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Immunological Studies of Aspartate Transcarbamylase. II. Effect of Ligands on the Conformation of the Enzyme*

Roland von Fellenberg, Martha R. Bethell,† Mary Ellen Jones,‡ and Lawrence Levine

ABSTRACT: Antibodies can be used to measure the dissociation of *Escherichia coli* aspartate transcarbamylase into its subunits (Bethell *et al.*, 1968b). The reactivity of the sulfhydryl groups with *p*-hydroxymercuribenzoate is influenced by ligands which stabilize different allosteric conformations of aspartate transcarbamylase at 25° (Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 553). We have studied the nature of the *p*-hydroxymercuribenzoate dissociation of aspartate transcarbamylase at 0° using antibodies to detect the amount of dissociation. We have found that the *p*-hydroxymercuribenzoate at 0° depends upon the concentration of *p*-hydroxymercuribenzoate and aspartate transcarbamylase. Stereospecific ligands change the rate of *p*-hydroxymercuribenzoate dissociation. Carbamyl phosphate or the aspartate analog, succinate, added singly, increases the rate of *p*-hydroxymercuribenzoate dissociation. The maximal effects of carbamyl phosphate and succinate are additive . Carbamyl phosphate increases the binding of succinate to aspartate transcarbamylase. In phosphate buffer, aspartate, below 1 mM, also increases the rate of aspartate transcarbamylase dissociation, but, as the concentration of aspartate is raised, the enhancement of *p*-hydroxymercuribenzoate dissociation decreases and under certain circumstances aspartate transcarbamylase is stabilized against *p*-hydroxymercuribenzoate dissociation. The exact effect of aspartate is dependent upon the orthophosphate concentration. Cytidine triphosphate, adenosine triphosphate, uridine triphosphate, guanosine triphosphate, and pyrophosphate stabilize aspartate transcarbamylase toward *p*-hydroxymercuribenzoate. A direct effect of the ligands, carbamyl phosphate plus succinate or cytidine triphosphate, on aspartate transcarbamylase or its subunits was studied with antibodies to aspartate transcarbamylase, or to the catalytic subunit, or to the regulatory subunit of aspartate transcarbamylase. These studies may indicate that the major conformational effect occurs in the subunit to which the ligand binds. Some effects of urea are also discussed.

Dissociation of *Escherichia coli* ATCase[1] can be measured quantitatively using antibodies against the native enzyme and its subunits (Bethell *et al.*, 1968b). Kinetic data suggest (Gerhart and Pardee, 1962, 1964) and recent experiments demonstrate conformational changes in ATCase resulting from interaction with ligands (Gerhart and Schachman, 1968; Changeux and Rubin, 1968). In this paper we offer further evidence based on serologic studies for conformational changes in ATCase due to ligand binding. According to the predictions of Monod *et al.* (1965) substrates favor a loose, and feedback inhibitors a tight quaternary structure of an allosteric enzyme. Thus, it might be expected that the substrates would favor the dissociation of the enzyme into its subunits and the feedback inhibitor would inhibit that dissociation. In the present paper we have studied the dissociation of the native enzyme into its subunits and the effect of ligands on the dissociation.

In the preceeding paper (Bethell *et al.*, 1968b) it was demonstrated that changes in the complement fixing activities are very sensitive indicators of changes in ATCase

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† Present address: Department of Biology, University of Massachusetts, Boston, Mass. Part of this work was done by M. R. B. in partial fulfillment of the requirements for the Ph.D. degree at Brandeis University, and she gratefully acknowledges fellowship support from the National Institutes of Health (1F 1GM31310).

‡ Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, N. C.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: ATCase, aspartate transcarbamylase; CAP, carbamyl phosphate; *p*-HMB, *p*-hydroxymercuribenzoate.

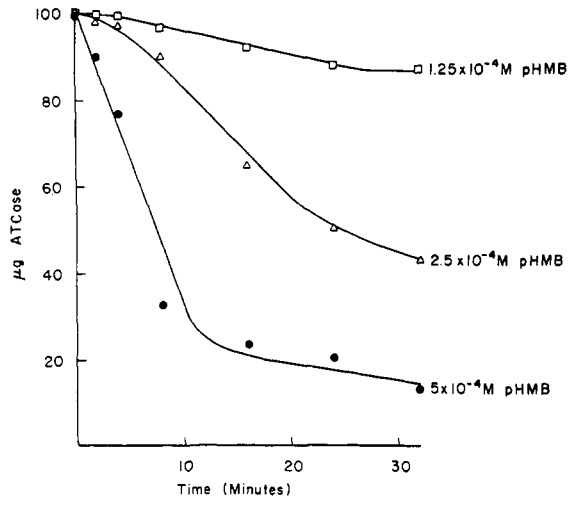

FIGURE 1: Native enzyme (micrograms) remaining after incubation of ATCase for varying periods of time with three different concentrations of *p*-HMB. Incubation: ATCase (100 μg/ml) in 0.04 M phosphate buffer (pH 7.0) at 0°.

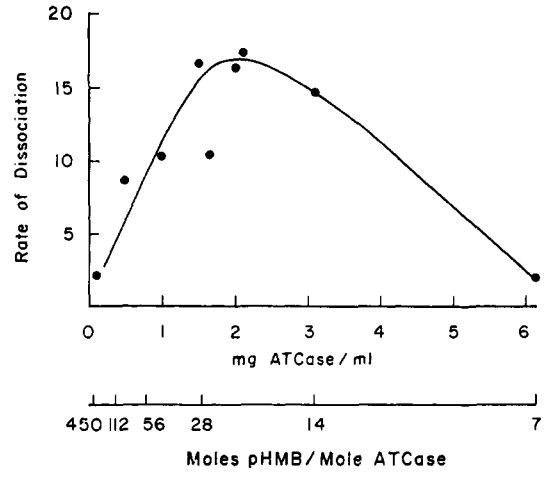

FIGURE 2: Rate of dissociation of ATCase by 1.5×10^{-4} M *p*-HMB as a function of ATCase concentration. Rate of dissociation is recorded as micrograms of ATCase dissociated per minute. Maximal rates were calculated from the ATCase remaining after incubation with *p*-HMB for 2, 4, 8, 16, 24, and 32 min. Incubation in 0.04 M phosphate buffer (pH 7.0) at 0°.

conformation. Thus, we have studied the effect of substrates, the feedback inhibitor, and other inhibitors on the complement fixing activities of ATCase and its subunits with their homologous antisera. Significant changes in the complement fixing activities resulting from ligand binding could be demonstrated.

Experimental Procedures

Materials

ATCase and the antisera against ATCase, catalytic, and regulatory subunits were the same as described in the previous paper (Bethell *et al.*, 1968b). CAP, succinate, aspartate, CTP, ATP, GTP, and UTP were commercial products. Iodoacetate, iodoacetamide, *o*-iodosobenzoate, *N*-ethylmaleimide, and *p*-HMB were also obtained from commercial sources.

Methods

Microcomplement fixation was carried out as described by Levine (1967). All ATCase dissociation experiments with *p*-HMB were carried out at 0° in 0.04 M phosphate buffer (pH 7.0) unless otherwise stated. After incubation of ATCase with *p*-HMB for a given time the reaction was stopped by dilution with the isotonic Tris buffer containing 0.1% bovine serum albumin used for complement fixation (Levine, 1967); the extent of dilution depended upon the quantity of ATCase being dissociated. Calculations of the amounts of native enzyme remaining in a partially dissociated mixture were made from its maximum complement fixation as described in the preceeding paper (Bethell *et al.*, 1968b). For titration of sulfhydryl groups, the spectrophotometric method of Boyer (1954) was used.

Results

Dissociation of ATCase into Its Subunits. As has been shown by Gerhart and Pardee (1962), the sensitivity of ATCase to the feedback inhibitor CTP can be abolished by different treatments. Heating the enzyme or performing the assay in 0.8 M urea were as effective in removing this inhibition as treatment of the enzyme with silver ions or mercurials. It is now well established that *p*-HMB dissociates the enzyme into the regulatory and catalytic subunits (Gerhart and Schachman, 1965, 1968). In contrast, alkylating agents and other substances which react with sulfhydryl groups had no effect on the feedback inhibition. In our study, iodoacetate, iodoacetamide, *o*-iodosobenzoate, and *N*-ethylmaleimide did not dissociate the enzyme into its subunits as measured by alteration of the antigenic properties of ATCase.

In order to see whether this narrow specificity of the reaction for mercurials could be ascribed to a relative inaccessability of the sulfhydryl groups in the ATCase molecule for the inactive sulfhydryl reagents, urea, at concentrations which have been shown to abolish feedback inhibition of the enzyme without dissociating it, was examined. The irreversible effects of urea on the enzyme, measured by the activity of the urea-treated enzyme with anti-ATCase, was first studied. Up to a concentration of 3.0 M urea, no irreversible alterations of the ATCase antigenicity was observed. At higher concentrations, the activity of ATCase with anti-ATCase decreased sharply.

The previously inactive sulfhydryl reagents were again tested for their ability to dissociate ATCase in the presence of 2.5 M urea. It was thought that under these incubation conditions, the "masked sulfhydryl groups" might now be available for reaction. ATCase was incubated with a 1500 M excess of iodoacetate, iodoacetamide, *o*-iodosobenzoate, or *N*-ethylmaleimide in the presence of 2.5 M urea. When the products of these incubations were assayed with anti-ATCase, no alteration in the antigenic properties of ATCase could be observed. The *N*-ethylmaleimide-treated ATCase was still

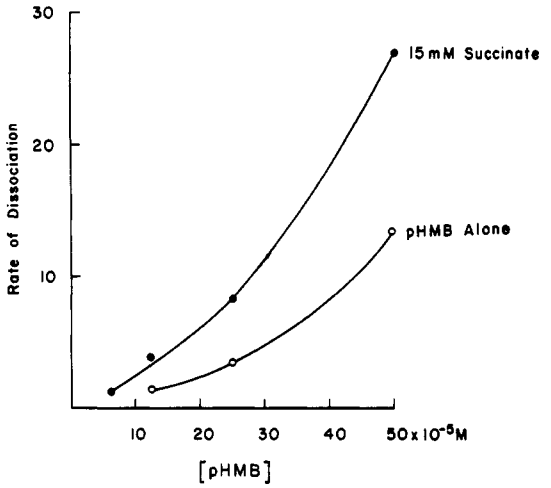

FIGURE 3: Rate of dissociation of ATCase (micrograms of ATCase dissociated per minute) by varying concentrations of *p*-HMB in the presence and absence of 15 mM succinate. Maximal rates were calculated from the ATCase remaining after incubation with *p*-HMB for 2, 4, 8, 16, 24, and 32 min. Incubation: ATCase (160 μg/ml) in 0.04 M phosphate buffer (pH 7.0), 0°.

capable of reacting with *p*-HMB to form 30 *p*-HMB–mercaptide bonds, as estimated by the method of Boyer (1954). The product, after back-titration with *p*-HMB, had lost its activity with anti-ATCase as the result of dissociation into subunits.

Rate of Dissociation of ATCase by p-HMB. In Figure 1, the rates of dissociation of ATCase by three different *p*-HMB concentrations are plotted. It can be seen that the concentration of *p*-HMB not only influences the maximal rate, but also the shape of the dissociation curve. At the highest *p*-HMB concentration (1500 M excess) no lag period is observed and the initial rate is linear. With a 750 M excess of *p*-HMB a lag period can be observed and the dissociation becomes sigmoidal.

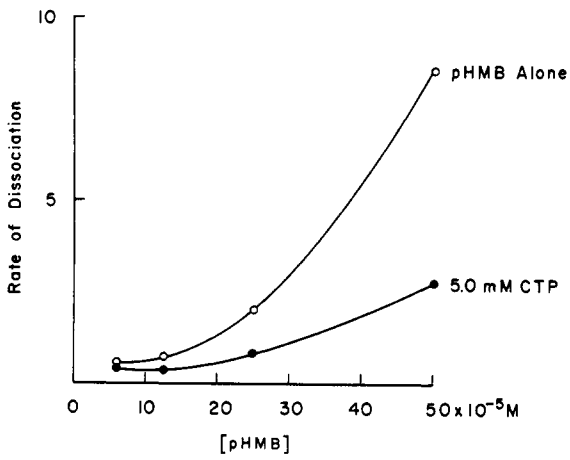

FIGURE 4: Rate of dissociation of ATCase (micrograms of ATCase dissociated per minute) by various concentrations of *p*-HMB in the presence and absence of 5 mM CTP. Maximal rates were calculated from the ATCase remaining after incubation with *p*-HMB for 2, 4, 8, 16, 24, and 32 min. Incubation: ATCase (100 μg/ml) in 0.04 M phosphate buffer (pH 7.0) at 0°.

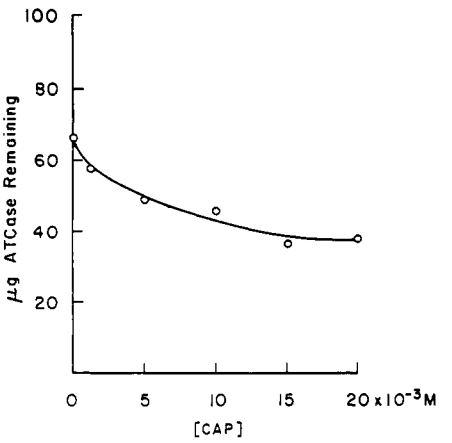

FIGURE 5: ATCase (micrograms) remaining (after incubation with 1.75×10^{-4} M *p*-HMB) in the presence and absence of varying concentrations of CAP. Incubation: ATCase (100 μg/ml) in 0.04 M phosphate buffer (pH 7.0) at 0° for 15 min.

In Figure 2, the reaction was studied at a constant concentration of *p*-HMB as a function of ATCase concentration. Two points are clear. The rate of the reaction is dependent not only upon *p*-HMB (Figure 1) but also upon the concentration of ATCase and therefore the reaction is bimolecular at 0°. The optimal ATCase concentration is between 5×10^{-6} and 6.6×10^{-6} M when *p*-HMB is 1.5×10^{-4} M. Secondly, the maximum rate is observed when the molar ratio of *p*-HMB to ATCase is 20–28, a figure which coincides with the number of moles of *p*-HMB, *i.e.*, 28, which must react with ATCase to cause dissociation (Gerhart and Schachman, 1968). As the ratio of *p*-HMB becomes less than 20, *i.e.*, when the ATCase concentration is greater than 2 mg/ml, the rate unexpectedly falls. The only explanation for this fall is that the reaction, under our conditions, requires that 20 of 28 μmoles of *p*-HMB be bound before ATCase dissociates at a maximum rate so that if insufficient *p*-HMB is present to dissociate the enzyme, the result is a decreased rate. It is apparent that at least 7 moles of *p*-HMB/mole of ATCase must react in order for dissociation to occur at a measurable rate under these conditions.

Dissociation of ATCase by *p*-HMB at 0° is, therefore, quite different from dissociation of ATCase with *p*-HMB at 20–25° as studied by Gerhart and Schachman (1968). At 25° the reaction of ATCase with *p*-HMB has no lag, and the rate of the reaction is independent of *p*-HMB and ATCase, *i.e.*, it is pseudo-first order. In contrast at 0° dissociation has a lag period and it is bimolecular. It is possible that at 0° the rate-limiting step in the subunit dissociation reaction can be ascertained, and the mechanism of the reaction studied.

Effect of Succinate, CAP, Urea, and CTP on the Rate of Dissociation. Succinate, as well as CAP, increases the rate of dissociation of ATCase in *p*-HMB. In Figure 3, the effect of 15 mM succinate on the maximal rate of dissociation is plotted for three *p*-HMB concentrations. The maximal rates are doubled in the presence of 15 mM succinate. In similar experiments CAP also in-

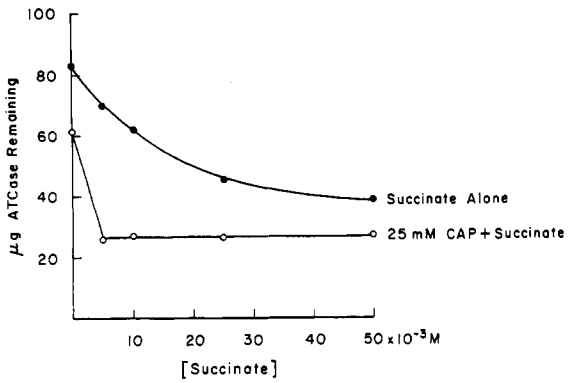

FIGURE 6: ATCase (micrograms) remaining after incubation with 1.75×10^{-4} M *p*-HMB in the presence and absence of varying concentrations of succinate alone and varying concentrations of succinate plus 25 mM CAP. Incubation: ATCase (100 μg/ml) in 0.04 M phosphate buffer (pH 7.0) at 0° for 15 min.

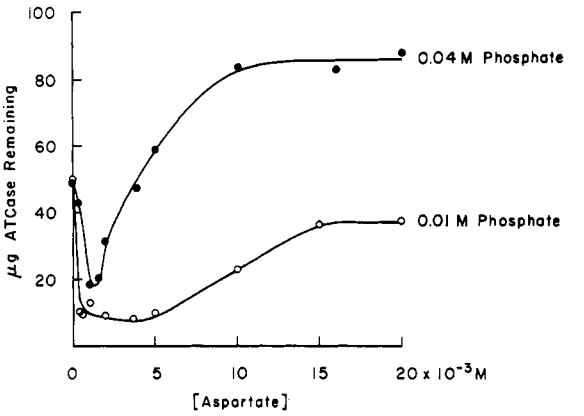

FIGURE 7: ATCase (micrograms) remaining after incubation with 1.75×10^{-4} M *p*-HMB in the absence and presence of varying concentrations of aspartate. Incubation: ATCase (100 μg/ml) in either 0.01 or 0.04 M phosphate buffer (pH 7.0) at 0° for 15 min.

creased the rate of dissociation. As already mentioned, the presence of 0.8 M urea in the enzyme assay abolishes the feedback inhibition of the enzyme by CTP. The effect of this concentration on ATCase is completely reversible, as far as antigenic properties of ATCase are concerned. Urea at this concentration, however, increases the rate of the *p*-HMB dissociation 1.7 times. The enhancement of dissociation by 0.8 M urea is equal to that of 10 mM succinate or 15 mM CAP. In contrast to this, the presence of 5 mM CTP diminishes the rate of dissociation by two-thirds as shown in Figure 4.

Effect of Varying Concentrations of Ligands on the Dissociation of ATCase by p-HMB. In the preceding experiments it was demonstrated that the rates of dissociation can be altered by ligands, or nonspecifically by urea. In order to compare the effects of different concentrations of ligand on the dissociation within one experiment, a constant amount of ATCase was incubated with an appropriate *p*-HMB concentration for a constant time (15 min). The effect of varying concentrations of ligand was then ascertained by comparing the amount of native enzyme remaining after incubation in the presence of ligand with that remaining in the incubation mixture in the absence of ligand. Figure 5 shows that the addition of CAP increases the rate of dissociation of ATCase by *p*-HMB. A maximal effect of CAP is observed, under these conditions, when the CAP concentration is 15 mM. When the concentration of CAP is 15 mM or higher, the amount of ATCase dissociated is 1.7 (Figure 5) or two times (Figure 6) as great as the amount of enzyme dissociated by *p*-HMB alone. The data in Figure 6 show the effect of varying concentrations of succinate on the dissociation. With increasing concentrations of succinate, the amount of undissociated ATCase remaining decreases. The maximal enhancement is reached at 50 mM succinate. At this concentration of succinate the amount of ATCase dissociated is twice the amount dissociated with *p*-HMB alone. When one studies the effect of various succinate concentrations on ATCase in the presence of CAP and *p*-HMB, it is observed that the individual maximal effects of CAP and succinate are nearly additive. The more dramatic change, however, is that 5 mM succinate now gives a maximal response.

Since it could be demonstrated that succinate alone influences the dissociation of ATCase, aspartate, the natural substrate of the enzyme, was tested in the same way. In Figure 7 the effect of increasing concentrations of aspartate on the dissociation of ATCase in the presence of 0.04 M phosphate and 0.01 M phosphate is plotted. Aspartate favors the dissociation at low concentrations. The maximal enhancement is reached at 1 mM aspartate in the presence of 0.04 M phosphate and at 0.25 mM aspartate in the presence of 0.01 M phosphate. At higher concentrations of aspartate the enhancement of dissociation becomes smaller and at concentrations above 5 mM aspartate in 0.04 M phosphate, the dissociation of ATCase is actually inhibited so that the rate of dissociation is less than with *p*-HMB alone. In the presence of 0.01 M phosphate and with 15 mM aspartate (or more) the rate of ATCase dissociation is not further decreased by increasing the aspartate and has not yet returned to the rate with *p*-HMB alone.

The effect of different trinucleotides on the dissocia-

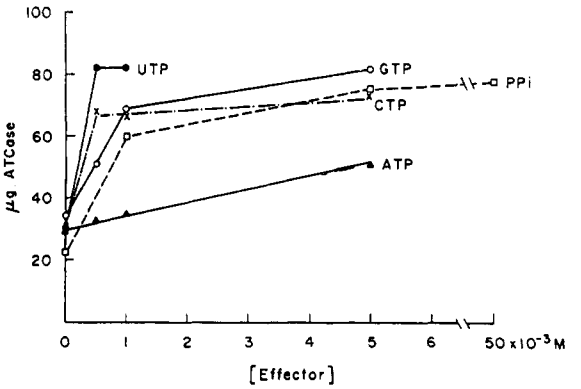

FIGURE 8: ATCase (micrograms) remaining after incubation with 2.5×10^{-4} M *p*-HMB in the presence of varying concentrations of UTP, GTP, CTP, PP_i, and ATP. Incubation: ATCase (86 μg/ml) in 0.04 M phosphate buffer (pH 7.0) at 0° for 15 min.

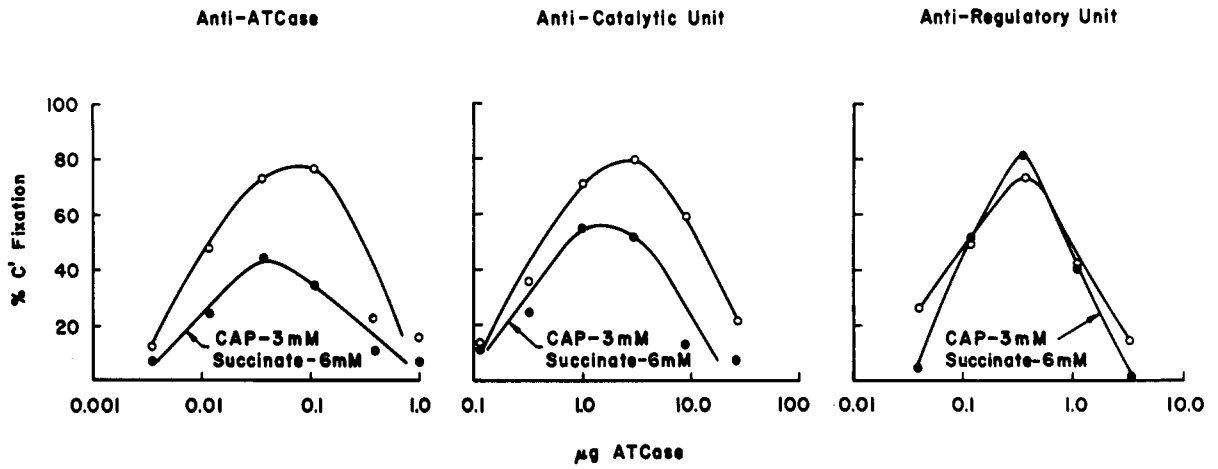

FIGURE 9: Fixation of complement by ATCase (○) and ATCase plus 3 mM CAP and 6 mM succinate (●) with anti-ATCase (left), anticatalytic unit (center), and antiregulatory unit (right). C′ stands for complement.

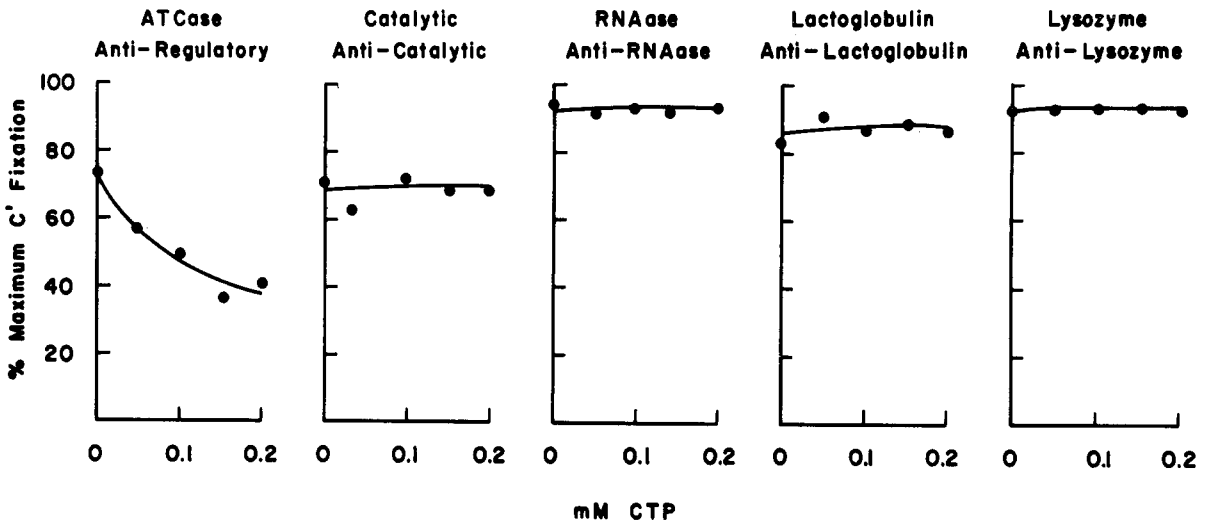

FIGURE 10: Maximum fixation of complement by ATCase, catalytic unit, ribonuclease, β-lactoglobulin, and lysozyme with designated antisera in the presence of varying concentrations of CTP. C′ stands for complement.

tion of ATCase by *p*-HMB is shown in Figure 8. All of the trinucleotides in concentration ranges of 0.5–5 mM protect the enzyme from dissociation. UTP, which is not a feedback inhibitor, is the most effective inhibitor of *p*-HMB dissociation of ATCase, followed by CTP, the best feedback inhibitor and GTP. ATP, the allosteric activator, inhibits dissociation to a small extent. Pyrophosphate, which can be considered an analog to the nucleotide triphosphates, and which has been shown to be an effective inhibitor of ATCase whose inhibition is competitive with CAP (Kleppe, 1966) also is an effective inhibitor of dissociation of ATCase. Indeed, it is as effective as GTP.

Conformational Changes in ATCase and Its Subunits Resulting from Their Interaction with CAP and Succinate, and CTP. The complement fixing activity of ATCase was assayed with anti-ATCase, anticatalytic, and antiregulatory subunit in the presence of a mixture of 6 mM succinate and 3 mM CAP. Complement fixation is diminished when measured with anti-ATCase (Figure 9). A smaller, but still significant decrease in serologic activity can be observed in the assay with anticatalytic subunit. However, no change in complement fixation occurs in the assay with antiregulatory subunit. A decrease in complement fixation was also observed when catalytic subunit was assayed with anticatalytic subunit in the presence of the same concentration of CAP and succinate.

The same experiment was also carried out with ATCase in the presence of CTP. A decrease in complement fixation was only observed when ATCase was assayed with antiregulatory subunit. Figure 10 shows the effect of increasing concentrations of CTP on the per cent complement fixed at the peak point of the complement fixation curve. The largest decrease in complement fixation is reached at a concentration of 0.15 mM CTP. The catalytic subunit anticatalytic subunit immune system was not changed in the presence of CTP, nor were the complement fixing properties of three unrelated immune systems.

Discussion

Based on kinetic studies, a variety of treatments, such as heat, urea, *p*-HMB, and X-irradiation, can alter the catalytic properties of ATCase (Gerhart and Pardee, 1962; Kleppe and Spavin, 1967), *i.e.*, ATCase loses its sensitivity to the feedback inhibitor CTP. Heat, urea, and *p*-HMB also lead to loss of the cooperative effects of aspartic acid (Gerhart and Pardee, 1962). Treatment with urea, *p*-HMB, heat (Bethell *et al.*, 1968b), and X-irradiation (unpublished data) also destroys the serologic activity of ATCase and concomitantly unmasks antigenic determinants of free catalytic subunit. For the purposes of this discussion, we would like to consider these effects as nonspecific since they alter the catalytic and antigenic properties of ATCase irreversibly. The effects of the substrates, substrate analogs, and inhibitors will be considered specific since they do not alter the antigenic or catalytic properties of the native enzyme irreversibly. It should be noted, however, that urea, at low concentrations (0.8 M), can reversibly alter the structure of ATCase as demonstrated by the fact that 0.8 M urea does not decrease the serological activity of ATCase and, like CAP or succinate, it enhances the dissociation of ATCase by *p*-HMB.

In our experiments, succinate or CAP, alone, enhances the dissociation of ATCase by *p*-HMB. The concentration of succinate which maximally enhances dissociation is lowered, at least tenfold (from 50 to 5 mM or less), when CAP is present also. This result agrees with the succinate binding studies of Changeux *et al.* (1968) who have observed that the amount of succinate bound to ATCase is increased at least tenfold, by the addition of CAP.[2] The maximum succinate concentration used by Changeux *et al.* (1968) was 3 mM. Our results also agree with those of Gerhart and Schachman (1968) who found that 5 mM succinate, in the presence of CAP, gives a maximum enhancement of the rate of *p*-HMB binding to ATCase. The novel results of the present study are: (1) that succinate *or* CAP added singly enhance *p*-HMB dissociation of ATCase; (2) the maximal effect of succinate (50 mM) and CAP (15 mM), singly, appears to be additive when these same amounts of succinate and CAP are added simultaneously; and (3) CAP apparently increases the binding of succinate, at least tenfold, but it is *not essential* for succinate binding nor for a maximal effect of succinate on ATCase conformation, *i.e.*, the same maximum effect can be observed with high concentrations of succinate alone.

The effects of aspartate on the dissociation of ATCase by *p*-HMB in the presence of phosphate buffer are particularly striking (Figure 7). At low (below 1 mM) concentrations of aspartate, dissociation is markedly enhanced by aspartate. At higher aspartate concentrations (above 2 or 5 mM aspartate with 40 or 10 mM phosphate, respectively), a second effect, that of increasing stabilization of ATCase toward *p*-HMB dissociation, is observed. Both the phosphate and the aspartate concentration are important in both allosteric effects. Enhancement of ATCase dissociation is maximal when both aspartate and phosphate concentration are low, but the point at which the curve turns appears to be related to the product of the phosphate and aspartate concentration, *i.e.*, the curve turns upward in 40 mM phosphate when the aspartate concentration is 1–1.5 mM and in 10 mM phosphate when the aspartate is 5 mM. The presence of an amino group may be necessary for the second allosteric effect, *i.e.*, stabilization of ATCase toward *p*-HMB, since it was not observed with the highest level of succinate studied here, in the presence or absence of CAP. (Higher levels of succinate would have to be studied, however, to be sure the amino group was necessary.)

This result was not unexpected because of the findings of Kleppe (1966) and those of Bethell *et al.* (1968a). Kleppe (1966) had observed that relatively high concentrations of aspartate inhibit the formation of carbamyl aspartate in the presence of certain anions, particularly phosphate, pyrophosphate, or chloride ions. Kleppe (1966) explained this inhibition by postulating formation of an enzyme–aspartate–anion complex, which has lowered enzyme activity, since orthophosphate, pyrophosphate, and chloride are competitive inhibitors with respect to CAP with either ATCase or the catalytic subunit of ATCase. To explain the fact that high aspartate concentrations augment the anion inhibition of ATCase, he suggested that the anions bind more tightly to the ATCase–aspartate complex than to free enzyme.

Bethell *et al.* (1968a) have recently found in kinetic studies that the CAP saturation curve for ATCase is sigmoidal, so that the binding of this substrate to ATCase, as well as the binding of aspartate to ATCase, should and does produce a conformational change in ATCase structure, as described above. Anions, such as orthophosphate, which inhibit the enzymatic activity of the catalytic subunit and ATCase by competing with CAP (Kleppe, 1966), might, therefore, be expected to have some influence on the conformation of ATCase, just as succinate, which inhibits the enzymatic activity of the catalytic subunit and ATCase by competing with aspartate (Gerhart and Pardee, 1968), can produce some (but not all) of the conformational changes observed by aspartate (Gerhart and Schachman, 1968, and this paper). It would be important, therefore, to repeat the experiment of Figure 7 in another buffer to attempt to ascertain what effects are due to the aspartate alone, what effects are produced by orthophosphate alone, and what effects, if any, require that they both be present.

In other experiments, not reported here, it has been observed that an increase in the amount of orthophosphate used as buffer (from 0.04 to 0.1 M) markedly decreased the rate of ATCase dissociation by *p*-HMB. No other ligands were present. This effect is the opposite of the CAP stimulation of ATCase dissociation by *p*-HMB. However, since most experiments of this paper,

[2] Changeux *et al.* (1968) found that ≤ 0.09 mole of succinate bound/mole of ATCase in the absence of CAP and 0.87 mole of bound/mole of ATCase in the presence of 4 mM CAP. They suggested that succinate is not bound to ATCase unless CAP is present and that CAP might be essential for succinate to bind to ATCase. We have assumed that the 0.09 mole of succinate bound/mole of ATCase in the absence of CAP was a significant figure, in which case their results would mean that CAP decreases the binding constant for succinate but is not essential for succinate binding.

the preceding paper (Bethell *et al.*, 1968b), and those of Gerhart and Schachman (1968) were carried out in 0.04 M phosphate buffer and *no* experiments were carried out in the absence of phosphate anion, we do not know if the orthophosphate buffer has modified the effect of CAP and the other ligands as well as the effects of aspartate.

CTP has been shown to decrease the rate of reaction of *p*-HMB with ATCase (Gerhart and Schachman, 1968). We have studied the effect of CTP and other nucleotides and phosphate compounds on the dissociation of ATCase by *p*-HMB. Of the phosphate compounds studied all inhibited the *p*-HMB dissociation of ATCase. The order of effectiveness in inhibiting the dissociation of ATCase by *p*-HMB was UTP > CTP ≥ GTP = PP_i > ATP.[3] This order is obviously not related to feedback inhibition (CTP > GTP ≫ UTP) or activation which is observed with ATP only (Gerhart and Pardee, 1962, 1964). Inorganic pyrophosphate appears (Kleppe, 1966) to bind principally to the catalytic subunit, although, as a nucleotide analog, it is conceivable that it could also bind to the regulatory subunit. Bethell *et al.* (1968a) have recently found that when the aspartate concentration is high that CTP can strongly inhibit ATCase if the CAP concentration is low; CTP under these conditions competes with CAP. In fact, the CTP inhibition, at low CAP concentrations, is biphasic (Bethell, 1967). The results suggest that CTP inhibits first (when CTP is 0.2 mM or less) by binding at the regulatory subunit, and, secondly, at higher concentrations (above 1 mM), CTP might act like the inorganic phosphates and competes at the CAP binding sites as do inorganic ortho- and pyrophosphate (Kleppe, 1966). In fact all four ribotrinucleotides, including ATP, are good inhibitors of ATCase at concentrations above 1 mM when CAP is at half-saturating concentrations, *i.e.*, 0.2 mM (Bethell, 1967). These observations may aid somewhat in explaining the unusual results observed in Figure 8 where all the various phosphates protect ATCase against *p*-HMB dissociation, even those which are not known to interact with the regulatory subunit. If it is assumed that protection toward *p*-HMB is conferred by binding of phosphate analogs at either the regulatory subunit, *i.e.*, CTP, GTP, and perhaps ATP (Gerhart and Pardee, 1962, 1964), or at the catalytic subunit, *i.e.*, UTP (Bethell, 1967), PP_i and P_i[3] (Kleppe, 1966), and perhaps high levels of ATP, GTP, and CTP (Bethell, 1967), then the protective effects need not be directly related to the ability of the phosphates to bind to the regulatory subunit. Without further studies of the inhibition caused by phosphate compounds (Bethell, 1967) and the ability of the phosphate compounds to bind to ATCase and its subunits (Gerhart and Schachman, 1965, 1968), it is premature to try to rationalize these effects in detail.

Dissociation of ATCase followed by exposure of sulfhydryl groups has been suggested as a possible explanation for the mechanism of the reaction of ATCase with *p*-HMB (Gerhart and Schachman, 1968). This mechanism links all-or-none reactivity with dissociation of the protein. Our data do not exclude the all-or-none reactivity with dissociation. Two lines of evidence, however, make this mechanism questionable: (1) the rates of dissociation at 0° depend upon the *p*-HMB concentration (Figures 1, 3, and 4) and (2) the rates of dissociation at 0° are markedly inhibited at low molar ratios of *p*-HMB/ATCase. Our data would favor a mechanism in which the reaction with *p*-HMB changes the conformation without dissociation, followed by a slow dissociating reaction.

While the above effects of ligands on the dissociation of ATCase by *p*-HMB can be explained by changes in conformation of the native enzyme, the experimental approach is indirect, *i.e.*, a change in the rate of *p*-HMB dissociation. In an effort to look for direct alterations of antigenic structure resulting from antigen–ligand interaction, we have looked at the serologic activities of the native enzyme and its subunits in the presence of certain ligands. The observed decreases in serologic activity with CAP plus succinate and also with CTP agree with the known specificity of ligand interaction. The addition of CAP plus succinate in the serologic reaction mixture altered the antigenic activities of the native enzyme and catalytic subunit when measured with antinative enzyme and anticatalytic subunit, but not when assayed with antiregulatory subunit. In the presence of CTP, only the antiregulatory immune system was affected. These results suggest that the major conformational change appears, by the immunochemical technique, to have taken place in the subunit to which the ligand binds. A possible explanation, although remote, for this decreased serologic activity in the presence of ligands is that the active ligands mask an antigenic determinant and therefore prevent its interaction with an antibody.

[3] In another experiment, it was found that when the orthophosphate concentration is raised from 40 (the concentration of orthophosphate used as a buffer for all the samples of Figure 8) to 100 mM that the dissociation of ATCase is decreased. It was found that the additional 60 mmoles of orthophosphate/ml decreases ATCase dissociation to the same degree as 5 mmoles of pyrophosphate/ml (see Figure 8). From this data, one might expect orthophosphate to be as effective or slightly less effective than ATP as an inhibitor of ATCase dissociation by *p*-HMB.

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Amine Oxidase. XII. The Association and Dissociation, and Number of Subunits of Beef Plasma Amine Oxidase*

Frances M. Achee, Charles H. Chervenka,† Richard A. Smith, and Kerry T. Yasunobu‡

ABSTRACT: Previous studies on the sedimentation of the beef plasma amine oxidase indicated that it might be an associating–dissociating system. This was confirmed by the studies reported here. A reinvestigation of the molecular weight of the native enzyme by the methods of gel filtration and sedimentation equilibrium gave a (minimum) molecular weight of 166,700 and 170,000, respectively, compared with the average value obtained previously of 260,000. In addition, the sedimentation velocity pattern of the enzyme was symmetrical at pH 7.0, but was sharpened considerably in acidic or alkaline solutions. Whether this is due to a pH-dependent association–dissociation process or charge effect was not ascertained definitively. The sedimentation velocity patterns of the enzyme were also dependent upon time of storage and upon concentration of the enzyme solution. After 8 weeks at 4°, multiple boundaries were observed in the ultracentrifuge pattern which exhibited $s_{20,w}$ values corresponding to monomer, dimer, and trimers. This may be due to the fact that with time and at high protein concentrations, the association of the monomer becomes slowly irreversible. The enzyme in its monomeric form of mol wt 170,000 was not easily dissociated into subunits. Acid, base, 8 M urea, and 6 M guanidine hydrochloride failed in this regard, indicating the presence of covalent bonds. Guanidine hydrochloride (6 M) in combination with 0.1 M mercaptoethanol was found to be efficient for the dissociation process. A molecular weight of 87,000 was obtained from the studies of the reduced enzyme by combined sedimentation and diffusion measurements and by the sedimentation equilibrium studies, suggesting the monomer is composed of two polypeptide chains of equivalent size.

The *S*-sulfo derivative was hydrolyzed by trypsin and the fingerprint pattern disclosed that there were about 38 peptides. From the known lysine and arginine content of the enzyme, it is concluded tha the enzyme consists of two identical subunits.

In a previous paper (Yamada *et al.*, 1964), the molecular weight of the amine oxidase from beef plasma was reported to be 261,000. However, there was an increase in the sedimentation coefficient with increasing protein concentration, suggesting the presence of reversible association of a monomeric unit to polymers. The molecular weight of the native enzyme has been reinvestigated by the methods of sedimentation equilibrium and Sephadex gel filtration, and a value of the molecular weight of the monomeric unit has been obtained. Results of some dissociation and molecular weight studies indicate that the monomer consists of two polypeptide chains which are covalently linked by disulfide groups. Other preliminary studies on the association of plasma amine oxidase are also presented.

Materials and Method

Materials. Reagent grade guanidine hydrochloride and 2-mercaptoethanol were purchased from Eastman Organic Chemicals. The guanidine hydrochloride was recrystallized from methanol as described by Reithel and Sakura (1963). Bovine serum albumin and *p*-mercuribenzoate (sodium salt) were purchased from the Nutritional Biochemical Corp. Bovine γ-globulin was obtained from Pentex, Inc. Acetic acid was a product of Mann Research Laboratories. Sephadex G-200, G-15, and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Inc. Cytochrome *c* was prepared as described earlier (Matsubara and Yasunobu, 1961). Cellulose for thin-layer chromatography (Sigmacell, type 19) was purchased from Sigma Chemical Co. Trypsin was obtained from Worthington Biochemical Corp.

Crystalline beef plasma amine oxidase was prepared

* From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii 96822. *Received July 12, 1968.* Supported in part by Grant MH-10280-0-04 from the National Institute of Health, a grant from the Hawaii Heart Association, and the American Heart Association.

† To whom requests for reprints should be addressed.
‡ Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.