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Linkage between Reactivity of Sulfhydryl Groups and Subunit Interactions in Aspartate Transcarbamoylase*

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In previous studies on the reaction between aspartate transcarbamoylase and *p*-hydroxymercuribenzoate it was shown that dissociation of the enzyme into catalytic and regulatory subunits was tightly coupled to the formation of mercaptide complexes with sulfhydryl groups on the regulatory chains. The mechanism of that process has been analyzed with the aim of eliciting information regarding the strength of the six noncovalent bonds between the catalytic and regulatory chains. According to the proposed model, one such bond in the native enzyme is continuously breaking and forming with an equilibrium constant for dissociation of about 10^{-7} M. When the mercurial is present, this bond cannot reform because of the reaction between the mercurial and cysteine residues exposed while the contact between the catalytic and regulatory chains is transiently disrupted. The kinetic treatment based on these assumptions accounts for (*a*) the pseudo-first order reaction of all 24 sulfhydryl groups on the regulatory chains of the enzyme, (*b*) the observation that the enzyme and the large intermediate formed during the dissociation process do not contain bound mercurial, and (*c*) the linkage between the degradation of the enzyme and the formation of mercaptide complexes of the regulatory subunits. Moreover, the large (6-fold) increase in the mercurial-promoted dissociation of aspartate transcarbamoylase upon the addition of active site ligands is interpreted in terms of weakening of the bonds between the catalytic and regulatory subunits. The ligand-promoted decrease in the strength of each such bond is estimated to be 1.0 kcal/mol. However, this value must be considered tentative because of the difficulty in partitioning energy among different types of interchain interactions in the enzyme.

In earlier studies from this laboratory the regulatory enzyme, aspartate transcarbamoylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli* was shown to dissociate into free catalytic (C) subunits and mercaptide complexes of the regulatory (R) subunits when the native enzyme composed of two C and three R subunits is treated with *p*-hydroxymercuribenzoate (1-3). Additional experiments demonstrated that the 24 sulfhydryl groups located on the three R subunits reacted with excess mercurial according to a pseudo-first order reaction and the rate was enhanced 6- to 8-fold by the binding of the ligands, carbamoyl phosphate and succinate, or the bisubstrate ligand, *N*-(phosphonacetyl)-L-aspartate, to the active sites (2, 3). This large increase in the pseudo-first order rate constant has served as a sensitive indicator of the gross conformational change associated with the ligand-promoted allosteric transition of ATCase[1] from the constrained, T-state to the relaxed, R-state.

A subsequent analysis of the mechanism of the process showed that the dissociation of ATCase, designated as C_2R_3, proceeds via a sequence of reactions involving the formation of the R subunit-deficient species, C_2R_2 (4-8), and $R(PMB)_8$ followed by the degradation of C_2R_2 into 2 C and 2 $R(PMB)_8$ where $R(PMB)_8$ is the mercaptide complex of the R subunit in which all sulfhydryl groups have reacted with the mercurial (9). Individual rate constants were measured for the two stages of the dissociation process. Moreover, a kinetic analysis showed that, both in the absence and presence of various active site ligands, the rate of reaction of the sulfhydryl groups on the R subunits was coupled exactly to the rate of dissociation of ATCase to generate C_2R_2 (9). Since the dissociation of an R subunit from ATCase involves the rupture of two "bonds" linking a catalytic (c) and a regulatory (r) chain in the enzyme, the reaction of the sulfhydryl groups appears to be linked to the rupture of c:r bonding domains. Hence, kinetic studies of the rate of reaction of the sulfhydryl groups with PMB in the absence and presence of active site ligands should provide valuable information regarding the change in the c:r bond strengths corresponding to the T→R transition in ATCase.

In this paper we present a model which accounts for the pseudo-first order kinetics for the reaction of all 24 thiols in ATCase and the linkage between the strength of the c:r bonds and the reactivity of the cysteines on the six r chains. The model permits a calculation of the weakening of these interchain interactions corresponding to the gross conformational change in ATCase from the T- to the R-state.

EXPERIMENTAL BASIS FOR THE MODEL

The proposed model for the reactivity of the sulfhydryl groups in ATCase must account for the following observations.

1) The reaction of ATCase with various mercurials, *e.g.* PMB (1-3), neohydrin (10), 2-chloromercuri-4-nitrophenol (9, 11), results in dissociation of the enzyme into subunits. Although there are six cysteine residues on the two C trimers,

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[1] The abbreviations used are: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; c, catalytic polypeptide chain; r, regulatory polypeptide chain; PALA, *N*-(phosphonacetyl)-L-aspartate; C_2R_3, ATCase molecule designated in terms of its subunit structure; C_2R_2, enzyme-like molecule lacking one R subunit; PMB, *p*-hydroxymercuribenzoate.

the dissociation of ATCase involves the reaction of only the 24 thiols located on the six r chains (2, 3, 9). The four cysteine residues at positions 109, 114, 137, and 140 in the amino acid sequence of the r chains (12) are implicated in the binding of each of the six zinc ions in ATCase (13–17). Since x-ray diffraction studies (17) place the metal ions near the c:r bonding domains, the sulfhydryl groups which chelate the zinc ions must also be near the c:r interface.

2) The reaction of the 24 thiols in the three R subunits in ATCase appears to be "all or none" in the sense that mercaptide complexes were observed for only the dissociated R subunits (2). In solutions containing both intact ATCase and subunits produced by limited amounts of PMB, no mercurial could be detected on the undissociated ATCase (2).[2] Therefore, the model must account for the rapid dissociation of an R subunit once the first sulfhydryl group of an R subunit reacts with PMB.

3) The sulfhydryl groups of isolated R subunits react at least 100-fold more rapidly with PMB than the cysteine residues in the native enzyme.[3]

4) The rates of formation of mercaptide complexes and the dissociation of ATCase into C_2R_2 are identical (9). This coincidence of rates is observed both for ATCase in the T-state (in the absence of ligands) and for the enzyme in the R-state (in the presence of active site ligands). These observations suggest that the rupture of the c:r bonding domains leads to exposure of thiols allowing for their rapid reaction with PMB until dissociation of the R subunit occurs (9).

5) The bisubstrate analog, PALA, which causes the T→R transition in ATCase (18, 19), also weakens the c:r bonds (20, 21). In addition, there is a 6-fold increase in the reactivity of the thiols on the R subunits in ATCase toward PMB when PALA is present (3, 9). Other active site ligands which shift the allosteric equilibrium of ATCase toward the R-state also weaken the c:r bonding domains and cause an enhancement in the reactivity of the cysteine residues in ATCase (3, 9). Hence there appears to be an inverse correlation between the strength of the c:r bonds and the reactivity of the sulfhydryl groups in the R subunits of ATCase.

6) The local environment at the metal ion-binding sites in ATCase is altered when the enzyme is converted to the R-state by the binding of PALA. This conformational change was demonstrated by circular dichroism (16) and by difference spectroscopy (22) with ATCase derivatives in which the zinc ions normally present in the enzyme were replaced by either cadmium (16) or nickel (22) ions.[4] Hence the effect of ligand binding at the active sites of the c chains is propagated a considerable distance in ATCase to the interfaces between the c and r chains. Moreover, other regions in the r chains are affected as well as shown by the change in the visible absorption spectrum of chromophores on the r chains in hybrid ATCase molecules containing native C subunits and nitrated R subunits (24).

MODEL FOR THE REACTION BETWEEN ATCase AND MERCURIALS

To account for the observations enumerated above, we assume that the reaction between PMB and sulfhydryl groups of an R subunit in ATCase occurs at a significant rate only if a c:r bond is disrupted. Thus, in Fig. 1, ATCase with all six c:r bonds intact would not react at a measurable rate with PMB. In contrast, broken ATCase with only five c:r bonds intact and one c:r bond disrupted would react at a significant rate because of the presumed exposure of one or more cysteine residues. Once the first sulfhydryl group has reacted to form a mercaptide complex, the c:r bond cannot be reformed. The resultant complex would then contain a partially reacted R subunit which is bound through only a single c:r bond. Hence this R subunit would dissociate at a rate determined by the rate of rupture of a c:r bond to yield C_2R_2 and a free partially reacted R subunit[5] which would combine very rapidly with the PMB to yield $R(PMB)_8$.

The rate of disappearance of ATCase can be written as

$$-\frac{d[\text{ATCase}]}{dt} = k[\text{PMB}][\text{ATCase}]_{\text{broken}} \quad (1)$$

where k is the intrinsic rate constant for the reaction of an exposed sulfhydryl group in ATCase containing one broken c:r bond. This species, shown in Fig. 1, is designated $\text{ATCase}_{\text{broken}}$, and its concentration is readily expressed as

$$\frac{[\text{ATCase}]_{\text{broken}}}{[\text{ATCase}]_{\text{intact}}} = K_b \quad (2)$$

where K_b is the equilibrium constant for the disruption of a c:r bond. Since undissociated ATCase can be represented by the two forms, $[\text{ATCase}]_{\text{broken}}$ and $[\text{ATCase}]_{\text{intact}}$, we can write

$$[\text{ATCase}] = [\text{ATCase}]_{\text{broken}} + [\text{ATCase}]_{\text{intact}} \quad (3)$$

where [ATCase] designates the total concentration of ATCase. Rearranging Equations 2 and 3 leads to

$$[\text{ATCase}]_{\text{broken}} = \frac{K_b[\text{ATCase}]}{1 + K_b} \quad (4)$$

and combining Equations 4 and 1 yields

$$-\frac{d[\text{ATCase}]}{dt} = \frac{k[\text{PMB}]K_b[\text{ATCase}]}{1 + K_b} \quad (5)$$

For experiments in which PMB is in excess (2, 3, 9), we can write for the degradation of ATCase,

$$-\frac{d[\text{ATCase}]}{dt} = k_{\text{obs}}[\text{ATCase}] \quad (6)$$

where k_{obs} is the measured pseudo-first order rate constant described by

$$k_{\text{obs}} = \frac{k[\text{PMB}]K_b}{1 + K_b} \quad (7)$$

According to the model the dissociation of ATCase into

[2] The observation in the ultracentrifuge experiments of Gerhart and Schachman (2) that no mercurial was bound to the undissociated enzyme was made before the discovery that C_2R_2 was an intermediate in the mercurial-mediated degradation process. It is likely that the rapidly migrating material with a sedimentation coefficient of about 11.3 S which was designated as undissociated protein was actually a mixture of ATCase and C_2R_2. As judged by spectral measurements on the moving boundary, less than 1 PMB was bound/mol of protein.

[3] S. Subramani and H. K. Schachman, unpublished observations.

[4] Recent extended x-ray absorption fine structure measurements (23) on the zinc enzyme have shown that mean distance from zinc to the sulfur atoms is 2.34 ± 0.03 Å. No change in this distance was detected upon the addition of PALA but, as the authors point out, changes in bond angles would not have been observed.

[5] It is possible, of course, that a single partially reacted r chain is released due to the rupture of the r:r bond. Although this alternative cannot be rigorously eliminated on the basis of the available experimental evidence, it should be noted that a C_2R_2-derivative in which the r chains were covalently cross-linked also disproportionates at approximately the same rate as C_2R_2 containing noncovalently linked R dimers (21). Hence, rupture of the r:r bonds is not required for the dissociation of C_2R_2, and we assume, therefore, that the mercurial-mediated breakdown of ATCase proceeds via the release of an intact R dimer. We have no information as to the number of cysteine residues which react with PMB while the R subunit is still linked to the protein by one c:r bond. Nor is it known whether the rupture of the second c:r bond linking the partially reacted R subunit occurs at the same rate as that for the breakage of the first c:r bond.

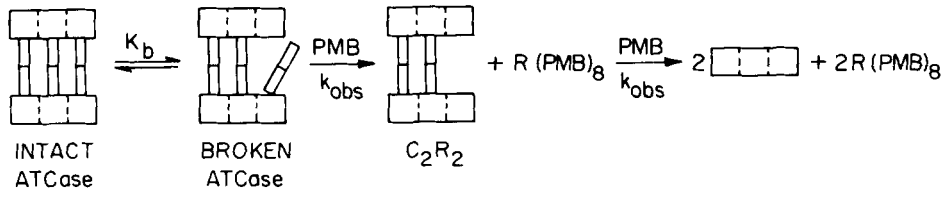

FIG. 1. **Scheme for the reaction between ATCase and PMB.** Native ATCase is considered to be an equilibrium mixture between intact ATCase containing six c:r bonds and broken ATCase containing only five intact c:r bonds and one disrupted c:r bond. The equilibrium constant is designated as K_b. Only broken ATCase can react with PMB at a significant rate to yield C_2R_2 and the mercaptide complex, R(PMB)$_8$. C_2R_2 reacts with additional PMB to give 2 C + 2 R(PMB)$_8$. C subunits, which are trimers of c chains, are designated by the *horizontal rectangles* and the R subunits, which are dimers of r chains, are designated by the *vertical rectangles*.

subunits in the presence of excess mercurial follows pseudo-first order kinetics and the observed rate constant is related both to the intrinsic rate constant for the reaction between PMB and exposed sulfhydryl groups in broken ATCase and to the equilibrium constant for the disruption of a c:r bond.

RELATIONSHIP BETWEEN RATE OF PMB DISSOCIATION OF ATCase AND STRENGTH OF C:R BONDING DOMAIN

An interesting consequence of this treatment is that the model can be used to calculate the change in the c:r bond strength accompanying the ligand-promoted conformational transition from the T- to the R-state.

Experiments with C_2R_2 which contains only four c:r bonding domains as compared to six in ATCase have demonstrated that C_2R_2 is less stable than native ATCase in solutions of low ionic strength (20). As a consequence C_2R_2 disproportionates into C_2R_3 and free C subunits and the rate-limiting reaction in this process has been shown to depend on the rate of rupture of two c:r bonds (21). Moreover, the rate of disproportionation of C_2R_2 is increased about 300-fold upon the addition of PALA. Thus, the strength of the c:r bonds is markedly weakened when PALA is added to C_2R_2, which like native ATCase is converted to the R-state upon the binding of active site ligands (21). We infer, therefore, that the c:r bonds in ATCase are also weakened by the addition of PALA. Accordingly the equilibrium constant for the rupture of a c:r bond in ATCase saturated with PALA can be written as K_b^{PALA} and the observed pseudo-first order rate constant, k_{obs}^{PALA}, for the PMB-mediated dissociation of liganded ATCase is

$$k_{obs}^{PALA} = \frac{k[PMB]K_b^{PALA}}{1 + K_b^{PALA}} \quad (8)$$

In Equation 8 we assume that the intrinsic rate constant, k, for the reaction of exposed sulfhydryl groups in ATCase containing one disrupted c:r bond is the same for both the liganded and unliganded species.

Combining Equations 8 and 7 (for the unliganded species) yields

$$\frac{k_{obs}^{PALA}}{k_{obs}^{unliganded}} = \frac{K_b^{PALA}(1 + K_b^{unliganded})}{K_b^{unliganded}(1 + K_b^{PALA})} \quad (9)$$

Because both $K_b^{unliganded}$ and K_b^{PALA} are small compared to 1, Equation 9 reduces to

$$\frac{k_{obs}^{PALA}}{k_{obs}^{unliganded}} = \frac{K_b^{PALA}}{K_b^{unliganded}} \quad (10)$$

Thus, the ratio of the pseudo-first order rate constants for the dissociation of ATCase in the presence and absence of PALA provides a value for the relative equilibrium constants for the rupture of the c:r bonds in the R- and T-states. Various experimental studies (3, 9) have yielded a value of about 6 for $k_{obs}^{PALA}/k_{obs}^{unliganded}$. Hence we calculate from $-RT\ln K_b^{PALA}/K_b^{unliganded}$ that each c:r domain in the R-state of ATCase (with bound PALA) is weaker than the corresponding domain in the T-state (unliganded) by 1.0 kcal/mol.

DISCUSSION

In contrast to many other oligomeric proteins, which in neutral solutions dissociate readily and reversibly into subunits, ATCase exhibits virtually no association-dissociation equilibria with its subunits or its 12 constituent polypeptide chains (19, 25). Nor is there significant exchange of its C or R subunits when the native enzyme is incubated with free radioactive subunits (20). This striking stability has been attributed to the multiplicity and intrinsic strengths of the interchain bonds. Each c chain is linked to two other c chains in the same C trimer as well as to one r chain. Similarly, each r chain is bonded noncovalently to another r chain in the same R dimer (as well as to a c chain). Thus, there are six c:c, six c:r, and three r:r bonds (26) as well as some slight contact between the two C subunits (17) which in large measure are separated from each other by a central aqueous cavity (27). Because mercurials like PMB cause the rapid dissociation of ATCase into intact C subunits and mercaptide complexes of the R subunits (2, 3, 9), the mechanism of that process has been analyzed here with the aim of providing information regarding the strength of the c:r bonds.

The basic postulate is that a single c:r bond in native ATCase is continuously breaking and reforming with an equilibrium constant for dissociation estimated at about 10^{-7} M (28). Although a single bond may be ruptured for a significant period of time, little dissociation or exchange of R subunits occurs because two c:r bonds in the same R dimer must be broken in the same time interval. According to our mechanism the reformation of a c:r bond can be prevented by the reaction of mercurials with cysteine residues in the R subunits which are exposed when the contact between the c and r chains is transiently disrupted. Because the cysteine residues are located at the interface between the c and r chains (17) and the sulfhydryl groups of isolated R subunits react with mercurials extremely rapidly, we have assumed that the much slower reaction of the same thiols in ATCase occurs only in that fraction of the enzyme molecules having a disrupted c:r bonding domain. These broken ATCase molecules containing five intact c:r bonds are presumed to be in equilibrium with intact ATCase. Hence reaction of the former with mercurial would lead progressively to the dissociation of ATCase into C_2R_2 which in turn reacts with additional PMB to form free C subunits and mercaptide complexes of the R subunits.

The model for the reaction of ATCase with PMB accounts for (*a*) the pseudo-first order kinetics observed for the reaction of all 24 sulfhydryl groups of the R subunits of ATCase, (*b*) the observation that ATCase and C_2R_2 do not contain bound mercurial, (*c*) the coupling between the disappearance of ATCase and the reaction of the sulfhydryl groups with PMB to form mercaptide complexes, and (*d*) the correlation be-

tween the strength of the c:r bonds in C_2R_2 and the reactivity of the thiols with PMB. In the model as proposed, the intrinsic reactivity of all four sulfhydryl groups in the r chains in broken ATCase is considered to be identical.

Active site ligands like PALA which weaken the c:r bonds would increase the fraction of enzyme molecules in which a c:r bond is ruptured. Hence the reaction of ATCase with PMB would be expected to be faster in the presence of PALA. Earlier work has shown that there is indeed a 6- to 8-fold enhancement of the rate of reaction of ATCase with PMB upon the addition of active site ligands (2, 3, 9). Moreover, there is an excellent correlation between the estimates of the ligand-promoted T $\rightarrow$ R conversion evaluated from the changes in the sedimentation coefficient of the enzyme and the enhanced reactivity of the sulfhydryl groups (19). Thus, the model accounts for the T $\rightleftharpoons$ R equilibrium in terms of the changes in the c:r bond strength and in the reactivity of the sulfhydryl groups. In addition, the correlation between weakening of the c:r bonds and enhanced reactivity of the sulfhydryl groups is observed for ATCase in the presence of carbamoyl phosphate (3, 9). The rate of dissociation of ATCase into C_2R_2 and the overall rate of reaction of the thiols in the R subunits of the enzyme are increased to the same extent by the addition of the substrate which also causes a significant weakening of the c:r bonds (9). This ligand-promoted weakening of the intersubunit interactions was also observed in studies of the dissociation of ATCase at low concentrations of sodium dodecyl sulfate (29).

Equation 10 also accounts for the results with an inactive mutant form of ATCase which contains weaker c:r bonds (30). This mutant enzyme, $ATCase_{231}$, which contains a single alteration in the c chains (a glycine residue is replaced by an aspartate residue (31)) has such weak c:r bonds that no stable C_2R_2 species has yet been detected (30). Moreover, preliminary experiments indicate that the C_{231} subunits in $ATCase_{231}$ exchange readily with radioactively labeled, wild type C subunits.[6] When $ATCase_{231}$ is treated with PMB there is a rapid dissociation into subunits and no intermediates are observed (32). The pseudo-first order rate constant, 39×10^{-3} s^{-1}, for the dissociation is 10-fold greater than that observed for the wild type enzyme in the absence of ligands (32). According to the model, this very large rate, which is identical to that for the formation of mercaptide complexes, corresponds to a weakening of the c:r bonds in the mutant by 1.38 kcal/mol compared to those in the wild type enzyme. Hybrid enzyme molecules containing one native C and one succinylated C subunit along with three native R subunits also have much weaker c:r bonds due presumably to the destabilization resulting from the negative charges on the modified C subunits. This hybrid reacts with PMB at a much faster rate than the native enzyme.[7]

ATCase from the inactive *pyrB*231 mutant is not only unusual in its higher reactivity with PMB in the absence of ligands, but also it exhibits a 2.6-fold *decrease* in its PMB-promoted rate of dissociation in the presence of saturating amounts of carbamoyl phosphate (32). In contrast, the rate of dissociation of the wild type enzyme is *increased* 3-fold by the addition of the substrate (3). If the model proposed here is correct, the c:r bonds in the mutant enzyme are strengthened by the addition of carbamoyl phosphate as contrasted to the weakening observed with the wild type enzyme. As yet no experimental evidence is available to test this prediction of the model. Nor are there any known ATCase species (mutants or hybrids) with stronger c:r bonds than those in the native, wild type enzyme. Further tests of the model are clearly needed to determine whether species with stronger c:r bonds do exhibit decreased reactivity of their sulfhydryl groups with PMB.

Although many observations on the wild type enzyme and the inactive mutant, $ATCase_{231}$, can be interpreted in terms of the relationship between the reactivity of the sulfhydryl groups and intersubunit interactions, it is important to recognize significant limitations in the model. We have attributed the ligand-promoted increase in the rate of dissociation of the enzyme to weakening of the c:r bonds. These bonds are certainly implicated in an important way in mediating the T$\rightarrow$R transition in ATCase and their rupture occurs when the enzyme is treated with mercurials. However, other parts of the ATCase molecules are also affected when the enzyme is converted from the T- to the R-state or is dissociated into subunits. Hence, it is probably an oversimplification to attribute the change in the rate of the PMB-promoted dissociation solely to alterations in the strength of c:r bonds. Clearly the limited contacts between the two C subunits observed in recent crystallographic studies (17) must be broken during the dissociation process. At present there are no estimates of the magnitude of the interactions between the two C subunits. Nor is there any information about differences in these contacts for ATCase in both the T- and R-states. Without such evidence and data for the changes in the strengths of the c:c and r:r bonds accompanying the ligand-promoted swelling (2, 19) and dissociation (2, 3, 9) of the enzyme, our assignment of the more rapid degradation of ATCase in the presence of active site ligands to a weakening of only the c:r bonds must be considered as tentative.[8]

Another limitation of the model is the lack of experimental evidence regarding the value of the intrinsic rate constant, k, in Equation 1 for the reaction between exposed sulfhydryl groups in ATCase containing one disrupted c:r bond. We assume that this rate constant is the same for both unliganded and liganded species (Equation 8) and that it is much less than that for the rupture of a c:r bond. Clearly data are needed to test the validity of these assumptions.

According to the analysis of the PMB-promoted dissociation of ATCase, each c:r bond is weakened by 1.0 kcal/mol when the enzyme is converted from the T- to the R-state. This value, calculated from the 6-fold increase in the rate of dissociation of ATCase in the presence of PALA, is significantly less than the weakening of the c:r bonds (1.7 kcal/mol) evaluated from studies of the rate of disproportionation of C_2R_2 in the absence and presence of PALA (21). It should be noted, however, that the disproportionation studies on C_2R_2 were performed on solutions of much lower ionic strength than the kinetic experiments on the dissociation of ATCase with PMB. As shown by the relative stability of C_2R_2 in solutions of moderate ionic strength (50 mM Tris-HCl) as compared to the rate of disproportionation at low ionic strength (4 mM Tris-HCl), the c:r bonds in C_2R_2 must be significantly stronger at higher ionic strengths. Whether the effect of PALA on the strength of these c:r bonds is also dependent on ionic strength is not known.

As indicated above, there are numerous, relatively weak noncovalent interactions among the 12 polypeptide chains in

[6] J. M. Ritchey, I. Gibbons, and H. K. Schachman, unpublished observations.

[7] M. N. Blackburn and H. K. Schachman, unpublished observations.

[8] No effort was made to apply the model to interpret the results (33) on the effect of PALA on the rate of reaction of ATCase with PMB at pH 6.0 because most of the relevant details on the properties and dissociation of ATCase under these conditions are not available. In addition, as shown in 1963 by Gerhart and Pardee (34), the enzyme at pH 6.0 is almost devoid of cooperativity and activity.

ATCase. Hence, it is hazardous to partition the energies among discrete types of bonds which are likely to be interrelated. Nonetheless, the simple model proposed here to account for the effect of active site ligands on the PMB-promoted dissociation of ATCase does provide a useful, tentative value of the change in the strength of the c:r bonds accompanying the allosteric transition of the enzyme.

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