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Spin-Labeling Studies of Aspartate Transcarbamylase. I. Effects of Nucleotide Binding and Subunit Separation*

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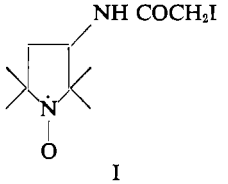
ABSTRACT: Aspartate transcarbamylase has been reacted with the spin-labeling reagent 4-α-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl and the effects of ligand binding, pH, and separation into subunits on the electron spin resonance spectrum of the labeled protein have been investigated. CTP and ATP binding cause broadening of the electron spin resonance spectrum while UTP and succinate (a substrate analog) and carbamyl phosphate have no effect. Increasing pH and decomposition into subunits by *p*-hydroxymercuribenzoate result in narrowing of the spectrum. It was also found that the course of the labeling reaction was affected by the presence of ligands. The conformational change measured by changes in the labeled protein electron spin resonance in the presence of CTP did not correlate well with the predictions of the simple allosteric models for heterotropic effects.

The spin-labeling technique, in which conformation changes in biopolymers are followed by means of changes in the electron spin resonance spectrum of a bound free-radical label, has been shown by McConnell and coworkers to be very useful in studying the allosteric transitions accompanying oxygen binding in hemoglobin (Ogawa and McConnell, 1967; McConnell and Hamilton, 1968; McConnell *et al.*, 1969). In these investigations the protein was reacted with the reagent *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I) and a change in the electron spin resonance spectrum of the label bound at the β93 cysteine was observed which correlated with the degree of oxygenation in agreement with the predictions of the sequential model for allosteric transitions of Koshland *et al.* (1966) (referred to as KNF in this paper).



I

The aim of the present work is to use this technique to study the binding of allosteric effectors in a more complex system which exhibits heterotropic as well as homotropic effects. An ideal protein for such a study is the regulatory enzyme aspartate transcarbamylase (ATCase) from *Escherichia coli* which exhibits all of the general features of allosterism, including cooperativity in substrate binding, and inhibition and activation through binding at sites distinct from the catalytic site. With the exception of hemoglobin it is the most extensively studied protein in regard to its allosteric properties and is available in gram quantities from a special strain of *Escherichia coli* by a relatively simple isolation procedure (Gerhart and Holoubek, 1967).

ATCase is the first enzyme concerned only with pyrimidine biosynthesis and is subject to feedback inhibition by the end product cytidine triphosphate (CTP), as a means of turning off synthesis of intermediates when levels of the end product are sufficiently high in the cell. It is activated in a manner analogous to the CTP inhibition by adenosine triphosphate (ATP). This has been suggested to be of importance in maintaining the balance between the supply of purines and pyrimidines in the nucleic acid synthesis pool (Gerhart and Pardee, 1962). Also binding of the substrates aspartate and carbamyl phosphate (CAP)[2] is cooperative through interactions between catalytic sites on different subunits.

Considerable progress has been made toward understanding the structure and mechanism of catalysis by ATCase in recent years. Gerhart and Schachman (1965) have shown that the enzyme can be separated by heat or mercurials into 2 distinct types of subunits, one of which binds only the substrates and is fully catalytically active (catalytic C) and another that has no activity and binds the inhibitor

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[2] Abbreviations used are: CAP, carbamyl phosphate, PMB, *p*-hydroxymercuribenzoate.

CTP (regulatory R). They determined molecular weights and ligand binding constants for the subunits and on the basis of these proposed a structural model for the enzyme consisting of 2 C subunits with 2 catalytic sites each and 4 R subunits with 1 regulatory site each. The R subunit has recently been sequenced (Weber, 1968b) and preliminary X-ray studies on the intact enzyme have been reported (Wiley and Lipscomb, 1968). The results of these studies suggest that the enzyme has a threefold axis of symmetry and is composed of 6 identical regulatory chains and 6 identical catalytic chains, in conflict with the earlier findings.

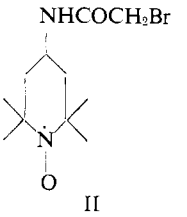
Recently two research groups have reported detailed investigations of the conformation changes that accompany binding of the allosteric ligands. One approach used by Gerhart and Schachman (1968) involved the observation of changes that occur in the sedimentation velocity of the protein, and its reaction rate with *p*-hydroxymercuribenzoate, the reagent used to separate the C and R subunits, as a result of changes in the accessibility of potential sites of attack in different conformational states. The sedimentation coefficient decreases by about 3.6% in the presence of the substrate CAP and succinate, an inactive analog of the other substrate aspartate. This change can be partially reversed by CTP, but CTP alone has no effect. Similarly, the rate of reaction with PMB increases in the presence of CAP and succinate and this increase can be partially reversed by CTP although CTP itself does not affect the rate.

McClintock and Markus (1968) used changes in the rate of reaction of ATCase with proteolytic enzymes in the presence of the allosteric ligands as a measure of conformational state. In these studies it was found that the rate of digestion by several such enzymes was increased by the substrate aspartate and decreased by both the inhibitor CTP and the activator ATP.

All of these results, with the exception of the effect of ATP on digestability, are consistant with allosteric activation resulting in a more open (relaxed) protein structure and inhibition in a more constrained structure. From a comparison of ligand binding curves with the state functions for the changes in sedimentation coefficient and reaction rate with PMB in the presence of CAP, succinate, and CTP, Changeux and Rubin were able to calculate a set of parameter values to fit the behavior of ATCase to the Monod *et al.* (1965) (MWC) concerted allosteric model. However, McClintock and Markus (1969) found that the state function determined from the effect of the substrate aspartate on digestability was homologous with plots of activity and binding as a function of aspartate concentration and thus concluded that in regard to the homotropic effects the behavior of ATCase was more consistant with the KNF sequential model.

The spin-labeling technique offers a number of unique advantages as a probe to investigate the details of these allosteric conformation changes. The observed changes in the electron spin resonance spectrum can be unambiguously related to local conformational changes in the region of the protein where the label is attached and, to the extent that the location of the label can be determined, the portion of the protein undergoing the conformation change can also be specified. In most cases where this technique has been used it has been found that the labeling agent does not seriously alter the specific activity of the enzyme and thus perturbation of the label spectrum can easily be followed under the normal conditions of catalysis. From the nature of the change in the electron spin resonance spectrum it may be possible to reach conclusions as to how the protein tertiary structure is modified by ligand binding.

In this paper the results of electron spin resonance studies on intact ATCase labeled with the reagent, 4-α-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl (II) are presented.



II

This labeled protein shows no loss of activity or susceptability to CTP and the nitroxide electron spin resonance spectrum is sensitive to heterotropic allosteric effects. The effects of CTP, ATP, UTP, and substrate binding, pH changes, and reaction with PMB have been investigated. It has also been found that the type of electron spin resonance spectrum exhibited by the labeled ATCase can be modified by carrying out the labeling reaction in the presence of various ligands. These results are discussed in terms of their relation to the other studies of conformational effects in ATCase and the MWC and KNF allosteric models. Future papers in this series will be concerned with the results of investigations with other labeling agents and labeling of individual subunits, in addition to further explorations with this labeled enzyme.

Materials and Methods

Chemicals. PMB and ATP were purchased from Sigma Chemical Co., UTP from California Biochemical Research, CTP from Pierce Chemical Co., and *N*-ethylmaleimide from Aldrich Chemical Co.

The spin-labeling reagent II was prepared by a method similar to that used by Ogawa and McConnell (1967), and which has been described previously by Buckman *et al.* (1969).

Enzyme. ATCase was isolated from a special strain of *E. coli* following the procedure of Gerhart and Holoubek (1967). Bacteria were grown at 35° in 100 l. of media to a density of about 200 klett units/ml and disrupted by sonic oscillation. The strain of *E. coli* was kindly provided by Dr. John Gerhart. The purified enzyme was stored frozen at −15° in pH 7.0, 0.04 M potassium phosphate buffer containing 2×10^{-3} M 2-mercaptoethanol and 2×10^{-4} M sodium EDTA.

Protein concentration in crude extracts was determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a reference standard. The concentration of purified ATCase was determined from measurement of the optical density at 280 nm using value of 0.59 for the absorbance of 1-mg/ml solution (Gerhart and Schachman, 1968). Activity of the enzyme was measured using the colorimetric assay for carbamyl aspartate of Gerhart and Pardee (1962). A maximum specific activity of 7900 units/mg was obtained for the purified preparation. In the presence of 5×10^{-3} M aspartate and 3.6×10^{-3} M CAP the ATCase

was inhibited 54% by 5×10^{-4} M CTP under the usual assay conditions.

Enzyme purity was also checked by gel electrophoresis and the ultracentrifuge sedimentation velocity pattern. Acrylamide gels (7.5%) run in pH 7.0 barbitol buffer at 25–30 V/cm showed, in addition to the ATCase band, a small slower moving band (1 to 5%) and occasionally traces of a component running just ahead of ATCase. Relative concentrations were measured with a densitometer. ATCase was also denatured to the C and R peptide chains with sodium dodecyl sulfate and the denatured protein run on sodium dodecyl sulfate containing acrylamide gels. The molecular weights of the chains from their movement on the gels relative to reference standards agree with those found by Weber (1968b) using this method.

In the analytical ultracentrifuge with schlieren optics the ATCase preparation had a major component with a sedimentation coefficient $s_{20,w} = 11.2$ S in good agreement with the value of 11.7 S for ATCase determined by Gerhart and Schachman (1965) and a minor component (<5%) with $s_{20,w} = 16.5$ S.

Reaction of ATCase with 4-α-Bromoacetamido-2,2,6,6-tetramethylpiperidine-N-oxyl. The labeling reaction was carried out by allowing a solution of ATCase (5–10 mg/ml) in pH 7.0, 0.04 M phosphate buffer to stand 2–3 days at 4° with approximately 1 mg of the labeling agent II for each 10 mg of protein. The nitroxide was initially largely insoluble, but dissolved within about 6 hr. Prolonged stirring caused some precipitation of the protein. Excess label was removed by dialysis against the same buffer. To block sulfhydryl groups which might be potential labeling sites experiments were also carried out in which the enzyme was reacted with an equivalent amount of *N*-ethylmaleimide for 1 to 5 days at 3° before introduction of II.

When the labeled enzyme was stored at 4° for long periods (2–3 weeks) in the presence of the stabilizer 2-mercaptoethanol the intensity of the nitroxide signal decreased appreciably, indicating that the mercaptan may be able to reduce the nitroxide under these conditions. This problem could be avoided by storing the labeled enzyme at −15° in the absence of stabilizers. The presence of the stabilizers in the buffer did not effect the course of the labeling reaction itself.

When ATCase preparations about 1-month or more old are reacted with II the electron spin resonance spectrum of the product often begins to show the appearance of label with greater mobility (explained in Results section). The usual spectrum observed for fresh enzyme preparations could be obtained from this older protein by several precipitations by dialysis against pH 5.9, 0.01 M phosphate buffer [the last step in the Gerhart and Holoubek (1967) ATCase purification procedure] before or after the labeling reaction. The protein samples which gave the altered electron spin resonance spectrum were identical with fresh ATCase in specific activity, inhibition by CTP, and acrylamide gel electrophoresis behavior. Storing the protein at −15° did not appear to retard this behavior.

To determine the amount of label bound the enzyme was first denatured by titration to pH 12 converting all of the label electron spin resonance spectrum into the type showing a high degree of motional freedom. This type of spectrum can be approximately duplicated by solutions of free nitroxide in viscous medium (~55% sucrose) and the concentration of bound nitroxide can then be estimated by comparison with such a solution of known concentration.

Separation of the Subunits. The C and R subunits of native and spin-labeled ATCase were separated by reaction with excess PMB and isolated on a column of DEAE-Sephadex A-50 using the procedures described by Gerhart and Holoubek (1967). In the experiment in which the isolated subunits from spin-labeled protein were examined for electron spin resonance signals the 2-mercaptoethanol stabilizer was not used to prevent possible nitroxide reduction. The R subunit had the correct sedimentation coefficient in the subunit mixture and appeared in the correct place on the Sephadex column, but appeared to have aggregated when examined in the analytical ultracentrifuge and by gel electrophoresis after isolation.

CTP Binding Study. Binding of CTP was investigated using a series of solutions containing 1.5 mg/ml of ATCase and concentrations of CTP from 4.5×10^{-6} M to 2.1×10^{-4} M. The CTP concentration in these solutions was determined from the absorbance at 271 nm before addition of the protein. The protein was then sedimented from the solution in a Beckman Model L2-65 preparative ultracentrifuge and the concentration of CTP in the supernatant again measured. The difference between the starting and final concentrations was taken to be the amount of bound CTP. The solutions were spun 40 hr at 23° at 35,000 rpm in a swinging-bucket rotor or 21 hr in a fixed-angle rotor. Both methods gave consistant results. A protein blank (~3%) was subtracted from the measured optical densities after centrifugation.

Effect of Ligand Binding on the Electron Spin Resonance of Labeled ATCase. Samples for the ligand binding electron spin resonance studies were prepared by adding 1- to 10-μl aliquots of a concentrated solution of the ligand to 0.20 ml of enzyme solution (5–12 mg/ml) in pH 7.0, 0.04 M potassium phosphate buffer without stabilizers. A separate solution was made up for each ligand concentration and these were stored in ice until the spectra were run.

All electron spin resonance spectra were run at 23° in a Varian aqueous sample cell on a Varian Model V-4502 X-band spectrometer.

Results

Effect of Ligands on the Labeling Reaction. Figure 1A shows a typical electron spin resonance spectrum of the ATCase reacted with II used in these investigations (the labeled protein will be referred to hereafter as BrA-ATCase). The spectrum is similar to that reported by Ogawa and McConnell (1967) for oxyhemoglobin labeled with the spin label I and is indicative that most of the label has little residual motional freedom relative to the peptide chain to which it is attached. The heights of lines 2, 3, and 4 in the spectrum relative to 1 and 5 tend to increase when older enzyme preparations are labeled indicating that the 3 central lines of the spectrum are due in part of the labeling at sites where the nitroxide has much higher mobility relative to the protein. Exclusive labeling at such sites would give a spectrum similar to 1C with lines 1 and 5 completely absent.

The course of the labeling reaction is also affected by adding saturating concentrations of various ligands for the enzyme to the reaction mixture. The spectrum in Figure 1A

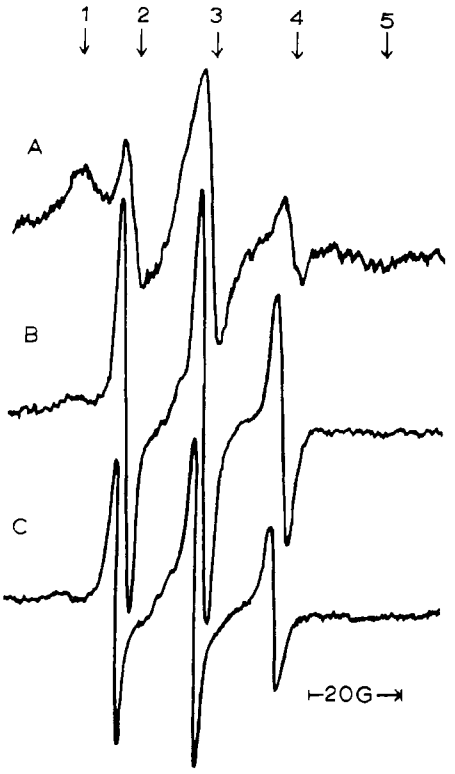


FIGURE 1: Electron spin resonance spectra of ATCase labeled with II in the presence of various ligands, recorded at 23° in pH 7.0, 0.04 M phosphate buffer: (A) 5×10^{-3} M CTP; (B) 5×10^{-3} M succinate and 5×10^{-3} M CAP; (C) 5×10^{-3} M ATP. The spectrum of ATCase labeled with II in the absence of ligands is identical with 1A except for a small variation in the relative heights of lines 2, 3, and 4 and was therefore not pictured.

is that of fresh enzyme labeled with II in the presence of 5×10^{-3} M concentration of CTP. The spectrum of enzyme labeled in the absence of any ligands is similar except for a slight increase in label mobility reflected in an increase in the heights of lines 2 and 4 relative to 1, 3, and 5. The molar ratio of bound label to enzyme is about 0.4 to

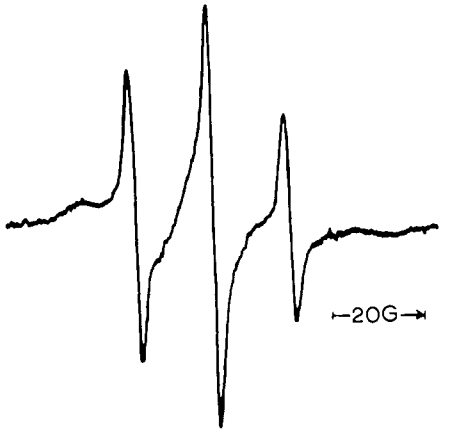


FIGURE 2: Electron spin resonance spectrum of ATCase labeled with 4-α-iodoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl, recorded at 23° in pH 7.0, 0.04 M phosphate buffer.

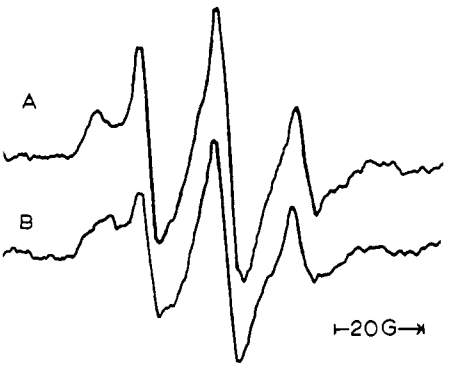


FIGURE 3: Effect of CTP on the electron spin resonance spectrum of BrA-ATCase (labeled in the presence of CTP): (A) no CTP; (B) 10^{-3} M CTP.

1.3 in both cases and the spectra show the same high sensitivity to the binding of certain allosteric ligands. Carrying out the labeling reaction in the presence of the same concentrations of ATP or CAP-succinate results in the very different spectra shown in Figure 1B,C as well as the incorporation of much larger amounts of label. These spectra are no longer sensitive to allosteric ligand binding and are indicative that the nitroxide is attached primarily at a new class of sites where motional freedom is much higher.

When the enzyme was reacted with *N*-ethylmaleimide for 1 day before reacting with II, the electron spin resonance spectrum was reduced in intensity by about one-third but otherwise unchanged. Extending the reaction time with the *N*-ethylmaleimide to 5 days caused no further loss of intensity.

The iodoacetamide label corresponding to II was also reacted with ATCase. It gave a labeled protein with the electron spin resonance spectrum in Figure 2. Here again there is a large amount of the more mobile type label and the spectrum shows no sensitivity to the binding of any of the allosteric ligands.

BrA-ATCase samples prepared in the absence of any

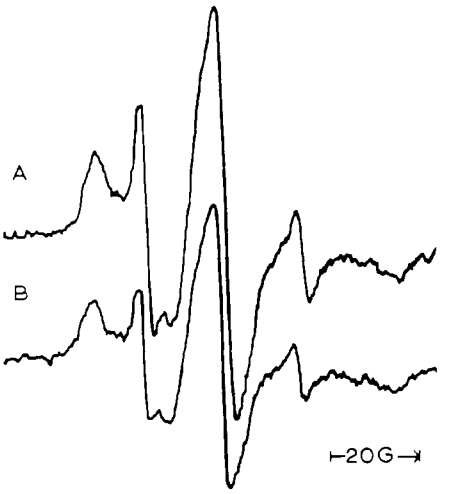


FIGURE 4: Effect of ATP on the electron spin resonance spectrum of BrA-ATCase (no ligands present during the labeling reaction): (A) no ATP; (B) 10^{-3} M ATP.

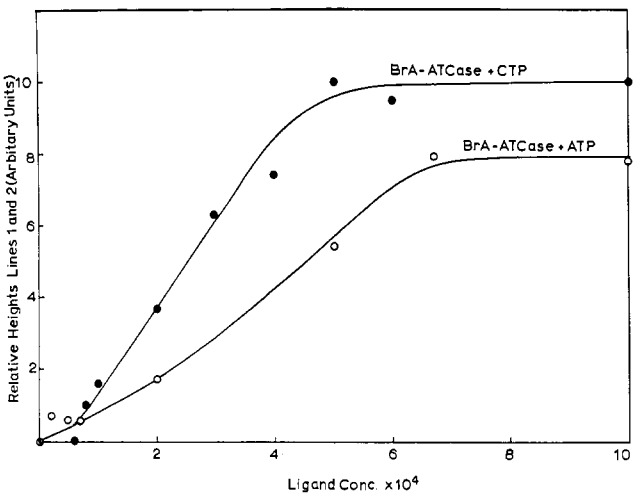


FIGURE 5: Change in the electron spin resonance spectrum of BrA-ATCase with increasing CTP concentration (solid circles) and ATP concentration (open circles). The change in the electron spin resonance is represented graphically by the relative heights of lines 1 and 2 in arbitrary units. The initial relative heights for the two protein samples used in these experiments were different and were converted into a common origin for direct comparison of the data. Spectra were recorded at 23° in pH 7.0, 0.04 M phosphate.

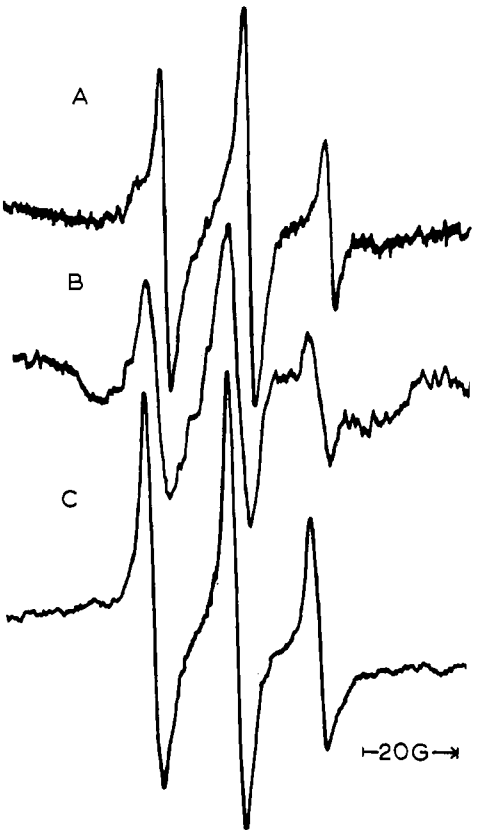


FIGURE 6: Electron spin resonance spectra of the C subunit labeled with II, recorded at 23° in pH 7.0, 0.04 M phosphate: (A) spectrum of BrA-ATCase after addition of 10^{-3} M PMB to separate the subunits; (B) spectrum of the C subunit after isolation from labeled intact BrA-ATCase; (C) spectrum of C subunit separately labeled with II.

ligands or in the presence of CTP behave identically in the experiments described in the remainder of the paper. In general enzyme labeled in the latter manner was used. When compared with native ATCase the labeled enzyme shows no loss of activity or CTP inhibition, and no changes in acrylamide gel electrophoresis behavior or sedimentation velocity.

Effect of Ligand Binding on the Electron Spin Resonance Spectrum of BrA-ATCase. The effect of the allosteric inhibitors CTP and UTP, the activator ATP, and the substrate analog succinate on the electron spin resonance spectrum of the labeled protein have been studied. Concentrations of succinate from 10^{-5} to 5×10^{-3} M in the presence of 5×10^{-3} M CAP gave only small random variations in the BrA-ATCase spectrum.

Both CTP and ATP cause a pronounced general broadening of the BrA-ATCase spectrum as is shown in Figures 3 and 4-A, B. Figure 5 is a plot of the spectral change as a function of ligand concentration. The relative heights of lines 1 and 2, the parameter which shows the largest variation, is taken as a measure of the change. The ordinate is in arbitrary units with the 2 curves adjusted to a common origin. It was necessary for purposes of comparison to plot the data in this way, because as indicated above the relative height of lines 1 and 2 in the spectra varies somewhat with the age of the enzyme preparation labeled and was for this reason slightly different initially in the BrA-ATCase used in these two experiments. At the concentrations of protein used in the experiment the CTP-induced change is half-complete at 2.5×10^{-4} M ligand and that induced by ATP at 3.8×10^{-4} M. The maximum change induced by ATP is 80% of that induced by CTP. The spectral changes accompanying the binding of both ligands show an isobestic point at the minimum between peaks 1 and 2.

It was also found that the spectral change observed with CTP could be reversed by the addition of succinate and CAP. At a CTP concentration of 3×10^{-4} M and an enzyme concentration of 5.5 mg/ml, 10^{-3} M CAP and 10^{-3} M succinate were sufficient to completely reverse the CTP-induced change.

A concentration of 10^{-2} M UTP, which is the weakest inhibitor of ATCase of any of the nucleotide triphosphates examined by Gerhart and Pardee (1962), has no discernable effect on the electron spin resonance spectrum of BrA-ATCase.

Decomposition of BrA-ATCase into Subunits. When BrA-ATCase is titrated with PMB in the concentration range where it causes separation of the protein into subunits a change in the electron spin resonance spectrum is observed corresponding to an increase in label mobility (Figure 6A). The change in the electron spin resonance spectrum with the amount of PMB added closely follows the appearance of the C and R subunits in the sedimentation velocity pattern, recorded parallel to the electron spin resonance spectra. Figure 7 is a plot of the increase in the height of line 2, taken as a measure of the appearance of label with greater motional freedom as a function of PMB concentration. For a BrA-ATCase concentration of 10 mg/ml the intact protein is no longer in evidence in the ultracentrifuge at a PMB concentration of 6×10^{-4} M and the electron spin resonance change is complete at 7×10^{-4} M PMB.

If a large excess of PMB is added to a solution of BrA-ATCase and the solution is quickly transferred to the electron

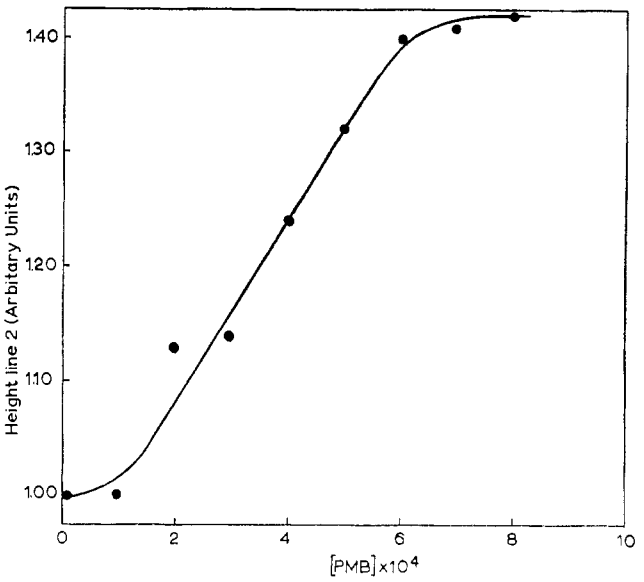


FIGURE 7: The effect of PMB on the electron spin resonance spectrum of BrA-ATCase (labeled in the presence of CTP) measured in terms of the increase in the height of line 2 (arbitrary units). The enzyme solution was allowed to equilibrate 45 min after each addition of PMB before recording the electron spin resonance spectrum.

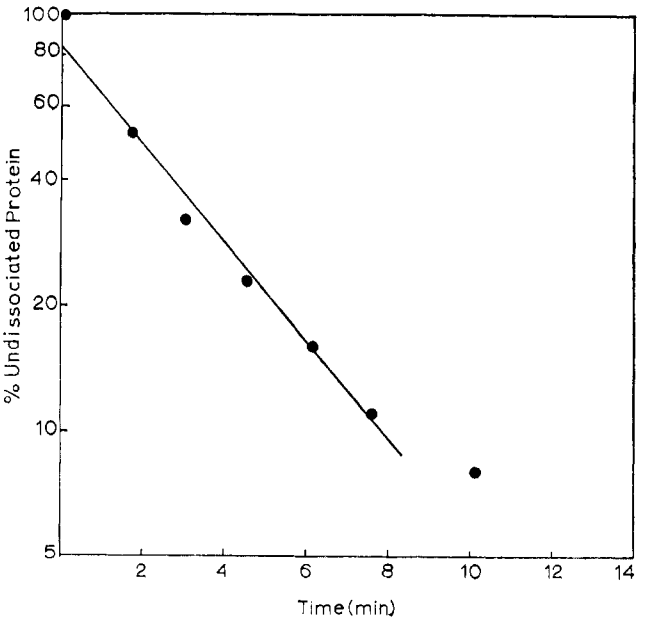


FIGURE 8: Analysis of the reaction of PMB with ATCase in terms of pseudo-first-order kinetics. The per cent undissociated protein was calculated from the appearance of more mobile spin label as subunit separation takes place. The change in the height of electron spin resonance line 2 was again taken as a measure of the increase in radical mobility. The reaction mixture contained only BrA-ATCase and 10^{-3} M PMB. PMB was added in the form of a 10^{-2} M stock solution in 5×10^{-3} M KOH. The buffer was pH 7.0, 0.04 M phosphate and the reaction temperature 23°.

spin resonance cavity the spectral change can also be used to follow the kinetics of dissociation, if it is assumed that the rate of appearance of the more mobile label is proportional to the rate at which the subunits are released. This requires that in the reaction between PMB and ATCase there exist no partially dissociated intermediates, as has been demonstrated by Gerhart and Schachman (1965). Figure 8 shows the change in the concentration of undissociated protein with time, measured in terms of the change in the height of line 2 of the BrA-ATCase electron spin resonance spectrum, plotted on a semilog scale. The plot is linear over the range of 50 to 90% reaction and the apparent first-order rate constant calculated from the half-life is 6.1×10^{-3} sec^{-1}. A value of 5×10^{-3} sec^{-1} was found by Gerhart and Schachman (1968) from a study of the increase in absorption at 250 nm corresponding to the formation of PMB–mercaptide bonds.

When the subunits of the PMB-dissociated BrA-ATCase were separated by passage through a DEAE-Sephadex column, precipitated from the pooled chromatography fractions with ammonium sulfate, and redissolved, only the C subunit gave an electron spin resonance signal. This signal (Figure 6B) was similar to that in the unseparated subunit mixture, except that some intensity was lost in the isolation process. Addition of 5×10^{-3} M CTP to the labeled subunit resulted in some additional narrowing of the electron spin resonance spectrum.

When native C subunit was reacted with II under the same conditions as the intact enzyme an electron spin resonance spectrum similar to that for the C subunit isolated from the labeled enzyme was obtained as shown in Figure 6C.

Effect of pH on the Electron Spin Resonance Spectrum of BrA-ATCase. To investigate whether any pH-dependent conformation changes could be detected in BrA-ATCase. electron spin resonance spectra were recorded for a 5.5-mg/ml solution of BrA-ATCase in potassium phosphate buffer between pH 6.03 and 10.37. A narrowing of electron spin resonance hyperfine lines 2, 3, and 4, indicating increased motional freedom for the label, was observed beginning at pH 7.9 and increasing very rapidly above pH 10. This is shown as a plot of the ratio of the heights of lines 1 and 2 in the electron spin resonance spectrum *vs.* pH in Figure 9. The observed changes were fully reversible. The titration was not extended beyond these limits because of problems of denaturation at higher pH and precipitation at lower pH.

Binding of CTP to Native ATCase. A study of the binding of CTP to ATCase was undertaken for comparison with the electron spin resonance changes observed in the presence of CTP. CTP binding has previously been studied by Gerhart and Schachman (1965) using an analytical ultracentrifuge method and by Changeux *et al.* (1968) using equilibrium dialysis. It was felt worthwhile to do this binding study again by another method because of the uncertainty that presently exists as to the number of binding sites per molecule of ATCase and also in order to have binding data for conditions as close as possible to those for the electron spin resonance experiments. As outlined in the Materials and Methods section the bound CTP for various total CTP concentrations was determined from the difference between the amount of CTP in solution before and after sedimenting the protein in a preparative ultracentrifuge. In Figure 10 the moles of CTP bound per mple of protein is plotted as a function of the total CTP concentration. In Figure 11, the data are presented in the form of a Scatchard plot which was analyzed

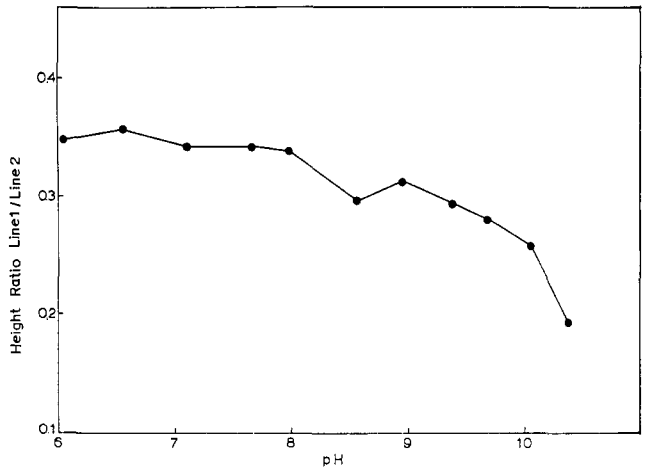


FIGURE 9: The effect of pH on the electron spin resonance spectrum of BrA-ATCase (labeled in the presence of CTP). measured in terms of the change in the relative heights of lines 1 and 2.

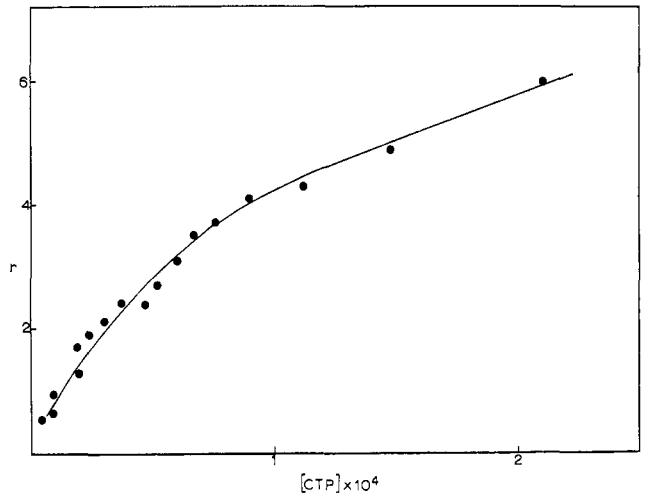


FIGURE 10: Binding of CTP to native ATCase. The moles of CTP bound per mole of enzyme is plotted against total CTP concentration at 23°.

using the method of Scatchard *et al.* (1957). The curve can be reproduced by a set of 3 tight binding sites with a dissociation constant of 9.0×10^{-3} M and a set of 6 weak sites with a dissociation constant of 1.8×10^{-4} M.

Discussion

Sites of Attachment of the Bromoacetamide to ATCase. A general problem in labeling studies such as this is relating the information about conformational states obtained from the probe to the location of the probe in the protein. Except for determination of the type and number of amino acid residues to which the probe binds this is necessarily limited by the state of knowledge about the secondary and tertiary structure of the protein, which in the case of ATCase is still at a very early stage of development.

The electron spin resonance spectrum of BrA-ATCase corresponds to labeling primarily at sites of the type that McConnell refers to as having intermediate immobilization (Ogawa and McConnell, 1967) where motion of the label is considerably restricted by interactions with the surrounding protein structure, but not completely quenched. Such sites are usually considered to be in hydrophobic regions inside the protein. The spectrum due to label with much higher mobility superimposed on this spectrum when older enzyme preparations are reacted with II is usually taken as an indication of labeling of residues on the surface of the protein (Stone *et al.*, 1965). The dependence of this effect on the age of the ATCase preparation and the fact that it can be reversed by further purification indicates that it is due to labeling of some minor peptide impurity formed through slow degradation and not surface labeling of ATCase itself. Higher label to enzyme ratios or longer reaction times do not have any significant effect on the amount of label bound indicating that there are no much less reactive secondary labeling sites.

The absence of any electron spin resonance signal from the isolated R subunit indicates that the labeling agent attacks only the C subunit. It is unlikely that the label is in the immediate proximity of the catalytic site since it has no effect on enzymatic activity.

Haloacetamide spin labels such as II are known to be general alkylating agents, the type of amino acid residues attacked depending on the nature of the protein (Hamilton and McConnell, 1968). Amino acid analysis indicates that there are at least 3 cysteines in the C subunit (Weber, 1968a) and these are one likely possibility. That the intensity of the electron spin resonance spectrum of BrA-ATCase was reduced by only one-third when an attempt was made to block these cysteines with *N*-ethylmaleimide could be taken as evidence for heterogeneous labeling, except that a change in

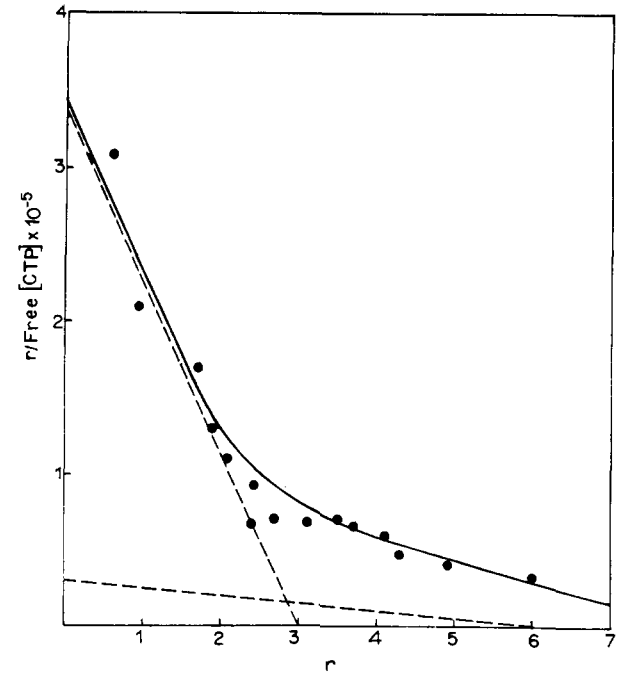


FIGURE 11: Scatchard representation of the CTP binding data.

the appearance as well as the intensity of the spectrum would be expected as one class of sites is eliminated unless the environment of the label at each site is identical. The blocking agent could also merely be exerting a steric effect on the ability of II to bind to the protein by reacting with a cysteine near the actual spin-labeling site.

Although these results are ambiguous in regard to the uniqueness of the labeling site, the low amount of label incorporated, the high sensitivity of the spectrum to ligand binding, the isobestic point associated with the spectral changes, and the high degree of label immobilization (nonspecific labeling is generally of the highly mobile type), all tend to suggest that II reacts with the enzyme at a single class of sites in the labeled product giving only the immobilized electron spin resonance spectrum. Other experiments to determine the location of the label more precisely are in progress.

The increase in label mobility observed when BrA-ATCase is separated into its subunits cannot be due simply to a decrease in the correlation time of the fragment to which the label(s) are attached because the catalytic subunit has a molecular weight in the range of 100,000 and its rotational frequency would still be too low relative to the hyperfine anisotropy of the nitroxide electron spin resonance to have any effect on the observed line widths. One reasonable explanation for this spectral change is that the primary site(s) of attachment of the label are in the region where the subunits are joined and when they are separated nitroxides which were surrounded by protein structure become surface labels with greater freedom of rotation. This explanation is attractive because the CTP-induced conformation change which is reflected in changes in label mobility must be transmitted between the R and C subunits through this region and also this part of the protein should be open enough to admit the spin label since it is the site of attack of PMB. Another possible explanation for the narrowing of the label electron spin resonance spectrum on reaction with PMB is a change in the conformation of the C subunit as a result of separation. The observation of higher activity after subunit separation (Gerhart and Pardee, 1964) is consistent with a conformation change leading to a more open structure in which the label has greater mobility.

There seems to be a paradox between the effect of CTP on the labeling reaction with II and its effect on the electron spin resonance spectrum of the labeled protein. The increase in specificity for labeling at the site sensitive to CTP binding suggests that CTP induces a more open structure in the region of this site, but the decrease in label mobility when CTP is added to BrA-ATCase, as well as the results of the other studies of the effect of CTP on conformation, are more consistant with a tightening up of the surrounding structure. To resolve this contradiction one could consider that the amino acid residues to which the spin label binds are more exposed in the presence of CTP, but that after reaction the label slips into a hydrophobic pocket to become immobilized.

The effect of succinate-CAP and ATP in giving a preponderance of highly mobile label in the reaction of ATCase with II is probably the result of activation of some other labeling site rather than a decrease in accessibility of the CTP sensitive sites, since the overall degree of labeling is much higher. The spectrum of the immobilized label is masked too much to judge accurately if it has decreased at all in intensity.

Electron Spin Resonance Spectral Changes Accompanying Ligand Binding. The first question to be asked in relation to the changes in the electron spin resonance of BrA-ATCase in the presence of CTP is whether they are directly related to the allosteric conformation change or just the result of a local interaction between the label and the CTP binding site. Because the label is bound only to the C subunit and does not affect feedback inhibition it is very unlikely that it is situated close enough to interact directly with this site. The lack of effect of UTP on the spectrum further supports this. UTP is the weakest inhibitor of ATCase of the nucleotide triphosphates which were tested (8% maximum). No binding studies of this nucleotide have been done, however, the work of Gerhart and Pardee (1964) suggests that the ribose and triphosphate are the primary reguirements for binding and thus UTP probably still binds fairly strongly to ATCase although it is not very effective in inducing the allosteric conformation change. If the spin label were interacting locally with the binding site rather than feeling a transmitted conformation change then one would expect UTP to have an effect also. The decrease in label mobility observed in the presence of CTP is also consistant with the change being related to the allosteric transition. Gerhart and Schachman (1965) have proposed that CTP binding results in a more constrained protein structure in which the active site is less accessible and the reported studies of the effect of CTP and substrates on conformation are in agreement with this. Such a conformation change would be expected to increase steric interactions of a spin label located inside the enzyme and result in broadening of the electron spin resonance spectrum. That only one conformation change is observed in both the CTP and ATP binding experiments can be inferred from the isobestic point in the spectra.

The failure to detect both heterotropic (inhibitor) and homotropic (substrate cooperativity) allosteric effects using the same spin label probe suggests that, contrary to the predictions of the MWC model, in ATCase these two types of conformation changes are not related in the sense that one is just the reverse of the other and therefore must be transmitted through the same region of protein structure. The reversal of the effect of CTP on the electron spin resonance spectrum by succinate and CAP is in agreement with the ability of large substrate concentrations to overcome CTP inhibition by reducing the ability of CTP to bind to the enzyme (Changeux *et al.*, 1968) and with the results of previous studies of ligand-induced conformation changes in which reversal of an effect attributed to substrate binding by CTP has been observed.

Another labeling agent sensitive to succinate binding has been found and experiments with this nitroxide are in progress.

That the broadening of the electron spin resonance spectrum of BrA-ATCase by CTP is the direct result of a conformation change resulting in a more compact protein structure and reduced ability to bind substrates is difficult to reconcile with the fact that the allosteric activator ATP causes the same sort of broadening. A similar observation has been made by McClintock and Markus (1968) in their finding that both CTP and ATP lowered the susceptibility of ATCase to attack by proteolytic enzymes, an effect they ascribe to a less open protein structure. The mechanism proposed for ATP activation is that ATP binds to the same allosteric site as CTP, but brings about a conformation change leading to increased substrate binding by virtue of differences in the base part of the two nucleotide triphosphates. The principle evidence for this is the effect of ATP in decreasing CTP binding competitively

(Changeux *et al.*, 1968). In terms of this mechanism it is somewhat puzzling that all of the other purine and pyrimidine bases examined by Gerhart and Pardee (1962) are inhibitors. One would expect at least GTP to have an effect similar to ATP if the function of this activation is to maintain a purine–pyrimidine balance (GTP is a moderately good inhibitor). The present results could be explained if ATP did act as a weak inhibitor when bound to the CTP site and if this effect were overcome by activation through binding at some other allosteric site. In this case the change observed in the electron spin resonance with ATP binding would be just the masked conformation change due to binding at the CTP site. That the conformation change is complete at a concentration comparable to that required for CTP binding and much lower than that required for activation, also is consistant with such a mechanism. Binding studies to examine this further are in progress. The very different effects of ATP and CTP on the sites of attachment of the labeling agent to the enzyme indicate that their overall effect on conformation cannot be the same.

Comparison with the Allosteric Models. To test whether the conformation change observed with CTP binding to BrA-ATCase could be fit to either of the simple allosteric models the curve for the electron spin resonance change was compared with a plot of the fractional saturation of binding sites with CTP concentration. Such a comparison is complicated by the apparent existence of 2 types of CTP binding sites. In view of the high concentration of CTP required to saturate the weaker sites relative to the concentrations required for maximum inhibition it is unlikely that these sites are associated with the feedback inhibition. It is most probable that they correspond to the binding of CTP to the active site as a competitive inhibitor for CAP, which has been observed by Porter *et al.* (1969) in kinetic studies with the isolated C subunit. The dissociation constant from these studies of 3.7×10^{-4} M is in fair agreement with the value of 1.8×10^{-4} M from the CTP binding experiment. Since it can also be concluded from the binding data that there are 6 such sites this would mean that the enzyme has 6 catalytic sites which is reasonable in view of Weber's (1968b) findings that there are 6 C and 6 R peptide chains per molecule.

The 3 tight CTP binding sites per ATCase obtained from the low CTP concentration part of the Scatchard plot is also interesting in view of Weber's findings and the threefold axis of symmetry proposed by Wiley and Lipscomb (1968). While the results of the present study are very close to those of Changeux *et al.*, $K = 1.3 \times 10^{-5}$ M, 3.8 binding sites, the fit of the binding data with 3 binding sites per ATCase molecule rather than 4 is more consistant with the new model for enzyme structure. The presence of 6 R chains would mean that 2 are required for each CTP allosteric site.

Figure 12 shows a plot of the fractional saturation of the two types of CTP binding sites (given the symbol $\bar{Y}$ by MWC) as a function of CTP concentration and the corresponding change in the relative state function, $\bar{T}r$ (the relative change in the 2-state conformational equilibria induced by binding a particular allosteric ligand in the MWC model), measured in terms of the electron spin resonance broadening. The binding curves were calculated using the dissociation constants from the Scatchard plot and the total value of r ($r = n\bar{Y}$) for each CTP concentration from the relationships in the appendix. For comparison with the electron spin resonance data the total CTP concentration for a given value of $\bar{Y}$ was converted into a CTP concentration corresponding to the higher protein concentration at which the electron spin resonance experiments were performed. The $\bar{T}r$ curve follows the $\bar{Y}_1$ curve at low CTP and crosses it at close to saturation. It preceeds the $\bar{Y}_2$ curve and the conformation change is complete at approximately 62% saturation of these weaker binding sites. The lack of homology of $\bar{T}r$ with either binding function excludes explanation of the electron spin resonance observations in terms of the KNF sequential allosteric model. Changeux and Rubin (1968) have analyzed the data from the effect of ligands on the PMB reaction rate and the changes in sedimentation coefficient in terms of the MWC concerted model. If the values they have calculated for the parameters L and d[3] are used to plot $\bar{T}r$ *vs.* CTP a curve is obtained which preceeds $\bar{Y}_1$. Using $K_1 = 9.7 \times 10^{-6}$ M from the present binding study as the value of the dissociation constant from the less active conformational state in the MWC model other reasonable values of L and d which satisfy the experimental $\bar{T}r$ curve cannot be found. As mentioned earlier the failure to observe an effect of succinate on the electron spin resonance of BrA-ATCase is also inconsistant with this 2-state model.

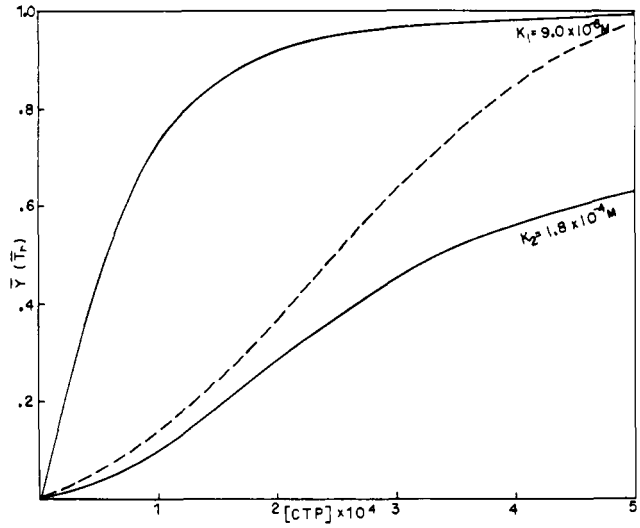


FIGURE 12: Change in $\bar{Y}_1$ and $\bar{Y}_2$, the fractional saturation of the 2 types of CTP binding sites with CTP concentration (method of calculation given in the Appendix) (———) and the corresponding change in $\bar{T}r$, the relative state function for CTP binding, measured in terms of the change in the electron spin resonance spectrum of BrA-ATCase (— — —).

Even if one of these models is a reasonable approximation of the behavior of ATCase there are several possible explanations for the failure of the electron spin resonance data to be consistant with the predictions of the model. What is observed in this experiment is actually the result of a decrease in the rotational freedom of the spin label as a result of local steric interactions in some part of the catalytic subunit, probably not too near the active site, and it does not necessarily follow that this local effect must be proportional to the active-site

[3] In the MWC model L is the conformational equilibrium constant for the 2 states of the enzyme with high and low substrate affinity in the absence of ligands and d is the ratio of the dissociation constants for the inhibitor from the 2 states.

conformation change even though both are the result of CTP binding to the allosteric site. One way in which this situation could arise is as a result of an inhibition mechanism where the addition of each molecule of CTP to the enzyme causes an additive conformation change decreasing equally the ability of substrates to bind to all of the catalytic sites. This would be a symmetry preserving model like that of MWC with the addition of intermediary states. In such a case it is possible that even though addition of each CTP causes a proportional change in activity only the last CTP changes the protein conformation enough to result in an appreciable increase in the local steric interactions to which the label electron spin resonance responds.

Another more simple explanation of the failure of the electron spin resonance data to fit the models is that at low CTP concentrations, both types of CTP binding sites affect steric interactions in the region where the label is attached and in opposite directions. That is, the broadening of the electron spin resonance spectrum caused by CTP binding to the allosteric site is partially offset by an effect increasing label mobility from binding at the secondary sites, the net effect being that the electron spin resonance change would occur at a higher CTP concentration than the allosteric transition. If the weaker binding of CTP is to the active site, the narrowing of the electron spin resonance spectrum of the labeled C subunit by CTP suggests that this might be possible, although there is no way to tell at present how closely related the conformation of the subunit is to that of the intact enzyme.

Another factor to keep in mind in comparing the electron spin resonance and CTP binding data is that the binding experiments were done with unlabeled enzyme and there could conceivably be small differences in behavior between labeled and unlabeled protein. In view of the complete absence of any effect of the label on feedback inhibition it was not felt that such perturbations would be large enough to justify the large expenditure of enzyme required to repeat the experiment with labeled material.

If the changes in the electron spin resonance of BrA-ATCase are directly related to the active-site conformation change the results suggest that the CTP allosteric sites must be close to saturation to induce the conformation change.

Effect of pH on the Electron Spin Resonance Spectrum of BrA-ATCase. ATCase exhibits two activity maxima (Gerhart and Pardee, 1964). The lower one is strongly dependent on aspartate concentration changing from pH 6.9 to 8.9 with increasing aspartate. An enhancement of the sigmoid character of the reaction velocity–substrate concentration curve is also observed with increasing pH in this range. The second maxima occurs at pH 10.2, is independent of aspartate, and is accompanied by a loss of sigmoid kinetics. The relationship of these maxima to cooperativity effects indicates that conformational changes in the enzyme play at least a partial role in determining their position. It has been suggested (Weitzman and Wilson, 1966) that the pH 10.2 maximum is the result of a weakening of subunit interactions, because of the loss of cooperativity and higher activity in the isolated C subunit.

The changes in the electron spin resonance of the BrA-ATCase in the direction of increased label mobility over this same pH range indicate that conformation changes do indeed occur and these changes are consistant with the more open protein structure expected for higher activity.

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Appendix

The binding curves corresponding to the two CTP dissociation constants were calculated from the following relations.

$$I_f = \frac{rK}{n - r} \tag{1}$$

$$\frac{r_1 K_1}{n_1 - r_1} = \frac{r_2 K_2}{n_2 - r_2} \tag{2}$$

$$r = r_1 + r_2 \tag{3}$$

In these expressions I_f is the concentration of free CTP, K_1 and K_2 the dissociation constants, r_1 and r_2 the moles of CTP bound to each site per mole of enzyme, and n_1 and n_2 the number of binding sites of each type. The first equation is a rearrangement of the Scatchard equation and the second follows from the fact that I_f must be the same for each binding relation. Equations 2 and 3 were combined and the resulting quadratic solved for r_1 and r_2 for various values of r. These values were then converted into $\bar{Y}$ by multiplying by n.

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Effect of Nucleotides and Pyrophosphate on Spin Labels Bound to S_1 Thiol Groups of Myosin*

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ABSTRACT: Titration of myosin with the spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide, indicates the presence of two rapidly reacting thiol groups per molecule which are presumably the so-called S_1 thiol groups. Labeling of these groups results in the well-known increase in Ca^{2+}-activated adenosine triphosphatase activity and loss of K^+-activated adenosine triphosphatase characteristic of the reaction of myosin with sulfhydryl modifiers. Strong immobilization of the spin label accompanies this reaction as indicated by electron spin resonance spectra. The addition of adenosine triphosphate, inosine triphosphate, adenosine diphosphate, or pyrophosphate increases the mobility of spin labels bound to S_1 groups, the maximum changes in electron spin resonance spectra being obtained in the presence of 2 moles of pyrophosphate/mole of enzyme. The effect of adenosine triphosphate is also observed in the absence of activating divalent or monovalent cations and after all enzymatic activity has been inhibited with *N*-ethylmaleimide. Tryptic digestion of spin-labeled myosin to heavy meromyosin (HMM) or digestion of HMM to subfragment 1 does not alter the electron spin resonance spectrum or the spectral change produced by adenosine triphosphate. The present results suggest that each of two heavy chains of myosin contains one S_1 thiol group and one enzymatic site. The change in electron spin resonance spectra produced by adenosine triphosphate is interpreted to represent a change in the structure of myosin near the nitroxide radicals bound to S_1 thiol groups. Since adenosine triphosphate produces no gross change in the structure of myosin as measured by optical rotatory dispersion (Gratzer, W. B., and Lowey, S. (1969), *J. Biol. Chem. 244*, 22), it is concluded that the conformational change is confined to a relatively small region of the molecule and that the S_1 groups are located close to the adenosine triphosphate binding site.

It is generally believed that there are two classes of thiol groups in myosin whose blocking affects its ATPase activity. Reaction of thiol groups in one class (S_1)[1] with reagents such as NEM[2] or *p*-mercuribenzoate results in the increase of the Ca^{2+}-stimulated ATPase activity and inhibition of the K^+-EDTA activated activity (Kielley and Bradley, 1956). Additional reaction of thiol groups in the second class (S_2) leads to inhibition of both types of ATPase activity. Recent work in our laboratory has shown that, notwithstanding these differences between S_1 and S_2, blocking of either group individually has the same effect on ATPase activity (Seidel, 1969). The technique of spin labeling (Stone *et al.*, 1965; Hamilton and McConnell, 1968) provides a method for probing the environment of the thiol groups involved in these effects and investigating their relationship to the binding and hydrolysis of ATP. The method involves binding of a

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† This work was done during the tenure of an Established Investigatorship of the American Heart Association, Inc.

‡ Part of this work was presented in partial fulfillment of the requirements of the degree A.B. with honors in biochemical sciences, Harvard University.

[1] The S_1 thiol groups are those rapidly reacting thiol groups whose modification results in an increase in Ca^{2+}-activated ATPase activity and loss of K^+-ATPase activity. The S_2 groups are those which on modification lead to a loss of Ca^{2+}-activated ATPase of S_1-modified myosin.

[2] Abbreviations used are: NEM, *N*-ethylmaleimide; HMM, heavy meromyosin; TES, *N*-tris(hydroxymethyl)methyl-2-ethanesulfonic acid.