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The Thiol Group in the Catalytic Chains of Aspartate Transcarbamoylase

(enzyme activity/sulfhydryl group/allosteric enzyme/mercurials)

DAVID R. EVANS, CECIL H. McMURRAY, AND WILLIAM N. LIPSCOMB

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT **The allosteric enzyme aspartate transcarbamoylase (EC 2.1.3.2) was previously shown to consist of two functionally distinct types of polypeptide chains. X-ray diffraction and chemical studies showed that there are six copies of both catalytic (C) and regulatory (R) chains, and that the intact molecular complex (C_6R_6) has D_3 symmetry. Organomercurials react preferentially with the four thiol groups on each R chain, dissociating the molecular complex. We show that 2-chloromercuri-4-nitrophenol reacts specifically and rapidly with the single C-chain thiol, which is believed to be near the catalytic site. This reaction inactivates the enzyme in solution and does not dissociate the molecular complex. Spectrophotometric titration and mercury analysis indicates that six molecules of this mercurial are firmly bound to the enzyme (R_6C_6), and crystallographic studies establish that only six sites, related by D_3 symmetry, are modified.**

The known low reactivity of this C-chain thiol with other sulfhydryl reagents, the unusual structural requirements in the reaction with 2-chloromercuri-4-nitrophenol, and the spectral properties of the resulting derivative provide insight into the environment of this thiol. Probably, at least one positively charged group of the enzyme is nearby, and the environment of this thiol is at least partially hydrophobic.

Allosteric enzymes participate in one of the major mechanisms, which have evolved in living organisms, for coordination of the myriad chemical reactions within cells. Many of these enzymes, like aspartate transcarbamoylase (EC 2.1.3.2), catalyze the first committed step of an important biochemical pathway. As a consequence of this strategic location, allosteric enzymes control the flow of metabolites through the pathway in response to requirements of the organism. It is generally believed that these enzymes undergo conformational changes that are induced by binding of effectors (allosteric inhibitors or activators) to specific sites distinct from the catalytic site. These conformational changes are transmitted through the molecule, altering the environment of the catalytic site. However, the detailed mechanism of this fundamental process, essential for rapid and precise regulation of intracellular metabolism, is not understood.

Aspartate transcarbamoylase (ATCase) is an allosteric enzyme (1, 2) that catalyzes the first step in pyrimidine biosynthesis: the reaction of aspartate and carbamoylphosphate to yield carbamoyl-aspartate and phosphate. The activity of ATCase is modulated by several allosteric effectors. Cytidine triphosphate, the end-product of the pathway, is a potent feedback inhibitor. Also, the enzyme is activated by adenosine triphosphate. This inhibition by a pyrimidine and stimulation by a purine is one mechanism for achieving balance in the synthesis of nucleic acids.

ATCase has a molecular weight of 310,000 (3) and contains two functionally distinct types of polypeptide chains, regulatory (R) and catalytic (C) (2, 3). The first indication of the hexameric nature (R_6C_6) of ATCase occurred when the x-ray study (4) demonstrated the simultaneous presence of both three-fold and two-fold molecular symmetry, and accurate molecular weights of the R and C chains were determined (5, 6). It is firmly established that the molecular symmetry is D_3 (7). This hexameric nature has been amply confirmed in recent studies (6, 8–10). There are four cysteines on each R chain (5, 11) and one cysteine on each C chain (11, 12). Reaction of the thiols of the R chain with mercurials (3) causes dissociation of ATCase into two catalytic subunits (C_3) and three regulatory subunits (R_2) (3–5). The cysteine of each catalytic chain in the intact ATCase molecule is unusually unreactive towards conventional reagents such as mercurials, *N*-ethylmaleimide, iodoacetic acid, and others (13, 14). This residue is more reactive in isolated catalytic subunits and reacts slowly with *p*-hydroxymercuribenzoate (11, 13) and with 5,5′-dithiobis-(2-nitrobenzoate) (13). Permanganate rapidly oxidizes the C-chain thiol in both isolated catalytic subunits (14) and intact enzyme (Jacobson, G. R. & Stark, G. R., unpublished data). All of these reactions cause a loss of catalytic activity.

The stimulus of this investigation has been the necessity for preparation of a simple heavy-atom derivative of known composition and occupancy for the x-ray crystallographic study now in progress in our laboratory. We have succeeded in preparing a mercurial derivative of the C-chain thiol under conditions that do not result in dissociation of the ATCase molecule. During this study we became aware of the critical nature of both reaction conditions and the detailed structure of the organomercurial. A more immediate result is a preliminary exploration of the environment of this thiol group, which is most probably near the active site of ATCase.

METHODS AND MATERIALS

ATCase was isolated from *Escherichia coli* by the procedure of Gerhart and Holoubek (15). Large quantities of cells were grown at the New England Enzyme Center from a special mutant strain kindly provided by J. C. Gerhart. The protein was assayed, by a method developed in our laboratory by F. A. Quiocho, by continuously monitoring the enzyme-catalyzed release of protons at pH 8.3 on a Radiometer TT2 pH stat.

Abbreviations: ATCase, aspartate transcarbamoylase: C, catalytic chain; R, regulatory chain; PHMB, *p*-hydroxymercuribenzoate; MNP, 2-chloromercuri-4-nitrophenol.

The assay mixture consisted of 30 mM aspartate and 4.8 mM carbamoylphosphate. Protein concentration was measured either spectrophotometrically at 280 nm, with an absorption coefficient of 0.59 $(mg/ml)^{-1}\ cm^{-1}$ (3), or by the Lowry *et al.* (16) procedure. All spectrophotometric measurements were made on either a Zeiss PMQ II or a Cary model 14 spectrophotometer. *p*-Hydroxymercuribenzoate (PHMB) was purchased from Sigma Chemical Co., and 2-chloromercuri-4-nitrophenol (MNP) was synthesized by the method of McMurray and Trentham (17). Mercury and zinc were analyzed by atomic absorption in a Perkin Elmer model 303 spectrometer with the aid of zinc chloride or mercury acetate standards. Electrophoresis on 78 × 150 mm cellulose acetate strips was performed in a Shandon electrophoretic chamber with 50 mM Tris-citrate (pH 7.8) running buffer (3).

RESULTS

In attempts to modify specifically the single thiol residue on the catalytic chain of ATCase, we reacted the protein with about 20 different organomercurials under various conditions. After passing the protein over a G-25 Sephadex column to remove unreacted mercurial, we established, upon analysis for mercury, that all of these compounds reacted extensively with ATCase. While most compounds did not alter the catalytic activity of the enzyme, complete inactivation was produced by a few, most notably 2-chloromercuri-4-nitrophenol (MNP). The MNP derivative was typically prepared by reaction of ATCase with a stoichiometric amount of the mercurial (6Hg:R_6C_6) in 40 mM triethanolamine–HNO_3, 100 mM NaCl at pH 7.5.

Atomic absorption measurements on solutions (7–10 mg/ml) of the MNP derivative were used to determine the amount of mercury bound to the enzyme. Duplicate samples from four different experiments were analyzed: the average ratio of bound mercury per mol of protein for these eight determinations was 6.2 ± 0.6. The mercurial remains tightly bound to the protein after passage over a G-25 Sephadex column or after exhaustive dialysis, but the inactivation is rapidly reversed by 2-mercaptoethanol. This behavior is characteristic of mercury–thiol bonds.

ATCase contains six tightly bound atoms of zinc (18–20). If ATCase that has been dissociated by reaction with excess PHMB is reconstituted by addition of thiols, the organic portion of the mercurial is split away and each zinc ion is replaced by one mercuric ion (20). However, we have found, upon analysis, that the MNP derivative of ATCase contains six equivalents of zinc in addition to the six equivalents of mercury. Hence, the Hg of MNP does not replace the Zn of ATCase under these conditions.

The observation that 6 mol of MNP are bound per mol of protein simply demonstrates that all of the mercurial present in the reaction mixture has reacted with ATCase. The actual stoichiometry of the reaction was established by the following experiments.

Mercurinitrophenols absorb strongly in the visible region of the spectrum (17). ATCase can be titrated spectrophotometrically with MNP by taking advantage of the chromophoric changes that occur at 410 nm (pH 7.0) on binding of this mercurial to the enzyme. Aliquots of the mercurial were added to the protein, and the reaction was allowed to go to completion (at least 3 min) between successive additions. The resulting titration curve (Fig. 1*a*) has a well-defined breakpoint that occurs on the addition of six equivalents of the mercurial. The slope before the breakpoint reflects a substantial decrease in absorption of the chromophore as the mercurial reacts rapidly with six sites on the molecule. The complete inactivation that occurs on addition of six equivalents of MNP suggests that the initial site of reaction is the thiol group on each of the six catalytic chains. After the breakpoint, the chromophoric changes are much smaller. Nevertheless, the slope in this region of the curve differs significantly from the control (mercurial added to buffer, Fig. 1*c*) and is the result of reaction of excess mercurial with the R-chain thiols. In another experiment, the C-chain thiol in intact ATCase was oxidized by permanganate (Jacobson, G. R. & Stark, G. R., unpublished data), and a similar titration was performed (Fig. 1*b*). There is no break-point, and the slope of the curve indicates that the mercurial is reacting with the sulfhydryl groups on the regulatory chains. This result verifies that MNP reacts initially with the C-chain thiol.

We have found a striking nonlinear correlation between enzymatic activity and the extent of reaction of this thiol with MNP. The observed inactivation is always greater than that expected from the fraction of C-chain thiols that have reacted with MNP, and is a function of the aspartate concentration in the assay.

The conclusion that MNP reacts with the C-chain sulfhydryl group, unlike most other mercurials, is reinforced by examination of the products of the reaction of ATCase with these different types of mercurial (MNP and PHMB) by cellulose acetate electrophoresis (Fig. 2). ATCase that has been reacted with a stoichiometric amount of MNP migrates as a single band indistinguishable from native ATCase. By contrast, reaction with PHMB under the same conditions produces significant dissociation. This result is very likely

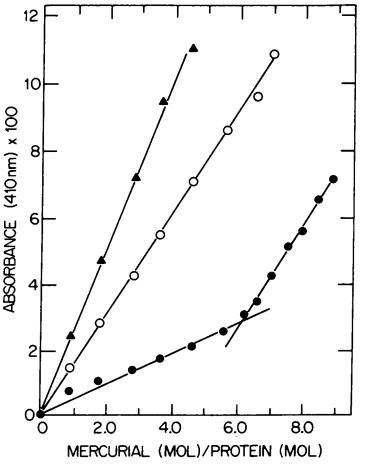

FIG. 1. Spectrophotometric titration of ATCase with 2-chloromercuri-4-nitrophenol. (*a*) ●: To determine the stoichiometry of the reaction of MNP with ATCase, aliquots of the mercurial (1.07 mM in 1.0 mM NaOH) were added to a solution containing 0.64 mg/ml in 40 mM triethanolamine$^+$ NO_3^-, 100 mM NaCl (pH 7.0). The reaction was monitored at 410 nm, and sufficient time (as much as 10 min for the slowly reacting R chains) was allowed between successive additions for the reaction to go to completion. (*b*) ○: This titration is the same as in (*a*) except that the thiol group on the catalytic subunit had been oxidized by permanganate. (*c*) ▲: A control in which equivalent aliquots of the mercurial were added to the same buffer, but containing no protein.

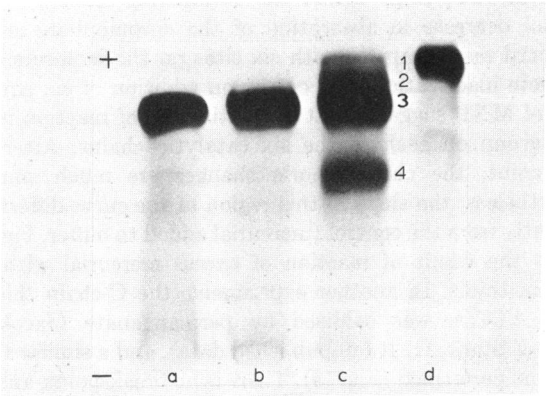

FIG. 2. Cellulose acetate electrophoresis of the products of the reaction of ATCase with mercurials. Electrophoresis on 75 × 150 mm cellulose acetate sheets (Celagram, Shandon Instrument Co.) was done for 20 min at a constant current of 10 mA with 50 mM Tris-citrate (pH 7.8) buffer. Bromphenol blue was added to each sample to serve as a marker. Aliquots (1–2 μl) of the following samples were spotted: (*a*) ATCase (20 mg/ml), (*b*) ATCase reacted with MNP, (*c*) ATCase reacted with 20 mg/ml of PHMB, (*d*) C subunit (8 mg/ml). The samples of modified ATCase were both prepared by reaction (30 min) of the protein with a stoichiometric amount (6Hg:R_6C_6) of the mercurial in 40 mM triethanolamine$^+NO_3^-$–100 mM NaCl (pH 7.5). The components of the resulting banding pattern are labeled *1–4* and are discussed in the text. The three overlapping bands in (*c*) are resolved at lower protein concentrations.

because of the unusual concerted fashion in which the R-chain thiols react. Reaction of one thiol group on the regulatory chain facilitates reaction of the other R-chains thiol on the same molecule, so that this group of residues appears to react in an "all or none fashion" (11). Assuming this concerted mechanism, the reaction of 6 of the 24 R-chain thiols with a stoichiometric amount of PHMB (6Hg:R_6C_6) would be expected to result in dissociation of 25% of the enzyme molecules. The electrophoretic pattern (Fig. 2*c*) provides clear evidence of dissociation. In addition to the dense band (Fig. 2*c*, band *3*), corresponding to unreacted ATCase, there are

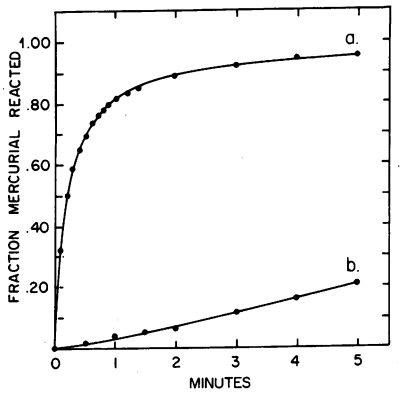

FIG. 3. Rate of reaction of ATCase with mercurials. Reaction of ATCase with mercurials was followed spectrophotometrically at 410 nm for (*a*) MNP and at 250 nm for (*b*) PHMB. In each case the reaction was initiated by addition of a stoichiometric amount of the mercurial (1 mM in 1 mM NaOH) to a solution consisting of 0.59 mg/ml of ATCase in 40 mM triethanolamine$^+NO_3^-$–100 mM NaCl (pH 7.0).

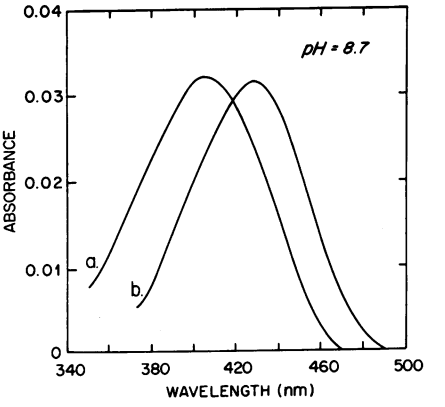

FIG. 4. Comparison of absorption spectra of free and bound MNP at pH 8.7 (*a*) 1.8 μM MNP: (*b*) 1.5 μM MNP derivative of ATCase. There is a large pH dependence of the MNP absorption spectrum. The titration (Fig. 1) was performed at pH 7.0, and the spectra at this pH shows a substantial difference in the absorption of the free and bound species at 410 nm.

three smaller bands. The slowest migrating species (band *4*) is the regulatory subunit. Immediately preceding ATCase, there are two partially overlapping bands. The most rapidly migrating species (band *1*) has the same mobility as isolated catalytic subunit (Fig. 2*d*). The other species, which has a mobility between that of native ATCase and the catalytic subunit, has not been reported previously. This band may correspond to an intermediate that occurs when ATCase is dissociated by PHMB (Fig. 2*c*, band *2*).

It is apparent that these two types of mercurial, represented by PHMB and MNP, react in very different ways with the ATCase molecule. A clue to the unusual affinity of the mercurinitrophenol for the C-chain thiol is provided by study of the relative rates of reaction of these two mercurials with the enzyme. Each reaction mixture consisted of a stoichiometric molar ratio (6:1) of mercurial to protein. The reaction was followed spectrophotometrically at 250 nm for PHMB and 410 nm for MNP. There is a striking difference in reaction kinetics (Fig. 3): MNP reacts much more rapidly with the enzyme than does PHMB. Furthermore, the reaction of MNP and ATCase closely conforms to second-order kinetics as expected if the C-chain thiols are reacting with the mercurial in an independent fashion. The second-order rate constant is 325,000 M^{-1} min^{-1}, in terms of molarity of RC units. By contrast, PHMB reacts sluggishly with the enzyme and significantly deviates from a second-order relationship. On completion of the reaction the total change of absorption at 250 nm is equivalent to six mercury thiol bonds formed per ATCase molecule, from the extinction coefficient derived by Boyer (21). In the reaction of ATCase with mercurials there is competition between R- and C-chain thiols for a limiting amount of reagent. For most mercurials, the R-chain thiols react rapidly and are preferentially modified. By contrast, MNP reacts rapidly (Fig. 3) with the sulfhydryl group on the catalytic chain. This result suggests that inactivation of ATCase by MNP is a consequence of the unusual reactivity of the C-chain thiol with this mercurial, and cannot be accounted for by a relatively slow rate of reaction of this compound with the thiol groups on the regulatory chain.

The MNP derivative of ATCase is intensely colored as a result of the characteristic absorption of this mercurial in the visible region. There is a substantial shift in the absorption

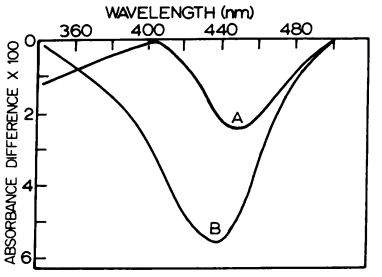

FIG. 5. Comparison of difference spectra produced on the binding of carbamoylphosphate and CTP to the MNP derivative of ATCase. Spectra were determined with a Cary 14 spectrophotometer with a 0.1 slide wire. The derivative was prepared before the spectra were obtained. ATCase was 3.5 μM; MNP was 15 μM. The buffer was 0.1 M triethanolamine$^+$Cl$^-$ (pH 7.5). (*A*) Difference spectra produced by 5.0 mM CTP. (*B*) Difference spectra produced by 4.0 mM carbamoylphosphate. The observed difference in absorbance is negative.

spectrum (Fig. 4) when the mercurial is bound to the enzyme. Subsequent experiments have shown that the change in absorption is made up of three components. Compared with the free mercurial there is a shift in the absorption band from 405 to 430 nm, an increase in the molar extinction coefficient from $1.74 \times 10^4\ M^{-1}\ cm^{-1}$ to $2.09 \times 10^4\ M^{-1}\ cm^{-1}$, and a shift in the pK_a of the phenolate from 6.5 to 8.0. We conclude that these changes are consistent with a change in the environment of the chromophore on binding to the enzyme.

The environment of the bound chromophore is perturbed by the addition of either CTP or carbamoylphosphate, as shown by the difference spectra (Fig. 5) obtained at pH 7.5. Other spectra (not shown) demonstrated that on addition of carbamoylphosphate in the presence of CTP, only the difference due to carbamoylphosphate was observed. Addition of CTP to carbamoylphosphate produced difference spectra that indicated carbamoylphosphate and CTP were binding competitively. Since phosphate compounds (such as acetylphosphate and other competitive inhibitors of carbamoylphosphate) give difference spectra identical to the spectrum shown by enzyme-bound CTP, this site is probably the carbamoylphosphate site on the catalytic subunit and not the functional CTP binding site on the R chain.

DISCUSSION

Despite the higher intrinsic reactivity of the R-chain thiols with most organomercurials, it has been possible to modify specifically the C-chain thiol with 2-chloromercuri-4-nitrophenol. This result illustrates that in modification studies that attempt to exploit the difference in reactivity of classes of thiol groups, the structure of the mercurial and choice of reaction conditions are crucial. The MNP derivative crystallized in both crystal forms (P321 and R32) under investigation in our laboratory. The derivative proved instrumental in obtaining the phases of the protein, and, by virtue of its known location on the catalytic chain, aided in interpretation of the electron density map.

Since there are six copies of each type of chain, the abrupt change in slope of the titration curve (Fig. 1*a*) occurring as it does at a molar ratio of 6, argues persuasively that a single class of sites on the enzyme has been modified. This conclusion is confirmed by crystallographic studies (7) that have shown that there is one mercury per RC pair in the asymmetric unit. Furthermore, the loss of activity that occurs on formation of the MNP derivative and the failure of the permanganate-oxidized ATCase to react specifically with this mercurial indicate that the sulfhydryl group on the C-chain thiol has been modified.

The observation that the thiol group on the isolated catalytic subunit will react with several sulfhydryl reagents, whereas the same residue in the intact complex is completely unreactive, has promoted the suggestion that this residue may be masked by the regulatory chains due to its location at the interface between R and C chains. However, our study argues that this residue is not buried upon assembly of the intact enzyme, since it still reacts readily with MNP. Preliminary interpretation of the low-resolution electron density map supports this conclusion in the sense that the density believed to be associated with the R chains is not near the Hg bound to the C-chain thiol. The difference in the reactivity of the C-chain thiol in isolated catalytic subunit and native enzyme can probably be attributed to a change in conformation that occurs in the catalytic subunit as it is incorporated into the ATCase complex.

The intensity of the absorption of the mercurial in the visible region makes these compounds excellent probes of the environment of the protein (17). The spectral changes observed when the mercurial binds to the enzyme are characteristic of the shift that occurs when it passes to a medium of low dielectric constant, suggesting that this site of the

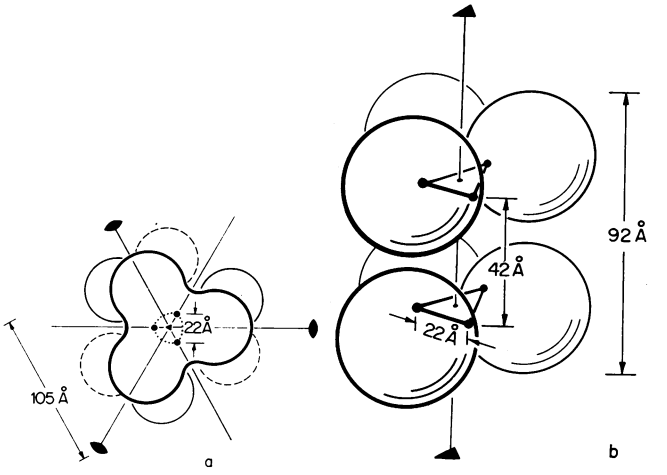

FIG. 6. Schematic representation of the ATCase molecule based on the 5.5-Å electron density map (7) showing the position of the bound mercury atoms. (*a*) View down the 3-fold axis. Three of the six mercury atoms bound to the ATCase molecule are represented by *black dots* in this view. They are located in a plane perpendicular to the three-fold axis and separated from each other by 22 Å. The *heavily outlined* part of the diagram incloses the catalytic subunit. The regulatory chains most closely associated with these C chains are represented by circles formed by *solid lines*. The second catalytic subunit is hidden from view and is located beneath the one shown in the diagram. The regulatory chains associated with this second catalytic subunit are represented by *dashed lines*. (*b*) View perpendicular to the 3-fold axis. The relative position of the two planes containing the mercury atoms are more clearly illustrated in this diagram in which the catalytic chains are idealized as spheres and the R chains have been omitted for clarity. The planes are separated from each other by 42 Å along the 3-fold axis. Molecular dimensions of 92 Å and 105 Å are only approximate.

enzyme is partially hydrophobic. Also, this chromophore is perturbed by the binding of carbamoylphosphate and various other phosphates, and the differences in the mode of binding of these compounds are of considerable interest. Although these studies suggest that MNP may be close to the carbamoylphosphate site, indirect interaction cannot be ruled out at this time.

Under the conditions at which the reaction is done, the phenolic hydroxyl group (pK 6.5) is almost completely ionized. Also, we have found that at pH values less than 7.5 the rate of reaction of this mercurial with the C-chain thiol drops precipitously, resulting (at pH 6) in reaction of MNP with the R-chain thiols. We feel that it is no coincidence that permanganate, the only other reagent that has been reported (Jacobson, G. R. & Stark, G. R., unpublished data) to react with this residue in the intact enzyme, is also negatively charged. It is reasonable to suppose that in the vicinity of the C-chain thiol there may be one or more positively charged groups that promote the reaction of negatively charged inhibitors. However, charge alone is not a sufficient criterion since PHMB, which contains a negatively charged carboxyl group, has no affinity for this residue.

It is interesting to define the location of the C-chain thiol in the structural model based on the 5.5-Å electron density map (7). A 3-fold axis passes through the center of the molecule, and three catalytic chains are arranged around this axis to form a thick disc, the catalytic subunit. Two such subunits are stacked, although there is probably little or no contact between them, one above each other (Fig. 6). The regulatory chains are clustered in pairs around the outer edge of the molecule. Three mercury atoms are located 22 Å apart in a plane perpendicular to the 3-fold axis. Two such configurations of heavy atoms are separated by 42 Å along this axis. Thus, according to the preliminary structural model (7), the C-chain thiols are located on the inner surface of the catalytic subunit.

A great deal of circumstantial evidence is accumulating that suggests that the C-chain thiol is in the general vicinity of the active site. This includes (*i*) loss of catalytic activity, although Vanaman and Stark (13) have shown that this residue is involved directly in neither binding nor catalysis, (*ii*) the masking effect of substrate analogs on the reaction of this thiol with mercurials and other inhibitors (11, 13, 23), (*iii*) failure of the MNP derivative to bind succinate (22), and (*iv*) the suggestion of Benisek (14) that the rapid oxidation of this residue occurs because permanganate is a structural analog of phosphate, a competitive inhibitor of ATCase. However, many of these observations can be interpreted alternatively as long-range effects mediated by conformational changes. Confirmation that the C-chain thiol is in the vicinity of the active site must await further crystallographic studies of complexes of the enzyme with substrate analogs.

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