Alteration of the Allosteric Properties of Aspartate Transcarbamoylase by Pyridoxylation of the Catalytic and Regulatory Subunits†

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ABSTRACT: Extensive modification of aspartate transcarbamoylase from *Escherichia coli* with pyridoxal 5′-phosphate followed by reduction of the Schiff base with sodium borohydride caused only partial inactivation of the enzyme. Under comparable conditions, virtually complete loss of enzyme activity is obtained with the free catalytic subunits. The pyridoxylated, intact enzyme containing more than 60% of the bound pyridoxamine phosphate on the regulatory subunits exhibited considerable cooperativity, inhibition by CTP, and activation by ATP. When the modification was performed in the presence of the ligands which bind to the catalytic sites, the resulting product had virtually the same activity as the native enzyme, but it exhibited significantly reduced cooperativity and virtually no inhibition by CTP. The pyridoxylation of the regulatory subunits within the intact enzyme was enhanced markedly in the presence of ligands as compared with the reactivity of these subunits when the modification was performed in the absence of the active site ligands. Both types of pyridoxylated derivatives exhibited the ligand-promoted conformational changes characteristic of the native enzyme. Spectrophotometric studies of inactive pyridoxylated catalytic subunits and intact enzyme showed that the substrate (carbamoyl phosphate) bound strongly but that the substrate analogue (succinate) did not bind. Both the pyridoxylation experiments in the presence and absence of ligands and the spectral behavior of a hybrid containing one native and one pyridoxylated catalytic subunit indicated that ligand binding was accompanied by a conformational change in the intact enzyme molecules.

In addition to catalyzing the first committed reaction in the biosynthesis of pyrimidines, the enzyme aspartate transcarbamoylase (ATCase)[1] (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) also serves as a major control point in this metabolic pathway (Yates and Pardee, 1956; Gerhart and Pardee, 1962). The mechanism by which ATCase fulfills these two roles has been the subject of extensive study over the past few years (Gerhart, 1970; Jacobson and Stark, 1973a; Schachman, 1972, 1974). The control of ATCase occurs via the modulation of its activity by its substrates, carbamoyl phosphate (CAP) and aspartate, and the nucleotide effectors CTP and ATP (Gerhart and Pardee, 1962). In contrast to most enzymes, ATCase exhibits a sigmoidal, rather than hyperbolic, dependence of activity on substrate concentration. This cooperativity has been interpreted in terms of the allosteric model of Monod et al. (1965) by Changeux and Rubin (1968). The enzyme also exhibits feedback inhibition by CTP and is activated by ATP. These two types of allosteric effects, termed homotropic and heterotropic, respectively, are generally attributed to an equilibrium between two distinct conformational states of the enzyme (Monod et al., 1965). The constrained (or low-affinity) state is stabilized by the inhibitor and the relaxed (or high-affinity) state is favored by the activator and the substrates.

Much evidence has been accumulated indicating that ATCase contains two catalytic (C) trimeric subunits which are "cross-linked" by three regulatory (R) dimers (Gerhart and Schachman, 1965; Weber, 1968; Wiley and Lipscomb, 1968; Meighen et al., 1970; Rosenbusch and Weber, 1971; Richards and Williams, 1972; Cohlberg et al., 1972; Nagel and Schachman, 1975). This structure, designated C_2R_3, contains six active sites as shown by the binding of the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA) (Jacobson and Stark, 1973b). ATCase-like molecules lacking one R subunit, i.e., C_2R_2, exhibit allosteric properties (Yang et al., 1974) as do hybrid molecules containing one active and one inactive C subunit and three R subunits (Gibbons et al., 1974). In contrast, complexes containing only one C subunit and three R subunits (CR_3) exhibit neither homotropic nor heterotropic effects (Mort and Chan, 1975). Similarly ATCase-like molecules containing extensively succinylated R subunits do not exhibit allosteric properties (Nagel and Schachman, 1975). In an effort to gain further insight into the structural requirements for cooperativity and inhibition, we initiated a series of experiments to perturb the properties of ATCase by chemical modification.

Since both the catalytic and regulatory sites have an affinity for phosphate-containing compounds, we utilized pyridoxal 5′-phosphate (PLP) as a modifying agent. Greenwell et al. (1973) showed that the binding of PLP to the isolated C subunit was competitive with CAP, and they isolated a

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[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; N (subscript), native; P (subscript), pyridoxylated; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; CAP, carbamoyl phosphate; PMB, *p*-mercuribenzoate; PMBS, *p*-mercuriphenylsulfonate; PALA, *N*-(phosphonacetyl)-L-aspartate.

stable inactive derivative by reducing the Schiff base with $NaBH_4$. In subsequent studies, Kempe and Stark (1975) have shown that this procedure leads to the modification of a single lysyl residue in each catalytic polypeptide chain. As shown below, the reaction of the intact enzyme differs markedly from that of the isolated C subunits. Moreover the modification of the different polypeptide chains in the enzyme is affected by the presence of the ligands and the various derivatives exhibited altered kinetic and physical properties.

The inactivation of ATCase or its C subunit might arise from the inability of the enzyme to bind one or both of the substrates, CAP or aspartate, or result from a blockage of the catalytic step by the pyridoxamine 5′-phosphate (PMP) moiety at the active site. To distinguish between these alternatives, we investigated the ligand-binding properties of the inactivated enzyme through the use of the technique of difference spectroscopy. These studies showed that CAP binds to the pyridoxylated sites, but the inactive enzyme was unable to bind succinate, a nonreactive analogue of aspartate. The difference spectra obtained in these experiments showed that the covalently bound PMP also responded to the binding of ligands and in this manner served as a reporter group for the micro-environment of the active site (Cortijo and Shaltiel, 1972). We therefore extended these studies to hybrid molecules that contained both native and pyridoxylated catalytic chains in order to examine possible communication effects between the C subunits of ATCase.

Experimental Procedure

Materials

ATCase was prepared by the method of Gerhart and Holoubek (1967) and the subunits were isolated by DEAE-cellulose chromatography after dissociation with neohydrin (Kirschner, 1971). L-Aspartic acid, CAP, PMB, and PMBS were obtained from Sigma Chemical Co. The CAP was reprecipitated from cold ethanol prior to use (Gerhart and Pardee, 1962). Succinic acid was purchased from Eastman Organic Chemicals. PLP (A grade) and PMP were obtained from Calbiochem. $NaBH_4$ was purchased from Metal Hydrides, Inc. PALA was kindly provided by G. R. Stark.

Methods

Protein concentrations were generally determined spectrophotometrically using ϵ_{280nm} (cm^2 mg^{-1}) of 0.59 for ATCase, 0.72 for C, and 0.32 for R subunits. Alternatively, the protein concentration was determined from synthetic boundary measurements with the ultracentrifuge (Richards and Schachman, 1959; Babul and Stellwagen, 1969). The enzyme was assayed by the method of Porter et al. (1969). Polyacrylamide gel electrophoresis with 7% gels was conducted with the buffer system of Jovin et al. (1964). PLP concentrations were determined spectrophotometrically with ϵ_{388nm} (M^{-1} cm^{-1}) of 6.6×10^3 in 0.1 N NaOH (Peterson and Sober, 1954). The incorporation of PLP into ATCase or its subunits was determined from the amount of covalently bound phosphate according to the method of Ames and Dubin (1960). Potassium phosphate, PLP, and PMP were all used as standards.

Spectral measurements were performed at 18 °C with a Cary 14 spectrophotometer equipped with a high-intensity tungsten source in addition to the standard ultraviolet-light source. Difference spectra were obtained with use of a 0–0.1 expanded-scale slide wire and with tandem cylindrical cells or semi-micro rectangular cells (Hellma). The sensitivity of the instrument and the slit width were adjusted to minimize noise consistent with the required spectral resolution.

Sedimentation experiments were performed with a Beckman-Spinco Model E ultracentrifuge equipped with a split-beam photoelectric scanning absorption optical system (Schachman and Edelstein, 1966). Difference sedimentation was performed according to Gerhart and Schachman (1968). All experiments were conducted at approximately 20 °C.

Pyridoxylation of ATCase and C Subunits. The reaction of PLP with ATCase and free C subunits was performed at 0 °C in 0.02 M triethanolamine–HOAc buffer at pH 8, containing 0.2 mM EDTA. Protein concentrations were 10 mg/ml and aliquots of a freshly prepared solution of PLP (10–20 mM) in the same buffer were added to give the desired concentration. The samples were then incubated in the dark for 15 min. During this time, a 1.0 M slurry of $NaBH_4$ was prepared in ice-cold water. Reduction of the protein–PLP complex was accomplished by the addition of a volume of the $NaBH_4$ slurry equal to one-tenth that of the reaction mixture. The intense yellow color of the PLP was bleached over a period of 1–2 min. After an additional 15 min incubation the solutions were dialyzed at 0 °C in the dark against 1 l. of the reaction buffer containing 0.2 M NaCl followed by several dialyses against 0.04 M potassium phosphate buffer at pH 7. During all stages of work with these derivatives, the exposure of the samples to light was kept to a minimum (Ritchey et al., in preparation). When the chemical modification was performed in the presence of ligand, the concentrations of CAP and succinate were 20 and 50 mM, respectively. Pyridoxylated C subunits (C_P) were reacted with R subunits to give ATCase-like molecules, $C_PC_PR_3$, and hybrid molecules, $C_NC_PR_3$, were prepared by the method of Gibbons and Schachman (1976).

Results

Extent and Location of Pyridoxylation of ATCase. Analysis of five different ATCase samples modified to varying extents (6–17 PMP residues bound per ATCase molecule) yielded an extinction coefficient (at 326 nm) of 5.8×10^3 M^{-1} cm^{-1} at pH 7. Greenwell et al. (1973) obtained a value of 5.35×10^3 M^{-1} cm^{-1} for C_P at pH 8 with an absorbance maximum at 322 nm. Spectral titration of PMP showed a similar shift in maxima and extinction coefficient with pH.

As shown in Figure 1a, pyridoxylation of ATCase in the presence of the active site ligands, CAP and succinate, did not differ significantly from that in the absence of ligands in terms of the total amount of bound PMP.[2] Since both the isolated C (Greenwell et al., 1973) and R subunits [M. N. Blackburn, P. Koga, and H. K. Schachman (unpublished data)] are modified readily with PLP and sodium borohydride, we measured the amount of PMP bound to the two types of subunits to determine whether preferential labeling of either the C or R subunits occurred and whether the relative affinities of the different subunits were affected by ligands.

[2] When modification of the enzyme was performed with high concentrations of PLP, there was more extensive pyridoxylation if the ligands were present; e.g., at an 85-fold molar ratio of PLP to ATCase, 28 residues of PMP were incorporated in the presence of CAP and succinate as compared with 19 when the ligands were absent.

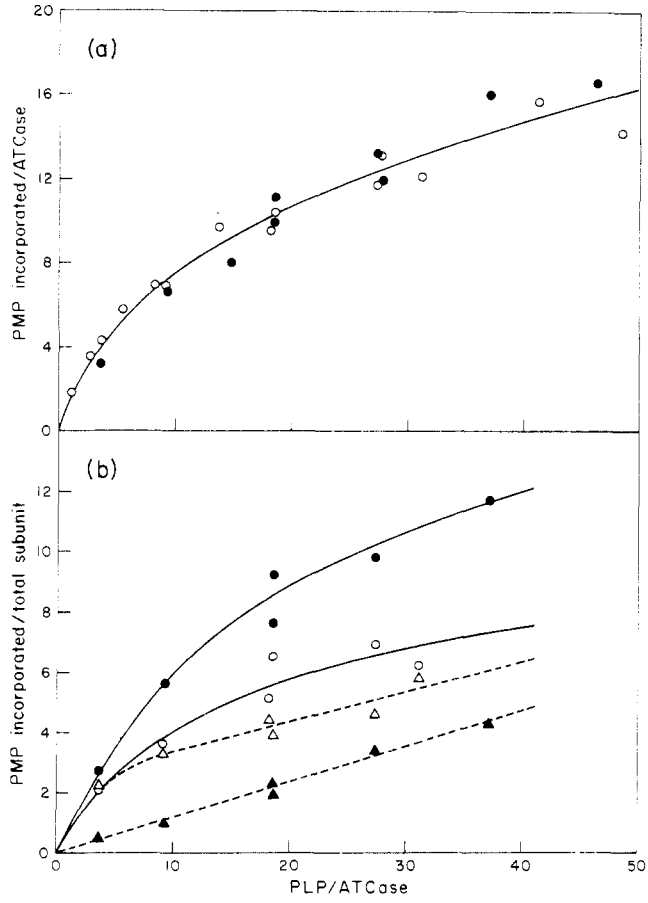

FIGURE 1: Incorporation of PLP into ATCase and distribution of the bound PMP on the C and R subunits. ATCase was modified with PLP as described under Methods. Excess reagents were removed by dialysis against 0.04 M potassium phosphate (pH 7) or 0.05 M imidazole-imidazole·HCl, 2×10^{-4} M EDTA (pH 7). (a) Amount of PLP incorporated into ATCase was determined by measuring the covalently bound phosphate by the method of Ames and Dubin (1960) or with use of a molar extinction coefficient of 5.8×10^3 M^{-1} cm^{-1} for the bound PMP (see Results). The data are presented as the number of bound PMP residues per ATCase molecule as a function of the molar ratio of PLP per ATCase. Incorporation of PLP into ATCase in the presence of 0.02 M CAP plus 0.05 M succinate is represented by (●), and (○) represents the results of pyridoxylation experiments in the absence of these ligands. (b) Distribution of bound PMP on the C and R subunits of ATCase was determined in the ultracentrifuge by analysis of the C and R subunit boundaries with light of 326 nm. The pyridoxylated enzyme was diluted to give an absorbance of 0.5–0.8 at 326 nm in 0.04 M potassium phosphate buffer (pH 7) and dissociated into its subunits by treatment with a 1.5-fold molar ratio of PMBS to total sulfhydryl groups. Data obtained with ATCase pyridoxylated in the presence of 0.02 M CAP plus 0.05 M succinate, filled symbols, ● and ▲. Open symbols represent data obtained with enzyme modified in the absence of these ligands ((△, ▲) C subunit; (○, ●) R subunit). The distribution of PMP is expressed as the moles bound per total subunit in ATCase; i.e., two C subunits and three R subunits.

The distribution of the bound PMP on the subunits was measured by sedimentation velocity experiments on the pyridoxylated enzyme which had been dissociated into modified C and R subunits by the addition of mercurials such as PMB or PMBS (Gerhart and Schachman, 1965). Analysis of the boundaries was conducted with the split-beam photoelectric scanning absorption optical system (Schachman and Edelstein, 1966) with light of wavelength 326 nm. At this wavelength, the total absorbance is due solely to PMP. Sedimentation velocity patterns for two samples pyridoxylated in the presence and absence of CAP and succinate are shown in Figure 2. Although the total amount of bound

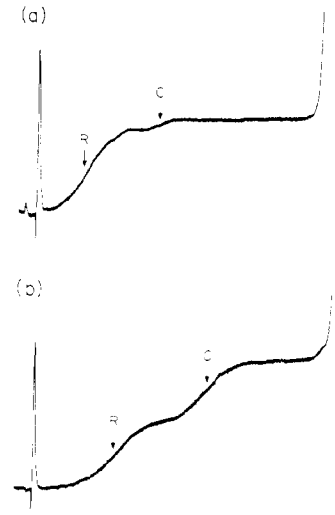

FIGURE 2: Determination of distribution of bound PMP on C and R subunits. ATCase was pyridoxylated in the presence and absence of 0.02 M CAP plus 0.05 M succinate with the incorporation of 3.2 and 3.9 residues of PMP per ATCase molecule, respectively. The enzyme was dissociated in 0.04 M potassium phosphate buffer by treatment with a 1.5-fold molar ratio of PMBS to total sulfhydryl groups. After 1 h, the samples were analyzed at 20 °C in an ultracentrifuge equipped with a split-beam photoelectric-scanning absorption system. The wavelength of the incident light was 326 nm and the rotor speed was 60 000 rpm. The boundaries due to the C and R subunits are designated. The direction of sedimentation is from left to right.

PMP was approximately the same for the two samples (3.2 and 3.9 mol of PMP bound per mole of ATCase in the presence and absence of ligands, respectively), the distributions varied significantly. Modification in the presence of CAP and succinate resulted in the almost exclusive labeling of the R subunits with a small fraction of the PMP on the C subunits. In contrast, the sample modified in the absence of these ligands showed extensive pyridoxylation of both types of subunits. The amount of PMP bound to the subunits is presented in Figure 1b as a function of the concentration of PLP in the reaction mixture. Binding of CAP and succinate to the active sites of the C subunits not only provided protection against modification of this subunit but also led to an increase in the reactivity of the R subunit. Some modification of the C subunits is still observed, however, in the presence of CAP and succinate. Under these conditions, very little enzyme activity is lost and this level of pyridoxylation probably represents nonspecific modification of sites other than the active site. A similar effect was observed with isolated C subunits at high concentrations of PLP (Greenwell et al., 1973).

Although the R subunits account for only 32% of the weight of ATCase molecules, the R subunits were the major site of pyridoxylation. In the absence of CAP and succinate, more than 60% of the bound PMP was located on the R subunits when high concentrations of PLP are used for the modification. When the ligands were present during the pyridoxylation more than 80% of the PMP was bound to the R subunits even at low levels of total modification.

Kinetics of ATCase Pyridoxylated in the Presence of Ligands. Although the C subunits within ATCase were pyridoxylated even in the presence of saturating concentrations of CAP or CAP plus succinate, the ability of the modified enzyme to catalyze the enzymatic reaction was not impaired. As seen in Table I, V_{max} and the apparent K_m for aspartate were essentially unaltered. Even after the incor-

Table I: Kinetics of Pyridoxylated ATCase.[a]

Species	V_{max}[b]	K_m[c] (mM ASP)	n_H[d]	Inhibition by CTP[e] (%)
Native enzyme	11	7	1.6	52
6.6 PMP/ATCase	12	7	1.5	38
10 PMP/ATCase	11	6	1.4	22
16 PMP/ATCase	10	6	1.2	7

[a] ATCase was pyridoxylated in the presence of 0.02 M CAP plus 0.05 M succinate. Assays were performed by the procedure of Porter et al. (1969). [b] Values are μmoles of carbamoyl aspartate formed per hr/μg of protein; V_{max} obtained by extrapolation of plots of velocity/substrate concentration *vs.* velocity to infinite aspartate concentration (Eadie, 1942). [c] Concentration of the substrate, aspartate, required for half-maximal velocity. [d] Determined from maximal slope of the Hill plot (Brown and Hill, 1922). [e] The CTP concentration was 0.5 mM. Inhibition measured at 5 mM aspartate.

poration of 16 PMP groups per ATCase molecule, of which about four were bound to the two C subunits, the derivative retained 90% of its original activity. As the extent of pyridoxylation increased, the Hill coefficient (n_H) decreased essentially as a linear function of the degree of modification, and n_H for the derivative with 16 PMP residues bound per ATCase molecule was 1.2. Although the cooperativity was reduced, the substrate concentration required to produce one-half the maximal velocity (K_m) was not significantly altered. Values of 7 and 6 mM aspartate were obtained for the native enzyme and the most heavily modified derivative, respectively.

Pyridoxylation of ATCase also desensitized the enzyme to CTP. Thus, while CTP inhibits native ATCase by 50–60% (at an aspartate concentration of 5 mM), the derivative with 16 PMP residues bound per ATCase molecule showed only slight inhibition of 7%. Direct measurement of the binding of CTP showed that 0.2 and 3.4 molecules were bound per molecule of pyridoxylated and native enzyme, respectively. Since the residual inhibition was not decreased further at greater levels of modification, it can be attributed probably to competitive binding at the active sites (Kleppe, 1966).

Partial Inactivation of ATCase by Pyridoxylation in the Absence of Ligands. Although there was very little loss of enzymic activity at moderate levels of pyridoxylation in the presence of CAP and succinate (or CAP alone), there was some inactivation at greater extents of modification. As seen in Figure 3, the enzyme activity was reduced to 65% of that of the native enzyme when the molar ratio of PLP to ATCase was 85. Under these circumstances, 28 PMP groups were incorporated per enzyme molecule.

In contrast the enzyme activity was decreased markedly when the pyridoxylation was performed in the absence of ligands which bind to the C subunits. However, even under these conditions complete loss of activity was not observed. As seen in Figure 3, the decrease in activity as a function of the ratio of PLP to ATCase followed a biphasic curve. At low levels of PLP (less than 6 molecules per ATCase molecule), the activity decreased in approximately a linear fashion with PLP concentration to a value about 65% that of the native enzyme. Further increases in the concentration of PLP in the reaction mixture led to slightly more inactivation. The derivative produced by pyridoxylation with a molar ratio of PLP to ATCase of 60 still exhibited about 50% of the original activity.

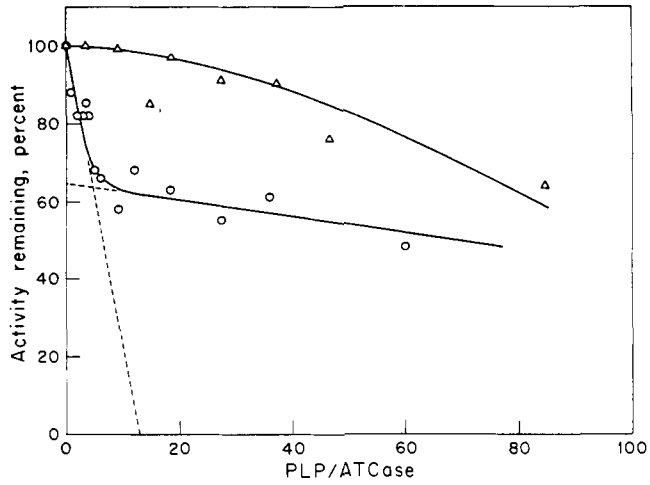

FIGURE 3: Inactivation of ATCase by pyridoxylation. ATCase was modified with PLP as described in Methods, in the presence (△) or absence (○) of 0.02 M CAP plus 0.05 M succinate. The modified enzyme was assayed according to the method of Porter et al. (1969). Data are presented as V_{max} relative to the native enzyme (specific activity equal to 12 μmol of carbamoyl aspartate formed per h/μg of protein).

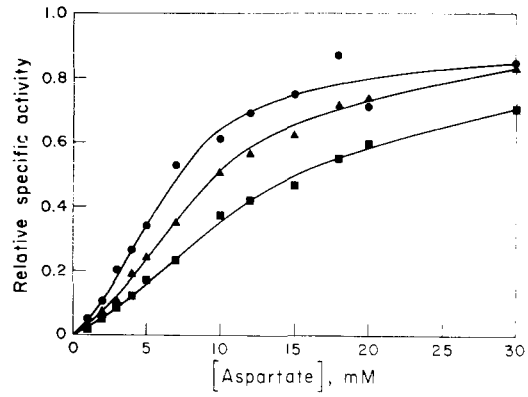

FIGURE 4: Kinetics of ATCase pyridoxylated in the absence of active site ligands. Assays were performed according to Porter et al. (1969). Data plotted as specific activity relative to the maximal velocity vs. aspartate concentration. Native ATCase is represented by ●, and derivatives containing 7 and 12 PMP residues per protein molecule are designated by ▲ and ■, respectively.

Kinetics of Partially Inactivated ATCase. Figure 4 shows the aspartate saturation curves for native ATCase and for two partially inactivated derivatives produced by pyridoxylation in the absence of ligands. For both derivatives, the curves were sigmoidal, indicating that considerable cooperativity was preserved even after partial inactivation. The Hill coefficients calculated from the data in Figure 4 were between 1.4 and 1.5 for the pyridoxylated derivatives as compared with 1.6 for the native enzyme. As seen in Figure 4, the apparent K_m increased from approximately 7 mM aspartate for the native enzyme to about 10 and 15 mM for the derivatives containing 7 and 12 PMP per molecule, respectively. Both of these derivatives were inhibited by 0.5 mM CTP almost to the same extent as the native enzyme (about 50% compared with 60% for unmodified ATCase). Stimulation of enzymatic activity by ATP was about 50% for the derivatives as compared with 20% for the native enzyme. This enhanced activation is probably attributable to the reduced activity of the derivatives at low aspartate stemming from their higher apparent K_m values and the change in K_m resulting from the addition of ATP.

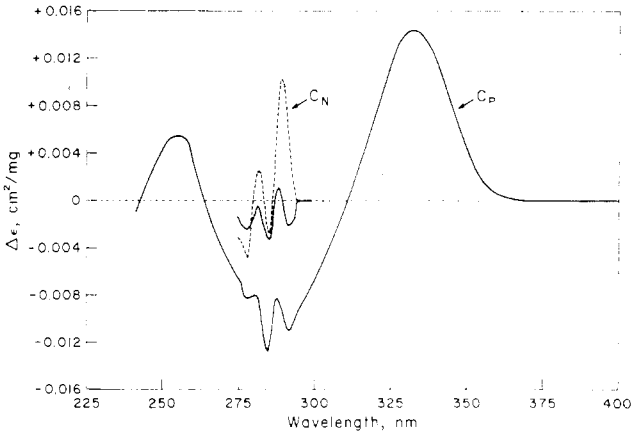

FIGURE 5: Effect of CAP and succinate on the spectra of C_N and C_P. Difference spectra were measured in tandem cylindrical cells at 18 °C; the buffer was 0.04 M potassium phosphate (pH 7). The concentration of C_N was 1.5 mg/ml and the difference spectrum promoted by the addition of CAP (1.8 mM) is designated by (—). The subsequent addition of succinate (5.4 mM) yielded the difference spectrum designated by (- - -). Analogous results are shown for C_P containing 1.2 PMP residues per c chain; this derivative had less than 3% residual activity. In these experiments the protein concentration was 2.9 mg/ml, CAP was 1.5 mM and succinate was 3 mM. The data are presented as the change in absorbance for a solution of C subunit at 1 mg/ml.

Distribution of Bound PMP on the C Subunits of Pyridoxylated ATCase. Since isolated C subunits were almost completely inactivated by pyridoxylation and the resulting derivative contains three bound PMP per C subunit (Greenwell et al., 1973), it was of interest to determine the distribution of PMP bound to the C subunits in the partially inactivated ATCase. Accordingly the pyridoxylated derivatives described above were dissociated by heating for 15 min at 68 °C in 4 mM potassium phosphate at pH 7 containing 4 mM β-mercaptoethanol and 2 mM EDTA (Rosenbusch and Weber, 1971). Since this procedure caused denaturation and precipitation of the R subunits, the resulting solution contained only C subunits. Electrophoretic analysis of the C subunits from an ATCase derivative formed at a ratio of 6 PLP per ATCase molecule showed bands corresponding to native and singly pyridoxylated C subunit. When the pyridoxylation of ATCase was performed with higher levels of PLP and the heat-treated derivative was subjected to electrophoretic analyses, two species were observed. One corresponded to protein containing two PMP per C subunit and the other was singly pyridoxylated C subunits.

Ligand-Promoted Conformational Changes in Pyridoxylated ATCase. Since the pyridoxylated ATCase derivatives prepared in either the presence or absence of CAP and succinate exhibited both homotropic and heterotropic effects, it was of interest to determine whether the derivatives undergo the ligand-promoted conformational change normally identified as the allosteric transition (Gerhart and Schachman, 1968). Accordingly measurements were made of the changes in sedimentation coefficient, $\Delta s/\bar{s}$, produced by the addition of ligands. As seen in Table II, similar values of $\Delta s/\bar{s}$, −3.6 and − 3.5%, were obtained for ATCase and the derivative produced by pyridoxylation in the presence of the two ligands. A slightly lower value, −2.9%, was obtained with the derivative prepared in the absence of the ligands. Table II also shows that the value of $\Delta s/\bar{s}$ is produced by CAP alone was significantly larger (−1.1%) for the enzyme pyridoxylated in the presence of ligands as compared with that for the native enzyme, −0.5%, and for the derivative formed in the absence of these ligands, −0.3%.

Table II: Ligand-Promoted Conformational Changes in Native and Pyridoxylated ATCase.

	$\Delta s/\bar{s}$[a]	
	CAP (%)	CAP + Succinate (%)
Native ATCase	−0.5	−3.6
ATCase pyridoxylated in presence of ligands[b]	−1.1	−3.5
ATCase pyridoxylated in absence of ligands[c]	−0.3	−2.9

[a] Change in sedimentation coefficient, $\Delta s/\bar{s}$, determined according to Gerhart and Schachman (1968). CAP concentration, 2 mM, and succinate concentration, 2 mM. [b] Pyridoxylated in the presence of 0.02 M CAP plus 0.05 M succinate. Total PMP incorporated, 11 residues per ATCase molecule with 1.8 on C and 9.2 on R subunits. [c] Total PMP incorporated was 10.4 residues per ATCase molecule with 3.9 on C and 6.5 on R subunits.

Effect of Ligands on the Spectra of C_P and $C_PC_PR_3$. Since CAP and succinate produce characteristic changes in the ultraviolet spectrum of the protein (Collins and Stark, 1969), it was possible to determine the ability of C_P and $C_PC_PR_3$ to bind CAP and succinate. As shown in Figure 5, the binding of CAP to the native C subunit produced a difference spectrum with peaks at 281.5 and 288.5 nm and troughs at 285 and 292 nm. The subsequent addition of succinate caused a large increase in the heights of the 281.5 and 289 nm peaks (the latter was shifted from 288.5 nm); the trough at 285 nm was hardly altered, although that at 292 nm was no longer observed. These spectra are essentially identical with those obtained by Collins and Stark (1969).

The most obvious features of the CAP difference spectrum for C_P were the large peak at 332 nm and the trough centered at approximately 285 nm due to the perturbation of the spectrum of the bound PMP. Superimposed upon this PMP trough is the normal protein difference spectrum for this ligand; the positions and relative magnitudes are not significantly altered by the bound PMP. The addition of succinate to the inactive C_P, however, did not result in a detectable change in the spectrum, indicating that succinate can no longer bind to the pyridoxylated catalytic sites.

The change in optical density at 332 nm upon the binding of CAP represented a 10% change in the total absorption at this wavelength. A value of 0.0147 $cm^2\ mg^{-1}$ for $\Delta\epsilon_{332}$ was obtained for C_P. An identical value, 0.0144 $cm^2\ mg^{-1}$, was obtained for a sample of C subunit with only 2.2 PMP bound per subunit after normalization to correspond to the inactivated subunit with all three active sites modified. The addition of succinate to this sample did not result in a further change in the absorbance at 332 nm, although binding of succinate to the open sites was clearly evident from the change in the protein difference spectrum at 285 and 288 nm. Titration of the partially pyridoxylated C subunit with PALA yielded a value of 0.015 $cm^2\ mg^{-1}$ for $\Delta\epsilon_{332}$ essentially identical with the measured spectral change obtained with CAP.

The effects of CAP and succinate on the spectra of ATCase-like molecules containing one and two C_P subunits are shown in Figure 6. As with C_P, CAP perturbs the PMP spectra to give rise to a peak at 332 nm and trough at about 285 nm. The height of the 332 nm peak obtained with

Table III: Effect of CAP on the Absorbance of C Subunits, ATCase, and Pyridoxylated Derivatives.

Species	$\Delta\epsilon$ (cm^2/mg of catalytic subunit)[a]	
	332 nm[b]	(288–285 nm)[c]
C_N		0.0054[d]
C_P[e]	0.0147	0.0044
C_NC_N[R]		0.0062[f]
C_NC_P[R]	0.0078	0.0085
C_PC_P[R]	0.0164	0.0078

[a] Values determined by difference spectroscopy in 0.04 M potassium phosphate buffer (pH 7). The data for ATCase and the ATCase-like hybrids have been normalized to the concentration of C subunits (67% by weight (Cohlberg et al., 1972)). [b] Maximum in the PMP difference signal. [c] Difference between the 288-nm peak and the 285-nm trough. [d] Determined by M. Springer (unpublished data). [e] Fully pyridoxylated C subunit containing 1.2 PMP residues per c chain. [f] Determined independently by Pigiet (1971) and Y. Yang (unpublished data).

$C_PC_PR_3$ (Figure 6a) is approximately double that with $C_NC_PR_3$ (Figure 6b). Table III summarizes the values for the difference extinction coefficients which were normalized to the concentration of C subunits to allow comparison between the intact enzyme and the isolated subunits. The difference extinction coefficient at 332 nm for the C_P was 0.0147 cm^2 mg^{-1}. For $C_NC_PR_3$ and $C_PC_PR_3$ values of 0.0078 and 0.0164 cm^2 mg^{-1}, respectively, were obtained. Thus the magnitude of the 332-nm difference peak was directly proportional to the concentration of PMP containing c chains and incorporation of the C subunits into ATCase-like molecules did not significantly alter the difference extinction coefficient obtained with saturating levels of CAP.

In addition to the large change in the PMP spectra, the binding of CAP also produced the normal protein difference spectra for this ligand; peaks at 282 and 289 nm and a trough at 285.5 nm were observed. As in the case of C_P, the reconstituted ATCase-like molecules, $C_PC_PR_3$, showed no spectral change upon the addition of succinate. Both the PMP and protein spectra for the hybrid species, $C_NC_PR_3$, however, respond to the binding of succinate. Although the C_P within the hybrid cannot bind succinate, the change in the spectrum of the bound PMP due to CAP plus succinate was slightly greater than that obtained for CAP alone.

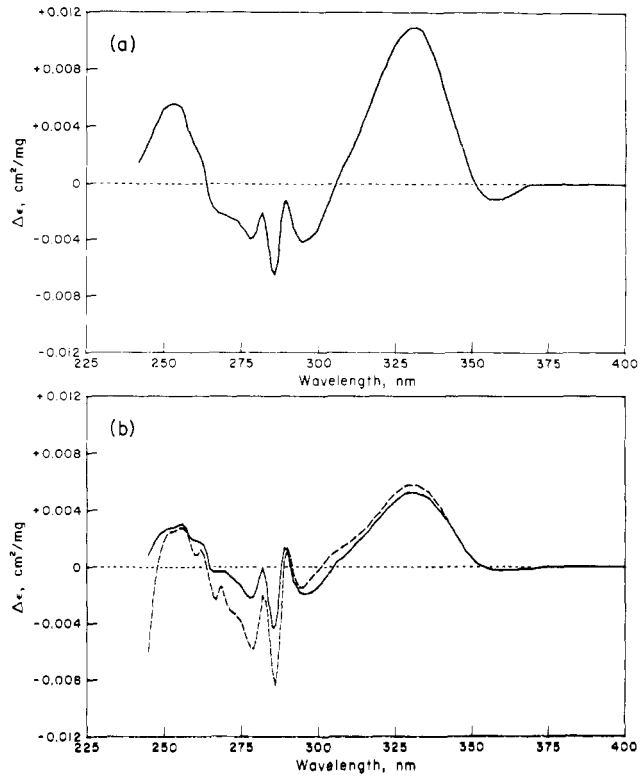

FIGURE 6: Effect of CAP and CAP plus succinate on the spectra of ATCase-like molecules containing one and two pyridoxylated C subunits. The enzyme containing two pyridoxylated C subunits ($C_PC_PR_3$) and the hybrid containing one pyridoxylated and one native C subunit ($C_NC_PR_3$) were obtained from the hybrid mixture as described by Gibbons et al. (1974). Difference spectra were measured as described in Figure 5. (a) Difference spectrum for $C_PC_PR_3$ obtained at a CAP concentration of 3 mM CAP and a protein concentration of 1.4 mg/ml is designated by (—). Addition of 5 mM succinate produced no further change in the spectrum. (b) Difference spectrum for the hybrid $C_NC_PR_3$ was obtained at a protein concentration of 2.7 mg/ml upon the addition of 3 mM CAP (—). The subsequent addition of succinate (5 mM) yielded the difference spectrum designated by (- - -). The data are presented in terms of the change in absorbance for protein solutions at 1 mg/ml.

Discussion

Effect of Ligands on the Pattern of Pyridoxylation of ATCase. When the active site ligands, CAP and succinate, were present during the pyridoxylation, there was almost no inactivation despite the extensive modification of both C and R subunits. This protection can probably be attributed to a direct effect of the ligands in blocking modification of the essential lysyl residue in the active site (Greenwell et al., 1973; Kempe and Stark, 1975). In addition these ligands must have an indirect effect on the entire enzyme molecule since the R subunits in ATCase exhibit an enhanced reactivity toward PLP as compared with the R subunits within ATCase when modification is performed in the absence of ligands. This gross effect is similar to the sixfold enhancement of the reactivity of the 24 sulfhydryl groups of the three R subunits when the enzyme is treated with the mercurial, PMB, in the presence of CAP and succinate (Gerhart and Schachman, 1968; Blackburn and Schachman, in preparation). This increase in reactivity has been correlated with the conformational change ($\Delta s/\bar{s}$ of −3.5%) identified as the allosteric transition, and it seems likely that the increased reactivity of the R subunits toward PLP is a manifestation of this same transition.

Kinetic Properties of Pyridoxylated ATCase. The functional characteristics of the enzyme pyridoxylated in the presence and absence of CAP and succinate are distinctly different. Modification in the absence of protecting ligands leads to the inactivation of two to three of the six catalytic chains in the native enzyme. However, even though the enzyme contains both functional and nonfunctional active sites, the ability of the pyridoxylated enzyme to respond allosterically to the substrate, aspartate, and the nucleotide effectors (CTP and ATP) is not significantly reduced. The degree of cooperativity in the binding of aspartate, as measured by the Hill coefficient, is only slightly reduced to between 1.4 and 1.5 as compared with 1.6 for the native enzyme. Gibbons et al. (1974) have recently shown that ATCase-like molecules containing only three functional active sites are cooperative. This enzyme species was prepared by hybridization of C_N and C_P with R subunits, and hence all of the active sites were located in one of the catalytic subunits. Analysis by disc gel electrophoresis of the partially inactivated C subunits prepared by pyridoxylation of the in-

tact enzyme has shown that in this case both C subunits were partially pyridoxylated.

When ATCase was pyridoxylated in the presence of CAP or CAP plus succinate, the active sites were protected against modification. Greater than 90% of the original activity was still present even though 16 PMP residues were bound per ATCase molecule. However, the modification of the regulatory subunits blocked the transmission of the allosteric signals between the active sites. Both the homotropic response to aspartate and the heterotropic effect of CTP were lost essentially as linear functions of the extent of pyridoxylation; the two effects were not, however, abolished simultaneously. Derivatives with 16 PMP residues bound per ATCase molecule did not show inhibition by CTP; however, the Hill coefficient was 1.2, indicating that some cooperativity remained.

Ligand Binding to the Pyridoxylated Enzyme. Kinetic studies by Greenwell et al. (1973) indicated that inhibition of activity of the isolated C subunit by PLP was due to competitive binding with the substrate CAP. Additionally G. R. Jacobson and G. R. Stark (cited by Greenwell et al., 1973) showed that CAP displaced PLP bound as a Schiff base to the C subunit.[3] As shown above, CAP protected the catalytic sites of ATCase from inactivation by pyridoxylation. Hence it seemed likely that the inactivation of ATCase and the isolated C subunits was attributable to the covalently bound PMP blocking the binding of the substrate CAP However, the CAP difference spectra obtained for C_P and $C_PC_PR_3$ showed clearly that this substrate binds to the inactivated derivatives. Furthermore, the similarity of the protein difference spectra for C_N and C_P suggests that the bound PMP does not seriously distort the CAP binding site. The magnitude of the 332-nm difference signal due to the binding of CAP is the same, within the estimated precision of the measurements, for each of the pyridoxylated derivatives when normalized to the concentration of pyridoxylated catalytic chains. The spectral response of the pyridoxylated protein to CAP appears to be due to a local change within the active sites of the C subunits even when these subunits have been recombined with native R subunits to form ATCase-like molecules. Thus the PMP difference spectra for C_P and $C_PC_PR_3$ were very similar. Also the presence of the C subunit in the hybrid molecule, $C_NC_PR_3$, or native chains in the partially pyridoxylated C subunit does not alter the shape or magnitude of this signal. In contrast to this, it can be seen from Table III that the protein difference signal, as measured by the difference between the 288-nm peak and the 285-nm trough, is significantly greater in ATCase than in the isolated catalytic subunits.

The inability of either C_P or $C_PC_PR_3$ to bind succinate indicates that the inactivation of the enzyme by pyridoxylation is due directly or indirectly to blockage of the aspartate binding site. Kempe and Stark (1975) have shown that pyridoxylation of the C subunit leads to the modification of a single lysyl residue per c chain. Whether this lysyl residue is directly involved in the binding of the aspartate is not known. It is possible that the bound PMP blocks the aspartate site sterically. It seems unlikely, however, that the modified lysyl residue participates directly in the binding of CAP since, as shown by the spectral changes, C_P interacts strongly with CAP.

In the case of the hybrid, $C_NC_PR_3$, CAP can bind to all six catalytic sites in the molecule; the substrate analogue, succinate, however, binds to only the native subunit. Despite the fact that succinate cannot bind to the pyridoxylated subunit, a small additional increase in the magnitude of the 332-nm peak over that due to CAP was observed upon the addition of succinate. Although the measured increase in absorbance (0.0015 unit) upon the addition of succinate (in the presence of CAP) was small, it should be noted that spectra could be readily reproduced to a precision of approximately 0.0005. Also no spectral change (less than 0.0003) was detected upon the addition of succinate to the inactive molecule, $C_PC_PR_3$. Hence the increase in the PMP difference signal in $C_NC_PR_3$ was not due to the increase in ionic strength contributed by the succinate. This slight shift in the absorbance of the bound PMP must therefore reflect a conformational change in the inactive subunit that results from the binding of succinate to the active catalytic subunit. As such this observation provides evidence for the communication of interactions from one C subunit, through the "cross-linking" regulatory subunits, to the other C subunit.[4]

Acknowledgments

We thank Celine Tung for excellent technical assistance and T. D. Kempe and G. R. Stark for communicating their results prior to publication. Also we are grateful to S. Shaltiel for suggesting the procedure used for the reduction of the Schiff base and helpful discussion of the spectral properties of pyridoxylated derivatives.

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[3] Analysis by difference spectroscopy of the formation of the Schiff base with intact ATCase indicated that addition of CAP decreased the amount of bound PLP. These results are complicated, however, by the binding of PLP to both the C and R subunits.

[4] Recent studies by Yang and Schachman (unpublished data) with hybrid ATCase-like molecules containing native and nitrated-pyridoxylated C subunits demonstrate conclusively that the binding of succinate to sites on the native subunit produces a conformational change in the inactive subunit.

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