The release of Zn$^{2+}$ from aspartate transcarbamoylase (ATCase; c$_{AT}$) upon challenge by p-hydroxymercuriphenylsulfonate (PMPS) has been studied using the sensitive, high-affinity metallochromic indicator 4-(2-pyridyldiazoresorcinol at pH 7.0. When the —SH group of each catalytic (c) chain is protected, 1 Zn$^{2+}$ is released for every 4 eq of PMPS added to ATCase, with titration of the 24 —SH groups of regulatory (r) chains. Moreover, the release of Zn$^{2+}$ is a linear function of PMPS added, indicating that the rate-limiting step in Zn$^{2+}$ release is mercurial attack on the 1st of the 4 r —SH groups bonded tetrahedrally to Zn$^{2+}$ in an r chain near c-r contacts. Dissociation of ATCase is linked to Zn$^{2+}$ release and mercaptide formation; e.g., upon addition of 4 eq of PMPS to ATCase in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, 4/6 of ATCase is dissociated to c$_3$ and r$_2$ subunits at ~83% of the rate of Zn$^{2+}$ release, with no accumulation of the c$_3r$ intermediate as is observed in KPO$_4$ buffer. Adding <4 eq PMPS/ATCase, the release of Zn$^{2+}$ is first-order in [PMPS] and is virtually independent of [ATCase] with an activation energy of 18 kcal/mol. With large excesses of PMPS, stopped-flow traces show a lag period followed by pseudo first-order release of Zn$^{2+}$ from ATCase and the reaction order in [PMPS] = ~1.3. Under these conditions, PMPS has a chaotropic effect on ATCase; the activation energy for Zn$^{2+}$ release is much lower than that obtained with phosphate or active-site ligand from 6.6 to ~12 kcal/mol. A reasonable explanation of the observed kinetic data is that the organomercurial reagent binds reversibly to nitrogenous side chain groups in an ATCase molecule prior to the rate-limiting reaction with a sulfhydryl group.

The regulation of ATCase$^*$ (carbamoylphosphate:L-aspar-

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Mercurial-promoted Zn$^{2+}$ Release from <i>Escherichia coli</i> Aspartate Transcarbamoylase$^*$

(Received for publication, May 10, 1984)

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The release of Zn$^{2+}$ from aspartate transcarbamoylase (ATCase; c$_{AT}$) upon challenge by p-hydroxymercuriphenylsulfonate (PMPS) has been studied using the sensitive, high-affinity metallochromic indicator 4-(2-pyridyldiazoresorcinol at pH 7.0. When the —SH group of each catalytic (c) chain is protected, 1 Zn$^{2+}$ is released for every 4 eq of PMPS added to ATCase, with titration of the 24 —SH groups of regulatory (r) chains. Moreover, the release of Zn$^{2+}$ is linear function of PMPS added, indicating that the rate-limiting step in Zn$^{2+}$ release is mercurial attack on the 1st of the 4 r —SH groups bonded tetrahedrally to Zn$^{2+}$ in an r chain near c-r contacts. Dissociation of ATCase is linked to Zn$^{2+}$ release and mercaptide formation; e.g., upon addition of 4 eq of PMPS to ATCase in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, 4/6 of ATCase is dissociated to c$_3$ and r$_2$ subunits at ~83% of the rate of Zn$^{2+}$ release, with no accumulation of the c$_3r$ intermediate as is observed in KPO$_4$ buffer. Adding <4 eq PMPS/ATCase, the release of Zn$^{2+}$ is first-order in [PMPS] and is virtually independent of [ATCase] with an activation energy of 18 kcal/mol. With large excesses of PMPS, stopped-flow traces show a lag period followed by pseudo first-order release of Zn$^{2+}$ from ATCase and the reaction order in [PMPS] = ~1.3. Under these conditions, PMPS has a chaotropic effect on ATCase; the activation energy for Zn$^{2+}$ release is much lower than that obtained with phosphate or active-site ligand from 6.6 to ~12 kcal/mol. A reasonable explanation of the observed kinetic data is that the organomercurial reagent binds reversibly to nitrogenous side chain groups in an ATCase molecule prior to the rate-limiting reaction with a sulfhydryl group.

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Relocation of ATCase into C trimers and R dimers have shown that the rates of protein dissociation (12, 23) and of organomercurial-mercaptide bond formation (19) are closely linked. In some studies presented here, the treated of Zn\(^{2+}\) during the organomercurial reaction with ATCase was monitored by using the very sensitive, high-affinity metallochromic indicator 4-(2-pyridylazo)resorcinol (24–26). The large change in extinction coefficient at 500 nm that accompanies Zn\(^{2+}\) binding to this indicator has allowed studies of the reaction of ATCase with substrateconcentric measurements of the mercurial reagent PMPS. In this way it was found that 1 Zn\(^{2+}\) is released for every 4 eq of the mercurial added to the enzyme. Moreover, the use of the indicator PAR facilitated kinetic measurements of Zn\(^{2+}\) release from ATCase, which were performed in the absence and presence of inhibitory buffers of mercurial-thiol reactions (27) such as Tris/HCl and KPO\(_4\), previously used (12, 19, 23). Evidence is presented that phosphate causes the accumulation of the intermediate lacking one R dimer, C\(_R\), during the dissociation of ATCase by mercurial attack (23, 28).

**EXPERIMENTAL PROCEDURES**

**Materials**—Several preparations of wild-type ATCase, purified by the method of Holoubek and Holoubek with the mutant ATCase\(_{C_{228G}}\), isolated and purified as described by Wall et al. (30), were provided by Y. R. Yang at the University of California, Berkeley, and shipped at 0°C as protein suspensions in 3.6 M ammonium sulfate containing 5 mM 2-mercaptoethanol. These proteins were stored at 4°C as received and were collected as needed by centrifugation just prior to use. All other chemicals were of the highest grade commercially available.}

**Methods**—The Perkin-Elmer Model 300 spectrophotometer and black selfmasking semimic cuvettes of 1.0-cm path length were used for UV difference spectra (ATCase ± PALA in different buffer systems, ±0.02 A scale), for spectrophotometric titrations of ATCase with mercurial reagents and for kinetic measurements of reactions when these were > 40 s in duration (0.1 A scale; autorange), and for protein concentration with (28) was monitored by molar extinction coefficient at 280 nm for ATCase. Temperature was controlled (±0.1°C) in this and other instruments used for kinetic measurements by circulating water (± 0.05°C) through thermostable blocks; YSI thermistor probes were used to directly monitor solution temperatures.

Stock solutions of freshly dialyzed ATCase tested negatively for free Zn\(^{2+}\), using 10^4 M PAR and subsequent 1 mM EDTA addition at 500 nm. Also, the extent of PALA binding to ATCase preparations in 40 mM Hepes/KOH, pH 7.0, or in 40 mM KPO\(_4\), pH 7.0, was measured by spectrophotometric titrations (33) to be the theoretical 6.0 eq of PALA bound per ATCase molecule, monitoring peak-trough absorption changes at 289–285 nm. These measurements checked the concentrations of both the stock PALA solutions (30 mM) and the competent catalytic sites of ATCase.

Quantitative measurements of organomercurial-promoted Zn\(^{2+}\) release from ATCase in the spectrophotometer, 10^4 M PAR was used (monitoring absorbance changes at 500 nm) and the reactions were initiated by adding a small volume of PMPS to the thermostated ATCase solution (~1.0 ml); mixing was by cell inversion. In the few experiments using PMS, equal volumes (0.50 ml) of protein with 10^4 PAR and the mercurial reagent with PAR were equilibrated at the same temperature and then mixed; alternatively, reactions with 0.2 mM PMS were initiated by adding 15 μl of ATCase. At > 8-fold excess [PAR] to [Zn\(^{2+}\)] at pH 7.0 (20°C), ΔA = 6.6 × 10^4 M^−1 cm^−1 at 500 nm for (PAR);Zn\(^{2+}\) formation. To obtain first-order rate constants, the absorbance time data (collected continuously at 5, 10, 15, or 20 s/chart division) were fitted by means of a nonlinear least-squares program to the equation 

\[ A(t) = (A_0 - A_	ext{eq})e^{-kt} + A_	ext{eq}, \]

where \(A_0\), \(A_	ext{eq}\), and \(k\) are the instantaneous time, infinite time, and zero-time absorbances, respectively.

Buffer and protein solutions were degassed under vacuum before use to avoid interference from bubble formation during the observation of small absorbance changes by spectrophotometry in many kinetic experiments. This was especially critical for stopped flow and light scattering experiments.

The stopped-flow spectrophotometer (1.0-cm light path) was used for kinetic experiments lasting < 40 s has been described by Rhee and Chock (34). In measurements of the kinetics of Zn\(^{2+}\) release by stopped-flow, PAR was added at the same concentration to both solutions to be mixed to avoid possible artifacts associated with the formation or dissociation of PMPS-PAR complexes.

Difference sedimentation velocity measurements were performed as described previously (21), using dialyzed enzyme samples at a protein concentration of 3.9 mg/ml, Schlieren optics, and a speed of 52,000 rpm. For Δs measurements of ATCase ± PALA in two-cell experiments, the stoichiometry of PALA binding was determined separately by a spectrophotometric titration (see above). Values of Δs were computed by the procedure of Howlett and Schachman (15), the data Δs/Δt vs time and in r vs time for Δs and sedimentation coefficient calculations, respectively, were fitted separately by a linear least-squares regression, where r is the radial distance (in centimeters) from the center of the protein boundary to the axis of rotation, \(f\) is the average r value for the two boundaries, and Δs is the difference between r values at a given time after reaching speed. Photographs were taken at 4- to 6-min intervals and those taken at 12- to 15 min (after separation of the small amount of dimer in ATCase samples) were used for calculations.

Light scattering measurements at a 90° angle were made at 360 nm using a Perkin-Elmer Model 650-40 fluorescence spectrophotometer (equipped with a Model 057 X-Y recorder) with excitation and emission slits of 2 and 8 nm, respectively.

Polyacrylamide slab gel electrophoresis (7% running gel with 1 cm of 3% stacking gel) was in a Bio-Rad Protein slab gel unit (16-cm cell), using the Tris/glycine system of Jovin et al. (35) as in Ref. 23. Samples of ~3.5 μg of protein/channel were loaded in 10% gels, 0.013% bromphenol blue; after electrophoresis, gels were stained with Coomassie Blue R-250 and photographed after destaining.

**RESULTS**

The association of Zn\(^{2+}\) with PAR was not rate-limiting in our studies of Zn\(^{2+}\) release from ATCase at pH 7.0 and the affinity of PAR for Zn\(^{2+}\) was such that essentially all Zn\(^{2+}\) released from ATCase was converted to the highly absorbant (PAR);Zn\(^{2+}\) complex (ΔA = 6.6 × 10^4 M^−1 cm^−1 at 500 nm). 2

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2 Quantitative studies of J. B. Hunt, S. H. Neece, and A. Ginsburg on the interaction of Zn\(^{2+}\) with the metallochromic indicator PAR will be published elsewhere.
Zn$^{2+}$ Release from Aspartate Transcarbamoylase

97% of that expected for the release of the ultraviolet absorption of the protein solution. As did the plot of \( F_\text{on} \) vs. PMPS in the presence of PALA. The break in the plot occurred at 23.5 PMPS/ATCase, which is the theoretical absorbance change for complete reaction, and was calculated assuming that complete reaction releases 6 Zn$^{2+}$ ions per ATCase with \( \Delta A\text{on} = 6.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \), or for \( \Delta A\text{on} = 24 \) mercaptide bonds with PMPS are formed per ATCase molecule. The line at \( < 22 \) PMPS/ATCase is the linear least-squares best fit to both sets of data points. The increase in \( \Delta A\text{on} \) after the break in the plot (indicated by the dashed line) is due to the absorbance of PMPS at 250 nm.

The sensitivity of PAR to the addition of free Zn$^{2+}$ (yellow to orange color change) and the relative insensitivity of PAR to the presence of mercurial or other reagents also were important properties in the present studies.

Release of Zn$^{2+}$ during the Titration of ATCase(PALA)$_6$ with the Mercurial Reagent PMPS—Previous studies (19) showed that when ATCase is saturated with the high-affinity bisubstrate analog PALA, the —SH group of each c chain is protected from reaction with excess PMB. Fig. 1 shows plots of the absorbance changes (recorded after completion of any time-dependent absorbance changes) during the titration of the ATCase(PALA)$_6$ complex with PMPS. The absorbance increase at 500 nm in the presence of PAR was a measure of Zn$^{2+}$ release from the protein and the absorbance increase at 250 nm was indicative of mercaptide bond formation (36). The break in the plot of \( \Delta A\text{on} \) occurs at 23.5 PMPS/ATCase or at ∼4 PMPS/regulatory chain, indicating that the 4 sulfhydryl groups of each r chain reacted with PMPS but that the sulfhydryl group of each c chain (2) did not react with PMPS in the presence of PALA. The break in the plot of \( \Delta A\text{on} \) occurred at precisely the same value of PMPS/ATCase as did the plot of \( \Delta A\text{on} \), and the total quantity of Zn$^{2+}$ released (based on calculations with standard Zn$^{2+}$) was 97% of that expected for the release of 6 Zn$^{2+}$/ATCase (based on the ultraviolet absorption of the protein solution).

An interesting feature of Fig. 1 is the linearity of the plot of \( \Delta A\text{on} \) versus PMPS added during titration of ATCase with PAR. This result indicates that even with the protein sulfhydryl groups in great excess (at < 6 PMPS/ATCase), the attack of these groups by PMPS is not random. Rather, for each 4 PMPS molecules provided, 1 Zn$^{2+}$ ion is released. An explanation of such a result is that once the first of the 4 —SH groups in a Zn$^{2+}$ binding site of an r chain reacts with the mercurial reagent, the other 3 —SH groups of the same Zn$^{2+}$ cluster rapidly form mercaptide bonds with PMPS. The fact that the mercurial-promoted dissociation of ATCase proceeds 1 molecule at a time (see below and Ref. 12) further suggests that once 1 of the 6 Zn$^{2+}$ binding sites of ATCase is disrupted, —SH groups of the other 5 Zn$^{2+}$ sites are much more susceptible to attack by mercurials than are the r chain sulfhydryl groups of Zn$^{2+}$ sites in intact ATCase.

Kinetics of Reaction of 4.0 eq of PMPS with ATCase(PALA)$_6$ (4th Mercurial Reagent/Available Protein —SH Groups)—Fig. 2A shows first-order rate plots based on absorbance increases at 500 and 250 nm during the reaction of ATCase in Hepes buffer, pH 7.0, with sufficient PMPS to react with only 1/4th of the available r chain sulfhydryl groups. In these experiments the sulfhydryl group of each c chain was protected from attack by PMPS by the binding of PALA. The reaction is clearly first-order in PMPS, the limiting reagent, and the half-time for the reaction measured as mercaptide formation (74 s) or Zn$^{2+}$ release (77 s) at 19.7 °C is the same within experimental error.

First-order rate constants for the reaction of limiting PMPS with excess sulfhydryl groups of ATCase(PALA)$_6$ are listed in Table I for a wide range of initial PMPS/ATCase ratios. For the experiments with 2.15 μM ATCase(PALA)$_6$ no significant change in the value of \( k \) was observed for an 8-fold change in the PMPS/ATCase ratio. This was the case whether Zn$^{2+}$ release or mercaptide bond formation was monitored. (Of course, if plots such as those of Fig. 2A are truly linear, then changing the PMPS/ATCase ratio merely means looking at different linear segments of the same first-order plot.) However, a remarkable feature of the data in Table I is that the value of \( k \) is fairly insensitive to the total ATCase concentration. A 4-fold increase in [ATCase(PALA)$_6$] gave at most a 50% increase in the pseudo first-order rate constant.

Fig. 2B shows a first-order rate plot for the light scattering decrease accompanying the reaction of 4.0 eq of PMPS with ATCase(PALA)$_6$ under the conditions of Fig. 2A. The log of the light scattering decrease (R$_{500}$-R$_{500}$ vs. time) was linear for at least 85% of the decrease (data incompletely shown in Fig. 2B). The measured half-time (93 s) for the light scattering decrease was ∼1.2-fold greater than that for Zn$^{2+}$ release or mercaptide formation (Fig. 2A). The total light scattering decrease was approximately 12%, which is the value expected for 1/4th dissociation of ATCase (M, 300,000) into 2 C trimers of M, 99,000 and 3 R dimers of M, 34,000 (8). The addition of 5 mM 2-mercaptoethanol at the end of the light scattering decrease in the experiments of Fig. 2B produced (within 1 min) a 97% return to the original light scattering value of intact ATCase.

ATCase samples treated with 1.6-fold excess PMPS were completely dissociated but possibly contained aggregated R subunits since the value of R$_w$ was 65% or ∼88% of that expected for complete dissociation. When 5 mM 2-mercaptoethanol was added to protein samples after complete dissociation with 1.6-fold excess PMPS, the light scattering value...
A complex at 500 nm, to provide 4 PMPS ions per ATCase molecule.

The rate of Zn\(^{2+}\) release was followed by the formation of Zn\(^{2+}\)-PAR expansion of 0-0.1 of the light scattering decrease following the addition of 4.0 eq of PMPS (Fig. 2B) indicates that all of the mercaptoethanol to mercurial-dissociated ATCase produces some aggregated ATCase species in addition to assembled ATCase.

The total light scattering decrease after addition of 4.0 eq of PMPS to ATCase (Fig. 2B) indicates that all of the mercaptoethanol to mercurial-dissociated ATCase produces some aggregated ATCase species in addition to assembled ATCase.

The mobility of the mercurial reagent in mercaptide linkage to r chain sulfhydryl groups of 1/4th of the ATCase population increased within 1 min to 110-120% of the original value and remained constant for at least 10 min. This was consistent with polycrystalline gel patterns which showed that the addition of 2-mercaptoethanol to mercurial-dissociated ATCase produces some aggregated ATCase species in addition to assembled ATCase.

For complete dissociation of C\(_2\)R\(_3\) to 2 C\(_4\)R\(_4\) and 12 eq of PMPS, protein patterns show increasing amounts of C\(_2\)R\(_3\) (exr) and C\(_4\)R\(_4\) trimer (Fig. 3, center channels). In contrast, the C\(_2\)R\(_3\) intermediate is not visible in samples of ATCase(PALA)\(_6\) in 40 mM Hepes, pH 7.0, buffer (± 1 m urea) before or after treatment with 4, 8, or 12 eq of PMPS (Fig. 3).

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was tested by including 1 mM N-ethylmaleimide in an incubation mixture at room temperature containing 10^{-4} M PAR, ATCase(PALA)$_s$, and 4 eq of PMPS in HEPES, pH 7.0, buffer. The initial rapid release of 1/4th of the protein-bound Zn$^{2+}$ was followed by a very slow release of almost all of the remaining Zn$^{2+}$ over a period of 3 days, at which time polyacrylamide slab gel electrophoresis showed that nearly complete dissociation of ATCase had occurred. A control incubation to which 1 mM N-ethylmaleimide was added to ATCase(PALA)$_s$ in the absence of PMPS produced no Zn$^{2+}$ release or measurable dissociation of the enzyme. Thus, an initial attack on an r chain sulfhydryl group requires the avid mercurial reagent. Once cr contacts in an ATCase molecule are disrupted, the mercurial reagent can be displaced slowly by the irreversible reaction of sulfhydryl groups with N-ethylmaleimide, thereby freeing the mercurial to attack masked sulfhydryl groups in Zn$^{2+}$ binding sites in intact ATCase molecules.

**Influence of Effectors of ATCase on Its Reaction with 4 eq of PMPS**—Table II gives first-order rate constants for Zn$^{2+}$ release at pH 7.0 upon addition of 4.0 eq of PMPS to ATCase at 24.5 or 30.0°C in the absence and presence of various effectors of the enzyme. In the absence of the bisubstrate analog PALA, smaller total absorbance changes (~20% less) were observed for this reaction, as expected if some of the PMPS reacted with the sulfhydryl group of each c chain in the absence of PALA. The reaction in HEPES buffer was faster in the presence of PALA, but only by a factor of 2, rather than the factor of 6 observed previously for the reaction of excess PMB with ATCase in a mixed Tris/KPO$_4$ buffer (19). Also, the rate of Zn$^{2+}$ release upon the addition of 4.0 eq of PMPS to the inactive mutant ATCase$_{231}$ in HEPES buffer was about the same as that measured with the wild-type enzyme (Table II). This is in marked contrast to the 12-fold rate enhancement (compared with the wild-type enzyme) observed by Wall and Schachman (37) for the reaction of excess PMB with ATCase$_{231}$ in Tris/KPO$_4$ buffer.

The activation energy for the reaction of ATCase with 4.0 eq of PMPS in HEPES buffer is the same in the absence and presence of PALA (Fig. 4). Furthermore, rate measurements at two temperatures gave the same activation energy of 18 kcal/mol in KPO$_4$ buffer as in HEPES buffer. This is despite the inhibitory action of phosphate, where the substitution of 40 mM KPO$_4$, pH 7.0, for HEPES buffer caused an large decrease in the rate of Zn$^{2+}$ release in the absence or presence of PALA (Fig. 4; Table II). The substitution of the mixed KPO$_4$/Tris, pH 7.0, buffer used previously (19) further inhibited the mercurial reaction (Table II), but increased the solubility of PMB so that measurements with 4 eq of PMB were possible. Comparable rates of Zn$^{2+}$ release from ATCase were obtained with 4 eq of PMPS and PMB (Table II).

The presence of the inhibitor CTP (0.21 mM) or the activator ATP (0.1 mM) in HEPES buffer decreased the first-order k for Zn$^{2+}$ release from ATCase in the absence of PALA (Table II). However, the bisubstrate analog PALA produced a 3-4-fold increase in the rate of Zn$^{2+}$ release from ATCase in the presence of CTP, ATP, or phosphate. The inhibitory effects of ligands on the rate of Zn$^{2+}$ release from ATCase upon challenge with a substoichiometric amount of PMPS in Table II can be due to inhibition of the mercurial reaction (27) as well as to effects from ligand-protein interactions. However, the effects of PALA on the first-order k for Zn$^{2+}$ release from ATCase (Table II) must be due to the binding of this high-affinity bisubstrate analog to catalytic sites (2) since the free concentrations of this ligand were
negligible. It is possibly significant also that PALA increased the rate of Zn$^{2+}$ release the most when it was bound in the presence of 1 M urea or Tris/HCl, occurring at -0.5 mM. The presence of 1 M urea had little effect on the rate of Zn$^{2+}$ release produced by the addition of 4 eq of PMPS to ATCase in Hepes buffer, increasing $k$ ~10% in the absence of PALA and decreasing $k$ ~10% in the presence of saturating PALA (Table II). 2 Light scattering measurements, performed as in Fig. 2B with 1 mM urea present in Hepes buffer, pH 7.0, gave about the same total decrease ($R_0 = 11.4\%$) as in the absence of urea and the dissociation reaction produced by the addition of 4.0 eq of PMPS to 3.3 mM ATCase(PALA)$_6$ was apparently first-order with $t_0 = 111$ s at 18.7°C. The first-order release of Zn$^{2+}$ measured under the same conditions in the presence of 10$^{-4}$ M PAR (as in Fig. 2A) was ~1.3-fold faster than the light scattering change in 1 M urea. Thus, 1 M urea in Hepes buffer decreased slightly the rates of both Zn$^{2+}$ release (Table II) and dissociation of ATCase saturated with PALA.

3 PALA binds to ATCase with high affinity in the presence of 1 M urea and 40 mM Hepes/KOH, pH 7.0, as evidenced by UV difference spectral measurements. The shape and amplitude of the spectral changes upon the addition of 8 eq of PALA to ATCase were the same as in the absence of 1 M urea (33) with the exception of a small (0.5 nm) red shift. Some positive cooperativity in the spectral changes was observed during the titration of ATCase in 1 M urea with PALA. At 1 eq of PALA/catalytic site, 88.9% of the maximum absorption change had occurred.

The PALA-promoted conformational change of ATCase in Hepes/KOH, pH 7.0, buffer was measured by difference sedimentation velocity (Table III). The value of -3.6% for $\Delta s/s$ (uncorrected for bound ligand) produced by saturating PALA in 40 mM Hepes/KOH, pH 7.0, buffer was the same as that measured in KPO$_4$ buffer (15). Furthermore, the addition of 2 and 4 eq of PALA to ATCase in Hepes buffer produced 54 and 100% of the total negative change in $\Delta s/s$, respectively, as observed previously in KPO$_4$ buffer (15, 21). Thus, the magnitude of the enhancement in the reactivity of r chain sulphydryl groups produced by PALA binding of ATCase (Tables II and IV below) is not a simple function of the protein conformation, as measured by sedimentation coefficient changes.

Kinetics of Zn$^{2+}$ Release in the Reaction of ATCase with Excess PMPS—Previous kinetic studies of the reaction of ATCase with organomercurials (PMB, rather than PMPS) have utilized phosphate or Tris/HCl buffers, or a mixture of these. In the present study, the reaction of ATCase with excess PMPS was found to be much more rapid in Hepes/KOH than in KPO$_4$ or Tris/HCl buffers. Since phosphate, Tris, and Cl$^-\text{ are all expected to form complexes with PMPS and the rates of mercurial-sulphydryl reactions have been shown to be sensitive to the presence of complexing agents (27), the effect of the concentration of Tris/HCl on the rate of the reaction of PMPS with ATCase was investigated. For a constant [PMPS] = 0.4 mM and initial [ATCase] = 2 $\mu$M at pH 7.0, a plot of log $k$ versus log [Tris/HCl] had a slope of -1.07, where $k$ is the pseudo first-order rate constant. At a constant [Tris/HCl] = 40 mM at pH 7.0, a plot of log $k$ versus log [PMPS] had a slope of 1.40 over the range [PMPS] = 0.3-1.1 mM.

Bromide ion also was found to inhibit markedly the reaction of PMPS with ATCase. In Hepes/KOH, pH 7.0, buffer at constant [PMPS], a plot of log $k$ versus log [Br$^-\text{] had a slope of -1.04, and at constant [Br$^-\text{] a plot of log $k$ versus log [PMPS] had a slope of 1.40 for [PMPS] = 0.2-0.6 mM. Half-saturation for the inhibition of the reaction of excess PMPS with ATCase by either Br$^-\text{ or Tris/HCl occurred at ~0.5 mM. Most of the experiments using excess PMPS were performed in the noninhibiting Hepes/KOH, pH 7.0, buffer and were therefore sufficiently rapid to require stopped-flow mixing. The stopped-flow spectrophotometer had the advantage of allowing observation to begin immediately at the time of mixing, whereas about 10 s at the beginning of each reaction were lost in the other kinetics experiments. A typical oscilloscope trace and the corresponding computer-generated first-order rate plot are shown in Fig. 5A. In experiments over a

![Figure 4](image-url)  

**Fig. 4. Arrhenius plots for Zn$^{2+}$ release in the reaction of 4.0 eq of PMPS with ATCase at pH 7.0.** First-order rate constants, $k$, were obtained from absorbance changes at 500 nm for the release of Zn$^{2+}$ in the reaction of 2.14 $\mu$M ATCase with 8.6 $\mu$M PMPS, at pH 7.0, using 10$^{-4}$ M PAR. For the plots designated Hepes (□) and Hepes + PALA (○) reactions were in 40 mM Hepes/KOH, pH 7.0, buffer in the absence and presence of 50 $\mu$M PALA, respectively. For the plots designated $P_i$ (●) and $P_i$ + PALA (●) reactions were in 40 mM KPO$_4$, pH 7.0, buffer and in the absence and presence of 50 $\mu$M PALA, respectively. Each point is the average value of $k$ for at least two experiments performed under the same conditions. The Arrhenius activation energies in kilocalories/mol obtained from absorbance changes at 500 nm for the reaction of 2.14 $\mu$M ATCase with 8.6 $\mu$M PMPS, at pH 7.0, using 10$^{-4}$ M PAR (as in Fig. 4A) was apparent first-order with $t_0 = 111$ s at 18.7°C. The first-order release of Zn$^{2+}$ measured under the same conditions in the presence of 10$^{-4}$ M PAR (as in Fig. 2A) was ~1.3-fold faster than the light scattering change in 1 M urea. Thus, 1 M urea in Hepes buffer decreased slightly the rates of both Zn$^{2+}$ release (Table II) and dissociation of ATCase saturated with PALA.

| Sector 1 | Sector 2 (Reference) | $\Delta s$ | $(\Delta s/s)_{\text{ATCase}}$ |
|---------|----------------------|--------|-----------------|
| 2.0     | 0.0                  | -0.21  | -1.9            |
| 5.0     | 0.0                  | -0.41  | -3.8            |
| 6.0     | 0.0                  | -0.39  | -3.8            |
| 8.0     | 0.0                  | -0.39  | -3.6            |
| 4.0     | 0.0                  | 0.01   | 0.0             |
| 5.8*    | 0.0*                 | -0.39  | -3.6            |
FIG. 5. Stopped-flow measurements of the kinetics of Zn\(^{2+}\) release from ATCase(PALA) at 20 °C upon the addition of excess PMPS in 40 mM Hepes/KOH buffer, pH 7.0. A, oscilloscope tracings from a stopped-flow spectrophotometer recorded during the reaction of ATCase with a 100-fold excess of PMPS. Solutions of PMPS and of ATCase with PALA, each containing 40 mM Hepes/KOH, pH 7.0, and 10\(^{-4}\) M PAR, were degassed and then equilibrated at 20 °C before mixing equal volumes of the two solutions in the stopped-flow apparatus; after mixing, concentrations were 0.6 mM PMPS, 0.25 \(\mu\)M ATCase, and 10\(^{-4}\) M PAR. The upper tracing is a record of transmittance at 500 nm which changed from 1.00 to 0.80 during the reaction. The lower tracing is a first-order rate plot generated by the computer of the stopped-flow spectrophotometer. The first-order rate constant on which the half-time of 659 ms is based, was calculated from the linear portion of the first-order rate plot over the period from 0.4 to 2.8 s. A rate plot based on absorbance values, calculated from the digitally-recorded transmittance values, gave essentially the same half-time for the experiment. B, plots of the log of the pseudo first-order rate constant for Zn\(^{2+}\) release from ATCase upon reaction with excess PMPS versus log of PMPS concentration. The data were obtained by stopped-flow experiments at 20 °C and pH 7.0 such as that shown in A. Values of k were obtained from the linear portion of the first-order rate plot, following the lag period at the beginning of the reaction. All solutions contained 0.25 \(\mu\)M ATCase and 30 \(\mu\)M PALA after mixing, and 10\(^{-4}\) M PAR. The upper plot is from experiments in 40 mM Hepes/KOH, pH 7.0, buffer; the line is a linear least-squares fit to the data (O) obtained at 500 nm. The triangles are data points from experiments monitored at 250 nm for mercaptide formation. The data of the lower plot (C) were obtained at 500 nm in 40 mM KPO\(_4\), pH 7.0.

wide range of [PMPS] there was a lag period which seemed to constitute a constant fraction of a half-life. That is, at higher [PMPS], the overall rate was faster and the lag period shorter. The lag period was observed whether or not PALA was present and also when KPO\(_4\), pH 7.0, buffer was substituted for Hepes/KOH. A similar lag period was seen in traces recorded at 250 nm. For experiments such as that shown in Fig. 5A, the lag period was followed by first-order behavior.

TABLE IV

| Buffer present | Ligand present | [PMPS] (mM) | k (s\(^{-1}\)) |
|----------------|----------------|-----------|--------------|
| A              | PALA           | 1.60      | 0.76         |
| KPO\(_4\)      | None           | 1.60      | 0.34         |
| KPO\(_4\)      | PALA, CTP      | 1.60      | 0.68         |
| Hepes          | PALA           | 1.60      | 3.10         |
| Hepes          | PALA           | 0.60      | 1.31         |
| Hepes          | None           | 0.60      | 1.62         |
| B              | None           | 0.040     | 0.0014       |
| KPO\(_4\)      | PALA           | 0.040     | 0.0057       |
| KPO\(_4\)/Tris, 25 mM | None | 0.20 | 0.0046 (0.0045) |
| KPO\(_4\)/Tris, 25 mM | PALA | 0.20 | 0.0228 (0.024) |
| KPO\(_4\)/Tris, 50 mM | None | 0.20 | 0.0028 (0.0028) |
| KPO\(_4\)/Tris, 50 mM | PALA | 0.20 | 0.016 (0.016) |

Pseudo first-order rate constants were obtained by stopped-flow for the reaction of ATCase with excess PMPS under a variety of conditions, using the linear portion of first-order plots such as shown in Fig. 5A. The rate constant for a given concentration of PMPS was found to depend on the buffer used and the effectors present; Table IV, A, presents some representative data. As in the case of the reaction of ATCase with 4 eq of PMPS (excess ATCase sulfhydryl groups), Zn\(^{2+}\) release is more rapid in Hepes/KOH than in KPO\(_4\), pH 7.0, buffer. In phosphate buffer, the presence of PALA increases the rate 2.2-fold which is less than observed at lower PMPS concentration (Table I). The addition of CTP in the presence of PALA decreases the rate 1.3-fold. Surprisingly, the rate of Zn\(^{2+}\) release from ATCase at high [PMPS] in Hepes/KOH, pH 7.0, buffer is decreased by the presence of PALA even though [PMPS]/[PALA] is 1200.

For the reaction of large excesses of PMPS with ATCase in KPO\(_4\), buffer with PALA present, the first-order rate constant based on the absorbance-time trace at 250 nm agreed well with that obtained at 500 nm. With PALA absent and in Hepes buffer even with PALA present, however, the absorbance at 250 nm continued to increase after completion of the absorbance change at 500 nm (i.e., Zn\(^{2+}\) release). The most reasonable explanation for the slow absorbance change at 250 nm is mercaptide formation with the active-site sulfhydryl group on each c chain.

Fig. 5B shows log-log plots of the pseudo first-order rate constant versus [PMPS] for the reaction of excess PMPS with ATCase in the presence of PALA and Hepes/KOH or KPO\(_4\), buffer, pH 7.0. For Hepes/KOH buffer, the apparent order of the reaction in [PMPS] is 1.24 for 0.1–1.5 mM PMPS monitored at either 500 or 250 nm. In KPO\(_4\), buffer over a more limited range of 0.6–1.6 mM PMPS, the apparent order
of the reaction [PMPS] is 1.36 (monitored at 500 nm). The data of Fig. 5B obtained in phosphate buffer fit the empirical equation:

\[ k (s^{-1}) = 0.6038 \times [\text{PMPS}] + 0.217 \times [\text{PMPS}]^2 + 0.09 \]

where [PMPS] is millimolar concentration.

The PMPS-mercaptide bonds formed with r chain sulphhydryl groups are thermodynamically very stable but kinetically quite labile. These bonds are rapidly disrupted by reaction with 2-mercaptoethanol. Also, the addition of the organomercurial neohydrin (2 or 5 mM) in 10-fold excess over PMPS present at the end of experiments such as that shown in Fig. 5A gave an absorption decrease at 250 nm that was complete within the time of stopped flow mixing (2–3 ms). This indicates that neohydrin rapidly displaces PMPS in mercaptide bond formation.

Pseudo first-order rate constants were obtained at several temperatures for the reaction of 100-fold excess PMPS ([PMPS] = 0.60 mM) with ATCase in Hepes/KOH, pH 7.0, buffer in the absence and presence of PALA. The data from these experiments are presented as Arrhenius plots in Fig. 6A. Below 37 °C, the rate of the reaction of ATCase with excess PMPS is decreased by the addition of PALA, rather than 6-fold increased as under the conditions of Blackburn and Schachman (19) using a mixed KPO4/Tris, pH 7.0, buffer (see Table IV).

Considerably lower activation energies were obtained in Hepes buffer for the reaction of excess PMPS with ATCase (Fig. 6A) than for the reaction of 4 eq of PMPS with ATCase (Fig. 4). Activation energies of ~7 and 10 kcal/mole were obtained in the absence and presence of PALA, respectively, for the reaction of ATCase in Hepes buffer upon addition of a 100-fold excess of PMPS (Fig. 6A). Under these conditions, PALA appears to stabilize Zn2+ bonding sites, possibly by protecting against thermal melting of cr contact regions in ATCase.

Arrhenius plots for the reaction of ATCase in 40 mM KPO4, pH 7.0, buffer with 6.7-fold excess PMPS are shown in Fig. 6B. It is clear that the presence of phosphate increased the activation energy (\(E_a\)) over that observed in Hepes buffer (Fig. 6A). Under the conditions of Fig. 6B, PALA produced a 4-fold increase in the rate of Zn2+ release (Table IV, B) and decreased \(E_a\) from 13.8 to 12 kcal/mol. For Zn2+ release from the inactive mutant ATCase231 in KPO4 buffer, an activation energy of 14.1 ± 0.5 kcal/mol was measured as in Fig. 6B. Thus, the wild-type and ATCase231 mutant enzymes are indistinguishable by this criterion.

Since previous studies (12, 23) of the reaction of excess PMB (~4-fold PMB to ATCase —SH groups) employed a mixture of KPO4/Tris buffer at pH 7.0, experiments were conducted under similar conditions using ~23-fold PMPS or PALA (~4-fold ATCase —SH groups, Table IV, B). A 6-fold enhancement in the rate of Zn2+ release with PMPS or PMB was produced by saturating PALA in the presence of 25 or 50 mM Tris in 40 mM KPO4, pH 7.0, buffer, as had been observed previously for the enhancement in the rate of ATCase —SH group reactivity by PALA binding (19, 23). In fact, the pseudo first-order rate constants in the absence and presence of saturating PALA for Zn2+ release from ATCase in KO4, 50 mM Tris in Table IV, B, are in excellent agreement with those reported by Blackburn and Schachman (19). No significant difference was observed using PMPS or PMB as the mercurial reagent (Table IV, B).

The results given in Table IV suggest that the magnitude of the increase in the sulfhydryl group reactivity of ATCase promoted by active site ligand binding is dependent on the amount of excess organomercurial and the buffer components present. For example, with 267- and 6.7-fold PMPS/ATCase —SH groups in KPO4 buffer, the effect of PALA was to increase the rate of Zn2+ release 2- and 4-fold, respectively. The addition of Tris to KPO4 buffer further inhibited the mercurial reaction and increased to a factor of 6 the effect of PALA on the rate of Zn2+ release.

**DISCUSSION**

Titrations of ATCase(PALA)_e with PMPS in the absence and presence of the high-affinity metallocromatic indicator PAR indicated that Zn2+ release and mercaptide formation are coincidental. Furthermore, the release of Zn2+ from the ATCase(PALA)_e complex was a linear function of the mer-
curial added with 1 eq of Zn$^{2+}$ released for every 4 eq of PMPS added during the titration of the 24 sulfhydryl groups of r chains in the enzyme molecule. At limiting PMPS (with ATCase—SH groups in excess) the release of Zn$^{2+}$ in PMPS is first order in mercurial concentration. These observations indicate that the rate-limiting step for Zn$^{2+}$ dissociation from ATCase is the mercurial attack on one of the 4 —SH groups involved in tetradeid coordination of Zn$^{2+}$ in an r chain of an ATCase molecule (7, 9, 13). Once the first of the 4 —SH groups in a Zn$^{2+}$ binding site reacts with the organomercurial reagent, the other 3 —SH groups react rapidly which gives a linear release of $10n^2$ for every 4 eq of PMPS added.

The x-ray crystallographic analyses (9, 10) of the ATCase structure places the Zn$^{2+}$ binding site in each r chain very near the r:r contact region. In fact, Monaco et al. (9) noted that all substructures of loops ending with cysteinyl residues 109, 114, 137, and 140 that are coordinated to Zn$^{2+}$ in each r chain participate in the interactions between the r and c chains. This explains the earlier observations of Gerhart and Schachman (11, 12) who showed that PMB produces dissociation of ATCase into C trimers and R dimers. Furthermore, the mercurial-promoted dissociation of ATCase was shown also in those studies to proceed 1 molecule at a time (with any intact ATCase present containing no mercurial in mercaptide linkage), suggesting that once one r:r contact is disrupted, the other 5 Zn$^{2+}$ sites become more susceptible to mercurial attack and consequently to r:r contact disruption. In the present studies, we have corroborated this result by polyacrylamide gel electrophoresis during titration of ATCase with PMPS (Fig. 3) and by light scattering measurements of the reaction of 4 eq of PMPS with ATCase(PALA)$_{10}$/ in Hepes buffer at pH 7.0 (Fig. 2B), in which almost exactly 1/4th dissociation of ATCase was observed. Under these conditions, there appears to be no accumulation of the C$_{R2}$ intermediate as there is in KPO$_4$, pH 7.0, buffer (Fig. 3). Thus, the rate-limiting reaction of the 1st of 4 —SH groups in a Zn$^{2+}$ binding cluster triggers the rapid reaction of the other 23 —SH groups of the 6 r chains in an ATCase molecule. This catastrophic effect of organomercurial reagents on ATCase results in the dissociation of ATCase 1 molecule at a time.

We have used the sensitive metallochromic indicator PAR in the present kinetic studies to monitor the mercurial-promoted release of Zn$^{2+}$ from ATCase, both under conditions of limiting organomercurial reagent (ATCase sulfhydryl groups in excess) and of limiting ATCase with the mercurial reagent in excess. PMPS rather than PMB was used because PMB has a limited solubility, although PMB when tested gave similar rates to those obtained with PMPS (Tables II and IV). These organomercurial compounds have a single labile ligand which is displaced by reaction with thiols. Khalifah (27, 39) has shown that the kinetics of organomercurial-thiol reactions can be influenced profoundly by facile replacement of the mercurial labile ligand prior to reaction with the thiol. For this reason, the noninteracting Hepes/KOH buffer at pH 7.0 was chosen for many of our experiments. However, some studies were conducted also in the presence of organomercurial inhibitors at pH 7.0 (Tris/HCl with $K_i = 0.5 \text{ mm}$, Br$^-$ with $K_i = 0.5 \text{ mm}$, KPO$_4$, and mixtures of Tris/HCl/KPO$_4$) in order to directly compare the results from this and previous studies (19, 23).

Kahlifah (27, 39) observed that the effect of a particular organomercurial inhibitor is unpredictable, being a function of the stability of the inhibitor-mercurial complex and the nature of the thiol group under attack. In the present studies, the enhancement in the rate of PMPS attack on ATCase produced by binding the high-affinity bisubstrate analog PALA at enzyme catalytic sites also was dependent on the mercurial inhibitor(s) present (Tables II and IV). The PALA-promoted conformational change of ATCase ($\Delta G^\circ = -3.6\%$; Table III), however, is the same in Hepes/KOH, pH 7.0, buffer as in KPO$_4$, pH 7.0, buffer, which acts as a mercurial inhibitor.

Under the conditions of limiting [PMPS] and excess [ATCase —SH groups], the reaction of PMPS with ATCase is clearly first order in [PMPS]. No lag time was noted in these experiments, but observations began after the first 5–10 s of the reaction. With excess [ATCase —SH groups], the reaction rate is insensitive to the concentration of protein (Table I) although the reaction becomes first order in ATCase as the limiting reactant with excess PMPS (or PBM). The Arrhenius activation energy for the reaction of limiting [PMPS] with excess [ATCase —SH groups] is quite large, 18 kcal/mol. The fact that this value is the same in Hepes/KOH and KPO$_4$ buffers suggests that there is no fundamental difference for the mercurial-ATCase reaction in the two buffers even though phosphate binds to ATCase (40) and stabilizes an accumulation of the C$_{R2}$ intermediate (Fig. 3), which reacts with the mercurial reagent at about the same rate as does intact ATCase, C$_{R3}$ (23). Note also that the reaction with excess [ATCase —SH groups] in phosphate buffer is first order in PMPS, the limiting reagent, just as it is in Hepes buffer. Certainly, the major inhibitory effect of phosphate on the mercurial reaction with thiols of ATCase is due to the binding of phosphate to PMPS (27, 39).

Several quantitative changes in kinetic behavior were observed as the concentration of PMPS was increased. At high concentrations of PMPS, the reaction becomes first order in [ATCase], after an initial lag period, and the order of the reaction with respect to [PMPS] increases to a value between first and second (Fig. 5). In addition, the apparent Arrhenius activation energy is decreased substantially from that for the case of limiting [PMPS]. Furthermore, the effect of PALA binding to ATCase on increasing the reaction rate with mercurials appears to be reversed at high [PMPS] at temperatures < 37°C in Hepes buffer (Fig. 6A). PALA and phosphate appear to stabilize ATCase during the attack by excess PMPS, producing an increase in the Arrhenius activation energy over that observed in Hepes buffer. In contrast, the inactive mutant ATCase$_{23a}$ appears to not be stabilized against mercurial attack by the presence of phosphate.

In stopped-flow experiments under the conditions of excess [PMPS] to [ATCase—you SH groups], the initial lag observed was a constant fraction of a half-life whether the buffer was in Hepes or phosphate at pH 7.0, or whether the reaction was monitored at 300 nm for Zn$^{2+}$ release or at 250 nm for mercaptide formation. This initial lag could be due to a steady-state accumulation of a more reactive species. The evidence is against the dissociation intermediate C$_{R2}$ being a more reactive species: (a) Subramani and Schachman (23) found that the rate of mercurial attack on C$_{R2}$ and on intact ATCase to be approximately the same; (b) the accumulation of C$_{R2}$ in KPO$_4$, buffer is not reflected in proportionately longer lag times. We propose below that a more reactive species could be generated in a pre-equilibrium step by mercurial attack on nonthiol groups of ATCase that then enhance the susceptibility of Zn$^{2+}$-thiol clusters to attack by PMPS.

Many of the discrepant observations may be rationalized by again noting that organomercurials may form complexes with a variety of moieties other than sulfhydryl groups, including nitrogenous side chains in the protein itself. Since Hepes apparently binds to PMPS weakly, whereas amines such as Tris bind to PMPS rather well, it seems reasonable to suggest that ATCase may serve simultaneously
as a reactant and as an inhibitor in Hepes buffer. For example, the sequestering of PMPs by lysyl ε-amino acid groups (without an accompanying absorbance change at 250 nm) would lower the concentration of free [PMPs] available for reaction with sulfhydryl groups (with an accompanying absorbance change at 250 nm) as illustrated in Reactions 1 and 2:

\[
\text{R-HgOH + ATCase-NH}_2 \xrightleftharpoons[k_{-1}]{k_1} \text{R-Hg-NH}_2-\text{ATCase} + \text{H}_2\text{O} \tag{1}
\]

\[
\text{R-HgOH + ATCase} - \text{SH} \xrightleftharpoons[\text{slow}]{k_2} \text{R-Hg-S-ATCase} + \text{H}_2\text{O} \tag{2}
\]

where HgOH is the hydroxyl complex of PMPs and ATCase-NH\textsubscript{2} and ATCase-SH are simply ATCase molecules written so as to indicate the group with which PMPs reacts.

If Reactions 1 and 2 are occurring simultaneously, then increasing [ATCase] would tend to increase the rate of Reaction 2 directly but at the same time decrease the rate of Reaction 2 indirectly by lowering [R-HgOH] because of its utilization in Reaction 1. The two effects could nearly cancel, leading to an apparent independence on [ATCase] under conditions of limiting [PMPs] as is actually observed. Since

\[
\text{rate} = k_2 \left[\text{R-HgOH}\right] [\text{ATCase} - \text{SH}] \tag{3}
\]

but if \(k_1/k_2\) in Reaction 1 is large and substitutions for the mass distribution of [R-Hg]\textsubscript{TOTAL} are made under conditions of limiting [R-Hg]\textsubscript{TOTAL}

\[
\left[\text{R-HgOH}\right] = \frac{1}{K} \left[\text{R-Hg-S-ATCase}\right] - \left[\text{R-Hg-S-ATCase}\right] \tag{4}
\]

then

\[
\text{rate} = \frac{k_2 \left[\text{R-Hg-S-ATCase}\right]\left[\text{ATCase} - \text{SH}\right]}{[\text{ATCase-NH}_2]} \tag{5}
\]

so that the observed pseudo first-order rate of the mercurial reaction becomes

\[
d\ln \left[\text{R-HgOH}\right] = \frac{k_2 \left[\text{ATCase} - \text{SH}\right]}{K \left[\text{ATCase-NH}_2\right]} \tag{6}
\]

The proposed inhibitory effect of ATCase would not be observed in any single kinetic experiment even if ATCase were the limiting reagent, since the concentration of competing amino acid side chains would remain constant. The presence of such protein inhibitory groups also would not affect the order of the apparent reaction in a single kinetic experiment.

With [ATCase] limiting at high [PMPs], the mercurial reaction will be pseudo first-order in [ATCase]; Equation 3. It is conceivable that the binding of PMPs to a non-thiol group of ATCase could accelerate the reaction of PMPs with the thiol groups, presumably by means of conformational effects, rather than retarding the reaction by decreasing [PMPs]. This mode of attack by PMB was proposed earlier by Pigiet (41) to explain the second-order path implied by the rate law of Gerhart and Schachman (12), in which the reaction order in [PMB] was greater than 1 at low [PMB] and was 1 at high [PMB]. However, the earlier studies on the reaction of ATCase with PMB (11, 12) were severely restricted by the sensitivity of measurements of mercaptide formation (36) and by the limited solubility of PMB.

It certainly would not be surprising that the high concentrations of the organomercurial PMPs used in the present studies could have a chaotropic effect. Saturation of "helper" sites in a pre-equilibrium step (reaction 1) could explain the observed initial lag in the pseudo first-order reaction of ATCase with excess [PMPs] and also the change in the apparent reaction order with respect to [PMPs], which indicates that more than 1 mercurial molecule is involved in the attack on ATCase thiols when [PMPs] is in excess. In this case, Reactions 1 and 2 are as written and

\[
d\ln [\text{ATCase} - \text{SH}] = \frac{k_1 [\text{R-HgOH}^2]}{\left(k_1 + k_2 [\text{R-HgOH}]\right)} \tag{7}
\]

Such a chaotropic effect at high [PMPs] could decrease the Arrhenius activation energy from 18 to ~7 kcal/mol. The bisubstrate analog PALA stabilizes ATCase against this action of the organomercurial in Hepes buffer until increasing temperatures melt out such structures. In inhibitory buffers containing KPO\textsubscript{4}, however, PALA promotes a conformational change that enhances the rate of reaction of ATCase thiols with excess PMPs or PMB (19, 23). Indeed, the Arrhenius activation energy for the reaction of excess [PMPs] with ATCase in KPO\textsubscript{4} buffer (13.8 kcal/mol) is higher than that in Hepes buffer and is decreased to 12 kcal/mol by saturating PALA. The additional effects of amines such as Tris on the fold enhancement in the reaction rate of ATCase with excess [R-Hg] given by PALA binding could relate to differences in facile ligand exchange of the Tris-substituted mercurial reagent with groups exposed on the protein in the R- and T-state conformations of ATCase (2, 15).

In summary, the studies of the kinetics of Zn\textsuperscript{2+} release from ATCase using the metallochromic indicator PAR have been found to correlate exactly with reaction rates of organomercurial compounds with ATCase sulfhydryl groups, since the action of the mercurial is necessary to disrupt Zn\textsuperscript{2+} binding clusters in ATCase. The mercurial-promoted dissociation of ATCase occurs in an "all or none" or "zipper" fashion, suggesting that once one Zn\textsuperscript{2+} site is disrupted in an ATCase molecule, the other five Zn\textsuperscript{2+}-thiol clusters are destabilized. Thus, the intactness of Zn\textsuperscript{2+} binding clusters in an ATCase molecule is essential for thermodynamic stabilization of the c chain interactions responsible for the allosteric properties of this enzyme. Studies on the kinetics of Zn\textsuperscript{2+} release and uptake by isolated R subunits are in progress.

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