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Allosteric Interactions in Aspartate Transcarbamylase. II. Evidence for Different Conformational States of the Protein in the Presence and Absence of Specific Ligands*

John C. Gerhart and Howard K. Schachman

ABSTRACT: In the previous paper it was shown that the antagonistic and cooperative effects observed in the multiple binding of ligands by aspartate transcarbamylase (ATCase) are indirect. In order to determine whether these allosteric effects are mediated by the protein itself through changes in its tertiary or quaternary structure, we conducted physical-chemical studies of the conformational state of the enzyme both in the presence and absence of specific ligands such as substrates or feedback inhibitors. The effect of ligands on the reactivity of the sulfhydryl groups of the enzyme was examined as a probe for possible changes in the conformation of the enzyme. Spectrophotometric titration of ATCase with *p*-mercuribenzoate (PMB) yielded a reaction end point of 27 ± 1 PMB per ATCase molecule.

Upon the addition of PMB to form mercaptide complexes the enzyme dissociated into two catalytic and four regulatory subunits. In partially reacted mixtures the undissociated enzyme bound no PMB whereas the dissociated products were fully reacted. Moreover, in this *all-or-none* reaction of the sulfhydryl groups of the enzyme, virtually all of the PMB was bound to the regulatory subunits and practically none (5% or less) of the PMB was complexed to the catalytic subunits. Of the 32 half-cystines (found by cysteic acid analysis) in the intact ATCase molecules, 24–28 were in the four regulatory subunits and 8 in the two catalytic subunits. Although the rate of reaction of the isolated regulatory subunits with PMB was too rapid for measurement by conventional spectrophotometry, the rate for the intact enzyme was easily determined. Pseudo-first-order reaction velocity constants were determined for solutions containing the ligands at differing concentrations. The addition of both carbamyl phosphate and the substrate analog, succinate (which bind to the catalytic subunits), led to a sixfold increase in the rate of reaction of the sulfhydryl groups in the regulatory subunits of ATCase. Succinate alone had no effect, and the effect of carbamyl phosphate alone was much less than that resulting from the addition of both ligands. This enhancement of the reactivity of these sulfhydryl groups of ATCase was opposed by the addition of the regulatory metabolite, cytidine triphosphate (CTP), or its analog, 5-bromocytidine triphosphate (BrCTP). This antagonism between substrates and feedback inhibitor was highly specific and only partial in character. In the absence of carbamyl phosphate and succinate, CTP (and BrCTP) had almost no effect on the reactivity of ATCase toward PMB. Companion studies of the gross morphology of the enzyme were made by sedimentation velocity measurements. In the presence of both carbamyl phosphate and succinate there was a 3.6% reduction in the sedimentation coefficient of ATCase. Both ligands were required and the magnitude of the reduction was a function of the concentration of succinate (at a fixed concentration of carbamyl phosphate). As in the experiments on the reactivity of the sulfhydryl groups, CTP opposed the effect of carbamyl phosphate and succinate in reducing the sedimentation coefficient of ATCase; again the antagonism was only partial In the absence of the other ligands CTP had virtually no effect. The observed changes in the reactivity of sulfhydryl groups and in the hydrodynamic behavior appear to result from modification in the conformation of the undissociated protein and to be the indirect effect of ligands bound to the enzyme. It appears that in the presence of carbamyl phosphate and succinate the intact enzyme exists in a swollen (or anisometric) conformation which exhibits an enhanced reactivity toward PMB, perhaps because of its greater rate of dissociation into catalytic and regulatory subunits. When these ligands are absent, the intact enzyme appears to exist in a more compact conformation; the presence of CTP favors this compact, slowly reacting conformational state of ATCase.

In the preceding paper (Changeux *et al.*, 1968) evidence was given that aspartate transcarbamylase[1] (ATCase) from *Escherichia coli* mediates indirect effects in the multiple binding of ligands. These effects include: (1) homotropic effects such as the cooperative binding of substrate-like ligands (*e.g.*, succinate, a nonreactive analog of the substrate aspartate) and (2) heterotropic effects such as the partial antagonism between the feedback inhibitor, cytidine triphosphate (CTP), and succinate. It was concluded that the binding of ligands occurs at topographically distinct sites which are in fact located on different subunits, and consequently the cooperative and antagonistic effects must be indirect, *i.e.*, allosteric, effects which are mediated by the protein itself. This mediation could occur through alterations of the tertiary and quaternary structures of the protein, as proposed by Monod *et al.* (1963, 1965) and Koshland *et al.* (1966).

This paper deals with physical and chemical studies on the conformation of ATCase in the presence and absence of ligands for which the enzyme mediates homotropic and heterotropic effects. Since the sulfhydryl groups of ATCase appear to be located in regions of the protein molecule which are important for the association of the subunits (Gerhart and Schachman, 1965), it seemed likely that changes in their chemical reactivity might be an index of alterations in the quaternary structure of the protein. Thus studies were conducted on the effect of various specific ligands on the rate of the reaction of the sulfhydryl groups of ATCase with the mercurial, *p*-mercuribenzoate. To complement these measurements on the fine structure of the enzyme, parallel measurements were made on its gross morphology as revealed by hydrodynamic studies on its molecular size, shape, and volume. Accordingly experiments were conducted on the effect of various ligands on the sedimentation coefficient of ATCase.

From these studies it was concluded that the enzyme molecules exist predominantly in different conformational states depending upon the presence or absence of ligands of the substrate binding sites. For example, in the presence of both succinate and carbamyl phosphate the rate of reaction of the sulfhydryl groups of the enzyme was approximately sixfold greater than that for the enzyme in the absence of these ligands. Accompanying this enhanced reactivity of the sulfhydryl groups in the presence of these ligands was an apparent swelling of the enzyme molecules as inferred from the 3.6% decrease in the sedimentation coefficient. The feedback inhibitor alone had only a small effect on the reactivity of the sulfhydryl groups and the sedimentation coefficient of the enzyme. However, it was very effective in opposing the changes in the enzyme molecules which are observed when the substrate-like ligands are added.

The changes in the chemical reactivity and sedimentation coefficient of the enzyme were found to occur only under conditions similar to those for which the homotropic and heterotropic effects are observed by kinetic and equilibrium dialysis measurements (Gerhart and Pardee, 1964; Changeux *et al.*, 1968). It seems therefore that the conformational changes observed experimentally are equivalent or related to those mediating the allosteric effects.

Experimental Procedures

Materials

Purified ATCase and its separated subunits were prepared as described previously (Gerhart and Holoubek, 1967). Protein concentrations were determined spectrophotometrically at 280 mμ based on absorbance values 0.59 and 0.72 for 0.1% solutions of ATCase and catalytic subunit, respectively (Gerhart and Schachman, 1965). The protein concentration of the regulatory subunit was determined colorimetrically (Lowry *et al.*, 1951). Purified rabbit muscle aldolase was kindly supplied by Dr. Stuart Edelstein. Dilithium carbamyl phosphate, potassium succinate, cytidine triphosphate (CTP), and 5-bromocytidine triphosphate (BrCTP) were prepared and used as described previously (Changeux *et al.*, 1968).

p-Hydroxymercuribenzoate (PMB) was obtained as the sodium salt from Sigma Chemical Co. Stock solutions (0.010 M) were prepared by dissolving PMB in 5×10^{-3} M KOH. The concentration of PMB in the stock solution was measured spectrophotometrically based on a molar extinction coefficient at 232 mμ of 1.69×10^4 at pH 7 (Boyer, 1954). The purity of PMB was estimated to be better than 95% on the basis of titration with 2-mercaptoethanol or cysteine hydrochloride, according to the method of Boyer (1954).

Methods

Measurement of Rates of Reaction of PMB with ATCase. The reaction of PMB with ATCase was followed spectrophotometrically at 250 mμ, the wavelength at which PMB is known to undergo the maximal increase in absorbance due to formation of a mercaptide complex with sulfhydryl groups (Boyer, 1954). The reaction was initiated and recorded as follows. In a quartz cuvet of 1.5-ml capacity was placed 0.8 ml of a solution containing 2.5×10^{-4} M PMB, 2.5×10^{-2} M Tris-HCl buffer (pH 7.0), and 5×10^{-2} M

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[1] Carbamyl phosphate:L-aspartate carbamyl transferase (EC 2.1.3.2). Abbreviations used: ATCase, aspartate transcarbamylase; CTP, cytidine triphosphate; PMB, *p*-hydroxymercuribenzoate; BrCTP, 5-bromocytidine triphosphate; UTP, uridine triphosphate.

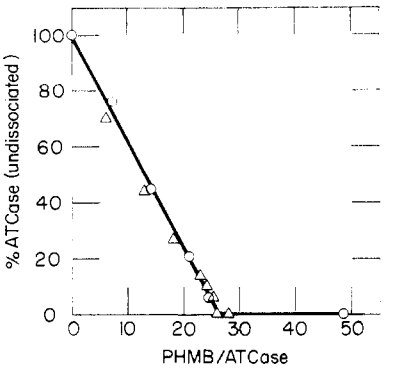

FIGURE 1: Titration of ATCase with PMB as measured by the dissociation of the enzyme into subunits. Samples were prepared to contain 0.04 M potassium phosphate buffer (pH 7.0), 5.2 mg of ATCase, and PMB at a concentration from 0 to 8×10^{-4} M, in a volume of 0.6 ml. After 30 min at room temperature, the sample was introduced into one compartment of a 12-mm double-sector ultracentrifuge cell equipped with a metal-filled epoxy centerpiece, and a comparable solution minus ATCase was introduced into the reference compartment. The solutions were centrifuged at 60,000 rpm at 22° and boundary movement was observed with schlieren optics. Photographs were obtained throughout the centrifugation and analyzed by measurement of the area of the boundary representing undissociated ATCase (with a sedimentation coefficient approximately 11.4 S). The value for the area was corrected for radial dilution of the protein during sedimentation and compared to the area obtained in a separate experiment with a comparable solution of ATCase having no PMB. The ratio of areas representing the fraction of undissociated ATCase is plotted (○) *vs.* the molar ratio of PMB to ATCase. Alternatively, samples were diluted tenfold in 0.04 M potassium phosphate (pH 7.0) after 30-min incubation at room temperature and examined in the ultracentrifuge with absorption optics at a wavelength of 280 mμ. The absorbance corresponding to the fast-sedimenting species (undissociated ATCase) was measured directly from the traces, corrected for radial dilution, and compared to that obtained with an untreated ATCase sample; the ratio calculated in this manner is represented by △. ○ = schlieren optics; △ = absorption optics.

potassium phosphate buffer (pH 7.0), and then the appropriate volumes of solutions of potassium succinate, dilithium carbamyl phosphate, BrCTP, and water were added to give a total volume of 0.95 ml. The solution was mixed and the cuvet was placed in a Cary 14 recording spectrophotometer in the sample chamber thermostated at 21.5° and a zero reading at 250 mμ was measured against a reference cuvet of the same solution. The A_{250} of the solution (read against buffer) was approximately 0.95 in the absence of BrCTP, and as high as 1.8 in its presence (1.4×10^{-4} M BrCTP). The reaction was initiated by the addition of 0.05 ml of a solution of ATCase (or subunits) usually at a protein concentration of 6 mg/ml in 4×10^{-2} M potassium phosphate buffer (pH 7.0). (The solution of ATCase had been dialyzed to reduce 2-mercaptoethanol and Na-EDTA to less than 10^{-6} M.) The cuvet was immediately shaken and the recording of absorbance was begun within 15 sec after the addition of enzyme. In control experiments with cysteine hydrochloride, the increase in absorbance was found to be proportional to the concentration of sulfhydryl groups up to 2×10^{-4} M (at which concentration PMB was exhausted). The increase in extinction (on the basis of the molar concentration of sulfhydryl groups) was 7.6×10^{3}, in agreement with the value reported by Boyer (1954). At the protein concentrations used in these experiments, 13% or less of the PMB was consumed during the reaction.

Sedimentation Velocity Measurements. Sedimentation experiments were performed with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm and a rotatable light source for Rayleigh interference optics. Ultracentrifuge patterns were photographed on Metallographic plates when the schlieren optical system was used and on Spectroscopic II G plates with Rayleigh optics. Calculations of sedimentation coefficients were based on the movement of the maximum refractive index gradient as measured with a Gaertner two-dimensional microcomparator. In some experiments equivalent boundary positions were also calculated from the interference patterns (Richards and Schachman, 1959). For the experiments with extremely dilute solutions of ATCase (less than 20 μg/ml) the split-beam photoelectric-scanning absorption system (Schachman and Edelstein, 1966) was used with light having a wavelength of 235 mμ. All sedimentation coefficients were calculated from the slope of the straight line (fitted by the method of least squares) of the logarithm of boundary position *vs.* time. These plots were essentially straight lines since the dependence of sedimentation coefficient on concentration was so slight (Gerhart and Schachman, 1965). The observed sedimentation coefficients were corrected to values ($s_{20,w}$) corresponding to a solvent with the viscosity and density of water at 20° (Svedberg and Pedersen, 1940).

For measurements of the effect of various ligands on the sedimentation coefficient, two ultracentrifuge cells were used in a single experiment. One of the two cells contained a 1° quartz wedge as the upper window and the other cell had a conventional quartz window with parallel surfaces. The use of the wedged window led to an elevation of the schlieren pattern on the photographic plate, thereby permitting measurements to be made on two different solutions in the same ultracentrifuge experiment. One of the cells contained the buffered solution of the enzyme and the specific ligands while the other was filled with a control solution of the enzyme devoid of the ligands. In order to maintain nearly identical viscosities, densities, and ionic strengths for the two solvents, glutarate was added to the control when the effect of succinate on ATCase was being

examined. Potassium phosphate was used as a control for carbamyl phosphate and uridine triphosphate (UTP) for CTP.

Most sedimentation velocity experiments were performed with cells containing double-sector, metal-filled epoxy centerpieces. Some of the experiments on the effect of ligands were conducted with cells having single-sector Kel-F centerpieces. The optical paths were usually 12 mm although for the dilute solutions cells of 30 mm thickness were used.

When two different solutions were examined simultaneously the reference on the photographic plate for measurements of boundary positions was obtained from the slot opening in the side hole of the An-O (or An-E) analytical ultracentrifuge rotor. The precision in locating the center of the reference hole was increased by replacing the cup normally supplied with the rotor with one having a much narrower opening (0.006 in.). With this opening in the side of the rotor each photographic pattern contained a thin black line which was used as a reference for measuring boundary positions.

Measurements of the rotor temperature were performed with the rotor temperature indicator and control unit (RTIC) supplied with the ultracentrifuge. In addition, at the beginning and conclusion of each experiment readings were obtained through the use of external leads which bypassed the mercury cup in the base of the vacuum chamber. Checks on the temperature of the rotor were performed also with a contact thermocouple connected with a sensitive galvanometer.

Concentrations of the various sedimenting species were determined from the ultracentrifuge patterns in a variety of ways. For the schlieren patterns, areas corresponding to the various boundaries were measured from enlarged traces. With the interference patterns (which were used only occasionally for this purpose) direct fringe counting was performed (Richards and Schachman, 1959). When the photoelectric scanner was used the recorder deflection (in a vertical direction) was measured for each boundary (Hanlon *et al.*, 1962). This deflection gave a value directly proportional to the absorbance of the various sedimenting species and the absolute absorbance was evaluated from calibration curves obtained with solutions of measured optical densities. Frequently only spectral ratios were required and these were determined from successive recorder traces obtained with light of different wavelengths (Schachman, 1963).

Results

Stoichiometry of the Dissociation Reaction of ATCase with Respect to PMB. Previous experiments (Gerhart and Pardee, 1962; Gerhart and Schachman, 1965) had shown that in the presence of PMB, ATCase (of mol wt 3.1×10^5) dissociated asymmetrically into two catalytic subunits (each of 1.0×10^5 mol wt) and four regulatory subunits (each of 2.7×10^4 mol wt). The stoichiometry with respect to PMB was determined by examination in the ultracentrifuge of a series of samples of ATCase treated with different molar equivalents of PMB. Since undissociated ATCase, catalytic subunit, and regulatory subunit sedimented at sufficiently different rates (approximately 11.7, 5.8, and 2.8 S, respectively), they were readily distinguished in a mixture by either schlieren or absorption optics. As shown in Figure 1, the complete dissociation of ATCase required 26 ± 2 molecules of PMB per molecule of ATCase.

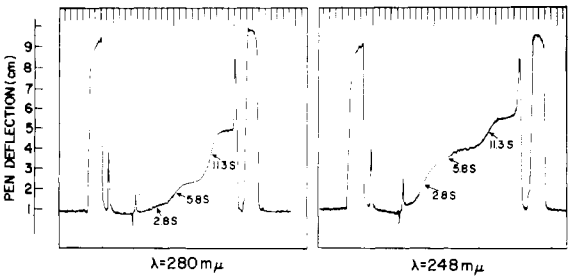

FIGURE 2: Binding of PMB to ATCase and the regulatory and catalytic subunits. Samples were prepared as described in the legend of Figure 1, incubated for 30 min at room temperature, diluted tenfold in 0.04 M potassium phosphate (pH 7.0), and centrifuged at 60,000 rpm at room temperature. The pen deflection is directly proportional to optical density with a 1.0-cm deflection corresponding to an absorbance of 0.14 for an optical path of 10 mm.

The All-or-None Reaction of ATCase with PMB. The linear relationship (Figure 1) between the per cent of undissociated ATCase and the molar ratio of PMB to ATCase indicates that the various reaction mixtures contain only unreacted (and undissociated) ATCase and fully reacted dissociation products of ATCase, rather than a significant amount of partially reacted molecules. For example, when 13–14 molecules of PMB were present per molecule of ATCase, it was found that 50% of the ATCase molecules was undissociated and 50% was dissociated, each molecule having reacted presumably with 26 ± 2 PMB molecules. To test further for the absence of partially reacted intermediates, a direct measurement of the extent of binding of PMB to undissociated ATCase was made in the ultracentrifuge by the use of absorption optics. A series of samples of ATCase treated with different concentrations of PMB were prepared as in the previous experiment and examined in the ultracentrifuge with light of 280 and 248 mμ. At 280 mμ the light absorption was due almost entirely to the protein, whereas at 248 mμ the absorption by protein was approximately one-half its 280-mμ value and PMB absorbed strongly whether alone or as its mercaptide complex. Thus, with light of 248 mμ it was possible to determine whether undissociated ATCase had an increased absorption due to bound PMB. Figure 2 presents two sedimentation velocity patterns of a partially dissociated sample of ATCase. These patterns were recorded in rapid sequence with

TABLE I: Binding of PMB by ATCase and the Catalytic and Regulatory Subunits during the Dissociation Reaction.

Protein	Moles of PMB/ Mole of Protein	% Dissociated	A_{280}/A_{248} 11.7 S	5.8 S	2.8 S
		Experimental Values			
ATCase	0	0	1.67		
	6	30	1.7	1.8	0.17
	12.6	56	1.6	1.5	0.17
	18	73	1.6	1.8	0.19
	22.9	87	1.7	1.9	0.16
	25.6	100			0.19
Catalytic subunit	0			2.40[a]	
Regulatory subunit	0				0.72[b]
		Calculated Values[c]			
	1		1.48	1.65	0.37
	2		1.33	1.25	0.27
	4				0.19
	6				0.16

[a] Determined from a separate centrifugation using purified subunit. [b] Calculated by difference from the ratios for ATCase and catalytic subunit. [c] Calculated using molar extinction coefficients for PMB mercaptide as 1.4×10^4 at 248 mμ and 0.1×10^4 at 280 mμ, and the subunit weight ratios and molecular weights given in Table II. Procedure: Samples were prepared and centrifuged as described in the legend of Figure 2; sedimentation velocity patterns were obtained with the photoelectric scanner using light of wavelengths 280 and 248 mμ. Traces of the patterns of optical density (recorder deflection) *vs.* distance along the axis of sedimentation were obtained in close time sequence at the two wavelengths (see the example of Figure 2) and analyzed for the absorption contributed by each of the three sedimenting species, *i.e.*, ATCase at approximately 11.7 S, catalytic subunit at 5.8 S, and regulatory subunit at 2.8 S. The absorption of ATCase was estimated from traces made 30 min after reaching speed, whereas the absorptions of catalytic and regulatory subunits were estimated from traces made 60 min after reaching speed; at that time the separation of the boundaries was more complete.

light of 280 and 248 mμ. The relative absorption at the two wavelengths was measured from the recorder deflection across each boundary. Table I summarizes a series of dissociation experiments with different molar ratios of PMB to ATCase. As shown there, the relative absorbance (A_{280}/A_{248}) of undissociated ATCase did not vary regardless of the extent of dissociation. Even when 87% of the population was dissociated, the undissociated ATCase (11.7 S) still had a value of 1.7 for A_{280}/A_{248}. From these experiments it was concluded that PMB does not bind to undissociated ATCase. The lower limit of detection was estimated to be one to two PMB per ATCase molecules, since two PMB per ATCase molecules would lower the absorption ratio of 1.33, a value significantly lower than that found experimentally. Thus ATCase appears to react with PMB in an *all-or-none* fashion, *i.e.*, a molecule of ATCase in the course of dissociation reacts with 26 ± 2 PMB molecules or does not react at all.

Location of the PMB Binding Sites in ATCase. Since the dissociation of ATCase led to the formation of two kinds of protein subunits which could be distinguished by their sedimentation rates, it was possible to estimate the amount of PMB bound to each. The sedimentation velocity patterns, such as those shown in Figure 2, were analyzed as described above and the results are given in Table I. In all samples, the absorption ratio (A_{280}/A_{248}) for the catalytic subunit was approximately 1.8, a value slightly lower than that of 2.4 found for the purified (and presumably mercurial free) catalytic subunit. On the basis of these two absorption ratios and the known absorption of PMB as a mercaptide complex, it was calculated (Table I) that each catalytic subunit binds a maximum of 0.5–1 PMB molecule in a mercaptide complex. Consequently, of the 26 ± 2 PMB molecules consumed per ATCase molecule in the dissociation reaction, only 1 to 2 of these could be accounted for as being bound to the two catalytic subunits of an ATCase molecule. In contrast, as shown in Table I, the absorption ratio of the regulatory subunit (approximately 0.17) departed greatly from that expected for protein alone (approximately 0.72). The increase of absorption at 248 mμ was consistent with the binding of four to six PMB molecules per regulatory subunit. This estimate was based on a molar extinction coefficient (at 248 mμ) of 14×10^3, that of PMB in mercaptide linkage (Boyer, 1954). Thus, the four regulatory subunits of an ATCase molecule could account for the binding of 16–24 PMB molecules in the course of the dissocia-

TABLE II: Half-Cystine Content of ATCase and Its Two Separated Subunits.

Determination	ATCase	Catalytic Subunit	Regulatory Subunit
1. Micromoles of cysteic acid residues per milligram of protein[b]	0.106 ± 0.003[a]	0.038 ± 0.004[a]	0.245 ± 0.011[a]
2. Weight fraction of subunit in ATCase[c]		0.68	0.32
3. Micromoles of cysteic acid residues contributed by each kind of subunit to 1 mg of ATCase	(0.104)[d]	0.026	0.078
4. Per cent of total cysteic acid residues of ATCase contributed by each kind of subunit		25	75
5. Moles of cysteic acid contributed by each kind of subunit to 1 mole of ATCase		8.0	24.5
6. Assumed molecular weight for ATCase and subunits[e]	3.1×10^5	1.0×10^5	2.7×10^4
7. Moles of cysteic acid per mole of protein	32.8 ± 0.9	3.8 ± 0.4	6.6 ± 0.3

[a] Average deviation of four independent analyses. [b] Protein was determined as follows. Cysteic acid was evaluated relative to glutamic acid, alanine, and leucine residues in a sample of oxidized protein. Then in a separate analysis of unoxidized protein, the content of glutamic acid, alanine, and leucine was determined per milligram of residues recovered from the column (recoveries were estimated in the range 0.85–0.95 of protein present before hydrolysis). [c] Determined from areas corresponding to the different components observed with the schlieren optical system during sedimentation of ATCase dissociated by PMB (H. K. Schachman and J. C. Gerhart, unpublished observations). [d] Sum of entries for catalytic and regulatory subunits; compare with 0.106, the determined value for ATCase. [e] Taken from Gerhart and Schachman (1965). Procedure: A sample of 5 mg of protein in 0.3 ml of 0.04 M potassium phosphate (pH 7.0) was evaporated to dryness *in vacuo* at room temperature and treated with 2 ml of performic acid overnight at 0° as described by Moore (1963). Oxidized samples were then treated with HBr, taken to dryness, and hydrolyzed in 1.0 ml of 6 N HCl for 24 hr at 109°. Hydrolysates were taken to dryness and analyzed on the Beckman–Spinco automatic amino acid analyzer. Approximately 1 mg of hydrolyzed protein was used/analysis. Thanks are due Mrs. Frances Hamburg for performing the analyses.

tion reaction. Clearly the regulatory subunits contributed most, if not all, of the 26 ± 2 groups of ATCase reacting with PMB.

An indication of the chemical nature of the groups reacting with PMB was obtained by spectrophotometric titration according to the method of Boyer (1954). The titration depends on an increase of the absorption of PMB (at 250 mμ) upon reaction with sulfhydryl groups. Figure 3 shows the titration of a fixed amount of PMB with increasing amounts of ATCase. The end point indicated by the change of slope on the graph corresponded to a ratio of 27 ± 1 PMB molecules per molecule of ATCase. This value agrees closely with the end point determined previously in the sedimentation studies of the dissociation reaction. At the spectrophotometric end point, the increase of absorption beyond that contributed separately by protein and PMB was approximately 7.5×10^3 based on the molar concentration of PMB. This value is in close agreement with the molar absorption increment of 7.6×10^3 at 250 mμ reported by Boyer (1954) for the reaction of PMB with sulfhydryl-containing compounds such as cysteine. The increment at other wavelengths was also measured and found to agree closely with the known values for the PMB–sulfhydryl (mercaptide) complex. Thus, it is concluded that the reactive groups identified spectrophotometrically are sulfhydryl groups and are the same as those found in the sedimentation studies as being located mostly, if not entirely on the regulatory subunits.

Amino acid analysis provided a direct means to determine whether sufficient cysteinyl residues were present in ATCase to account for the 26–28 sulfhydryl groups indicated by the above experiments. As shown in Table II, ATCase appears to contain 32–34 half-cystines/molecule, as inferred from the analysis of cysteic acid after performic acid oxidation of the protein. From the analysis of the cysteic acid content of the subunits after oxidation (Table II), it was found that approximately 75% of the cysteic acid content of ATCase was contributed by the regulatory subunits, even though they comprise only one-third of the weight of ATCase (Gerhart and Schachman, 1965). Thus, the four regulatory subunits of an ATCase molecule appear to contribute 24–26 half-cystines (*i.e.*, approximately 6.6 half-cystines/regulatory subunit). This result taken together with the evidence from the ultracentrifuge studies and the spectrophotometric titration, shows that the 24–26 half-cystines of the four regulatory subunits are derived from cysteinyl residues.

The remainder of the 32–34 cysteic acid/ATCase molecule found in hydrolysates of the oxidized protein were accounted for by analysis of the catalytic subunits. As seen in Table II, a value of 3.8 ± 0.4 molecules of

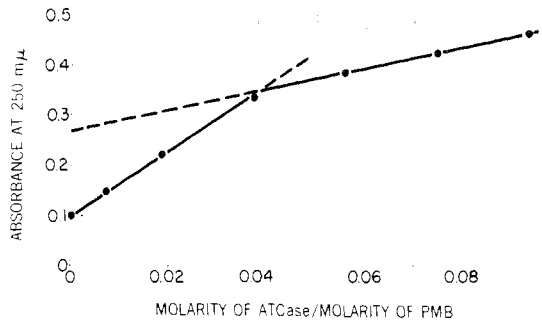

FIGURE 3: Titration of sulfhydryl groups of ATCase, according to the method of Boyer (1954). Cuvets contained 2.14×10^{-5} M PMB in 0.04 M potassium phosphate (pH 7.0), and ATCase at concentrations up to 2.06×10^{-6} M (0.64 mg/ml). Absorption at 250 mμ was determined after 20 hr at room temperature (25°). The ordinate gives the absorbance at 250 mμ and the abscissa gives the molar ratio of ATCase to PMB. The end point in the titration is indicated by the intersection of the two straight lines drawn through the experimental points.

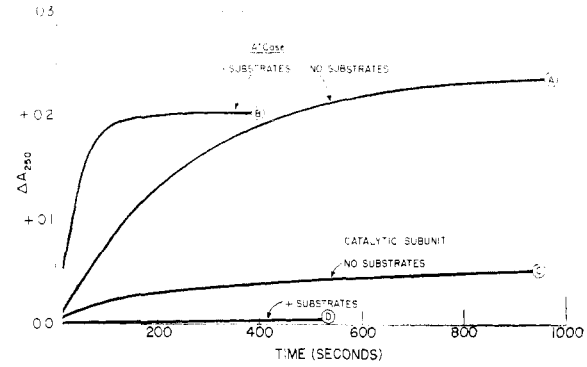

FIGURE 4: Increase of light absorption as a function of time of reaction of PMB with ATCase and the catalytic subunit. Reaction mixtures contained 2×10^{-4} M PMB. The reactions were initiated by the addition of protein and the changes of absorption at 250 mμ were recorded automatically on a Cary 14 spectrophotometer. Curve A: 0.3 mg of ATCase/ml (0.97×10^{-6} M). With no substrate present. The absorption at zero time was determined by numerical addition of the absorptions of separately prepared solutions of protein and of PMB. Curve B: same as curve A but the solution contained in addition 2×10^{-3} M potassium succinate and 1.8×10^{-3} M dilithium carbamyl phosphate. Curve C: 0.2 mg of catalytic subunit/ml (2×10^{-6} M), with no substrate present. The absorption at zero time was determined as described above for ATCase. Curve D: the conditions were the same as for curve C but in addition the solution contained 2×10^{-3} M potassium succinate and 1.8×10^{-3} M dilithium carbamyl phosphate. The ordinate represents the change in the absorbance at 250 mμ, and the abscissa represents the time after the initiation of the reaction (in seconds).

cysteic acid per molecule of protein was found, corresponding to approximately 8 half-cystines in the 2 catalytic subunits of ATCase. Since the evidence from the ultracentrifuge studies showed very little binding of PMB to the catalytic subunits, these half-cystines could be ascribed to nonreactive sulfhydryl groups in the form of eight cysteinyl residues or to disulfide bonds in the form of four cystinyl residues. Results described in a later section indicate that these half-cystines probably exist as relatively unreactive cysteinyl residues.

The Effect of Ligands on the Rate of Reaction of ATCase (and the Subunits) with PMB. The rate and extent of reaction of ATCase with PMB was followed spectrophotometrically at 250 mμ where the formation of a PMB–sulfhydryl (mercaptide) complex is accompanied by a large absorption increment. During the reaction, 10–15% of the total PMB was consumed and the rate of reaction was easily measured, as shown in Figure 4 (curve A). Under these conditions of almost tenfold excess of PMB, it was estimated that approximately 32 PMB–mercaptide bonds were formed/molecule of ATCase after 30-min reaction (assuming a molar extinction increment at 250 mμ of 7.6×10^3 for PMB–mercaptide formation). After 4 hr, this value approached 38 PMB–mercaptide bonds/ATCase molecule. Curve C shows the reaction of PMB with catalytic subunit. Approximately eight PMB–mercaptide bonds per two molecules of catalytic subunit (for comparison with one molecule of ATCase) were formed after 30-min reaction. This value increased to 12 after 4 hr. Thus very little of the spectral change observed with ATCase could be attributed to the catalytic subunits, a conclusion consistent with the previously described experiments. Attempts to determine directly the rate and extent of reaction of purified regulatory subunit with PMB have been complicated for two reasons. First, the rate of reaction was too fast to measure under the conditions used for ATCase. Second, the extent of the reaction could not be determined accurately since the absorption of the protein decreased concomitantly with the increase of absorption due to PMB–mercaptide formation.[2] Despite these uncertainties, it is estimated that at least 16 mercaptide bonds/4 regulatory subunits were formed upon reaction with PMB.

The rate of reaction of ATCase with PMB was markedly increased when the substrate (carbamyl) phosphate and the substrate analog (succinate) were

[2] Preparations of the regulatory subunit in the presence of excess 2-mercaptoethanol invariably exhibited an unusual absorption spectrum extending to about 330 mμ. In addition, the extinction coefficient at 280 mμ was unexpectedly high (1.2 for a 0.1% solution) when compared with that of native ATCase (0.59 for a 0.1% solution) and the purified catalytic subunit (0.72 for a 0.1% solution). Upon reaction with neohydrin, a mercurial which is transparent to ultraviolet light of wavelength greater than 240 mμ, the absorption of the protein (from 250 to 290 mμ) decreased to about 40% of its initial value. The initial absorption was restored upon addition of 2-mercaptoethanol.

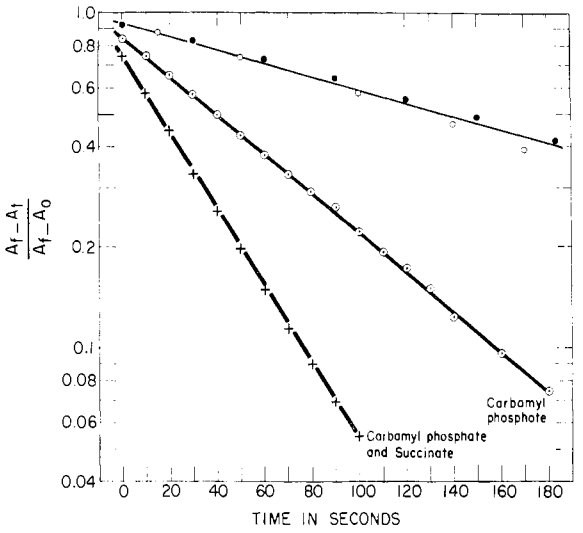

FIGURE 5: Analysis of the reaction of PMB with ATCase in terms of pseudo-first-order kinetics. Data were obtained as described in the legend of Figure 4. The values for the absorption at 250 mμ were determined at zero time (A_0), at various times during the reaction (A_t), and after the reaction was essentially complete (A_f). The results were plotted according to the equation, log $(A_f - A_t)/(A_f - A_0) = -kt/2.3$. ○ and ● represent experiments with 2×10^{-3} M potassium succinate and with no ligands, respectively. Results for these experiments were plotted after subtraction of a slower reaction (at one-seventh the rate) which represented about 30% of the total reaction after 4 hr (see text). The symbol, ⊙, represents experiments with solutions containing 1.8×10^{-3} M dilithium carbamyl phosphate. For these experiments A_f was determined after 15-min reaction. + represents experiments on solutions containing 2×10^{-3} M potassium succinate and 1.8×10^{-3} M dilithium carbamyl phosphate. For these experiments A_f was determined after 15 min. ● = no subtrates; ○ = succinate.

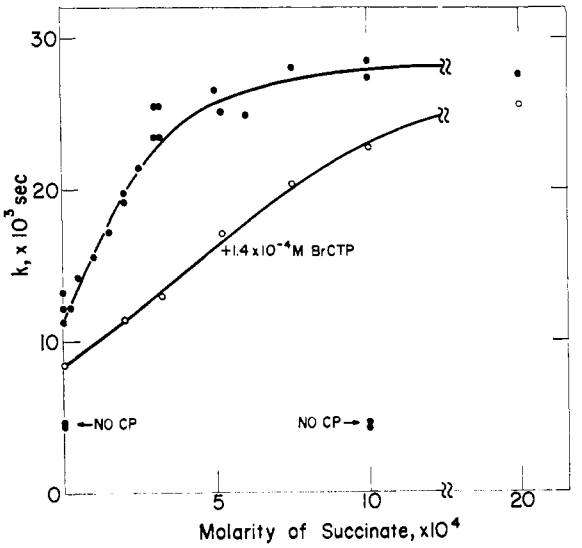

FIGURE 6: The effect of ligands on the rate of reaction of ATCase with PMB. Rate constants (k) for the pseudo-first-order reaction of ATCase with PMB were determined as described in the legend of Figure 5. The curve represented by ● gives the results for solutions containing 1.8×10^{-3} M dilithium carbamyl phosphate and varying concentrations of succinate. The curve represented by ○ gives the results for solutions containing 1.8×10^{-3} M dilithium carbamyl phosphate, 1.4×10^{-4} M BrCTP, and varying concentrations of succinate as indicated. In a few experiments indicated by ● the reaction velocity constants were determined for solutions which did not contain carbamyl phosphate. These are identified on the figure.

present. This is shown by the comparison of curves B and A in Figure 4. When both these ligands were present, the absorption increase was virtually completed within 5 min and corresponded to the formation of 26–28 PMB–mercaptide bonds/molecule of ATCase. In the subsequent 30 min, very little increase of absorption occurred, corresponding at most to one to two additional PMB–mercaptide bonds. These ligands had just the opposite effect on the rate of reaction of the purified catalytic subunit with PMB, as shown by the comparison between curves D and C in Figure 4.

This enhancement of the rate of reaction of ATCase with PMB was investigated further in order to determine whether other small molecules (ligands and nonligands of ATCase) were effective. Glutarate or D-aspartate in place of succinate had no effect; *N*,*N*-dimethylcarbamyl phosphate (kindly supplied by Dr. George Stark) in place of carbamyl phosphate had no effect. These compounds were known to act neither as substrates nor as competitive inhibitors of ATCase. Moreover, succinate in the absence of carbamyl phosphate had no effect on the rate of reaction. This finding is particularly significant in view of the evidence from equilibrium dialysis experiments (Changeux *et al.*, 1968) that succinate is not bound to ATCase in the absence of carbamyl phosphate. Since only ligands of ATCase caused an increase in the rate of reaction of ATCase with PMB and since the same ligands had the opposite effect on the rate of reaction of the catalytic subunit, we conclude that it was the reactivities of the proteins (and not the reactivity of PMB) which were influenced by the ligands. The effect of carbamyl phosphate and succinate is especially noteworthy since these ligands, in binding to the catalytic subunits of ATCase, diminish their reactivity toward PMB and yet enhance the reactivity of the regulatory subunits toward PMB.

The reaction of ATCase with PMB was found to follow pseudo-first-order kinetics even though 28 sulfhydryl groups/ATCase molecule were involved. Therefore the results were treated quantitatively in

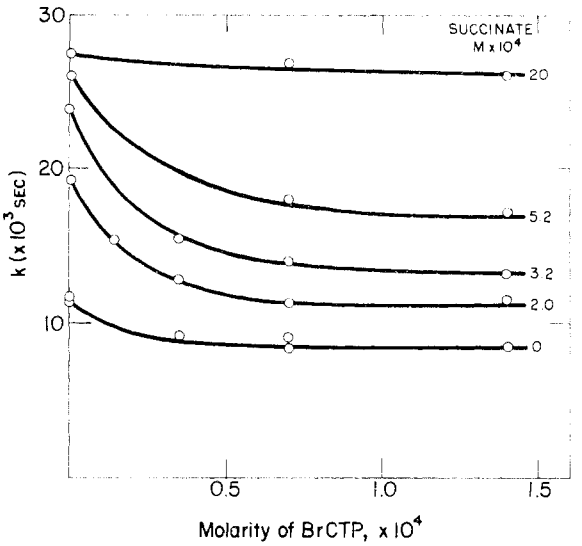

FIGURE 7: Partial antagonism by BrCTP of the effect of succinate on the reaction of ATCase with PMB. Pseudo-first-order rate constants were determined as described in Figure 5 and plotted on the ordinate as a function of the concentration of BrCTP, plotted on the abscissa. The different curves represent experiments done at different succinate concentrations as indicated in the figure.

terms of the reaction velocity constant (k) defined by

$$\log \frac{(A_t - A_t)}{(A_t - A_0)} = -kt/2.3,$$

where A_0 and A_t represent the initial and final absorbance, and A_t represents the absorbance at time (t). In all experiments, the consumption of PMB did not exceed 13% of the initial amount, and thus treatment of the data according to pseudo-first-order kinetics (which neglects changes of PMB concentration during the reaction) seems appropriate. As seen in Figure 5, the semilogrithmic plots of some of the data of Figure 4 are linear over 90% of the reaction. In the absence of ligands[3] or in the presence of succinate alone, k was found to be approximately 5×10^{-3} sec^{-1}. In the presence of carbamyl phosphate alone, this value increased to 12×10^{-3} sec^{-1}. When both carbamyl phosphate and succinate were present, k increased to 26×10^{-3} sec^{-1}.

The effect of succinate was explored further in a series of experiments in which the carbamyl phosphate concentration was maintained at 1.8×10^{-3} M, an amount at least ten times that required to produce its maximal effect. Figure 6 shows the dependence of the reaction velocity constant (k) on succinate concentration; k became independent of succinate at concentrations greater than 10^{-3} M. This enhancement of the reactivity of the enzyme was half complete at approximately 2×10^{-4} M succinate.

The Effect of Inhibitors on the Reactivity of ATCase with PMB. As shown in the preceding section, only those ligands which bind specifically to the catalytic subunits of ATCase affected the reactivity of the enzyme toward PMB. This relationship between specificity of binding and effect on reactivity of sulfhydryl groups applies also to ligands which bind to the regulatory subunits of the intact enzyme. For example, the feedback inhibitor (CTP) reduced the enhancement of reactivity promoted by succinate and carbamyl phosphate. In contrast, UTP, which neither inhibits nor activates the catalytic activity (nor does UTP compete with known inhibitors or activators), had no effect on the rate of reaction of ATCase with PMB. In order to study the effect of inhibitors, BrCTP was substituted for CTP since it had a lower extinction coefficient at 250 mμ and bound slightly more strongly to ATCase. With BrCTP, pseudo-first-order kinetics were still obtained and the extent of the reaction was unchanged (approximately 28 mercaptide bonds formed/ATCase molecule). As shown in Figure 7, BrCTP opposed the stimulatory effect of succinate (at constant concentration of carbamyl phosphate) and also reduced slightly the basal reaction rate obtained with carbamyl phosphate alone. Thus, the antagonistic effect of BrCTP appeared directly primarily toward succinate, rather than carbamyl phosphate, just as had been found in the binding studies and kinetic studies with ATCase (Changeux *et al.*, 1968; Gerhart and Pardee, 1964). In addition BrCTP did not completely prevent the stimulation by succinate, but only reduced it to a limiting value beyond which additional BrCTP had no effect. This limiting value was determined by the concentration of succinate, not BrCTP, and, in fact, at high succinate concentrations, almost no reduction was caused by BrCTP.[4] This "partial" antagonism has a striking similarity to that found between BrCTP and succinate in the binding studies (Changeux *et al.*, 1968) and between CTP and aspartate in the kinetic studies (Gerhart and Pardee, 1964).

The data from experiments with high concentrations of BrCTP (1.4×10^{-4} M) were cross-plotted in Figure 6 to illustrate the effect of BrCTP on the dependence of the reactivity of the enzyme on succinate. As seen there, the reactivity of the sulfhydryl groups of the enzyme increases with succinate concentration to a maximum value close to that obtained in the absence of BrCTP. However, when BrCTP is present, higher

[3] In the absence of carbamyl phosphate there appeared to be two classes of reactive groups, one equivalent to 28 PMB–mercaptide bonds/ATCase molecule and reacting with $k = 4.9 \times 10^{-3}$ sec^{-1}, and a second class equivalent to approximately 10 PMB–mercaptide bonds and reacting with $k = 0.7 \times 10^{-3}$ sec^{-1}. This second class disappeared almost completely in the presence of carbamyl phosphate, with k less than 0.1×10^{-3} sec^{-1}. Presumably carbamyl phosphate protected the catalytic subunits from the attack of PMB.

[4] The specificity of the BrCTP effects is cited as evidence that BrCTP was affecting the reactivity of ATCase, and not the reactivity of PMB.

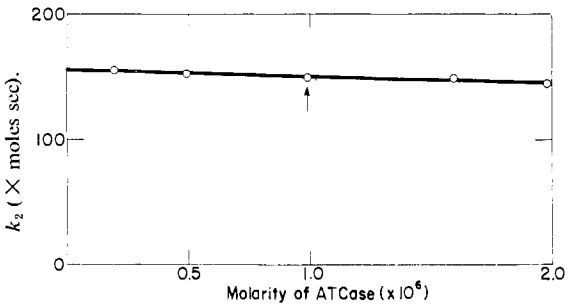

FIGURE 8: The rate of reaction of ATCase with PMB, as a function of protein concentration. In all experiments the solutions contained 1×10^{-3} M potassium succinate, 1.8×10^{-3} M dilithium carbamyl phosphate, and varying concentrations of ATCase as indicated. The arrow on the graph corresponds to the concentration of ATCase used for most of the other experiments. At the highest concentration of ATCase approximately 26% of the PMB was consumed in the reaction and therefore corrections for this consumption were taken into account by analyzing all data in terms of second-order, rather than pseudo-first-order, rate constants.

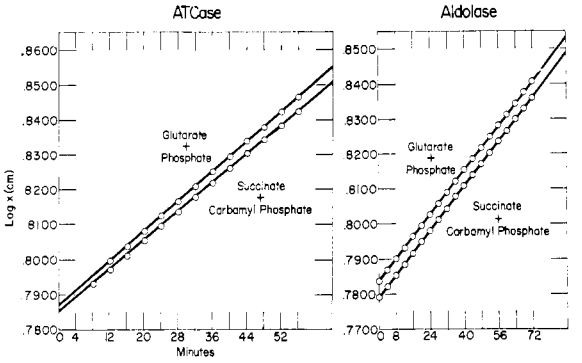

FIGURE 9: Effect of succinate and carbamyl phosphate on the sedimentation coefficient of ATCase and aldolase. Samples contained 2×10^{-3} M potassium succinate, 1.8×10^{-3} M dilithium carbamyl phosphate, 0.04 M potassium phosphate at pH 7.0, and 5.0 mg of protein/ml (either ATCase or aldolase). Reference solutions were prepared containing the same components at the same concentration except for 2×10^{-3} M potassium glutarate in place of succinate and 1.8×10^{-3} M potassium phosphate at pH 7.0 in place of carbamyl phosphate. Sample and reference solutions were analyzed simultaneously in same ultracentrifuge rotor at 60,000 rpm at 20°. Single-sector Kel-F cells were used, one of which contained an upper wedged window and the other having conventional windows. Sedimentation coefficients were determined from the movement of the maximum ordinate of the schlieren patterns. In the figure the ordinate represents the logarithm of the distance of the boundary (in centimeters) from the axis of rotation and the abscissa gives the time in minutes.

concentrations of succinate are required to attain an equivalent enhancement of reactivity. Again, studies of a structural property (*i.e.*, chemical reactivity) gave results very similar to those obtained in the analysis of a functional property (kinetic behavior).

Preliminary Studies of the Mechanism of Reaction of ATCase with PMB. From the evidence that the reaction of ATCase with PMB followed pseudo-first-order kinetics, it was expected that the rate constant for the reaction would not depend on protein concentration. In order to test the effect of protein concentration, the kinetics were followed over a tenfold range of initial ATCase concentration. Since a substantial fraction of PMB was consumed at high protein concentrations, data were treated in terms of second-order kinetics. The calculated velocity constant as shown in Figure 8 was indeed independent of protein concentration. This result does not eliminate the possibility that protein dissociation into subunits is an important factor in the mechanism of the reaction of ATCase with PMB (*e.g.*, as a rate-limiting step).

Since PMB was present in tenfold excess in the previous experiments, no information was obtained regarding the reaction order for PMB. In preliminary experiments, a low reaction order was found for PMB and the data did not fit a simple first-order dependence on PMB concentration.[5] It seemed clear that the data did not fit a rate equation based on a high-order term for PMB as would be required if many of the sulfhydryl groups of ATCase had to react in a concerted fashion to give the all-or-none reaction with PMB.

The Effect of Ligands on the Sedimentation Coefficient of ATCase. In preliminary studies of the effect of specific ligands on the sedimentation rate of ATCase it was found that the change in the sedimentation coefficient of the enzyme upon the addition of the ligands was only slightly greater than the experimental errors incurred in individual experiments. Accordingly all subsequent experiments were performed in a manner which permitted a direct comparison between the different solutions which were examined simultaneously. One solution contained the ligands, succinate and carbamyl phosphate; the other (reference) solution had equivalent concentrations of the dicarboxylic ion glutarate (an analog having negligible affinity for ATCase) in place of succinate and phosphate in place of succinate and phosphate in place of carbamyl phosphate. Through this analysis of two samples in a single ultracentrifuge experiment the precision in measuring the effect of the ligands (relative to the control) was enhanced greatly. The effect of errors in measuring the temperature and speed of the rotor in separate experiments was eliminated and also inaccuracies in locating the relative boundary positions on the photographic plates were

[5] The dependence of reaction velocity on PMB concentration was approximated by the equation: $d \ln p/dt = k_1 PMB^2/(k_2 + PMB)$, where p = mercaptide product, $k_1 = 220$ M^{-1} sec^{-1}, and $k_2 = 9 \times 10^{-5}$ M.

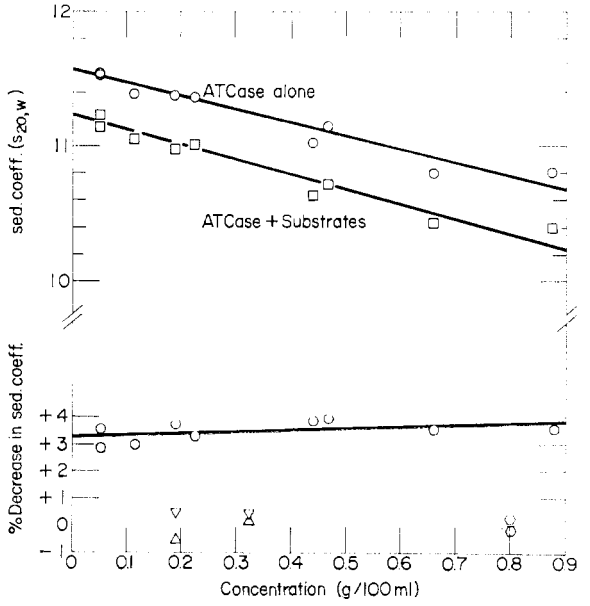

FIGURE 10: Effect of protein concentration on the sedimentation coefficient of ATCase in the presence and absence of ligands. Samples contained 0.04 M potassium phosphate at pH 7.0, 2×10^{-3} M potassium succinate, 1.8×10^{-3} M dilithium carbamyl phosphate, and ATCase at the concentrations indicated. Samples were analyzed in pairs by examining a solution containing the ligands in one cell and the reference solution in a second cell in a single experiment. A reference solution contained potassium glutarate in place of succinate and potassium phosphate in place of carbamyl phosphate. All of the centrifuge experiments were performed at 20° and 60,000 rpm. At the top of the figure is plotted the actual sedimentation coefficient as a function of concentration for the samples of ATCase in the absence and in the presence of the specific ligands (succinate and carbamyl phosphate). ○ represents ATCase in the reference solvent and □ represents the results with solutions containing succinate and carbamyl phosphate. At the bottom of the figure is a plot of the per cent decrease in sedimentation coefficient as measured in each single experiment with a pair of solutions. ○ represents the calculated results from each of the solutions in a given experiment. Also at the bottom of the figure are experiments which serve as additional controls; ▽ represents experiments in which ATCase was sedimented in the presence of carbamyl phosphate alone; △ represents experiments on ATCase in which only succinate was present in the solution; and ◌ represents experiments on aldolase in which both carbamyl phosphate and succinate were present in the solution.

reduced substantially. Since the two solvents had essentially the same ionic strengths, viscosities, and densities, the change in the sedimentation coefficient of ATCase can be attributed to a specific effect of the ligands on the gross size and shape of the enzyme molecules.

As seen in Figure 9A, ATCase in the presence of both succinate and carbamyl phosphate sedimented at a rate 3.5% slower than the enzyme in the reference solution. When carbamyl phosphate was omitted the sedimentation coefficients of the enzyme in the two solutions (succinate *vs.* glutarate) were virtually identical. If carbamyl phosphate was present alone the reduction in the sedimentation rate of ATCase (compared to the enzyme in phosphate) was only about 0.5%. Thus both ligands are required for the maximal effect. In this respect the sedimentation studies can be related to the findings of Changeux *et al.* (1968) which showed that succinate is bound to ATCase only when carbamyl phosphate is also present. The results of the sedimentation studies are also similar to those from the kinetic experiments which showed that the maximal enhancement of the reactivity of the sulfhydryl groups of ATCase was obtained when both succinate and carbamyl phosphate were present.

An additional test of the precision of the sedimentation measurements was provided by examining the effect of succinate and carbamyl phosphate on the enzyme, aldolase. The results in Figure 9B show clearly that aldolase in the presence of the ligands which are specific for ATCase sediments at the same rate as in the reference solution (containing glutarate and phosphate). From these experiments with aldolase (see Figure 10) the precision in measuring changes in sedimentation coefficients was found to be about ±0.5%.

In contrast to ATCase, the catalytic subunit did not undergo a reduction in sedimentation rate when succinate and carbamyl phosphate were added. In fact, there was an increase of about 1.8% in the sedimentation coefficient. Both ligands were required for this effect. These results suggest that the decreased sedimentation coefficient of ATCase in the presence of the specific ligands is related to changes of the gross structure of the whole enzyme molecule and perhaps can be attributed to the rearrangement of the two catalytic and four regulatory subunits within the intact molecules.

Sedimentation velocity studies of the effect of ligands were also conducted over a range of ATCase concentrations. As seen in Figure 10 the dependence of sedimentation coefficient on protein concentration (both in the presence and absence of the ligands) was essentially linear. No evidence of dissociation of the enzyme into subunits was obtained either from the appearance of the boundaries or from the concentration dependence of the sedimentation coefficient. Even at protein concentrations of 3–20 μg/ml the sedimentation coefficient of ATCase was about 11.7 S (Schachman and Edelstein, 1966), a value consistent with those obtained at higher concentrations (Figure 10). Thus there was no evidence (over the concentration range accessible to ultracentrifugal measurements) that succinate and carbamyl phosphate promoted a reduction in the sedimentation rate of ATCase by perturbing an association–dissocia-

tion equilibrium in the direction of dissociation into subunits. Instead, the reduction in the sedimentation coefficient in the presence of ligands can be attributed to an increase in the frictional coefficient of the undissociated enzyme molecules (Svedberg and Pedersen, 1940; Schachman, 1959). This increase in the frictional coefficient could arise from either a swelling of the molecules (to give a larger effective hydrodynamic volume) or a change in their shape (from an essentially spherical form to one that was more elongated or flattened).

As seen in Figure 10 the dependence of the sedimentation coefficient of ATCase on concentration is slightly greater for the solutions containing succinate and carbamyl phosphate than in the reference solvent. This result, too, can be ascribed to the increased hydrodynamic resistance which the "swollen" ATCase molecules experience when the ligands are present.

Most of the reduction in the sedimentation rate of ATCase occurred upon the addition of succinate (although the presence of carbamyl phosphate was essential). In order to determine the effectiveness of succinate in promoting the change in the hydrodynamic behavior of ATCase a series of experiments were performed at varying succinate concentrations (with a fixed concentration of carbamyl phosphate). The results in Figure 11 show that the decrease in sedimentation coefficient attained a limit at concentrations of 1–2 $\times$ 10^{-3} M succinate. At approximately 2 $\times$ 10^{-4} M succinate the decrease in sedimentation coefficient was half the limiting value obtained at tenfold higher levels of succinate. This value of 2 $\times$ 10^{-4} M succinate is virtually identical with that required for half-completion of the enhancement of the reactivity of the sulfhydryl groups of ATCase (see Figure 6).

Studies were also conducted to determine the effect of the inhibitor (CTP) on the sedimentation rate of the intact enzyme. For these experiments solutions of ATCase containing uridine triphosphate were used as a control since UTP, unlike CTP, has no inhibitory activity. The results of these experiments showed that within experimental error ($\pm$0.5%) the enzyme had the same sedimentation rate in either CTP or UTP solutions (at concentration of 1 $\times$ 10^{-3} M). Even though CTP was bound to the enzyme (and UTP presumably was not) the sedimentation coefficient was affected so little as to escape detection by this technique. When, however, the ligands which bind to the catalytic subunits of the intact enzyme were also present in the solution, the effect of CTP (*vs.* UTP) was readily observed. Under these circumstances the addition of CTP led to an increase in the sedimentation coefficient, *i.e.*, the effect of succinate and carbamyl phosphate in causing a reduction in the sedimentation coefficient of the enzyme was much less if the inhibitor was also present. This counteraction by the inhibitor of the effect of the ligands binding to the catalytic subunits was only partial. As seen in Figure 11, the effect of succinate and carbamyl phosphate was reduced approximately 50% by 10^{-4} M CTP and only about 70% by a tenfold higher concentration of CTP. In this regard,

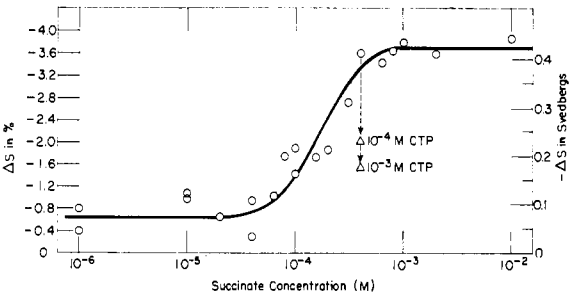

FIGURE 11: Dependence of the sedimentation coefficient of ATCase on the concentration of the substrate analog, succinate. All samples contain 0.04 M potassium phosphate at pH 7.0, 1.8 $\times$ 10^{-3} M dilithium carbamyl phosphate, 4.2 mg of ATCase/ml (1.4 $\times$ 10^{-5} M), and potassium succinate at the concentrations indicated. Each sample was centrifuged in a single-sector ultracentrifuge cell simultaneously with a reference solution containing the same amount of potassium phosphate and ATCase, but no carbamyl phosphate or succinate. In place of succinate at each of the concentrations indicated on the graph the reference solution contained an equivalent amount of potassium glutarate. The ordinate gives the change in the sedimentation coefficient (Δs) in per cent and the abscissa represents the succinate concentration in molarity. All of the centrifuge experiments were performed at about 20° and the speed was 60,000 rpm. In some experiments the reference solution was placed in the cell containing the wedged window, whereas in others this cell contained the sample of ATCase at different concentrations of succinate. In all calculations of the per cent decrease in sedimentation coefficient the reference solution for that particular experiment was used as a standard. The sedimentation coefficients of ATCase in the reference solutions were very reproducible and gave values of about 11.6 S.

the results of these sedimentation studies are similar to those on the effect of BrCTP in opposing the enhancement of the reactivity of the sulfhydryl groups caused by the addition of succinate and carbamyl phosphate (see Figure 7). Although the data from the sedimentation studies are much less complete than those from the kinetic analysis of the reaction of PMB with ATCase, the results in Figure 11 are formally analogous to those in Figure 6.

Discussion

Different Conformational States of ATCase in the Presence and Absence of Specific Ligands. The results presented above demonstrate that both the rate of reaction of ATCase with PMB and the sedimentation coefficient of ATCase are markedly affected by the presence of specific ligands of the substrate binding sites of the enzyme. For example, the rate of reaction of the sulfhydryl groups is increased sixfold (over the control lacking ligands) when both succinate and

carbamyl phosphate are present. Similarly the sedimentation coefficient is decreased 3.5% under the same conditions. If only carbamyl phosphate is present with the enzyme, the change (relative to the control) in both the rate of reaction with PMB and the sedimentation coefficient is much smaller. If only succinate is present with the enzyme, no change is detected. This specific pattern of effects is in accord with the findings of Changeux *et al.* (1968) that succinate binding for ATCase is measurable only when carbamyl phosphate is present. As shown in Figure 12 the changes in both measurements occur at low concentrations of succinate (below 10^{-3} M) and attain their maximum value within the concentration range found previously for the saturation of the binding sites of ATCase by succinate (Changeux *et al.*, 1968). These observations indicate that the changes in both the rate of reaction of ATCase and PMB, and the sedimentation coefficient of ATCase were due to the interaction of the enzyme with its specific ligands and were a reflection of altered properties of the protein (or the protein ligand complexes).

These changes in the properties of the enzyme in the presence of specific ligands are more likely an indication of profound alterations in the structure of the protein itself, rather than simply the result of the presence of the ligands on the surface of an "unmodified" protein. For example, the *reduction* in the sedimentation coefficient could conceivably be attributed to bound ligands protruding from the surface of the enzyme and thereby increasing the frictional coefficient of the protein–ligand complex. This possibility is unlikely, however, since it was found that the catalytic subunit of ATCase did *not* undergo a reduction in sedimentation rate when these same ligands were added. On the contrary the sedimentation coefficient actually increased by about 1.8%. As a further example, with regard to the change in reactivity of ATCase toward PMB (an observed sixfold *increase* in the rate of reaction upon addition of both ligands), again the effect could be attributed to the influence of ligands on the reactivity of local sulfhydryl groups of an "unmodified" protein. If this were the case, the effect of the ligands in enhancing the reactivity of these sulfhydryl groups would be *direct*, involving modification of the local environment perhaps by the introduction of charged groups. This possibility seems unlikely since almost none of the 28 highly reactive sulfhydryl groups of an ATCase molecule were located on the catalytic subunits, where succinate and carbamyl phosphate were bound. The few slowly reacting sulfhydryl groups detected on the catalytic subunits are in fact affected in the opposite direction by ligands. As shown in curves C and D of Figure 4, the addition of succinate and carbamyl phosphate led to a definite reduction in their reactivity, as if the ligands provided steric or ionic protection for the sulfhydryl groups. Since almost all of the highly reactive sulfhydryl groups are located on the regulatory subunits, it seems likely that ligands of the catalytic subunits enhance the reactivity of these sulfhydryl groups *indirectly*, as a consequence of the modification of the structure of the protein itself.

Moreover, if local and direct effects of ligands were largely responsible for the observed changes of sedimentation coefficient and chemical reactivity of ATCase, then one would expect a proportional relationship between the fraction of binding sites occupied by ligand, and the amount of change in sedimentation coefficient or reactivity toward PMB. As shown in Figure 12, this expectation was not fulfilled. The observed changes in both chemical reactivity and sedimentation coefficient attained half their limiting and maximum value when only 15% of the binding sites was occupied by succinate. This critical result will be discussed and analyzed in detail in the following paper (Changeux and Rubin, 1968) and is mentioned here only as evidence that structural changes of the protein mediate the observed effects of ligands on the chemical reactivity and sedimentation coefficient of ATCase.

Since sedimentation velocity experiments revealed no change in the state of aggregation of ATCase upon the addition of ligands, we can conclude that the enzyme exists in two or more *undissociated* forms which differ at least by 3.6% in sedimentation coefficient. As discussed above, this reduction in sedimentation rate is attributable to an increase in the frictional coefficient (by 3.6%) of ATCase upon the addition of succinate and carbamyl phosphate.[6] Provided that the various forms of ATCase are spherical, the change of frictional coefficient indicates a 3.6% increase in the effective radius of the protein (Scheraga and Mandelkern, 1953), *i.e.*, an 11% increase of hydrodynamic volume. By this reasoning, we can conclude that succinate and carbamyl phosphate enrich the ATCase population (in a manner unspecified) for a more swollen form of the protein. If the forms of ATCase cannot be considered spherical, the change in sedimentation coefficient would indicate that in the presence of the ligands, the protein molecules are more elongated (or flattened) relative to the protein in the absence of ligands.

Since the pseudo-first-order rate constant for the reaction of ATCase with PMB did not depend on protein concentration, we can conclude that the enzyme exists in two or more undissociated forms which differ at least by sixfold in their rate of reaction with PMB (depending upon the presence or absence of succinate and carbamyl phosphate).[7] Moreover, the observation of pseudo-first-order kinetics indicates that the different forms of the enzyme equilibrate

[6] The 3.6% decrease of sedimentation coefficient could conceivably be attributed to an increase (approximately 1%) in the partial specific volume of the protein as a consequence of the binding of ligands. This possibility seems unlikely since these ligands are probably more dense than the protein and partial specific volumes are generally additive (Rosenberg and Klotz, 1955). In addition when the same ligands were added to the catalytic subunit, an increase in the sedimentation coefficient was produced. Thus, we conclude that it is the frictional coefficient, and not the partial specific volume, which is affected.

[7] Of the sixfold difference in the reactivity of the forms of ATCase, about 28% of the difference is produced by carbamyl phosphate alone, and the remaining 72% is produced by the subsequent addition of succinate.

rapidly with one another compared to their rate of reaction with PMB. The measured rate constant would thus contain not only a term for PMB concentration but also terms for the reactivities of the different forms and terms for their relative concentrations in the total ATCase population.

It is important to note that the change in the sedimentation coefficient and the change of the reactivity toward PMB have almost an identical dependence on succinate concentration. As seen in Figure 12 both properties attain their half-maximal change at approximately 2×10^{-4} M. This similar concentration dependence may indicate that the changes of both properties originate from the *same* structural transitions in the protein. The more swollen conformation of ATCase, which predominates in the presence of succinate and carbamyl phosphate and is characterized by a reduced sedimentation coefficient, might also have more exposed sulfhydryl groups which react rapidly with PMB.

It is striking that ligands affect the physical properties of the enzyme in the same way as they affect catalytic activity (Gerhart and Pardee, 1964) and ligand affinity (Changeux *et al.*, 1968). This similarity is particularly apparent from the following comparison. The reactivity of ATCase with PMB was enhanced by succinate and this enhancement was only partially reduced by BrCTP. The limit of reduction obtained with excess BrCTP depended on the concentration of succinate (see Figure 7). Likewise, the catalytic activity of ATCase was only partially inhibited by excess CTP, to an extent depending on the amount of aspartate present. The conformational changes which mediate the ligand effects on the reactivity of ATCase toward mercurials are thus likely to be those which mediate changes of catalytic activity as well. The exact relationship between ligand binding and changes of conformation of the protein will be considered in terms of the model of Monod *et al.* (1965) in the following paper (Changeux and Rubin, 1968).

Possible Mechanisms for the Reaction of ATCase with PMB. The all-or-none reaction of proteins with mercurials has been explained previously by mechanisms in which the rate of reaction of the numerous sulfhydryl groups of a single protein molecule is limited by an initial reaction such as (1) the formation of the first mercaptide bond, after which all other groups react rapidly (a "wedge" or "zipper" model, *cf.* Madsen and Gurd, 1956; Boyer, 1958); (2) an isomerization of the protein to expose all sulfhydryl groups; or (3) a dissociation of the protein into subunits with exposed sulfhydryl groups (Keresztes-Nagy *et al.*, 1965). Although there are insufficient data for ATCase to permit us to discriminate among these mechanisms, we will consider the third one in more detail here because it explicitly links all-or-none reactivity with dissociation of the protein, namely, the two main characteristics of the reaction of ATCase with PMB. According to the provisions of this proposal, the 28 sulfhydryl groups of ATCase would be buried in the undissociated molecule and become exposed for reaction with PMB only as a consequence of the dissociation reaction, which would constitute the rate-limiting step. Since ATCase is known to be a highly undissociated protein, it is conceivable that its dissociation rate is slow (see, for example, the dissociation of tryptophan synthetase with half-times in the range of several hundred seconds or more (Creighton and Yanofsky, 1966)).

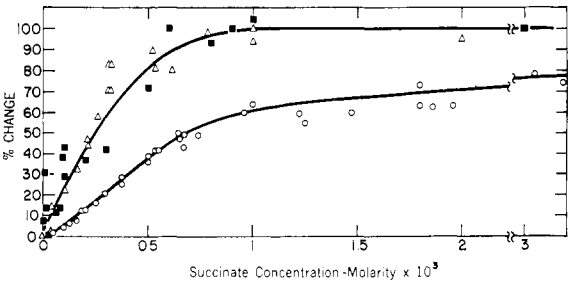

FIGURE 12: Summary of the extent of conformationa change and of ligand saturation of ATCase, as a function of the concentration of succinate. Data from Figures 6 and 11 are replotted as per cent change after normalization, with 100% change on the ordinate representing the maximal change observed due to the addition of succinate (carbamyl phosphate being present throughout). For Δs measurements the range extended from a 0.6% reduction (no succinate) to a 3.6% reduction in sedimentation coefficient; for rate constants of the PMB reaction, the range extended from $k = 12 \times 10^{-3}$ sec^{-1} (no succinate) to 28×10^{-3} sec^{-1}. For comparison, data for the binding of succinate by ATCase were replotted from Changeux *et al.* (1968) with 100% change on the ordinate representing the complete saturation of one molecule of ATCase by four molecules of succinate. $\Delta = k \times 10^3$ sec, $\circ = N$; $\blacksquare = \Delta s$, $\circ$ = fractional saturation of ATCase by succinate.

According to this mechanisms, the rates measured spectrophotometrically for the reaction of ATCase with PMB would be limited and determined by the rate of dissociation of ATCase into subunits, which would then react very rapidly with PMB. Indeed, separated regulatory subunits were found to react with PMB at a rate too fast to measure in these experiments. This postulated mechanism of dissociation prior to reaction would not be apparent from the test of the dependence of reaction rate on protein concentration, since the dissociation *rate* of the protein would not depend on protein concentration. Furthermore, even though one might expect to detect a dissociated and therefore fast-reacting fraction of the population at the beginning of the reaction, this is not feasible in the case of ATCase since so little of the protein exists freely in the dissociated form (even at 3 μg/ml, no dissociated material has been detected).

Presumably, the ATCase conformation of higher reactivity in the presence of ligands is one which dissociates faster. Perhaps the swollen form indicated by the sedimentation velocity data is one with relatively

weak bonds between subunits and which therefore dissociates rapidly.

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