Concerted Allosteric Transition in Hybrids of Aspartate Transcarbamoylase Containing Different Arrangements of Active and Inactive Sites†

Ian Gibbons,‡ James M. Ritchey, and H. K. Schachman*

ABSTRACT: Various hybrids of aspartate transcarbamoylase of *Escherichia coli* were constructed from native regulatory subunits and mixtures of active and inactive (pyridoxylated) catalytic chains in specific arrangements within the two catalytic subunits. The kinetic and physical properties of these well-defined hybrids were studied in order to determine the effects of reducing the number of substrate binding sites and distributing the active and inactive chains in different ways. Experiments on enzyme-like molecules containing six, four, three, two, and one active sites showed that the Hill coefficient decreased and the apparent K_m increased as the number of active chains in the hybrids was reduced. The maximum inhibition and activation by the nucleotide effectors, CTP and ATP, were independent of the composition of the enzyme-like molecules. Two hybrids were of particular interest since one contained two active sites in one catalytic subunit and none in the other, and the second hybrid had one active site in each catalytic subunit. These two hybrids exhibited identical kinetic behavior despite the markedly different structural arrangements. The ligand-promoted conformational changes of the hybrids monitored both by sedimentation velocity measurements and the reactivity toward *p*-hydroxymercuribenzoate were similar to those of the native enzyme. These results indicate that there are no discrete "cooperative units" within the enzyme molecules but rather that the allosteric transition promoted by ligands is fully concerted. The various kinetic and physical properties can be accounted for satisfactorily in terms of the two-state model of Monod et al. (Monod, J., Wyman, J., Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88).

The cooperativity exhibited by aspartate transcarbamoylase (ATCase,[1] EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) accounts in part for the regulation of pyrimidine biosynthesis in *Escherichia coli* (Gerhart and Pardee, 1962). In addition, regulation is achieved by the response of the enzyme to cellular levels of metabolites which differ chemically from the substrates (Gerhart, 1970). These characteristics of allosteric enzymes, termed homotropic and heterotropic effects, respectively (Monod et al., 1965), are manifested with ATCase by the sigmoidal dependence of activity on aspartate concentration and by the change in shape of the saturation curve and its shift to higher or lower aspartate concentrations upon the addition of the nucleotide effectors, CTP and ATP (Gerhart and Pardee, 1962). These phenomena have been interpreted in terms of a ligand-promoted conformational change of the enzyme from a constrained (or low-affinity) state to a relaxed form having a high affinity for substrate. The various models proposed to account for the behavior of allosteric enzymes (Monod et al., 1965; Koshland et al., 1966) differ in the number of conformational states required and the extent to which the allosteric transition between these states is concerted.

In this paper we present the results of studies aimed at determining whether the allosteric transition in ATCase is concerted. The approach involved examining the properties of well-defined hybrids of ATCase-like molecules containing different arrangements of active and inactive catalytic polypeptide chains. The native enzyme is composed of two trimeric catalytic subunits (C) and three regulatory dimers (R) organized in a structure in which each c chain in one C subunit is linked to a c chain in the other C subunit via an R subunit (Cohlberg et al., 1972; Schachman, 1972). In this structure, designated C_2R_3 or c_6r_6 (Weber, 1968; Wiley and Lipscomb, 1968; Meighen et al., 1970; Rosenbusch and Weber, 1971), there is apparently no direct contact between the two C subunits (Richards and Williams, 1972; Cohlberg et al., 1972; Evans et al., 1973). Inactive C subunits were made by specific modification of active site lysyl residues with pyridoxyl 5′-phosphate and sodium borohydride (Greenwell et al., 1973). These inactive pyridoxylated subunits (C_P) still bind the substrate (carbamoyl phosphate) but no longer are capable of binding the other substrate (aspartate) or the nonreactive analogue (succinate) (Blackburn and Schachman, (1976)). In a previous paper from this laboratory Gibbons et al. (1974) demonstrated that a hybrid containing one native C and one inactive C_P subunit along with three native R subunits ($C_NC_PR_3$) exhibited both the homotropic and heterotropic effects as well as the conformational transition characteristic of the native enzyme. The present paper extends these observations to

† From the Department of Molecular Biology and the Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720. *Received October 28, 1975.* This investigation was supported by NIH Research Grant GM 12159 from the National Institute of General Medical Sciences and Training Grant CA 05028 from the National Cancer Institute and by National Science Foundation Research Grant GB 32812X.

‡ Present address: Department of Biochemistry, University of Bristol, Bristol BS8 1TD, United Kingdom.

[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; c, catalytic polypeptide chain; r, regulatory polypeptide chain; N (subscript), native subunit; n (subscript), native chain; P (subscript), pyridoxylated subunit; p (subscript), pyridoxylated c chain; T (subscript), tetrahydrophthaloylated subunit; C_2R_3, ATCase; C_2R_2, R-deficient ATCase. Hybrid C subunits are specified by their content of native and pyridoxylated chains; e.g., C_{nnp} has two native chains and one pyridoxylated chain. Hybrid ATCase molecules are specified by the composition of the two C subunits; e.g., $C_{nnp}C_PR_3$ has one C_{nnp}, one C_P subunit, and three R subunits; CAP, carbamoyl phosphate; PALA, *N*-(phosphonacetyl)-L-aspartate; PMB, *p*-hydroxymercuribenzoate.

Table I: Hybridization Procedure.

Desired Hybrid	Components Used for Hybridization	Isomers[a]
C_{nnp}	$(C_N + C_{P,T})$ or $(C_T + C_P)$	One
C_{npp}	$(C_T + C_P)$	One
$C_NC_NR_3$	$C_N + R$ or $(C_N + C_{P,T}) + R$	One
$C_{nnp}C_{nnp}R_3$	$C_{nnp} + R$	Three
$C_NC_PR_3$	$(C_N + C_{P,T}) + R$	One
$C_{nnp}C_PR_3$	$(C_{nnp} + C_{P,T}) + R$	One
$C_{npp}C_{npp}R_3$	$C_{npp} + R$	Three
$C_{npp}C_PR_3$	$(C_{npp} + C_{P,T}) + R$	One
$C_PC_PR_3$	$C_P + R$ or $(C_N + C_{P,T}) + R$	One

[a] When two asymmetric C subunits (e.g., C_{nnp}) are combined with R to form ATCase-like molecules, the product is almost certainly a mixture of three isomers which differ according to the relative orientations of the C subunits.

other hybrids to permit the comparison of ATCase-like molecules having the same number but different distributions of active sites. Since the hybrids with different arrangements of the active chains within or between the two C subunits have similar kinetic and physical properties, it appears that the entire molecule responds to ligands in a concerted fashion.

Materials and Methods

Chemicals. Carbamoyl phosphate (CAP) as the dilithium salt was crystallized from ethanol, dissolved in buffer, and frozen for storage (Gerhart and Pardee, 1962). Because of the instability of aqueous solutions of CAP they were used immediately after thawing and proteins were not exposed to concentrations greater than 4 mM CAP for more than 3 h. L-Aspartate and succinate were used as the potassium salts, and succinate was crystallized from water and ethanol before use. *N*-(Phosphonacetyl)-L-aspartate (PALA) as the dilithium salt was the kind gift of Dr. George R. Stark.

Subunits. C_N and R were isolated from native ATCase as described by Kirschner (1971). C_P was prepared according to Greenwell et al. (1973) by reacting C_N with pyridoxyl 5′-phosphate and reducing the Schiff base with sodium borohydride. The preparations of C_P contained 1.2 residues of pyridoxamine 5′-phosphate per c chain and had about 2% of the enzymic activity of C_N. Manipulations with pyridoxylated derivatives were conducted in darkened rooms, and vessels were covered with aluminum foil in order to exclude light. This procedure was necessary in order to prevent light reactivation of C_P (J. M. Ritchey, I. Gibbons, and H. K. Schachman, manuscript in preparation).

Hybrids. Acylation of C subunits with 3,4,5,6-tetrahydrophthalic anhydride and the subsequent deacylation of the various species were performed as described by Gibbons et al. (1974). The various *intra*-subunit hybrids were constructed by mixing the appropriate acylated and unacylated subunits, dissociation of the proteins in 6.5 M urea and 0.1 M dithiothreitol at 0 °C for 15 min, dialysis against buffer, fractionation on DEAE-Sephadex, and deacylation of the purified hybrids (Meighen et al., 1970; Pigiet, 1971; Gibbons et al., 1974). ATCase-like hybrids were obtained and purified according to Gibbons et al. (1974) by mixing the appropriate parental C species with R subunits. Table I summarizes the procedure employed for the preparation of the desired hybrids which were assayed for enzymic activity by the method of Porter et al. (1969). The hybrids were free (<5% contamination) of other members of the corresponding hybrid sets as revealed by electrophoresis on cellulose acetate strips (Gibbons et al., 1974). The preparations of ATCase-like hybrids contained about 5% aggregates (Gerhart and Schachman, 1965) and less than 5% R-deficient molecules (C_2R_2) (Yang et al., 1974) as measured by scanning the stained 5% polyacrylamide gels (Jovin et al., 1964).

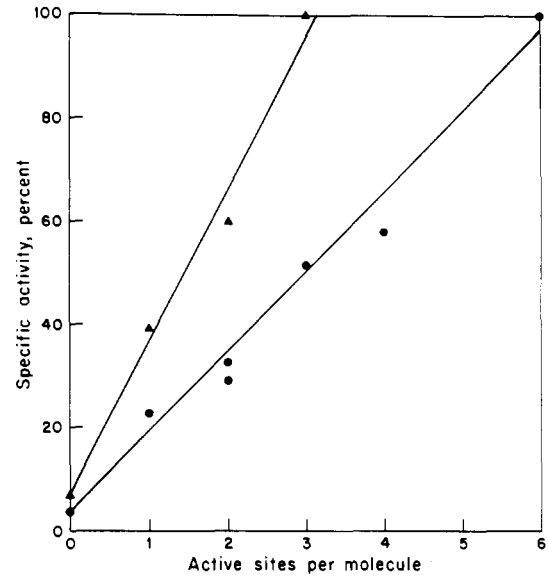

FIGURE 1: Effect of the number of native sites on the relative specific activity of ATCase and catalytic subunit. Values for ATCase-like molecules with structure, C_2R_3, designated by ●, for subunits with structure, C (*intra*-subunit hybrid set), designated by ▲. The V_{max} values were obtained by extrapolation of the Eadie plots as shown in Figures 2b and 3b and were 11.6 and 28.3 μmol of carbamoyl aspartate per h per μg of protein for native ATCase and catalytic subunit, respectively.

Measurement of Conformational Changes. Ligand-promoted changes in the sedimentation coefficient ($\Delta s/\bar{s}$) were performed as described by Gerhart and Schachman (1968). The protein concentration was 2–4 mg/ml and the sedimentation experiments were performed at 60 000 rpm and room temperature (20 °C). When CAP was the only ligand, an equimolar concentration of phosphate was added to the reference solution to compensate for the contribution of CAP to the viscosity and density of the solution. For measurements of the change caused by succinate, 2 mM CAP was present in both the experimental and reference solutions, and glutarate was added to the reference cell as a compensating ligand. Measurements of the effects of PALA involved no compensating ligand.

The effect of ligands on the rate of the pseudo-first-order reaction of the ATCase-like molecules with PMB was measured at 20 °C (Gerhart and Schachman, 1968). Analysis of the data was performed with a computer fitting program (M. N. Blackburn and H. K. Schachman, manuscript in preparation) so as to resolve the rate due to the reaction of the r chain thiols and the much slower rate of reaction of the c chain cysteinyl resudue. When both CAP and succinate were present, the reaction of the latter groups was negligible (Gerhart and Schachman, 1968).

Results

Kinetic Properties. As shown in Figure 1 the relative specific activities (as measured by V_{max}) varied linearly with the number of native c chains in both the C subunits and in the ATCase-like molecules. These results, presented in an alternative fashion in Table II as turnover numbers per ac-

Table II: Kinetic Properties of Hybrids and Related Species.

Species	Active Sites	Turnover Number[a] $\times 10^{-3}$ (min^{-1})	K_m[b] (mM)	n_H[c]	Inhibition by CTP[d] (%)	Activation by ATP[e] (%)
$C_NC_NR_3$	6	9.9	7	1.75	80	158
$C_{nnp}C_{nnp}R_3$	4	8.0	11	1.37		
$C_NC_PR_3$	3	9.4	12	1.38	75	122
$C_{npp}C_{npp}R_3$	2	8.5	15	1.34	75	152
$C_{nnp}C_PR_3$	2	7.5	15	1.36	75	152
$C_{npp}C_PR_3$	1	11.1	18	1.25	68	130
$C_PC_PR_3$	0		40	1.16	68	156
C_N	3	15.7	7	1.00		
C_{nnp}	2	13.6	7	1.00		
C_{npp}	1	16.3	8	1.00		
C_P	0		8	1.00		

[a] Assays conducted as described in Figure 2 and in Materials and Methods; V_{max} values determined from plots like Figure 2b; units are number of carbamoyl aspartate molecules formed per minute per active site; corrections were made for the contribution of the pyridoxylated sites. [b] Aspartate concentration corresponding to half maximal velocity. [c] Hill coefficient corresponding to activity between 10 and 50% of V_{max}. [d] CTP concentration of 0.5 mM; aspartate concentration extrapolated to zero. [e] ATP concentration of 2 mM; aspartate concentration extrapolated to zero.

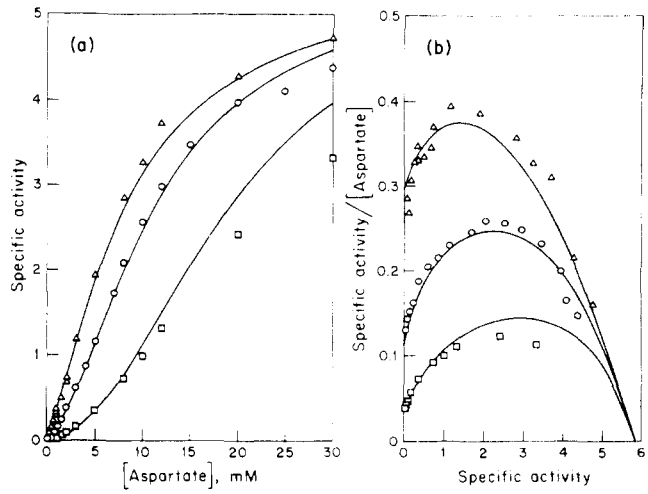

FIGURE 2: Kinetics of hybrid $C_NC_PR_3$. Assays were performed at 30 °C in 50 mM imidazole-acetate, pH 7, containing 0.2 mM EDTA and 4 mM carbamoyl phosphate. (a) Specific activity (μmol of carbamoyl aspartate formed per h per μg of protein) as a function of aspartate concentration (mM). (b) The same data replotted as specific activity aspartate concentration vs. specific activity (Eadie plot). Without nucleotide designated by ○, with ATP (2.0 mM) by △, and with CTP (0.5 mM) by □. The lines were drawn according to the $\bar{Y}$ function of Monod et al. (1965) (specific activity is given by $5.8\bar{Y}$). In each case $n = 3$, $c = 0.05$, $K_R = 6.5$ mM. $L = 7$ for no nucleotide, 2 with ATP, and 35 with CTP. A maximum specific activity per site of 1.93 was used in the calculations.

tive site, show that at saturating substrate concentrations each native chain contributes to the total activity independently.

All members of the *intra*-subunit hybrid set (C_{nnn}, C_{nnp}, C_{npp}, C_{ppp}) exhibited Michaelis-Menten kinetics with virtually identical K_m values (Table II). Like the native subunit, C_N, the *intra*-subunit hybrids were not affected by the nucleotides CTP and ATP.

The ATCase-like hybrids (all having the structure C_2R_3), as seen in Table II, exhibited both homotropic and heterotropic effects. Figure 2 illustrates the properties of one such hybrid, $C_NC_PR_3$. The specific activity varied in a sigmoidal fashion with aspartate concentration, and the nucleotide effectors, CTP and ATP, inhibited and activated the hybrid by increasing and decreasing, respectively, the apparent K_m. Neither nucleotide affected V_{max}, but the sigmoidality was increased by CTP and decreased by ATP. These effects are shown in Figure 2b where the data are plotted as specific activity/[aspartate] vs. specific activity (Eadie, 1942).

Table II, which summarizes the kinetic properties of the different hybrids, shows that the Hill coefficient decreased and the apparent K_m increased as the number of active chains in the ATCase-like molecules was reduced. The maximum inhibition by CTP and activation by ATP were virtually the same for all species (activation is difficult to measure accurately and the experimental errors are larger).

Figure 3 shows the kinetic data for the two "isomeric" hybrids, $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$, both of which contain the same number of active and inactive (pyridoxylated) chains. Despite the different arrangements of the active chains in these two ATCase-like hybrids, the Hill coefficients, apparent K_m values, turnover numbers, and inhibition and activation by CTP and ATP, respectively, were virtually identical. These results are summarized in Table II.

Physical Properties. All the ATCase-like hybrids had physical properties very similar to the native enzyme. In particular the sedimentation coefficients and electrophoretic mobilities on cellulose acetate strips and in 5% polyacrylamide gels were identical. Also, as shown in Table III, the hybrids exhibited the ligand-promoted conformational changes characteristic of the native enzyme (Gerhart and Schachman, 1968).

Saturating levels of CAP alone (2 mM) had a small effect on the sedimentation coefficient. For most species $\Delta s/\bar{s}$ was −0.5%. The changes promoted by the subsequent addition of succinate were much larger (about −2.7%). As shown in Figure 4, the succinate concentration required to promote a given percentage change in the sedimentation coefficient was significantly higher for the hybrid, $C_NC_PR_3$, than for the native enzyme, $C_NC_NR_3$. With the bi-substrate analogue, PALA, which binds tightly to both the active and pyridoxylated sites (Greenwell et al., 1973), maximal values for $\Delta s/\bar{s}$ were obtained for all the ATCase-like species. These values were virtually the same for all the species, even including the molecule, $C_PC_PR_3$, which had very little enzyme activity.

Table III also summarizes the data for the ligand-promoted changes in the reactivity of the thiol groups of various ATCase-like species. As shown by Gerhart and Schachman (1968) when excess PMB was added to the native enzyme, pseudo-first-order kinetics (with a single rate) were observed for the 24 sulfhydryl groups per molecule which are located on the r chains (4 thiol groups per chain). The single cysteinyl group on each of the six c chains either reacted more slowly or failed to react with PMB (when both CAP and succinate were present). In the absence of ligands, similar results were obtained with the hybrid, $C_NC_PR_3$, and with the virtually inactive complex, $C_PC_PR_3$. Moreover, as shown in Table III, these species like the native enzyme exhibited a marked increase (more than twofold) in the pseudo-first-order rate constant upon the addition of CAP. Upon the subsequent addition of succcinate, there was an additional increase (almost threefold) in the rate constant for both $C_NC_NR_3$ and $C_NC_PR_3$. When the increase in the rate constant for $C_NC_PR_3$ caused by succinate (in the presence of CAP) is plotted as a function of the suc-

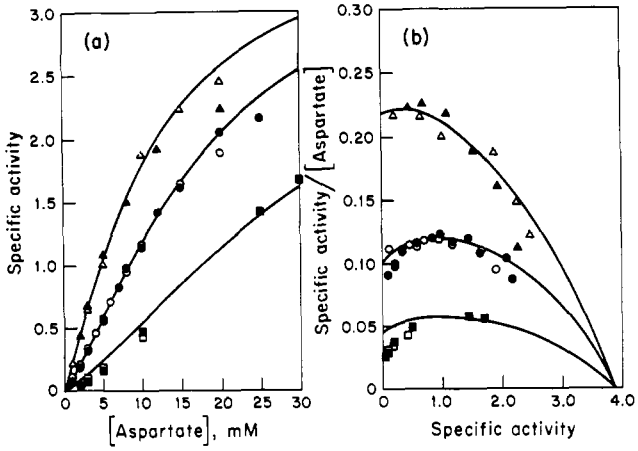

FIGURE 3: Comparison of the kinetics of the hybrids $C_{nnp}C_PR_3$ (open symbols) and $C_{npp}C_{npp}R_3$ (solid symbols). Measurements were conducted as in Figure 2. Without nucleotides designated by ○ and ●, with ATP by △ and ▲, and with CTP by □ and ■. The specific activity refers to the data for $C_{nnp}C_PR_3$ and the values for $C_{npp}C_{npp}R_3$ were decreased by 11% so that the V_{max} values for both hybrids were the same (cf. footnote 3). Theoretical curves were drawn as described for Figure 2 with identical parameters except for the number of active sites (n = 2).

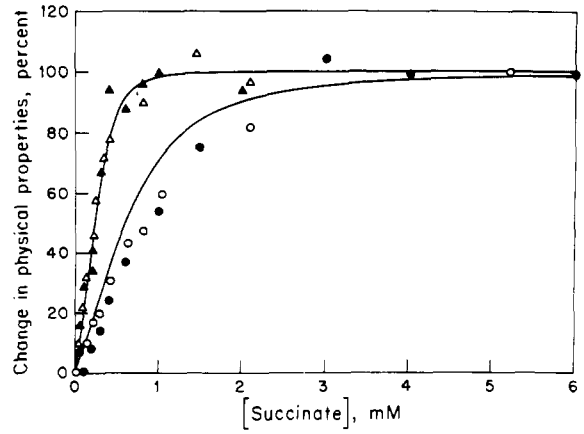

FIGURE 4: Conformational transition in ATCase and in hybrid $C_NC_PR_3$. The percent of the maximum change in sedimentation coefficient (ATCase, ▲ (from Gerhart and Schachman, 1968)); $C_NC_PR_3$ (●), or reactivity with PMB (ATCase, (△); $C_NC_PR_3$, (○) as a function of succinate concentration. Conditions described in Table III and in Materials and Methods. The curves are drawn according to the $\bar{R}_{rel}$ function of Monod et al. (1965), using values of L = 7, c = 0.05, K_R = 0.48 mM, and n = 6 for ATCase and n = 3 for $C_NC_PR_3$.

cinate concentration (Figure 4), the change in reactivity is seen to parallel closely the percentage change in sedimentation coefficient. For $C_PC_PR_3$ the addition of succinate caused no change in the reactivity observed with CAP alone.

Discussion

Kinetic Properties of Hybrids. As shown by Greenwell et al. (1973), mixed populations of C subunits pyridoxylated to different extents exhibited a loss of enzymic activity directly proportional to the degree of modification of the active sites. This observation was confirmed and extended by the studies on the individual members of the *intra*-subunit hybrid set; all of them followed Michaelis–Menten kinetics and had values of V_{max} which varied linearly with the number of active sites (Figure 1). These results indicate that the chains in the isolated C subunits act independently and that a pyridoxamine 5′-phosphate residue attached to one chain does not interfere with the activity of the nonpyridoxylated sites in the same trimer. Do such pyridoxylated chains affect the active chains in the ATCase hybrids? Is their effect on the active chains in the same C subunit in ATCase similar to their influence on the active chains in the other C subunit? Direct interactions may be expected in $C_{nnp}C_{nnp}R_3$ and $C_{npp}C_{npp}R_3$, for example, since the active and inactive chains are in contact with each other. In the hybrid, $C_NC_PR_3$, however, the active chains are not in direct contact with the inactive chains and effects of one type of chain on the other must be mediated by the entire molecule, presumably through the R subunits.

Like the hybrids of the C subunit the various ATCase-like species exhibited enzymic activities at high substrate concentrations (V_{max}) which were proportional to the number of active sites regardless of the arrangements of those sites within the molecules. Thus each catalytic chain contributed equally to V_{max}. However there was a significant decrease in n_H and an increase in the apparent K_m as the number of active sites per molecule was reduced (Table II). The decrease in n_H is to be expected as the number of active sites capable of cooperativity is diminished. The increases in the apparent K_m of the hybrids relative to ATCase can be attributed to the reduction of remaining active sites in each molecule that are converted from a low-affinity form to a conformation with higher affinity as a result of the allosteric transition accompanying the binding of ligands to the first site (or sites).

Table III: Ligand-Promoted Conformational Changes in ATCase Hybrids.

Species	Active Sites	$\Delta s/\bar{s}$ (%)[a] CAP[c]	Succinate[d]	PALA[e]	$k^b \times 10^3$ No Ligand	CAP[f]	Succ
Native enzyme	6	−0.5	−3.0	−3.1	3.0[g]	9.0[g]	25[g]
$C_NC_NR_3$	6	−0.5			4.1	10	23
$C_{nnp}C_{nnp}R_3$	4	−0.5					
$C_NC_PR_3$	3	−0.5	−2.7	−3.4	4.2[g]	10[g]	29[g]
$C_{nnp}C_PR_3$	2	−0.1	−2.3	−2.8			
$C_PC_PR_3$	0	−0.5		−3.1	6.2[g]	13[g]	14[g]

[a] Measurements made at room temperature (20 °C) in 40 mM potassium phosphate buffer at pH 7 containing 0.2 mM EDTA and 2 mM β-mercaptoethanol. [b] Pseudo-first-order rate constant (s^{-1}) with 0.2 mM PMB at 20 °C. Solutions contained 40 mM potassium phosphate at pH 7 and 25 mM Tris. [c] Concentration of 2 mM. [d] CAP present in these solutions and in reference solutions; succinate concentration, 3 mM. [e] Saturating levels, 0.9 mM. [f] Concentration of 2.1 mM. [g] Data from Blackburn and Schachman (1976).

The derivatives, $C_{npp}C_PR_3$ and $C_PC_PR_3$, are particularly interesting with respect to their values of n_H and K_m. As shown by Greenwell et al. (1973), pyridoxylation of C subunits followed by reduction of the Schiff base does not lead to complete inactivation.[2] Thus $C_PC_PR_3$ instead of being totally devoid of activity had a V_{max} about 4% of that of the native enzyme. Similarly $C_{npp}C_PR_3$ exhibited more activity than expected for a hybrid containing only one active chain. This activity of the pyridoxylated derivatives stemmed both

[2] As shown in Table II, C_P has the same K_m as the unmodified C subunits, indicating that the remaining enzymic activity is attributable to unmodified sites rather than to an intrinsic activity of pyridoxylated sites.

from incomplete modification (Greenwell et al., 1973) and from reversal of the pyridoxylation reaction by a light-dependent process (J. M. Ritchey, I. Gibbons, and H. K. Schachman, manuscript in preparation). Experiments with different preparations indicated that individual sites which should have been inactivated by pyridoxylation had a small probability (between 0.02 and 0.07) of being active. This was of little consequence for those hybrids containing three or four active sites. However, the residual activity had a significant impact on the kinetic behavior of $C_{npp}C_PR_3$; this hybrid had a measured n_H of 1.25, whereas a molecule with only one active site should have a Hill coefficient of 1.0. Even the preparation of $C_PC_PR_3$ with only 4% of the activity of the native enzyme would contain molecules possessing more than one active site; in a binomial distribution of species there would be 78% having no active sites, 20% with one active site, 2% with two active sites, and about 0.2% with more than two active sites. The observed kinetic properties of $C_PC_PR_3$ thus corresponded largely, but not exclusively, to molecules with one active site. When the estimated activity due to species containing two active sites is subtracted from the measured activity of $C_PC_PR_3$, the corrected data corresponded to a Hill coefficient of about 1.0. Thus the observed values for n_H of $C_PC_PR_3$ and $C_{npp}C_PR_3$ are doubtless too high because of the lack of complete inactivation of the pyridoxylated polypeptide chains. It is striking that the apparent K_m of $C_PC_PR_3$ was very high compared with the native enzyme (40 vs. 6.5 mM). This high value is partially representative of the intrinsic substrate affinity of the constrained state of the enzyme. If the derivative contained only one active c chain, the apparent K_m would have represented the population of molecules prevailing in the absence of substrates, i.e., principally the constrained form. Similarly, if the hybrid, $C_{npp}C_PR_3$, had not contained any molecules with two active chains, its apparent K_m would probably have approached that of $C_PC_PR_3$.

As seen in Table II both the maximum inhibition by CTP and activation by ATP were unchanged (within experimental error) by incorporation of pyridoxylated chains in place of native c chains in the ATCase-like molecules. Neither the number nor the location of the inactive chains influenced the heterotropic effects manifested at low substrate concentrations. Also the nucleotide effects vanished at high aspartate concentration as shown in Figures 2 and 3 for different hybrids. Thus the response of the hybrids to the nucleotide effectors was similar to that of the native enzyme at very low and high substrate concentrations.

The kinetic properties of $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$ are particularly noteworthy. Despite the different locations of the two active chains in these ATCase-like molecules, their allosteric properties summarized in Table II were indistinguishable.[3] Such behavior would be expected if the molecules responded to ligands by a concerted conformational change. In contrast a sequential progression of ligand-promoted conformational changes would probably have a significantly different effect on $C_{nnp}C_PR_3$ (where the active chains are in direct contact) from that on $C_{npp}C_{npp}R_3$ (where the active chains are separated from each other). Thus the kinetic properties provide strong evidence for the view that the ligand-promoted conformational change in these molecules is concerted.

Ligand-Promoted Conformational Changes in the Hybrids. In interpreting the homotropic and heterotropic effects manifested by the ATCase hybrids, we tacitly assumed that the various molecules undergo the same type of ligand-promoted allosteric transition as the native enzyme. This assumption is based on the premise that subunit interactions are not drastically altered as a consequence of the introduction of the pyridoxamine 5′-phosphate moieties. That the various hybrids (and even $C_PC_PR_3$) are similar to native ATCase in their response to ligands is seen by the values of $\Delta s/\bar{s}$ and the pseudo-first-order rate constants summarized in Table III.

As shown by the spectral changes described in the previous paper (Blackburn and Schachman, (1976)), CAP binds strongly to pyridoxylated sites in addition to active sites in c chains in hybrids like $C_NC_PR_3$. This binding promotes a small conformational change in the various hybrids as revealed by the values of $\Delta s/\bar{s}$ and the pseudo-first-order reaction rate constants for the reaction of the thiol groups with PMB. Even the almost inactive derivative, $C_PC_PR_3$, showed the same changes in these physical parameters upon the addition of CAP. Succinate, a nonreactive analogue of the second substrate, aspartate, does not bind to pyridoxylated chains (Blackburn and Schachman, (1976)) and, as shown in Table III, it had no effect on the cysteinyl residues of the R subunits in $C_PC_PR_3$. However, succinate did have a significant effect on those hybrids (like $C_NC_PR_3$) which contained active sites. This is shown in Figure 4 where the values of $\Delta s/\bar{s}$ and the rate data for both $C_NC_PR_3$ and the native enzyme are presented.

The effect of the bi-substrate analogue, PALA, on the various ATCase-like molecules is particularly striking. With the native enzyme, PALA causes a physical transition similar to that promoted by CAP and succinate (G. J. Howlett and H. K. Schachman, manuscript in preparation). All the species tested, including $C_PC_PR_3$, displayed essentially the same values of $\Delta s/\bar{s}$ upon the addition of saturating values of PALA. Thus PALA which is known to bind to pyridoxylated sites (Greenwell et al., 1973) can promote a gross conformational change even in molecules which cannot bind succinate.

The various ATCase-like molecules containing pyridoxylated and native c chains were remarkably similar in (1) turnover number per active site; (2) maximum inhibition by CTP and activation by ATP; (3) change in sedimentation coefficient due to saturating concentrations of ligands; and (4) reactivity of thiol groups toward PMB in both the absence and presence of ligands. Hence the active chains in the various molecules must be capable of existing in both constrained and relaxed conformations and, upon the addition of ligands, they are converted from one form to the other. Also the similarities in terms of these diverse properties indicate that the strengths of the *inter*-subunit bonding domains are unaltered by the chemical modification and that the mediation of both homotropic and heterotropic effects by a conformational change is unimpaired. Moreover, since the active c chains in hybrids like $C_{npp}C_{npp}R_3$ and $C_{nnp}C_PR_3$ (and other ATCase-like species) have turnover numbers per active site characteristic of ATCase other than the free C subunits (or C_{nnp} or C_{npp}), the quaternary constraint in the ATCase-like molecules must be the same as in the native enzyme.

[3] These two hybrids were prepared at different times from different preparations of pyridoxylated C subunits. The slight differences (11%) in V_{max} for $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$ can be attributed to variations in the extent of pyridoxylation of the subunits and to small errors in the measurement of the low enzymic activities. Hence the values plotted in Figure 3 for these hybrids were normalized to the same value of V_{max} (3.6 μmol of carbamoyl aspartate per h per μg of protein).

Aspartate (or succinate) promotes the conversion of the enzyme in the presence of CAP to a relaxed state as revealed by the kinetic and physical measurements. A demonstration of this conversion with the hybrid, $C_NC_PR_3$, and the native enzyme is presented in the Hill plots shown in Figure 5. The lower line, with a slope of 1.0, representing the state of both $C_NC_PR_3$ and $C_NC_NR_3$ at very low substrate concentration, corresponds to a K_m of 40 mM aspartate. The upper line shows data for the relaxed form of $C_NC_NR_3$ at high substrate concentration. The K_m evaluated from this line is 6.5 mM. Between the low and high substrate concentrations, the data depart from the lower line toward the upper one as both $C_NC_NR_3$ and $C_NC_PR_3$ are converted from the low-affinity state to one with a higher affinity. Also shown in Figure 5 are the data for the highly charged hybrid, $C_NC_{P,T}R_3$, which fit on the upper line. These results indicate that this noncooperative species[4] (Gibbons et al., 1974) exists in a form resembling the relaxed state of the native enzyme. It is noteworthy that the value of K_m (40 mM) for ATCase and $C_NC_PR_3$ in the state corresponding to low substrates is close to that observed[5] for the virtually inactive molecules, $C_PC_PR_3$.

Are there Distinct Cooperative Units in ATCase? In the light of the unusual architecture of ATCase and the considerable speculation on cooperative dimers in hemoglobin (Edelstein, 1975), there has been interest in determining whether distinct parts of the ATCase molecules act as cooperative independent entities (Markus et al., 1971; Chan, 1975; Diesendorf, 1975). The only feasible models for ATCase are those including two or three sites. One of these involving a combination of at least two functional c and two r chains (as in c:r:r:c) bonded to each other from one C subunit to that beneath it can be ruled out since the hybrid $C_NC_PR_3$ is cooperative (Gibbons et al., 1974). Similarly a cooperative unit composed of two or three c chains in a C subunit can be eliminated since $C_{npp}C_{npp}R_3$ exhibits cooperativity. The remaining possibility implicating two sites in one C subunit and one site in the other is unlikely since the kinetic properties of the hybrids $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$ are similar. The whole molecule, then, must be involved in the allosteric transition promoted by ligands.

Can a Two-State Model Account for the Properties of the Pyridoxylated ATCase-Like Molecules? Since an intermediate state model (Koshland et al., 1966) would only account adequately for the similarities in properties of $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$ if an arbitrary set of assumptions were made about various subunit interactions, it seemed appropriate to examine the applicability of the two-state model of Monod et al. (1965). According to this model

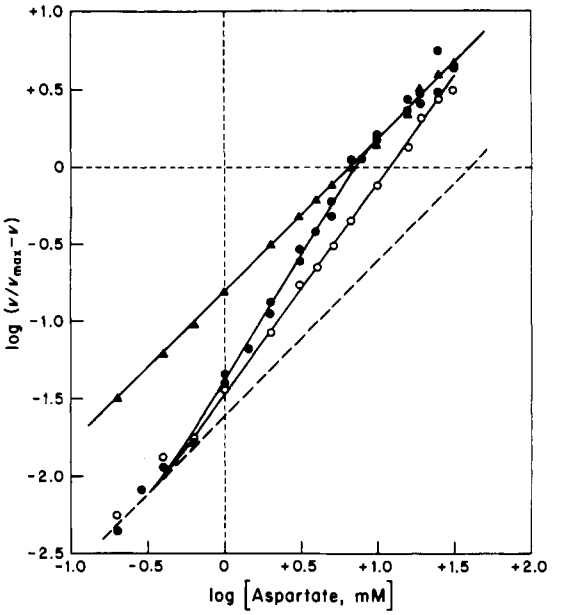

FIGURE 5: Kinetics of the species $C_NC_NR_3$, $C_NC_PR_3$, and $C_NC_{P,T}R_3$. The data are plotted according to the method of Hill (1952). The line representing the behavior of $C_NC_{P,T}R_3$ (▲) has a slope of 1. The dashed line is the extrapolation of the data for both $C_NC_NR_3$ (●) and $C_NC_PR_3$ (○) at low aspartate concentrations (<1 mM) and also has a slope of 1.

(cf. Rubin and Changeux, 1966), there are only two forms of the enzyme, T and R, with the former (constrained state) having the lower affinity for substrate and the latter (relaxed state) being the high-affinity form. The equilibrium between the T and R states is defined by L ([T]/[R]). Inhibition by CTP is due to its stronger binding to the T form and the consequent shift of L to a high value; in an analogous way ATP is presumed to activate ATCase by binding preferentially to the R form, thereby lowering the value of L (Changeux et al., 1968). In testing the model for the various hybrids, we assumed that the active sites in the R and T forms have the same specific activities and that the number of aspartate (or succinate) binding sites, n, is equal to the number of unmodified sites in the complexes. Since, as shown above, pyridoxylation does not appear to alter the interactions between subunits we assumed that the same value of L (in the presence of saturating CAP) applies to all the species. In a similar vein we assumed that c, the ratio of affinities of the native active sites for aspartate (or succinate) in the R and T states, and K_R, the intrinsic dissociation constant of the complex between the R form and aspartate, are all unchanged despite the presence of pyridoxylated chains in the complexes.

For the two-state model (Monod et al., 1965; Changeux and Rubin, 1968) the relative state function, $\bar{R}_{rel}$, representing the fraction of protein molecules in the R conformation in the presence of varying concentrations of ligand compared with the fraction at saturating concentrations of ligand, can be described by

$$\bar{R}_{rel} = \frac{(1 + Lc^n)[(1 + \alpha)^n - (1 + \alpha c)^n]}{(1 - c^n)[(1 + \alpha)^n + L(1 + \alpha c)^n]} \qquad (1)$$

In eq 1, c is K_R/K_T where K_R and K_T are the intrinsic dissociation constants for the substrate (or analogue) from the R and T states of the enzyme, respectively, and α is a measure of the ligand concentration defined as α = [ligand]/

[4] As shown by Gibbons et al. (1974), $C_NC_{P,T}R_3$ had a Hill coefficient of 1.0. Moreover a relatively large value of $\Delta s/\bar{s}$ was observed upon the addition of CAP, and the effect of succinate was considerably smaller than that obtained with the native enzyme. Thus, although all the $C_NC_{P,T}R_3$ molecules were not in the relaxed state in the presence of CAP, a significant fraction of the population of molecules existed in that form. Presumably the charge repulsion due to the acyl groups destabilizes the constrained form of the molecules relative to the relaxed state.

[5] The data at very low aspartate concentrations are subject to experimental error, and therefore the placement of the asymptote is only approximate. Consequently the values of K_m for ATCase, $C_NC_PR_3$, and $C_PC_PR_3$ may vary slightly from the reported 40 mM. This value of 40 mM for K_m can also be calculated from the theory for a two-state model (Monod et al., 1965) according to the relationship, $(1/K_m) = (1 + Lc)/(1 + L)K_R$, where the various terms are defined in connection with eq 1 and 2.

K_R. The saturation function, $\bar{Y}$, representing the fraction of specific sites in the total protein population occupied by the ligand, is represented by

$$\bar{Y} = \frac{\alpha(1+\alpha)^{n-1} + Lc\alpha(1+\alpha c)^{n-1}}{(1+\alpha)^n + L(1+\alpha c)^n} \quad (2)$$

These equations were employed to calculate theoretical curves for the kinetic behavior of the hybrids, $C_NC_PR_3$ (Figure 2), and $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$ (Figure 3), as well as for the conformational transition of ATCase and $C_NC_PR_3$ (Figure 4). For the calculation of the theoretical kinetic curves the appropriate K_m values were employed.

In calculating curves for the specific activity vs. aspartate concentration, we assumed that the velocity of production of carbamoyl aspartate was equal to $1.93n\bar{Y}$, where the value, 1.93, represents the V_{max} of ATCase per active site (i.e., 11.6 μmol of carbamoyl aspartate per h per μg of protein for the enzyme with six active sites) and the values of $\bar{Y}$ were calculated from eq 2. The value, 6.5 mM aspartate, for K_R was obtained by extrapolation of the asymptote of the Hill plot (Figure 5) for ATCase at high substrate concentration. Values of $L = 7$ and $c = 0.05$ were found to provide a good fit of the kinetic data for ATCase in the absence of nucleotides. For the kinetic data in the presence of CTP, a value of L of 35 gave a good fit as did $L = 2$ for the kinetics in the presence of ATP (the other parameters, c, K_R, and the specific activity per site, were not altered). When the values of L, c, and K_R deduced for the native enzyme were used to calculate theoretical curves for $C_NC_PR_3$ (with $n = 3$), excellent fits of the experimental data were obtained. Figure 2 shows these theoretical curves for the production of carbamoyl aspartate at varying aspartate concentrations in the absence of nucleotides and in the presence of either CTP or ATP. Similarly, excellent fits of the experimental data for $C_{nnp}C_PR_3$ and $C_{npp}C_{npp}R_3$ (with $n = 2$) were obtained as shown in Figure 3.

Equation 1 was used to calculate $\bar{R}_{rel}$ as a function of the succinate concentration (in the presence of CAP) with $L = 7$, $c = 0.05$, and a value of $K_R = 0.48$ mM, taken from Changeux and Rubin (1968). Both $\Delta s/\bar{s}$ and the change in the pseudo-first-order rate constant were employed as a measure of $\bar{R}_{rel}$. As seen in Figure 4 the theoretical curve for ATCase accounts very well for the experimental data. For the hybrid, $C_NC_PR_3$, the fit is not as satisfactory but it is clear that the model predicts that a higher succinate concentration is required to promote the conformational change in the hybrid containing three ligand-binding sites. A better fit of the data for $C_NC_PR_3$ could be achieved by a slight change in the value of K_R. However, it is not our goal here to calculate independent sets of parameters for each hybrid; rather, it is our purpose to demonstrate that the parameters evaluated from the behavior of the native enzyme can account satisfactorily for the properties of the hybrids containing fewer active sites. In this regard it is worth noting that, according to the model and the parameters evaluated above, the binding of a single ligand is not sufficient to promote the conformational change to the relaxed state. With a hybrid capable of binding two ligand molecules, the model predicts that the relaxed state is produced upon the addition of sufficient ligand. As seen in Table III, the value of $\Delta s/\bar{s}$ upon the addition of succinate and CAP to $C_{nnp}C_PR_3$ was −2.3%, a value only slightly less than that found for the native enzyme.

References

Blackburn, M. N., and Schachman, H. K. (1976), *Biochemistry*, preceding paper in this issue.

Chan, W. W.-C. (1975), *J. Biol. Chem. 250*, 668.

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.

Cohlberg, J. A., Pigiet, V. P., Jr., and Schachman, H. K. (1972), *Biochemistry 11*, 3396.

Diesendorf, M. (1975), *Biopolymers 14*, 19.

Eadie, G. S. (1942), *J. Biol. Chem. 146*, 85.

Edelstein, S. J. (1975), *Annu. Rev. Biochem. 44*, 209.

Evans, D. R., Warren, S. G., Edwards, B. F. P., McMurray, C. H., Bethge, P. H., Wiley, D. C., and Lipscomb, W. N. (1973), *Science 179*, 683.

Gerhart, J. C. (1970), *Curr. Top. Cell. Regul. 2*, 275.

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.

Gibbons, I., Yang, Y. R., and Schachman, H. K. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 4452.

Greenwell, P., Jewett, S. L., and Stark, G. R. (1973), *J. Biol. Chem. 248*, 5994.

Hill, T. L. (1952), *J. Chem. Phys. 20*, 1259.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.

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

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

Markus, G., McClintock, D. K., and Bussel, J. B. (1971), *J. Biol. Chem. 246*, 762.

Meighen, E. A., Pigiet, V., and Schachman, H. K. (1970), *Proc. Natl. Acad. Sci. U.S.A. 65*, 234.

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

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

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

Richards, K. E., and Williams, R. C. (1972), *Biochemistry 11*, 3393.

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

Rubin, M. M., and Changeux, J.-P. (1966), *J. Mol. Biol. 21*, 265.

Schachman, H. K. (1972), in Protein-Protein Interactions, Jaenicke, R., and Helmreich, E., Ed., West Berlin, Springer-Verlag, pp 17–54.

Weber, K. (1968), *Nature (London) 218*, 1116.

Wiley, D. C., and Lipscomb, W. N. (1968), *Nature (London) 218*, 1119.

Yang, Y. R., Syvanen, J. M., Nagel, G. M., and Schachman, H. K. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 918.