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Propagation of conformational changes in Ni(II)-substituted aspartate transcarbamoylase: Effect of active-site ligands on the regulatory chains

(allosteric enzymes/communication between subunits/cooperativity/spectral probes/metalloproteins)

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ABSTRACT **Although the importance of ligand-promoted conformational changes in allosteric enzymes has been recognized, it often has been difficult to determine whether the effects of binding are propagated to remote positions in different chains. Efforts were made, therefore, to demonstrate that changes due to ligand binding to the catalytic chains of aspartate transcarbamoylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) of *Escherichia coli* are "communicated" to the regulatory chains. For these studies the endogenous zinc in the latter chains was replaced by nickel, which served as a discriminating spectral probe. The Ni(II)-enzyme was constructed by dissociating the native enzyme, separating the catalytic and regulatory subunits, removing Zn(II) from the latter, replacing it with Ni(II), and reconstituting the enzyme from native catalytic and Ni(II)-containing regulatory subunits. Ni(II) derivatives containing either six Ni(II) or five Ni(II) and one Zn(II) possess the allosteric properties of the native enzyme and exhibit absorption bands at 360 and 440 nm due to charge transfer transitions. Smaller bands were also observed at 665 and 720 nm from *d–d* transitions, which are consistent with tetrahedral geometry in the coordination sphere of nickel. Binding of the bisubstrate ligand *N*-(phosphonacetyl)-L-aspartate to the catalytic subunit of Ni(II)-aspartate transcarbamoylase perturbed the Ni(II) chromophore, giving rise to two difference spectral bands (at 390 and 465 nm). Spectral titrations showed that the conformational changes at the metal-ion-binding sites were complete even though about one-third of the active sites were unoccupied. This propagation of conformational changes is in accord with other evidence indicating that the allosteric transition in aspartate transcarbamoylase is concerted.**

The allosteric properties of regulatory enzymes are generally attributed to ligand-promoted conformational changes whereby the oligomeric proteins are converted from a constrained or low-affinity state to a relaxed conformation having a higher affinity for substrates (1). For the regulatory enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase; carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli* the local conformational changes resulting from the binding of substrates to some catalytic (c) chains are thought to be propagated throughout the molecule, thereby affecting the active sites on other chains. This hypothesis has been proposed to account for the observed sigmoidal dependence of enzyme activity on substrate concentration (2, 3) in ATCase, which is composed of two catalytic (C) trimers and three regulatory (R) dimers (see ref. 4 for a review of the evidence regarding the structure designated as C_2R_3 or c_6r_6). According to this view, inhibition and activation of the enzyme caused by the binding of CTP and ATP, respectively, to the regulatory chains are attributed to the local conformational changes in those chains being transmitted to the catalytic chains. This interpretation received support in the recent demonstration (5) with specially constructed ATCase-like molecules containing nitrated catalytic (C_{NIT}) subunits and native R subunits. With this derivative, $C_{NIT}C_{NIT}R_3$, it was shown that the binding of CTP and ATP caused different conformational changes, which were readily detected by opposite alterations in the absorption spectrum of the nitrotyrosyl chromophores on the C_{NIT} subunits. This paper presents analogous studies on ATCase in which the effect of the binding of active-site ligands to the C subunits is monitored by chromophores located in the R subunits.

ATCase as normally isolated contains six zinc ions, each of which is bound to a regulatory polypeptide chain in a distorted tetrahedral configuration by four cysteinyl residues (6–10). Recent crystallographic studies have shown that the metal ions are located near the bonding domains between the C and R subunits (10). The metal ion binding sites in the R subunits are of particular interest because metal ions are required for the integrity of the oligomeric structure (7). Moreover, the sulfhydryl groups that chelate the metal ions have been shown to undergo a 6- to 8-fold increase in reactivity toward *p*-hydroxymercuribenzoate upon the addition of substrate analogs (3, 11). It seemed plausible, therefore, to postulate that there would be alterations in the metal-binding sites during the allosteric transition of ATCase. Hence efforts were initiated to replace Zn(II) with a metal from the first transition series such as Co(II), Ni(II), or Cu(II). Such complexes would have electronic absorption bands in the visible region of the spectrum that presumably could be used to monitor conformational changes resulting from binding of ligands to the C subunits.

Nickel was selected because its radius is comparable to that of zinc (12), it has a high affinity for sulfhydryl groups (13–15), it can form tetrahedral complexes (16, 17), and it exhibits little tendency to catalyze the oxidation of sulfhydryl groups (18, 19). As shown below, Ni(II)-ATCase is fully active, possesses the allosteric properties characteristic of the native enzyme containing Zn(II), and exhibits absorption bands in the visible region of the spectrum that are perturbed by the binding of ligands at the active sites located at a considerable distance (about 20 Å) from the metal ions. The Ni(II) ions thus serve as a sensitive probe for monitoring conformational changes propagated throughout the enzyme molecules as they are converted from the taut, T, state to the relaxed, R, conformation.

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Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; c, catalytic polypeptide chain; r, regulatory polypeptide chain; C_2R_3, subunit description of ATCase in terms of two C and three R subunits; PALA, *N*-(phosphonacetyl)-L-asparate.

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MATERIALS AND METHODS

ATCase and C and R subunits were prepared according to the procedure described by Yang *et al.* (20). Metal standards were obtained from Matheson, Coleman and Bell; Chelex-100 from Bio-Rad; and nickel nitrate from Mallinckrodt. The bisubstrate analog *N*-(phosphonacetyl)-L-aspartate (PALA) was kindly provided by G. R. Stark of Stanford University.

All buffer solutions used in the preparation of Ni(II)-ATCase were passed over a Chelex-100 column. Plastic laboratory equipment was employed routinely after being washed for 24 hr in 20 mM EDTA.

Metal determinations were made with a Model 403 Perkin–Elmer atomic absorption spectrophotometer kindly made available by A. H. Rubin. Sedimentation experiments were performed with a Beckman model E ultracentrifuge. Protein concentrations were determined with the ultracentrifuge equipped with a synthetic boundary cell and Rayleigh interference optics (21). The ligand-promoted change in sedimentation coefficient, $\Delta s/s$, caused by the addition of PALA was measured by difference sedimentation velocity experiments, and the observed value of $\Delta s/s$ was corrected for the buoyancy contributed by the bound PALA (21). Absorption spectra and difference spectra were obtained with a Cary 118 spectrophotometer interfaced to a Digital Equipment Corporation PDP 11/40 computer. Preparations of the Ni(II) derivative were analyzed electrophoretically in 5% polyacrylamide gels with the Tris–glycine system of Jovin *et al.* (22). The method of Bothwell (23) was used to locate the enzymically active species in the polyacrylamide gels by staining for phosphate released by the catalytic formation of carbamoyl aspartate from carbamoyl phosphate and aspartate.

Enzyme assays were performed at 30°C by the method of Davies *et al.* (24) with a 50 mM imidazole–acetate buffer at pH 7.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. The kinetic data were analyzed by a nonlinear least squares regression with a program generously provided by Michael L. Johnson and Gary K. Ackers. With this program the values of V_{max}, L (the allosteric equilibrium constant, [T]/[R]), and $K_{R(Asp)}$ (the dissociation constant for the enzyme–aspartate complex in the R state) were allowed to float, whereas the value of c (the ratio of affinities of the T and R states for aspartate) was fixed at 0.05 (25). The Hill coefficients, n_H, were determined from the maximal slopes of plots of $\log[v/(V_{max} - v)]$ *vs* log[aspartate].

RESULTS

Preparation and Characterization of Ni(II)-ATCase. Both the R and C subunits purified from native ATCase after dissociation of the enzyme with neohydrin (20) were dialyzed for 24 hr at 4°C and pH 8.0 against 10 mM Tris·HCl buffer containing 2 mM 2-mercaptoethanol and 10 mM EDTA. Analysis of the metal ion content of preparations of R subunit treated in this way showed that more than 95% of the zinc had been removed. The apo-R subunits were then dialyzed for 36 hr at 4°C against 10 mM Tris·HCl buffer at pH 8.0 containing 5 mM 2-mercaptoethanol and 0.5 mM $Ni(NO_3)_2$. These preparations of Ni(II)-R and metal-free C subunits at about 3 mg/ml were then mixed at a molar ratio of 6:1, and the resulting solution was dialyzed for 24 hr at 4°C against 10 mM Tris·HCl (pH 8.0) containing 5 mM 2-mercaptoethanol and 0.5 mM $Ni(NO_3)_2$. All buffers used for dialysis were first degassed and then saturated with N_2. In addition, N_2 was continuously bubbled through the solution during dialysis. After formation of Ni(II)-ATCase the sample was concentrated to approximately 4 ml with an Amicon filtration vessel containing a PM 30 membrane, and the resulting solution was passed twice through a Sephadex G-200 column (2.5-cm diameter, 36-cm length) that had been washed previously with 10 mM Tris·HCl (pH 8.0) containing 5 mM 2-mercaptoethanol and 10 mM EDTA.

Metal analysis of the various preparations for both nickel and zinc showed that all contained six ions per enzyme molecule. One had six Ni(II) and less than 0.2 Zn(II), another contained five Ni(II) and one Zn(II), and others were mixtures of about three Ni(II) and three Zn(II). The nickel content seemed to depend on the effectiveness of the removal of zinc from laboratory equipment and buffer solutions. Prolonged dialysis (24 hr) of the Ni(II)-ATCase preparations against solutions containing 5 mM EDTA did not cause a decrease in the nickel content.

Ultracentrifugal studies of different samples of Ni(II)-ATCase showed a single, sharp, symmetrical boundary with a sedimentation coefficient of 11.4 S. Electrophoretically, a major component was observed in all preparations and the mobility of Ni(II)-ATCase was the same as that observed with the native enzyme. Most preparations had a component that migrated in the polyacrylamide gels slightly more rapidly than ATCase. The mobility of this component was characteristic of the R-deficient species (C_2R_2) frequently observed in preparations of native ATCase and in reconstitution mixtures containing an excess of C subunits (26). Some preparations contained a small amount of a more slowly migrating species that is generally observed in reconstituted ATCase and has been attributed to aggregates such as dimers (26). In addition to these minor components there was a very small amount of material in the Ni(II)-ATCase preparations that had an electrophoretic mobility slightly less than that of the principal component. This material was enzymically active as shown by the formation of inorganic phosphate in the gels that were soaked in carbamoyl phosphate and aspartate. Unlike the other components, this material was not stable; reexamination of samples of Ni(II)-ATCase several days after their preparation showed only the major species and C_2R_2 with the occasional presence of aggregates.

Enzymic Properties of Ni(II)-ATCase. All preparations, regardless of the nickel content, exhibited both the homotropic and heterotropic effects characteristic of the native enzyme (Fig. 1*A*). The cooperativity illustrated by the sigmoidal dependence of enzyme activity on the concentration of aspartate is emphasized in Fig. 1*B* by the marked curvature in plots of the kinetic data in the form of specific activity divided by aspartate concentration as a function of specific activity (27). The values of the inhibition by CTP, 56%, and of the activation by ATP, 122%, observed at 5 mM aspartate are virtually identical to those obtained with native ATCase.

Studies of different preparations of Ni(II)-ATCase containing various amounts of nickel showed that the specific activities, V_{max}, were similar to the specific activity of the native enzyme (25). However, the cooperativity, as indicated by the Hill coefficient, $n_H = 1.4$, was lower for Ni(II)-ATCase than that, $n_H = 1.7$, exhibited by native ATCase. This decreased cooperativity for Ni(II)-ATCase was also shown by smaller values for the allosteric equilibrium constant, L, compared to those for the native enzyme. The estimates of L (in the legend of Fig. 1) were determined by curve fitting of the experimental data in terms of the two-state model of Monod *et al.* (1). As seen in Fig. 1, the theoretical curves account satisfactorily for the kinetic data obtained both in the absence and in the presence of the effectors, CTP and ATP. The changes in L caused by these effectors were similar for Ni(II)-ATCase and the native enzyme (25).

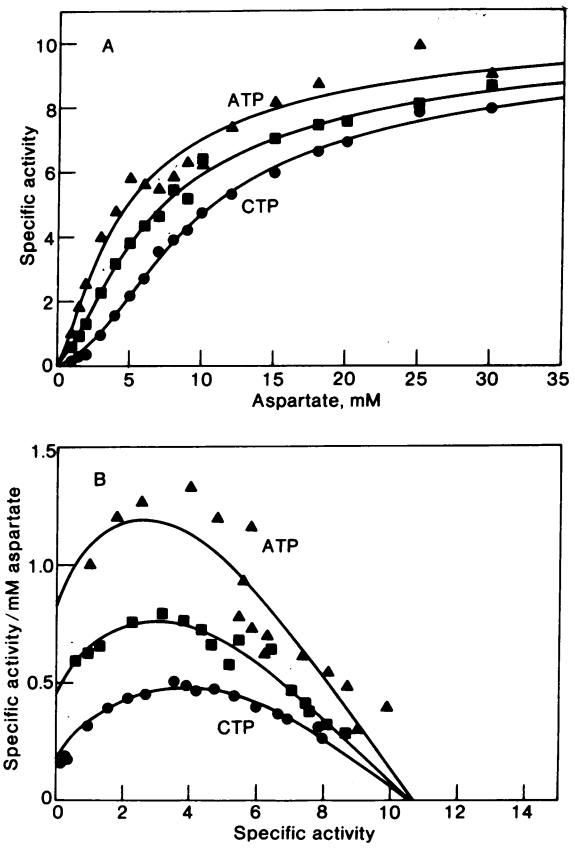

FIG. 1. Enzyme kinetics of Ni(II)-ATCase in the absence and presence of CTP and ATP. The concentration of carbamoyl phosphate was 4 mM; specific activities at 30°C are given as μmol of carbamoyl aspartate formed per hr per μg of protein. Values for the nickel derivative containing five Ni(II) and one Zn(II) per enzyme molecule in the absence of effectors are designated by ■, in the presence of CTP (0.5 mM) by ●, and in the presence of ATP (2 mM) by ▲. The curves are theoretical fits based on the two-state model (1, 25) according to the procedure described in *Materials and Methods*. For all computed curves the value of c was 0.05. The values of L were 2.5, 7.6, and 1.7 for the enzyme in the absence of effectors and in the presence of CTP and ATP, respectively. The corresponding estimates of the parameter $K_{R(Asp)}$ were 7.5, 9.9, and 5.2 mM. (*A*) Saturation curves in terms of specific activity as a function of aspartate concentration. (*B*) Plot of data as specific activity/[aspartate] vs. specific activity (27).

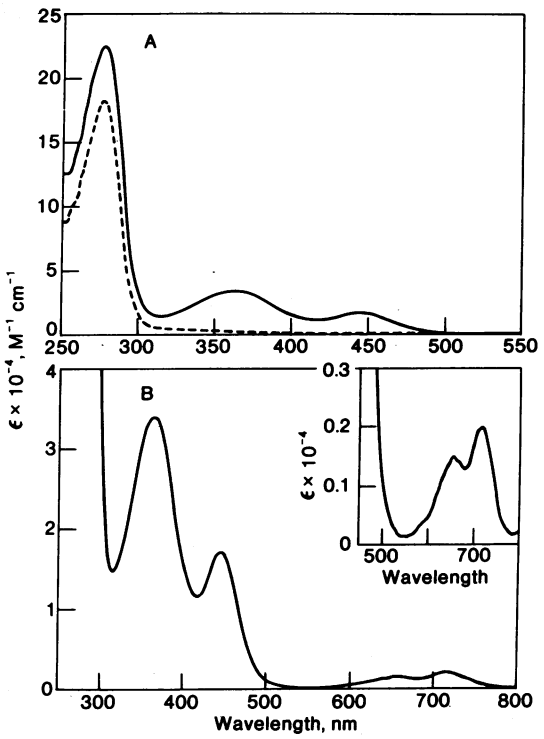

FIG. 2. Absorption spectra of Ni(II)-ATCase and native enzyme. Molar extinctions are based on $M_r = 3.1 \times 10^5$ for ATCase. (*A*) Spectrum for Ni(II)-ATCase containing six nickel ions (and a trace amount of zinc) per enzyme molecule is shown by —, and that for native enzyme is illustrated by ---. (*B*) Spectrum of same preparation of Ni(II)-ATCase enlarged for the visible region to show two bands at 360 and 440 nm due to charge transfer transitions. *Inset* in *B* represents a further enlargement of the spectrum, showing two bands at 665 and 720 nm resulting from *d–d* transitions.

Spectral Properties of Ni(II)-ATCase. Replacement of zinc ions in ATCase by nickel ions caused a slight increase in the extinction coefficient of the enzyme in the ultraviolet region of the spectrum (Fig. 2*A*). Moreover, the Ni(II) derivative exhibited absorption bands in the visible region. The two bands at 360 and 440 nm with the relatively large molar extinction coefficients (per nickel ion), $\epsilon_{360} = 5500$ and $\epsilon_{440} = 2800$ M^{-1} cm^{-1}, are attributable to charge transfer transitions (12). As seen in Fig. 2*B* there were two smaller bands at 665 and 720 nm, which are due to *d–d* transitions. The magnitude of the extinction coefficients per nickel ion (ϵ_{665} and ϵ_{720} about 250 and 330 M^{-1} cm^{-1}, respectively) and the location of the *d–d* transition bands are consistent with tetrahedral geometry in the metal ion coordination sphere (16, 17, 28).

Ligand-Promoted Conformational Changes in Ni(II)-ATCase. Because the homotropic and heterotropic effects manifested by Ni(II)-ATCase are indicative of ligand-promoted conformational changes, it was of interest to determine directly the extent of the local and gross changes that occur upon the addition of active-site ligands (3, 21, 29). Hence measurements were made on the effect of the bisubstrate analog PALA on the sedimentation coefficient of Ni(II)-ATCase. As with the native enzyme (21), there was a *decrease* in the sedimentation coefficient, $\Delta s/s = -3.6\%$, upon the addition of 13 mol of PALA per mol of Ni(II)-ATCase. A similar value of $\Delta s/s$ was obtained when PALA was added to the nickel derivative in the presence of 4 mM carbamoyl phosphate. These results indicate that Ni(II)-ATCase undergoes a gross conformational change, such as a "swelling" of the molecules, upon the binding of PALA at the active sites.

Both local and gross effects of binding PALA to Ni(II)-ATCase were also seen by difference spectroscopy. As shown by Collins and Stark (30, 31), the binding of active-site ligands to isolated C subunits caused a change in the ultraviolet absorption leading to maxima at 282 and 289 nm in the difference spectrum. Similar spectral shifts were obtained with intact ATCase, and the magnitude of the change in absorbance was directly proportional to the amount of PALA added, with the maximum change occurring upon the addition of 6 mol of PALA per mol of enzyme (32). Ni(II)-ATCase exhibits the same two maxima in the ultraviolet difference spectrum (Fig. 3). In addition, the difference spectrum caused by a saturating amount of PALA shows peaks at 390 and 460 nm amounting to 2 and 3%, respectively, for $\Delta A/A$. These maxima in the difference spectrum correspond to red shifts in the absorption bands at 360 and 440 nm resulting from perturbations in the charge transfer bands of the nickel complex with the protein.

In the titration of Ni(II)-ATCase with PALA (Fig. 4) it was found that the change in absorbance in the ultraviolet ($\Delta A_{290-286}$) was proportional to the degree of saturation of the

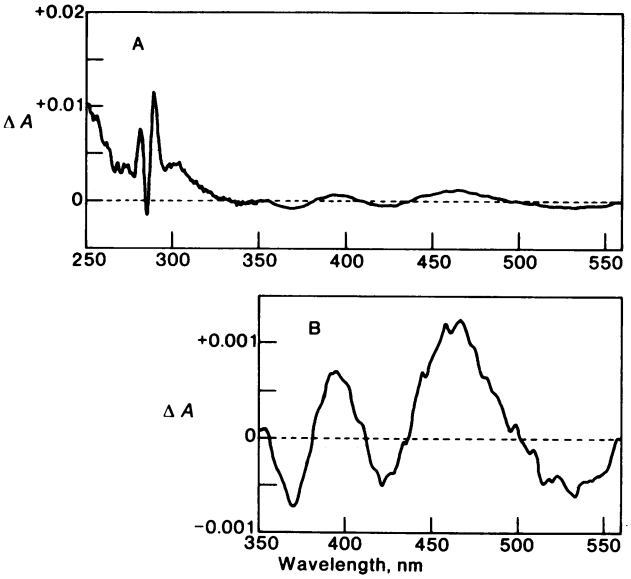

FIG. 3. Difference spectrum produced by the binding of PALA to Ni(II)-ATCase. The difference in absorbance (ΔA) between the protein solution containing the ligand and the reference protein solution is shown on the ordinate. Concentration of Ni(II)-ATCase (same preparation as in Fig. 1) was 1.53 mg/ml, and PALA concentration was 3.8 μM. In computer acquisition of data, 500 values were recorded between 250 and 350 nm and 730 values between 340 and 560 nm. The data between 250 and 350 nm were smoothed twice and those between 340 and 560 nm were smoothed 50 times. *B* is an enlarged plot of the difference spectrum from 350 to 560 nm.

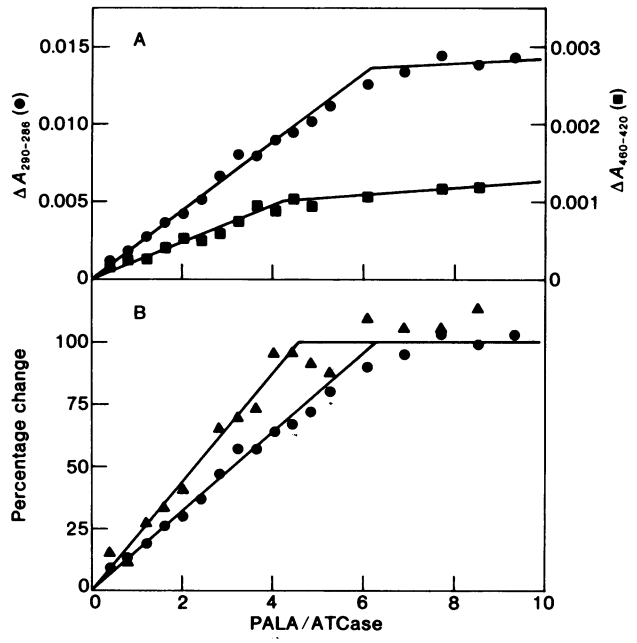

FIG. 4. Spectral titration of Ni(II)-ATCase with PALA. Difference spectra such as that shown in Fig. 3 were obtained at different molar ratios of PALA/ATCase. In the ultraviolet region of the spectrum the differences between the absorbances at 290 and 286 nm ($\Delta A_{290-286}$) were measured. For the visible region of the spectrum, the values of ΔA_{390} were calculated from the absolute change in the absorbance with a baseline constructed between the two minima at 370 and 425 nm (see Fig. 3). Also, measurements were made of the differences in absorbances between 460 and 420 nm ($\Delta A_{460-420}$). (*A*) Experimental results for $\Delta A_{290-286}$ are designated by ● with the scale on the left and for $\Delta A_{460-420}$ by ■ with the scale on the right. (*B*) Normalized results in terms of percentage change in optical density relative to maximum change. Results for percentage change in ΔA_{390} are designated by ▲ and for percentage change in $\Delta A_{290-286}$ by ●.

active sites, with the maximum value being attained at six PALA per ATCase. In contrast, the spectral changes in the visible region for both the 390 and 460 nm difference bands were virtually complete, when only slightly more than four of the six active sites were occupied by PALA.†

Thus the difference spectrum and the titration curves in Figs. 3 and 4 show both the local perturbation of aromatic residues in the C subunits of Ni(II)-ATCase caused by the binding of PALA to the active sites and the propagation of that effect to the metal-binding sites in the R subunits. The gross conformational change represented by the difference spectrum in the visible region of the spectrum is complete even though only about four of the six active sites of the enzyme were occupied by the ligand.

DISCUSSION

In earlier studies on native ATCase (3, 21) it was shown that the binding of active-site ligands caused a 3.5% decrease in the sedimentation coefficient of the enzyme. Moreover these ligands, though binding to the C subunits, caused a marked increase in the reactivity of the 24 sulfhydryl groups located on the 6 r chains (3, 11). These observations constituted evidence for a gross conformational change in the enzyme that affected regions of the protein remote from the sites of the bound ligand. However, a quantitative interpretation of the enhanced reactivity of the sulfhydryl groups in terms of the putative T $\rightleftarrows$ R equilibrium for ATCase was complicated because the experimental method used to detect the allosteric transition (i.e., titration of the sulfhydryl groups with a mercurial) caused dissociation of the enzyme into free C and R subunits. The dissociation process involving a series of reactions is complex, and the rates of various reactions relative to that for the allosteric transition have not as yet been determined. Hence efforts were initiated toward developing a direct method for detecting changes in the R subunits within intact ATCase as a consequence of binding ligands to the C subunits. As seen in Figs. 3 and 4, this goal has been achieved by replacing the zinc in ATCase by nickel, which serves as a sensitive spectral probe for monitoring the allosteric transition of Ni(II)-ATCase.

Of the various divalent metals from the first transition series, Ni(II) seemed ideally suited for replacing Zn(II) in ATCase.‡ The order of decreasing stability for 1:1 complexes between these metals and cysteine is Ni>Co>Fe>Mn (13–15), and the affinity of Ni(II) for cysteine is similar to that for Zn(II). In addition Ni(II) is a poor catalyst for the oxidation of sulfhydryl groups as judged by the effects of the metal ions on the reoxidation of reduced Taka-amylase, for which the order was Cu≫Co>Fe>Mn>Ni> control (18). On the basis of these two criteria, Ni(II) is an excellent potential substitute for Zn(II). In general, tetra-coordinated nickel complexes with chelates possessing sulfhydryl groups are found to have square planar stereochemistry (16, 17). However, sterically hindered chelates with sulfhydryl groups can form tetrahedral complexes with nickel. Although further investigation of the geometry is re-

† The data in Fig. 4*A* for $\Delta A_{460-420}$ are given directly as the measured values, whereas those for ΔA_{390} are normalized relative to the average maximum change measured from 4 to 10 PALA per ATCase. Similarly, the data for $\Delta A_{290-286}$ were normalized in Fig. 4*B*.

‡ Derivatives of ATCase containing other metals such as Hg(II), Cd(II), and Mn(II) have been prepared and shown to exhibit allosteric properties (6, 7, 9, 33). However, these preparations do not have spectral properties of the type shown by Ni(II)-ATCase and are therefore not as useful for observing conformational changes propagated to the metal-binding sites.

quired, the magnitude and the location of d–d transition bands in Ni(II)-ATCase (Fig. 2) are consistent with the view that the metal ion coordination is tetrahedral (16, 17, 28). Perhaps the folding of the regulatory polypeptide chain provides the necessary constraint on the orientation of the sulfhydryl groups for the formation of a tetrahedral complex of Ni(II) with the protein.

One preparation of Ni(II)-ATCase contained six nickel ions and trace amounts of zinc per enzyme molecule, another contained five Ni^{2+} and one Zn^{2+} per molecule, whereas others contained three ions of each metal. Various criteria indicate that Ni(II) occupies the same metal-binding sites as Zn(II). First, there is an absolute requirement for metal ions for the formation of stable C_2R_3 oligomers (7). Second, Ni(II), like Zn(II), could not be removed from the reconstituted ATCase by prolonged dialysis against EDTA (7). Third, the total metal content [Ni(II) plus Zn(II)] in the reconstituted enzyme was constant (6 mol per molecule) regardless of the ratio of nickel to zinc in the various preparations.

Although Ni(II)-ATCase exhibits the characteristic homotropic and heterotropic effects of the native enzyme (Fig. 1), the cooperativity of the nickel derivative was less than that of native ATCase. This is evident in both the lower Hill coefficient and the smaller values for L in the absence and presence of effectors. It should be noted that ATCase reconstituted from C and R subunits often shows slightly less cooperativity than the native enzyme. Also, the nickel derivative is formed from apo-R subunits with a smaller yield than is obtained with zinc ions. A lower efficiency in the reconstitution of ATCase would be expected if the nickel ions catalyzed the oxidation of some sulfhydryl groups in the r chains, thereby rendering them incapable of association with C subunits. It is also possible that the conformation of the r chains when nickel is bound to them is slightly different from that for the chains containing zinc. If the nickel ions caused some type of "strain" in the secondary and tertiary structure of the polypeptide chains, the interaction with C subunits might be less effective and the resulting Ni(II)-ATCase might have properties, such as the cooperativity, that differ from the native enzyme.

As seen in Figs. 3 and 4, the two absorption bands in the visible region of the spectrum are sensitive to the binding of the active-site ligand PALA. The local effects of ligand binding to the C subunits in Ni(II)-ATCase cause a gross conformational change as shown by the 3.6% decrease in the sedimentation coefficient of the enzyme. This alteration in the quaternary structure, which is attributable to a "swelling" of the protein molecules or to a change in their shape resulting in an increased frictional coefficient (3, 21), is accompanied by a change at the metal-binding sites. Thus the conformational change promoted by binding of ligands to the C subunits is propagated about 20 Å to the metal ions in the unliganded R subunits. Moreover, the changes at all of the metal-binding sites precede those at the active sites, attaining the maximum value with only about two-thirds of the active sites being occupied by ligand. These results, coupled with those on different hybrid molecules (29) and the demonstration that CTP and ATP in binding to the R subunits in ATCase have opposite effects on chromophores located on the C subunits (5), provide additional support for the view that the allosteric transition in ATCase is concerted (25).

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