The Separate Effects of Coenzyme Components May Not Be Additive

ROLES OF PYRIDOXAL AND INORGANIC PHOSPHATE IN ASPARTATE AMINOTRANSFERASE APOENZYMES*

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Both cytosolic and mitochondrial aspartate transaminase can be resolved of pyridoxal phosphate. The resulting apoenzymes still bind individual structural components of the coenzyme. The separate contributions of coenzyme components to protein thermal stability have been independently assessed for phosphate ions (P_i) and for the pyridoxal or pyridoxamine components of the coenzyme. \(^{31}\)P NMR and differential scanning calorimetry reveal that the thermodynamic contributions of binding are not additive and are dissimilar for the two isozymes. High and low affinity sites for P_i binding are present in both apoenzymes with only the low affinity site being present in the holoenzyme forms. The contribution of both bound phosphates to increasing temperatures (T_m) and enthalpies (\(\Delta H_D\)) of denaturation differ between the isozymes and within sites. In either isozyme occupancy of the high affinity site by P_i produces only a 4- or 5-degree increase in the T_m value with respect to P_i-free apoenzyme. By contrast, in the mitochondrial apoenzyme, the presence of P_i at the second low affinity site increases the calorimetric parameters from T_m = 47 °C and \(\Delta H_D = 4.7 \text{ cal g}^{-1}\) to T_m = 62 °C and \(\Delta H_D = 7 \text{ cal g}^{-1}\). For cytosolic apoenzyme the respective changes are from 66 to 69.5 °C and 5.2 to 5.8 \text{ cal g}^{-1}. Addition of pyridoxal, but not pyridoxamine, displaces the high affinity P_i in both apoenzymes. This shows that the pyridine ring and P_i groups of pyridoxal-P bind exclusive of each other when they are not covalently linked as an ester, as in the coenzyme. The observation has been exploited as a method to prepare completely dephosphorylated mitochondrial apoenzyme. Electrostatic effects, structural differences in the phosphate binding pockets, and steric effects can be invoked to account for the P_i and pyridine binding behavior in the two proteins.

Determination of the interactions of coenzymes and enzymes often rely on the measurement of affinities of separate components of the coenzyme or its analogs for the enzyme. From these studies inferences are derived as to the importance of various atomic elements of the coenzyme in ligand binding, protein stabilization, and in catalytic function. In several cases the simultaneous addition of separate coenzyme components restores part of the function of the complete coenzymes (1). In pyridoxal-P\(^1\)-dependent enzymes much work has been carried out using this approach to determine the function of the different structural parts of pyridoxal-P. This coenzyme can easily be divided in two parts: the vitamin B\(_6\) component, pyridoxal (or pyridoxamine), and inorganic phosphate. Hence, studies of the binding of these two components or their analogs by apoenzymes and their effect on apoenzyme activity have been extensive (1). In the aspartate transaminase system, which is the best characterized of this family of enzymes, most of this work has been carried out with the cytoplasmic isozyme. Since the structure of the cytoplasmic and mitochondrial isozymes are known to 2.7 Å resolution (2, 3), these studies can be designed to reveal the subtle differences in the interaction of pyridoxal-P with these two structurally related proteins.

Differences in phosphate binding have long been recognized as features distinguishing the two transaminase isozymes. One of the earliest noted differences involved the effect of inorganic phosphate concentration on the catalytic activity of the apoenzymes in the presence of the pyridine part of the coenzyme. Specifically, it was found that P_i inhibited the transamination between keto acid and pyridoxamine by the cytoplasmic apoenzyme while it was required for this reaction with the mitochondrial apoenzyme (4). Additional insight into the differences in the environment of the cofactor phosphate moiety between the mitochondrial and cytoplasmic holoenzymes was gained through \(^{31}\)P NMR studies of bound coenzyme (5, 6). More recently it has been reported that binding of pyridoxal-P to the mitochondrial apoenzyme occurs by a cooperative mechanism and that the intersubunit cooperativity was reduced by the presence of P_i (7). No cooperativity was found in similar studies with the cytoplasmic enzyme. Experiments conducted to analyze contributions of the various coenzyme structural elements to the thermodynamic stability of the cytoplasmic enzyme (8) showed that coenzyme components stabilize the apoenzyme and appear to exert their effect in an additive manner. By contrast, mitochondrial apoenzyme is destabilized in the presence of the dephosphorylated coenzyme (9). Since both apoenzymes are stabilized by pyridoxal-P, the unusual destabilization induced in the mitochondrial enzyme by the pyridine moiety of the coenzyme is further evidence for the differences in coenzyme-enzyme interaction with these two isozymes. This study employs both DSC and \(^{31}\)P NMR techniques to further probe the different effects of phosphate and pyridoxal binding to the cytosolic

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1. The abbreviations used are: pyridoxal-P, pyridoxal 5'-phosphate; pyridoxamine-P, pyridoxamine 5'-phosphate; P_i, inorganic phosphate; DSC, differential scanning calorimetry; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane.
and mitochondrial isozymes of aspartate transaminase. The results provide an explanation for the observed thermal de
stabilization of the mitochondrial apoenzyme upon binding of pyridoxal.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—The α subform of cytoplasmic aspartate transaminase and the C subform of the mitochondrial isozone were isolated from pig heart as previously described (10, 11). A poenzyme was prepared from each holoenzyme by standard procedures (11, 12). The activities were assayed spectrophotometrically using a coupled assay as already published (10). Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm using molar absorbances of 125,000 M
-1 cm
-1 (11) and 140,000 M
-1 cm
-1 (13) for the mitochondrial and cytosolic enzymes, respectively. The preparation of P
-1-free apoenzymes is described below (see "Results").

**NMR Experiments**—Fourier transform 31P NMR spectra were acquired on an IBM NR-80 spectrometer operating at 32.4 MHz. Spectra were accumulated at 25°C using 10-mm sample tubes. All NMR samples were in 0.01 M Tris-cacodylate buffer, 0.1 M KCl, pH 6.5, containing 10
-3 M EDTA. Each sample also contained approximately 10% D
2O in order to provide a field-frequency lock. No decoupling irradiation was employed. A 9-Hz line-broadening function was applied to the free induction decay signal prior to Fourier transformation, and chemical shifts were referenced to 85% phosphoric acid. Due to the difference in sensitivity between the two techniques, the concentrations of enzyme in the NMR experiments were at least 25 times higher than for the DSC experiments.

**Calorimetry**—Differential scanning calorimetric thermograms were recorded with a Microcal MC-1 differential scanning calorimeter. 1-ml samples were placed in each cell (sample and reference) with a calibrated microsyringe. All samples were dialyzed against the buffer (0.01 M Hepes, 0.1 M KCl, pH 8.0) used as reference material. The sample was then degassed prior to delivery into the cells. The calorimeter directly monitors the differential heat capacity of the sample cell with respect to the reference cell as a function of temperature. A scan rate of 1°C min
-1 was used over a temperature range of 20–100°C. Protein concentrations ranging from 1.2-1.6 mg/ml were used. Absolute values of the differential heat capacity were obtained by using a calibration pulse which is a design feature of the instrument. The enthalpy of denaturation, ΔH
D, was obtained by relation of the areas under the peak to the area of a precalibrated trace. The values of ΔH
D were then normalized per mg of protein (14). The transition temperatures (T
m) were obtained as the temperatures of the maximum differential heat capacity in the thermograms.

**RESULTS**

**Holoenzymes**—In the holoenzyme form, the cofactor pyridoxal-P is securely held by a Schiff base linkage to Lys-258 and a number of noncovalent interactions with other enzyme residues. The phosphate moiety is stabilized by electrostatic and hydrogen-bonding interactions and the dipolar field of α-helix 5 (15). A characteristic 31P NMR chemical shift is associated with this phosphate, whose signal appears, respectively, at approximately 3.2 and 4.0 ppm (downfield from the phosphoric acid reference) in mitochondrial and cytosolic holoenzymes at pH 6.5. Differences in the environment of this phosphate between the holoenzymes have been previously noted. Specifically, the phosphate chemical shift was found to be quite pH dependent in the mitochondrial enzyme (5) but much less so in the cytoplasmic isozone (6). The addition of free P
i to each of the holoenzymes causes the appearance of a distinct second resonance, at about 1 ppm, in the 31P NMR spectra. The chemical shift of this additional peak is concentration dependent and at high concentrations is indistinguishable from free P
i. Such behavior is indicative of a weakly bound phosphate in rapid exchange with unbound P
i.

The denaturation temperatures of the mitochondrial and cytosolic holoenzymes are determined by DSC to be 70.6 and 79.5°C, respectively. The areas under the transition curves give the partial specific enthalpies ΔH
D for the denaturation transitions. These are 6.4 cal g
-1 and 7.0 cal g
-1 for the cytosolic and mitochondrial isozymes, respectively. Neither T
m nor ΔH
D values are altered by the introduction of inorganic phosphate (50 mM) into the sample medium (Table I).

**Apoenzymes**—Most standard procedures for removing co-factor from the holoenzyme involve conversion of pyridoxal-P to the aminic form and subsequent displacement by a high concentration of phosphate buffer at low pH. Other procedures (16) seem to render apoenzyme preparations which are less stable.

DSC thermograms of standard preparations of apoenzyme extensively dialyzed against P
i-free buffer reveal a reduction in thermal stabilities of this form of the enzymes with respect to holoenzyme. They exhibit denaturation temperatures (T
m) of 47°C for the mitochondrial and 66°C for the cytosolic apoenzyme. The respective ΔH
D values are 4.7 and 6.2 cal g
-1 (Table I).

These apoenzyme forms retain bound P
i, as revealed by 31P NMR. At pH 6.5, the resonance peak for this high affinity phosphate appears at 2.25 ppm for apo-mitochondrial aspartate transaminase and 2.47 ppm for the cytosolic apoenzyme. The NMR resonances for the two isozymes exhibit a similar, although not identical, pH dependence (Fig. 1). Repetitive dialysis or passage through mixed bed ion exchange resins fails to remove the P
i ligand from this high affinity site in both isoforms.

**Apoenzymes and Inorganic Phosphate**—The addition of free P
i to the apoenzymes produces an additional narrower second resonance in the 31P NMR spectra, which, similar to the

| Enzyme form | T
m (°C) | ΔH
D (cal g
-1) | Chemical shift (ppm) |
|-------------|--------|----------------|----------------------|
| Holoenzyme  |        |                |                      |
| Cytoplasmic | 79.5   | 6.4            | 4.0                  |
| Cytoplasmic + P
i (50 mM) | 79.5 | 6.5 | 4.0 (<1) |
| Mitochondrial | 70.6 | 7.0 | 3.2 |
| Mitochondrial + P
i (50 mM) | 70.6 | 7.0 | 3.2 (<1) |
| Apoenzyme   |        |                |                      |
| Cytoplasmic | 66     | 5.2            | 2.47                 |
| Cytoplasmic + 100 mM P
i | 66.5 | 5.8 | 2.47 (<1) |
| Cytoplasmic dephosphorylated | 61 | 2.6 |
| Cytoplasmic + 25 mM pyridoxal | 70 | 5.0 | 2.47 (<1) |
| Cytoplasmic + 10 mM P
i + 25 mM pyridoxal | 70 | 5.1 | 2.47 (<1) |
| Cytoplasmic + 25 mM pyridoxamine | 66 | 5.3 | 2.47 |
| Mitochondrial | 47    | 4.7            | 2.25                 |
| Mitochondrial + 10 mM P
i | 62 | 7.0 | 2.25 (<1) |
| Mitochondrial dephosphorylated | 43.5 | 4.4 |
| Mitochondrial + 25 mM pyridoxal | 45.5 | 5.0 | -1 |
| Mitochondrial + 10 mM P
i + 25 mM pyridoxal | 46 | 5.0 | <1 |
| Mitochondrial + 25 mM pyridoxamine | 47 | 4.7 | 2.25 |
| Mitochondrial + 19 mM P
i + 25 mM pyridoxal | 62 | 7.0 | 2.25 (<1) |

* Chemical shifts referenced to 85% phosphoric acid.
*a Apoenzyme prepared by acetate/ammonium sulfate method (see "Results").
*a Apoenzyme prepared by incubation with excess pyridoxal and dialysis.
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Pi bound to apoenzyme of mitochondrial (●) and cytoplasmic (○) isozymes. Conditions: 0.01 M Tris-cacodylate, 0.1 M KCl, 10^{-3} M EDTA at variable pH values.

Fig. 1. The effect of pH on the 31P NMR chemical shift of Pi bound to apoenzyme of mitochondrial (●) and cytoplasmic (○) isozymes. Conditions: 0.01 M Tris-cacodylate, 0.1 M KCl, 10^{-3} M EDTA at variable pH values.

The 31P NMR spectra represent an enzyme sample (3 × 10^{-4} M) to which has been added: a, 0; b, 0.25; c, 0.5; d, 1.1; e, 1.6; and f, 2.1 molar eq of Pi/enzyme monomer. Similar results can be obtained with apocytoplasmic isozyme. Conditions: 0.01 M Tris-cacodylate, 0.1 M KCl, 10^{-3} M EDTA, pH 6.5. Spectrum of nondephosphorylated apoenzyme (standard apo preparation) is similar to d. Addition of Pi to the latter form of apoenzyme results in spectra identical to e and f at the same molar concentrations of Pi.

Holoenzyme, reveals a low affinity binding site for phosphate. If the addition is of 1 eq of Pi/apoenzyme monomer, the integrated areas of the two NMR peaks for each isozyme are identical, indicating that the stoichiometry of high affinity Pi is one phosphate dianion/enzyme monomer, as expected. This conclusion has been verified by preparing Pi-free apoenzyme (described below) and monitoring its titration with Pi via NMR. Solutions of both Pi-free apoenzymes, mitochondrial and cytoplasmic, at a concentration of 3 × 10^{-4} M were titrated with increasing amounts of Pi, ranging from 1.2 × 10^{-4} to 5 × 10^{-4} M. Complete reconstitution of the high affinity Pi signal was achieved after addition of 1 mol of Pi/enzyme subunit (Fig. 2). These enzyme-Pi complexes exist in slow exchange conditions, and the chemical shifts are identical to those observed for the intrinsic Pi found in the initial apoenzyme forms. Further additions of Pi give rise to the weakly bound phosphate resonance (Fig. 2). The existence of a secondary Pi binding site is inferred from observed concentration-dependent shifts in the 31P NMR spectra, as shown in Fig. 3. As the Pi concentration is increased, the chemical shift positions gradually approach the limiting value observed for free Pi at that pH. Such fast exchange behavior is indicative of a relatively weak enzyme-Pi complex and is distinct from that observed in the presence of stoichiometric amounts of phosphate. A much higher affinity for Pi is exhibited by the mitochondrial enzyme, as evidenced by the correspondingly greater chemical shift dependence of the Pi resonance (Fig. 3). Dialysis against Pi-free buffer will remove such low affinity Pi from its weak binding site in both isozymes. This treatment restores the protein species to their original forms, having identical calorimetric and NMR spectral characteristics as prior to the addition of excess Pi.

The calorimetric behavior of the two extensively dialyzed apoisozymes upon addition of Pi is quite dissimilar. In the mitochondrial enzyme, thermograms reveal a steady elevation in thermal stability (T_m) accompanying increased Pi concentration (Fig. 4). The denaturation temperature ascends from an initial value of 47 °C for preparations without added phosphate (and without the 1-ppm phosphate resonance line in the NMR spectrum) to a limiting value of approximately 62 °C at a Pi concentration of 10 mM. Further increases in Pi concentration have no effect. These T_m values are a measure of the degree of stability imparted to the mitochondrial apoenzyme structure by the ligand Pi at its binding site. Apparently, thermal resistance to unfolding depends on the degree of occupancy of the low affinity site by Pi. A reciprocal plot of T_m versus Pi concentration yields an approximate K_d of 5 × 10^{-5} M for the low affinity phosphate species. In addition to the T_m values, the enthalpies of denaturation (ΔH_d) are likewise dependent on the amount of phosphate present (Table I). Moreover, the ΔH_d value for mitochondrial apoenzyme in the presence of 10 mM phosphate (7.0 cal g^{-1}) is identical to that for holoenzyme (Table I). This is not likely just an ionic strength effect due to increasing concentrations of Pi, since in

Fig. 2. The effect of adding inorganic phosphate to dephosphorylated apomitochondrial aspartate aminotransferase. The 31P NMR spectra represent an enzyme sample (3 × 10^{-4} M) to which has been added: a, 0; b, 0.25; c, 0.5; d, 1.1; e, 1.6; and f, 2.1 molar eq of Pi/enzyme monomer. Similar results can be obtained with apocytoplasmic isozyme. Conditions: 0.01 M Tris-cacodylate, 0.1 M KCl, 10^{-3} M EDTA, pH 6.5. Spectrum of nondephosphorylated apoenzyme (standard apo preparation) is similar to d. Addition of Pi to the latter form of apoenzyme results in spectra identical to e and f at the same molar concentrations of Pi.

Fig. 3. Concentration-dependent changes in the 31P chemical shift of inorganic phosphate in the presence of mitochondrial (●) and cytosolic (○) holoenzymes (A) and apoenzymes (B). Conditions: 0.01 M Tris-cacodylate, 0.1 M KCl, 10^{-3} M EDTA, pH 6.5. Protein concentration: 5 × 10^{-4} M. The chemical shifts presented in B (relative to 85% phosphoric acid) are those for the resonance line that appears at Pi concentrations in excess of 1 eq/mol of apoenzyme monomer. For details see “Results.”
all our experiments we used buffers containing 0.1 M KCl.

By contrast, the thermostabilizing effect of P_i on apocytosolic enzyme is minor (Table I). Concentrations as high as 100 mM P_i increase the observed denaturation temperature from 66 to 69.5 °C, an increment of only 4 °C, as compared with the 15 °C difference for the mitochondrial enzyme.

**Apoenzymes and Pyridoxal**—Calorimetric studies also reveal dramatic differences between the two apoenzymes with respect to pyridoxal binding. A modest rise in the T_m from 66 to 70 °C was observed upon addition of pyridoxal (25 mM) to the cytosolic apoenzyme. Conversely, the presence of this same concentration of pyridoxal results in a lowering of the T_m value of mitochondrial apoenzyme stabilized by excess P_i (from 62 to 48 °C), with a concomitant decrease in transition enthalpy (Fig. 4 and Table I). This apparent destabilization induced by pyridoxal could not be observed when apoenzyme was obtained by standard procedures which has only the high affinity P_i bound. Addition of pyridoxal to these extensively dialyzed preparations gave a narrower thermal transition (∆H = 5.0 cal g⁻¹) but a similar T_m value of 45.5 °C (Table I). However, ³¹P NMR experiments provide additional information concerning this process. Upon addition of increasing concentrations of pyridoxal there is a gradual elimination of the ³¹P resonance at 2.25 ppm which is characteristic of the high affinity P_i. Simultaneously a new resonance at around 1 ppm appears which is characteristic of the low affinity P_i (Fig. 5A). Higher concentrations of pyridoxal induce a complete removal of the 2.25-ppm resonance and a further shift of the 1-ppm ³¹P resonance line toward the limiting value characteristic of free phosphate at this pH. This behavior is analogous to that observed for the weak binding site when titrating holo-

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![Figure 4](https://example.com/figure4.png)

**Fig. 4.** The effect of inorganic phosphate on the thermal stability of mitochondrial apoenzyme and of the presence of 25 mM pyridoxal on the stabilization induced by P_i addition to the same apoenzyme. Conditions: 0.01 M Hepes, 0.1 M KCl, pH 8.0. enzyme concentrations: 1.5 mg/ml (transitions a–d and f), and 1 mg/ml (transition e). Phosphate was added to samples to yield final concentrations of: (a) no addition (the apoenzyme contains 1 eq/molecule of intrinsic phosphate at its high affinity binding site, as determined by NMR); (b) 3 × 10⁻³ M; (c) 8 × 10⁻⁴ M; (d) 1.3 × 10⁻⁴ M; (e) 10⁻⁵ M; (f) 10⁻⁵ M inorganic phosphate plus 25 mM pyridoxal.

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** The displacement of high affinity P_i from mitochondrial and cytosolic apoenzymes by increasing concentrations of pyridoxal, as revealed by ³¹P NMR. A, mitochondrial apoenzyme (9 × 10⁻⁴ M) in the presence of 0, 5, 10, and 30 mM pyridoxal. B, cytosolic apoenzyme in the presence of 0, 10, 20, and 30 mM pyridoxal. Conditions: 0.01 M Tris-cacodylate, 0.1 M KCl, 10⁻⁵ M EDTA, pH 6.5.

Phosphate-free Apoenzymes—By taking advantage of the fact that pyridoxal will readily dislodge high affinity P_i from mitochondrial and cytosolic apoenzymes, as revealed by ³¹P NMR, apoenzyme forms with increasing concentrations of P_i.

For the cytoplasmic isozyme the lower field resonance line at 2.47 ppm (high affinity P_i) can also be removed with addition of pyridoxal, yet the concentrations of pyridoxal required are much higher than for the mitochondrial apoenzyme (Fig. 5B). 25 mM pyridoxal displaces all tightly bound P_i from mitochondrial enzyme; by contrast, 30 mM pyridoxal displaces only about 35% in the cytoplasmic enzyme. Clearly, the efficiency of P_i displacement by pyridoxal is considerably lower for the cytoplasmic enzyme. Verification of specific binding of pyridoxal at the enzyme-active site was made by determining its effect on the enzyme specific activity. The cytoplasmic apoenzyme was incubated with increasing concentrations of pyridoxal, and after reduction of the Schiff base with cyanoborohydride and further dialysis, the specific activity of the enzyme was determined upon addition of an excess (0.2 × 10⁻³ M) of pyridoxal-P. After incubation of apoenzyme (1.5 mg/ml) with 5 mM pyridoxal the enzyme lost more than 95% of its initial reconstitutable activity.

Apoenzymes and Pyridoxamine—In contrast to pyridoxal, pyridoxamine, the dephosphorylated aminic form of the coenzyme, exerts no apparent effect on either apoenzyme. At concentrations up to 25 mM, pyridoxamine induces no discernible displacement of high affinity P_i via NMR nor does it alter enzyme thermal stabilities (Table I). This result reaffirms earlier observations concerning the effect of pyridoxamine on apocytosolic enzyme, in which it was concluded that very little thermal stability originated from interaction of enzyme with the pyridine ring of the cofactor (8). Instead, the observed stabilization induced by native coenzyme was attributed to the Schiff base and to interactions with the phosphoryl group.
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was stable for at least a week when stored at 4 °C and regained full catalytic activity upon incubation with pyridoxal-P.

This method, however, was not successful for preparing dephosphorylated cytosolic apoenzyme, presumably due to its instability over the course of several dialyses. An alternative procedure was, therefore, used, with the intent of avoiding phosphate altogether (16). Cytosolic holoenzyme initially in sodium acetate buffer was adjusted to a concentration of approximately 5 mg/ml. After treatment with a 2-fold molar excess of cysteine sulfinate, the solution pH was reduced to 4.6 with 0.5 N acetic acid and then stirred for 30 min at room temperature in the presence of 50% saturating ammonium sulfate. After rechilling to 0 °C, the apoenzyme was precipitated with 75% saturating ammonium sulfate, centrifuged, and recovered in cold buffer. The entire process was repeated and the product finally dialyzed against a large volume of chilled buffer (0.1 M KCl, 0.01 M Hepes, pH 8.0).

Thermograms of Pi-free (31P NMR evidence) apomitochondrial enzyme exhibit a denaturation temperature of 43-5 °C, a value approximately 3 °C lower (Table I) than that for Pi-bound apoenzyme (47 °C). This represents a direct determination of the contribution of high affinity P* to the thermodynamic stability of this species. Thermograms of this enzyme equilibrated in 10 mM pyridoxal medium reveal a Tm of 45.5 °C, affording an assignment of the approximately 2 °C increase in the transition temperature to the Schiff base linkage and pyridine ring binding to the apoenzyme. Since association of the pyridine ring component has been shown to be of minor thermodynamic impact (8), the 2-degree increment of stability may be ascribed, in its major part, to the Schiff base contribution.

Cytosolic apoenzyme prepared without phosphate exhibits a Tm of 61 °C. Upon stoichiometric reconstitution by P*, of the high affinity binding site, the Tm increases to 66 °C. Therefore, the contribution of such phosphate to the thermal stability of the cytosolic apoenzyme is about 5 °C.

DISCUSSION

Most previous work on the properties of coenzyme binding used analogs of the coenzymes in which either individual atomic components are omitted or used individual molecular components. In pyridoxal phosphate-dependent enzymes this type of work has been carried out in great detail, and replacements at every position of the pyridine ring of the coenzyme have been tried with a variety of apoenzymes. In many cases, however, it has been noticed that replacements at the C-5' position are most sensitive in perturbing either the binding affinity of the analog or its ability to mimic the natural coenzyme in regenerating catalysis (17). Studies with aspartate transaminase have been limited to the cytoplasmic isozyme which shows extreme sensitivity to the type of substituents on the coenzyme analog. Indeed, in this isozyme, the phosphate ester bond was originally postulated to be one of the mandatory pivots necessary for a rotation of the coenzyme along a C-2'-C-5' axis during catalysis (18). Even though such rotation is not included in present mechanisms (2, 5, 17), some movement along the C-5-C-5' bond is considered a necessity for the mechanism of catalysis in both transaminase isozymes.

Previous studies with cytoplasmic apoenzyme revealed no correlation between the binding of inorganic phosphate and that of pyridoxal or its analogs. Yet, it has long been recognized that the phosphate component is a strong contributor to the binding of the coenzyme (8). It was also believed that the transaminase apoenzyme, if freed of inorganic phosphate, would be a very unstable molecule to work with. On the other hand, the mitochondrial enzyme was detected to differ from its cytoplasmic counterpart in that it was more difficult to prepare free of bound phosphate ions. 31P NMR studies revealed that even after extensive dialysis against Tris-chloride buffer 1 mol of P, remained bound. The latter could be displaced by extensive dialysis against arsenate (5).

Crystallographic studies of the apoenzymes have revealed that P* can occupy the pocket where the coenzyme phosphate ester fits. In holoenzyme this phosphate seems to interact with Arg-266, Ser-255, and Thr-109 (also Ser-107 in the mitochondrial enzyme) and is further stabilized by the dipolar field generated by the proximal end of the α-helix extending between residues 108 and 124 (15). Thus, a priori there are no apparent major structural reasons for the disparity of properties of P* binding among the isozymes. That this disparity in properties is not attributable to the high affinity bound P* is suggested by our 31P NMR and DSC studies. First, the pH dependence of the bound P* NMR resonances exhibits a similar chemical shift range and apparent pK values in the two isozymes (Fig. 1). Secondly, DSC experiments reveal a comparable increment of thermal stability (∆Tm) in both isozymes after binding the high affinity P*, (Table I). The Tm increments of 3 to 5 degrees, although appreciable, probably only reflect the thermal stability differences between apoenzymes containing P*, and those containing other anions such as chloride. The dephosphorylated apoenzyme probably retains a buffer anion(s) at the apoenzyme's phosphate-binding site.

The affinity of the coenzyme phosphate-binding pocket for P* is reduced in the presence of pyridoxal, especially in the mitochondrial apoenzyme. We propose that this is primarily due to electrostatic effects. In the absence of pyridoxal, dianionic phosphate ion may be stabilized by a positively charged Lys-258. Indeed, X-ray maps of the mitochondrial holoenzyme have shown that when the coenzyme is in a pyridoxamine phosphate form Lys-258 is located in the proximity of the coenzyme phosphate-binding pocket hydrogen bonding to Tyr-70* and the C4'-amino group of pyridoxamine-P (15). A similar situation may also occur for the mitochondrial apoenzyme. Upon addition of pyridoxal there is Schiff base formation between pyridoxal and Lys-258 which should induce a reorientation of Lys-258 away from the P* binding site. The loss of the free amino group and movement away from its original position should induce electrostatic rearrangements, most notably the delocalization of the apoenzyme's stabilizing charge in its pocket for the dianionic P*. Steric interference of the C5'-hydroxyl group of pyridoxal may also assist in displacing the P* from its apoenzyme pocket. That no such displacement is observed with pyridoxamine may be explained on its inability to form a Schiff base, which would leave the effect of Lys-258 on bound P* essentially undisturbed. Indeed, the presence of pyridoxamine can contribute an extra positive charge to a protein electrostatic environment which would be unfavorable for the release of dianionic phosphate.

NMR experiments reveal distinctions in the response of the two apoisozymes to the addition of pyridoxal (Fig. 5). P* displacement by pyridoxal takes place with a several-fold greater efficiency in the mitochondrial apoenzyme than in the cytosolic, as judged by the concentration of pyridoxal necessary to bring about comparable P* release. The apoenzyme's structural basis for this difference is unclear. Some possibilities include undetected structural differences in their respective coenzyme binding domains. A small difference such as a variation in the proximity of active site lysine (Lys-258) to
the phosphate-binding pocket in each apoenzyme could explain our observations.

The unexpected role of a second weak phosphate binding site on coenzyme binding and overall protein thermal stability further distinguishes the two isozymes. As seen from the NMR data of Fig. 3, a much greater affinity for this second Pi is exhibited by the mitochondrial enzyme, as compared to the cytosolic. However, for a given isozyme the data for its holo and apo forms are markedly similar, indicating no apparent influence of coenzyme binding on its affinity at this secondary site. The dissimilar binding affinity between the two isozymes for the second Pi may have a direct bearing on our observed differences in the thermostabilizing effect of Pi detected by DSC. In the cytoplasmic isozyme, excess Pi plays a minor role in protecting the apoenzyme against thermal denaturation. This contrasts with a dramatic dependence of both $T_m$ and $\Delta H_m$ values on phosphate ion concentration for mitochondrial apoenzyme, which suggests an influence of Pi binding on the protein structure. The amount of phosphate ion required to induce maximum resistance to thermal denaturation (10 mM) agrees with the concentration required in our $^{31}P$ NMR results to effect saturation of the low Pi affinity site (6-10 mM). Interestingly, the analogous binding of excess Pi to the holoenzyme form of the same isozyme (Fig. 3A) is without discernible effect as monitored by DSC (Table I).

In the mitochondrial apoenzyme displacement of the high affinity Pi, from its pocket by pyridoxal leads to apparent migration of Pi to the low affinity site. Yet, the chemical shift value of 1.0 ppm of the low affinity Pi-protein complex gradually shifts, with increasing concentration of pyridoxal (up to 30 mM) toward the limiting value of 0.8 ppm characteristic of free Pi (Fig. 5A). This suggests that not only does pyridoxal displace Pi from its high affinity site, but it also competes with the weaker secondary binding site. By contrast, in cytosolic apoenzyme the $^{31}P$ chemical shift of displaced high affinity Pi, resulting from additions of pyridoxal is indistinguishable from free Pi at all concentrations of pyridoxal tested (Fig. 5B).

Early clues to structural differences between the two apoenzymes were revealed in the catalytic work of Wada and Morino (4). They showed that Pi enhanced the rate of half-transamination of pyridoxamine and ketoadic catalyzed by the mitochondrial apoenzyme, whereas in the cytoplasmic apoenzyme that same reaction was inhibited by Pi. These results may be rationalized in part in light of the present results. High affinity phosphate can coexist with pyridoxal at the catalytic concentrations needed in cytoplasmic apoenzyme but not in the mitochondrial apoenzyme. Hence, in the transamination of pyridoxamine, displacement of the protein-bound reaction product, pyridoxal, is only observed in the mitochondrial apoenzyme, through competition with the high affinity phosphate site. This can give the kinetic appearance of an activation. Such a mechanism would be even more likely if in the mitochondrial isozyme the required ternary complex pyridoxamine-keto acid-aphenzyme were Pi free.

Evidence that there is competition between vitamin B$_6$ compounds and the weak Pi binding site has also been recently reported when a dephosphorylated analog of pyridoxamine was used as an affinity label of both apoenzymes (19, 20). N-Bromoacetylpdioxamine inactivation was inhibited by millimolar concentrations of inorganic phosphate.

The protein location of the second low affinity Pi binding site is uncertain. A reasonable hypothesis can be made from the known structure of the active site region. The guanidinium groups of Arg-386 and Arg 282* are likely candidates. (The latter arginine arises from the second subunit in each dimeric apoenzyme.) In crystals of cytoplasmic holoenzyme (1) these residues have been detected as binding acetate ions, and the latter are also known to be competitive with phosphate ions in both isozymes (21). The guanidinium groups are seen 6 A apart, and the phosphate ion may reside at one of these sites or be shared by them. There are also the protein sites to which the carboxyl groups of the substrates are thought to bind in each holoenzyme. The proposed hypothesis is attractive from an electrostatic point of view, since the presence of Pi could neutralize the guanidinium charges and induce charge rearrangements.

In conclusion, it appears that although there are similarities in the present electron density maps of the two isozymes, discrete differences may exist in the orientation of some of their active site amino acid residues. Such differences may be detectable when greater resolution maps and refinement of the x-ray diffraction data are available. Apparently, such structural disparities between the isozymes are manifested as variations in the competition of pyridoxal with Pi, and on a differing thermal stability in the apoenzyme upon phosphate binding. Electrostatic rearrangements induced by the presence of ligands such as Pi may affect charged active site residues differently in each isozyme. Protein domain movements induced by ligand binding have been detected through the use of difference electron density maps in both isozymes (2, 15). These two proteins possess only 48% amino acid sequence homology and since electrostatic changes may be transmitted through movement of entire protein domains, if these domains contain dissimilar structural entities (i.e. amino acid residues) the movements may affect distant regions of the protein in different ways. Thus, although each isozyme's active site ligand recognition may be by the same type of amino acid residues, the consequences of the transmitted conformational message could have a dissimilar structural effect on regions of the protein which differ in amino acid sequences.

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