and E-CP-Asp is the ternary complex of aspartate transcarbamylase with carbamyl phosphate and L-Asp. Following Cleland (1977), the equation for a single ionizable group is

$$\log Y = \log(Y_{s} + Y_{s}[K_{s}(H)])/(1 + K_{s}(H))$$

(Eq. 4)

where $Y_s$ is either $V_{\text{max}}$ or $V_{\text{max}}/K_{s}^{n}$, $Y_s$ and $Y_{s}$ are the fitted values of the appropriate parameter for the appropriate protonated and unprotonated species, and $K_{s}$ is the appropriate microscopic dissociation constant ($K_{s}$ for $V_{\text{max}}$ plots; $K_{s}$ in $V_{\text{max}}/K_{s}^{n}$ plots). The general equation for the ionization of two independent groups is

$$\log Y = \log(Y_{s} + Y_{s}[K_{s}(H)])/(1 + K_{s}(H))$$

(Eq. 5)

where $K_{s}$ and $K_{s}$ are macroscopic acid dissociation constants, which are equal to the microscopic constants $K_{s}$ and $K_{s}$ when $pK_{s} - pK_{s} > 1.5$. When $pK_{s} - pK_{s} < 1.5, K_{s} = K_{s} + K_{s}$ and $K_{s}K_{s} = K_{s}K_{s}$. As in Equation 4, $Y_s$, $Y_s$, and $Y_{s}$ are the fitted values of $V_{\text{max}}$ or $V_{\text{max}}/K_{s}^{n}$ for different ionization states of the protein. The higher the index, the lower the protonation state of the protein. Since plots of both log $V_{\text{max}}$ and log $V_{\text{max}}/K_{s}^{n}$ had two maxima for Y165F, these curves were fit to Equation 6, which assumes that ionization in the low pH region does not affect ionization in the high pH region.

$$\log Y = \log(Y_{s} + Y_{s}[K_{s}(H)])/(1 + K_{s}(H))$$

(Eq. 6)

For simplicity, $Y_{s}$, $Y_{s}$, $Y_{s}$, and $Y_{s}$ were assumed to have values of zero, such that $Y_{s}$ and $Y_{s}$ correspond to the singly protonated low and high pH forms. Correlation of the “group” $pK_{s}$ values resolved from such analyses with specific ionizable residues in the enzyme active site is a complex and often assumption-fraught exercise (Knowles, 1976; Brodiehurst, 1994). It minimally requires that one demonstrate or assume that each apparent $pK_{s}$ corresponds to a single side chain and not the sum of several overlapping ionizations, that these side chains are in the active site itself, that for each activity maximum in the pH dependence there is only one ionization state of the active site that is enzymatically active, and that the kinetic mechanism of the enzyme does not change appreciably with pH. As this study demonstrates, however, comparative analysis of the quantitative pH dependence of different mutants and states of assembly of the enzyme does allow one to examine many aspects of the involvement of electrostatics in the mechanism of aspartate transcarbamylase without specific side chain assignments.

RESULTS

Aspartate Titration Curves—Selected sets of data illustrating the effects of pH on L-Asp saturation curves are shown in Fig. 2. The effects of the mutations on both activity and affinity are much greater than the effects of pH, i.e. while both the $K_{s}^{n}$ and $V_{\text{max}}$ of the low activity mutants (E50A and Y165F) span a 4–5-fold range dependent on pH, they both differ by an order of magnitude in their affinity and activity from the wild-type and Y240F enzymes (see also Fig. 3). The pronounced substrate inhibition seen at pH 8.3 and higher in the wild-type enzyme and Y240F is a striking feature of these data. Substrate inhibition either does not occur in the low activity mutants or occurs only at aspartate concentrations greater than 350–400 mM.

The effects of mutating the two Tyr residues in the C1-c4 interface are quite different, with Y240F increasing affinity for L-Asp and catalytic activity above the levels seen in the wild-type enzyme and Y165F having greatly reduced affinity and activity relative to the native enzyme. Removing the r2 subunits of Y165F largely restores its catalytic activity and partially restores L-Asp affinity (see also Fig. 5). E50A, the inter-domain mutant, shows reduced affinity and activity, similar to Y165F. The effects of removing the r2 subunits of E50A were not examined.

pH Dependence of $K_{s}^{n}$, $V_{\text{max}}$, and $n_{H}$—The values of the kinetic parameters derived by fitting the titration curves of the wild-type enzyme and the mutants to Equations 1 or 3 are plotted as a function of pH in Fig. 3. The kinetic properties of the wild-type enzyme and Y240F are very similar over the entire pH range, although the maximal velocity and substrate affinity of Y240F are larger by factors of 1.5 and 2, respectively, than the values for the wild-type enzyme, while its Hill coefficient never exceeds a value of 1.7. Both wild-type and Y240F show maximal values for $V_{\text{max}}$ at pH 9.5 and for $K_{s}^{n}$ at pH 9.0. Y165F and E50A both display low activity and affinity over the entire pH range. Although the pH dependence of $n_{H}$ for E50A is similar to the wild-type enzyme, the maximum $n_{H}$ for Y165F, as well as the largest values of $V_{\text{max}}$ and $K_{s}^{n}$ for both E50A and Y165F are at or near pH 7 (the $K_{s}^{n}$ maximum is at pH 7.5 for Y165F). pH 7 is commonly considered to be the functional pH optimum for the T form of aspartate transcarbamylase, based on measurements of the pH dependence of velocity at single aspartate concentrations (see “Discussion”). Although the low activity and affinity of Y165F and a value of ~1 for $n_{H}$ above pH 7.5 suggests that its ability to undergo the
The low substrate affinity and catalytic activity of Y165F and the unusual pH dependence of these parameters must depend upon subunit interactions in the holoenzyme. When the regulatory subunits are removed, both substrate affinity and \( V_{\text{max}} \) increase by approximately a factor of six, and the pH dependence of these parameters becomes similar to those of wild-type.
wild-type enzyme and 7.5 fitting to Equation 5 are 7.8 with Fig. 5). p catalytically active but substrate affinity is lowest (compare dependence on pH. Substrate inhibition is strongest between derived by fitting to Equation 3 have a reverse bell-shaped groups outside the active site, particularly those involved in E50A plateau at high pH, and hence can be fit to one p there are at least two active species of this holoenzyme. The log Ka values resolved by this analysis may reflect the aggregate interpretation becomes more complicated, since the individual (1992). When this approach is applied to the holoenzyme, in- there are at least two active species of this holoenzyme. The log values for substrate inhibition derived by c3 (Turnbull et al., 1979). The first type of plot identifies groups involved in binding and/or catalysis; the second identifies groups involved only in catalysis. This approach has been applied to c3 by Leger and Hervé (1988) and Turnbull et al. (1992). When this approach is applied to the holoenzyme, interpretation becomes more complicated, since the individual pKa values resolved by this analysis may reflect the aggregate behavior of several titrating groups and/or the behavior of groups outside the active site, particularly those involved in assembly and the T → R transition. Fits to two pKa values

| pKa | log Vmax/Kmapp | log Vmax/Kmapp |
|-----|----------------|----------------|
| c3  | 6.46 ± 0.18    | 7.32 ± 0.08    |
| c6r6| 5.81 ± 0.42    | 6.34 ± 0.25    |

From Turnbull et al. (1992), in 0.1 M MES, 0.051 M N-ethylmorpholine, and 0.061 M diethanolamine with 0.2 mM EDTA and 2 mM β-mercaptoethanol.

c3, with maxima for Vmax and Kmapp at 8.5 and 9.5, respectively (see Fig. 5 and Table I).

Substrate Inhibition—As shown in Fig. 4, values of log Kc derived by fitting to Equation 3 have a reverse bell-shaped dependence on pH. Substrate inhibition is strongest between pH 8 and 9, where the wild-type enzyme and Y240F are most catalytically active but substrate affinity is lowest (compare with Fig. 5). pKa values for substrate inhibition derived by fitting to Equation 5 are 7.8 ± 0.2 and 9.5 ± 0.2 for the wild-type enzyme and 7.5 ± 0.2 and 10.0 ± 0.2 for Y240F.

Apparent pK Values—pKa values of ionizable groups involved in substrate binding and catalysis are often derived by examining the pH dependence of log Vmax/Kmapp and log Vmax (Cleland, 1977; Dixon et al., 1979). The first type of plot identifies groups involved in binding and/or catalysis; the second identifies groups involved only in catalysis. This approach has been applied to c3 by Leger and Hervé (1988) and Turnbull et al. (1992). When this approach is applied to the holoenzyme, interpretation becomes more complicated, since the individual pKa values resolved by this analysis may reflect the aggregate behavior of several titrating groups and/or the behavior of groups outside the active site, particularly those involved in assembly and the T → R transition.

Plots of log Vmax and log Vmax/Kmapp versus pH for wild-type, Y240F, Y165F, and E50A holoenzymes and Y165F c3 are shown in Fig. 5. Plots of log Vmax for wild-type enzyme, Y240F, and E50A plateau at high pH, and hence can be fit to one pKa. In contrast, the double maxima in both Y165F plots indicate that there are at least two active species of this holoenzyme. The log Vmax and log Vmax/Kmapp profiles for the holoenzymes are obviously different from those of wild-type c3 (Turnbull et al., 1992) and Y165F c3.

Although the results for the wild-type, Y240F, and E50A holoenzymes can be fit reasonably well to a single ionizable groups, they were fit to both one and two groups so that these fits could be compared with those derived for c3 by others. The fitted values of the parameters are given in Tables I and II. Table II reports the fitted Y values as log Kcat and log Kcat/Km to facilitate comparison between the different assembly states of the enzyme.

Fitting the log Vmax curves of both wild-type and Y240F holoenzymes to either one or two groups identifies a group with an apparent pKa of 7.8–8. The fitted values of Kcat for individual species given in Table II indicate that this group is unprotonated in the major active species. Fitting log Vmax/Kmapp of the wild-type and Y240F holoenzymes to one pKa identifies a group with an apparent pKa in the range of 6.4–7.1, while the two group fit identifies a second group with a pKa of 7.8. Both fits indicate that in the active species one of these groups is unprotonated (Table II). The major differences between E50A and the wild-type and Y240F holoenzymes are that the second pKa is shifted down to 7.2 in the fits to log Vmax, and shifted up to 9.4 in the fits to log Vmax/Kmapp.

Test of the Reverse Protonation Model—Fig. 6 compares the fit to the experimental values of Vmax/Kmapp values for one- and two-group fits in the presence and absence of reverse protonation. Reverse protonation is the participation in the catalytic mechanism of a species in which the ionization state of two groups is the reverse of what would be predicted from their pKa values. The model for catalysis in the catalytic trimer proposed by Turnbull et al. (1992) postulates reverse protonation of the catalytically productive binary complex with carbamyl phosphate in c3. The curves in all panels of Fig. 6 are calculated by assuming that only one or two of the several protonation states of the protein is catalytically active (see the legend to Fig. 6). The curves in Fig. 6, a and b, were calculated assuming there is no reverse protonation (i.e. the group with the lower pKa ionizes at the lower pH). These curves show that either species X alone (in the one pKa fits) or species X + XH+ (in the two pKa fits) as resolved from fitting the log Vmax/Kmapp plots reproduce the original pH dependence of Vmax/Kmapp. In contrast, the curves shown in Fig. 6, c and d, which were calculated assuming reverse protonation, do not generate the plateau at high pH seen in the wild-type and Y240F data. Note, however, that for the catalytic trimer of mutant Y165F, the reverse protonation model clearly fits the data better.

Thermodynamic Coupling of the Active Site to the 240s Loop and the cr Assembly Interface—Since changes in pK values correspond to changes in free energies of ionization (ΔG = 2.303 RT pK), the change in the pK value of a given group that results from mutating a second group can be thought of as a measure of the interaction energy (ΔG) between the two groups. By analogy to Loewenthal et al. (1993), ΔGactive site = mutation site = –RT ln(Kmapp/Kmappmutant) = 1.364 ΔpK, where ΔpKa is the change in the pKa derived from the pH dependence.
of the wild-type versus the mutant enzyme. Similarly, a change in the pK value of a functional group that results from assembling the holoenzyme from its subunits is a measure of the net sum interaction energy between the group and the subunit interfaces. The active site pK shifts caused by assembly and mutation thus reflect long range energetic coupling within the molecule.

The pK values derived for wild-type and mutant catalytic subunits and holoenzymes are linked by the following thermodynamic cycle.

\[
\begin{align*}
\text{mut} & \quad c_3^\text{mut} \quad \text{assembly} \\
\rightarrow & \quad c_6^{\text{mut}} \\
\end{align*}
\]

**Scheme II.**

The only mutant for which data are available for both \(c_3\) and \(c_6\) is Y165F. Although these data yield values that only crudely approximate real free energies, examination of those thermodynamic cycles that balance provides further evidence for long range coupling within the molecule. For example, consider the thermodynamic cycles for pK values derived from \(\log V_{\text{max}}/K_m\) plots that link pK values \(c_3^\text{mut}, c_3^\text{wt},\) and \(c_6^\text{wt}\) with pK values \(c_6^\text{mut}\) and \(c_6^\text{mut}\).

Here the subscripts denote which pK values are being used to calculate the \(\Delta G\) along each pathway, e.g. \(\Delta G_{2\rightarrow 2}^\text{mut}\) along the upper edge of the cycle is the interaction energy estimated from the shift in pK in the wild-type enzyme as it assembles (pK of \(c_3^\text{wt} \rightarrow \text{pK}_{2}^\text{wt}\) of \(c_6^\text{mut}\)). All possible linkages among the titrating groups can be examined in this way. Using these linkages, one
Electrostatics and pH Dependence of Aspartate Transcarbamylase

### Table II

| Type of Mutation | Y_a (X/H) | Y_b (X/H) | Y_c (X) | K<sub>app</sub> (mM) |
|------------------|-----------|-----------|---------|------------------|
| WT (c<sub>f</sub>) | 71 ± 7 | 0.95 ± 0.39 | 0.74 ± 0.02 | 80 ± 4 |
| Y240F (c<sub>f</sub>) | 16 ± 2 | 0.20 ± 0.02 | 0.74 ± 0.02 | 80 ± 4 |
| E50A (c<sub>f</sub>) | 71 ± 7 | 0.95 ± 0.39 | 0.74 ± 0.02 | 80 ± 4 |

### Table III

| Type of Mutation | Y_a (X/H) | Y_b (X/H) | Y_c (X) | K<sub>app</sub> (mM) |
|------------------|-----------|-----------|---------|------------------|
| WT (c<sub>f</sub>) | 71 ± 7 | 0.95 ± 0.39 | 0.74 ± 0.02 | 80 ± 4 |
| Y240F (c<sub>f</sub>) | 16 ± 2 | 0.20 ± 0.02 | 0.74 ± 0.02 | 80 ± 4 |
| E50A (c<sub>f</sub>) | 71 ± 7 | 0.95 ± 0.39 | 0.74 ± 0.02 | 80 ± 4 |

**a** K<sub>a</sub> values are all expressed as turnover/active site, so that c<sub>f</sub> and c<sub>f</sub><sub>6</sub> can be compared.

**b** The subscript of H corresponds to the pK<sub>a</sub> of the ionizable group; for example H<sub>1</sub> is the proton with a pK<sub>a</sub> of pK<sub>a</sub>.

**c** From Turnbull et al. (1992), in 0.1 mM MES, 0.051 M N-ethylmorpholine, and 0.051 M diethanolamine with 0.2 mM EDTA and 2 mM β-mercaptoethanol.

**d** K<sub>app</sub> value calculated from k<sub>cat</sub>K<sub>app</sub> = Y<sub>b</sub> for species H<sub>1</sub> and/or H<sub>2</sub>.

### Figure IV. 

The one exception to this global coupling is the linkage in the binary complex between pK<sub>a</sub> in c<sub>f</sub> and the highest pK<sub>a</sub> in c<sub>f</sub> and c<sub>f</sub><sub>6</sub>. The finding of an independent set of pK<sub>a</sub> values in the binary complex, but not in the Michaelis complex, may be indicative of the T to R conformational switch. Data from equilibrium isotope exchange kinetics indicate that the T to R transition accompanies the binary to Michaelis complex transition, possibly beginning just before or simultaneously with the binding of the first asparate. (Hsuanyu and Wodler, 1987) Residue Tyr-165 shifts from an interchain to an intrachain interaction when the enzyme switches from the T to the R state. This shift will change the way residue Tyr-165 is energetically coupled to the rest of the molecule and the observed loss of this one independent set of pK<sub>a</sub> values may be a marker for the allosteric switch.

### DISCUSSION

This study represents the first detailed analysis of the pH dependence of the kinetic parameters of both the holoenzyme and a set of single-site mutants. Two levels of questions are addressed: the effects of assembly on the catalytic mechanism of the holoenzyme and the effects of mutations.

### Assembly and the Catalytic Mechanism

There are obvious qualitative differences in the pH dependence of the kinetic parameters of the holoenzyme and catalytic subunit. Whereas both log V<sub>max</sub> and log V<sub>max</sub>K<sub>m</sub> for c<sub>f</sub> have a bell-shaped dependence on pH (Leger and Herve, 1988; Turnbull et al., 1992), log V<sub>max</sub> for the holoenzyme is a half-bell, and

---

**Tyr-165**

\[ \text{CF assembly} \quad \text{--- active site} \]

**Scheme IV.**

---

such that changes in one of the three "pairwise" relationships lead to compensatory changes in the other two. It is unlikely that these three regions of the molecule represent an isolated three site coupling, however. More likely, these results reflect global long range coupling throughout the enzyme that can profoundly and independently influence the electrostatics at the active site.
log $V_{\text{max}}/K_m$ plateaus above pH 8. These differences raise the question of whether the catalytic mechanisms of the $c_5$ subunit and holoenzyme differ, or whether such differences result simply from modest quantitative shifts in $pK_a$ values of the same functional groups.

Two models of the catalytic mechanism of the catalytic trimer have been proposed. In their analysis of the pH dependence of the catalytic trimer, Leger and Hervé (1988) suggested that groups with $pK_a$ values of 7.1 and 9.1 in the binary complex of $c_5$ with carbamyl phosphate are involved in binding L-Asp and that a group with an $pK_a$ of 9.5 in the ternary complex with carbamyl phosphate is protonated. Turnbull et al. (1992) proposed that binding of L-Asp involves a group that must be protonated for binding with a $pK_a$ of 7.3 in the binary complex with carbamyl phosphate and a $pK_a > 9$ in the ternary complex. Catalysis was proposed to involve two groups: one with a $pK_a$ of 9.1 in the binary complex and 7.2 in the Michaelis complex that must be deprotonated and a second group with a $pK_a$ of 9.5 in the Michaelis complex that must be protonated. A novel feature of this model is reverse protonation of the catalytically productive form of the binary complex with carbamyl phosphate. This in turn requires that the catalytically productive form be a minor species ($<0.1\%$).

One possible explanation for the change in shape of the $V_{\text{max}}/K_m^\text{app}$ profile for $c_6r_6$ relative to $c_3$ is that L-Asp is a sticky substrate for $c_6r_6$ with a rate of dissociation less than $k_{\text{cat}}$. This seems unlikely as values of $k_{\text{cat}}$ and $K_m^\text{app}$ are similar for the holoenzyme and catalytic subunit, and the possibility that L-Asp is a sticky substrate for the catalytic subunit has been ruled out by Turnbull et al. (1992). Instead, the fits reported in Tables I and II and shown in Fig. 5 suggest that there are significant differences between the carbamyl phosphate bound binary complexes of $c_6r_6$ and $c_3$. This would not be surprising, since subunit interactions in the holoenzyme, particularly those between the catalytic subunits at the $c_3r_6$ interface, constrain active site residues in the holoenzyme to a greater extent than they are constrained in $c_3$.

On the other hand, the log $V_{\text{max}}$ plot for the holoenzyme can be fit with the same model used to fit $c_5$, the fitted $pK_a$ values differ by $\approx 1\ pH$ unit (7.9 versus 7.2; 16.5 versus 9.5; from Table I), and the turnover number and $K_m^\text{app}$ of the putative active species are similar (750 versus 680 s$^{-1}$ and 16 versus 8 mM; from Table II). At the saturating substrate concentrations at which $V_{\text{max}}$ is determined, domain closure in the holoenzyme creates a highly constrained active site in which the interactions of many side chains with the substrates are optimized. Since domain closure would be expected to be at least as efficient in $c_5$, this step in the catalytic mechanism might be expected to be the same in both proteins. The half-bell log $V_{\text{max}}$ profile of $c_5r_6$ can be accounted for simply by the one pH unit increase in the $pK_a$ of the more basic group involved in catalysis. Modeling indicates that a very small change in the relative positions of two charged side chains is sufficient to generate a $pK_a$ change of this magnitude (Oberoi et al., 1995).

#### Effects of Mutations

Mutation and Mechanism—Mutant E50A was originally designed to impede domain closure by eliminating interdomain salt bridges between Glu-50, Arg-167, and Arg-234 in the R state (Newton and Kantrowitz, 1990). It has been shown to have a random kinetic mechanism (Lee et al., 1995). The similarities between Y165F and E50A may indicate that domain closure is also difficult in Y165F, and this may in turn give rise to a random kinetic mechanism, lack of substrate inhibition, and a shift in the pH optimum for $V_{\text{max}}$. However, a change in

---

**Fig. 6.** Calculated pH dependence of $V_{\text{max}}/K_m^\text{app}$ of specific protonation species. Solid lines are calculated $V_{\text{max}}/K_m^\text{app}$ values from the species distributions and $Y$ values for the fits to two $pK_a$ values; dashed lines are calculated values from the fits to a single $pK_a$ value. Symbols are experimental values: WT (○), E50A (●), Y240F (□), Y165F $c_5r_6$ (■), Y165F $c_3$ (▲). The units are mmol/mg/h for $V_{\text{max}}$ and mM for $K_m^\text{app}$. a and b demonstrate reasonable agreement between the experimental data for the wild-type enzyme and Y240F with both the one-group model in which $X$ is the active species and the two-group model in which species $XH^+_1$ and $X$ contribute to productive binding. Only the second model fits the data for E50A. Also shown is the comparison for the four group model with two active species for Y165F. c and d show that the $V_{\text{max}}/K_m^\text{app}$ values calculated from $Y_{\text{app}}$ and the distribution of $XH^+_1$, the catalytically productive species in the model of Turnbull et al. (1992), do not agree with the experimental data for the wild-type enzyme, Y240F and E50A. The calculated values have been scaled to account for the fact that $XH^+_1$ is a minor species generated by reverse protonation.
kinetic mechanism (the order and relative rates of binding and catalytic events) does not imply a change in the catalytic mechanism (the specific residues involved in facilitating catalysis and their functions). It may be, however, that a change in kinetic mechanism from ordered to random substrate binding results in residues involved in the carbamyl phosphate portion of the active site influencing the apparent pH dependence of aspartate binding.

The T to R Transition—Since the mutated residues are located in regions of the holoenzyme whose three-dimensional structure changes during the T → R transition and the T → R transition and the ionization state of the protein are known to be linked (Gerhart and Pardee, 1964; Thiry and Hervé, 1978), the results presented here allow a number of long standing questions regarding aspartate transcarbamylase to be addressed.

The first is the question of whether pH alone can induce the T to R switch in some mutants. In principle, it would be possible for mutants that appear T-like at one pH to become R-like at a different pH, and vice versa. However, since the variations in substrate affinity and catalytic activity with pH of any individual mutant are much less than those produced by mutation, radical changes in quaternary structure with pH appear unlikely. Instead, the Hill coefficients of the mutants vary less with pH than the wild type, suggesting their ability to undergo the T → R transition is reduced, or that in some way the structural-energetic “distance” between the T and R states, which gives rise to cooperativity, is diminished in the mutant enzymes. This is turn bears on two other questions that have frequently been raised.

1) Can mutations “lock” the enzyme into the T or R state? If the mutant enzyme exhibits cooperativity, manifested either by having a Hill coefficient \( >1 \) in Equation 3 or by curvature in a Hanes plot (see also Allewell and LiCata (1995)), then it obviously cannot be locked in the T or R state. Many mutants are noncooperative under some conditions and cooperative under other conditions. Mutants that have previously believed to be locked into one conformation have later been found to be cooperative under other solution conditions; for example E50A of this study and E239Q. This question can be answered in the affirmative only after an exhaustive examination of the behavior of the mutant.

2) Can pH dependences at high and low aspartate concentrations be used as a conformational probe of the enzyme (i.e. as a T versus R state indicator)? It has been known for many years that in wild-type aspartate transcarbamylase the pH dependence of velocity at very low [L-Asp] has a maximum at pH 7, while at high [L-Asp] the velocity maximum shifts to pH 8.3 (Gerhart and Pardee, 1964). These optima are generally assigned to the T and R states of the enzyme, respectively. The exact positions of these optima depend on solution conditions and on the specific substrate concentrations used to determine them. Since \( K_{\text{app}} \), \( V_{\text{max}} \), and \( n_\text{H} \) for aspartate transcarbamylase all change with pH, the apparent velocity at a single [L-Asp] over a range of pH cannot be related to any single kinetic property of the enzyme. When full titration curves are collected as a function of pH, as in this study, it can be seen that there is not a one to one correlation between the pattern of pH dependence and the allosteric state of the enzyme. Both the \( V_{\text{max}} \) and the \( K_{\text{app}} \) of the wild-type enzyme and Y240F are maximal at about pH 9 (Fig. 3). These are opposing effects, i.e. catalysis is most rapid at pH 9 and slowest at pH 6–7, while binding is tightest at pH 6–7 and weakest at pH 9. For Y165F and E50A both the highest \( V_{\text{max}} \) and the weakest \( K_{\text{app}} \) occur near pH 7 (pH 7.5 for Y165F \( K_{\text{app}} \)). Examining the pH dependence in the usual way (i.e. at one low and one high [L-Asp]) will yield a composite of these two opposing effects that varies with each pH. Surely the weakest \( K_{\text{app}} \) is not associated with the R state of the enzyme, so it is reasonable to assume that the high pH maxima of wild-type and Y240F reflect an R state enzyme? Likewise, associating the pH 7 maxima of the low activity mutants with a T-like state would require that the highest \( K_{\text{app}} \) be associated with that state. In addition, how can classifying E50A as either a T-like or R-like be rationalized with the nearly exact equivalence of the magnitude and pH dependence of \( n_\text{H} \) for E50A and the wild-type enzyme? It would appear then, that although a cursory examination makes this diagnostic seem quite attractive, without additional types of information, the position of the pH maxima for an aspartate transcarbamylase mutant should not be used as an indicator of the conformational state of the enzyme.

The question of whether mutations can push aspartate transcarbamylase into a stable intermediate state (i.e. different from T or R) is also frequently raised. The term “intermediate state” is quite broad and can be operationally defined in a number of ways. Structural methods are the best way to address the question of intermediates. Attempting to establish intermediate forms from functional studies and mutational analyses in a system where cooperativity is still characterized only by a Hill coefficient is inadvisable (see Ackers et al. (1992) for an example of the type of approach necessary).

Long Distance Electrostatic Interactions—The relationship between changes in residues distant from the active site and changes in the pH dependence of enzymatic activity has been a topic of interest to enzymologists and protein engineers for some time (cf. Thomas et al. (1985) and Russell and Fersht (1987)), as has been the challenge of predicting these interactions from electrostatic modeling (Gilson and Honig, 1987; Sternberg et al., 1987). Analysis of the crude thermodynamic cycles linking holoenzyme assembly, enzyme activity, and residue Tyr-165 point toward long range energetic coupling networks within aspartate transcarbamylase that are manifested in the enzyme’s pH dependence.

The mutations characterized in this study yield further information about the nature of long distance coupling in the molecule. The smallest distances between any atom of the bisubstrate analog PALA and any atom of Tyr-240 and Tyr-165 are 16 and 14 Å, respectively. If the intramolecular interactions that produce the observed pK\(_a\) shifts depended only upon distance, the effects of removing these tyrosines should be nearly identical, while the pK\(_a\) shift caused by altering Glu-50, which is much closer to the active site, should be larger than either of the other two substitutions. This is clearly not the case. The significant quantitative difference between the two nearly equidistant Tyr to Phe substitutions demonstrates specific path dependence of this coupling, as opposed to either distance dependence or a dependence on the type of substitution.

These functional studies allow us to observe pK\(_a\) changes at the active site upon mutating sites elsewhere in the protein. Electrostatic modeling allows us to look at the same interaction in the opposite direction. Recent calculations using a multigrid approach to solving the nonlinear Poisson-Boltzmann equation (Oberoi and Allewell, 1993) indicate that active site ligation induces a \( \Delta pK_a \) of \( <0.3 \) pH units for Tyr-240, but causes the pK\(_a\) values of both Glu-50 and Tyr-165 to shift by \( >1.5 \) pH units. Hence, both experimental studies of the pH dependences of the functional parameters and electrostatic calculations predict a specific, strong interaction between the active site of the enzyme and residues Tyr-165 and Glu-50 and only a weak interaction with residue Tyr-240. These correla-
ties of experiment and calculation support the premise that the interactions between the sites of mutation and the active site are predominantly electrostatic and suggest that engineering the activity of enzymes as complex as aspartate transcarbamylase by altering specific long range interactions predicted by modeling may be an achievable goal.

Acknowledgments—We thank Drs. Evan Kantrowitz and James Wild for generously providing the mutants used in this study. We also thank Dr. Larry Wackett for a critical reading of the manuscript.

REFERENCES

Acker, G. K., Doyle, M. L., Myers, D., and Daugherty, M. A. (1992) Science 255, 64–63
Allsop, N. (1987) Nature 328, 316–330
Brocklehurst, K. (1994) Nature 94, 515–518
Burz, D. S., and Allewell, N. M. (1982) J. Mol. Biol. 155, 161–168
Davis, B. J. (1964) J. Mol. Biol. 7, 235–354
Dixon, M., Webb, E. C., Thorne, C. J. R., and Tipton, K. F. (1979) in Methods Enzymol. Vol. 54, pp. 48–55, Academic Press, New York
Dixon, M., Webb, E. C., Thorne, C. J. R., and Tipton, K. F. (1979) Enzymes, 3rd Ed., pp. 138–164, Longman, London, United Kingdom
Gouaux, J. E., Lipscomb, W. N., and Kantrowitz, E. R. (1989) Biochemistry 28, 7989–8003
Hervé, G. (1989) in Allosteric Enzymes (Hervé, G., ed) pp. 61-79, CRC Press, Boca Raton, FL
Henzato, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Edwards, B. F. P., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C., and Lipscomb, W. N. (1982) J. Mol. Biol. 160, 219–263
Hsuanyu, Y., and Wedler, F. C. (1987) Arch. Biochem. Biophys. 259, 116–120
Johnson, M. L., and Frasier, S. G. (1985) Methods Enzymol. 130, 301–342
Kantrowitz, E. R., and Lipscomb, W. N. (1980) Trends Biochem. Sci. 5, 53–59
Ke, H. M., Honzatko, R. B., and Lipscomb, W. N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4037–4040
Knie, B. L., and Alliswell, N. M. (1975) Biochemistry 14, 224–230
Krause, K., L. Vodz, K. W., and Lipscomb, W. N. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 164–167
Krause, K. L., Vodz, K. W., and Lipscomb, W. N. (1987) J. Mol. Biol. 193, 527–533
Ladjimi, M. M., and Kantrowitz, E. R. (1987) J. Biol. Chem. 262, 312–318
Ladjimi, M. M., and Kantrowitz, E. R. (1988) Biochemistry 27, 276–283
Lee, B. H., Ley, B. W., Kantrowitz, E. R., O’Leary, M. H., and Wedler, F. C. (1995) J. Biol. Chem. 270, 15620–15627
Lege, D., and Hervé, G. (1988) Biochemistry 27, 4293–4298
Lipscomb, W. N. (1992) Proc. Robert A. Welch Found. 36, 143–144
Lipscomb, W. N. (1994) Adv. Enzymol. Relat. Areas Mol. Biol. 68, 67–151
Loewenthal, R., Sancho, J., Reinikainen, T., and Fersht, A. R. (1993) J. Mol. Biol. 232, 574–583
McCarty, M. P., and Allewell, N. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6824–6828
Middleton, S. A., and Kantrowitz, E. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5866–5870
Middleton, S. A., Stebbins, J. W., and Kantrowitz, E. R. (1989) Biochemistry 28, 1617–1626
Newton, C. J., and Kantrowitz, E. R. (1990) Biochemistry 29, 1444–1451
Nowlan, S. F., and Kantrowitz, E. R. (1980) J. Biol. Chem. 255, 14712–14716
Oberoi, H., and Allewell, N. M. (1993) Biophys. J. 65, 48–55
Oberoi, H., Trikha, J., Yuan, X., and Allewell, N. M. (1995) Proteins Struct. Funct. Genet. In press
Pastra-Landis, S. C., Evans, D. R., and Lipscomb, W. N. (1978) J. Biol. Chem. 253, 4624–4630
Prescott, L. M., and Jones, M. E. (1969) Anal. Biochem. 32, 408–419
Russell, A. J., and Fersht, A. R. (1987) Nature 328, 496–500
Sternberg, M. J. E., Hayes, F. R. F., Russell, A. J., Thomas, P. G., and Fersht, A. R. (1987) Nature 330, 86–88
Tauc, P., Leconte, C., Kerbiriou, D., Thiry, L., and Hervé, G. (1982) J. Mol. Biol. 156, 155–168
Thiry, L., and Hervé, G. (1978) J. Mol. Biol. 125, 515–534
Thomas, P. G., Russell, A. J., and Fersht, A. R. (1985) Nature 317, 375–376
Turnbull, J. L., Waldrop, G. L., and Schachman, H. K. (1992) Biochemistry 31, 6562–6569
Waldrop, G. L., Turnbull, J. L., Parmentier, L. E., O’Leary, M. H., Cleland, W. W., and Schachman, H. K. (1992a) Biochemistry 31, 6568–6591
Waldrop, G. L., Turnbull, J. L., Parmentier, L. E., O’Leary, M. H., Cleland, W. W., and Schachman, H. K. (1992b) Biochemistry 31, 6592–6597
Wales, M. E., Hoover, T. A., and Wild, J. R. (1988) J. Biol. Chem. 263, 6109–6114
Wild, J. R., and Wales, M. E. (1990) Annu. Rev. Microbiol. 44, 193–218
Wild, J. R., Loughrey, C. S., and Corder, T. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 46–50
Xie, X. G., Van Vliet, F., Ladjimi, M. M., Cunin, R., and Hervé, G. (1990) Biochemistry 29, 8491–8498
Yang, Y. R., Kirschner, M. W., and Schachman, H. K. (1978) Methods Enzymol. 51, 31–41
Yates, R. A., and Pardee, A. B. (1956) J. Biol. Chem. 221, 757–770