Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Sokolovsky, M., Harrell, D., and Riordan, J. F. (1969), *Biochemistry 8*, 4741.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3583.
Svensson, H. (1950), *Acta Chem. Scand. 4*, 399.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Vallee, B. L., and Riordan, J. F. (1969), *Annu. Rev. Biochem. 38*, 733.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245*, 3565.
Vincent, J. P., Lazdunski, M., and DeLaage, M. (1970), *Eur. J. Biochem. 12*, 250.
Weber, K. (1968a), *Nature (London) 218*, 1116.
Weber, K. (1968b), *J. Biol. Chem. 243*, 543.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Local and Gross Conformational Changes in Aspartate Transcarbamylase†

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ABSTRACT: Aspartate transcarbamylase and the free catalytic subunits isolated from it are known to undergo different, gross conformational changes upon the addition of the same, specific ligands. However, there has been little direct evidence indicating how, and to what extent, the catalytic subunits within the intact enzyme change upon the addition of ligands. In an effort to study the ligand-promoted conformational changes of the subunits within aspartate transcarbamylase we have prepared an enzyme-like complex composed of native regulatory subunits and modified catalytic subunits containing nitrotyrosyl chromophores whose spectrum was sensitive to the local environment. This modified enzyme exhibited the homotropic and heterotropic effects characteristic of native aspartate transcarbamylase. The spectral changes in the complex on binding ligands were similar to the changes observed for the free nitrated catalytic subunits. These spectral shifts representing local conformational changes were in marked contrast to the gross changes in hydrodynamic behavior which were in opposite directions for the intact enzyme and isolated catalytic subunit. The spectral change of the isolated subunit at varying succinate concentrations could be described by a hyperbola and was closely linked to the gross change in conformation revealed by difference sedimentation measurements. In contrast the enzyme-like complex had a titration curve for the spectral change which was sigmoidal in shape and which was clearly separated from the curve relating the change in sedimentation coefficient to succinate concentration. These results indicate that the local and gross conformational changes in the enzyme molecules are weakly linked. The local changes in the catalytic chains are sequential and strongly linked to ligand binding, whereas the gross conformational change appears to be concerted and is weakly linked to the binding of ligands.

Difference sedimentation experiments (Kirschner and Schachman, 1971a) have revealed that the free catalytic subunit of aspartate transcarbamylase undergoes a ligand-promoted conformational change opposite in direction to that exhibited by the native enzyme (Gerhart and Schachman, 1968; Kirschner and Schachman, 1971b). Whereas the isolated catalytic subunits change to a more compact or isometric conformation, the intact enzyme becomes more swollen or anisometric upon the binding of the same ligands. Although these hydrodynamic data show that the conformational change in the whole enzyme is not merely the sum of the changes observed for the isolated subunits, they provide virtually no information as to the relationship between the conformational changes in the isolated catalytic subunits and those for the same subunits within the enzyme complex. To investigate such a possible relationship we required a sensitive technique which could be employed selectively on both the isolated catalytic subunits and on these same subunits within intact enzyme molecules.

As shown in the preceding paper (Kirschner and Schachman, 1973), nitration of the catalytic subunit under controlled conditions produced a derivative having enzymic and physical properties similar to the native protein. Moreover, the nitrotyrosyl residues in the modified protein had an absorption spectrum which was altered markedly upon the addition of only those stereospecific ligands which promote conformational changes in the native subunit. This nitrated derivative combined with native regulatory subunits to form a reconstituted enzyme-like complex. Thus the nitrotyrosyl chromophore should serve as a sensitive spectral probe for the examination of conformational changes in the catalytic subunits both in the isolated state and within the intact enzyme molecules.

Gerhart and Schachman (1968) showed that the binding of succinate (in the presence of carbamyl phosphate) and the

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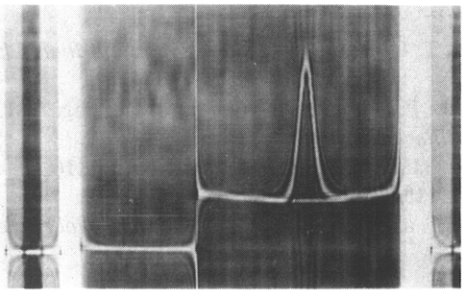

FIGURE 1: Sedimentation velocity pattern of $C^N(R)$. The reconstituted enzyme-like complex at a concentration of 8 mg/ml was sedimented at 56,000 rpm in 0.04 M potassium phosphate buffer (pH 7), containing 2 mM mercaptoethanol and 0.2 mM EDTA, 20°. The schlieren phase plate was set at an angle of 65° and the photograph was taken 28 min after reaching speed.

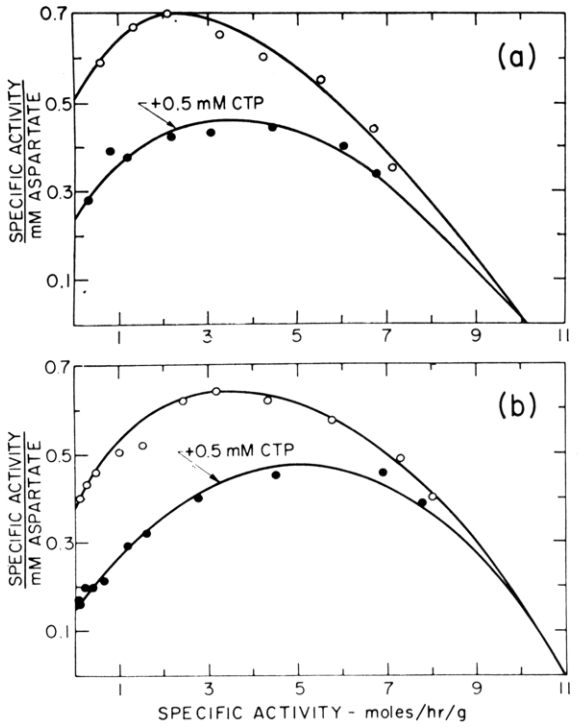

FIGURE 2: Kinetics of aspartate transcarbamylase and $C^N(R)$. The concentration of enzymes was 1 μg/ml. The enzyme activity was measured at various concentrations of aspartate according to the assay procedure of Porter *et al.* (1969). The buffer was 0.05 M imidazole acetate at pH 7. Data are plotted as specific activity divided by aspartate concentration on the ordinate *vs.* specific activity on the abscissa. Specific activities are given as moles carbamyl aspartate formed per hour per gram of protein. At the top (a) are the results with $C^N(R)$ and the bottom curves (b) were obtained with native enzyme. Data represented by open circles were obtained with carbamyl phosphate and varying amounts of aspartate in the assays. For the data represented by closed circles the corresponding solutions contained in addition CTP at a concentration of 0.5 mM.

conformational changes accompanying its binding to aspartate transcarbamylase are clearly separable (*i.e.*, weakly linked) in terms of the concentration of succinate required to produce a given degree of saturation and a corresponding change in conformation. In contrast, as shown in the preceding paper (Kirschner and Schachman, 1973), there appeared to be a strong linkage between binding and conformational changes in the isolated catalytic subunit. Do these observations indicate that the individual catalytic subunits in aspartate transcarbamylase show conformational changes weakly linked to binding? Or is the binding to the catalytic subunits closely linked to local conformational changes in those subunits and are these changes weakly linked to a different, gross transition involving the entire enzyme complex?

As shown here, the spectral change in the nitrated catalytic subunits within aspartate transcarbamylase molecules occurs proportionately to binding. In this respect the catalytic subunits within the enzyme complex are similar to the free subunits. However, the conformational changes within the subunits in the enzyme do not coincide with the gross conformational transition measured by difference sedimentation. In general the data support a model in which local conformational changes in the individual catalytic subunits are strongly linked to ligand binding but are weakly linked to a gross transition affecting the volume and shape of the enzyme molecule and the packing of the subunits within it.

Experimental Section

Aspartate transcarbamylase, catalytic subunits and regulatory subunits were prepared as described in the preceding paper (Kirschner and Schachman, 1973). A nitrated derivative of the catalytic subunit with 85% remaining enzymic activity and 0.7 nitrotyrosine/chain (Kirschner and Schachman, 1973) was used for the reconstitution of enzyme-like molecules. The complex containing nitrated catalytic subunits was prepared by mixing an excess of regulatory subunit with nitrated catalytic subunit at 2 mg/ml in 0.04 M potassium phosphate buffer at pH 7 containing 0.1 M mercaptoethanol; the solution was then dialyzed against 0.1 M Tris-HCl (pH 8.5)–0.1 M KCl containing 2 mM mercaptoethanol and 0.2 mM EDTA. The resultant solution was then fractionated by separating the reconstituted enzyme-like molecules from the excess regulatory subunit on a DEAE-cellulose column identical with that used for the separation of catalytic and regulatory subunits (Kirschner, 1971). The regulatory subunit emerged at 0.1 M KCl and the reconstituted enzyme was eluted with 0.15 M KCl. On addition of 0.5 M KCl no further protein was eluted, indicating that all of the nitrated catalytic subunit had been reconstituted.

Assays were performed by the method of Porter *et al.* (1969) and inhibition constants for succinate were obtained as described in the previous paper (Kirschner and Schachman, 1973). Similarly other methods were used as described in the previous paper (Kirschner and Schachman, 1973).

Results

Properties of Enzyme Reconstituted from Nitrated Catalytic Subunit. The nitrated catalytic subunit, C^N, upon the addition of native regulatory subunit, R, was converted quantitatively into an enzyme-like complex, $C^N(R)$,[1] having a sedimentation coefficient of 11.7 S, a value identical to that found for the native enzyme (Gerhart and Schachman, 1965). Figure 1 shows a representative sedimentation velocity pattern for $C^N(R)$ with the single, sharp, symmetrical boundary characteristic of aspartate transcarbamylase.

Since the preparation of C^N was obtained by limited nitration of the catalytic subunit with tetranitromethane at pH 6.7

[1] We have used C^N to represent nitrated catalytic subunit and $C^N(R)$ to represent an aspartate transcarbamylase like complex containing nitrated catalytic subunits.

TABLE I: Properties of C^N and $C^N(R)$.[a]

	C^N	Native Catalytic Subunit	$C^N(R)$	Native Enzyme
V_{max} (mole/hr per g)	24	28	10.1	11.1
CTP inhibition (%)			44	56
Maximum Hill coefficient	1.0	1.0	1.31	1.6
$\Delta s/s$ (succinate and carbamyl phosphate) (%)	+1.1	+1.4	−2.9	−3.6

[a] Enzyme activities were obtained from experiments illustrated in Figure 2 for $C^N(R)$ and native enzyme. The data obtained for C^N and unmodified catalytic subunit were obtained from assays performed at 0.01 M aspartate according to the method of Porter *et al.* (1969). It was assumed that the K_m values for C^N and native catalytic subunit were 0.02 M. Inhibition by 4 mM CTP was measured at an aspartate concentration of 5 mM. Difference sedimentation experiments were performed as described by Kirschner and Schachman (1971a).

in the presence of both succinate and carbamyl phosphate, about 85% of the enzymic activity was preserved (Kirschner and Schachman, 1973). As seen in Figure 2, the reconstituted enzyme-like complex, $C^N(R)$, was not only almost as active as native aspartate transcarbamylase, but it also exhibited the cooperative kinetic behavior characteristic of the enzyme. The Eadie plots in Figure 2 for $C^N(R)$ and aspartate transcarbamylase also show the inhibition caused by the addition of CTP to the assay mixtures.

The maximum velocity, V_{max}, for $C^N(R)$, obtained from the intercept of the Eadie plot with the abscissa, was slightly less than that for the native enzyme, as shown in Table I. This reduction may be in about the same proportion as that observed for C^N relative to the native catalytic subunit. Heterotropic interactions, as revealed by the CTP inhibition listed in Table I, were somewhat less for $C^N(R)$ than for the native enzyme. In the third row of Table I are listed the Hill coefficients at 50% saturation which indicate that the homotropic interactions exhibited by $C^N(R)$ were also somewhat less than those for native aspartate transcarbamylase. This decrease in homotropic interactions was reflected in the smaller effect of succinate and carbamyl phosphate on the sedimentation coefficient of $C^N(R)$, $\Delta s/s = -2.9\%$, as compared to the native enzyme, $\Delta s/s = -3.6\%$. There was a similar decrease in the effect of succinate and carbamyl phosphate on the sedimentation coefficient of C^N as compared to native catalytic subunit. Thus, $C^N(R)$ showed clearly the two major allosteric effects characteristic of the native enzyme, feedback inhibition with CTP and cooperativity with the substrate aspartate, as well as the large decrease in sedimentation coefficient with succinate and carbamyl phosphate. Quantitatively all these effects for $C^N(R)$ were somewhat reduced as compared to the native material. It is not clear whether this decrease in allosteric properties in $C^N(R)$ was due to the modification of the catalytic subunits or to problems with the regulatory subunits or the conditions of reconstitution.

Spectral Characteristics of $C^N(R)$. When regulatory subunit and C^N were mixed to form $C^N(R)$, an absorbance change occurred in the nitrotyrosyl spectrum. This absorbance change occurred more rapidly (within less than 15 sec) than can be measured without resort to rapid kinetic techniques. The spectrum of $C^N(R)$ obtained after removal of excess regulatory subunit, is shown in Figure 3. For comparison the spectrum of C^N as well as the spectrum of $C^N(R)$ in the presence of succinate and carbamyl phosphate are shown. All spectra were normalized for the absorbance at 390 nm, which is the isosbestic point for the nitrotyrosyl phenol–phenolate spectra in C^N as well as in nitrotyrosine. The pK of the nitrotyrosyl groups in $C^N(R)$ calculated from the spectrum was 6.7, while the corresponding pK for C^N was 6.3. Thus aggregation of the nitrated catalytic subunits into an enzyme-like complex caused the nitrotyrosyl groups on the catalytic subunits to become more protonated.

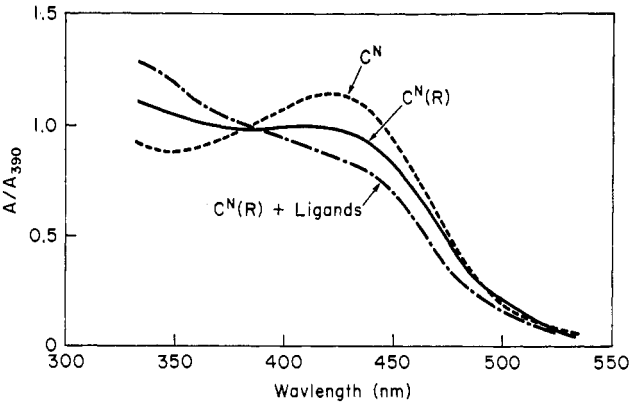

FIGURE 3: Spectrum of $C^N(R)$. The absorbance of $C^N(R)$ in the presence and absence of 2×10^{-3} M succinate and carbamyl phosphate are plotted on the ordinate as A/A_{390} so as to normalize the spectra relative to the absorbance at 390 nm. For comparison the spectrum of C^N (in the absence of ligands) is also included. The concentration of both proteins was approximately 4 mg/ml. The buffer was 0.04 M potassium phosphate at pH 7 containing 2 mM mercaptoethanol and 0.2 mM EDTA.

Effect of Ligands on the Spectrum of $C^N(R)$. As shown in Table II, the addition of saturating concentrations of car-

TABLE II: Spectral Changes in $C^N(R)$.[a]

Ligand	Concn (M)	$\Delta A_{430}/A_{430}$ (%) $C^N(R)$	$\Delta A_{430}/A_{430}$ (%) C^N
Carbamyl phosphate	5×10^{-4}	+3	0
Succinate	1×10^{-2}	0	0
Phosphate	4×10^{-2}		
Succinate	1×10^{-2}	−13	−15
Carbamyl phosphate	2×10^{-3}		
CTP	2×10^{-4}	−3	
ATP	2×10^{-4}	0	

[a] Spectral changes were measured at 430 nm and expressed as per cent change of original absorbance. All experiments were conducted in 0.04 M potassium phosphate buffer at pH 7.0 containing 2 mM mercaptoethanol and 0.2 mM EDTA. Data for C^N were obtained from the preceding paper (Kirschner and Schachman, 1973). The experiments with CTP and ATP were performed with 2×10^{-3} M carbamyl phosphate in the same buffer. All protein concentrations were 4 mg/ml.

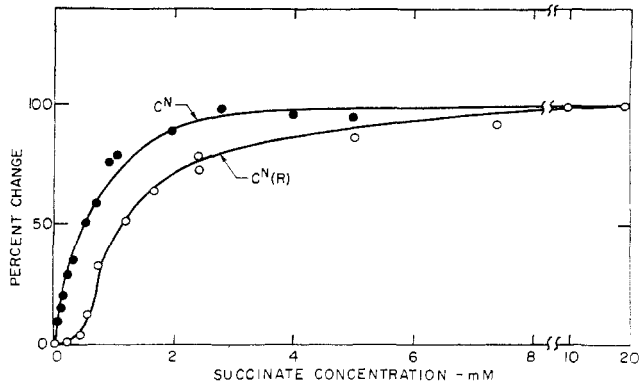

FIGURE 4: Titration of spectral change in $C^N(R)$ at varying succinate concentrations. The results of a series of difference absorbance measurements are plotted as per cent maximal change *vs.* succinate concentration. The data for C^N are replotted from Kirschner and Schachman (1973). The maximal change in absorbance at 430 nm was 15% for C^N and 16% for $C^N(R)$. The buffer in both cases was 0.04 M potassium phosphate at pH 7 containing 2 mM mercaptoethanol, 0.2 mM EDTA, and 4×10^{-3} M carbamyl phosphate.

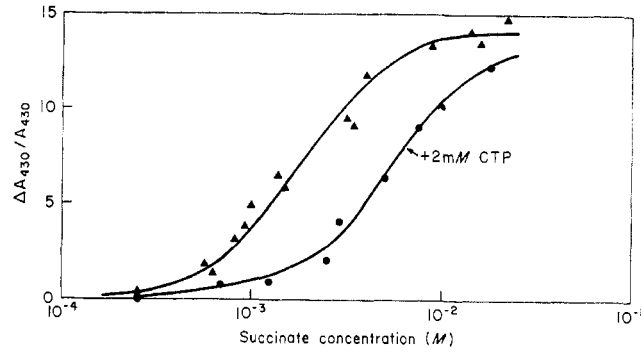

FIGURE 5: Effect of CTP on the succinate titration of $C^N(R)$. The data are plotted as per cent change in absorbance at 430 nm *vs.* the logarithm of the succinate concentration. The data in the absence of CTP are represented by closed triangles and in the presence of 2 mM CTP by closed circles.

bamyl phosphate to $C^N(R)$ caused an increase in absorption at 430 nm of 3%, which corresponds to a decrease in the pK of the nitrotyrosyl residue. This result is to be contrasted to the effect of carbamyl phosphate on C^N, where no change in the spectrum was observed. Table II also shows that succinate in the presence of phosphate, at a concentration sufficient to yield approximately 25% saturation of the enzyme (Collins and Stark, 1969) produced no change in the spectrum of $C^N(R)$ or C^N. In contrast, as shown in Figure 3, succinate and carbamyl phosphate together at concentrations of 2×10^{-3} M caused a significant decrease in the absorbance of $C^N(R)$ at 430 nm and an increase in absorbance at 360 nm. Saturating concentrations of succinate and carbamyl phosphate caused a 13% decrease in absorbance at 430 nm. Correcting for the specific effect of carbamyl phosphate gave a resulting spectral change of 16% due to succinate in the presence of carbamyl phosphate. Though this value is similar in magnitude to the effect observed for C^N (see Table II) the corresponding shift in the pK of the nitrotyrosyl residues amounted to only 0.2 pH unit in $C(R)^N$ as compared to 0.4 pH unit in C^N. This difference arises because the pK (6.7) for unliganded $C^N(R)$ is closer to the pH at which the experiments were conducted (pH 7.0) than is the pK (6.3) for unliganded C^N. Hence a smaller shift in pK of $C^N(R)$ can produce a change in absorbance equal to that of C^N.

As shown in Table II the inhibitor, CTP, which binds to the regulatory subunit, caused a decrease in the absorbance of the nitrotyrosyl residues on the catalytic subunits in $C^N(R)$. To prevent contributions from CTP binding to the carbamyl phosphate sites on the catalytic subunits (Bigler and Atkinson, 1969) as a result of nonspecific competition which occurs generally with compounds containing phosphate (Kleppe, 1966; Porter *et al.*, 1969), we added saturating concentrations of carbamyl phosphate to the solutions. In contrast, ATP had no measurable effect on the spectrum of the nitrotyrosyl residues in $C^N(R)$ as shown in Table II.

Effect of Succinate Concentration on the Spectrum of $C^N(R)$. Figure 4 shows a titration curve for the change in absorbance of $C^N(R)$ at 430 nm as a function of succinate concentration. For comparison the analogous data obtained with C^N (Kirschner and Schachman, 1973) are also presented. The data are plotted as per cent maximal change in absorbance, which for C^N was 15% and for $C^N(R)$ was 16% (after subtracting the contribution of carbamyl phosphate), *vs.* the concentration of succinate. The titration curve for $C^N(R)$ was distinctly sigmoidal and its half-titration point was shifted to higher succinate concentration as compared to the half-titration point for C^N. The Hill coefficient at 50% saturation for $C^N(R)$ was 1.68, while the titration curve for C^N was readily fit by a hyperbola (Hill coefficient of 1).

Figure 5 shows the effect of CTP on the saturation curve for succinate as determined by the change in absorbance at 430 nm. For this plot the change in absorbance was plotted *vs.* the logarithm of the succinate concentration, in order to provide a direct comparison of the titration curves in the presence and absence of CTP. As is readily observed CTP shifted the saturation curve to higher concentrations of succinate from a half-titration value of 1.5 mM in the absence of CTP to 7 mM in the presence of 0.2 mM CTP. At the highest concentrations of succinate the inhibition by CTP was almost completely reversed. These results are very similar to the observed reversal by aspartate of CTP inhibition of the enzymic activity of the native enzyme (Gerhart and Pardee, 1962).

Effect of Ligands on the Sedimentation Coefficient of $C^N(R)$. As shown in Table I the addition of both succinate and carbamyl phosphate to solutions of $C^N(R)$ led to a reduction of 2.9% in the sedimentation coefficient of the native complex. The same ligands caused an increase in the sedimentation coefficient of C^N. In this respect, except for slight differences in magnitude, C^N and $C^N(R)$ behaved in a manner identical with the native catalytic subunit and aspartate transcarbamylase, respectively (Kirschner and Schachman, 1971b). Figure 6 shows the percentage change in the sedimentation coefficient of $C^N(R)$ as a function of succinate concentration (at a constant level of carbamyl phosphate). In this titration curve for $\Delta s/\bar{s}$, $C^N(R)$ again behaved similarly to the native enzyme with a half-titration value of 0.3 mM for the former and 0.2 mM for the latter (Gerhart and Schachman, 1968).

For comparative purposes we have included in Figure 6 the results of the spectral titration of $C^N(R)$ as the percentage change in A_{430} (from Figure 4) as a function of the logarithm of the succinate concentration (again at a constant level of carbamyl phosphate). It is clear from Figure 6 that the curve for the spectral change is displaced to the right of the curve for $\Delta s/\bar{s}$ with a half-titration concentration of 1.5 mM for the former and 0.3 mM for the latter. In those plots of per cent change *vs.* the logarithm of succinate concentration the spectral curve was much steeper than that for $\Delta s/\bar{s}$. When these

same data were plotted in terms of succinate concentration (rather than logarithm of concentration), the curve for the spectral change was distinctly sigmoidal (see Figure 4) while the curve for $\Delta s/s$ had the shape of a normal saturation curve. These contrasting responses of spectral and hydrodynamic properties to the level of succinate were illustrated further by Hill plots which gave 1.68 and 0.91, respectively, for the Hill coefficients at 50% saturation.

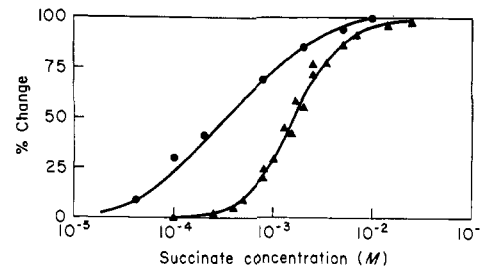

FIGURE 6: Effect of succinate on the sedimentation coefficient (●) and spectrum (▲) of $C^N(R)$. Difference sedimentation experiments were conducted as described by Kirschner and Schachman (1971a). The concentration of protein was 2 mg/ml. In each experiment the concentration of carbamyl phosphate was 2×10^{-3} M in one sector plus a given concentration of succinate; the other sector contained 2×10^{-3} M phosphate and the corresponding concentration of glutarate. The data are plotted as the per cent maximal change due to succinate after subtraction of the effect of carbamyl phosphate which was −0.6% *vs.* the logarithm of the succinate concentration. Data for the spectral change of $C^N(R)$ are replotted from Figure 4.

Disscussion

Properties of $C^N(R)$. Upon the formation of an enzyme-like complex from native regulatory subunits and nitrated catalytic subunits there was a decrease in absorbance at 430 nm of 14% corresponding to an increase in the pK of the nitrotyrosyl residues of 0.4 pH unit. Although the shift in pK on the association of C^N with regulatory subunits may have little functional significance, nonetheless it is interesting that the chromophores are so located that they are affected both by aggregation of the subunits into an enzyme-like complex and by the binding of specific ligands to either C^N or $C^N(R)$. Conjecture as to the relationship, if any, between the ligand-promoted changes in conformation of the catalytic subunits and the changes accompanying their assembly into the quaternary structure of aspartate transcarbamylase will have to await further knowledge of the three-dimensional structure of the enzyme (Wiley *et al.*, 1971), and information of the location of the nitrotyrosyl residues in the amino acid sequence of the catalytic polypeptide chains.

$C^N(R)$ exhibits the two principal allosteric properties of native aspartate transcarbamylase: feedback inhibition by CTP and cooperative kinetics with the substrate, aspartate (Gerhart and Pardee, 1962). There were slight differences in behavior between $C^N(R)$ and the native enzyme with the former being less inhibited by CTP and showing less cooperativity as measured by the Hill coefficient at 50% saturation by aspartate. Similarly the reduction in the sedimentation coefficient of $C^N(R)$ upon the addition of succinate and carbamyl phosphate was about 20% less than for the native enzyme. Some of these differences may be attributable to the presence of mercury in $C^N(R)$ instead of zinc which is a normal constituent of aspartate transcarbamylase (Rosenbusch and Weber, 1971; Nelbach *et al.*, 1972). In the past, preparations of aspartate transcarbamylase reconstituted from isolated catalytic and regulatory subunits have differed from the native enzyme presumably from changes in the metal content or slight alterations in the subunits during their isolation. Thus it would be premature to conclude that the slightly altered properties of $C^N(R)$, compared to the native enzyme, stem from an effect of the nitrotyrosyl residues on intersubunit interactions.

Ligand-Promoted Conformational Changes in C^N and $C^N(R)$. Although different types of evidence have indicated that succinate and carbamyl phosphate cause conformational changes in aspartate transcarbamylase (Gerhart and Schachman, 1968; McClintock and Markus, 1968) and in the isolated catalytic subunits (Collins and Stark, 1969; Kirschner and Schachman, 1971b), there has been little information concerning the ligand-promoted changes in the catalytic subunits within intact enzyme molecules. In this regard it is of interest that the spectral change of the nitrotyrosyl chromophores in $C^N(R)$ is in the same direction as was found in C^N. However, without knowledge of the location of the chromophores in relation to the ligand binding sites is it not clear whether the spectral change is the result of a conformational change or the direct effect of the binding of ligands. Several lines of evidence support the view that the shift in the nitrotyrosyl spectrum upon the addition of ligands is associated with alterations in the three-dimensional structure of the catalytic subunits. First, spectral changes in C^N occurred only with those ligands which produced conformational changes as measured by other techniques such as difference sedimentation. Other ligands, which bind at the same site without producing a conformational change, had no effect on the spectrum of the nitrotyrosyl residue. Second, the presence of succinate during the reaction with tetranitromethane did not prevent the nitration of the tyrosyl residues. The rate of nitration was decreased to an extent consistent with a probable shift of the pK of the groups due to succinate binding. In addition, the presence of ligands did not protect the nitrotyrosyl groups from reduction with dithionite. Third, the nitrotyrosyl groups both in the protonated and in the deprotonated forms had little influence on the values of K_m and V_{max} for the catalytic subunit. Fourth, the nitrotyrosyl spectrum in the intact enzyme responded to the binding of carbamyl phosphate while the same ligand had no effect on the spectrum of the groups in the nitrated catalytic subunit. When carbamyl phosphate was added to the intact enzyme there was a significant decrease in the sedimentation coefficient (Gerhart and Schachman, 1968; Kirschner and Schachman, 1971a); however, carbamyl phosphate had virtually no effect on the catalytic subunit (Kirschner and Schachman, 1971b). Fifth, the nitrotyrosyl spectrum of $C^N(R)$ was altered by the addition of CTP which binds to sites on subunits which do not contain the chromophores. In summary, this evidence, though indirect, indicates that the nitrotyrosyl groups do not interact directly with succinate and carbamyl phosphate at the active site but instead to conformational changes in the catalytic subunits.

Since the spectral changes at 430 nm are due to the protonation and deprotonation of a single chromophore which is sensitive to small alterations in its local environment, the similarity of the spectral changes in C^N and in $C^N(R)$ upon the addition of specific ligands does not constitute unequivocal evidence that the isolated subunits and those within the intact enzyme molecules undergo the same type of conformational change. Thus it is of interest that the ligand-promoted changes in the near-ultraviolet absorption spectrum of the catalytic subunits (Collins and Stark, 1969) are very similar to those observed for the intact enzyme (Pigiet, 1971). The catalytic

TABLE III: Dissociation of Succinate from Catalytic Subunit.[a]

Parameter	K (mM)	Reference
Optical rotation[b]	0.2	(Pigiet, 1971)
Sedimentation coefficient[c]	0.2	(Kirschner and Schachman, 1971b)
ΔA_{430} of nitrated catalytic subunit[d]	0.37	(Kirschner and Schachman, 1973)
Uv difference spectrum[e]	0.74	(Collins and Stark, 1969)
Inhibition constant[f]	0.38	(Kirschner and Schachman, 1973)
Direct binding[g]	0.55	(Changeux *et al.*, 1968)

[a] The values listed are apparent dissociation constants. Carbamyl phosphate was maintained at a constant level in all experiments. Corrections for the bound succinate in the calculation of K were made only for the equilibrium dialysis measurements. [b] 0.04 M Potassium phosphate buffer at pH 7 containing 4×10^{-4} M EDTA, 10^{-4} M dithiothreitol, and 4×10^{-3} M carbamyl phosphate at 25°. [c] 0.04 M Potassium phosphate buffer at pH 7 containing 2×10^{-4} M EDTA, 2×10^{-3} M mercaptoethanol, and 2×10^{-3} M carbamyl phosphate at 20°. [d] 0.04 M Potassium phosphate buffer at pH 7 containing 2×10^{-4} M EDTA, 2×10^{-3} M mercaptoethanol, and 2×10^{-3} M carbamyl phosphate at 21°. [e] 0.02 M Glycylglycine buffer at pH 6.9 containing 2×10^{-4} M EDTA, 2×10^{-3} M mercaptoethanol, and 4.9×10^{-3} M carbamyl phosphate. [f] 0.04 M Potassium phosphate buffer at pH 7 containing 4×10^{-3} M carbamyl phosphate, and varying aspartate concentrations at 20°. [g] 0.04 M Potassium phosphate buffer at pH 7 containing 2×10^{-4} M EDTA, 2×10^{-3} M mercaptoethanol, 0.02 M Tris, and 8×10^{-3} M carbamyl phosphate at 21°.

subunits are responsible for about 84% of the absorbance of aspartate transcarbamylase; hence this similarity in the effect of ligands constitutes additional evidence for the notion that the catalytic subunits within the complex undergo the same conformational changes as in the isolated state.

Two other methods, which have been used for the study of conformational changes in the isolated catalytic subunits and the intact enzyme, have given different results. The ligand-promoted changes in the sedimentation coefficient (Kirschner and Schachman, 1971b) and the optical rotatory dispersion (Pigiet, 1971) were opposite in direction for aspartate transcarbamylase and the isolated catalytic subunits. It is difficult to interpret the extremely small changes in optical rotation but certainly the change in sedimentation coefficient of the enzyme complex could be attributed to a loosening of the structure of the aspartate transcarbamylase molecules even while the putative changes in the catalytic subunits within them are the same as those measured on the isolated subunits.

Local Conformational Changes in the Catalytic Subunit and Their Relationship to the Binding of Ligands. All of the methods used for the study of conformational changes in the isolated catalytic subunits as a function of succinate concentration (in the presence of carbamyl phosphate) have yielded data which were readily fit by hyperbolic curves. As shown in Table III, the apparent dissociation constants (corresponding to the succinate concentration at which the half-maximal change occurred) are in general agreement despite the differences in properties on which they are based. Moreover, they are close to the value of the binding constant determined by equilibrium dialysis (Changeux *et al.*, 1968).[2] The dissociation constant obtained by difference spectroscopy in the ultraviolet region of the spectrum (Collins and Stark, 1969) was higher than the other values but this may be due to differences in the temperature and buffer used for this particular study (Kirschner and Schachman, 1973). These results indicate that the conformational changes in the isolated catalytic subunits are linked closely to the binding of ligands. Moreover, the hyperbolic relationships and studies with hybrid catalytic subunits containing native and chemically modified, inactive polypeptide chains showed that the chains respond independently to the addition of ligands (V. P. Pigiet, Jr., and H. K. Schachman, unpublished results).

Upon the incorporation of C^N into enzyme-like molecules the spectral response of the chromophores changed from a hyperbolic to a sigmoidal dependence on succinate concentration. In addition, the level of succinate required to produce a half-maximal change in absorbance at 430 nm was increased significantly (see Figure 4). In these two respects the spectral response is very much like the binding data of Changeux *et al.* (1968). This comparison in the spectral behavior of C^N *vs.* $C^N(R)$ is also similar to the relationship of the enzymic activities of the isolated catalytic subunits *vs.* intact aspartate transcarbamylase. The concentration of succinate required for half-saturation of the binding sites of aspartate transcarbamylase (Changeux *et al.*, 1968) was 7×10^{-4} M whereas that required for the half-maximal change in the spectrum of $C^N(R)$ was 1.5×10^{-3} M. A strict comparison between $C^N(R)$ and native aspartate transcarbamylase may not be justifiable since there were slight differences in their properties. In addition, the binding data for the native enzyme, especially at high succinate concentrations, are inadequate. Despite these reservations it seems likely that the local changes in the conformation of the catalytic subunits in $C^N(R)$ are closely linked to the binding of the ligands to these subunits.

Linkage between Local and Gross Conformational Changes in Aspartate Transcarbamylase. As pointed out by Gerhart and Schachman (1968), the large changes in the properties of aspartate transcarbamylase upon the addition of specific ligands must be due to profound alterations in the three-dimensional structure of the enzyme itself rather than simply the result of the presence of the ligands on the surface of an otherwise "unmodified" protein. The decrease in sedimentation coefficient of the intact enzyme is indicative of a more swollen (or elongated) conformation with a greater frictional coefficient and was in marked contrast to the increase observed for the isolated catalytic subunits. The same ligands also led to a marked enhancement of the reactivity of the sulfhydryl

[2] These experiments yielded only two succinate binding sites per catalytic subunit, whereas more recent studies with a variety of ligands have shown clearly that each subunit possesses three binding sites (Hammes *et al.*, 1970; Rosenbusch and Weber, 1971; Collins and Stark, 1971). The third binding site may have been missed in the earlier studies because of the weak binding of succinate or because the older preparations suffered a partial loss of their binding capacity. In view of the discrepancy between the number of polypeptide chains and the number of binding sites per subunit, the value for the binding constant obtained with this material must be treated with caution. It is of interest that the more recently reported dissociation constant, 0.11 mM, obtained by Rosenbusch and Weber (1971) with a different buffer and lower temperature, is not too different.

groups of the regulatory subunits even though the ligands were bound to the catalytic subunits. If these gross changes in the conformation of aspartate transcarbamylase were the result of local and direct effects of the binding of the ligands, one would expect a proportional relationship between the fraction of binding sites occupied by ligands and the amount of change in sedimentation coefficient and in reactivity toward mercurials. This expectation was not fulfilled; on the contrary, the chemical reactivity of the sulfhydryl groups and the sedimentation coefficient changed to half their limiting and maximal values when only 15% of the binding sites were occupied by succinate. Hence it appeared that the gross conformational change in the enzyme could be ascribed to a transition between a compact and a loose structure and that the change from one to the other did not coincide with but actually preceded the saturation of the binding sites with succinate (Monod *et al.*, 1965; Gerhart and Schachman, 1968; Changeux and Rubin, 1968).

The availability of enzyme-like molecules containing a sensitive chromophore furnished an opportunity to explore further the transition between the two conformational states. As shown in Figure 6, the sedimentation coefficient changed at a much lower concentration of succinate than was required to produce an equivalent percentage change in the spectrum of the nitrotyrosyl chromophores. At a succinate concentration corresponding to a 50% change in $\Delta s/s$ there was only 7% of the maximal change in absorbance at 430 nm. There was also a marked difference in the shapes of the curves describing the change in physical properties as a function of succinate concentration.[3] Hence we can conclude that the local and gross conformational changes in the enzyme molecules are not strongly linked. Binding of succinate in the presence of carbamyl phosphate caused conformational changes in the catalytic subunits in direct proportion to the amount of ligand which is bound. These changes, both in the isolated catalytic subunits and those within intact enzyme molecules, appear to be sequential in that each ligand bound makes an equivalent and separate contribution to the conformation of the polypeptide chains in the subunits. Although these chains respond independently in the isolated subunits, they become interdependent when the subunits are subject to the quaternary restraint of the intact enzyme. This difference is evident from the change in the hyperbolic response of the spectrum in the isolated catalytic subunits to a sigmoidal dependence of the spectral change on succinate concentration for the intact enzyme-like molecules (see Figure 4). These local conformational changes in the catalytic chains which are strongly linked to ligand binding are in turn accompanied by a gross conformational change in the whole enzyme molecules. The gross conformational change is weakly linked to the binding of ligand and appears to represent a different type of transition involving, for example, changes in the packing and orientation of the catalytic subunits relative to each other and the regulatory subunits. Since the binding of ligands produced changes in the optical rotation at 233 nm (Pigiet, 1971) and in the sedimentation coefficient (Kirschner and Schachman, 1971b) of the isolated catalytic subunit which are in the opposite direction to those produced in aspartate transcarbamylase, we conclude that the gross conformational change in the complex is not merely the sum of the changes in the constituent catalytic subunits. In contrast, the local conformational changes are additive and are related to the saturation of the binding sites.

A linkage must occur between the local and gross conformational changes since the ligands which bind to the catalytic subunits promote changes in the whole complex. Hence reagents which produce a gross conformational change should have a reciprocal effect on the catalytic subunits. Evidence for this is shown in Table II; CTP which binds to the regulatory subunits caused a change in the absorbance of the nitrotyrosyl groups on the catalytic subunits. The addition of CTP, as shown in Figure 5, caused an antagonistic effect in the spectral response of the nitrotyrosyl chromophores to varying succinate concentration (in the presence of carbamyl phosphate). This effect of CTP on the succinate titration of the spectral change of $C^N(R)$ is similar to its effect on the kinetic behavior of the native enzyme (Gerhart and Pardee, 1962). The binding of carbamyl phosphate (in the absence of succinate) produces both a conformational change in aspartate transcarbamylase (Gerhart and Schachman, 1968) and a spectral change in $C^N(R)$. However, this same ligand has a negligible effect on the conformation of the isolated catalytic subunit (Kirschner and Schachman, 1971a) and on the spectrum of the nitrated catalytic subunit (Kirschner and Schachman, 1973). Thus the effect of carbamyl phosphate on the spectrum of $C^N(R)$ may be attributed to the change in the conformation of the entire complex and a reciprocal effect on the catalytic chains rather than a local contribution of the ligand at the binding site.

The linkage of two different levels of conformational change in an oligomeric protein has been discussed recently by Perutz (1970). According to his proposal hemoglobin generates a large number of intermediate states by the superposition of two general classes of conformations with bulk states involving the orientation of the subunits relative to each other and local states involving oxy and deoxy forms. This view includes the principal elements of the models of both Monod *et al.* (1965) and Koshland *et al.* (1966). The microscopic forms in these two states have different local conformations and hence different affinities for ligands. However, the major difference in ligand affinity stems from the transition between the two gross conformations of the entire molecule. Such a model seems appropriate for aspartate transcarbamylase where local conformational changes in the catalytic subunits are linked to transitions in the entire enzyme molecule between two canonical forms.

Obviously discussions of conformational changes and two-state *vs.* intermediate state models are susceptible to misunderstanding because of the subtlety and vagueness of the terms. The existence of different levels of transitions in an oligomeric enzyme poses pitfalls in investigations of these transitions through the use of a single probe. Through the

[3] The initial flatness of the spectral titration of $C^N(R)$ with succinate (Figure 4) may be misleading since opposing changes could be occurring. On the one hand, there is the direct decrease in absorbance at 430 nm due to the local conformational change accompanying the binding of the ligands to the nitrated catalytic polypeptide chains within the enzyme complex. On the other hand, there *may be* an increase in absorbance due to the overall conformational change in the complex revealed by the difference sedimentation experiments. Since a major part of the gross transition occurs before appreciable saturation of the binding sites, such a purported increase in absorbance would contribute disproportionately to the first part of the titration curve of ΔA_{430} *vs.* succinate concentration. It should be recalled that the incorporation of nitrated catalytic subunits into an enzyme-like structure caused a decrease in absorbance at 430 nm. Hence a relaxation of that structure upon the addition of ligands (Gerhart and Schachman, 1968) could lead to the postulated increase in absorbance. Such an increase would be masked by the opposing, larger decrease due to local effects with the result that the curve appears flat at low succinate concentration.

use of several probes such as difference sedimentation which is sensitive to gross changes in macromolecules and difference spectroscopy with a specific chromophore, which is sensitive to local changes, some of the ambiguity may be eliminated. It is clear that aspartate transcarbamylase undergoes both local and gross conformational changes upon the addition of specific ligands. These changes appear to be weakly linked with the gross change being concerted and occurring when only a fraction of the binding sites are occupied and the local changes being sequential and associated strongly with ligand binding. As yet we have no direct evidence that binding of ligand to one catalytic subunit can promote local conformational changes in the other. Presumably this occurs through the linkage between local and gross changes, but as yet direct experimental evidence regarding the interdependence of local conformational changes in the two catalytic subunits is lacking.

References

Bigler, W. N., and Atkinson, D. E. (1969), *Biochem. Biophys. Res. Commun. 36*, 381.

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 531.

Changeux, J.-P., and Rubin, M. M. (1968), *Biochemistry 7*, 553.

Collins, K. D., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1869.

Collins, K. D., and Stark, G. R. (1971), *J. Biol. Chem. 246*, 6599.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Gerhart, J. C., and Schachman, H, K. (1968), *Biochemistry 7*, 538.

Hammes, G. G., Porter, R. W., and Wu, C-W. (1970), *Biochemistry 9*, 2992.

Kirschner, M. W. (1971), Ph.D. Thesis, University of California, Berkeley, Calif.

Kirschner, M. W., and Schachman, H. K. (1971a), *Biochemistry 10*, 1900.

Kirschner, M. W., and Schachman, H. K. (1971b), *Biochemistry 10*, 1919.

Kirschner, M. W., and Schachman, H. K. (1973), *Biochemistry 12*, 2987.

Kleppe, K. (1966), *Biochim. Biophys. Acta 122*, 450.

Koshland, D. E., Jr., Némethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

McClintock, D. K., and Markus, G. (1968), *J. Biol. Chem. 243*, 2855.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Nelbach, M. E., Pigiet, V. P., Jr., Gerhart, J. C., and Schachman, H. K. (1972), *Biochemistry 11*, 315.

Perutz, M. F. (1970), *Nature (London) 228*, 726.

Pigiet, V, P. (1971), Ph.D. Thesis, University of California, Berkeley, Calif.

Porter, R. W., Moldebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1846.

Rosenbusch, J. P., and Weber, K. (1971), *J. Biol. Chem. 246*, 1644.

Wiley, D. C., Evans, D. R., Warren, S. G., McMurray, C. H., Edwards, B. F, P., Franks, W. A., and Lipscomb, W. N. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 285.