Taking the value from Fisher et al. (1981) of 22 kcal mol^{-1} for $\Delta H°$, at temperatures in the range of 25–35 °C:

$$\Delta T_0 \simeq 20\Delta pK \quad (10)$$

using the value of T_0 of 43 °C reported in that same paper in eq 9, we calculate a ΔpK_0 of 0.90, corresponding to a pK_{HE} = 8.50 in excellent agreement with the value observed here derived from free energy studies at a constant temperature. It is clear then that the implicit two-state process responsible for the unusual temperature-dependent $\Delta C_p°$ reported in our earlier paper is fully accounted for by a step in which the binding of NADPH drives a proton off of a functional group on the enzyme.

One major problem does remain. It is by no means easy to account for an ionization of an amino acid residue having either a $\Delta H°$ of 19 kcal mol^{-1} or a $\Delta S°$ of +23 eu regardless of its pK. The excessively large $\Delta H°$ could be accounted for by the forced ionization of *two* protons from highly basic amino acid residues, one proton being released to the solvent and the other being transferred intramolecularly to a residue having a low ionization enthalpy. Since amino acid residues which have ionization $\Delta H°$'s of 11–13 kcal mol^{-1} typically have small negative ionization $\Delta S°$'s, while those of low ionization enthalpy can have positive $\Delta S°$'s of neutralization of about +20 eu, the unusual combination of a large positive enthalpy change and a large positive entropy change could be accommodated by such a mechanism.[3] In any case, it would appear that the process we designate as $EH^+ \rightleftharpoons E + H^+$ must involve the ionization of a protonated enzyme group which is very tightly coupled to some other process having very large energetic properties. Any such change must in all probability involve very substantial alterations in protein structure.

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[3] Since ionizable groups in proteins are not infrequently found to have pK's which differ considerably from those of the corresponding free amino acids, perhaps we should not be astonished at finding "unusual" values for $\Delta H°$ and $\Delta S°$ for ionizable groups on enzymes.

Ionization State of the Coenzyme 5′-Phosphate Ester in Cytosolic Aspartate Aminotransferase. A Fourier Transform Infrared Spectroscopic Study†

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ABSTRACT: In order to determine the ionization state of the 5′-phosphate of bound pyridoxal phosphate, a Fourier transform infrared spectroscopic study of cytosolic aspartate aminotransferase has been carried out. Dianionic and monoanionic phosphate monoesters give rise to two bands each in the infrared spectrum [Shimanouchi, T., Tsuboi, M., & Kyogoku, Y. (1964) *Adv. Chem. Phys. 8*, 435–498]. These bands can be identified in infrared spectra of the free coenzyme in solution. Due to interfering bands arising from the protein, only the band assigned to the symmetric stretching of the dianionic phosphate is observed in holoenzyme solutions. The integrated intensity of this band does not change with pH in the range 5.3–8.6, while for free pyridoxal phosphate, the integrated intensity of the same band changes with pH according to the pK value expected for the 5′-phosphate group in solution. Moreover, the value of the integrated intensity for the bound cofactor is close to the value given by free cofactor at pH 8–9. These results suggest that the 5′-phosphate of the bound cofactor remains mostly dianionic throughout the investigated pH range and disfavor other interpretations in terms of ionization of the phosphate group on the basis of the nuclear magnetic resonance ^{31}P chemical shift–pH titration curve of holoenzyme [Schnackerz, K. D. (1984) in *Chemical and Biological Aspects of Vitamin B_6 Catalysis* (Evangelopoulos, E. A., Ed.) Part A, pp 195–208, Alan R. Liss, New York]. Instead, it seems likely that the ^{31}P chemical shift may sense the ionization of the pyridoxal phosphate–Lys-258 Schiff's base, as proposed for the mitochondrial isozyme [Mattingly, M. E., Mattingly, J. R., & Martinez-Carrion, M. (1982) *J. Biol. Chem. 257*, 8872].

A large variety of enzymes contain a bound phosphoryl group, usually attached to a serine or histidine residue, or as part of substrates or cofactors. A knowledge of the ionization state of the bound phosphoryl group is essential to understand the possible roles played by this group in catalytic events or in protein structure. In a given phosphoryl-containing enzyme, it is desirable to know if the phosphoryl group is dianionic or monoanionic, if the ionization state is affected in the steps of the catalytic process, or by the presence of ligands, substrates, and pH, and, in this last case, if its pK is perturbed by the protein environment.

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Many ^{31}P NMR studies have been addressed to understand the roles of phosphoryl groups in phosphoryl-containing enzymes [for a review see Rao (1984) and Vogel (1984)]. Although valuable information has been derived from those studies, it is apparent that, in many cases, the ionization state of the phosphoryl group may not be obvious on the basis of ^{31}P chemical shift data.

The above-mentioned ^{31}P chemical shifts for protein-bound phosphoryl groups have often differed from those obtained for model compounds in solution. For instance, the phosphorus of the phosphoserine residue of alkaline phosphatase resonates at 8.4 ppm (Chlebowski et al., 1976), while the resonance of the phosphoaspartate intermediate in detergent-solubilized ATPase has been reported to appear at −17 ppm (Fossel et al., 1981). On the other hand, values for small molecular weight monoanionic and dianionic phosphate monoesters in solution are about 0 and 4 ppm (Gorenstein, 1975).

The observation of the ^{31}P chemical shift–pH-titration curve does not necessarily imply protonation of the phosphoryl moiety, as the phosphoryl group could be sensing the protonation of a nearby residue in the protein.

Examples of this kind of behavior can be found in cases such as the ^{31}P chemical shift titration for the cytidine 3′-monophosphate–RNase complex which can only be accounted for by assuming two ionization processes with $pK_1 = 4.7$ and $pK_2 = 6.7$ (Gorenstein et al., 1976). The pK_1 has been associated with the ionization of the phosphate group and pK_2 to the ionization of a protonated histidine residue that is hydrogen bonded to the phosphate. Another example is provided by serine proteases. After inactivation by modification of the active site serine residue with diisopropyl fluorophosphate (DIFP), ^{31}P chemical shift titrations are observed in spite of the fact that the DIP linkage cannot undergo ionization (Porubcan et al., 1979). In these cases, it was proposed that the chemical shift was reflecting the active site histidine titration, given that the apparent pKs were identical with those obtained for the histidine residue by ^1H NMR.

Monoanionic and dianionic phosphate give rise to strong bands in the infrared spectrum. Therefore, it appears that, in principle, infrared spectroscopy could be a valuable technique to determine the ionization state of protein-bound phosphate groups, and in fact, Matthies (1977) has used infrared spectroscopy to study the interaction of cytidine 2′-phosphate and cytidine 5′-phosphate with ribonuclease A.

Aspartate aminotransferase is a vitamin B_6 dependent enzyme composed by two identical subunits and containing 1 mol of pyridoxal phosphate/mol of subunit. Aspartate aminotransferase exists as two isozymes localized in the cytosol and mitochondria. They show about 50% sequence homology and a very similar folding pattern according to X-ray crystallographic data (Arnone et al., 1982). The cytosolic isozyme was one of the first proteins to be studied by ^{31}P NMR (Martinez-Carrion, 1975). The chemical shift of the bound pyridoxal phosphate was found to change only slightly with pH, its value being similar to the one for the phosphate of the free cofactor in solution when in the dianionic state. On the other hand, in the mitochondrial isozyme (Mattingly et al., 1982), the chemical shift is more sensitive to pH changes and the presence of several ligands, although the size of the chemical shift changes (about 1 ppm) is still smaller than that for free cofactor (about 3.5 ppm). The interpretation of these small chemical shift changes is unclear. We have postulated a likely retention of the dianionic phosphate (Mattingly et al., 1982; Martinez-Carrion et al., 1984; Martinez-Carrion, 1986), while recently, an interpretation in terms of protonation of the phosphate group has been advanced (Schnackerz, 1984). In this report we use Fourier transform infrared spectroscopy to independently assess the degree of ionization of the phosphate ester in cytosolic aspartate aminotransferase.

Experimental Procedures

The α-subform of cytosolic aspartate aminotransferase was obtained from pig hearts as previously described (Martinez-Carrion et al., 1965). Activity was assayed spectrophotometrically by using published procedures (Slebe & Martinez-Carrion, 1978). Apoenzyme was prepared according to Jenkins (Jenkins & D'Ari, 1966a). Apoenzyme obtained by using this method contains inorganic phosphate bound in the active site (Iriarte et al., 1985); phosphate was displaced by arsenate upon three dialysis against a hundredfold volume of 50 mM sodium arsenate, pH 6.8, followed by dialysis against 50 mM KCl to remove the nonbound arsenate (Mattingly et al., 1982). Protein concentrations were calculated from the absorbance at 280 nm by using a molar absorptivity of 140000 M^{-1} cm^{-1} for a dimer of M_r 94000 (Martinez-Carrion et al., 1967), or (in solutions of concentration about 250 mg/ml) as concentration of pyridoxal phosphate by using a molar absorptivity of 6600 M^{-1} cm^{-1} at 388 nm, in 0.1 M NaOH (Peterson & Sober, 1954).

Protein films were prepared by drying, on a calcium fluoride window, 25 μL of a protein solution. The process was carried out in a container with high humidity maintained by a saturated solution of potassium nitrate. The starting solutions for film preparation were about 100 mg/mL in protein and 50 mM KCl, and pHs ranging from 5.8 to 8.9 were obtained by titrating an initial solution at pH 5.8 with 100 mM sodium arsenate, pH 12. Films were pressed against another calcium fluoride window, and the sandwich was mounted in the infrared cell. After the infrared spectrum was recorded, the visible spectrum was taken without dismounting the infrared cell. Aspartate aminotransferase in solution presents a pH dependent visible spectrum (maximum at 430 nm at acid pH and 360 nm at basic pH) which has been attributed to the protonation of the pyridoxal phosphate–Lys-258 Schiff base; in fact, from the absorbance/titration curve, a pK of 6.3 can be calculated for the ionization of that group in the presence of chloride ions (Jenkins & D'Ari, 1966b). Once corrected for absorption due to calcium fluoride windows, visible spectra of films were practically identical with those obtained in solution. An operational value of pH was, therefore, assigned to films by comparison of the relation absorbance at 430 nm/absorbance at 360 nm with the values of this ratio in solution. After infrared and visible spectra were taken, holoenzyme films were dissolved in 100 mM phosphate buffer, pH 7.5, and apoenzyme films in 50 mM Hepes buffer, pH 8.0. No cloudiness was observed, and the specific activity (activity after addition of coenzyme for the apoenzyme) was found to be higher than 85% of the initial specific activity in all cases and almost identical in most of the holoenzyme samples.

Protein solutions used to obtain infrared spectra were about 250 mg/mL protein and 50 mM KCl. pH was adjusted as previously indicated. Calcium fluoride windows and 50-μm spacers were employed in all cases. UV–vis spectra of protein solutions were obtained as previously indicated for protein films.

Infrared spectra were obtained at room temperature in a Sirius 100 (Mattson Instruments) Fourier transform infrared spectrometer at 2-cm^{-1} resolution. A total of 2500 interferograms was added and Fourier transformed to obtain spectra of films. A total of 5000 interferograms was added to obtain spectra in solution and 500 for spectra of pyridoxal phosphate,

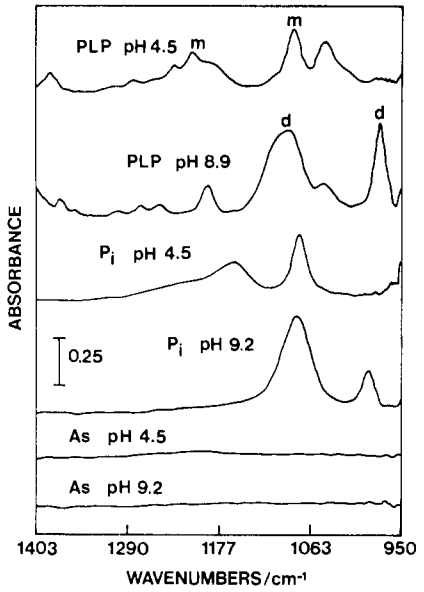

FIGURE 1: (Top to bottom) Infrared spectra of pyridoxal phosphate (PLP), inorganic phosphate (P_i), and arsenate (As) in aqueous solution at the indicated pHs. Concentration is, in all cases, 100 mM. Absorption due to water and calcium fluoride windows has been subtracted.

inorganic phosphate, and arsenate in solution. Absorbance due to calcium fluoride windows and water was subtracted. The criteria for water subtraction was to obtain clearly defined base lines, or at least direct discreet minima, before and after the band due to the symmetric stretching of the dianionic phosphate (see Results). No smoothing was applied to spectra of enzyme, pyridoxal phosphate, inorganic phosphate, or arsenate; however, previous to the subtraction process, the spectra of water and calcium fluoride windows were smoothed by using a Savitsky–Golay algorithm with 25 points.

Integrated intensities of the band at 975–977 cm^{-1} were calculated after tracing linear base lines connecting the minima at both sides of the band or tangent to both the pre- and postband base lines in those cases where these were clearly observed. As films may differ in thickness, the integrated intensity of the band in film spectra has been referred to the absorbance of the band at 2962 cm^{-1} after tracing a base line as shown in Figure 1. Water subtraction prior to this base-line tracing leads to practically the same results.

Band positions were calculated by using the program provided by Mattson Instruments which implies polynomial fitting to the three highmost points. They were found to be reproducible, at least, to ±0.4 cm^{-1}. Values given in Table I have been rounded to the nearest wavenumber.

Results

Infrared Spectra of Pyridoxal Phosphate, Inorganic Phosphate, and Arsenate in Solution. Bands due to dianionic and monoanionic 5′-phosphate of pyridoxal phosphate are clearly observed in aqueous solution after subtraction of the absorbance due to water and calcium fluoride windows (Figure 1). The assignation of the bands is done on the basis of their wavenumbers (see Table I) and of their pH titration behavior. The intensity of the bands assigned to the phosphate ester in Figure 1 changes with pH according to the pK value expected for a phosphate monoester such as the 5′-phosphate group (results not shown).

Inorganic phosphate (Figure 1) shows bands in the same region. Apoenzyme prepared by using regular methods contains inorganic phosphate bound in the active site (Iriarte et al., 1985). However, since arsenate can be substituted for

Table I: Wavenumbers of Bands Arising from Monoanionic and Dianionic Phosphate Monoesters

	characteristic positions[a] (cm^{-1})	free cofactor in solution[b] (cm^{-1})	bound cofactor[b] (cm^{-1})
dianionic degenerate stretching	1100	1090	
dianionic symmetric stretching	980	976	975 (pH 5.3), 977 (pH 8.6)
monoanionic symmetric stretching	1080	1083	
monoanionic antisymmetric stretching	1230	~1230[c]	

[a] Shimanouchi et al. (1964). [b] This work. [c] The presence of weak, but narrow, bands in this region precludes the precise calculation of the position of the band.

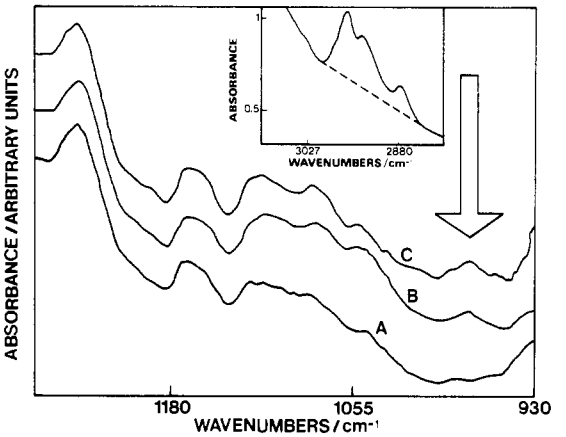

FIGURE 2: Infrared spectra of (A) film of arsenate apoenzyme (pH of the starting solution 6.0) and (B) film of holoenzyme. Operational pH 5.7. (C) Holoenzyme in solution, 5.4 mM in pyridoxal phosphate, pH 5.8. Absorbance is given in arbitrary units. Spectra have been drawn so that the bands in the 1200–1000-cm^{-1} region show the same apparent intensity, approximately. (Inset) Spectra of film A in the C–H stretching region showing the base line used to calculate the intensity of the band at 2962 cm^{-1} at its maximum.

phosphate upon dialysis against arsenate solution (see Experimental Procedures) and arsenate shows only a very weak absorption in the phosphate region (Figure 1), arsenate apoenzyme was used throughout this study.

Infrared Spectra of Holo- and Apoaspartate Aminotransferase. Infrared spectra of holoaspartate aminotransferase films show relatively strong absorption in the 1250–1000-cm^{-1} region (Figure 2). This absorption also appears in spectra of arsenate apoenzyme (Figure 2) and is, therefore, to be mainly attributed to the protein, rather than to the cofactor. In addition, difference spectra (spectrum of holoenzyme minus spectrum of apoenzyme) in this region were difficult to interpret due to the fact that no clear base lines could be obtained. Below 1000 cm^{-1} holoenzyme gives rise to a band at 975–977 cm^{-1} which is not present in the spectra of arsenate apoenzyme. Since monoanionic phosphate does not absorb below 1000 cm^{-1} (Table I and Figure 1), this band should correspond to the symmetric stretching of the dianionic phosphate of the cofactor.

The band at 975–977 cm^{-1} cannot usually be detected with samples of holoenzyme solutions at 100 mg/mL, probably due to an unfavorable signal to noise ratio below 1000 cm^{-1}. However, it is clearly observed with samples of 250 mg/mL (Figure 2).

Spectra of both holoenzyme and arsenate apoenzyme do not

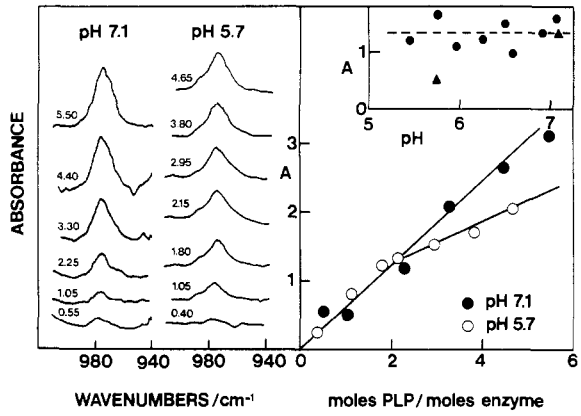

FIGURE 3: (Left) Titration of arsenate apoenzyme with pyridoxal phosphate. Operational pHs were calculated on the basis of the visible absorption spectra of films of apoenzyme undersaturated with pyridoxal phosphate. The pH values of the starting solutions were 8.7 and 6.1 for films with operational pHs 7.1 and 5.7, respectively. Spectra shown are in the difference spectra of apoenzyme plus pyridoxal phosphate minus spectrum of apoenzyme; the subtractions were carried out on the basis of the intensity of the band at 2962 cm^{-1}. Absorbance is given in arbitrary units, although the values have been referred to the intensity of the band at 2962 cm^{-1}. Numbers alongside the curves are moles of pyridoxal phosphate per mole of enzyme. (Right) Plot of integrated intensity of the band arising from the symmetric stretching of the dinanionic phosphate vs. moles of pyridoxal phosphate per mole of enzyme ratio. (Inset) Effect of pH on the integrated intensity of the band assigned to dianionic phosphate. (Circles) Bound cofactor. (Triangles) Free cofactor (values calculated from the postsaturation slopes of the plot in the right). *A* stands for the integrated intensity (in cm^{-1}) divided by the intensity of the band at 2962 cm^{-1}. PLP, pyridoxal phosphate.

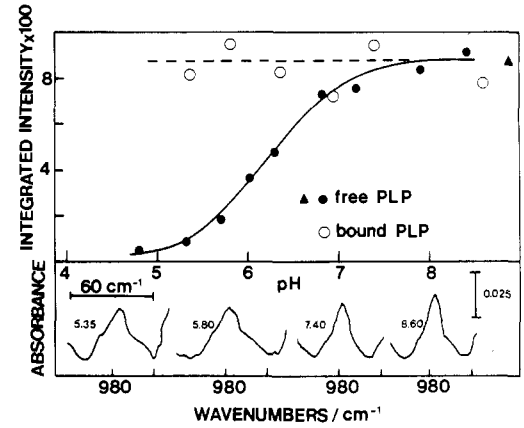

FIGURE 4: (Top) Effect of pH on the integrated intensity (in cm^{-1}) of the band due to dianionic phosphate. Values have been referred to 1 mM solution and 50-μm path length. Actual concentrations were 40 (●) and 100 mM (▲) for free cofactor and 5.4 mM in pyridoxal phosphate for holoenzyme solutions. The values of integrated intensities have been corrected for the contribution of the small bands seen in spectra of arsenate apoenzyme. (Bottom) Effect of pH on the band due to symmetric stretching of dianionic phosphate in spectra of holoenzyme in solution (5.4 mM in pyridoxal phosphate). PLP, pyridoxal phosphate.

essentially change with pH (a small change in the position of the band attributed to the symmetric stretching of the dianionic phosphate will be described). It is of interest that the band at 975–977 cm^{-1} is observed even at pH 5.3 at an intensity identified with that of pH 8.5.

Titration of Arsenate Apoenzyme with Pyridoxal Phosphate in Films. Films of arsenate apoenzyme plus pyridoxal phosphate at several enzyme/pyridoxal phosphate molar ratios were prepared and their infrared spectra recorded. The results are shown in Figure 3.

At an operational pH of 7.1, a steady increase in the integrated intensity of the band at 975–977 cm^{-1} is observed, while at pH 5.7 a break at the stoichiometric ratio of 2 mol of pyridoxal phosphate/mol of enzyme is noticed. In addition, the integrated intensity does not seem to change with pH (Figure 3), within experimental error.

These results are consistent with the assignment of the band at 975–977 cm^{-1} and can be explained by assuming that the 5′-phosphate binds mainly as dianionic phosphate, while the ionization state of the same group in the free cofactor in films is pH dependent, as it is in solution.

pH Titration of Holoenzyme in Solution. Infrared spectra of holoaspartate aminotransferase in the region 1250–950 cm^{-1} show little change with pH in the range 5.3–8.6. Throughout that pH range the band assigned to the symmetric stretching of dianionic phosphate is present (Figure 4). The integrated intensity of that band remains constant with pH within experimental error, its value being close to the one expected for 100% dianionic phosphate (Figure 4). Some change is, however, observed in the shape and position, the band becoming somewhat narrower (Figure 4) and displaced to higher wavenumbers as the pH is increased (Table I). It should be noted that the p*K* of the pyridoxal phosphate–Lys-258 Schiff's base (calculated from the absorbance at 430 vs. pH titration curve) is not significantly altered at the concentrations used (about 250 mg/mL) from its value in diluted enzyme solutions (6.3) (results not shown).

Discussion

Cytosolic aspartate aminotransferase is a dimer containing one pyridoxal phosphate per subunit, attached by a convalent linkage (Schiff base) to Lys-258. Crystallographic studies have shown the coenzyme phosphate group interacting with Arg-266, Ser-255, Ser-257, Thr-109, and Tyr-70 from the other subunit, as well as with the helix dipole of the 108–123 helix (Arnone et al., 1982). ^{31}P NMR studies (Martinez-Carrion, 1975) showed that the chemical shift of the cofactor phosphate was only slightly affected by pH or by the presence of several ligands, the value being close to the chemical shift observed for the dianionic 5′-phosphate of the free cofactor in solution. Recently, these minor changes with pH observed in the chemical shift have been redetermined with greater precision by using more modern NMR instrumentation and interpreted as being due to ionization of the phosphate group (Schnackerz, 1984). A p*K* of 6.2 was calculated for that ionization, although the size of the change (0.5 ppm) is small when compared with the one observed for free coenzyme in solution (3.5 ppm). However, as stated in the introduction, in many cases, ^{31}P chemical shift titrations may be induced by nearby groups in the enzyme. This FTIR study was addressed to determine whether there is an actual ionization of the phosphate group or the chemical shift titration curve is originated by some other group(s) in the enzyme. Initial experiments were carried out with protein films in order to avoid interference due to the absorption of water. Yet, these results were later confirmed in concentrated protein solutions.

The phosphoryl moiety of phosphate monoesters gives rise to four bands in the infrared spectrum, two for the monoanionic ionization state and two for the dianionic state (Table I). Due to the presence of protein bands in the investigated spectral region, we have been able to observe only one of those bands, the one arising from the symmetric stretching of the dianionic phosphate.

Given that no bands due to monoanionic phosphate can be observed in holoenzyme spectra, estimates of the amounts of dianionic and monoanionic phosphate at several pHs must rely on the comparison of the intensity of the observed band with the intensity of the same band in the free cofactor in solution.

Due to several possible sources of error, these calculations should be regarded as "semiquantitative". The use of a linear base line could lead to underestimation of the integrated intensities, while the presence of small bands not due to phosphate (perhaps the shoulder seen in some of the spectra in Figure 4) would produce overestimation. In addition, interactions with groups within the protein might affect the intensity of the band. Yet, in spite of that, the following results disfavor an ionization of the phosphate group in coenzyme-bound cytosolic aspartate aminotransferase:

(A) The infrared band assigned to dianionic phosphate is present in spectra of holoenzyme at pH 5.3, that is, one whole pH unit below the apparent pK calculated from the ^{31}P chemical shift titration curve for bound coenzyme.

(B) In titration experiments of apoenzyme with added free pyridoxal phosphate in films, the presaturation slopes, in plots of intensity of the band due to dianionic phosphate vs. the moles of cofactor per mol of enzyme ration (Figure 3), are practically identical at pH 5.7 and 7.1. The postsaturation slopes, however, reflect the ionization of the free cofactor, being identical with the presaturation slope at pH 7.1 and smaller at pH 5.7.

(C) The integrated intensity of the band due to the symmetric stretching of the dianionic phosphate in solutions of holoenzyme does not change with pH, within experimental error, and its value is close to the intensity of the same band for the free cofactor at pH 8–9. By contrast, the intensity of the band for the free cofactor in solution changes with pH according to the known pK of the 5′-phosphate group in solution (Figure 4).

Although we cannot rule out the presence of small amounts of monoanionic phosphate (a proton might be shared between the phosphate group and a protein group, residing most of the time on the protein group), the results presented here strongly suggest that the phosphate group of the bound cofactor remains mostly dianionic throughout the pH range 5.3–8.6. Given these conditions, it appears plausible that the observed small changes in chemical shift with pH could be originated by the ionization of other group(s) in the protein. The Schiff-base linkage formed between the carbonyl group of the cofactor and Lys-258 undergoes protonation with a pK identical (in the presence of chloride ions) with the apparent pK calculated from the ^{31}P chemical shift titration curve. It seems likely that this is the pK being reflected by the phosphate group ^{31}P NMR.

Unfortunately, no general theory for the ^{31}P chemical shifts is available, and their interpretation remains largely empirical. Although several factors may affect ^{31}P chemical shifts, the effect of the O–P–O bond angle has been experimentally demonstrated (Gorenstein, 1975). It does not seem likely that the O–P–O bond angle senses the electrostatic field created by the protonated Schiff's base, although we cannot rule out the possibility that the effect is transmitted through the rearrangement of charged residues present in the active site. We favor, however, a mechanism in which the O–P–O bond angle is perturbed as a consequence of the coenzyme ring reorientation caused by the protonation of the Schiff's base, as we have already proposed for the mitochondrial isozyme (Mattingly et al., 1982; Martinez-Carrion et al., 1984; Martinez-Carrion, 1986). Large changes in coenzyme ring position have been observed in crystallographic studies of the enzyme in the presence of substrates or inhibitors (Arnone et al., 1982). Although smaller changes associated with protonation of such Schiff's base may not be detected at the present degree of resolution of the crystallographic maps, they are supported by polarized light spectrophotometric studies in crystals (Markarov et al., 1980, 1981). Such rotation(s) of the pyridine could distort the O–P–O angle enough to be sensed by the ^{31}P chemical shift. Alternatively the electrostatic field rearrangement caused by Schiff's base protonation–deprotonation may cause an alteration in the phosphate binding pocket net charge, which could also be detected by NMR chemical shift changes.

In conclusion, we present evidence supporting that, in the active site of cytosolic aspartate aminotransferase, the 5′-phosphate group of the cofactor remains dianionic throughout the pH range 5.3–8.6. We, also, show that Fourier transform infrared spectroscopy can be a valuable technique to determine the ionization state of phosphoryl moieties in phosphoproteins.

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Registry No. PLP, 54-47-7; aspartate aminotransferase, 9000-97-9.

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A Probe of the Active Site Acidity of Carboxypeptidase A

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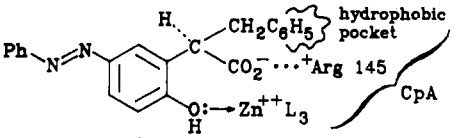
ABSTRACT: The substrate analogue 2-(1-carboxy-2-phenylethyl)-4-phenylazophenol is a potent competitive inhibitor of carboxypeptidase A. Upon ligation to the active site, the azophenol moiety undergoes a shift of pK_a from a value of 8.76 to a value of 4.9; this provides an index of the Lewis acidity of the active site zinc ion. Examination of the pH dependence of K_i for the inhibitor shows maximum effectiveness in neutral solution (limiting $K_i = 7.6 \times 10^{-7}$ M), with an increase in K_i in acid (pK_1 = 6.16) and in alkaline solution (pK_2 = 9.71, pK_3 = 8.76). It is concluded that a proton-accepting enzymic functional group with the lower pK_a (6.2) controls inhibitor binding, that ionization of this group is also manifested in the hydrolysis of peptide substrates (k_{cat}/K_m), and that the identity of this group is the water molecule that binds to the active site metal ion in the uncomplexed enzyme ($H_2OZn^{2+}L_3$). Reverse protonation state inhibition is demonstrated, and conventional concepts regarding the mechanism of peptide hydrolysis by the enzyme are brought into question.

Although details of the mechanism of peptide hydrolysis by the prototypical metalloenzyme carboxypeptidase A are controversial, it is widely accepted that the active site zinc ion functions by coordination to the oxygen atom of the substrate scissile carboxamide, thereby serving to render the peptide linkage more susceptible to nucleophilic addition. This interpretation is indicated strongly by crystallographic evidence for enzyme–pseudosubstrate complexes (Quiocho & Lipscomb, 1971) and by the inactivity of the Co^{3+}- (ligand-exchange inert) substituted enzyme in comparison with other metal ion modified derivatives of carboxypeptidase A (Van Wart & Vallee, 1978). Recently, a synergism test has been applied, in which the pattern of reactivity observed for normal and for thiocarboxamide substrates, acted upon by native and Cd^{2+}-substituted enzymes, substantiated a productive interaction between active site metal ion and substrate carbonyl group (Mock et al., 1981). Upon an assumption that the carboxamide cleavage mechanism for carboxypeptidase A does involve a Lewis acid role for the active site metal ion, it is reasonable next to inquire as to whether a *quantitative assessment* may be obtained for the magnitude of this interaction; i.e., how acidic is the active site zinc ion? This article provides an answer to that question by direct measurement with a suitably designed probe. As a bonus, additional evidence is provided that suggests a reverse protonation mechanism for activation of peptides by this enzyme.

The idea underlying the research to be described was construction of an active site directed agent (inhibitor) for carboxypeptidase A, which would serve as an *indicator* for the electron deficiency of the metal ion known to be present. L-Benzylsuccinic acid, $C_6H_5CH_2CH(CO_2H)CH_2CO_2H$, has been shown to be an avidly bound inhibitor for this enzyme, due to its successful mimicry of the specificity factors associated with good substrates for carboxypeptidase A (Byers & Wolfenden, 1973). Consequently, an analogue, 2-(1-carboxy-2-phenylethyl)-4-phenylazophenol, was synthesized and resolved to provide the appropriate L enantiomer (**1**).



1

When fitted as a surrogate substrate as depicted, **1** presents a *phenolic hydroxyl* as a potential ligand to the active site metal ion. This oxygen atom corresponds spatially to the carboxamide oxygen of an *N*-acylphenylalanine substrate.

As we subsequently describe, the special virtues of this molecule are that the correct (L) enantiomer of **1** is indeed a good competitive inhibitor and the azophenol visible absorbance undergoes a characteristic perturbation upon interaction with the metal ion. The relevant evidence to be developed concerns the pH dependence of the electronic spectrum of a complex between **1** and carboxypeptidase A, as well as a variation with pH in the value of the kinetically determined inhibition constant K_i for **1**.

Materials and Methods

Carboxypeptidase A (CpA),[1] was supplied by Sigma Chemical Co. (no. C 0386). The Allan form was chosen for its reported greater solubility. It was recrystallized by dialysis according to established procedures before use (Mock & Chen, 1980). Buffers employed in this work for kinetic analysis were (0.05 M each) as follows: Ammediol, pH 8.75–10; Tris, pH 7.25–8.75; Mes, pH 5.3–7.25; 2,6-pyridinedimethanol, pH

[1] Abbreviations: CpA, bovine pancreatic carboxypeptidase A; FAPP, *N*-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine; Ammediol, 2-amino-2-methyl-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide.

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