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An Intermediate Complex in the Dissociation of Aspartate Transcarbamylase

(thiols/mercurials/dissociation/central cavity/intermediate)

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ABSTRACT **The multisubunit enzyme aspartate transcarbamylase consists of six copies of two types of polypeptide chains, catalytic (C) and regulatory (R). A complex formed by the partial dissociation of this enzyme has been isolated. This species, which has the structure C_6R_4, is a likely intermediate in the stepwise dissociation of aspartate transcarbamylase induced by mercurials. The formation of the complex is the result of the release of a single regulatory dimer (R_2) from the parent molecule.**

The specific activity of the intermediate is essentially the same as that of aspartate transcarbamylase. By contrast, both homotropic and heterotropic interactions are reduced, but not abolished. These observations suggest that the allosteric transitions involved in the control mechanisms do not require the intact structure C_6R_6.

The allosteric enzyme aspartate transcarbamylase (ATCase, EC 2.1.3.2) from *Escherichia coli* has a molecular weight of 310,000 (1) and is composed of 12 polypeptide chains (2–5). The enzyme catalyzes the first step in the biosynthesis of pyrimidines, the carbamylation of aspartate by carbamyl phosphate, and is involved in the regulation of this pathway (6, 7). Sigmoidal substrate saturation curves have been observed, indicative of homotropic, i.e., cooperative, interactions between catalytic sites. Furthermore, enzymatic activity is modulated by several allosteric effectors. Cytidine triphosphate, the end product of the pyrimidine pathway, is a potent feedback inhibitor, while adenosine triphosphate activates the enzyme.

Reaction with mercurials results in the dissociation of ATCase into two functionally distinct types of subunit (1): catalytic subunits which are enzymatically active but which are insensitive to allosteric effectors, and regulatory subunits which bind nucleotides but are inactive. It was subsequently shown that the catalytic subunit is a trimer (C_3) composed of three catalytic polypeptide chains (C) each of molecular weight 34,000, and that the regulatory subunit is a dimer (R_2) composed of two regulatory chains (R) each of molecular weight 17,000 (2–5).

Consequently, six copies of these two different types of polypeptide chain are present in the ATCase complex. Crystallographic studies (2, 8–10) have shown that the molecule has D_3 symmetry and that the subunits are organized such that there are two catalytic subunits in a nearly eclipsed configuration and that the regulatory chains are clustered in pairs around the outer edge of the molecule. These results are consistent with chemical and electron microscopic studies (4, 11, 12).

Abbreviations: ATCase, aspartate transcarbamylase; C and R, catalytic and regulatory subunits, respectively, of ATCase; PHMB, *p*-hydroxymercuribenzoate; βME, 2-mercaptoethanol.

The x-ray diffraction study also revealed the existence of a large ellipsoidal (50 × 50 × 25 Å) cavity in the center of the molecule. The most obvious access to the central cavity appears to be through six relatively narrow channels, 15 Å in diameter, which can be seen to pass through regions of electron density most probably associated with both regulatory and catalytic chains. Furthermore, the internal location of the C chain thiol believed to be near the catalytic site (8–10, 13) implies that substrates must pass through these access channels and into the central cavity before catalysis can occur. These unusual structural features immediately suggest regulatory mechanisms which involve controlled access of substrates to the central cavity. On the other hand, these regulatory mechanisms could be mediated by conformational changes which do not involve these access channels. Some of our results, described below, are related to a distinction between these two modes of regulation, favoring no essential role of the access channels in the mechanisms.

ATCase is rich in sulfhydryl groups. One cysteine is present in each catalytic chain (14, 15), and four cysteines occur in each regulatory chain (3, 14). These R chain thiols have a number of interesting properties as follows:

1. The dissociation of the ATCase molecule is the result of the reaction of the R chain thiols with mercurials, causing the specific disruption of the domain of bonding between catalytic and regulatory chains (1, 14, 16).

2. The R chain thiols in the intact enzyme are relatively unreactive. While susceptible to attack by mercurials, these residues are impervious to conventional sulfhydryl reagents, such as *N*-ethylmaleimide, iodoacetic acid and others (14, 17). In contrast, these same thiols react readily in the isolated regulatory subunit, leading to the suggestion that they are "buried" in the intact molecule, possibly at the interface between C and R subunits (14).

3. The reaction of the R chain thiols can be described as "all or none," i.e., cooperative (14). Once initiated, the reaction of other cysteines on the same molecule is facilitated.

4. The reactivity of the R chain thiols is modified by substrates and allosteric effectors (14, 18). Carbamyl phosphate and the substrate analog succinate accelerate the rate of reaction 7-fold. This effect of ligands has been attributed to structural changes which render these thiols more or less accessible. Several investigators (14, 18–20) have employed the reactivity of the R chain thiols to monitor the conformational state of the enzyme.

5. ATCase contains six tightly bound ions of zinc. The metal ion is not required for catalysis (21, 22) but may be involved in mediating the homotropic interactions (23).

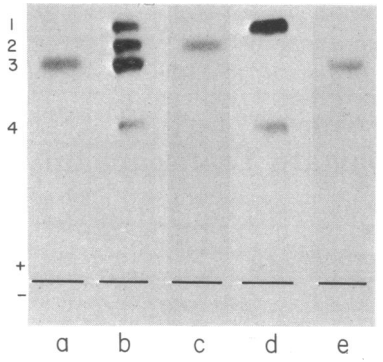

FIG. 1. Cellulose acetate electrophoresis of ATCase and intermediate. This photograph is a composite of several different cellulose acetate strips. In each instance a marker (usually a mixture consisting of ATCase, C subunit, R subunit, and intermediate) was subjected to electrophoresis simultaneously which allowed the individual strips to be lined up in register. Electrophoresis was carried out on 78 × 150-mm cellulose acetate strips at 10 mA for 15 min. A buffer system (1) consisting of 0.05 M Tris citrate, pH 7.8, was employed. The strips were stained with Ponceau S. (*a*) ATCase. (*b*) A mixture prepared by reacting ATCase with 6 equivalents of PHMB ($6Hg:C_6R_6$). (*c*) Purified intermediate. (*d*) Intermediate reacted with a 50-fold molar excess of PHMB. (*e*) Intermediate after incubation with a slight excess of Zn R subunit and 2 mM βME for 1 hr at 37°. The proteins are numbered in order of decreasing electrophoretic mobility: (*1*) catalytic subunit; (*2*) intermediate; (*3*) ATCase; and (*4*) regulatory subunit.

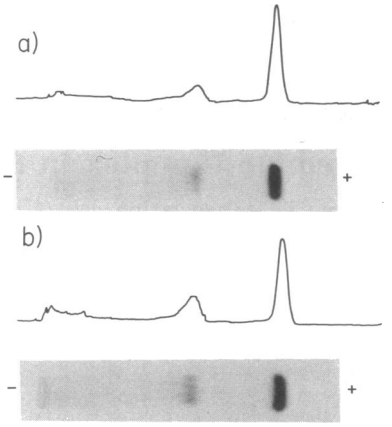

FIG. 2. Subunit composition of intermediate and ATCase. ATCase (*a*) and intermediate (*b*) were dissociated into constituent R and C subunits by reaction with a 50-fold molar excess of PHMB. The reaction was carried out in 0.05 M Tris·HCl pH 9.0, to minimize aggregation of the regulatory subunit. Cellulose acetate electrophoresis of the dissociated proteins was carried out as described in the *legend* of Figure 1. The strips were stained in Coomassie brilliant blue (27), cleared by immersion in Whitemore oil and scanned using a Joyce–Loebl scanning microdensitometer.

Furthermore, zinc has a definite structural role since it, or a similar metallic ion, is required for the reconstitution of the enzyme from subunits (21, 22). Also, zinc has been shown (12) to stabilize greatly the isolated regulatory dimer. Recent evidence suggests that the R chain thiols serve as ligands securing zinc to the regulatory chain (21, 22).

The R chain thiols of aspartate transcarbamylase are not unique. A number of other proteins have been shown to contain structural thiols which have many of these same properties. These proteins include phosphorylase (24), hemerythrin (25), formyltetrahydrofolate synthetase (26), and others.

Further studies are needed to elucidate the structural and functional significance of the R chain thiols, and the implications of the changes in reactivity of these residues upon ligand binding. In addition, the investigation of partially dissociated complexes may aid in evaluating the contribution of individual subunits in the allosteric transitions associated with regulation, as described below.

RESULTS

In a previous investigation (13) which dealt with the modification of the C chain thiol we observed that *p*-hydroxymercuribenzoate (PHMB), when added in stoichiometric amounts to the native enzyme ($6Hg:C_6R_6$), reacts preferentially with the R chain thiols, inducing partial dissociation of the molecule. The unexpected result was that, when subjected to cellulose acetate electrophoresis (Fig. 1*b*), the reaction mixture contained a fourth component, in addition to R and C subunits and unreacted ATCase. We proposed that this species, which had an electrophoretic mobility between that of ATCase and the catalytic subunit, was an intermediate in the dissociation of the ATCase molecule. At that time we initiated studies to characterize further this intermediate species and we present here a preliminary account of that investigation. We have isolated the intermediate by column chromatography using DEAE Sephadex and a linear pH gradient (pH 8.5–7.5). The purified protein (Fig. 1*c*) is stable when stored in 2 mM 2-mercaptoethanol (βME), 0.2 mM ethylenediamine tetraacetic acid, pH 7.0, but was found to disproportionate slowly into catalytic subunit and ATCase over a period of several months.

Our experiments in which the intermediate was formed using PHMB labeled with ^{14}C or ^{203}Hg indicate that the intermediate contains only small quantities of mercury, less than 1 mole of mercury per mole of ATCase. Judging from the slight proportional (10%) increase in enzymatic activity which occurs on the addition of mercaptoethanol, we conclude that this residual mercury is bound to the sulfhydryl group on the catalytic chain. The attendant loss of residual mercury did not change the electrophoretic mobility or any of the other measured properties of the intermediate. We therefore conclude that this species is a distinct complex, not simply a mercurial derivative of ATCase or one of its subunits.

The reaction of the intermediate with excess PHMB results in the complete dissociation of the molecule into catalytic and regulatory subunits, indicating that it is comprised of both types of polypeptide chain (Fig. 1*d*). Furthermore, incubation of the intermediate with regulatory subunit in the presence of zinc and βME results in complete conversion of the intermediate into ATCase (Fig. 1*e*). The fact that the native enzyme can be reconstituted from the intermediate is a good indication that this species is not an artifact resulting from denaturation or some other irreversible alteration.

We have examined the subunit structure of the purified intermediate by first completely dissociating the molecule into constituent R and C subunits by the addition of excess PHMB. The subunits were then separated by cellulose acetate electrophoresis. The stained and cleared cellulose acetate sheets were sealed between glass plates and scanned with a Joyce–Loebl microdensitometer. Two peaks were observed (Fig. 2), corresponding to catalytic and regulatory subunits. Integration of the densitometer trace showed that the inter-

mediate consisted of 76.5% catalytic subunit and 23.6% regulatory subunit, on a weight percentage basis. In a control experiment conducted simultaneously, ATCase was found to contain 66.7% catalytic subunit and 33.4% regulatory subunit. These values for the native enzyme are in excellent agreement with previously published values (1), and with those calculated by combining the molecular weights of the individual polypeptide chains (3, 4) with the known subunit structure C_6R_6 (catalytic chain 66.3% and regulatory chain 33.7% by weight).

The composition of the intermediate suggests that the subunit structure is $(C_3R_2)_x$. Sedimentation velocity experiments indicate that the intermediate is homogeneous and that its rate of sedimentation is only 6.5% slower than that of native ATCase ($S_{20,w}$ = 11.6). Thus, barring any anomalous changes in frictional coefficient, the molecular weight of the intermediate is just under that of native ATCase, indicating that x is 2 and that the subunit structure is C_6R_4. Other possibilities consistent with the composition, such as C_9R_6 and C_3R_2, for x = 3 and 1 respectively, cannot be considered plausible structures in view of this sedimentation velocity. Furthermore, this structure of the intermediate is supported by preliminary sedimentation equilibrium experiments and by gel filtration on Sephadex G-200.

A titrimetric assay which monitors the enzyme-catalyzed release of protons (F. A. Quiocho, unpublished results from this laboratory) was used to investigate the kinetic properties of the intermediate. The specific activity of the intermediate was essentially indistinguishable from that of the native enzyme when assayed at saturating levels of carbamyl phosphate and 30 mM aspartate.

The heterotropic interactions observed with the intermediate are less than those of the native enzyme. The effect of allosteric effectors was examined at half-saturating aspartate and saturating carbamyl phosphate. When the intermediate is assayed in the presence of 1 mM CTP the activity is reduced by 32%, whereas under comparable conditions there is a decrease of 61% in the activity of ATCase. Similarly 2 mM ATP activates the native molecule by 53%, while the activity of the intermediate increases by only 12%. A comparison of the substrate saturation kinetics of the intermediate and ATCase (Fig. 3) shows that the release of a regulatory dimer results in a significant decrease in the cooperative interaction between catalytic sites. The intermediate saturation curve, while much less sigmoidal, is nevertheless not hyperbolic. The affinity for aspartate is somewhat greater in the intermediate than in ATCase, as indicated by a shift in the apparent K_m from 13 mM in C_6R_6 to 8 mM in C_6R_4. Comparable data for the catalytic subunit, which exhibits no homotropic interactions, are also presented in Fig. 3.

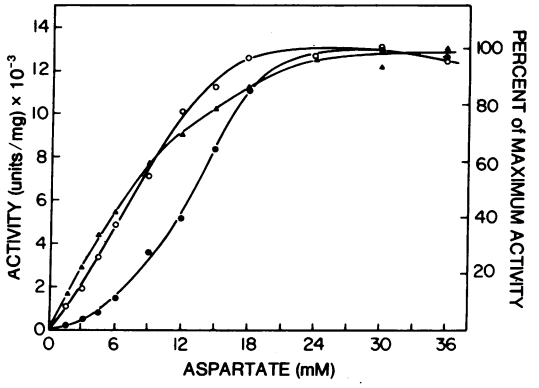

FIG. 3. Aspartate saturation curves of intermediate, ATCase, and catalytic subunit. Catalytic activity was measured by monitoring the release of protons at pH 8.3 using a Radiometer TT2 pHstat. All assays were conducted at saturating (4.8 mM) levels of carbamyl phosphate. The activity is expressed in μmole hr^{-1} mg^{-1} for ATCase (●) and intermediate (○). The specific activity for the catalytic subunit (▲) has been normalized (at 31 mM) to facilitate comparison with the other saturation curves. The actual specific activity of the catalytic subunit was found to be 1.45 greater than that of ATCase at saturating carbamyl phosphate and (30 mM) aspartate. The numbers on the left ordinate are the product of the experimental values and the indicated factor and maximum activity refers to the maximum observed in these experiments.

DISCUSSION

Partial dissociation of the ATCase molecule thus results in the formation of a complex which has the structure C_6R_4. The subunit structure, the conditions under which it is formed, the fact that mercurials induce further dissociation and that the parent molecule (C_6R_6) can be reconstituted from the intermediate by the addition of regulatory subunit, all lend credence to the hypothesis that this species is a true intermediate in the dissociation of the native enzyme.

The mercurial-induced dissociation of the ATCase complex has previously been described as "all or none." This conclusion has been based on the observation that the R chain thiols of the undissociated molecules have not reacted with mercurial, whereas in the dissociated molecules these residues have reacted fully. This description should now be expanded somewhat to take into consideration the existence of at least one intermediate species, which results from the stepwise removal of a regulatory dimer during dissociation. The identification of an intermediate is the first direct evidence that dissociation proceeds in a stepwise fashion, as suggested by Gerhart (28). The initial, and probably rate-limiting, event in dissociation is the reaction of one or more of the thiol groups on one of the regulatory chains in the ATCase molecule (C_6R_6). Reaction with PHMB ruptures this RC domain of bonding and at the same time would be expected to destabilize the RC domain of the other R chain in the same dimer. As a result of this destabilization, the R chain thiols at the remaining contact between the dimer and the complex react preferentially with PHMB. Disruption of this second RC domain of bonding results in the release of a regulatory dimer (R_2) and the formation of the intermediate (C_6R_4).

It is likely that other investigators have observed this species in studies dealing with the assembly of the ATCase molecule. Jacobson and Stark (29) have found that reconstitution of the native enzyme in the presence of excess catalytic subunit results in the formation of a species which was shown to be deficient in R chain. These authors (29) also refer to similar observations which have been made independently by J. M. Syvanen, G. Nagel and H. K. Schachman. It is possible this species is an intermediate in both the assembly and mercurial-induced dissociation of the ATCase molecule.

In addition to information which this intermediate may provide concerning dissociation and assembly of ATCase, this species has kinetic properties which may, to some extent, elucidate the regulatory mechanism. For example, removal of a regulatory dimer would be expected to increase significantly access to the central cavity. If the resulting intermediate still

retains moderate homotropic and heterotropic interactions, we would have to conclude that regulatory mechanisms based upon restricted access to the central cavity could not play a dominant role. It is, therefore, interesting to compare the kinetic properties of the intermediate with those of both ATCase, which exhibits strong homotropic and heterotropic interactions, and the catalytic subunit, which completely lacks these regulatory mechanisms.

The catalytic activity of the intermediate is essentially the same as that of ATCase. In contrast, we find that the catalytic subunit, when assayed under identical conditions (saturating carbamyl phosphate and 30 mM aspartate, pH 8.3), has a catalytic activity, expressed on the basis of concentration of active site, which is 1.45 times greater than that of ATCase or the intermediate. These results suggest that the conformation of the C_6R_4, at least in the vicinity of the active site, is more nearly like that in intact ATCase than in the isolated subunit.

As might be anticipated, the efficacy of allosteric effectors is markedly reduced in the R-deficient species C_6R_4. Neither CTP nor ATP modulate the activity of the intermediate as effectively as they do the native enzyme. However, the loss of heterotropic interactions is more pronounced than one-third, the reduction in the number of nucleotide binding sites.

This observation may suggest that desensitization is due partly to a reduction in the number sites and partly to a weakening of the heterotropic interactions between the remaining subunits in the complex due to the removal of a regulatory dimer. Alternatively, the observed inhibition or activation need not be proportional to the regulatory chain content of the complex, particularly in view of recent evidence (30–33) which has shown that functionally there are two classes of nucleotide-binding sites.

Comparison of the aspartate saturation curves* for the intermediate and for ATCase (Fig. 3) indicates that a large reduction in homotropic interaction accompanies the removal of a regulatory dimer. This loss of cooperativity is reflected in a decrease in the Hill coefficient from 4.0 for ATCase to 2.5 for the intermediate. It should be noted that the assays were conducted at pH 8.3 where the homotropic interactions are much larger (34), and consequently the Hill coefficients are greater than the values usually quoted (2–2.5) based on data obtained at pH 7.0. Nevertheless, moderate cooperative interactions do persist in the intermediate, judging from the shape of the saturation curve. The sigmoidal character of this curve is particularly obvious when compared to that of the catalytic subunit, which exhibits no cooperative interactions.

* All of the species studied, C_3, C_6R_6, and C_6R_4, exhibit substrate inhibition at high concentrations of aspartate. For the catalytic subunit, accurate values for K_m and V_{max} can be obtained by extrapolating the double reciprocal plot, a procedure which is not applicable to C_6R_6 or C_6R_4 because of the non-linearity of these plots. Consequently, the reported values for the K_m and Hill coefficients (the calculation of which depends on V_{max}) may be considered only as reasonable estimates. The values of the K_m in the *text* are minimum values and, while probably nearly correct, the actual parameter for C_6R_6 and C_6R_4 may be as high as 16 mM and 10 mM, respectively, and the Hill coefficients may be somewhat lower. However, the important point, that there exist significant differences between C_6R_6 and C_6R_4, in the properties described by these parameters, is quite apparent.

It is difficult to estimate the decrease in Hill coefficient expected to be associated with loss of regulatory dimer, but again the rather dramatic reduction in cooperativity may reflect in part a weakening of the homotropic interactions resulting from the destabilization of the native complex. The apparent K_m (half-saturating aspartate concentration in the absence of effector ligands) of the intermediate is 8 mM. The corresponding values for ATCase and catalytic subunits are 13 mM and 17 mM, respectively. Apparently, with regard to the binding of aspartate the intermediate is more effective than both ATCase and the catalytic subunit and more closely resembles the ATP-activated enzyme.

It is significant that the intermediate exhibits homotropic and heterotropic kinetics although both types of interaction are severely impaired. Evidently these allosteric transitions do not require that the ATCase molecule be whole and intact, and it seems likely that restricted access of substrates to the central cavity is not a major factor in the control mechanism. We do not, however, wish to rule out the possibility that there may be some functional significance for these highly unusual structural features: the central cavity, the access channels and the proximity of the active sites to this central cavity of ATCase.

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