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Hybridization of Native and Chemically Modified Enzymes, III. The Catalytic Subunits of Aspartate Transcarbamylase*

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Abstract. Succinylation of the catalytic subunits of ATCase yielded a relatively homogeneous, inactive electrophoretic variant which upon mixing with native regulatory subunits formed a complex the size of the native enzyme. Hybridization experiments with mixtures of this variant and the native catalytic subunits in the presence of excess regulatory subunits yielded three different molecular complexes which were separated and individually characterized. The number and properties of the various components indicated that each ATCase molecule contains two catalytic subunits. Hybridization was also effected at the intrasubunit level by dissociation and reconstitution of mixtures of the native and modified catalytic subunits. These experiments produced four components showing thereby that each catalytic subunit is composed of three polypeptide chains. The potential use of the various hybrids is discussed in relation to the unique properties manifested by regulatory enzymes.

In the preceding papers[1, 2] it was shown that hybridization of the native and succinylated forms of aldolase and glyceraldehyde-3-phosphate dehydrogenase (GPDH) provided strong evidence that each of these enzymes is composed of four identical polypeptide chains. The hybridization of the native and chemically modified aldolase was achieved only if the oligomeric structures were disrupted by a denaturing agent such as G-HCl. Such treatments lead to the destruction of both the quaternary and tertiary structures. In contrast, hybridization of the analogous two forms of GPDH was effected in solutions containing sodium chloride at high concentration (3 *M*) without, apparently, the disruption of the tertiary structure. Aspartate transcarbamylase (ATCase) from *E. coli*, the regulatory enzyme in the pathway leading to pyrimidine biosynthesis,[3, 4] seemed an appropriate enzyme for such hybridization studies since it is readily dissociated into discrete catalytic and regulatory subunits.[5] This paper presents results of hybridization experiments at both the intersubunit and intrasubunit levels. For the intersubunit hybridization the tertiary structure of the catalytic subunit remained intact whereas the intrasubunit hybridization required disruption of the tertiary structure.

When ATCase is treated with the mercurial, parahydroxymercuribenzoate, dissociation occurs to produce two types of subunits which are readily separated.[5]

Although the isolated catalytic subunits catalyze the formation of carbamyl aspartate, the allosteric properties characteristic of the native enzyme are conspicuously absent.[4, 5] The regulatory subunits, which function in the native enzyme to mediate the feedback inhibition by cytidine triphosphate, retain their ability to bind the inhibitor specifically. Thus both types of subunits apparently maintain their characteristic tertiary structures. The native enzyme can be rapidly reconstituted upon mixing the isolated catalytic and regulatory subunits after removal of the bound mercurial.

Experimental evidence from ligand binding studies,[6] molecular weight determinations,[5, 7] amino-terminal analyses,[7, 8] and X-ray diffraction analysis[9] led to the proposal of a tetramer as a model for ATCase. Recently, however, the determination of the amino acid sequence of the regulatory subunit,[10] revaluation of the molecular weights of the dissociated subunits,[10] and further consideration of the crystallographic studies[11] indicated that ATCase is a hexamer composed of six pairs of regulatory and catalytic polypeptide chains.[10, 11]

This communication presents the results of hybridization experiments aimed at resolving these apparently conflicting results. Studies were initiated on the native and succinylated catalytic subunits so as to investigate both the inter- and intrasubunit levels of ATCase; and the number and type of hybrids obtained support the view that ATCase contains two catalytic subunits each of which is composed of three polypeptide chains. The various hybrids proved to be readily separable and appear to be attractive species for evaluating the homotropic and heterotropic interactions in terms of subunit structure.[12]

Experimental. Materials: Native ATCase and the isolated catalytic and regulatory subunits were prepared as described by Gerhart and Holoubek.[13] Enzyme activity was determined according to the C^{14}-radioactivity assay of Porter *et al.*[14] Protein concentrations were determined spectrophotometrically on the basis of specific absorbance coefficients (0.1%, 1 cm) at 280 mμ of 0.59, 0.70, and 0.80 for ATCase, catalytic subunits, and regulatory subunits, respectively.

Succinylated catalytic subunits of ATCase were prepared according to the following procedure. Solid succinic anhydride (Eastman Organic Chemicals) was added to a 1.0% solution of the isolated catalytic subunits in 0.05 *M* Tris-chloride, pH 8.0, at room temperature. The pH was maintained at 8.0 by the manual addition of 1.0 *N* NaOH. After completion of the reaction, as indicated by the termination of the requirement for NaOH, the succinylated protein was dialyzed at 4°C against a phosphate buffer (0.04 *M* potassium phosphate, 2×10^{-4} *M* EDTA at pH 7) containing 2×10^{-4} *M* DTT.

Methods. Sedimentation velocity experiments were conducted at 60,000 rpm in a Spinco Model E ultracentrifuge equipped with a cylinder lens schlieren optical system. Photographic plates (Metallographic) were analyzed with the aid of a Gaertner microcomparator. A partial specific volume of 0.74 cc/gm was used in all calculations.

The ratio of the moles of succinic anhydride added per mole of lysyl residues was based on the presence of 42 lysyl residues per catalytic subunit[15] of molecular weight, 1.0×10^5. The percentage of free amino groups in the succinylated protein was estimated by colorimetric ninhydrin analysis according to the method of Moore and Stein[16] as modified by Fraenkel-Conrat.[17]

Zone electrophoresis experiments were performed routinely (200 v for 15 min) on 14.6 cm cellulose polyacetate strips (Gelman Sepraphore III) in phosphate buffer, in a Microzone Electrophoresis Cell, Model R-101 (Beckman Spinco). The protein was fixed and stained by immersion of the membrane in a solution of Ponceau-S (Beckman Spinco) for approximately 7 min; the membrane was rinsed in 5% acetic acid and then immersed

in 0.002% Nigrosin (Allied Chemical Co.) in 2% acetic acid for several hours. The membrane, after rinsing in 5% acetic acid, was dried at room temperature and stored.

Results. Preparation of succinylated catalytic subunit: Since limited succinylation of native enzymes can lead to relatively homogeneous electrophoretic variants,[1, 2] the isolated catalytic subunit of ATCase was reacted with succinic anhydride to produce a variant suitable for hybridization experiments. Figure 1 shows the sedimentation velocity and electrophoretic patterns for samples of the catalytic subunit reacted with 2.1 moles (A) and 3.5 moles (B) of succinic anhydride per lysyl residue in the protein. Ninhydrin analysis showed that 41 and 57 per cent of the lysyl residues in the subunit were succinylated in samples A and B, respectively. The succinylated species were essentially inactive with enzyme activities less than 2% that of the native catalytic subunit.

In both succinylated samples, the modified subunits sedimented as a single component (Fig. 1) with a sedimentation coefficient (5.8*S*) essentially identical to that for the native subunit. Thus limited succinylation did not disrupt the tertiary or quaternary structure of the catalytic subunit.

Electrophoresis of the succinylated species (Fig. 1) showed only a single component with mobilities of -1.7×10^{-4} and -1.8×10^{-4} cm²/v-sec for samples A and B, respectively, as compared to -1.0×10^{-4} cm²/v-sec for the native catalytic protein. Although sample B was apparently more homogeneous (i.e., migrated as a sharper band) than sample A, many electrophoresis experiments with sample B revealed a minor component with a mobility less than the major component[18]; thus sample A was used for all subsequent hybridization experiments.

Intra subunit hybridization: Sedimentation velocity experiments showed that treatment of the catalytic subunit (5.8*S*) with 2 *M* G-HCl produced a component sedimenting at 1.9*S* and aggregated material (about 50%). Subsequent removal of the denaturant by dialysis resulted in the precipitation of some protein; however, the supernatant contained a component with a sedimentation coefficient (5.8*S*) identical to the untreated catalytic subunit. Since these results indicated that the catalytic subunits dissociate into separate polypeptide

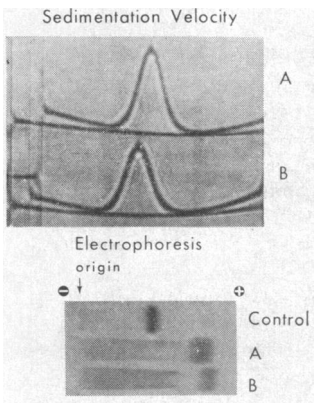

FIG. 1.—Sedimentation velocity patterns (upper) and electrophoresis patterns (lower) of the catalytic subunit of ATCase succinylated to different extents. The native catalytic subunit was reacted with 2.1 moles (sample A) or 3.5 moles (sample B) of succinic anhydride per lysyl residue as described in *Methods*. The protein concentration was 0.8% and the solvent for the ultracentrifuge experiments contained 0.5 *M* NaCl, 0.05 *M* DDT, in phosphate buffer, at 20°C. A double sector cell with a negative wedge window was used in conjunction with a second double sector cell having parallel windows so that two sedimentation velocity experiments could be conducted simultaneously. Sedimentation is from left to right. The sedimentation velocity pattern was obtained after 55 min at a rotor speed of 60,000 rpm.

Cellulose acetate electrophoresis was performed as described in **Methods**. The control was unmodified catalytic subunit, and the samples A and B refer to the succinylated catalytic subunits described above.

chains in 2 M G-HCl and that removal of the denaturant permitted reconstitution to the native catalytic subunit, this procedure was adopted for intrasubunit hybridization of the native and succinylated catalytic subunits.

In the hybridization experiments, solid DTT and G-HCl were added to a 0.5 per cent solution of the protein in phosphate buffer to give final concentrations of 0.1 and 2.0 M, respectively. The solution was then stored at 4°C for 15 minutes before dialysis overnight at 4°C against two changes of phosphate buffer containing 2×10^{-4} M DDT (100–200 vol of buffer per volume of sample). A small amount of precipitate produced by this procedure was removed in the clinical centrifuge and the supernatant saved for subsequent experiments.

The control experiments for the intrasubunit hybridization of native and succinylated catalytic subunits of ATCase are shown in Figure 2. The reconstituted succinylated catalytic subunit ($C_{S,Reconst.}$) migrated as a single sharp band well resolved from the reconstituted native catalytic subunit ($C_{N,Reconst.}$). Furthermore, these catalytic subunits reconstituted from their respective chains were indistinguishable from the undissociated succinylated (C_S) or native (C_N) catalytic subunits. No intermediate bands were detected in a mixture of native and succinylated catalytic subunits that either had not been dissociated and reconstituted ($C_N + C_S$) or had been dissociated and reconstituted prior to mixing ($C_{N,Reconst.} + C_{S,Reconst.}$).

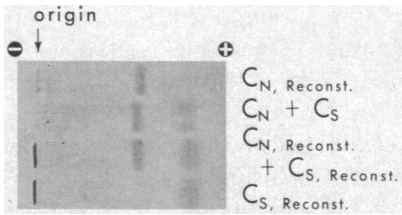

FIG. 2.—Controls for the intrasubunit hybridization of the native (C_N) and succinylated (C_S) catalytic subunits. The catalytic subunits were dissociated in 2 M G-HCl and the disorganized native and succinylated catalytic polypeptide chains subsequently were permitted to refold and reassociate during the dialysis procedure. In this way the respective subunits were reconstituted ($C_{N,econst.}$ and $C_{S,Reconst.}$). Cellulose acetate electrophoresis was performed as described in **Methods.**

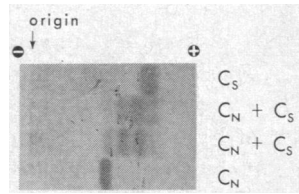

FIG. 3.—Intrasubunit hybridization of native, C_N, and succinylated, C_S, catalytic subunits. All samples were dissociated in 2 M G-HCl and then reconstituted by the dialysis procedure. The two hybrid sets, $C_N + C_S$, were mixtures of native and succinylated catalytic subunits at ratios of 0.6 and 0.9 for the upper and lower samples, respectively. Cellulose acetate electrophoresis was performed as described in **Methods.**

In Figure 3, results of the electrophoresis of hybrid sets of native and succinylated catalytic subunits are given. Four protein bands of different electrophoretic mobility can clearly be detected in mixtures of native and succinylated catalytic chains that had been reconstituted from G-HCl. The band migrating farthest toward the anode (-1.7×10^{-4} cm²/v-sec) corresponds to the succinylated catalytic subunit while the most cathodic band (-1.0×10^{-4} cm²/v-sec) corresponds to the native catalytic subunit. Comparison of these results with those for the controls leads to the conclusion that the two intermediate bands

with mobilities of -1.23×10^{-4} and -1.46×10^{-4} cm²/v-sec arose from hybridization of the native and succinylated catalylic subunits. Both the number and relative mobilities of the intermediate bands would be expected from hybridization of two molecules each composed of three similar polypeptide chains. Thus the four members of the hybrid set can be correlated with the structures: C_{nnn}, C_{nns}, C_{nss}, and C_{sss}, where $C_{nnn}(C_N)$ and $C_{sss}(C_S)$ represent the native and succinylated catalytic subunits, respectively. The intermediate members of the hybrid set thus represent catalytic subunits composed of two native and one succinylated catalytic chain (C_{nns}) or one native and two succinylated catalytic chains (C_{nss}).

Intersubunit hybridization: Since native catalytic and regulatory subunits can be recombined to form native ATCase molecules, it seemed of interest to determine whether the succinylated catalytic subunit could also combine with native regulatory subunits to form molecules of the size of the native enzyme. In these experiments, the native (C_{nnn}) or succinylated (C_{sss}) catalytic subunits were mixed with excess regulatory subunits (R) in phosphate buffer. Sedimentation velocity experiments showed a major component migrating at a rate characteristic of native ATCase (11.6*S*) in both cases. Boundaries for the excess regulatory subunit (2.8*S*) and a small amount of aggregate (16.4*S*) normally produced upon reconstitution of native ATCase were also observed. No components sedimenting at the rate of free catalytic subunits (5.8*S*) were observed. Thus, this experiment shows clearly that the succinylated catalytic subunits, like the native subunits, can combine completely with native regulatory subunits to form a species with a sedimentation coefficient identical to that of native ATCase.

Results of hybridization experiments conducted at the intersubunit level of ATCase are shown in the electrophoretic patterns in Figure 4. The top sample of native ATCase exhibits a single band with a mobility of -0.88×10^{-4} cm²/v-sec. Directly below is a band for native catalytic subunit (C_{nnn}). The addition of excess regulatory subunit (R) to C_{nnn} as shown in the third sample resulted in the disappearance of the band for C_{nnn} and the production of bands corresponding to native ATCase and excess regulatory subunit. The regulatory subunit, as shown in the bottom sample, is the most cathodic band (-0.65×10^{-4} cm²/v-sec).

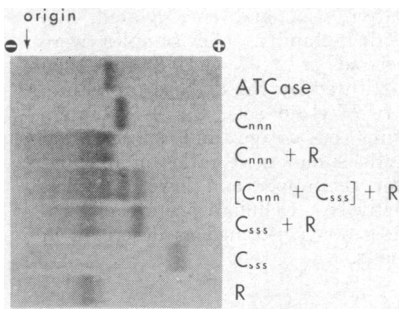

FIG. 4.—Intersubunit hybridization of the native (C_{nnn}) and succinylated (C_{sss}) catalytic subunits in ATCase. ATCase was reconstituted by the addition of excess regulatory subunits (R) to a solution containing native and/or succinylated catalytic subunits. Cellulose acetate electrophoresis was performed as described in **Methods.**

Similar results were obtained with the succinylated catalytic subunit (C_{sss}) shown as the most anodic band. The addition of excess regulatory subunit to C_{sss} as shown in the sample denoted by C_{sss} + R resulted in the disappearance of the band for C_{sss} and the production of a new band (-1.21×10^{-4} cm²/v-sec) as well as a band for the

excess regulatory subunit. This new band can be correlated with an ATCase molecule which contains succinylated catalytic subunits and native regulatory subunits.

If the native and succinylated catalytic subunits were initially mixed and then excess regulatory subunit added, as shown in the sample denoted by [C_{nnn} + C_{sss}] + R, not only are bands obtained for native ATCase (-0.88×10^{-4} cm^2/v-sec) and for ATCase containing succinylated catalytic subunits and native regulatory subunits (-1.21×10^{-4} cm^2/v-sec), but an intermediate band is obtained with a mobility of -1.04×10^{-4} cm^2/v-sec. The production of a single intermediate band with such a mobility would be expected only for an ATCase hybrid molecule that contains two catalytic subunits (one native and the other succinylated). Thus the three members of this hybrid set can be correlated with the structures $(C_{nnn})_2$ [R], C_{nnn} C_{sss} [R], and $(C_{sss})_2$ [R], that contain two native, one native and one succinylated, and two succinylated catalytic subunits, respectively, combined with regulatory subunits.[19]

Separation of the members of the intersubunit hybrid set: DEAE-Sephadex chromatography permitted further characterization of the members of the hybrid set and confirmation of the catalytic subunit structure established electrophoretically. Figure 5 shows plots of optical density at 280 mμ, enzyme activity, and specific versus fraction number for the hybrid set. The optical density plot clearly distinguishes four components; an initial small peak corresponding to the excess regulatory subunit, and three equally spaced peaks labeled I, II, and III.

Distinct enzyme activity peaks are present for both I and II; however, only a small shoulder of activity can be observed for the essentially inactive III. The corresponding specific activities for these fractionated components are 7.6, 3.8, and 0.1 units/mg, respectively. Although I and II are homogeneous with respect to enzyme activity as indicated by the plateaus in the specific activity plot, the high activity of II and the low activity of III preclude the formation of a discrete specific activity plateau for the last species. Consideration of the num-

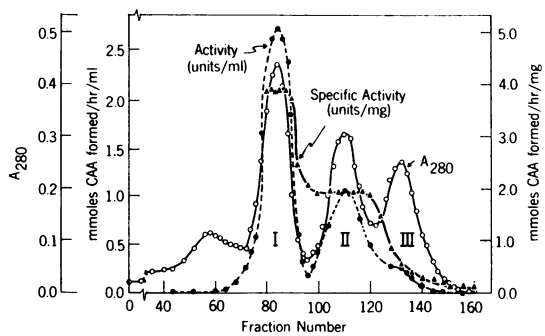

FIG. 5.—Separation of the members of the intersubunit hybrid set by DEAE-Sephadex chromatography. The hybrid set was produced by the addition of a small excess of regulatory subunits (R) to a solution containing approximately equal amounts of the native (C_{nnn}) and succinylated (C_{sss}) catalytic subunits. The sample was applied to a 1 × 40 cm column of DEAE-Sephadex A-50 preequilibrated with 0.15 *M* KCl, 0.05 *M* Tris-chloride, 0.001 *M* EDTA, pH 7.50. The fractions were eluted with a linear gradient of KCl from 0.15 to 0.75 *M* in 0.05 *M* Tris-chloride, 0.001 *M* EDTA, pH 7.50, over a range of 100 ml at a flow rate of 9 ml/hr. Fractions of 0.5 ml were collected and analyzed for absorbance at 280 mμ (—○—), enzyme activity as measured by carbamyl aspartate formation (see **Methods**) at saturating substrate concentrations of 50×10^{-3} *M* aspartate (—●—), and specific activity (—▲—). The major peaks were pooled into samples I (fractions 79 to 88), II (fractions 107 to 116), and III (fractions 131 to 140).

ber, position and expected elution order of the hybrid set members, together with their relative specific activities, leads to the conclusion that I, II, and III correspond to $(C_{nnn})_2$ [R], C_{nnn} C_{sss} [R], and $(C_{sss})_2$ [R], respectively. Furthermore, studies carried out on $(C_{nnn})_2$ [R] and $(C_{sss})_2$ [R] prepared directly from their constituent subunits exhibit specific activities of 7.6 and 0.1 units/mg, in excellent agreement with the values obtained for these species fractionated from the hybrid set.

Pooled fractions from each peak (I to III) were further characterized by cellulose acetate electrophoresis and sedimentation velocity experiments. The individual column fractions, I, II, and III, each exhibited one major electrophoretic band corresponding to $(C_{nnn})_2$ [R], C_{nnn} C_{sss} [R], and $(C_{sss})_2$ [R], respectively. Furthermore, the major component (>85%) in each pooled sample sedimented at 11*S* thus showing that all members of the hybrid set are of the same size as native ATCase. Thus the distribution of both protein and enzyme activity along with the physical properties of the individual members of the intersubunit hybrid set support the conclusion that there are two catalytic subunits in ATCase.

Discussion. The hybridization studies with the native and succinylated catalytic subunits indicate that each ATCase molecule contains two catalytic subunits which in turn are composed of three polypeptide chains. Thus there appear to be six polypeptide chains in ATCase involved in catalysis. This conclusion is in accord with the recently proposed model for ATCase as a hexamer comprising six pairs of catalytic and regulatory chains.[10, 11] However, the finding of six catalytic chains in each ATCase molecule is in conflict with the results from ligand binding studies,[6] end group analyses,[7, 8] and molecular weight determinations on the polypeptide chains of the dissociated catalytic subunits.[7] Binding data frequently give minimal estimates for the number of combining sites (and hence of polypeptide chains) due to contaminants in the preparations and partial inactivation of the enzyme; thus the value of two ligand binding sites per catalytic subunit[6] could easily be an underestimate. Similarly incomplete reactions in end group analyses may have led to misleading conclusions as to the number of aminoterminal alanyl residues per catalytic subunit.[7, 8] Recent molecular weight determinations[20] on the denatured polypeptide chains of the catalytic subunits in guanidine hydrochloride yielded a molecular weight of 3.5×10^4 rather than the higher value reported earlier.[7] This newer result, along with the molecular weights of the catalytic subunits (1.0×10^5) and ATCase (3.1×10^5), and the weight per cent of the catalytic subunits in ATCase (68%)[5] supports the conclusion that there are six catalytic chains per molecule of ATCase. Moreover, the characteristics of both the intersubunit and intrasubunit hybridization reactions and the stability and properties of the various hybrids, C_{nns}, C_{nss}, and C_{nnn} C_{sss} [R], indicate that each of the catalytic subunits exists as a tightly folded trimer within the ATCase molecules.

Although preliminary experiments have been initiated on the chemical modification of the isolated regulatory subunits, the results have been inconclusive. An electrophoretic variant was produced, but no reconstitution to form a complex of the size of ATCase was achieved when this derivative was mixed with the

native catalytic subunit. Modified regulatory subunits might be produced with unaltered sulfhydryl groups through succinylation of intact ATCase followed by separation of the succinylated subunits.

Hybrid molecules like C_{nnn} C_{sss} [R] should prove valuable in investigating whether the allosteric interactions (homotropic and heterotropic) characteristic of ATCase[4, 12] are manifested at the chain or subunit level. Activity measurements on $(C_{nnn})_2$ [R], (C_{nnn}) (C_{sss}) [R], and $(C_{sss})_2$ [R] showed that each subunit contributes enzyme activity independently at saturating substrate concentrations. Moreover, studies at varying substrate concentrations revealed that cooperative interactions (both homotropic and heterotropic) are preserved in the ATCase hybrid, (C_{nnn}) (C_{sss}) [R]. In this regard comparable kinetic studies with another hybrid, C_{nns} C_{nss} [R], should prove of interest in unravelling the structural features required in the mediation of feedback inhibition characteristic of regulatory enzymes.[4, 12, 21]

We would like to express our thanks to Miss Y. R. Yang for valuable contributions to this work.

Abbreviations used: ATCase, aspartate transcarbamylase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; G-HCl, guanidine hydrochloride; pHMB, parahydroxymercuribenzoate; phosphate buffer, 0.04 *M* potassium phosphate, 2×10^{-4} *M* EDTA, pH 7.0; EDTA, ethylene diaminetetraacetate; DTT, dithiothreitol.

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[18] This component has not yet been characterized; however, in previous studies of the succinylation of aldolase and GPDH, such a component was also observed and was shown to arise from dissociation of the protein. This species has occasionally been observed in very small amounts in sample A.

[19] The bracketed quantity, [R], refers to the total complement of regulatory chains in reconstituted ATCase without specification of the number or arrangement of these polypeptide chains within the regulatory subunits.

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