THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 3, Issue of February 10, pp. 1255-1262, 1981
Printed in U.S.A.

The Mechanism of Dissociation of Aspartate Transcarbamoylase by *p*-Mercuribenzoate*

(Received for publication, July 24, 1980)

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In previous studies on the reaction of the *Escherichia coli* regulatory enzyme, aspartate transcarbamoylase, with excess *p*-hydroxymercuribenzoate, pseudo-first order kinetics was observed, even though six of the reacting sulfhydryl groups are located on the two catalytic subunits and the remaining 24 thiols are on the three regulatory subunits. Moreover, upon the addition of active site ligands, the reactivity of the 6 thiols on the catalytic chains was markedly depressed and the 24 cysteine residues on the regulatory chains exhibited a 6-fold increase in rate. These observations of a single rate constant for the thiols seemed particularly unusual because of recent evidence that relatively stable enzyme-like molecules lacking one regulatory subunit are intermediates in the mercurial-promoted dissociation of aspartate transcarbamoylase into free catalytic and regulatory subunits. Since previous kinetic measurements of the formation of mercaptide complexes based on their absorption at 250 nm did not permit an unambiguous assignment of the rates to any particular species, we developed a procedure for determining both the nature and the concentration of various components formed during the process. The dissociation was stopped at designated times by chelating unreacted mercurial with diphenylthiocarbazone and the amounts of individual species were determined by electrophoresis. Two detectable sequential stages were observed during the dissociation of the enzyme. Measurements of the reaction of the sulfhydryl groups of the regulatory subunit-deficient species showed that the rates of the two stages of the overall process were virtually identical both in the absence and presence of active site ligands. Thus, a satisfactory explanation is provided for the observation of a single reaction rate for the many sulfhydryl groups of the enzyme. Moreover, the dissociation of aspartate transcarbamoylase by rupture of intersubunit "bonds" between catalytic and regulatory subunits is coupled exactly to the reaction of the cysteine residues on the regulatory chains.

Ligand-promoted changes in the reactivity of amino acid side chains in proteins have been used widely as a probe of conformational alterations. With the allosteric enzyme, aspartate transcarbamoylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli*, the increase in reactivity of the 24 sulfhydryl groups located on the regulatory polypeptide chains upon the addition of active site ligands which bind to the catalytic chains has served as a sensitive indicator of the gross conformational change associated with the allosteric transition of ATCase[1] (Gerhart and Schachman, 1968; Blackburn and Schachman, 1977). When ligands were absent, all 30 thiols on the enzyme (6 on the two catalytic trimers and 24 on the three regulatory dimers) reacted with excess *p*-hydroxymercuribenzoate according to first order kinetics. In the presence of saturating amounts of the bisubstrate analog, *N*-(phosphonacetyl)-L-aspartate, which causes the allosteric transition of the enzyme (Collins and Stark, 1971; Howlett *et al.*, 1977), the reactivity of the cysteine residues on the C subunits is markedly depressed and the pseudo-first order rate constant for the 24 sulfhydryl groups on the R subunits is increased 6-fold (Blackburn and Schachman, 1977).

In view of the large number of thiol groups in ATCase (C_2R_3) and their distribution on the different types of polypeptide chains in the enzyme (Gerhart and Schachman, 1965; Weber, 1968; Wiley and Lipscomb, 1968; Meighen *et al.*, 1970; Rosenbusch and Weber, 1971; Cohlberg *et al.*, 1972), the pseudo-first order kinetics with a single rate constant for the reaction of ATCase with excess PMB seemed unusual (Gerhart and Schachman, 1968). This observation appeared especially perplexing since, in the dissociation of ATCase into free C and R subunits (Gerhart and Schachman, 1965), the relatively stable R subunit-deficient molecules, C_2R_2, are found as an intermediate (Yang *et al.*, 1974; Bothwell and Schachman, 1974; Evans *et al.*, 1974, 1975). As shown in this paper, the dissociation of ATCase in the presence of mercurials is a two-stage process that can be described as follows:

$$C_2R_3 \xrightarrow{k_1} C_2R_2 + R\cdot(PMB)_x \quad (1)$$

$$C_2R_2 \xrightarrow{k_2} 2\,C + 2\,R\cdot(PMB)_x \quad (2)$$

where k_1 and k_2 are the pseudo-first order rate constants for the two stages of the reaction, and $R\cdot(PMB)_x$ is the mercaptide complex of the R subunits in which all sulfhydryl groups have reacted.[2]

* This work was supported by Public Health Service Research Grant GM 12159 from the National Institute of General Medical Sciences and by National Science Foundation Research Grant PCM76-23308. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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; MNP-ATCase, ATCase derivative in which the 6 cysteine residues on the catalytic chains were modified by reaction with 2-chloromercuri-4-nitrophenol.

[2] The six sulfhydryl groups on the two C subunits in ATCase also react with PMB. We presume that these groups can react when the

In all previous experiments, the reaction of ATCase with PMB was monitored by the formation of the R·(PMB)$_8$ complex at 250 nm, according to the method of Boyer (1954). However, it is clear that if each of the two steps in the reaction scheme is a pseudo-first order process, then the overall process monitored by the formation of mercaptide complexes should represent the sum of two pseudo-first order reactions. Nonetheless, only a single rate constant was detected (Gerhart and Schachman, 1968; Blackburn and Schachman, 1977). Was this observation due to the much more rapid dissociation of C_2R_2 (compared to C_2R_3) so that the first step was rate-limiting? Alternatively, did the two steps have the same rate constant? Distinguishing between these alternatives required the development of a method to study the rate constant for the two stages of the reaction independently, both in the absence and the presence of active site ligands. The second process was studied by the method of Boyer (1954) with purified C_2R_2. The rate constant for the first stage was determined by measuring directly the time course of the disappearance of ATCase, as well as the concomitant formation and dissociation of C_2R_2. This was performed by stopping the dissociation at designated times by the addition of diphenylthiocarbazone (dithizone), which is a strong chelating agent for unreacted PMB, and measuring the amounts of the various species after electrophoretic separation of C_2R_3, C_2R_2, and C subunits.

The dissociation of C_2R_3 to C_2R_2 in the first stage of the reaction involves the rupture of two of the six bonding domains (c:r domains) between the six c and r chains of ATCase. Is the rate of this dissociation process identical with the rate of reaction of the sulfhydryl groups? As shown below, the dissociation of the native enzyme is indeed coupled exactly to the rate of reaction of the thiols on the R subunits. An analysis of the two stages in the reaction mechanism is described, and it is shown that the rates of reaction of the sulfhydryl groups on C_2R_3 and C_2R_2 are remarkably similar in both the absence and the presence of various active site ligands. In this way, the pseudo-first order kinetics for the reaction of the sulfhydryl groups of ATCase with PMB is readily interpreted in terms of two sequential processes having similar rates.

MATERIALS AND METHODS[3]

RESULTS

Kinetic Analysis of the Dissociation of ATCase upon Reaction with PMB—The mercaptide complexes detected during the reaction of ATCase with PMB are generated by several different pathways which include the reaction of the thiol groups on both the C and the R subunits of ATCase and C_2R_2. The evaluation of the rates of reaction of the sulfhydryl groups by the method of Boyer (1954) therefore suffers from the inherent limitation that the measured values cannot be assigned unequivocally to any given protein species. This problem was circumvented by determining the nature and the concentration of the molecular species present during the reaction. Achieving this goal required stopping the reaction of ATCase with PMB instantaneously and removing the unreacted mercurial without altering the amounts of the various species in the reaction mixture. Sulfhydryl reagents could not be used to complex the unreacted mercurial because they regenerate the thiols on the C and R subunits, thereby leading to the reassembly of C_2R_2 and C_2R_3 (Gerhart and Schachman, 1965; Bothwell and Schachman, 1974). Therefore, we used a mercury-chelating reagent, diphenylthiocarbazone or dithizone (Fridovich and Handler, 1957), dissolved in an organic solvent that was immiscible with water. In this way, the unreacted PMB was complexed and partitioned into the organic phase (carbon tetrachloride) leaving the protein species in the aqueous layer. This "stopping" procedure was followed by electrophoretic resolution of the various proteins (see "Materials and Methods" in Miniprint). Mercurial which had already formed mercaptide complexes with the protein was not dissociated in this process.

Fig. 1*a* shows the pathway of dissociation of ATCase in a typical experiment involving treatment of the enzyme with excess PMB, stopping the reactions at selected times by the addition of dithizone in CCl_4, and separating the various protein species in the aqueous phase by electrophoresis in polyacrylamide gels.[4] The gel patterns show the progressive disappearance of C_2R_3 during the course of the experiment. Accompanying the decrease in the amount of C_2R_3 during the 1st min is the appearance of C_2R_2. Within the next minute or two, the concentration of C_2R_2 attains its maximal value, and upon further incubation of the reaction mixture, C_2R_2 is degraded with the result that virtually no C_2R_2 is detected at 14 min. The presence of C subunits is not observed until after a lag period of ~1 to 2 min, after which time the amount of C subunits increases rapidly during the remainder of the experiment. No R subunits (or their mercaptide complexes) are observed in the gel patterns because they are partitioned into the organic phase during the 'stopping' process.

Quantitative measurements of the amounts of the various species were obtained by scanning the stained gels with a densitometer and determining the areas corresponding to the different components. As seen in Fig. 1*b*, C_2R_3 disappears virtually completely within 900 s and the amount of C_2R_2 rises from zero at the beginning of the reaction to a maximum value at ~180 s. C_2R_2 then disappears over the following 1000 s. In contrast, the amount of C subunits increases slowly at first. Following this lag period, there is a more rapid formation of C subunits until ~200 s. Then, free C subunits accumulate over the following 1000 s and remain as the dominant species.

These results clearly are in accord with the scheme depicted in Reactions 1 and 2 with C_2R_2 as an intermediate in the dissociation of ATCase into free C and R subunits. As a test of the efficiency of the 'stopping' procedure, spectrophotometric experiments were performed with a mixture of 2-mercaptoethanol and PMB in order to determine quantitatively the extent and rapidity of removal of the unreacted mercurial. More than 97% of the mercurial was removed from the aqueous phase within ~10 s after adding the dithizone (see

C subunits are in C_2R_3 or C_2R_2 since these thiols can be modified preferentially, as shown later, with another mercurial, 2-chloromercuri-4-nitrophenol. Alternatively, these thiols could react once the free C subunits are released during the PMB-mediated dissociation of the enzyme. The rate of reaction of the sulfhydryl groups on the free C subunits appears to have approximately the same rate constant as that observed for ATCase (Blackburn and Schachman, 1977). Since these groups are not directly involved in the dissociation of ATCase to subunits, they are not considered in this reaction scheme. These sulfhydryls do not react in the presence of active site ligands and, as shown later, the elimination of these groups by modification with 2-chloromercuri-4-nitrophenol does not alter the reactivity of the SH groups on the R subunits. It also should be noted that the sulfhydryl groups of free R subunits react with PMB very rapidly (Gerhart and Schachman, 1968).

[3] Portions of this paper (including "Materials and Methods," some "Results," and Fig. 1-S) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M1538, cite author(s), and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[4] In preliminary experiments, EDTA was also found to effectively 'stop' the reaction of PMB with ATCase.

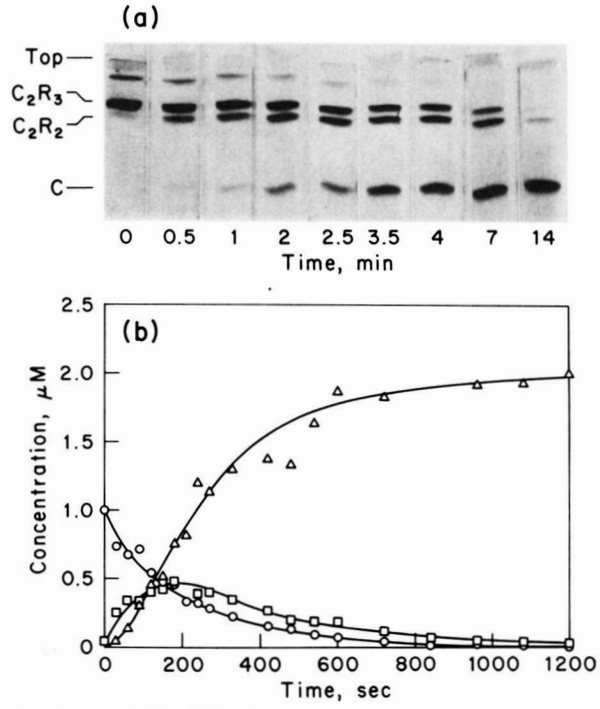

FIG. 1. **Analysis of the dissociation of ATCase by PMB.** The reaction was initiated by mixing equal volumes of ATCase at 0.6 mg/ml in 40 mM potassium phosphate, 50 mM Tris-Cl, at pH 7.0, with 0.40 mM PMB in the same buffer. All experiments were performed at 20 ± 0.5°C. The dissociation process was stopped at various times by the removal of 0.5-ml aliquots and adding them to tubes containing 4 ml of a solution of dithizone in CCl_4 (40 μg/ml). Rapid mixing of the aqueous and organic liquids was performed for 10 s by vigorous agitation of the contents of the tube with a Vortex stirrer. After the aqueous and organic phases had separated in all the tubes, 30-μl aliquots were removed from the aqueous layer in each tube; these samples were added to 3 μl of tracking dye and 20-μl aliquots were loaded onto cylindrical 7% polyacrylamide gels for electrophoresis. For the sample at zero time, the protein sample, prepared as described above, was mixed with an equal volume of buffer instead of PMB solution; the remainder of the procedure was identical with that for the other times. *a*, electrophoresis patterns for the reaction mixture at different times. The positions in the stained gels corresponding to ATCase (C_2R_3), C_2R_2, and C subunits are indicated on the figure. The band migrating above C_2R_3 is an aggregate of ATCase which also dissociates upon reaction with PMB. *b*, concentrations of the various species during the dissociation of ATCase. Gel patterns such as those in *a* were scanned, as described under "Materials and Methods," to obtain the concentrations of ATCase (○); C_2R_2 (□), and C subunits (△). The micromolar concentrations of each species are plotted as a function of time.

Miniprint). The accuracy of the method for determining the amounts of the various species at different times was assessed in control experiments in which myoglobin was used as an internal standard for all electrophoresis experiments. In this way, concentrations of the various components were normalized relative to the amount of myoglobin measured on densitometer traces of the stained gels. Identical results were obtained for the amounts of C_2R_3, C_2R_2, and C subunits in experiments both in the absence and presence of myoglobin. Therefore, myoglobin was not used in subsequent experiments.

The band above ATCase in the gel pattern in Fig. 1*a* corresponds to an aggregate of the native enzyme which also dissociates upon reaction with the mercurial.

Effect of Active Site Ligands on the PMB-promoted Dissociation of ATCase—In earlier studies (Gerhart and Schachman, 1968; Blackburn and Schachman, 1977), various active site ligands were found to enhance the reactivity of sulfhydryl groups on the R subunits while preventing the reaction of those groups on the C subunits. It was of interest, therefore, to determine whether these ligands would increase the rate of dissociation of C_2R_3 and whether the kinetics of the further dissociation of C_2R_2 into free subunits would be increased proportionately. Hence, experiments like that in Fig. 1 were conducted on purified ATCase in the presence of various ligands.

The time course for the mercurial-promoted dissociation of ATCase in the presence of saturating amounts of the bisubstrate ligand, PALA, is shown in Fig. 2*a*. As seen by compar-

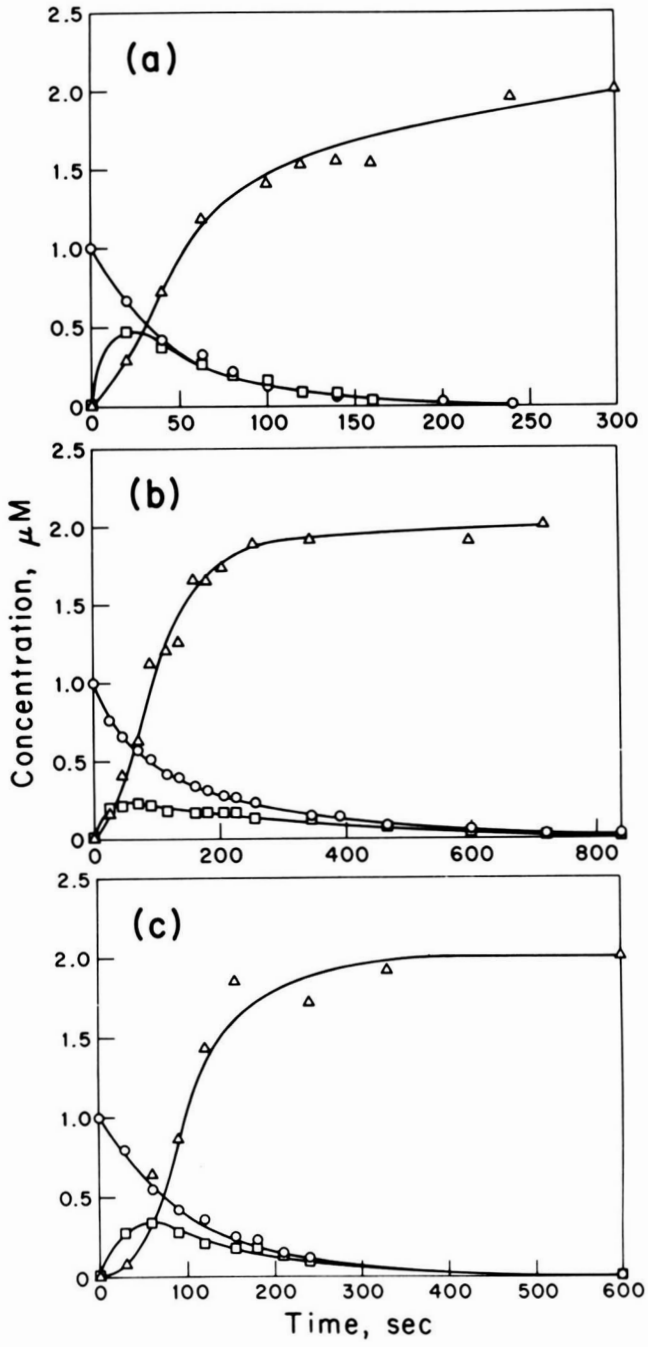

FIG. 2. **Analysis of the mercurial-promoted dissociation of ATCase in the presence of active site ligands.** Experiments were performed as described in Fig. 1 and the concentrations of ATCase, C_2R_2, and C subunits are plotted as a function of time. *a*, the solution contained a saturating amount of PALA (10 eq/mol of enzyme). *b*, experiment conducted in the presence of a subsaturating amount of PALA (1.5 eq/mol of ATCase). *c*, dissociation was performed in the presence of a saturating amount of carbamoylphosphate (3.1 mM).

ison of Fig. 1*b* and Fig. 2*a*, the general patterns of the dissociation of C_2R_3, the formation and disappearance of C_2R_2, and the production of free C subunits are similar in the absence and presence of the ligand. However, the rate of the overall process is markedly increased when PALA is present. Analogous kinetics (Fig. 2*b*) was obtained for the dissociation of ATCase in the presence of subsaturating amounts of PALA (~1.5 eq/mol of enzyme). Lowering the amount of PALA led to a decrease in the overall rate of dissociation of ATCase and, moreover, the maximum amount of C_2R_2 formed in the process was significantly less than that observed when all six active sites were occupied by PALA.

As shown in Fig. 2*c*, the substrate carbamoylphosphate also caused an increase in the rate of dissociation of ATCase upon the addition of PMB. The kinetics of the disappearance and formation of the various species was intermediate between those obtained in the presence of saturating and subsaturating amounts of PALA. When both carbamoylphosphate and succinate were present, the disappearance of C_2R_3 with time was even more rapid ($t_{1/2}$ ~ 30 s), and, therefore, accurate measurements of the formation and disappearance of C_2R_2 could not be made.

In all of these experiments, C_2R_2 appears as an intermediate which is subsequently degraded to free C and R subunits.

Rate Constant for the Dissociation of ATCase into C_2R_2 and R Subunits—Since all of the experiments indicated that the PMB-promoted dissociation of ATCase occurred via the two-stage process depicted in the Introduction, with C_2R_2 as an intermediate, the data for the disappearance of ATCase were analyzed in terms of a pseudo-first order rate equation. Fig. 3 shows that the results in the absence and presence of saturating amounts of PALA are readily fit by first order kinetics. In this way, a pseudo-first order rate constant, k_1, of 3.9×10^{-3} s^{-1}, was obtained for the dissociation in the absence of ligands and 17.6×10^{-3} s^{-1} for the reaction in the presence of a saturating amount of PALA.

Analogous first order kinetics was observed for the dissociation of C_2R_3 in the presence of saturating concentrations (3.1 mM) of carbamoylphosphate (Fig. 3) and the pseudo-first order rate constant was 8.6×10^{-3} s^{-1}. At subsaturating concentrations of carbamoylphosphate (1.6 mM), the kinetics could also be fit by a single rate constant which was intermediate between the values 3.9×10^{-3} s^{-1} and 8.6×10^{-3} s^{-1}, corresponding to no substrate and saturating amounts of substrate.

The addition of the aspartate analog succinate in the presence of carbamoylphosphate caused an additional increase in the rate of dissociation of ATCase (Fig. 3). Under these conditions, the disappearance of ATCase was more than 99% complete in less than 200 s and the calculated pseudo-first order rate constant was 23.7×10^{-3} s^{-1}.

In contrast to these results, the data for the dissociation of ATCase in the presence of subsaturating amounts of PALA (Fig. 2*b*) could not be fit by a single rate constant. However, the biphasic first order plot was readily analyzed in terms of two concurrent reactions with a fast pseudo-first order rate constant of 17.6×10^{-3} s^{-1} and a slow rate constant of 3.9×10^{-3} s^{-1}, with 40% of the protein dissociating with the high rate constant and 60% in the slower class.

The various rate constants (k_1) for the mercurial-promoted dissociation of ATCase in the absence and presence of active site ligands are summarized in Table I. Also listed there for comparative purposes are the values obtained earlier (Blackburn and Schachman, 1977) for the pseudo-first order rate constants (k) for the overall reaction of the sulfhydryl groups of ATCase with PMB. Both sets of measurements show that there is a marked increase in the rates when active site ligands are present and, moreover, there is a striking agreement between the two sets of values.

Rate of Reaction between C_2R_2 and PMB—In principle, the method described above for analyzing the conversion of C_2R_3 into C_2R_2 and R subunits could be applied to measure the rate of reaction of C_2R_2 with PMB. However, the mechanism of the dissociation with C_2R_2 is doubtless complex with concurrent reactions leading to intermediates like C_2R and CR_2. Both of these complexes are likely to be unstable (Bothwell and Schachman, 1980) because they contain only single relatively weak noncovalent "bonds" between the C and R subunits. Thus, intermediates might not be detectable. Nonetheless, the mercurial-promoted dissociation of C_2R_2 into C and R subunits could be studied readily by measuring directly the formation of the mercaptide complexes by the method of Boyer (1954).

Fig. 4 shows the amount of free sulfhydryl groups remaining as C_2R_2 reacts with PMB; the data are readily interpreted as

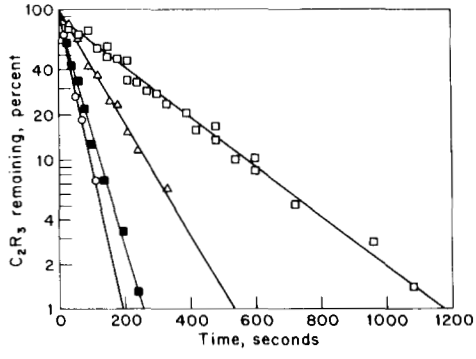

FIG. 3. **Analysis of the PMB-promoted dissociation of ATCase in terms of pseudo-first order kinetics and the effect of ligands on the rate constant.** Data for the disappearance of ATCase as a function of time were taken from experiments like those in Figs. 1 and 2 and plotted as the percentage of C_2R_3 remaining (on a logarithmic scale) *versus* time. The *lines* through the data are linear least squares fits. Results for the reaction are: in the absence of ligands, □; in the presence of carbamoylphosphate (3.1 mM), △; in the presence of a saturating amount of PALA, ■; and in the presence of both carbamoylphosphate (3.1 mM) and succinate (3.0 mM), ○. The corresponding pseudo-first order rate constants were 3.9×10^{-3} s^{-1}, 8.6×10^{-3} s^{-1}, 17.6×10^{-3} s^{-1}, and 23.7×10^{-3} s^{-1}, respectively.

TABLE I

Reaction of ATCase and C_2R_2 with PMB

Ligand[a]	Rate of dissociation of ATCase[b]	Rate of reaction of sulfhydryl groups[c]		No. of sulfhydryl groups reacting[d]	
		ATCase[e]	C_2R_2	ATCase	C_2R_2
	$k_1 \times 10^3$ (s^{-1})	$k \times 10^3$ (s^{-1})	$k_2 \times 10^3$ (s^{-1})		
None	3.9 ± 0.2	3.0 ± 0.2	3.9 ± 0.4	29	22–23
Carbamoylphosphate	8.6 ± 0.4	9.0 ± 0.5	12.9 ± 0.4	25	16–17
Carbamoylphosphate + succinate	23.7 ± 0.5	25.0 ± 0.5		25	16–17
PALA (saturating)	17.6 ± 0.5	18.0 ± 0.5	19.5 ± 1.5	25	16–17
PALA (subsaturating)	Biphasic	Biphasic	Biphasic		

[a] Ligand concentrations: carbamoylphosphate, 3.1 mM; succinate, 3.1 mM; PALA (saturating), 10 eq/mol of ATCase or C_2R_2; PALA (subsaturating), 1 to 3 eq/mol of ATCase or C_2R_2.

[b] Measured by the dithizone method as described in Fig. 1 and under "Materials and Methods."

[c] Experiments were performed as described by Blackburn and Schachman (1977) based on the method of Boyer (1954).

[d] The total number of sulfhydryl groups reacted/molecule of ATCase or C_2R_2 was calculated from the measured change in absorbance at 250 nm; the increment in the extinction coefficient was 7.6×10^4 M^{-1} cm^{-1}.

[e] Data reported by Blackburn and Schachman (1977).

a pseudo-first order process in precisely the same manner as observed previously for ATCase (Gerhart and Schachman, 1968; Blackburn and Schachman, 1977). A pseudo-first order rate constant, k_2, of 3.9×10^{-3} s^{-1} was calculated from the data in Fig. 4 for the reaction in the absence of ligands. When carbamoylphosphate (2 mM) was present in the reaction mixture, the rate increased to give a value of k_2 equal to 12.9×10^{-3} s^{-1}. An even larger increase was obtained (3.9×10^{-3} s^{-1} to 19.5×10^{-3} s^{-1}) when sufficient PALA was present to saturate the active sites in C_2R_2.

As shown in Table I, the rate constants for the reaction of C_2R_2 with PMB under different conditions are in excellent agreement with the analogous values for native ATCase.

When only 1.7 eq of PALA were present/mol of C_2R_2, the decrease in the number of sulfhydryl groups upon the addition of PMB exhibited biphasic kinetics (Fig. 5). The data could be fit accurately by two concurrent pseudo-first order reactions with a slow rate constant of 4×10^{-3} s^{-1} and a fast rate constant of 25×10^{-3} s^{-1}. The fraction of molecules in the slowly reacting class was estimated to be 32% and that reacting at the faster rate was 68%. These results are qualitatively similar to those observed by Blackburn and Schachman (1977) for native ATCase reacting with PMB in the presence of only 3.2 eq of PALA/mol of enzyme. Both sets of data are readily interpreted in terms of a mixed population of molecules, some of which are in the T state and react at rates corresponding to unliganded protein, and others in the R state which react with the rate constant characteristic of fully liganded protein. It should be noted that with C_2R_2 in the presence of 3.2 eq of PALA/mol of protein, the reaction with PMB followed pseudo-first order kinetics.

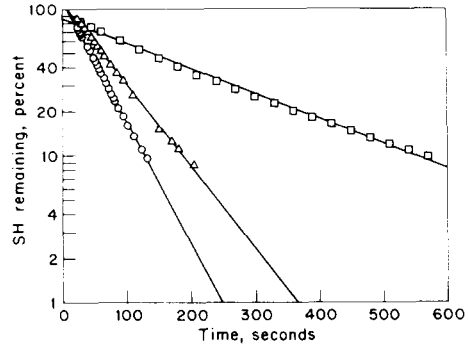

FIG. 4. **Analysis of the reaction of the sulfhydryl groups of C_2R_2 with PMB in terms of pseudo-first order kinetics.** Measurements were made by the method of Boyer (1954) and plotted as described by Blackburn and Schachman (1977) as the percentage of unreacted sulfhydryl groups (on a logarithmic scale) as a function of time. The *straight lines* for the different experiments were drawn with the calculated values of the pseudo-first order rate constants evaluated from a linear least squares fit of the data. Data are shown for experiments with no ligands (□), for solutions containing carbamoylphosphate (2 mM) (△), and for experiments with a saturating amount of PALA (10 eq/mol of C_2R_2) (○). The corresponding pseudo-first order rate constants were 3.9×10^{-3} s^{-1}, 12.9×10^{-3} s^{-1}, and 19.1×10^{-3} s^{-1}, respectively.

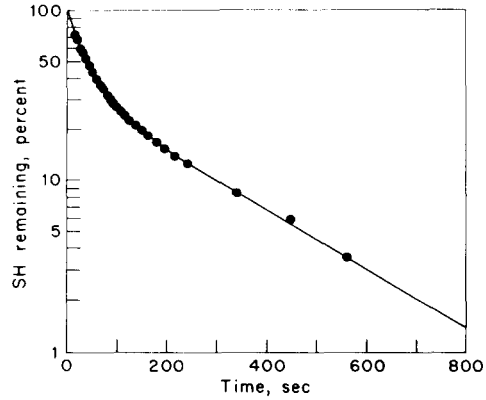

FIG. 5. **Biphasic reaction between C_2R_2 and PMB in the presence of subsaturating levels of PALA.** Data were obtained by the method of Boyer (1954) (as described under "Materials and Methods") and plotted in terms of pseudo-first order kinetics as outlined in Fig. 4. The data obtained in the presence of 1.7 eq of PALA/C_2R_2 were fit in terms of a fast rate constant of 25×10^{-3} s^{-1} and a slow rate constant of 4×10^{-3} s^{-1}, with 68% of the molecules reacting in the fast class and the remaining 32% reacting in the slow class.

Extent of Reaction of Sulfhydryl Groups in ATCase and C_2R_2—A complication in the analysis of the reaction of ATCase with PMB stems from the observation of Gerhart and Schachman (1968) that, in the absence of ligands, the six sulfhydryl groups on the two C subunits of ATCase react with PMB, in addition to the 24 thiols on the three R subunits. Experimentally, all 30 sulfhydryl groups in ATCase appear to react with an identical rate yielding a single pseudo-first order rate constant. Indeed, the rate of reaction of the cysteine residues on the free C subunits is comparable to that of unliganded ATCase. These sulfhydryl groups on the C subunits react much more slowly when active site ligands are present (Gerhart and Schachman, 1968). Thus, there is a significant decrease in the number of reactive sulfhydryl groups in ATCase when the reaction with PMB is conducted in the presence of active site ligands. This reduction from 29 to 25 thiols/ATCase was demonstrated by Blackburn and Schachman (1977). As seen in Table I, a comparable reduction was observed with C_2R_2. With this complex lacking one R subunit and 8 cysteine residues, only 22 to 23 sulfhydryl residues/C_2R_2 molecule react with PMB in the absence of ligands. When active site ligands were added, the number of reactive thiols decreased to between 16 and 17/molecule of C_2R_2.

In order to eliminate any ambiguity regarding the identity of the nonreacting and reacting sulfhydryl groups in ATCase, we conducted some measurements on an ATCase derivative in which the six sulfhydryl groups on the two C subunits were modified with 2-chloromercuri-4-nitrophenol (Evans *et al.*, 1972). With this enzymically inactive MNP-ATCase derivative,[5] prepared as described under "Materials and Methods" (see Miniprint), the reaction with PMB involves only the sulfhydryl groups on the R subunits. When the reaction of MNP-ATCase with excess PMB was analyzed, first order kinetics was observed (Fig. 1-S) and the pseudo-first order rate constant was 3.9×10^{-3} s^{-1}. Thus, the modification of the cysteine residues on the C subunits of ATCase did not affect the rate of reaction of the sulfhydryl groups on the R subunits. The 2-chloromercuri-4-nitrophenol derivative was also tested for its ability to undergo the conformational change associated with the allosteric transition characteristic of native ATCase. Although carbamoylphosphate binds to MNP-ATCase, succinate, which is the analog of the substrate aspartate, apparently does not bind. In addition, the affinity of MNP-ATCase for PALA is ~25-fold less than that of native enzyme. Thus, the reaction of MNP-ATCase with PMB was studied at a much larger concentration of PALA than used with native ATCase (1000 PALA/molecule of MNP-ATCase compared to 10 eq/mol of native enzyme). Under these conditions, the pseudo-first order rate constant (Fig. 1-S) was increased to 24

[5] As shown by Evans *et al.* (1972), the addition of 2-chloromercuri-4-nitrophenol to ATCase at a molar ratio of 6:1 caused inactivation without dissociation of the protein. Following this preferential reaction of the sulfhydryl groups on the C subunits of ATCase, additional mercurial causes the dissociation of the protein into modified C and R subunits.

$\times 10^{-3}$ s^{-1}. This 6-fold enhancement in the rate of reaction of the sulfhydryl groups on the R subunits of MNP-ATCase is exactly the same increase as that observed with native ATCase (Table I). Moreover, with subsaturating amounts of PALA, biphasic kinetics was observed with MNP-ATCase just as had been found with native enzyme (Blackburn and Schachman, 1977). Thus, the inactive derivative with 6 blocked cysteine residues on the c chains exhibited the same kinetics as the native enzyme both in the absence and presence of PALA. Moreover, despite the absence of enzyme activity, MNP-ATCase undergoes the same type of ligand-promoted gross conformational change as the native enzyme.[6]

DISCUSSION

Kinetic Analysis of the Dissociation of ATCase by PMB—As seen in Fig. 1, the technique based on the use of the mercury-chelating reagent, dithizone, followed by electrophoretic analysis of the mixture, was particularly useful for determining the amounts of C_2R_3, C_2R_2, and C subunits during the mercurial-promoted dissociation of ATCase. This rapid and efficient method for stopping the dissociation process by depleting the mixture of unreacted mercurial provided details about the sequence of reactions which could not be obtained merely by measuring the overall rate of reaction of the sulfhydryl groups.

The data in Figs. 1 and 2 show clearly that, both in the absence and presence of active site ligands, the R subunit-deficient species, C_2R_2, is an intermediate in the PMB-promoted dissociation of ATCase into subunits. These quantitative results for the formation of C_2R_2 in Stage 1 of the dissociation process and the subsequent breakdown of C_2R_2 in Stage 2 constitute an extension of earlier qualitative studies indicating that C_2R_2 is an intermediate in the overall reaction of ATCase with PMB (Bothwell and Schachman, 1974; Evans *et al.*, 1974, 1975). Moreover, the close correspondence between the rate of dissociation of ATCase measured by the dithizone method (3.9×10^{-3} s^{-1}) and that obtained earlier (Blackburn and Schachman, 1977) for the overall reaction of the sulfhydryl groups of the enzyme (3×10^{-3} s^{-1}) indicates that the dissociation of ATCase to C_2R_2 is coupled to the reaction of the sulfhydryl groups on the R subunits.[7] It is particularly striking that the rate of reaction of the sulfhydryl groups on C_2R_2 (3.9×10^{-3} s^{-1}) as measured by the method of Boyer (1954) is identical with the rate of dissociation of ATCase (3.9×10^{-3} s^{-1}) measured by the dithizone technique. These findings indicate that the two stages in the overall reaction of ATCase with PMB have the same rates. Thus, the values for the various rate constants (Table I) account for the earlier observations of Gerhart and Schachman (1968) that all of the thiols on the enzyme appear to react with a single rate constant, even though C_2R_2 is an intermediate in the process.

The remarkably close correspondence in the rates measured by the two very different methods (both in the absence and presence of active site ligands) confirms unambiguously the conclusion of Blackburn and Schachman (1977) that the rates of reaction of the sulfhydryl groups of ATCase under various conditions are inherent properties of the different conformational states of the enzyme. Moreover, the results for various experimental conditions (Table I) show that the dissociation of ATCase has the same rate constant as that for the reaction of the thiols on the R subunits of the enzyme. Thus, it is unlikely that there would be detectable amounts of undissociated enzyme molecules in which the sulfhydryl groups on the R subunits had reacted with PMB. In this respect, the process is all-or-none (Gerhart and Schachman, 1968), *i.e.* the reaction of 1 cysteine residue on a particular R subunit of ATCase is followed rapidly by the subsequent reaction of the other 7 thiols on that subunit with the formation of C_2R_2 and the mercaptide complex of the free R subunit (*cf.* Evans *et al.*, 1974).

The use of MNP-ATCase (Evans *et al.*, 1972) permitted us to circumvent the complication that 30 sulfhydryl groups in ATCase react with PMB in the absence of ligands and only 24 react in the presence of active site ligands (Gerhart and Schachman, 1968; Blackburn and Schachman, 1977; Table I). Modification of the 6 thiols on the two C subunits of ATCase with 2-chloromercuri-4-nitrophenol yielded a derivative in which only the 24 sulfhydryl groups of the R subunits of MNP-ATCase reacted with PMB both in the absence and presence of ligands. The pseudo-first order rate constant for the reaction of these thiols with PMB was also 3.9×10^{-3} s^{-1} and the rate was increased to 24×10^{-3} s^{-1} when a saturating amount of PALA was added. Thus, the inactive derivative showed the same enhanced reactivity of the sulfhydryl groups as the native enzyme. Sedimentation velocity experiments with a photoelectric scanning absorption optical system showed that the 2-chloromercuri-4-nitrophenol moiety remained bound to the protein both in the absence and presence of PALA.

Effect of PALA on the Rate of Dissociation of ATCase—The addition of saturating amounts of PALA increased the rate constant for the dissociation of ATCase to C_2R_2 to a value of 17.6×10^{-3} s^{-1}, which is close to the rate constant for the reaction of the thiols on C_2R_2 (19.5×10^{-3} s^{-1}) and that for the overall reaction of the sulfhydryl groups on ATCase with PMB (18×10^{-3} s^{-1}; Table I). Thus, the rates for the two stages of the reaction mechanism were similar even when PALA was present; in addition, the dissociation of C_2R_3 and the reaction of the sulfhydryl groups on its R subunits were coupled. Under these conditions, the concentration of C_2R_2 attained a maximum of 0.5 μM (Fig. 2*a*).

In the presence of subsaturating amounts of PALA, the dissociation of C_2R_3 to C_2R_2 and the subsequent dissociation of C_2R_2 to products were both biphasic. The overall reaction of sulfhydryl groups on ATCase should therefore be the sum of four exponentials. However the kinetics could be described as the sum of only two exponentials (Blackburn and Schachman, 1977) because the slow rates (no ligands) for C_2R_3 and C_2R_2 are similar, as are the fast rates (PALA present) for the reaction of the thiols of these species.

At subsaturating levels of PALA, the dissociation process is more complicated. The biphasic kinetics for ATCase has been interpreted in terms of the co-existence of molecules in both the T and R states which react with PMB at markedly different rates (Blackburn and Schachman, 1977). As yet, the allosteric equilibrium constant, $L = [T]/[R]$, has not been determined for C_2R_2; however, in view of the demonstrated lower cooperativity of C_2R_2 compared to ATCase (Yang *et al.*, 1974; Evans *et al.*, 1975), it is almost certain that L for C_2R_2 is less than that for ATCase. Hence, some of the C_2R_2 molecules generated by the dissociation of ATCase molecules in the T state would probably isomerize to the R state of C_2R_2. If the T $\rightarrow$ R transition is fast compared to the rate of reaction of the protein with PMB, the relative amounts of C_2R_2 mole-

[6] Ultracentrifuge experiments on MNP-ATCase showed that PALA caused a 2.9% decrease in the sedimentation coefficient. This value is very similar to that observed with the native enzyme.

[7] Studies of the crystal structure of ATCase suggest that the four sulfhydryl groups on each r chain are involved in chelating a zinc atom which is located near the interface between each c and r chain in ATCase (Monaco *et al.*, 1978). The location of these cysteine residues near the intersubunit contact region may account for the coupling between the dissociation of the enzyme and the reactivity of sulfhydryl groups on the R subunits.

cules in the T and R states would differ from the initial distribution of ATCase molecules in these two states. A conversion of C_2R_2 molecules toward the R state would account for an increase in the rate of its dissociation and explain why the concentration of this intermediate does not attain a value of 0.5 μM at subsaturating levels of PALA. Instead, a broad maximum is observed (Fig. 2*b*).

Effect of Other Active Site Ligands—In the presence of the substrate carbamoylphosphate, the rate of dissociation of ATCase is increased to 8.6×10^{-3} s^{-1}, a value in good agreement with the rate constant (9×10^{-3} s^{-1}) for the overall reaction of the sulfhydryl groups on the enzyme. However, the dissociation of ATCase into C_2R_2 appears to be rate-limiting since C_2R_2 reacted with PMB at a faster rate (12.9×10^{-3} s^{-1}) (Table I). This faster rate of dissociation of C_2R_2 relative to its rate of formation accounts for the observation that the maximum C_2R_2 concentration is less than 0.5 μM (Fig. 2*c*).

When both carbamoylphosphate and succinate were present, the rate constant for the dissociation of ATCase is increased to 23.7×10^{-3} s^{-1}, a value close to that observed for the rate of reaction of the sulfhydryl groups of the enzyme under these conditions (25×10^{-3} s^{-1}).

Ligand-promoted Conformational Changes in C_2R_2—In previous studies, it was shown that the change in the reactivity of the sulfhydryl groups of ATCase as a function of the degree of saturation of the active sites by PALA coincided with the gross conformational change measured by the decrease in sedimentation coefficient (Howlett *et al.*, 1977). Since C_2R_2 exhibits both the cooperativity and the feedback inhibition characteristic of the native enzyme, it was of interest to determine whether the reactivity of C_2R_2 toward PMB could be used as a probe of the gross conformational change in the molecule.

In the presence of saturating concentrations of carbamoylphosphate, the pseudo-first order rate constant for the reaction of the sulfhydryl groups in C_2R_2 increased ~3-fold to 12.9×10^{-3} s^{-1}, and the addition of PALA caused a 5-fold increment in the rate constant to 19.5×10^{-3} s^{-1}. These ligand-promoted enhancements in the reactivity of the thiols in C_2R_2 are remarkably similar to those measured for ATCase itself (Blackburn and Schachman, 1977). These results are further supported by the observation that C_2R_2 exhibited a 2.9% decrease in sedimentation coefficient in the presence of carbamoylphosphate and succinate,[8] which is to be compared to the 3.6% decrease reported for ATCase under the same conditions (Howlett and Schachman, 1977). It therefore seems likely that the gross conformational changes in C_2R_2 are at least qualitatively similar to those observed for ATCase and that the allosteric transition for C_2R_2 involves a change from a constrained (T) state to a relaxed (R) form, in the terminology of Monod *et al.* (1965).

In the presence of subsaturating levels of PALA, C_2R_2 also exhibits biphasic kinetics which could be interpreted in terms of a fraction of the molecules reacting with a slow rate constant of 4×10^{-3} s^{-1} (characteristic of the T state) and another fraction having a fast rate of 25×10^{-3} s^{-1} (approximately that of the R conformation).

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

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SUPPLEMENTARY MATERIAL TO

THE MECHANISM OF DISSOCIATION OF ASPARTATE TRANSCARBAMOYLASE BY *p*-MERCURIBENZOATE

Suresh Subramani and H. K. Schachman

This supplement provides experimental details of the measurement of the rate of reaction of the sulfhydryl groups of ATCase and an inactive derivative, MNP-ATCase, with PMB. Also, results are presented for the kinetics of the dissociation of MNP-ATCase in the absence and presence of active-site ligands. The application of the method used for measuring the rate of dissociation of ATCase into C_2R_2 and mercaptide complexes of the R subunits is illustrated for the analysis of the rate of dissociation of an aggregate of ATCase.

MATERIALS AND METHODS

Materials--ATCase was prepared according to the method of Gerhart and Holoubek (1967). Since the preparations contained small amounts of the R-subunit-deficient species, C_2R_2 (Yang et al., 1974), an excess of free R subunits was added to convert C_2R_2 into C_2R_3 and the resulting solution was fractionated on a Sephadex G-200 column. The resulting enzyme preparations frequently contained 5-10% aggregates (primarily dimers) of ATCase. C_2R_2 was prepared as described by Bothwell and Schachman (1974) and was 85-90% pure with ATCase as the remaining material. MNP-ATCase, in which only 6 sulfhydryl groups on the two C subunits of ATCase were modified with 2-chloromercuri-4-nitrophenol, was prepared by the method of Evans et al. (1972). The derivative had about 9% of the enzyme activity of native ATCase and complete restoration of activity was achieved by the addition of excess 2-mercaptoethanol. The MNP-derivative when analyzed either by electrophoresis on polyacrylamide gels or by sedimentation velocity measurements in a Beckman Model E ultracentrifuge exhibited properties of intact enzymes with no evidence for dissociation into subunits. Dilithium carbamoyl phosphate, PMB, and diphenyl thiocarbazone were obtained from Sigma Chemical Co. Succinate and CMNP were purchased from Eastman Organic Chemicals. PALA was kindly provided by Dr. G. R. Stark.

Methods--The rate of reaction of PMB with the sulfhydryl groups of C_2R_2 and MNP-ATCase was measured by the method of Boyer (1954) as described by Blackburn and Schachman (1977). Experimental data were analyzed in terms of pseudo-first-order kinetics according to Blackburn and Schachman (1977).

Electrophoresis of samples in polyacrylamide gels (7% with 2 cm of 2.5% stacking gel) was performed with the Tris-glycine system of Jovin et al. (1964). Coomassie brilliant blue G-250 was used for staining the gels, which were scanned after destaining (in 4% acetic acid) at 540 nm on a Gilford spectrophotometer equipped with a gel scanning attachment.

The ligand-promoted change in the sedimentation coefficient of MNP-ATCase was measured by a difference sedimentation velocity technique (Gerhart and Schachman, 1968; Howlett and Schachman, 1977) with two ultracentrifuge cells, one containing the reference solution in a cell with plane windows and the other containing the derivative and ligand in a cell with an upper wedged quartz window. Independent experiments on MNP-ATCase were performed with a Beckman Model E ultracentrifuge equipped with a split-beam photoelectric scanner (Schachman and Edelstein, 1973) and a monochromator.

The amounts of the different protein species (C_2R_3, C_2R_2 and C subunits) were evaluated quantitatively from the areas under the peaks. In some experiments, areas were also measured for bands corresponding to aggregates of ATCase. Measurements were made by weighing the trace of a peak corresponding to a specific component. Independent analyses of the areas corresponding to the various components were made occasionally by using a planimeter. Identical results were obtained by the two methods.

RESULTS

Test of the Rapidity and Efficiency of the Chelation of Free Mercurial--Since in many of the experiments significant amounts of C_2R_3 dissociated within a few minutes after adding PMB, it was important to demonstrate that the reaction could be "stopped" rapidly and completely when the dithizone was added. Accordingly an experiment was performed with 2-mercaptoethanol as a source of rapidly reacting sulfhydryl groups.

The reference and sample cuvettes were partially filled with 0.9 ml of 40 mM potassium phosphate buffer at pH 7.0 and placed in the compartment of a Cary 118 spectrophotometer. The solution in the sample cuvette also contained 0.5 mM 2-mercaptoethanol. The instrument was balanced at 250 nm to give a zero reading. An aliquot (0.1 ml) of 0.2 mM PMB in 40 mM potassium phosphate, 25 mM Tris-Cl at pH 7.0 was added to each cuvette and the optical density was measured as rapidly as possible. The resulting value of the optical density, 0.14, obtained within 20 s corresponded closely to that expected for the complete reaction of all the mercurial with sulfhydryl groups.

In a second experiment a 0.5 ml aliquot of the 0.2 mM PMB solution was added at zero time to the dithizone solution (40 μg/ml in carbon tetrachloride). The contents were mixed by vortexing for 5 s and the aqueous and organic phases were allowed to separate for 10 s. Then 0.1 ml of the aqueous layer was added to the two cuvettes. The time interval corresponded to 20 s. Measurements of the optical density yielded constant values of 0.004. Similarly the same optical density was obtained when the PMB solution was mixed with dithizone for 40 and 60 s before the layers were separated.

These experiments showed that only 2.9% of the mercurial remained in the aqueous phase after the treatment with the chelating reagent for as short a time as 15-20 s. Hence we conclude that the stopping reaction is both rapid and virtually complete since more than 97% of the PMB is removed within 20 s (or less).

Ligand-promoted Conformational Change of Inactive MNP-ATCase--The MNP-ATCase derivative (with only 9% of the enzyme activity of native ATCase and only 24 free sulfhydryl groups located on the R subunits) reacted with excess PMB according to pseudo-first-order kinetics as seen in Fig. 1-S. The rate constant, 3.9×10^{-3} s^{-1}, is virtually identical to that obtained with native ATCase (Table I). When PALA was added to MNP-ATCase at a molar ratio of 1000 the reaction rate was increased markedly (Fig. 1-S) and the pseudo-first-order rate constant was 24×10^{-3} s^{-1}, a value slightly greater than that observed with the native enzyme. Thus, despite its inactivity and the decreased affinity for PALA, the inactive derivative, MNP-ATCase, undergoes the ligand-promoted gross conformational change charcteristic of native ATCase.

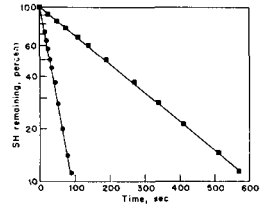

Figure 1-S. Pseudo-first-order kinetics for the reaction of the sulfhydryl groups on the R subunits of MNP-ATCase. Data were obtained by the method of Boyer (1954) and are plotted as percent of unreacted sulfhydryl groups (on a logarithmic scale) as a function of time. The straight lines are linear least-squares fits of the data with pseudo-first-order rate constants of 3.9×10^{-3} s^{-1} in the absence of ligands (■) and 23.7×10^{-3} s^{-1} in the presence of 1000 equivalents of PALA per mol of MNP-ATCase (●).

Mercurial-promoted Dissociation of an Aggregate of ATCase--Most preparations of purified ATCase contain a small amount of an aggregate with a sedimentation coefficient of about 16 S. In the reconstitution of ATCase from isolated C and R subunits at high concentration this aggregate is readily detected as one of the minor assembly products (Gerhart and Schachman, 1965). Preliminary molecular weight determinations of the partially purified aggregate yielded a value close to that expected for a dimer. In addition the aggregate exhibited a sigmoidal dependence of enzyme activity on aspartate concentration and its sedimentation coefficient was decreased about 3% upon the addition of a saturating amount of PALA (Y. R. Yang and H. K. Schachman, unpublished). Other aggregated species with molecular weights greater than a dimer are also observed in even smaller amounts.

The aggregate is readily observed upon electrophoresis in polyacrylamide gels (Fig. 1a) as a slowly migrating band. Upon the addition of PMB this band disappears and bands corresponding to C and R subunits are observed. With preparations containing both the aggregate and ATCase it was possible to measure the kinetics of disappearance of both species upon the addition of PMB. A pseudo-first-order plot for the dissociation of the aggregate yielded rate constants of 9.9×10^{-3} s^{-1} in the absence of ligands, 12.1×10^{-3} s^{-1} in the presence of 1.6 mM carbamoyl phosphate and 28.6×10^{-3} s^{-1} when the carbamoyl phosphate concentration was increased to 3.1 mM. This 2.9-fold increase in the rate of disappearance of the aggregate is similar to the 2.2-fold enhancement observed for ATCase under similar conditions (Fig. 3). Moreover, just as C_2R_3 dissociates into the intermediate, C_2R_2, the aggregate first forms an intermediate which migrates slightly faster on the gels. Further incubation of the reaction mixture leads to the dissociation of the intermediate into C_2R_3 and/or C_2R_2. Finally C and R subunits are obtained. When both carbamoyl phosphate (3.1 mM) and succinate (3.1 mM) are present, the aggregate dissociates completely within 20 s. The detailed structure of the aggregate and the intermediates formed during its dissociation, as well as the ligand-promoted conformational changes in them, merit further investigation.