Cooperative Interactions in Hybrids of Aspartate Transcarbamylase Containing Succinylated Regulatory Polypeptide Chains†

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ABSTRACT: Succinylated derivatives of the regulatory subunit of aspartate transcarbamylase of *Escherichia coli* were prepared by treating the intact enzyme with succinic anhydride followed by dissociation of the modified protein into catalytic and regulatory subunits which were separated by ion exchange chromatography. The succinylated regulatory subunits were used in hybridization experiments with native subunits to study the organization of the six regulatory polypeptide chains in the intact enzyme. Rapid mixing of succinylated and native regulatory subunits with native catalytic subunits yielded a four-membered hybrid set of reconstituted enzyme-like molecules; hence, the assembly process involves three regulatory combining units and the six regulatory polypeptide chains in the intact enzyme must be arranged as three dimeric subunits. When the modified and native regulatory subunits were incubated together for only brief periods (less than 1 min) followed by the addition of catalytic subunits, the resulting hybrid set was complex with no resolution of discrete species. Apparently the isolated regulatory dimers dissociate readily and reversibly into single polypeptide chains due to relatively weak intra-subunit bonding domains. In contrast, after reconstitution of enzyme-like molecules, the incorporated succinylated regulatory subunits did not exchange with free subunits. Enzyme-like molecules containing three extensively succinylated regulatory subunits show reduced binding of the inhibitor, CTP, and lack both the homotropic and heterotropic effects characteristic of native aspartate transcarbamylase. Preparations containing only slightly succinylated regulatory subunits showed only little inhibition by CTP and considerable cooperativity. The decrease in homotropic effects in these reconstituted molecules correlated with the reduction in the succinate-promoted change in the sedimentation coefficient. Reconstituted enzyme-like molecules containing regulatory subunits which had been extensively succinylated in the presence of CTP retained their binding capacity even though they were only slightly inhibited by CTP and exhibited reduced cooperativity. Hybrid molecules containing both native and succinylated regulatory subunits also possessed reduced allosteric behavior.

Aspartate transcarbamylase (ATCase)[1] from *Escherichia coli* has been the object of much study following the demonstration (Gerhart and Pardee, 1962) that the purified enzyme exhibits the homotropic and heterotropic effects characteristic of allosteric enzymes (Monod et al., 1965). In particular, the discovery of discrete subunits for catalysis and regulation (Gerhart and Schachman, 1965) has prompted many investigations aimed at determining the structure and function of the native enzyme (Gerhart, 1970; Schachman, 1972; Jacobson and Stark, 1973). It is now known that the enzyme is composed of six catalytic (c) and six regulatory (r) polypeptide chains (Weber, 1968; Wiley and Lipscomb, 1968; Meighen et al., 1970) arranged as two catalytic trimers (C) and three regulatory dimers (R) (Meighen et al., 1970; Rosenbusch and Weber, 1971; Nagel et al., 1972; Cohlberg et al., 1972). The isolated catalytic subunits, though fully active in catalyzing the formation of carbamyl aspartate from carbamyl phosphate and aspartate (Gerhart and Schachman, 1965; Gerhart and Holoubek, 1967), exhibit neither a sigmoidal dependence on aspartate concentration nor inhibition by CTP, the end product of the biosynthetic pathway; hence the regulatory subunits must be crucial in mediating the allosteric interactions. This paper describes the use of succinylated regulatory subunits (R_S) in elucidating the arrangement of the six regulatory polypeptide chains in the intact enzyme and their function in endowing the enzyme with its characteristic allosteric behavior.[2]

Reconstitution of enzyme-like molecules from native and succinylated subunits has been of value in determining the subunit composition of oligomeric proteins from the number of species in the hybrid set (Meighen and Schachman, 1970a,b). When this technique was applied to studies of the catalytic polypeptide chains of ATCase, it was found that the isolated catalytic subunits were trimers and that there were two such trimers per ATCase molecule (Meighen et al., 1970). An analogous study aimed at determining the organization of the six regulatory polypeptide chains in the intact enzyme is presented here. The hybrid set produced by rapid mixing of native regulatory subunits R_N, and R_S with native catalytic subunits, C_N, contained four species (Nagel et al., 1972). Hence the six regulatory chains are arranged as three dimers in ATCase. If R_N and R_S were incubated together for short periods prior to the addition of C_N, a

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[1] Abbreviations used are: ATCase, aspartate transcarbamylase; R, regulatory subunit; C, catalytic subunit; N (subscript), native; S (subscript), succinylated; r, regulatory polypeptide chain; c, catalytic polypeptide chain; CAP, carbamyl phosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate).

[2] A preliminary description of the results of this investigation was presented by Nagel et al. (1972).

more complex hybrid set was more observed due presumably to the dissociation of the regulatory dimers into single chains and the subsequent formation of mixed dimers. From the time dependence of the change in the hybrid set an approximate value of the dissociation constant of the R dimers was obtained.

Enzyme-like molecules containing native catalytic subunits and extensively succinylated regulatory subunits exhibited neither homotropic nor heterotropic effects. The role of the regulatory subunits in mediating the allosteric behavior of ATCase was studied with various derivatives by examining both their enzymic properties and the ligand-promoted conformational changes in the reconstituted enzyme-like complexes.

Experimental Procedure

Materials. ATCase was prepared by the method of Gerhard and Holoubek (1967) and stored as a precipitate in 3.6 M $(NH_4)_2SO_4$–0.01 M 2-mercaptoethanol, 2×10^{-4} M EDTA. Succinic anhydride was obtained from Eastman Kodak and the ^{14}C-labeled reagent (10^{-2} Ci/g) was obtained from New England Nuclear Corp. Reagent grade 1,4-dioxane (J. T. Baker) was redistilled just prior to use. Neohydrin, 1-(3-chloromercuri-2-methoxypropyl)urea, was purchased from K and K Laboratories and purified by precipitation from alkaline solution by the addition of HCl at 4°. DEAE-cellulose from Schleicher and Schuell was treated by the method of Peterson and Sober (1962). Dilithium carbamyl phosphate from Sigma Chemical Co. was purified as described by Gerhart and Pardee (1962). Succinic acid was obtained from Eastman Kodak and recrystallized. Glutaric acid from Mann Biochemicals was treated with decolorizing charcoal and recrystallized three times from 80% benzene–20% ethyl ether. L-Aspartic acid was obtained from Calbiochem and L-[^{14}C]aspartic acid (1.4 Ci/g) from New England Nuclear, Inc. CTP was purchased from P-L Biochemicals; concentrations were determined spectrophotometrically with E_{280} nm of 1.28×10^4 M^{-1} cm^{-1} at pH 2 (Bock et al., 1956).

Three buffers were used for most experiments. "Standard imidazole buffer" contained 0.05 M imidazole–imidazole acetate, 0.01 M 2-mercaptoethanol, and 2×10^{-4} M EDTA (pH 7). "Standard phosphate buffer" contained 0.04 M K_2HPO_4–KH_2PO_4, 0.01 M 2-mercaptoethanol, and 2×10^{-4} M EDTA (pH 7). "Standard Tris buffer containing zinc" was composed of 0.025 M Tris–Tris-acetate (pH 8), 0.01 M 2-mercaptoethanol plus 1% by volume of a solution containing 0.5 M imidazole–imidazole chloride (pH 7.5), and 0.02 M zinc acetate.

Methods. Protein concentrations were determined spectrophotometrically using E_{280nm} (cm^2 mg^{-1}) of 0.59 for ATCase, 0.72 for C, and 0.30 for R. Succinylation of the native enzyme was performed with dioxane solutions of [^{14}C]succinic anhydride with a specific radioactivity of 5×10^{-4} Ci/g. The anhydride concentration was 0.30 M when the final ratio of anhydride to lysyl residues was less than 1.5 and 0.48 M when more than 1.5 mol of anhydride was added per mol of lysyl residues. Calculations were made assuming 144 lysyl residues per ATCase molecule. The anhydride solution was added in aliquots of about 20 μl to a 5 mg/ml solution of ATCase in 0.05 M Tris–Tris-chloride (pH 8), with rapid stirring. The pH was maintained between 7.8 and 8 by manual addition of 1.0 M KOH. Each aliquot of anhydride had completely reacted in less than 1 min as indicated by a stable pH.

The total number of succinyl groups per r chain was determined from ^{14}C incorporation into isolated R_S. Between 0.2 and 0.5 mg of protein in 200 μl of buffer was used for each determination. The amino groups on the modified subunits were measured by the colorimetric method of Moore and Stein (1948) as modified by Fraenkel-Conrat (1957) with native R as a standard. The number of succinylated amino groups was calculated by difference on the basis of 11 amino groups per r chain (Weber, 1968).

Sulfhydryl determinations were performed by the method of Sedlak and Lindsay (1968) using 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2), and an E_{412nm} of 1.36×10^4 M^{-1} cm^{-1} for the 5-thio-2-nitrobenzoate ion (Ellman, 1959). Since sulfhydryl groups of both R_N and R_S were found to be very labile, the sulfhydryl content was determined after reconstitution to give modified enzyme. Enzyme activity was measured using L-[^{14}C]aspartate as described by Porter et al. (1969) at 30° in standard imidazole buffer. Measurements of ^{14}C radioactivity were made using either Nuclear-Chicago Unilux II or Mark I scintillation spectrometers with Bray's solution (10 ml/vial) as the scintillation fluid (Bray, 1960).

Zone electrophoresis was performed in a Beckman Model R-101 Microzone Electrophoresis Cell on 14.6-cm Gelman Sepraphore III cellulose acetate strips. Electrophoresis was usually performed in standard Tris buffer containing zinc at 300 V for 25 min. Gels were fixed and stained as described by Meighen et al. (1970).

Polyacrylamide disc gels were used as described by Ornstein (1964) and Davis (1964). The gels were prepared according to the "Tris system" of Jovin et al. (1964) except that the lower gel contained 5% acrylamide and 0.13% N,N'-methylenebisacrylamide. The upper buffer contained 6.32 g of Tris and 3.94 g of glycine per liter (pH 8.9) and the lower buffer was 0.1 M Tris–Tris-chloride (pH 8.1). Electrophoresis was performed until the tracking dye, Bromophenol Blue, had migrated to within 1 cm of the gel bottom. Analytical gels were 5 mm in diameter and from 5 to 10 cm in length. A current of between 1 and 2 mA per gel was applied for about 4 hr. Gels were stained for 2 to 3 hr in 0.25% Coomassie Brilliant Blue dissolved in 25% methanol–10% acetic acid. The gel background was destained electrophoretically in 5% methanol–7% acetic acid after the gel had been soaked in the same solution overnight. After destaining, some gels were scanned at 560 nm with a Gilford Model 222 photometer and Beckman DU monochromator equipped with a Gilford Model 2410 Linear Transport Accessory and a recorder. Preparative gels were 2.2 cm in diameter and 8 cm in length. A current of from 5 to 10 mA per gel was applied for 12 to 16 hr at 4°. Gels were removed from their glass tubes and kept at 4° while a duplicate gel was stained for 1 hr and destained by diffusion in 5% methanol–7% acetic acid. Bands of interest were sliced manually with a razor blade from the unstained gels using R_F values relative to the tracking dye calculated from the stained gel. Separated slices were minced with a glass rod and extracted overnight with mild shaking in 3 vol of standard phosphate buffer at room temperature. The mixture was then centrifuged and the supernatant, containing extracted protein, was removed with a pipet and filtered through Millipore filters.

Sedimentation studies were performed with a Beckman Model E ultracentrifuge equipped with schlieren and Rayleigh optics as well as a photoelectric scanning absorption optical system (Schachman and Edelstein, 1966). Double-

Table I: Extent of Succinylation of Regulatory Polypeptide Chains.

Anhydride Added per Protein Lysyl Residue (mol/mol)	Succinylated Residues per Regulatory Polypeptide Chain		Total Sulfhydryl Groups[c]
	Total[a]	Amino[b]	
0	0	0	28
1.0	2.5		28
2.8[d]	3.3 ± 0.1	3.0 ± 0.2	29
2.8	4.6 ± 0.2	4.3 ± 0.3	28

[a] Determined by ^{14}C incorporation into regulatory subunits. [b] Determined by the ninhydrin procedure of Fraenkel-Conrat (1957). [c] Determined by reaction of Nbs_2 with the modified enzyme or after R_S was reconstituted with C_N as described under Experimental Procedure. [d] Succinylation performed in the presence of 0.01 *M* CTP.

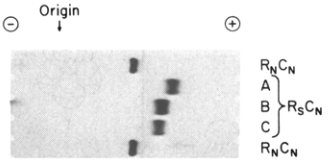

FIGURE 1: Electrophoresis of reconstituted ATCase containing succinylated regulatory subunits (R_S) and native catalytic subunits (C_N). R_SC_N samples A, B, and C contained an average of 4.6, 3.3, and 2.5 succinyl residues per regulatory polypeptide chain, respectively. Cellulose acetate electrophoresis was performed in 0.02 *M* K_2HPO_4–KH_2PO_4 and 10^{-4} *M* EDTA (pH 7.0), at 250 V for 20 min as described under Experimental Procedure.

sector cells with a 12-mm optical path were used throughout. When two samples in different cells were examined in the same experiment, one cell was fitted with a 1° positively wedged window (Gerhart and Schachman, 1968). Small changes in the sedimentation coefficient of native and modified ATCase produced by ligands were measured using the difference sedimentation technique described by Kirschner and Schachman (1971). Protein concentrations of from 3 to 5 mg/ml in standard imidazole buffer were routinely used; sample and reference ligands were present at a concentration of 10 m*M*. When no ligands were added to the protein in the sample and reference sectors, the apparent difference in sedimentation coefficient ($\Delta s/\bar{s}$) was −0.05%.

The binding of CTP to native and modified ATCase was measured using absorption optics and the sedimentation velocity technique (Steinberg and Schachman, 1966; Gerhart and Schachman, 1965). The concentration of unbound CTP was determined by measuring the A_{275nm} of the CTP plateau after the protein and protein–CTP complex boundaries had sedimented about halfway down the cell. The concentration of bound CTP was measured by the difference in the A_{275nm} of the CTP plateau in the absence and presence of protein. Traces taken at 36 min after reaching a speed of 56,000 rpm were analyzed in this manner. The total CTP concentration was 9.8×10^{-5} *M* and the total protein concentration was 8.7×10^{-6} *M* (2.7 mg/ml) in all experiments. Samples also contained 10^{-2} *M* dilithium carbamyl phosphate to reduce binding of CTP to the catalytic sites.

Subunit Separation and Reconstitution. Native and modified enzyme were dissociated with neohydrin and the subunits were separated by DEAE-cellulose chromatography as described by Kirschner (1971) and Schachman (1972). Elution of R_S required higher concentrations of KCl than were necessary for R_N. When 1 mol of anhydride was added per mol of protein lysyl residues, 0.15 *M* KCl was required to elute R_S. ATCase succinylated by adding 2.8 mol of anhydride per mol of lysyl residues required 0.20 *M* KCl to elute R_S. After collecting fractions of R_S, we added 2-mercaptoethanol to give a concentration of 0.01 *M*; then 10% by volume of a solution containing 0.5 *M* imidazole–imidazole chloride (pH 7.5) and 0.02 *M* zinc acetate was added. Subunit preparations were precipitated by dialysis vs. 3.6 *M* $(NH_4)_2SO_4$ (adjusted to pH with Tris)–0.01 *M* 2-mercaptoethanol for concentration and storage.

Reconstitution of ATCase-like molecules at concentrations of 2–10 mg/ml was accomplished merely by mixing the appropriate subunit preparations in standard Tris buffer containing zinc. Reconstituted enzyme containing R_S was routinely separated from either subunit which was in excess and from aggregated material by Sephadex G-200 chromatography. The four-membered hybrid set (see Results) was prepared with a device which permitted mixing of R_S and R_N for a very short time before they were added to a solution of native catalytic subunit (C_N). Two Cornwall syringes with spring-loaded plungers and 2-ml capacity were mounted on a Lucite block. The syringe tips were connected to a Hamilton three-way valve, and the syringes were loaded with solutions of R_S and R_N while the valve was closed. The plungers were depressed and the valve was opened simultaneously so that the mixture of R_S and R_N was injected directly from the valve exit into a rapidly stirred solution containing C_N. Both syringes were emptied in about 5 sec. The volume of the mixing valve was about 100 μl so that the effective time of mixing R_S and R_N was appreciably less than 5 sec.

Results

Preparation and Properties of Succinylated Regulatory Subunits. Succinylation of the native enzyme was performed under conditions where its quaternary structure remained intact so that the subunit contact areas were protected and the conformation of the R subunits was constrained by association with the C subunits. No dissociation into subunits was observed when less than 2.8 mol of succinic anhydride was added per mol of lysyl residues. However, addition of 3.0 mol of anhydride per mol of lysyl residues produced 5–10% dissociation of the enzyme with the extent increasing as greater amounts of anhydride were added. Control experiments showed that the small amount of 1,4-dioxane added, about 1% by volume, had no measurable effect of the sedimentation coefficient, electrophoretic mobility, or kinetic properties of the enzyme.

R_S was isolated from modified ATCase in yields of 70–100%. Table I summarizes the conditions for the succinylation and the extent of modification of the r chains. Four separate preparations of the most heavily modified derivative gave a mean deviation in total succinylated residues of only 5%. Measurements of the total number of succinyl residues incorporated and the number of modified amino groups indicated that lysyl amino groups were the major ones modified. The sulfhydryl content of the r chains was not affected by succinylation.

Modified regulatory subunits retained the ability to reconstitute with native catalytic subunits. When excess C_N was added to R_S, no free R_S could be detected by sedimentation velocity experiments or by chromatography on Sephadex G-200 columns. Figure 1 shows electrophoresis patterns of the reconstituted complexes, R_SC_N, formed from C_N and the modified R preparations described in Table I. The modified ATCase-like complexes produced slightly

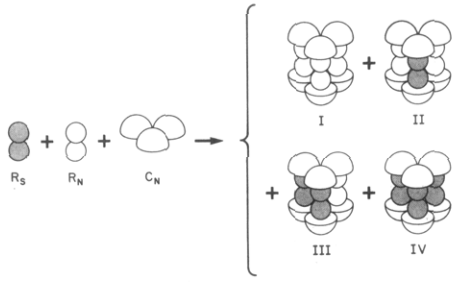

FIGURE 2: Schematic representation of four-membered hybrid set of ATCase-like molecules formed by reconstitution of native catalytic subunit (C_N) with both native, R_N (clear), and succinylated, R_S (stippled), regulatory subunits. According to this scheme regulatory subunits exist as dimers of regulatory polypeptide chains both in the isolated state and in the reconstitution process leading to ATCase.

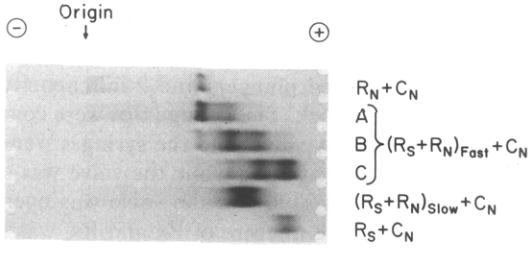

FIGURE 3: Hybrid sets of ATCase containing both R_N and R_S. "Fast" indicates that R_S and R_N were mixed for a very short time before being added to C_N. The device described under Experimental Procedure was used for this purpose. Samples A, B, and C were prepared by mixing different molar ratios of R_S to R_N before addition to C_N. These ratios were 0.5, 1.0, and 2.0 for A, B, and C, respectively. "Slow" indicates that R_S and R_N were mixed for 15 min before C_N was added; its preparation was otherwise identical with the fast sample B. In all cases C_N (4.5 mg/ml) was allowed to react with a 10% excess of regulatory protein. The volume of R_S (and R_N) was constant so that the concentration of the subunits was 1.5, 3.0, or 4.5 mg/ml. Fast samples were prepared at 4° while the slow sample was prepared at room temperature. Reconstituted ATCase containing only R_N and only R_S are shown as controls. Cellulose acetate electrophoresis was performed as described under Experimental Procedure at room temperature and 300 V for 23 min. Standard Tris buffer containing zinc was the solvent for both reconstitution and electrophoresis.

more diffuse bands than did the native enzyme, R_NC_N, due to charge heterogeneity from the succinylation. The relative electrophoretic mobilities for the different preparations corresponded to the extent of negatively charged succinyl groups incorporated.

Sedimentation velocity experiments on R_SC_N (sample A in Figure 1) showed a single, sharp boundary with an $s_{20,w}$ of 11.4 S in standard imidazole buffer containing 0.5 *M* NaCl. A control experiment with native enzyme gave an $s_{20,w}$ of 11.3 S.

Hybrids of ATCase Containing Both R_S and R_N. If the six r chains existed as three R dimers in the isolated state and reconstituted as such with C subunits to form ATCase-like molecules, the reaction of R_S and R_N with C_N should yield a four-membered hybrid set as illustrated in Figure 2. When R_S and R_N were mixed for a very short time, $(R_S + R_N)_{fast}$, and added to C_N a four-membered hybrid set was obtained. As seen in Figure 3, the amounts of the components in the hybrid set varied with the ratio of R_S to R_N in the reconstitution mixture. The pattern was unchanged after removal of aggregated material (usually about 5–10% of the protein) and excess subunits by Sephadex G-200 column chromatography. A similar pattern was obtained (Figure 5) on polyacrylamide gels where aggregates and subunits were separated clearly. The slowest band in the hybrid set in Figure 3 corresponded to native enzyme and the most rapidly migrating species contained three R_S subunits. The formation of these components and the two hybrids of intermediate mobility are in accord with the scheme depicted in Figure 2. Thus the reconstitution process involved three combining regulatory species and we conclude that the six r chains must exist in the enzyme as three R dimers.

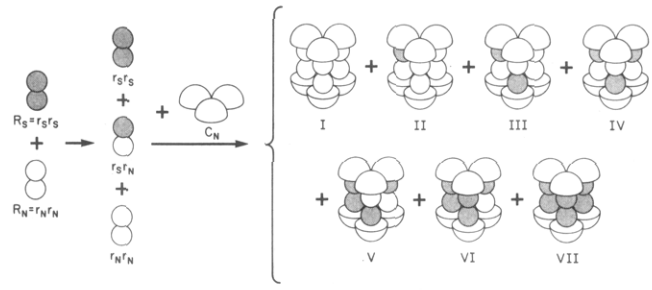

FIGURE 4: Schematic representation of a seven-membered hybrid set formed by mixing catalytic subunits with both succinylated (stippled) and native (clear) regulatory subunits. The formulation assumes that regulatory subunits (R_S and R_N) are dimers in equilibrium with regulatory polypeptide chains (r_S and r_N) and that there is sufficient time for a mixed dimer (r_Sr_N) containing one modified and one native chain to form.

However, when R_S and R_N were mixed for brief periods of time before reconstitution with C_N, different results were obtained as is shown by the "slow" sample in Figure 3. Premixing R_S and R_N followed by the addition of C_N gave a complex hybrid set of molecules which migrated within the limits defined by the native enzyme and that containing all R_S. Figure 4 provides an interpretation of this result. If R_S and R_N dimers were in equilibrium with monomeric polypeptide chains (r_N and r_S) and there was sufficient time to form mixed dimers (r_Sr_N) containing one succinylated and one native chain, reconstitution would produce a seven-membered hybrid set.

Inspection of the four-membered and complex hybrid sets in Figure 3 shows that the different hybrids were not formed in equal amounts when equivalent amounts of R_N and R_S were added to C_N. If R_N and R_S reconstitute with equal efficiency, the concentration distribution of the various species should be in the ratio 1:3:3:1 for a four-membered hybrid set and 1:6:15:20:15:6:1 for a seven-membered hybrid set (Meighen and Schachman, 1970a). Sample B in Figure 3, prepared with the aid of the device described under Experimental Procedure, does indeed show that the hybrids are present at higher concentrations than the fastest and slowest migrating species. Statistically predicted yields were not observed when hybridization was performed by injecting simultaneously separate solutions of R_S and R_N into a rapidly stirred solution of C_N. Presumably, local reconstitution occurred when this procedure was used; as a result higher proportions of the native enzyme and that containing all succinylated subunits were formed than in experiments involving reconstitution of a more homogeneous solution.

A quantitative test of the distribution of protein in the four bands was made by scanning stained polyacrylamide gels of the four-membered hybrid set such as that shown in Figure 5 and denoted "0" time. A plot of the absorbance vs. linear distance was recorded and the area corresponding to native ATCase was about 8% of the total area for the hybrid set. This is close to the expected value of 12% and indicates that R_N reconstituted with an efficiency no greater than that of R_S.

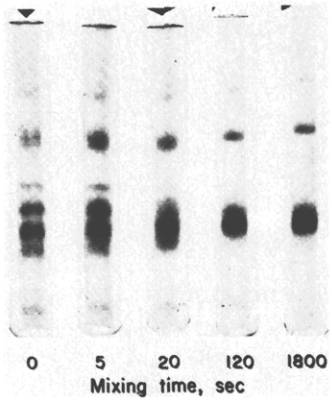

FIGURE 5: Kinetics of the transition from the four-membered to the complex hybrid set. One volume each of R_N and R_S (4.2 mg/ml) was mixed for the indicated times before two volumes of C_N (8.0 mg/ml) were added. The "0" time point was prepared with the aid of the device described under Experimental Procedure. Reconstitution was performed in standard Tris buffer containing zinc at room temperature. Analytical polyacrylamide disc gels, 10 cm in length, were used for electrophoresis as described under Experimental Procedure. Migration, toward the anode, is from top to bottom. The bands near the top of the gel are due to aggregated material formed during the reconstitution.

The complex hybrid set in Figure 3 is apparently devoid of a band corresponding to the native enzyme. As noted above, however, if reconstitution involved six regulatory chains combining randomly, only $1/64$ of the total protein would be expected statistically as native enzyme in the seven-membered hybrid set.[3] The difference in the distribution of species between slow and rapid mixing was exploited, as shown in Figure 5, to obtain an estimate of the dissociation rate of R dimers. R_N and R_S were mixed for various times before C_N was added. The polyacrylamide gels were scanned and the fraction of the native enzyme determined. After R_S and R_N had been mixed for about 20 sec prior to the addition of C_N, the amount of the native enzyme had fallen to 4% of the total protein, about one-half its original value. Premixing of R_N and R_S for 120 sec followed by addition of C_N produced virtually no detectable native enzyme.

Exchange of R Subunits between ATCase and Free R. Storage of the four-membered hybrid set in standard Tris buffer containing zinc for a month at 4° did not lead to an appreciable alteration of its electrophoretic pattern. These data indicate that reconstituted hybrids are not in rapid equilibrium with free subunits; otherwise the complex hybrid set should have formed. Subunit exchange was tested more directly by incubating reconstituted ATCase-like molecules containing R_S with free R_N for various periods of time (Figure 6). After 6 days at 4°, only a very small amount of hybrid could be detected electrophoretically. Since R_N retains the ability to reconstitute under these conditions and since it was present in slightly greater amounts than R_S, the amount of hybrid formed represents only a small fraction of the potentially exchangeable subunits which could be observed in this experiment. The small amount of hybrid species did not form by completion of

[3] It is tacitly assumed in the scheme illustrated by Figure 4 that both chains in the R dimers are succinylated to the same extent. If, however, the polypeptide chains within dimers were modified differently the distribution of members in the hybrid set would be skewed. As yet the electrophoretic resolution of the complex hybrid set is insufficient to warrant consideration of the effects of heterogeneity in the succinylation of the R dimers.

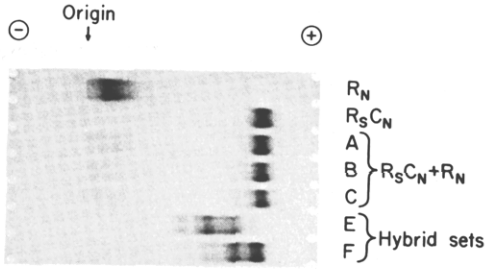

FIGURE 6: Regulatory subunit exchange. ATCase-like molecules (R_SC_N), 2.3 mg/ml, containing R_S were incubated for various times with R_N (1.0 mg/ml). Samples A, B, and C had been incubated in standard Tris buffer containing zinc for 0, 4, and 6 days at 4° before cellulose acetate electrophoresis was performed (Experimental Procedure) at 300 V for 25 min in the same buffer. Exchange is indicated by the formation of hybrid bands. Four-membered hybrid sets, E and F (samples B and C in Figure 3), are shown as controls. R_SC_N was prepared by mixing C_N with a 10% excess of R_S and the resulting material was then purified by Sephadex G-200 chromatography.

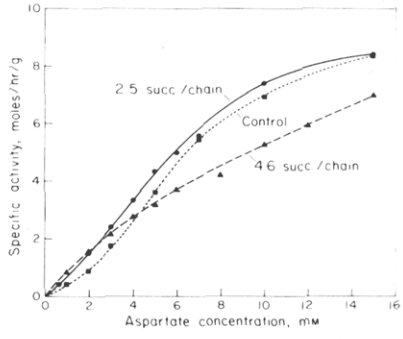

FIGURE 7: Kinetic properties of ATCase-like molecules containing R_S and C_N. The ordinate is the specific activity and the aspartate concentration is presented on the abscissa. Modified enzymes contained an average of 2.5 (●) or 4.6 (▲) succinyl residues per regulatory chain, samples C and A in Figure 1, respectively. Data for the native enzyme (■) are shown as a control. Kinetic assays were performed in standard imidazole buffer at 30° using L-[^{14}C]aspartate as described under Experimental Procedure. CTP inhibition data and Hill coefficients for these enzyme preparations are shown in Table II.

partially assembled ATCase-like molecules (Yang et al., 1974) since no hybrid bands were observed when R_N was added immediately before electrophoresis was performed.

Kinetic Properties of ATCase Containing R_S. The velocity of the reaction catalyzed by ATCase preparations containing regulatory subunits succinylated to different extents is shown in Figure 7 as a function of aspartate concentration. Both the heterotropic and homotropic effects, indicated respectively by CTP inhibition and Hill coefficients (Brown and Hill, 1922), were significantly reduced (Table II). The values of the maximal velocity, V_{max}, were determined from plots of v/[aspartate] vs. v (Eadie, 1942). V_{max} was found to be 12 ± 1 μmol of carbamyl aspartate/hr for reaction mixtures containing 1 μg of either the modified or native enzyme and there was no systematic trend with the degree of succinylation. Thus, differences in the saturation curves were not due to differences in the catalytic efficiency of the active sites. Since about 7% CTP inhibition was observed for the isolated catalytic subunit, the low levels of CTP inhibition observed for the modified enzymes may be due to competition at the active site (Kleppe, 1966) and not to a residual heterotropic effect.

Homotropic effects, although reduced, persist at low levels of succinylation as is indicated by the sigmoidal saturation curve of the less modified enzyme (Figure 7) and a Hill

Table II: Kinetic and CTP Binding Data for ATCase Containing R_S.

Succinylated Residues per Regulatory Chain	Hill Coeff.[a]	CTP Inhibition[b] (%)	r^c
0	1.7	55	2.9
2.5	1.5	10	0.5
3.3[d]	1.2	24	1.4
4.6	1.0	7	0.2

[a] Determined from the kinetic data in Figure 7. The results given are the maximum slopes of Hill plots (Brown and Hill, 1922). [b] Determined from the average inhibition of catalytic activity produced by 5×10^{-4} *M* CTP at aspartate concentrations between 3 and 7 m*M*. [c] *r* represents the average binding of CTP per ATCase-like molecule determined from sedimentation velocity studies using absorption optics as described under Experimental Procedure. The total CTP concentration was 9.8×10^{-5} *M* and the total protein concentration was 8.7×10^{-6} *M* in all experiments. [d] Succinylation performed in the presence of 0.01 *M* CTP.

Table III: Difference Sedimentation and Kinetic Data for Aspartate Transcarbamylase Containing R_S.[a]

Succinylated Residues per Regulatory Chain	$\Delta s/\bar{s}$ (%) I,[b] CAP vs. P_i	II,[b] Succinate + CAP vs. Glutarate + P_i	III,[c] Succinate vs. Glutarate	Hill Coeff.
0	−0.6	−3.6	3.0	1.7
2.5	−0.8	−2.7	1.9	1.5
4.6	−1.9	−2.5	0.6	1.0

[a] Difference sedimentation experiments were performed as described under Experimental Procedure. [b] Changes in the solvent viscosity and density caused by specific ligands were compensated by the addition of noninteracting ligands, potassium phosphate (P_i) for carbamyl phosphate (CAP) and glutarate for succinate. In this way both the reference solution and that under investigation were approximately identical in viscosity and density and the effect of each ligand at 10^{-2} *M* was investigated. [c] Calculated difference between the experimental values in I and II. If the effects of the ligands upon *s* are additive, these data given the magnitude of the effect of succinate (in the presence of CAP) on $\Delta s/\bar{s}$ for the native and modified enzymes. This experimental approach is necessary since succinate does not bind in the absence of CAP.

coefficient greater than 1.0. As the extent of succinylation was increased, however, homotropic effects also disappeared. As shown in Figure 7 and Table II, modification of 4.6 lysyl residues per r chain led to complexes which gave a hyperbolic saturation curve. In additon, the data from this heavily modified derivative in contrast to the other preparations gave linear Eadie and double reciprocal plots. Thus, ATCase-like species modified only in the R subunits have been produced which exhibit (a) reduced homotropic and very little heterotropic allosteric effects and (b) neither homotropic nor heterotropic effects.

Since succinylation could lead to the destruction of the binding sites for the inhibitor, the binding of CTP to both the native and modified enzymes was measured. As shown in Table II, both modified enzymes whose kinetics are shown in Figure 7 showed a greatly reduced ability to bind CTP. Thus, the data suggest that the binding sites for CTP were modified by succinylation. In order to determine whether the inhibitor binding sites could be protected, ATCase was allowed to react with a 2.8 molar excess of succinic anhydride in the presence of 0.01 *M* CTP. The data in

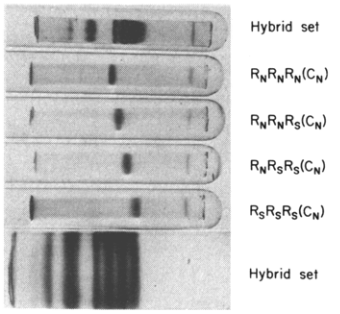

FIGURE 8: Resolution of the four-membered hybrid set formed by rapid mixing of R_N and R_S with C_N. ATCase containing all R_N and all R_S was prepared directly by reconstitution with C_N. The two species of intermediate mobility containing one and two R_S were prepared by extraction from sliced, unstained preparative gels; a stained example is shown at the bottom. Migration, toward the anode, is from left to right. Since the extent of protein migration is different on the small and large gels, an unfractionated hybrid set is also shown on a small gel as a control. Bands near the top of the gels result from aggregated species formed upon reconstitution. The use of polyacrylamide disc gels, both preparative and analytical, is described under Experimental Procedure.

Table I show that an average of 1.3 fewer residues per regulatory chain were succinylated in the presence of CTP than in its absence. Reconstitution of this derivative with C_N led to sample B in Figure 1. As expected, it had a lower electrophoretic mobility than sample A. The data in Table II indicated that CTP afforded partial protection of the regulatory binding sites. These results showed also that both homotropic and heterotropic effects are preserved in part when the enzyme is modified in the presence of CTP.

Ligand-Promoted Conformational Changes in ATCase Containing R_S. Upon addition of saturating amounts of carbamyl phosphate and the nonreactive aspartate analog, succinate, to the native enzyme, there is a 3.6% decrease in its sedimentation coefficient, *s* (Gerhart and Schachman, 1968). This decrease in *s* has been attributed to a change in the quaternary structure of the enzyme. Since this conformational change parallels the allosteric properties of the enzyme, it seemed of interest to examine the succinylated derivatives by difference sedimentation (Kirschner and Schachman, 1971). The percent changes in the sedimentation coefficient, $\Delta s/\bar{s}$, produced by carbamyl phosphate, CAP, and by both succinate and carbamyl phosphate are shown in Table III. The difference due to carbamyl phosphate increased and that due to the subsequent addition of succinate (column III) decreased markedly with increasing modification of the R subunits in parallel with the decrease in the Hill coefficient.

The change in *s* of −1.9% produced by CAP alone in the most heavily modified enzyme was much larger than that for the native enzyme (−0.6%). Analogous experiments with the modified enzyme in standard phosphate buffer which had an ionic strength about four times that of the imidazole buffer produced a similar change of −1.8%.

Isolation and Kinetic Properties of Hybrid Enzyme Molecules. Since incorporation of three heavily modified regulatory dimers led to enzyme-like molecules lacking allosteric properties, it seemed of interest to investigate the catalytic properties of hybrids containing both native and modified R dimers.

The intermediate species of the hybrid set were isolated using preparative polyacrylamide gels like those shown in Figure 8. Approximately 100 μg of each hybrid was obtained by extraction of slices of three unstained gels. Sam-

Table IV: Kinetic Properties of Hybrid Enzyme Molecules.

Succinylated Regulatory Dimers/ATCase Molecule	Hill Coeff.	Inhibition by CTP (%)
0[a]	1.7	55
1[b]	1.3	28
2[b]	1.1	18
3[c]	1.0	7

[a] Data obtained by assaying the native enzyme directly. [b] Data obtained from hybrid species isolated by extraction from sections of preparative polyacrylamide gels. [c] Data obtained from enzyme prepared by reconstituting heavily modified R_S with C_N.

ples of the purified hybrids were then examined separately and found to be free of contamination by other members of the hybrid set or by aggregated material. Neither native nor fully modified enzyme required purification and are shown as controls in Figure 8.

As seen in Table IV, the hybrids exhibited allosteric properties. In ATCase-like molecules containing two R_N and one R_S the Hill coefficient was 1.3 and the inhibition by CTP was 28% compared to 1.7 and 55%, respectively, for the native enzyme. A greater reduction in the allosteric properties was observed for the hybrid containing only one R_N and two R_S.

Discussion

Organization of the Regulatory Polypeptide Chains in ATCase. Since ATCase contains six r chains (Weber, 1968; Rosenbusch and Weber, 1971), the formation of a four-membered hybrid set upon rapid mixing of R_S and R_N with C_N demonstrates that the six r chains must be arranged as three R dimers within the ATCase molecules. This conclusion is in agreement with the finding of Cohlberg et al. (1972) who showed that covalently cross-linked R dimers could be incorporated into ATCase-like molecules and with the observation of G. Davies and G. Stark (personal communication) who showed that cross-linked R dimers could be detected by sodium dodecyl sulfate–polyacrylamide electrophoresis of ATCase which had been treated with dimethyl pimelimidate.

Equilibrium between Isolated R Subunits and r Chains. Sedimentation equilibrium experiments on isolated mercury-containing R subunits showed that the preparations were composed of dimers and monomers in reversible equilibrium (Cohlberg et al., 1972). In contrast, no dissociation of dimers was observed in analogous studies of the zinc-containing R subunits. However, in these experiments no special efforts were made to look for the dissociation of the zinc-containing R dimers, and Cohlberg et al. (1972) pointed out that a monomer–dimer equilibrium would have escaped detection if the association constant exceeded 10^7 M^{-1}. The equilibrium between R dimers and monomeric r chains is clearly demonstrated in Figure 5 by the time-dependent conversion of the four-membered hybrid set to a more complex mixture. Both R_N and R_S must have dissociated reversibly to single chains in order to produce mixed dimers, r_Nr_S, which then were incorporated into ATCase-like molecules. Assuming that dissociation of the R dimers is the rate-limiting step in the hybridization experiments we estimate from the patterns in Figure 5 at half-time, $t_{1/2}$, of about 20 sec for the dissociation of the dimers. Consideration of additional factors leads to a lowering of this estimate. In an equimolar mixture of R_N and R_S only one-half of the dissociation reactions produce hybrids upon reassociation of the single chains with the remainder leading to the original dimers. Also, as the concentration of hybrid dimers increases, the rate of the reverse reaction to form R_N and R_S becomes more significant. Thus, although the data available do not warrant a detailed evaluation of these factors, we conclude that, under the conditions used in these experiments, the $t_{1/2}$ for R dimer dissociation is probably less than 10 sec.

If the upper limit for the rate of association of the r chains to form R dimers is taken as 10^7 M^{-1} sec^{-1}, we estimate a maximal value for the association constant of about 10^8 M^{-1}. Hence, the free energy of formation of R dimers from r chains is between -10 and -11 kcal/mol. Since there are three r:r bonding domains implicated in the maintenance of the structure of ATCase, it is not surprising that no dissociation of ATCase has yet been observed. It should be noted, however, that it is hazardous to conclude that the strength of the r:r bonding domain in the isolated R subunits has the same value in the ATCase molecules. Some perturbation of the r:r domains is likely because of the constraints resulting from the bonding of the r chains to the c chains.

Stability of ATCase. As seen in Figure 6, there was very little exchange of subunits when ATCase-like molecules containing three R_S subunits were incubated for 6 days with free R_N. Since complexes containing R_S are likely to be less stable than native ATCase, we would expect even less exchange between free R subunits and those within the native enzyme. These results are in general agreement with those of Nelbach et al. (1972) who showed that less than 2% of the potentially exchangeable zinc ions in ATCase were replaced by $^{65}Zn^{2+}$ after 40 days of incubation. In contrast the zinc ions were removed readily from the isolated R subunits.

The lack of exchange of the R subunits is consistent with the observed great stability of ATCase and is attributable to the number and strength of the c:r bonding domains (Cohlberg et al., 1972). For exchange to occur, two such domains must be ruptured. Dissociation of the enzyme into subunits would require the disruption of six c:r bonding domains. As shown by Yang et al. (1974), ATCase-like complexes lacking one R subunit have considerable stability and disproportionate to ATCase and free C subunits relatively slowly. Thus four c:r bonding domains are sufficiently strong to confer considerable stability on the complex, C_2R_2, composed of two C and two R subunits. Since C_2R_2 disproportionates much more rapidly (I. Gibbons and H. K. Schachman, unpublished) in the presence of the bi-substrate analog, *N*-(phosphonacetyl)-L-aspartate (Collins and Stark, 1971), it would be of interest to measure the exchange of free R subunits with those in the intact enzyme when the bi-substrate analog is present. In this way the relative strengths of the c:r bonding domains for the two putative states of ATCase could be assessed.

Modification of the Binding Sites for CTP. Succinylation of only 2.5 residues per r chain was sufficient to cause nearly a complete loss of both CTP inhibition and binding (Table II). This destruction of the binding capacity can be attributed either to a selective modification of amino groups at or near the CTP binding sites or to a conformational change resulting from the increased negative charge on the protein. Considerable protection of the CTP binding sites was achieved by performing the succinylation in the presence of CTP. About 1.3 residues per r chain were protected

from modification (compare rows 3 and 4 in Table II) and the enzyme-like molecules reconstituted from the CTP-protected R_S subunits exhibited substantial inhibition by CTP (24%) and slight homotropic effect (Hill coefficient of 1.2).

It is of interest that the least modified derivative (2.5 succinylated residues per r chain) still exhibited considerable cooperativity as revealed by the Hill coefficient of 1.5. Until the precise locations of the modified residues in the polypeptide chains are identified it seems premature to speculate whether specific residues are crucial for the allosteric transition. It is likely that there is a general destabilization of the putative constrained or relaxed states of the enzyme due to steric and charge effects caused by the introduction of the succinyl groups. The apparent uncoupling of the homotropic and heterotropic effects in this derivative is probably attributable to the destruction of the CTP binding sites.

Conformational Changes in ATCase-Like Molecules Containing R_S. Since the most extensively modified R_S subunits combined with C_N to form enzyme-like complexes which displayed no allosteric effects, we conclude that incorporation of catalytic subunits into molecules with the general structure of ATCase is not, in itself, a sufficient condition for cooperativity. It is particularly interesting that the maximal velocity exhibited by this derivative is characteristic of the native enzyme rather than that of isolated catalytic subunits. This observation indicates that the catalytic sites in the modified enzyme molecules are in an environment like that of the native enzyme even though no cooperativity is observed. In this regard the results are somewhat similar to those for the R-deficient ATCase molecules (Yang et al., 1974). Even though one catalytic chain in each C subunit was not bonded to r chains, all the catalytic chains exhibited the same maximal velocity.

As shown by Yang et al. (1974), the R-deficient ATCase-like molecules (C_2R_2) exhibited allosteric properties (Hill coefficient of 1.4 and CTP inhibition of 36%) which are reduced compared to reconstituted or native ATCase (Hill coefficient of 1.6–1.7 and CTP inhibition of 55–60%). Thus neither the homotropic nor the heterotropic effects require the presence of three functional R subunits.[4] Similar results were obtained with hybrid molecules (Table IV) containing two native subunits, R_N, and one succinylated subunit, R_S. With $C_NC_NR_NR_NR_S$ a Hill coefficient of 1.3 and CTP inhibition of 28% were observed. Moreover, as seen in Table IV, hybrid molecules containing one R_N and two R_S exhibit both homotropic and heterotropic effects. Although the cooperativity (Hill coefficient of 1.1) and the CTP inhibition (18%) of $C_NC_NR_NR_SR_S$ were both markedly reduced compared to the native enzyme, they were significantly larger than those observed for ATCase-like molecules containing three R_S subunits. The Hill coefficient for $C_NC_NR_SR_SR_S$ was 1.0 and the CTP inhibition was only 7%. This low level of CTP inhibition is observed frequently with pure catalytic subunit, presumably due to the competition of CTP with carbamyl phosphate at the active sites. It should be noted that the kinetic measurements on $C_NC_NR_NR_NR_S$ and $C_NC_NR_NR_SR_S$ required the extraction of the proteins from the polyacrylamide gels and some loss in their allosteric properties may have occurred as a result of this manipulation. In contrast, $C_NC_NR_SR_SR_S$ was not subjected to this potentially hazardous treatment.

A reduction in the heterotropic effect exhibited by the hybrids containing R_S is not unexpected since these ATCase-like molecules have fewer CTP binding sites than the native enzyme. However, the more-or-less parallel decrease in the homotropic effects (as R_N is replaced by R_S) indicates that the allosteric behavior of ATCase is dependent on the total structure of the enzyme and that modification of one part of the molecule may influence other parts and alter the putative equilibrium between the constrained and relaxed states responsible for cooperativity and feedback inhibition. Mere destruction of the CTP binding sites would certainly cause a loss of feedback inhibition. But the concomitant decrease in cooperativity must be the result of indirect alterations in the structure of the whole enzyme molecule. Perhaps the constrained form of the enzyme is destabilized because of the negatively charged succinyl groups on the regulatory polypeptide chains with the result that the catalytic sites are partially in the high-affinity form even in the absence of the substrate, aspartate. Alternatively the conformational change of the enzyme from a form with low affinity for aspartate to the high-affinity state is prevented in part by stabilizing the former relative to the latter; i.e., the enzyme-like molecules containing R_S are "frozen" in the constrained state. Some evidence exists (see below) supporting each of these interpretations but as yet the data are too meager to permit definitive conclusions.

As shown earlier (Gerhart and Schachman, 1968) the sedimentation coefficient of native ATCase decreases about 3.6% upon the addition of the ligands, carbamyl phosphate and succinate. Similarly, binding of ligands to the catalytic chains is accompanied by a sixfold enhancement of the reactivity of the sulfhydryl groups of the regulatory chains. The concentration dependence of the ligand-promoted changes in the physical properties of the protein has been shown to be related to the effect of concentration of the substrate, aspartate, on the catalytic activity of the enzyme. Hence the conformational change in ATCase has been viewed as the allosteric transition from the constrained to the relaxed state of the enzyme. With the native enzyme only a small change (−0.6%) in the sedimentation coefficient is obtained when carbamyl phosphate is added (Table III). In contrast, ATCase-like molecules containing three extensively modified R_S subunits exhibit a large change in the sedimentation coefficient (−1.9%) upon the addition of carbamyl phosphate. As seen in Table III the change upon the subsequent addition of succinate is only −0.6% as contrasted to −3.0% for the native enzyme. On the basis of these observations alone it is tempting to speculate that the ATCase-like molecules containing R_S are partially converted to a swollen (relaxed) conformation by the addition of carbamyl phosphate; i.e., the constrained state is destabilized relative to the native enzyme. Hence, the active sites in enzyme-like molecules containing R_S are already in the high-affinity form when carbamyl phosphate is present and, as shown in Table III, no cooperativity is exhibited in kinetic studies with different concentrations of aspartate (Hill coefficient of 1.0). Molecules containing less-extensively succinylated R subunits (Table III) show intermediate values of $\Delta s/\bar{s}$ and Hill coefficient. Although this interpretation provides a satisfactory explanation for the sedimentation velocity data in terms of a conformational change and is consistent with the observed decrease in cooperativity for

[4] In the preceding paper in this series, Gibbons et al. (1974) showed that the homotropic and heterotropic properties were preserved in ATCase-like molecules containing one fully active and one inactive catalytic subunit along with three native regulatory subunits. Similarly, intra-subunit ATCase-like hybrids containing one inactive catalytic polypeptide chain in each subunit exhibited allosteric behavior.

these preparations, it does not account satisfactorily for all of the data unless an additional ad hoc assumption is made.

As seen in Figure 7, the K_m for the ATCase-like molecules containing extensively succinylated R subunits is significantly greater than the apparent K_m for the native enzyme. How do we account for the decreased affinity of these molecules for the substrate, aspartate? It is possible that these ATCase-like molecules containing R_S are in the relaxed state when carbamyl phosphate is present but their K_m is too high because of electrostatic repulsion from the negatively charged succinyl groups on the regulatory polypeptide chains. As shown in Figure 7 the enzyme-like molecules containing less extensively succinylated R subunits have an apparent K_m which is less than the native enzyme. Until the precise location of the succinyl groups and their spatial relationship to the active sites are known for the various derivatives, the validity of this interpretation cannot be assessed.

It seems less likely, but certainly not disproven, that the derivative containing extensively succinylated R subunits is "frozen" in the constrained state. Although such molecules would have a high K_m, as observed, it is difficult to understand why there would be a large change in their sedimentation coefficient upon the addition of carbamyl phosphate and a smaller subsequent change when succinate is added. It could be that the ligand-promoted change in the sedimentation coefficient is not related to that observed for the native enzyme. However, studies with other enzyme derivatives and with mutant enzymes (I. Gibbons, J. E. Flatgaard, and H. K. Schachman, unpublished) have consistently revealed a correlation between the changes in the sedimentation coefficient and the cooperativity of the preparations. Thus the sedimentation velocity data should probably be given more weight than the values of K_m which are more difficult to evaluate, especially in systems which show substrate inhibition and cooperativity.

Despite the present ambiguity in interpreting the available data it seems clear that the regulatory subunits serve more than a static role in mediating the conformational changes associated with the homotropic and heterotropic effects exhibited by ATCase. Dynamic changes in the regulatory subunits themselves and alterations in the bonding domains between them and the catalytic subunits seem to be essential.

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