# A Role for Zinc in the Quaternary Structure of Aspartate Transcarbamylase from *Escherichia coli**

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ABSTRACT: Aspartate transcarbamylase (ATCase) from *Escherichia coli* was found to contain zinc in the amount of 6 ± 1 atoms per molecule of protein ($3.1 \times 10^5$ daltons). $Zn^{2+}$ was not removed from the protein by prolonged dialysis against solutions of strong chelators and did not exchange detectably with free $^{65}Zn^{2+}$ during an exposure period of 40 days. The requirement of metal ions for the formation of intact ATCase could be demonstrated *in vivo*. *E. coli*, forced to synthesize enzyme in $Zn^{2+}$-deficient medium, contained only 30% of their transcarbamylase activity as ATCase, with the remaining 70% present as catalytic subunit, whereas bacteria grown with the normal excess concentration of $Zn^{2+}$ had less than 5% of their transcarbamylase activity as the catalytic subunit. When purified ATCase was treated with the mercurial *p*-mercuribenzoate (PMB), dissociation into catalytic and regulatory subunits occurred and $Zn^{2+}$ became easily removable from the protein by dialysis against chelators, or during the standard procedure employed for the isolation of subunits (Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2881). Regulatory subunit, obtained by this procedure, contained no detectable $Zn^{2+}$ but contained tightly bound mercuric ion in variable amounts ranging from 0.1 to 1.0 atom per polypeptide chain ($1.7 \times 10^4$ daltons). Mercuric ion appeared to be released from PMB during subunit isolation. The mercury content of regulatory subunit preparations correlated positively with their capacity to aggregate with catalytic subunit to form reconstituted ATCase. The isolated catalytic subunit also lacked $Zn^{2+}$ and, unlike the regulatory subunit, contained no detectable $Hg^{2+}$. This finding indicates that the catalytic activity of the enzyme does not depend on metal ions such as zinc or mercury. A modified procedure for subunit preparation yielded regulatory subunit containing approximately 1 zinc atom/polypeptide chain. Treatment of this material with EDTA yielded an apo-regulatory subunit, *i.e.*, material containing neither $Zn^{2+}$ nor $Hg^{2+}$. In reconstitution studies, the apo-regulatory subunit showed no detectable capacity to aggregate with catalytic subunit to form ATCase. However, subsequent addition of $Zn^{2+}$ allowed complete reconstitution of ATCase, which then contained firmly bound $Zn^{2+}$. Other metals of the IIb group, namely $Hg^{2+}$ and $Cd^{2+}$, were as effective as $Zn^{2+}$ in promoting reconstitution. Furthermore, transition ions such as copper, cobalt, and nickel also allowed reconstitution. These experiments demonstrate that zinc and certain analogous transition elements greatly stabilize the quaternary structure of ATCase. The stabilization appears to involve enhanced interchain affinity at both the r:r and r:c domains of bonding. The zinc complexing site may not be located exclusively in the regulatory subunit since preliminary experiments suggest that the catalytic subunit as well has affinity for metals. The complexes of metals with regulatory subunit or ATCase had notable spectral properties. When apo-regulatory subunit was treated with $Hg^{2+}$ to form a derivative analogous to the zinc complex, the resulting derivative had a greatly increased extinction at wavelengths below 300 nm (a 2.6-fold absorption increase at 276 nm compared to apo-regulatory subunit). The zinc regulatory subunit showed slightly increased absorption below 260 nm, in comparison to the metal-free subunit. Sulfhydryl groups of the protein are thought to participate in metal binding. The spectral characteristics of these complexes were preserved when regulatory subunit was incorporated into ATCase.

The regulatory enzyme, aspartate transcarbamylase (ATCase)[1] from *Escherichia coli*, contains two types of protein subunits, one the catalytic subunit which possesses active sites, and the other the regulatory subunit which possesses binding sites for inhibitors and activators (Gerhart and Schachman, 1965, 1968; Gerhart, 1970). Although the ligand binding specificities of ATCase are preserved in the separated subunits, the allosteric properties of ATCase exist only with the intact quaternary structure of the complex of the two proteins. This quaternary structure apparently provides not only the necessary proximity of substrate and effector binding sites in a single complex, but also the means for gross conformational changes in the enzyme associated with ligand binding (Gerhart and Schachman, 1968; Changeux and Rubin, 1968; Gerhart, 1970). These changes at the quaternary level ultimately devolve on changes of position and affinity at the intersubunit contact regions. Hence information is needed as to the nature and strength of these contact regions.

At least three contact regions, called "domains of bonding" (Monod *et al.*, 1965), are implicated in maintaining the quaternary structure of ATCase. These domains comprise: the isologous r:r domain between the two polypeptide chains of the regulatory subunit; the heterologous c:c domain between

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[1] Abbreviations used are: ATCase, aspartate transcarbamylase; PMB, *p*-hydroxymercuribenzoate.

chains within the trimeric catalytic subunits; and the heterologous r:c domains which act to cross-link the regulatory and catalytic chains within intact ATCase. Little is known about these domains in terms of their contribution to overall stability, and to transitions of quaternary structure. Also no knowledge is available as to the types of side chains which are involved in the respective bonding domains.

Inasmuch as ATCase was reconstituted from the isolated catalytic and regulatory subunits (Gerhart and Schachman, 1965), it has been tacitly assumed that protein–protein interactions alone were responsible for the stable domains of ATCase. However, several puzzling observations during this early work indicated that other constituents, and particularly metal ions, were involved in stabilizing ATCase. First, in all preparations of the regulatory subunit a substantial fraction of the protein was incapable of reconstitution (Gerhart and Schachman, 1965). Second, the reconstituted ATCase differed detectably from the native enzyme in its sensitivity to inhibition by CTP (Gerhart, 1970). Third, the preparations of the regulatory subunit invariably had a greater ultraviolet absorption than expected on the basis of the spectra of native ATCase and the purified catalytic subunit and the increased absorption was preserved in the reconstituted ATCase (Gerhart and Schachman, 1968). These observations prompted a search for metal ions in ATCase which revealed significant quantities of zinc.[2] Based on this preliminary observation $Zn^{2+}$ was added to the growth medium for the *E. coli* used as a source for ATCase. Since that time, the medium used by most workers studying ATCase has included $Zn^{2+}$ but, as yet, there has been no description of the biological experiments which demonstrate the requirement for $Zn^{2+}$ in the *in vivo* formation of ATCase. This communication describes, in addition to the *in vivo* experiments, the role of $Zn^{2+}$ (or other metal ions) in the stabilization of the domains of bonding of ATCase *in vitro*. Spectral characterization of the zinc, mercury, and metal-free derivatives of the regulatory subunit provides evidence for the presence of sulfhydryl groups in the metal binding sites. Similar findings have been reported recently by Rosenbusch and Weber (1971a,b).

## Experimental Section

### *Materials*

*Enzyme.* Purified ATCase was prepared as described by Gerhart and Holoubek (1967). Catalytic and regulatory subunits of ATCase were prepared according to the procedure of Gerhart and Holoubek (1967), except for modifications in the preparation of zinc-regulatory subunit and apo-regulatory subunit. These modifications are discussed in detail below in Methods.

*Chemicals.* Reagent grade chemicals were used unless otherwise specified. For work with trace metal ions, deionized tap water was further purified by distillation in a Corning AG1b still, followed by passage through a mixed-bed demineralizing resin (Barnstead Still and Sterilizer Co.). Spectrographically standardized sources of metals were obtained as the salts cadmium sulfate, cobalt nitrate, copper sulfate, magnesium sulfate, manganese sulfate and mercuric chloride, and zinc was obtained as metal rod, from Johnson-Matthey and Co. Ltd., through the Jarrell-Ash Co., Waltham, Mass. Ultra Pure calcium chloride was purchased from Alfa Inorganics. 1,10-Phenanthroline and diphenylthiocarbazone (dithizone) were the products of the G. F. Smith Chemical Co. *p*-Hydroxymercuribenzoate (PMB) (sodium salt) was obtained from Sigma Chemical Co., and was purified immediately before use by precipitation in 0.1 N hydrochloric acid, as described by Boyer (1954). Dilithium carbamyl phosphate was obtained and purified as described previously (Gerhart and Pardee, 1962). [$^{65}$Zn]Zinc chloride (0.1 mCi/$\mu$g) and [$^{203}$Hg]PMB (2 mCi/mmole) were purchased from Amersham-Searle. Carboxyl-labeled [$^{14}$C]PMB (10 mCi/mmole) was obtained from Schwarz BioResearch Co.

*Dialysis Tubing.* Dialysis tubing (Visking cellulose casing) was treated before use to reduce pore size (Callanan *et al.*, 1957; Craig, 1967) and prevent escape of regulatory subunit. Dry unwashed tubing was heated at 90° for 24 hr in a dry oven. The tubing was then washed by heating to 80° for 30 min in two successive changes of a solution of 5% sodium bicarbonate, followed by extensive rinsing in distilled water. The tubing was then soaked in a solution 0.01 M EDTA overnight (or longer for storage) before a final rinsing in three changes of demineralized distilled water.

*Buffers.* The buffer used most frequently for the preparation and storage of ATCase and its subunits contained 40 mM potassium phosphate (pH 7.0), 2 mM 2-mercaptoethanol, and 0.2 mM EDTA (Gerhart and Pardee, 1962); this will be referred to as "standard buffer." The buffer routinely used for assay of the activity of ATCase and catalytic subunit was 50 mM imidazole–acetate (pH 7.0). Other buffers will be described in the text.

### *Methods*

*Cell Growth.* For growth of *E. coli* in "low-zinc" medium, the procedures described by Torriani (1968) were followed. Autoclavable polypropylene and high-density polyethylene labware (Nalgene Corp.) and sterile plastic pipets (Falcon Plastics) were used throughout the experiments. The cell growth medium and diploid cell strain for production of ATCase were prepared and used as described by Gerhart and Holoubek (1967). Growth curves were followed for all cultures to determine precisely the time of uracil exhaustion. The following modifications were made for the removal of metal ions from the growth medium. The stock solutions of phosphate buffer, L-histidine, uracil, and D-glucose were purified individually by extraction with 0.003% dithizone in carbon tetrachloride. Dithizone was removed by three successive extractions with carbon tetrachloride, and the solutions were separately autoclaved in plastic flasks. Both ferrous sulfate and zinc sulfate were omitted from the medium. All other components were spectrochemically pure. With these precautions, $Zn^{2+}$ was reduced to the level of $1 \times 10^{-7}$ M in the medium, comparable to the level reached by Torriani (1968). The bacterial inoculum used for low-zinc ATCase preparation was obtained from cultures grown for 12 serial transfers in low-zinc medium.

*Sucrose Gradient Analysis of ATCase in Cell Extracts.* Cell extracts were prepared by sonic oscillation of washed *E. coli* suspended in 40 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol. After cell debris was removed by centrifugation, aliquots (approximately 0.2-ml volume) of each extract were layered individually on 5.5-ml gradients of 5–20% sucrose in the above buffer. The gradients were centrifuged in the Spinco Model L2 preparative ultracentrifuge, using the SW 50.1 rotor, at 20° for 6 hr at 49,000 rpm. Fractions were collected by the method of Martin and

[2] These initial experiments were conducted in collaboration with B. L. Vallee in 1965, and the authors are indebted to him for the preliminary results which provided the impetus for the studies reported here.

Ames (1961). Each fraction was assayed for ATCase activity by the colorimetric procedure (Gerhart and Pardee, 1962) with assay mixtures containing 40 mM imidazole–acetate (pH 7.0), 25 mM L-aspartate, and 4 mM carbamyl phosphate. Since drop size varied during fraction collection, the volume of fractions was measured with a calibrated plastic pipet.

*Preparation of Apo-Regulatory Subunit and the Zinc and Mercury Derivatives.* ATCase was dissociated with PMB and the catalytic and regulatory subunits separated on DEAE-Sephadex[3] according to the procedure of Gerhart and Holoubek (1967). To the pooled fractions obtained from the column step were added, first, 2-mercpatoethanol to a final concentration of 0.02 M and, second, zinc acetate (stock solution 0.05 M in water) to a final concentration of 2 mM. Addition of reagents in this order was important to avoid precipitation of zinc, which occurred in the absence of 2-mercaptoethanol. Occasionally, a slight turbidity formed on zinc addition, but quickly disappeared. When necessary, regulatory subunit was concentrated at this stage in the purification scheme by dialysis of the zinc treated material versus approximately 50 volumes of saturated ammonium sulfate (3.6 M) containing 50 mM 2-mercaptoethanol. This dialysis was performed with dialysis tubing treated as described earlier to ensure retention of protein. After dialysis for 24 hr at 4°, the precipitate was collected by centrifugation, suspended in a minimum volume of potassium phosphate (40 mM, pH 7.0) containing 2 mM 2-mercaptoethanol and dialyzed against 100 volumes of this same solution to reduce ammonium sulfate. The protein preparation obtained was identified as "zinc-regulatory subunit," and was stored routinely in plastic tubes at 4°. For periods of storage longer than a few days, zinc acetate was added to a final concentration of 0.2 mM.

A metal-free-regulatory subunit was prepared by dialyzing the zinc-regulatory subunit extensively against a solution of 0.05 M imidazole–acetate (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol. Dialysis was performed at 4° with three buffer changes, each for a period of 12 hr at a buffer to sample volume ratio of 500:1. In order to guard against the oxidation of protein sulfhydryl groups, dialysis buffers were purged with nitrogen prior to use, and precautions were taken to exclude air from the buffer during dialysis. The material obtained from this treatment was identified as "apo-regulatory subunit." It was stored in plastic tubes at 4°.

A mercury derivative of the regulatory subunit was prepared by dialyzing the zinc-regulatory subunit or the apo subunit (at approximately 2 mg of protein/ml) *vs.* a nitrogen-purged solution containing 0.2 mM mercuric acetate, 0.5 mM 2-mercaptoethanol, and 0.2 M sodium acetate (pH 5.5) for 24 hr (volume ratio 1:500) at 4°. In order to reduce the concentration of free metal ions the mercury-treated sample was then dialyzed *vs.* a nitrogen-purged solution of 0.05 M Tris-acetate (pH 7.0) for 48 hr (volume ratio 1:500 with one buffer change after 24 hr). This material was identified as "mercury-regulatory subunit" and was stored in plastic tubes at 4°.

*Metal Analyses.* For all work with trace metals, precautions to reduce contamination by zinc and other metals were followed, as recommended by Thiers (1957).

Dilute zinc standards were prepared immediately before use from a 0.1 M zinc chloride standard stock solution, 0.1 N in hydrochloric acid, prepared by dissolving spectrographically pure zinc metal rod in purified hydrochloric acid (Thiers, 1957).

For atomic absorption analysis of zinc, a Perkin-Elmer Model 303 atomic absorption spectrometer was used according to standard procedures.

Zinc was determined colorimetrically by the procedure of Kägi and Vallee (1958) with the following modifications. The volumes of samples and reagents were reduced 10-fold and the final aqueous mixture was extracted once with 3 ml of 0.0015% dithizone in carbon tetrachloride. Glass tubes of approximately 25-ml capacity were used so that the two phases could be mixed thoroughly by agitation on a Vortex Jr. mixer to facilitate the extraction. With these modifications, zinc in the amount of 40 nmoles yielded a final net $A_{525}$ of 1.2 (1-cm path length). Readings were proportional to the amount of zinc up to this level. EDTA interfered strongly and was removed from protein samples by dialysis. Samples containing more than 500 $\mu$g of protein stabilized emulsions of the aqueous and carbon tetrachloride phases; this problem was overcome by prior hydrolysis of protein samples in 6 N HCl for 16 hr at 110° in evacuated sealed tubes. Compensation for the amount of HCl introduced in the hydrolysate was achieved by reducing appropriately the volume of HCl in the reagent mixture.

Mercury was assayed colorimetrically by the procedure of Tyuma *et al.* (1966), with a tenfold reduction in scale.

*$^{65}$Zinc Exchange.* Exchange of radioactive zinc with zinc bound to ATCase was measured by a procedure similar to that described by Drum *et al.* (1969b). The buffer (see Results for details) for dialysis was prepared and made $1 \times 10^{-5}$ M in zinc chloride. Carrier-free [$^{65}$Zn]zinc chloride was added to give approximately $5 \times 10^{4}$ cpm/ml. A volume of 2.85 ml of this $^{65}$Zn–buffer solution was mixed with 0.15 ml of stock ATCase ($2 \times 10^{-4}$ M) to give a final ATCase concentration of $1 \times 10^{-5}$ M (0.31% protein). The entire 3 ml of this enzyme solution was placed in a dialysis bag attached to a short length of glass tubing in a silicone rubber stopper. The bag was then suspended in a graduated cylinder containing 60 ml of the $^{65}$Zn–dialysis buffer. Equilibration proceeded at room temperature, or 0°, with stirring. Sample aliquots of 100-$\mu$l volume were withdrawn at various times from the inside and outside solutions and counted as described later.

*Enzyme and Protein Analyses.* ATCase was assayed either by the [$^{14}$C]aspartate assay (Porter *et al.*, 1969) or by the colorimetric procedure of Gerhart and Pardee (1962). Protein was determined spectrophotometrically, using values of $E^{0.1\%}_{280nm}$ of 0.59 for ATCase and 0.72 for catalytic subunit. The extinction coefficient of the regulatory subunit depended on the type and amount of metal ion present, as will be discussed in the Results section. When ultraviolet-absorbing material interfered with direct spectrophotometric determination, ATCase protein was determined by the method of Lowry *et al.* (1951), using crystallized bovine serum albumin (Armour Pharmaceutical Co.) as a standard. Regulatory subunit protein was determined either by the Lowry procedure or by synthetic boundary formation in the analytical ultracentrifuge (Richards and Schachman, 1959). The values obtained by these methods agreed within 10%.

*Determination of Radioactivity.* $^{14}$C and $^{203}$Hg were measured by liquid $\beta$-scintillation counting in a Packard Tri-Carb scintillation spectrometer. Scintillation fluid used for $^{203}$Hg and $^{14}$C counting contained 500 g of Triton X100 detergent (Packard), and 5 g of Omnifluor (New England Nuclear), added per l. of toluene. $^{65}$Zn was determined by crys-

[3] Recently an improved method for separating subunits has been devised by M. Kirschner (reported in Cohlberg *et al.*, in preparation). This method substitutes neohydrin for PMB and DEAE-cellulose for DEAE-Sephadex.

TABLE I: Zinc Content of ATCase.[c]

| ATCase Prepn No. | Method of Zn Determination[a] | No. of Zn Determinations | Zn:ATCase (g-atoms/Mole)[b] |
|---|---|---|---|
| 1 | I | 17 | 6.1 ± 0.7 |
| 2 | I | 3 | 5.3 ± 0.2 |
| 2 | II | 8 | 5.2 ± 0.1 |
| 3 | I | 4 | 5.8 ± 0.3 |
| 4 | I | 2 | 5.8 ± 0.2 |
| 5 | I | 2 | 5.8 ± 0.2 |

[a] Zinc was analyzed either by atomic absorption spectroscopy (designated "I"), or by colorimetric dithizone assay (designated "II"), as described in Methods. [b] Error is reported as average deviation. A molecular weight of $3.1 \times 10^5$ for ATCase was assumed for this calculation. [c] ATCase was dialyzed for at least 2 days against standard buffer before zinc assays. Before colorimetric analysis EDTA was removed by dialysis against 0.04 M potassium phosphate (pH 7.0) as described in Methods.

TABLE II: Zinc Content of ATCase after Exposure to Chelators.[a]

| Condition of Treatment | Zinc:ATCase, (g-atoms/Mole)[b] Before Treatment | After Treatment |
|---|---|---|
| 1. Dialysis against 0.05 M Tris-acetate (pH 8.0)–2 mM *o*-phenanthroline | 5.2 ± 0.2 | 5.4 ± 0.2 |
| 2. Dialysis against 0.04 M potassium phosphate (pH 7.0)–0.01 M *o*-phenanthroline–0.1 M EDTA | 7.6 ± 0.1 | 7.5 ± 1.0 |
| 3. Dialysis against 0.1 M Na-EDTA (pH 9.0) | 5.2 ± 0.2 | 5.6 ± 0.4 |
| 4. Dialysis against 0.05 M Tris-acetate (pH 8.0)–1 mM 8-hydroxyquinoline | 6.2 | 5.8 ± 0.4 |
| 5. Dialysis against 0.05 M Tris-acetate (pH 8.0)–0.5 mM 8-hydroxyquinoline-5-sulfonate | 5.2 ± 0.2 | 5.8 ± 0.2 |
| 6. Passage through column of Chelex-100 in 0.04 M potassium phosphate (pH 7.0) | 6.5 ± 0.2 | 6.4 ± 0.2 |

[a] Conditions of dialysis included three buffer changes, each of 100-fold or greater volume excess; 24 hr or longer per buffer change; 4° except for conditions 4 and 6 at 25°. [b] Error given is average deviation for duplicate determinations.

tal scintillation counting in a Nuclear-Chicago Biospan $\gamma$ counting spectrometer.

*Sedimentation Velocity.* Sedimentation velocity experiments were conducted at 60,000 rpm in a Spinco Model E ultracentrifuge equipped with a cylinder lens schlieren optical system and a rotatable light source for Rayleigh interference optics. Photographic plates (Metallographic) were analyzed with the aid of a Gaertner microcomparator. The ultracentrifuge experiments were performed at constant temperature (19–22°) measured and controlled by the RTIC unit supplied by the manufacturer. Protein concentrations of the individual species were obtained from the measured areas of the schlieren patterns of the corresponding sedimenting boundaries; for the conversion of area to protein concentration a calibration factor was employed based on a separate experiment on ATCase of known protein concentration in which the area of the schlieren pattern was measured (Richards and Schachman, 1959; Schachman, 1963).

*Electrophoresis.* Zone electrophoresis experiments were performed on 14.6-cm cellulose polyacetate strips (Gelman Sephraphore III) in a Microzone electrophoresis cell, Model R-101 (Beckman-Spinco). The electrophoresis buffer was 20 mM potassium phosphate (pH 7.0) containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol unless specified otherwise. The protein was fixed and stained by immersion of the membrane in a solution of 0.2% ponceau S in 3% trichloroacetic acid, and 3% sulfosalicylic acid for approximately 7 min; the membrane was then rinsed in 5% acetic acid and immersed for several hours in 0.002% nigrosin (Allied Chemical Co.) dissolved in 2% acetic acid. After rinsing in acetic acid, the membrane was dried at room temperature and stored in a cellophane envelope.

## Results

*Zinc Content of ATCase.* The initial analysis of ATCase for trace elements was kindly performed by Dr. B. L. Vallee in 1965 and revealed the presence of zinc. Since then, our measurement of zinc by atomic absorption spectroscopy and by specific complex formation with dithizone has indicated the presence of approximately 6 ± 1 zinc atoms per molecule of enzyme, as shown in Table I. The five enzyme preparations studied were purified at different times over a period of 4 years, by the procedure described by Gerhart and Holoubek (1967). Observed variation in the zinc content measured from preparation to preparation was not significantly larger than the variation obtained among samples of a single preparation. Similar results on the presence of zinc in ATCase have been reported by Rosenbusch and Weber (1971a,b).

The presence of other metal ions in ATCase obtained under normal growth conditions has not been systematically investigated. However, experiments described in a later section indicate certain other metal ions, notably cadmium and mercury and a number of transition metal ions, could be incorporated into ATCase in place of zinc. Since the routine preparation of ATCase by the published procedure begins with bacteria grown with a relatively high concentration of zinc in their growth medium, the incorporation of zinc into ATCase may be favored over other metals.[4]

Further evidence for zinc as an integral part of native ATCase was provided by the high stability of the metal–protein complex. As shown in Table II, the zinc in ATCase was

[4] Rosenbusch and Weber (1971b) report the preparation of $Cd^{2+}$ containing ATCase from *E. coli* derepressed in zinc-deficient growth medium containing cadmium chloride.

not removed by dialysis of the enzyme for 3–10 days against solutions of chelators, such as EDTA and 1,10-phenanthroline, which have a known strong affinity for zinc.

Further information about the ATCase–zinc complex was obtained from zinc exchange experiments, in which ATCase was dialyzed for prolonged periods against solutions of [$^{65}$Zn]-zinc chloride, and tested for the incorporation of radioactive zinc. In the presence of 40 mM potassium phosphate buffer (pH 7) at 25°, a small amount of $^{65}Zn^{2+}$ was found to associate within 1 day with ATCase, to the extent of approximately one atom per ATCase molecule; however, the amount of associated $^{65}Zn^{2+}$ did not increase further during the following 18 days, nor approach the level expected for total exchange (*i.e.*, 78% of the zinc in ATCase replaced by $^{65}Zn^{2+}$). Over 95% of this small amount of radioactive zinc was removed from ATCase by subsequent dialysis of the protein against EDTA, or by a single passage of enzyme through a Chelex-100 column, although the total zinc content of ATCase remained approximately six atoms per molecule. Thus, the added radioactive zinc appeared complexed only weakly to ATCase, and not liganded in the manner observed for the zinc originally present in the enzyme. From these experiments, it could be concluded that less than 2% of the zinc in ATCase was replaced by $^{65}Zn^{2+}$ during 19-days exposure. In view of the unusual stability of this metal complex, conditions were sought which might affect protein structure and thereby enhance zinc exchange. The following conditions are known to affect heterotropic and homotropic interactions in ATCase (Weitzman and Wilson, 1966; Gerhart and Schachman, 1965; Gerhart, 1970): (1) 40 mM potassium phosphate (pH 7.0), 0°; (2) 40 mM potassium phosphate (pH 7.0)–25 mM potassium aspartate, 25°; (3) 40 mM potassium phosphate (pH 7.0)–1.0 M urea, 25°; (4) 50 mM potassium glycinate (pH 10.0), 25°.

Under all these conditions, the zinc content of ATCase remained unchanged in 40 days and zinc exchange was not enhanced with the possible exception of 1.0 M urea, which led to a 2% incorporation of $^{65}Zn^{2+}$ into ATCase in 40 days. The chelation and exchange experiments demonstrate the high kinetic stability of the zinc–ATCase complex. The absence of data on rates of association of zinc with ATCase or of equilibration of zinc–ATCase with chelators necessarily precludes an experimental estimate of the thermodynamic stability of this metal–protein complex.

*In Vivo Requirement for Zinc in the Formation of ATCase.* In order to investigate the importance of zinc for the original *in vivo* formation of ATCase, *E. coli* were grown and derepressed for ATCase synthesis in media containing or lacking $Zn^{2+}$. Cell extracts were analyzed on sucrose gradients to determine the fraction of enzyme activity sedimenting at the position characteristic of purified ATCase (12 S).

The growth medium routinely used for the bacterial production of ATCase (Gerhart and Holoubek, 1967) contained $1 \times 10^{-5}$ M zinc sulfate, a supplement probably providing $Zn^{2+}$ in excess of the bacterial requirement. For low-zinc growth conditions, zinc sulfate was omitted from the medium and precautions were taken to reduce the amount of zinc contamination, as described in Methods. The level of zinc remaining in low-zinc medium was $1 \times 10^{-7}$ M, as determined by atomic absorption spectrometry of growth medium concentrated tenfold by flash evaporation. This residual zinc was shown by calculation to be too low to support ATCase production at the normal level, as follows. The specially constructed strain of *E. coli*, described by Gerhart and Holoubek (1967), synthesizes up to 10% of its cell protein as ATCase.

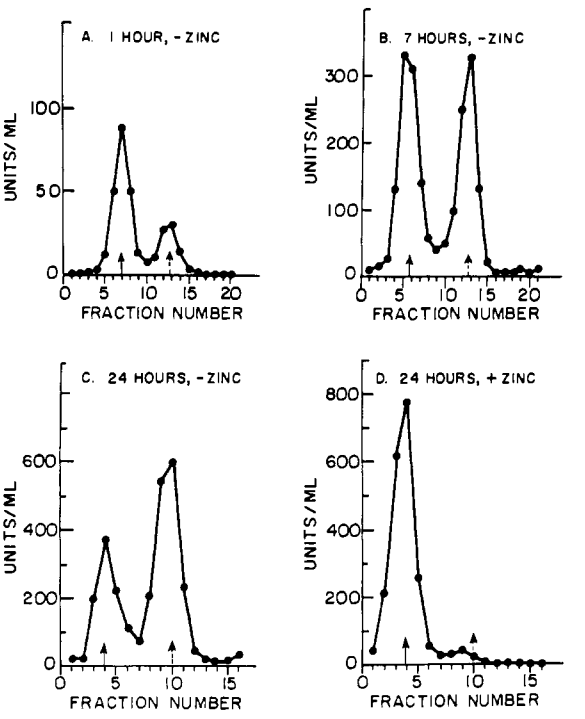


FIGURE 1: Sedimentation profiles of ATCase activity in extracts of cells grown with and without added $Zn^{2+}$. Cell extracts were prepared and analyzed by zone centrifugation as described in Methods. The centrifuge tubes contained 5.5-ml gradients of 5–20% sucrose in 40 mM potassium phosphate (pH 7.0)–1 mM EDTA–1 mM dithiothreitol. Fractions were assayed for ATCase activity in 40 mM imidazole-acetate (pH 7.0), 25 mM L-aspartate, and 4 mM carbamyl phosphate, by the colorimetric procedure. The volumes of all gradients were identical, but the number of fractions collected varied. The sedimentation positions of purified ATCase and catalytic subunit were determined in a separate sample and are designated in the figure by solid and broken arrows, respectively, on the abscissa. A unit of enzyme activity is defined as that amount which forms 1 μmole of carbamyl aspartate/hr.

Derepressed cells at a density of 2 mg wet weight of cells/ml (in late-log phase) would contain at least 10–15 μg of ATCase/ml of growth medium, which would be expected to contain the equivalent of 2 to 3 $\times 10^{-10}$ mole of $Zn^{2+}$, assuming six zinc atoms incorporated per ATCase molecule. This required amount of zinc would exceed that available in the medium by a factor of 2- to 3-fold. The growth rate of cells in the zinc-deficient medium was not significantly reduced during the derepression period, although it was reduced by 10–20% prior to the derepression period while uracil was present.

Examples of sedimentation profiles of ATCase activity in individual extracts are shown in Figure 1. Panels A, B, and C present the activity profiles of extracts from cells derepressed in low-zinc medium for 1, 7, and 24 hr, respectively. In Figure 1D, a representative extract from cells grown in high-zinc medium is also shown. When zinc was present, virtually all ATCase activity was present at the 12S position characteristic of purified ATCase, regardless of the duration of the derepression. In contrast, the cells grown in low-zinc medium contained not only 12S material, but significant amounts of enzymic activity sedimenting more slowly, at the 6S position. This 6S species was identified as catalytic subunit on the basis of its sedimentation and kinetic behavior. The percentage of total enzymic activity present as catalytic subunit was substantial after only 1 hr derepression and increased during the following 12-hr period, as shown in Figure 2, attaining a

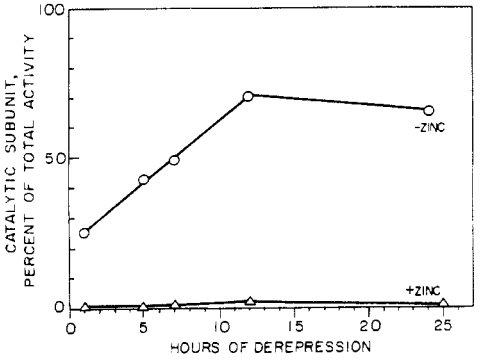


FIGURE 2: Percentage of total ATCase activity present as catalytic subunit in extracts of *E. coli* grown in the presence and absence of zinc. Sedimentation analysis of extracts containing ATCase activity is described in the legend of Figure 1, and in Methods. Total ATCase activity at the catalytic subunit (6 S) and ATCase (12 S) positions after centrifugation was calculated by multiplying the units of activity per ml times the fraction volume, and summing this product for all fractions comprising that peak. In order to simplify the calculation of these percentages, assay conditions were chosen (described in legend of Figure 1) in which ATCase and catalytic subunit had approximately the same specific activity, on a "per site" basis. The observed enzymic activities under the chosen assay conditions were $6.3 \times 10^3$ units per mg of catalytic subunit and $4.2 \times 10^5$ units per mg of ATCase. When ATCase is taken as containing 67% catalytic subunit by weight, and each catalytic subunit considered to have three active sites, the specific activities were calculated to be $2.1 \times 10^8$ units ($M^{-1}$ C-site) for catalytic subunit, and $2.2 \times 10^8$ units ($M^{-1}$ C-site) for ATCase.

TABLE III: Zinc and Mercury Content of ATCase Subunits.[a]

| Subunit | Method of Prepn[b] | g-atoms of Zinc/g-atoms of Mercury per Moles of Subunit[c] | Moles of Subunit[c] |
|---|---|---|---|
| Catalytic subunit | 1 | $\leq 0.07$ | $\leq 0.01$ |
| Regulatory subunit | 1 | $\leq 0.02$ | $0.40 \pm 0.35$ |
| | 2 | $1.4 \pm 0.2$ | $\leq 0.05$ |
| | 3 | $1.0 \pm 0.05$ | $\leq 0.05$ |
| | 4 | $\leq 0.02$ | $\leq 0.05$ |
| | 5 | $\leq 0.02$ | $1.1 \pm 0.1$ |

[a] Subunit preparations were dialyzed against standard buffer before zinc analysis by atomic absorption spectroscopy, and mercury analysis by colorimetric assay as described in Methods. [b] Subunits were prepared by: (1) the standard procedure of Gerhart and Holoubek (1967); 17 determinations on 8 different preparations; (2) the zinc treatment procedure described in Methods; 9 determinations on 5 different preparations; (3) as in 2 above, followed by passage of subunit over Chelex-100; 4 determinations on 2 different preparations; (4) apo-regulatory subunit procedure as described in Methods; (5) dialysis of the apo-regulatory subunit (entry 4) against the mercuric ion 2-mercaptoethanol complex, as described in Methods for mercury-regulatory subunit. [c] Values are reported with average deviation. Molecular weight of catalytic subunit is taken to be $1 \times 10^5$ daltons, and molecular weight of regulatory subunit is taken as the weight of the regulatory polypeptide chain, $1.7 \times 10^4$ daltons.

maximum of 70% of the total activity. During the period from 12 to 24 hr, the total transcarbamylase activity and cell density increased an additional twofold; however, the percentage of transcarbamylase activity as catalytic subunit remained constant at 70% of the total activity.

In view of these results, the M-56 medium originally used for ATCase production (Gerhart and Pardee, 1962) was probably zinc deficient. It contained no source of zinc other than as a metal contaminant, and derepressed cells routinely formed both catalytic subunit and ATCase. The presence of catalytic subunit explains the poor CTP inhibition observed for ATCase activity from highly derepressed bacteria (J. C. Gerhart, unpublished). Supplementation of this medium with $10^{-5}$ M zinc sulfate has resulted in the almost exclusive production of ATCase, accompanied by only trace amounts of catalytic subunit (Gerhart and Holoubek, 1967).

These experiments show clearly the essentiality of zinc ion for the *in vivo* formation or maintenance of ATCase as a complex of catalytic and regulatory subunits. Since catalytic subunit was itself present as a stable, active species, it seemed plausible that zinc might be required specifically at a late step of ATCase formation, namely the aggregation of catalytic and regulatory subunits. Preliminary experiments have been directed toward investigating this possibility, by determining whether catalytic and regulatory subunits which had accumulated *in vivo* under zinc-deficient conditions could be converted later to ATCase by subsequent addition of $Zn^{2+}$ to cells or extracts. In these experiments, bacteria were derepressed for ATCase for 18 hr in low-zinc medium and then supplemented with zinc sulfate ($10^{-5}$ M) for 30 min. These briefly supplemented cells were compared by zone centrifugation with control cells which received no zinc. Despite supplementation of these cultures with zinc, catalytic subunit still accounted for 70% of the total activity and was not converted into ATCase. Zinc uptake by the bacteria during this 30-min interval appeared appreciable; approximately 15% of the added zinc ion remained associated with cells after washing, as determined with radioactive zinc. Longer exposure periods (*e.g.*, 3 hr) also did not allow detectable conversion of catalytic subunit into ATCase, although a net increase in ATCase content occurred which was consistent with *de novo* protein synthesis. And finally, extracts from derepressed zinc-deficient cells were incubated with zinc ion ($10^{-5}$ M), but, as with intact cells, catalytic subunit was not converted into ATCase. Since subsequent *in vitro* experiments did show the importance of zinc for aggregation, we suggest that these *in vivo* results may have been affected by the unavailability of regulatory subunits due to either inactivation (*e.g.*, oxidation of sulfhydryl groups) or inadequate synthesis in the absence of zinc.

*Zinc and Mercury Content of the Regulatory and Catalytic Subunits.* Since purified ATCase contained six tightly bound zinc atoms per molecule, the regulatory and catalytic subunits were prepared and examined for their metal content and for their dependence on metal ions for structure and activity. Surprisingly, though, there was no detectable zinc in either the catalytic or regulatory subunits prepared by the PMB dissociation of ATCase and separation on DEAE-Sephadex by the procedure of Gerhart and Holoubek (1967). Complete isolation of the subunits was not necessary for zinc to be lost from the protein. Indeed, ATCase dissociated with PMB (without separation of the subunits) and then dialyzed against a chelator such as EDTA retained no significant amount of zinc. Thus, displacement of zinc from its tight complex with ATCase occurred when PMB reacted with the protein sulf-

hydryl groups and caused disruption of the bonding between catalytic and regulatory subunits. Analysis for zinc, as described in Table III, showed that less than 0.1 zinc atom was present per molecule of purified catalytic subunit. Both the absence of zinc in the purified subunit, together with the ineffectiveness of added zinc on the enzymic activity of either the catalytic subunit or ATCase, demonstrated that zinc has no role in catalytic activity. Mercury was also assayed in the catalytic subunit and was present in only negligible amount ($\leq$0.01 mercury atom per subunit molecule).

Isolated regulatory subunit, like catalytic subunit, did not contain significant amounts of zinc. However, in contrast to catalytic subunit, regulatory subunit was found to contain substantial amounts of mercury even after extensive dialysis against solutions of 2-mercaptoethanol. This residual amount of mercury found in regulatory subunit preparations varied widely, over a range from 0.1 to 1 mercury atom per $1.7 \times 10^4$ daltons of protein (*i.e.*, per polypeptide chain). In Table III, this material is entered as the first preparation of regulatory subunit, obtained by the published procedure of Gerhart and Holoubek (1967). The other preparations of regulatory subunit shown in Table III will be discussed later.

Mercury was retained in regulatory subunit primarily as $Hg^{2+}$ and not PMB, for when PMB labeled with $^{14}C$ in the 1-carboxyl position of the benzoate moiety was used for the dissociation of ATCase, the resulting regulatory subunit preparations contained little or no detectable $^{14}C$ after treatment with sulfhydryl compounds. Yet, the same preparations contained significant quantities of mercury, as determined by chemical assay. These results are presented in Table IV. Furthermore, when PMB labeled with $^{203}Hg$ in the mercury position of the molecule was used for dissociation of ATCase and preparation of subunits, $^{203}Hg$ label was retained in regulatory subunit in amounts comparable to those measured by chemical assay. These experiments suggest that the mercurial used for preparation of ATCase subunits was the source of mercuric ion present in regulatory subunit.

Mercuric ion was probably not present initially in the PMB samples since prior purification of the PMB by repeated acid precipitation as recommended by Boyer (1954) did not reduce the amount of mercury retained in the final regulatory subunit. More likely, $Hg^{2+}$ was released from PMB in the course of the subunit preparation procedure. Preliminary experiments showed that PMB released $Hg^{2+}$ with a half-life of about 2 days at 25°, in neutral buffered solutions (without protein) containing 2-mercaptoethanol (10 mM) and zinc ion (1 mM); mercuric ion was released, along with an organic product cochromatographing with [$^{14}C$]benzoate. Zinc ion greatly accelerated the rate of breakdown of PMB.

The variable content of mercuric ion in regulatory subunit preparations was thus probably due to uncontrolled experimental conditions which influenced both the rate of release of mercury from PMB, and the reactivity of sulfhydryl groups of the protein with mercuric ion. The mercury–carbon bond of organic mercurials is known to be weak, in the range of 15–19 kcal/per mole (Cottrell, 1954), and several examples have been documented in which mercuric ion is split from substituted aliphatic and aromatic mercurials in the presence of dithiol compounds (Benesch and Benesch, 1952; Daniel *et al.*, 1971).

Two characteristics of these mercury-containing regulatory subunits did provide information about the role of metals in ATCase. First, these preparations of regulatory subunit were capable of aggregating with catalytic subunits to form ATCase, even though the regulatory subunits contained mercury and no zinc. Preparations containing high mercury content were found in most cases to reconstitute to a greater extent than those with little mercury, as judged by the amount of regulatory subunit remaining free in the presence of excess catalytic subunit, after separation of test mixtures by zone electrophoresis. This rough correlation between mercury content and capacity to reconstitute[5] suggested that mercuric ion might substitute for zinc ion as a metal required for subunit association.

TABLE IV: Unequal Retention of Organic and Mercury Portions of PMB in Regulatory Subunit Prepared by the Standard Procedure.[a]

| Prepn No. | Mercury Atoms/ Regulatory Chain | [$^{14}C$]PMB Residues/ Regulatory Chain |
|---|---|---|
| 1 | 0.15 | $\leq$0.005 |
| 2 | 0.3 | $\leq$0.005 |
| 3 | 0.9 | 0.06 |
| 4 | 1.1 | 0.13 |

[a] Regulatory subunit was prepared by the procedure of Gerhart and Holoubek (1967) using $^{14}C$-labeled PMB for the initial dissociation of ATCase. Mercury was determined by the chemical assay described in Methods. The regulatory chain was taken to have a molecular weight of $1.7 \times 10^4$ grams per mole.

A second notable characteristic of regulatory subunit prepared from PMB-dissociated ATCase was its unexpectedly high extinction in the near-ultraviolet spectral region. On the basis of the difference spectrum between ATCase and catalytic subunit, the regulatory subunit was expected to have an extinction of approximately 0.35 $cm^{-1}$ $(mg/ml)^{-1}$ at 280 nm. Yet, in practice, the extinction values ranged from 0.4 to 1.2 $cm^{-1}$ $(mg/ml)^{-1}$. Preparations with high extinction contained high levels of mercury, approaching 1 mercury atom per polypeptide chain ($1.7 \times 10^4$ daltons). Likewise, preparations with low mercury content had low extinction values. The extinction could not be attributed to protein-bound PMB since PMB was not retained by the regulatory subunit, as discussed earlier for the [$^{14}C$]PMB experiments (Table IV).

In summary, these experiments with the purified subunits provided the following evidence and indications. First, PMB displaced zinc ion from ATCase, perhaps by directly disrupting a zinc-protein complex. Second, the regulatory subunit retained mercuric ion in a specific complex with high ultraviolet extinction. Third, the presence of mercuric ion in the regulatory subunit appeared to be a necessary, though not sufficient, condition for reconstitution with catalytic subunit to form ATCase. And fourth, the catalytic subunit remained stable and active, though it contained no zinc or mercury, indicating the absence of a role for these metals in catalysis in ATCase.

[5] Several preparations of regulatory subunit did contain high levels of mercury but failed to reconstitute well. Thus, other factors than metal content are important. Preliminary experiments indicated that the sulfhydryl groups of the regulatory subunit oxidized during storage, as determined by 5,5′-dithiobis(2-nitrobenzoic) acid titrations, and that oxidized preparations of subunit did not reconstitute well. Some oxidized preparations could be made to reconstitute by incubation with catalytic subunit at 35° in a solution of 50 mM Tris-HCl (pH 8.5) and 20 mM 2-mercaptoethanol for 30 min.

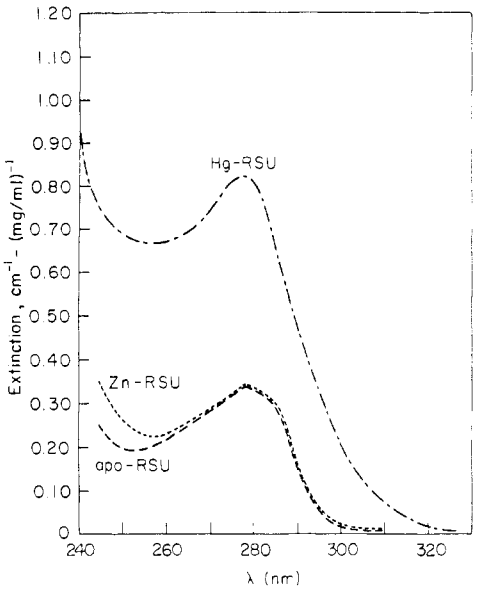


FIGURE 3: Absorption spectra of apo-regulatory subunit (apo-RSU) and the zinc (Zn-RSU) and mercury (Hg-RSU) derivatives of the regulatory subunit. Spectra were measured in 0.05 M Tris-HCl (pH 7.0) *vs.* a buffer blank using cells of 1.0-cm path length. Protein concentration for each sample was determined refractometrically as described in Methods. Apo-regulatory subunit and the two metallo-derivatives were prepared as described in Methods. Extinction is expressed in units of absorption per 1.0-cm path length for a solution of 1.0 mg/ml, *i.e.*, $cm^{-1}$ $(mg/ml^{-1})$.

*Preparation and Characterization of the Zinc-Regulatory Subunit and the Metal-Free Regulatory Subunit.* Although the previous experiments did provide circumstantial evidence for the structural role of metal ions in ATCase and for the nature of the liganding groups of the protein, the variability of the standard regulatory subunit preparations (Gerhart and Holoubek, 1967) hindered further study. Also, since these preparations did not contain zinc, but mercury, the question arose concerning their relation to native zinc-containing ATCase.

A zinc-containing regulatory subunit was successfully prepared by a modified procedure in which $Zn^{2+}$ was provided in excess at early stages when the subunit might have otherwise scavenged $Hg^{2+}$. As described in Methods, ATCase was dissociated with PMB and the freshly separated regulatory subunit was exposed to 2-mercaptoethanol and zinc acetate. As shown in Table III, metal analysis on such preparations showed the presence of approximately 1 zinc atom per $1.7 \times 10^4$ daltons of protein, and less than 0.05 mercury atom. As discussed later, this zinc-regulatory subunit uniformly reconstituted with excellent efficiency ($\geq 90\%$) with catalytic subunits to form an aggregate indistinguishable from native ATCase. For long periods of storage, zinc acetate was added to the regulatory subunit preparation to a final concentration of 0.2 mM. In contrast to the standard preparations of the regulatory subunit, the zinc subunit did not precipitate or lose the ability to reconstitute with the catalytic subunits, even after a storage period of several weeks. The affinity of zinc for regulatory subunit is not known except that the metal was retained upon passage of the protein over Chelex-100 under conditions which removed zinc from mixtures with bovine serum albumin.

The absorption spectrum in Figure 3 shows that the zinc-regulatory subunit had an extinction maximum at about 278 nm and a shoulder at 282 nm, a spectrum consistent with a protein containing no tryptophanyl residues (Donovan, 1969). Moreover, the subunit absorption spectrum agreed well with that expected for the regulatory subunit within native ATCase, as calculated from the difference in abosrption of the catalytic subunit and undissociated ATCase taking the weight fraction of the regulatory subunit in ATCase as 0.33 (Cohlberg *et al.*, in preparation). Thus, the zinc derivative of the regulatory subunit, in contrast to the previously obtained preparations, satisfied the spectral criteria for a "native" regulatory subunit.

Next, a metal-free derivative of the regulatory subunit was prepared by extensively dialyzing the zinc-regulatory subunit against a buffer containing EDTA and dithiothreitol, as described in Methods. The subunit prepared in this manner had less than 0.02 zinc atom and less than 0.05 mercury atom/polypeptide chain (Table IV). As shown in Figure 3 this apo-regulatory subunit, like the zinc-containing subunit, exhibited a spectrum characterized by low extinction in the near ultraviolet region. The extinction coefficient of 0.32 $cm^{-1}$ $(mg/ml)^{-1}$ at 280 nm for the apo subunit agreed well with the extinction calculated from the amino acid composition of the subunit (Changeux and Gerhart, 1968; Weber, 1968), namely 3 tyrosyl, 4 cysteinyl, and no tryptophanyl residues per polypeptide chain.[6] The spectra of the apo subunit and the zinc derivative were nearly identical at wavelengths above 260 nm, but differed significantly at lower wavelengths (*e.g.* approximately 30% at 250 nm) where the zinc derivative absorbed more strongly. The maximum difference in extinction occurred at approximately 215 nm. Similar spectral differences have been reported for the zinc- and metal-free forms of metallothionein (Kägi and Vallee, 1961).

*Preparation and Characterization of the Mercury-Regulatory Subunit.* Since experiments reported in a previous section of Results showed that the regulatory subunits prepared by the procedure of Gerhart and Holoubek (1967) contained mercury and reconstituted with catalytic subunits, efforts were made to prepare a well-defined mercury derivative of the regulatory subunit, starting from apo-regulatory subunit and mercuric ions, as described in Methods. This derivative contained 1.1 mercury atoms/polypeptide chain as shown in Table III. In contrast to the small effect of zinc on the absorption spectrum of the apo-regulatory subunit, the mercury derivative of the regulatory subunit differed markedly in its absorption properties (Figure 3). Moreover, it is significant that the spectrum for this derivative resembled closely the spectrum of the regulatory subunit obtained from the procedure of Gerhart and Holoubek (1967; Gerhart and Schachman, 1965, 1968).

At 275 nm, this mercury subunit had an extinction of approximately 0.83 $cm^{-1}$ $(mg/ml)^{-1}$, more than 2.5-fold greater than that of the apo-regulatory subunit. The difference spectrum for $Hg^{2+}$ and apo subunits had a maximum at 276 nm and a molar extinction coefficient of $8.7 \times 10^3$, calculated on the basis of one mercury atom per $1.7 \times 10^4$ daltons of protein. At wavelengths below 260 nm the extinction increased monotonically and reached the value of $1.2 \times 10^4$ at 230 nm. Similar large extinction values were obtained in

[6] If the extinction coefficients for the *N*-acetyl ethyl esters of tyrosine and cysteine were used, as determined in water (pH 7.0) (Bailey *et al.*, 1968), then the calculated extinction for the regulatory subunit would be 0.29 $cm^{-1}$ $(mg/ml)^{-1}$. On the other hand, if the extinction coefficient for the *N*-acetyl ethyl ester of tyrosine as determined in glycerol–water solution (50% v/v) (Horwitz *et al.*, 1970) is used in this calculation, the extinction value of the regulatory subunit would be 0.34 $cm^{-1}$ $(mg/ml)^{-1}$.

the range from 300 to 230 nm for model complexes of $Hg^{2+}$ and dithiothreitol at a 1:1 molar ratio at neutral pH values, whereas model complexes of $Hg^{2+}$ with monothiols such as 2-mercaptoethanol or cysteine failed to show high extinction even at molar ratios of 1:50. Although it seems plausible that a pair of sulfhydryl groups of the regulatory subunit are involved in a dithiol complexation of $Hg^{2+}$, further spectral studies with model compounds and with the cysteine-containing oligopeptides of the subunit are needed in order to identify the liganding groups of the protein.

*Requirement of a Metal for Subunit Association.* The availability of the metal-free regulatory subunit enabled us to test directly the role of metal ions in the association of subunits to form ATCase. Indeed, incubation of apo-regulatory and catalytic subunits resulted in the production of little or no ATCase, as shown by the faintness of the ATCase band on electropherograms of the reaction mixture (Figure 4). In contrast, addition of zinc to the subunit incubation mixture resulted in rapid and complete reconstitution as indicated by the appearance of a strong electrophoretic band at the position corresponding to native ATCase, with a concomitant disappearance of the electrophoretic band corresponding to free catalytic subunit. These results show clearly that zinc is required for the aggregation of subunits. A more quantitative description of the effect of zinc on the association of subunits was obtained by centrifugation of comparable reconstitution mixtures, as also shown in Figure 4. Analysis of these sedimentation patterns is presented in Table V. When reconstitution was carried out with apo-regulatory and catalytic subunits, almost all (86%) of the catalytic subunit remained free, that is, unassociated with the regulatory subunit. The absence of any detectable reaction boundaries between the various sedimenting species indicates the subunits were not in a rapidly reversible equilibrium with ATCase-like aggregates. Addition of zinc to the incubation mixture of apo-regulatory and catalytic subunits, on the other hand, resulted in the complete conversion of catalytic subunit into ATCase species (present as 12S and 16S species).[7] Under these reaction conditions, 90% of the total protein was present as ATCase. and the remaining 10% represented the excess regulatory subunit. Again, the sharpness of the boundaries for the various species indicates that a reversible equilibrium did not exist between the subunits and ATCase.

The small amount of ATCase-like material (15% of the total protein) apparent in sedimentation patterns of subunit reconstitution mixtures without zinc is most likely due to the incomplete removal of metal ions from the components of the reaction mixture. For, as shown in Table V, zinc was detected in these preparations at a low level, sufficient for the formation of ATCase amounting to approximately 10% of the total protein, assuming six zinc ions were required for each ATCase molecule. In preparations reconstituted with excess zinc, the amount of zinc incorporated into ATCase so firmly as to withstand dialysis against EDTA was approximately seven zinc atoms per ATCase molecule formed. Thus, the zinc ion

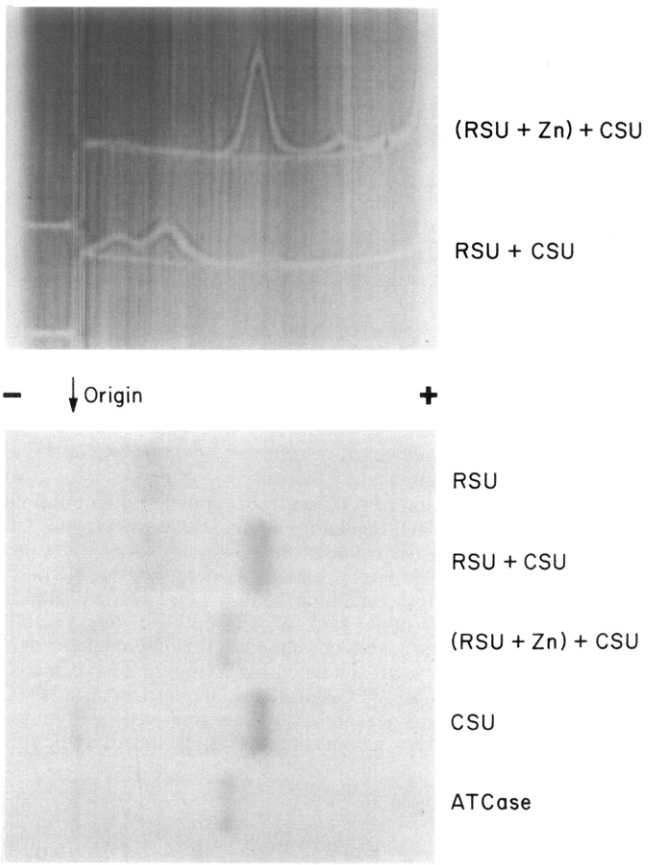


FIGURE 4: The dependence of the reconstitution of ATCase on the presence of zinc. Reaction mixtures (0.5 ml) of apo-regulatory subunit (1.8 mg) and catalytic subunit (2.4 mg) were incubated in potassium phosphate (0.04 M, pH 7.0) containing 2 mM 2-mercaptoethanol (no EDTA) either in the presence of zinc (2 mM zinc acetate) or in the absence of zinc. Each mixture was incubated at room temperature for 30 min and then dialyzed separately for 24 hr against 1 l. of standard phosphate buffer (*i.e.*, containing 0.2 mM EDTA). At the top are shown sedimentation patterns of these dialyzed reconstitution mixtures centrifuged simultaneously in a rotor equipped with two double-sector cells, one with a positive wedge window (upper pattern) and the other with regular windows (lower pattern). The protein concentration at the start of centrifugation for the sample in the presence and the absence of zinc was 4.2 and 3.8 mg per ml, respectively. Photographs were taken approximately 34 min after a rotor speed of 60,000 rpm was reached. The phase-plate angle was set at 65°. Sedimentation is from left to right. Electrophoresis of these same dialyzed reconstitution mixtures, together with control samples, was carried out at 200 V for 15 min at 25° on cellulose acetate membranes, as described in Methods. RSU indicates regulatory subunit and CSU indicates catalytic subunit.

required for the association of the subunits into ATCase became bound to ATCase in a complex similar in stoichiometry and stability to that observed for the native enzyme.

*Reconstitution of ATCase in the Presence of Metals Other Than Zinc.* When the mercury derivative of the regulatory subunit was incubated with catalytic subunit in the presence of 2-mercaptoethanol, the subunits reconstituted with good efficiency as shown in Figure 5. In fact, reconstitution appeared to be complete when the subunits were incubated at an approximate equimolar ratio (based on the polypeptide chain molecular weights), a reconstitution efficiency comparable with that of zinc regulatory subunit. The ATCase species formed upon reconstitution from the mercury subunit was found to contain approximately six mercury atoms per molecule of ATCase. The extinction coefficient (at 280 nm) of the

[7] The 16S component evident in this pattern is thought to represent ATCase also; it behaved as a discrete, stable species, and has been previously observed in reconstitution mixtures (Gerhart and Schachman, 1965) and in aged preparations of native ATCase. This 16S aggregate was enzymically active and exhibited normal CTP inhibition, as determined from enzyme activity studies on material isolated by zone centrifugation of the native enzyme in a sucrose density gradient. As a consequence, the total amount of ATCase-like material is summed from both the 12S and the 16S components. The 16S species is most likely a dimer of ATCase.

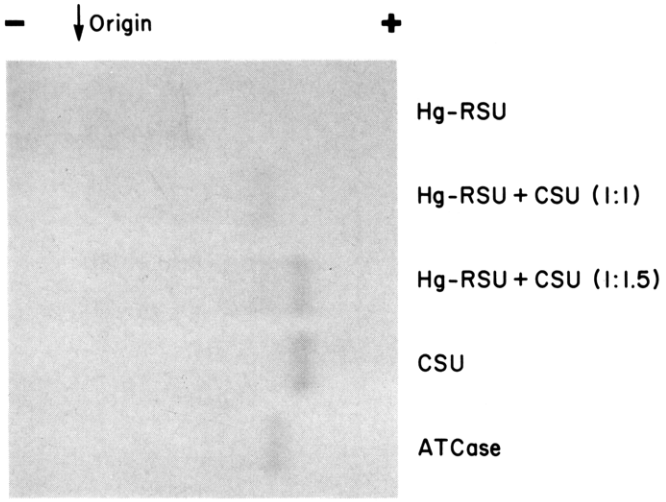


FIGURE 5: Reconstitution of ATCase from catalytic subunit and the mercury derivative of the regulatory subunit, as shown by electrophoresis. The mercury derivative of the regulatory subunit was prepared from the apo-regulatory subunit as described in Methods. Reconstitution mixtures containing 350 $\mu$g of regulatory subunit and either 700 $\mu$g (1:1 molar ratio of polypeptide chains) or 1000 $\mu$g of (1:1.5 molar ratio) catalytic subunit in 0.5-ml volume of standard phosphate buffer were incubated for 30 min at approximately 25° prior to electrophoresis. Cellulose acetate electrophoresis was conducted at 25° for 15 min and 200 V, as described in Methods. RSU indicates regulatory subunit and CSU indicates catalytic subunit.

mercury derivative of ATCase was 0.76 $cm^{-1}$ $(mg/ml)^{-1}$, significantly higher than that of native ATCase (0.59 $cm^{-1}$ $(mg/ml^{-1})$), but in agreement with the sum of the spectral contributions of the isolated subunits used in this reconstitution. Similar observations have been made with the regulatory subunits prepared by the standard procedures (Gerhart and Holoubek, 1967). For example, regulatory subunit preparations with high absorption, due to residual mercury, when recombined with catalytic subunits, produced ATCase molecules with higher than normal absorption.

Survey experiments have been conducted to determine whether other metals were effective in promoting the aggregation of the subunits. In these experiments mixtures containing 1.5 mg/ml of apo-regulatory subunit and 4.5 mg/ml of catalytic subunit (excess catalytic subunit) were incubated at room temperature for 30 min in 40 mM potassium phosphate buffer with each of various transition metal ions at 2 mM. On the basis of these preliminary experiments cadmium ($Cd^{2+}$), cobalt ($Co^{2+}$), nickel ($Ni^{2+}$), and copper ($Cu^{2+}$) were observed to promote the aggregation of subunits[4] as determined by the presence of material at the ATCase position in electropherograms.

## Discussion

*Stoichiometry and Stability of the Zinc–ATCase Complex.* Native ATCase contains zinc to the extent of six ions per molecule ($3.1 \times 10^5$ daltons of protein), a noteworthy amount since crystallographic (Wiley and Lipscomb, 1968), chemical (Weber, 1968), hybridization (Meighen *et al.*, 1970), and molecular weight (Rosenbusch and Weber, 1971a; Cohlberg *et al.*, in preparation) data show ATCase to have a hexameric quaternary structure. As a consequence, the repeating unit (*i.e.*, the protomer) in the native enzyme must contain one catalytic and one regulatory polypeptide chain, together with one zinc ion.

TABLE V: Dependence of ATCase Reconstitution on Zinc Ion. Quantitative Analysis of Reconstitution Mixtures Shown in Figure 5.

| Condition | Wt Percentage of Total Protein as Individual Component[a] | | | | $\mu$moles of Zinc/mg of Protein[b] |
|---|---|---|---|---|---|
| | 3 S | 6 S | 12 S | 16 S | |
| No zinc | 28 | 57 | 15 | nd[c] | 1.6 |
| Zinc added | 10 | nd[c] | 80 | 10 | 21.4 |
| Theoretical mixture with no association | 40 | 60 | 0[d] | | 0 |
| Theoretical mixture with complete association | 10 | 0 | 90[d] | | 17.5 |

[a] The distribution of components was estimated from area measurements of the boundaries in the schlieren patterns as described in Methods. Measurements were made from the photographic plates on patterns in which boundaries were resolved and the areas were then corrected for radial dilution. (Components were identified by sedimentation coefficients.) [b] Zinc was determined on aliquots of the samples used for centrifuge studies, dialyzed *vs.* 0.04 M potassium phosphate (pH 7.0) to remove EDTA. Zinc was determined by the dithizone method. The theoretical calculation for zinc content assumes six zinc atoms per molecule of ATCase, and no zinc for free regulatory subunit or catalytic subunit. [c] This component was not detectable. [d] Both 12S and 16S components are combined in these theoretical values.

The stability of the ATCase–metal complex is suggested by the results of the zinc-exchange experiments and competition experiments with metal chelators. With regard to the former, at neutral pH, less than 2% of the zinc in ATCase was replaced by $^{65}Zn^{2+}$ of the solvent medium in 40 days (bimolecular rate constant of exchange of less than $10^{-4}$ $M^{-1}$ $sec^{-1}$) under conditions where 78% $^{65}Zn^{2+}$ replacement would be expected at equilibrium. Since the zinc content of ATCase remains approximately six zinc atoms per molecule, this slow exchange demonstrates the high kinetic stability of the ATCase zinc complex, *i.e.*, its very slow rate of release of $Zn^{2+}$ to the medium. Generally, exchange reactions in stable complexes are thought to proceed through transition intermediates in which one complex contains two metal ions (Basolo and Pearson, 1966). There are two main possibilities for the step of the exchange reaction which might be rate limiting. On the one hand, simultaneous dissociation of several liganding groups from the central zinc atom of the complex may occur rarely, such that incoming $^{65}Zn^{2+}$ would seldom encounter free liganding groups for association. That is to say, even partial dissociation, not to mention full dissociation, of the complex would be limiting. This possibility would be characteristic of a metal complex of high stability. On the other hand, the metal complex may undergo partial dissociation at a significant rate, but may not be able to accommodate the incoming $^{65}Zn^{2+}$ in a transition state containing two zinc atoms, due to steric hindrance from the protein. This rate-limiting step would characterize a complex of lower thermodynamic

stability (compared to the prior case) but one in which the zinc is "buried" in the protein.

Since the exchange reactions of ATCase did not approach equilibrium despite long incubation periods, it is perhaps not surprising that chelators did not remove $Zn^{2+}$ from ATCase. For example, in the presence of 0.1 M EDTA at pH 9, ATCase lost no detectable amount of zinc in 10 days. As with the exchange results, the chelator experiments may either reflect the unusually high thermodynamic stability of the zinc–protein complex (*i.e.*, greater than the stability of the zinc–EDTA complex) or may reflect the inability of the reactant from the solvent medium (in this case, the chelator) to approach the protein surface to form partially liganded complexes with the occluded zinc atom. Kinetic problems are known to exist for zinc removal from another protein, carbonic anhydrase B (Lindskog and Nyman, 1964), from which zinc is only half removed in 9 days by 1 mM *o*-phenanthroline. Taken together, the exchange and chelator results for ATCase indicate the zinc–protein complex to have at least moderate thermodynamic stability and certainly high kinetic stability. Moderate thermodynamic stability for multidentate zinc complexes would be exemplified by an equilibrium association constant of $10^{10}$–$10^{12}$ $M^{-1}$ (Basolo and Pearson, 1966).

*Role and Location of Zinc in ATCase.* The results presented here demonstrate the essential role of metal ions in stabilizing the quaternary structure of native ATCase. In fact, this stabilization was larger than the present experiments could measure. On the one hand, residual stability of ATCase in the absence of zinc must be very small since subunits persisted in large quantities and no reaction boundaries were observed between the various protein species during sedimentation of subunit mixtures even though the experiments were done at a protein concentration of several milligrams per milliliter. On the other hand, the ATCase complexes formed in the presence of zinc must be very stable as shown in sedimentation experiments presented here in which ATCase was formed stoichiometrically from the available subunits. Moreover, the stability of zinc-containing ATCase in the form of native enzyme is so great that dissociation into subunits has not been detected in sucrose gradient zone centrifugation experiments at protein concentrations less than 0.1 μg/ml, *i.e.*, $3 \times 10^{-10}$ M ATCase (V. Pigiet, unpublished). The same conclusion has been reached from the negative results of experiments testing exchange between ATCase and radioactively labeled subunits (M. E. Nelbach, unpublished) or chemically modified subunits (Meighen *et al.*, 1970; Pigiet and Schachman, in preparation) despite incubation periods of 2 weeks. Thus, from a variety of experiments, the stability of metal containing ATCase appears many orders of magnitude greater than its metal-free analog. The difference of free energy of these two forms of ATCase must derive from the large free-energy change of binding zinc to the protein. Zinc binding by other proteins is known to occur with high affinity, in the range of $10^{10}$–$10^{12}$ $M^{-1}$ in the cases of carbonic anhydrase B, alkaline phosphatase, and carboxypeptidase A (see Lindskog, 1970, and Vallee and Wacker, 1971, for reviews). Given the earlier evidence for the stability of the zinc–ATCase complex and for the high zinc content of ATCase, zinc binding should be able to provide the thermodynamic driving force to effect the stable association of subunits in ATCase.

Zinc–protein complexes in α-amylase (Stein and Fischer, 1960), alkaline phosphatase (Simpson and Vallee, 1968), and liver alcohol dehydrogenase (Drum *et al.*, 1969a) have been assigned a role in quaternary structure, as suggested here for the role of zinc in ATCase. These cases are complicated by the simultaneous involvement of metal ions in catalytic activity, whereas ATCase has no metal requirement for catalytic activity as clearly shown by the full enzymic activity of the apo-catalytic subunit. However, before zinc can be assigned an unambiguous and exclusive structural role in ATCase, it must be shown to have no involvement in nucleotide binding by the regulatory subunit. Recently Rosenbusch and Weber (1971b) have reported unimpaired CTP binding by apo-regulatory subunit. Thus, zinc appears to be involved solely in the effective aggregation of regulatory and catalytic subunits into ATCase, a reaction essential for the regulatory function of this protein.

For ATCase to exist as a stable aggregate, the r:c, r:r, and c:c domains of bonding must each surpass a certain level of stability. The c:c domain is very stable even in the absence of $Zn^{2+}$ since the apo-catalytic subunit is a fully associated trimer even at concentrations of a few micrograms per milliliter (M. Springer and H. K. Schachman, unpublished results). Therefore, the stabilization of ATCase by $Zn^{2+}$ does not involve increases of interchain affinity at this domain. The r:r domain appears to be sufficiently strong in the absence of $Zn^{2+}$ to establish limited but detectable dimerization of apo-regulatory chains ($1.7 \times 10^4$ daltons each). However, Cohlberg *et al.* (in preparation) have found $Zn^{2+}$ addition causes regulatory subunit chains to associate fully to the dimer level, indicating a significant increase of interchain affinity at the r:r domain in the presence of $Zn^{2+}$. The effect of $Zn^{2+}$ on the r:c domains is less clear since formation of this domain has been studied only under conditions of mixing regulatory subunits and catalytic subunits to form ATCase, that is, conditions in which the r:r domain is simultaneously affected. Thus, although it seems likely that the r:c as well as the r:r domains are affected by $Zn^{2+}$, the contribution of stabilization at each domain to the overall stabilization of ATCase cannot be evaluated at present. Further reason for considering the effect of $Zn^{2+}$ on the r:c domain comes from preliminary binding studies in which catalytic subunit itself shows affinity for $Zn^{2+}$, suggesting that the zinc binding site in ATCase contains liganding groups from both subunits, and is therefore located in the r:c domain.

*Nature of the Liganding Groups of the Protein.* The liganding groups of ATCase appear capable of forming a specific complex with any member of the IIb group of metals. Zinc and mercury have been shown here to form one to one stable complexes with the regulatory polypeptide chain, and to effect reconstitution of ATCase containing 6 g-atoms of metal/mole. Cadmium behaves in an identical manner (Rosenbusch and Weber, 1971a,b).

Information on the chemical nature of liganding groups of the protein is afforded by spectral studies of the regulatory subunit. The derivative formed from $Hg^{2+}$ and apo-regulatory subunit absorbed 2.6-fold more strongly at 276 nm than did apo-regulatory subunit. The chromophore due to the mercury–protein interaction had a molar extinction coefficient of $8.7 \times 10^3$ at 276 nm. This chromophoric complex is the same as the complex required for subunit association since the $Hg^{2+}$-regulatory subunit and apo-catalytic subunit aggregated to form ATCase which preserved the unique absorption. Preliminary evidence for the participation of sulfhydryl groups in this complex is provided by studies of model compounds: at neutral pH values, the complex between $Hg^{2+}$ and dithiothreitol at 1:1 molar ratio absorbed as strongly at 276 nm as did the metal–protein derivative, whereas complexes of $Hg^{2+}$ and monothiols such as 2-mercaptoethanol absorbed much less strongly even at molar ratios of 1:50. The possibility

of a metal–dithiol complex in the protein is strengthened by the sequence data showing the four cysteinyl residues of the regulatory polypeptide chain located in two pairs in the C terminal third (Weber, 1968). Either of these pairs is found by model building to provide sulfhydryl groups at the required positions for a metal–dithiol complex. Rosenbusch and Weber (1971b) and V. Pigiet (unpublished data) further document the case for a metal–sulfhydryl complex in the regulatory subunit on the basis of comparisons of the spectra of the $Zn^{2+}$- and $Cd^{2+}$-regulatory subunits with spectra of complexes of these metals with 2-mercaptoethanol and metallothionine (Kägi and Vallee, 1961).

Aside from spectral evidence, sulfhydryl groups are implicated as ligands in the metal–protein complex by results from dissociation studies on ATCase (Gerhart and Schachman, 1968). Mercurials such as PMB and neohydrin rapidly split ATCase into catalytic and regulatory subunits in an all-or-none reaction with approximately 24 cysteinyl sulfhydryl groups per ATCase molecule (4 sulfhydryl groups for each of the 6 regulatory polypeptide chains). This reaction displaces zinc from the protein complex. In contrast to mercurials, alkylating agents such as iodoacetamide and *N*-ethylmaleimide fail to react with the sulfhydryl groups of ATCase and to cause dissociation, over a period of 2 days at room temperature (V. Pigiet, unpublished results). A similar specificity of reaction toward mercurials and alkylating agents is shown by the metal-liganded sulfhydryl groups of metallothionein and by model metal–sulfhydryl complexes (Kägi and Vallee, 1961). Thus, at least some of the sulfhydryl groups of ATCase behave like complexed sulfhydryl groups, rather than as free sulfhydryl groups, in terms of their specific reactivity. The involvement of sulfhydryl groups in binding zinc to the regulatory subunit is further supported by the finding that carboxymethylated regulatory subunit (in which all four sulfhydryl groups per polypeptide chain were derivatized) was unable to bind zinc (Cohlberg *et al.*, in preparation).

*Proposed Role for the Metal Complex in Structural Changes in ATCase.* Now that the importance of a metal complex is established for the stability of one or more of the kinds of domains of bonding in ATCase, the question arises whether *changes* of quaternary structure involve changes of liganding groups in the metal–protein complex. This question is interesting since changes of structure in ATCase are thought to be essential for its allosteric properties. The large magnitude of structural changes in ATCase (*e.g.*, a 3.6% reduction in sedimentation coefficient accompanying the binding of substrate analogs (Gerhart and Schachman, 1968; Kirschner and Schachman, 1971a,b) suggests rearrangements at the quaternary level. Such transitions of quaternary structure would involve changes in the bonding domains between subunits, and it is noteworthy that sulfhydryl groups of ATCase increase their reactivity with PMB 7-fold when substrate analogs are present (Gerhart and Schachman, 1968). In light of these effects, it seems plausible to propose that the metal–sulfhydryl complex, with its involvement in domain stability, undergoes changes in its coordination during structural transitions of the protein. A test of the role of the metal–protein complex in allosteric properties might be provided by ATCase analogs containing metals outside the IIb groups, on the chance that the foreign complex affects transitions of the protein between various quaternary structures. These analogs may show changes in their cooperative substrate binding and sensitivity to nucleotide activators and inhibitors. The $Co^{2+}$ derivative has the added advantage of affording spectroscopic information about the kinds of liganding groups in their coordination spheres and the symmetry relationships between these groups (Vallee and Wacker, 1970).

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# Studies of the Histidine Residues of Carbonic Anhydrases Using High-Field Proton Magnetic Resonance*

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ABSTRACT: A comparative study of the titration behavior of the histidine residues of the B and C isoenzymes of human and the B enzyme of bovine carbonic anhydrase has been carried out using 220-MHz proton magnetic resonance spectroscopy. In the case of the bovine enzyme, at least five and up to eight imidazole C-2 protons are observed to titrate with the aid of computer curve fitting. These resonances give a fairly narrow range of p$K$ values, with a highest value of 6.4. By contrast, four imidazole C-2 proton resonances are observed to titrate in the human B enzyme with widely spaced p$K$ values (obtained by curve fitting) of 5.9, 6.1, 6.9, and 7.2. For the human C enzyme, seven C-2 proton titration curves are seen, with five having p$K$ values in the range 5.9–6.6 and two others with values 7.1 and 7.2. While the number of titratable histidine residues found for the human B enzyme using the nuclear magnetic resonance (nmr) technique agrees with the number found using titrimetric methods, the nmr method shows a larger number of titrable histidines for the human C and bovine B enzymes than found by titration. The similarity of the two high p$K$ values in the two human isoenzymes indicates similar unusual environments for two histidine residues in these protein structures, which are probably absent in the bovine enzyme. Several nontitrating and poorly resolved resonances are also present in the spectra of each protein. These may correspond to histidine residues which are unable to titrate or are rigidly held within the protein structure. The addition of the inhibitor acetazolamide (Diamox) in saturating amounts to human B, a histidine-alkylated human B, and human C enzymes produced several effects which indicate inhibitor-induced conformational changes. Consideration of these effects, as well as the data for the three native enzymes, tend to indicate that the group with p$K \simeq 7$ which is essential for catalysis is probably not a histidine residue.

Carbonic anhydrases are widely distributed enzymes that catalyze the reversible hydration of carbon dioxide and other hydrolytic reactions. They consist of a single polypeptide chain and contain a zinc atom which is essential for both the catalytic activity and the binding of anionic and sulfonamide inhibitors. In most mammalian erythrocytes carbonic anhydrases are present in at least two forms, which differ principally in their specific catalytic activity, one being characterized as the low- and the other as the high-activity form. The pH–rate profiles and the pH-binding curves indicate that the same group with a p$K$ of approximately seven is involved in catalysis and binding of inhibitors for both the high- and low-activity forms (Edsall, 1968; see also discussion of Furth, 1968, and Bradbury, 1969).

It is believed that a water molecule bound to the zinc atom is the basis for the catalytic mechanism (Davis, 1961). However, the p$K$ of a water molecule bound to a zinc atom in model compounds is significantly higher than 7 (p$K \simeq 10$) (Hunt, 1963). Also, a simple nucleophilic attack by a zinc-bound hydroxyl group which has been formed from the water cannot account for the observed high catalytic rate of the carbon dioxide hydration (Wang, 1969).

Several histidine residues have been shown to be present in the active-site region (Whitney *et al.*, 1967a,b; Kandel *et al.*, 1968; Bradbury, 1969), and it has been suggested that one or more of these might be involved in the catalytic or binding

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