Eds.) pp 719-722, Walter de Gruyter, Berlin.
Mathews, R., Charlton, S., Sands, R. H., & Palmer, G. (1974) *J. Biol. Chem. 249*, 4326-4328.
Messing, J., & Vieira, J. (1982) *Gene 19*, 269-276.
Morningstar, J. E., Johnson, M. K., Cecchini, G., Ackrell, B. A. C., & Kearney, E. B. (1985) *J. Biol. Chem. 260*, 13631-13638.
Mukund, S., & Adams, M. W. W. (1990) *J. Biol. Chem. 265*, 11508-11516.
Ohnishi, T. (1987) *Curr. Top. Bioenerg. 15*, 37-65.
Ohnishi, T., Lim, J., Winter, D. B., & King, T. E. (1976) *J. Biol. Chem. 251*, 2105-2109.
Phillips, M. K., Hederstedt, L., Hasnain, S., Rutberg, L., & Guest, J. R. (1987) *J. Bacteriol. 169*, 864-873.
Prince, R. C., & Adams, M. W. W. (1987) *J. Biol. Chem. 262*, 5125-5128.
Rothery, R. A., & Weiner, J. H. (1991) *Biochemistry 30*, 8296-8305.
Rouault, T. C., Stout, C. D., Kaptain, S., Harford, J. B., & Klausner, R. D. (1991) *Cell 64*, 881-883.
Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., & Hartmann, U. (1972) *Eur. J. Biochem. 26*, 267-278.
Salerno, J. C., & Yan, X. (1987) in *Cytochrome Systems: Molecular Biology and Bioenergetics* (Papa, S., Chance, B., & Ernster, L., Eds.) pp 467-471, Plenum Press, New York.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Sato, S., Nakazawa, K., Hon-nami, K., & Oshima, T. (1981) *Biochim. Biophys. Acta 668*, 277-289.
Schröder, I., Gunsalus, R. P., Ackrell, B. A. C., Cochran, B., & Cecchini, G. (1991) *J. Biol. Chem. 266*, 13572-13579.
Simpkin, D., & Ingledew, W. J. (1980) *Biochem. Soc. Trans. 13*, 602-607.
Weiner, J. H., & Dickie, P. (1979) *J. Biol. Chem. 154*, 8590-8593.
Werth, M. T., Cecchini, G., Manodori, A. M., Ackrell, B. A. C., Schröder, I., Gunsalus, R. P., & Johnson, M. K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8965-8969.
Yao, Y., Wakabayashi, S., Matsubara, H., Yu, L., & Yu, C.-A. (1986) in *Iron-Sulfur Protein Research* (Matsubara, H., Katsube, Y., & Wada, K., Eds.) pp 240-244, Japan Science Society Press, Tokyo.

Inorganic Phosphate Binding and Electrostatic Effects in the Active Center of Aspartate Aminotransferase Apoenzyme†

Jose H. Martinez-Liarte,‡ Ana Iriarte, and Marino Martinez-Carrion*
School of Basic Life Sciences, University of Missouri—Kansas City, BSB 109, Kansas City, Missouri 64110-2499
Received September 18, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: The ionization state of the phosphate group bound at the aspartate aminotransferase apoenzyme's active site has been investigated utilizing Fourier-transform infrared spectroscopy following the band corresponding to the symmetric stretching of the dianionic phosphate. Unlike free phosphate, when inorganic phosphate is bound at the enzyme's active site, the integrated intensity value of the dianionic band does not change with pH within the studied range, and this value is similar to that for free dianionic phosphate at pH 8.3. From these results, we propose a dianionic state for the phosphate ion bound to cytosolic aspartate aminotransferase throughout the pH range of 5.7-8.3. The presence of other anions such as acetate and chloride or the substrate aspartate and its analogues produces a pH-dependent phosphate removal from the active site which is favored at low pH values. Elimination of the charged primary amine at the active-site Lys-258, through formation of a Schiff base with pyridoxal or chemical modification by carbamylation, also produces a pH-independent phosphate release. These results are interpreted as Lys-258 together with the active-site α-helix and other residues may be involved in stabilizing phosphate as a dianion in the apoenzyme phosphate pocket which anchors the phosphate ester of pyridoxal phosphate in the holoenzyme. It is proposed that the dianionic phosphate contributes to the apoenzyme's thermal stability through formation of strong hydrogen bond and salt bridges with the amino acid residues forming the phosphate binding pocket with assistance of Lys-258, and other active-site cationic components.

Aspartate aminotransferase (EC 2.6.1.1) is a dimeric enzyme composed of identical subunits with each active site containing pyridoxal 5′-phosphate (PLP) linked via an internal Schiff base to a lysine residue. This enzyme exists as two isozymes with different locations in the cell: mitochondria and cytosol. The apoenzyme forms of these isozymes are also known to bind ions at the active site where they both act as competitive inhibitors (Jenkins & D'Ari, 1966b; Martinez-Carrion et al., 1973; Cheng & Martinez-Carrion, 1972; Martinez-Carrion, 1985) and prevent binding of the coenzyme pyridoxal or pyridoxamine phosphate. In addition, phosphate ions induce both stabilization of this enzyme toward thermal denaturation (Iriarte et al., 1985) and resistance to proteolytic attack (Iriarte et al., 1984). Yet, little is known regarding the forces involved in the binding of ions including phosphate.

Fourier-transform infrared (FTIR) spectroscopy has been recently used by us to study the ionization state of the phosphate group in PLP in both cytosolic and mitochondrial aspartate aminotransferases (Sanchez-Ruiz & Martinez-Carrion,

† This work was supported by Grants GM-38341 and HL-38412 from the National Institutes of Health.
‡ Present address: Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Murcia, 30100 Murcia, Spain.

0006-2960/92/0431-2712$03.00/0

1986, 1990). In both cases, the 5′-phosphate group of bound cofactor remains mostly dianionic throughout the pH range of 5.3–8.6. Also, it has been shown that the FTIR technique can be useful in the study of phosphate-containing proteins such as those having phosphoserine residues (Sanchez-Ruiz & Martinez-Carrion, 1988).

The behavior of phosphoryl groups in the holoenzyme forms of aspartate aminotransferases has also been studied using ^{31}P NMR (Martinez-Carrion, 1975; Mattingly et al., 1982; Schnackerz, 1984; Schnackerz et al., 1989). Yet, the information provided could not be easily interpreted considering that the protein environment can modify the observed ^{31}P chemical shift in several ways (Gorenstein et al., 1976; Porubcan et al., 1979; Mattingly et al., 1982) and that changes in chemical shift can reflect a distortion of the tetrahedral symmetry of the phosphate group (Gorenstein, 1975). Hence, for phosphoryl compounds, FTIR spectroscopy was proposed, and utilized, as a method complementary to ^{31}P NMR.

Aspartate aminotransferase apoenzyme can be converted to the holoenzyme form by addition of PLP and PLP analogues (Braunstein, 1973; Gianfreda et al., 1974; Farach et al., 1983). Furthermore, all studies agree with a need of the phosphate component of the coenzyme for proper cofactor binding (Fonda & Auerbach, 1976; Martinez-Carrion, 1986). Spectroscopic analysis of the ionic state of phosphate bound to the apoenzyme was attempted by direct ^{31}P NMR studies (Mattingly et al., 1982; Iriarte et al., 1985), which produced much information regarding its effect on protein thermodynamic properties and stability, but was inconclusive for the characterization of its states of ionization.

Aspartate aminotransferase as synthesized in the cytoplasm or when studied in vitro exists in a highly ionic environment. Indeed, dicarboxylic acids and other anions, including phosphate, affect the properties of aspartate aminotransferases (Jenkins & D'Ari, 1966; Martinez-Carrion et al., 1971; Cheng & Martinez-Carrion, 1972; Bonsir et al., 1975; Jenkins, 1980; Relimpio et al., 1981; Iriarte et al., 1985). Therefore, the clarification of the consequences of anion binding to this protein is one important topic of investigation related to both the relationship between ions and proteins in solution and the potential involvement of ions present in the cytoplasm in the early stages of protein synthesis and folding prior to binding of coenzyme.

Previous FTIR spectroscopy studies with the aspartate aminotransferase holoenzymes followed the ionization behavior of the bound cofactor by monitoring the band originated by the symmetric stretching mode of the PLP 5′-phosphate ester dianion. Since free inorganic phosphate ions also show a similar band in the infrared region below 1000 cm^{-1} (Chapman & Thirlwell, 1964), in this work we employ FTIR spectroscopy to directly analyze the ionization states of the phosphate ions at the active site of the cytosolic apoenzyme and the possible changes induced by the presence of active-site-specific reagents, including substrates and inhibitory anions.

Experimental Procedures

The α-subform of the cytoplasmic isozyme of aspartate aminotransferase was purified from pig heart as previously described (Martinez-Carrion et al., 1967). Apoenzyme was prepared according to standard procedures (Jenkins & D'Ari, 1966a); apoenzyme obtained in this way has inorganic phosphate bound at the active site (Iriarte et al., 1985). When necessary, this phosphate was displaced by arsenate by dialysis against 50 mM sodium arsenate, pH 6.8, followed by dialysis against 50 mM KCl to remove the nonbound arsenate (Sanchez-Ruiz & Martinez-Carrion, 1986). Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm, using a molar absorptivity of 140 000 M^{-1} cm^{-1} (Feliss & Martinez-Carrion, 1970). Enzyme activities were assayed spectrophotometrically using a coupled assay (Martinez-Carrion et al., 1965). Reductive methylation was carried out according to the procedure of Roberts et al. (1988). Carbamylated apoenzyme was prepared as previously reported (Slebe & Martinez-Carrion, 1976).

Infrared spectra were obtained, at room temperature and 4-cm^{-1} resolution, in a Sirius 100 (Mattson Instruments) Fourier-transform infrared spectrometer equipped with a liquid nitrogen cooled mercury–cadmium–telluride detector. Calcium fluoride windows (1-mm thickness) and 50-μm Teflon spacers were employed in all the experiments.

Protein concentrations used were in the range of 140–180 mg/mL in 25 mM Tris–acetate or Tris–cacodylate buffer. pH was adjusted by microliter additions of 0.1 M HCl or 0.1 M NaOH. Infrared spectra of the protein solutions were obtained as already described (Sanchez-Ruiz et al., 1990). A total of 800 interferograms were added and Fourier-transformed to take the spectra. The speed of the interferometer moving mirror was 2.53 cm/s, and data were acquired in both directions (backward and forward) of the mirror. The total scan time was 4 min and 27 s. To prevent problems from the high dynamic range of the interferometer (Griffiths & de Haseth, 1986), a high value of amplifier gain (×4) and an optical filter (provided by Mattson Instruments) that blanked all the radiation above 2000 cm^{-1} were used (Sanchez-Ruiz & Martinez-Carrion, 1988).

Final solvent-corrected spectra were obtained by using either of the previously published methods of Sanchez-Ruiz and Martinez-Carrion (1988) and Sanchez-Ruiz et al. (1991) which gave identical results. Basically, in method 1, an air background spectrum was recorded, and then the interferograms for the sample were accumulated. The absorbance due to the aqueous solvent (25 mM Tris–acetate buffer) was then subtracted, obtaining clear base lines above and below the studied band. In method 2, pure solvent was used to obtain the background spectrum. This produces small water oversubtraction because the concentration of water in the aqueous solvent is higher than in the protein solution. The aqueous solvent spectrum was added to get clear base lines. In both cases, the aqueous solvent spectrum was smoothed by applying a 25-points Savitsky–Golay algorithm (Savitsky & Golay, 1964). No smoothing was used for the apoenzyme spectra. Band positions were calculated by polynomial fitting to the three highest points. They were found to be reproducible to ±0.4 cm^{-1}. Integrated intensities were estimated as previously described (Sanchez-Ruiz & Martinez-Carrion, 1986). The band intensities were normalized for protein concentration as indicated in the legends of the figures. These spectra were reproducible to within 5–10% error.

In the pH titration of apoenzyme in the presence of anions and pyridoxal, the following general procedure was used for the preparation of the samples. Apoenzyme was dialyzed against 25 mM Tris–cacodylate buffer, pH 8.3, and concentrated to about 40 mg/mL, using Centricon 30 microconcentrators (Amicon, Danvers MA). Individual samples for the infrared measurements were prepared using procedures similar to those previously described for determination of the pH and protein concentration of holoenzyme samples (Sanchez-Ruiz et al., 1986). Briefly, 200 μL of the 40 mg/mL solution was incubated for 15 min with 25 mM Tris–cacodylate buffer containing the desired ligand (100 mM) at different pH values followed by further concentration by ultrafiltration

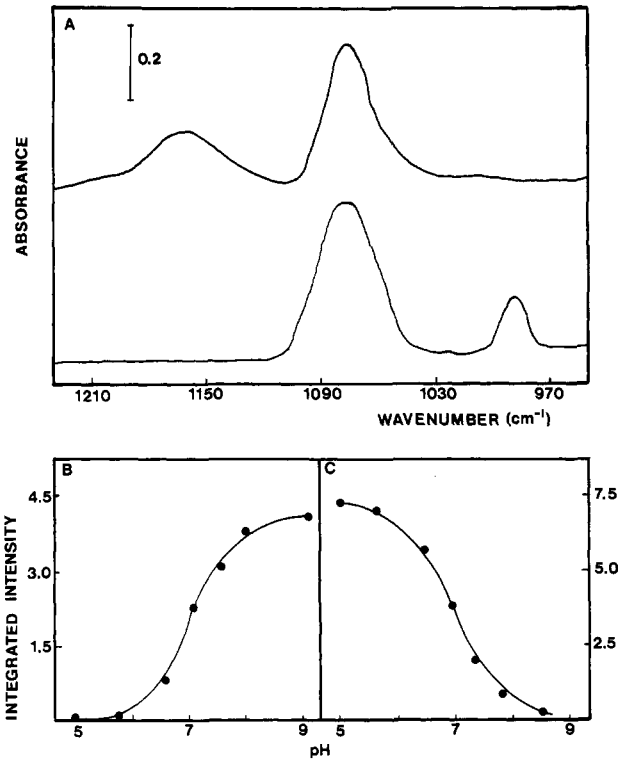

FIGURE 1: (A) Infrared spectra of 100 mM potassium phosphate in aqueous solution at pH 4.7 (top) and 9.3 (bottom). (B) Effect of pH on the integrated intensity (in cm^{-1}) of the band due to the symmetric stretching (991 cm^{-1}) of dianionic phosphate in solution. (C) Effect of pH on the integrated intensity of the band due to the antisymmetric stretching (1160 cm^{-1}) of monoanionic phosphate in solution.

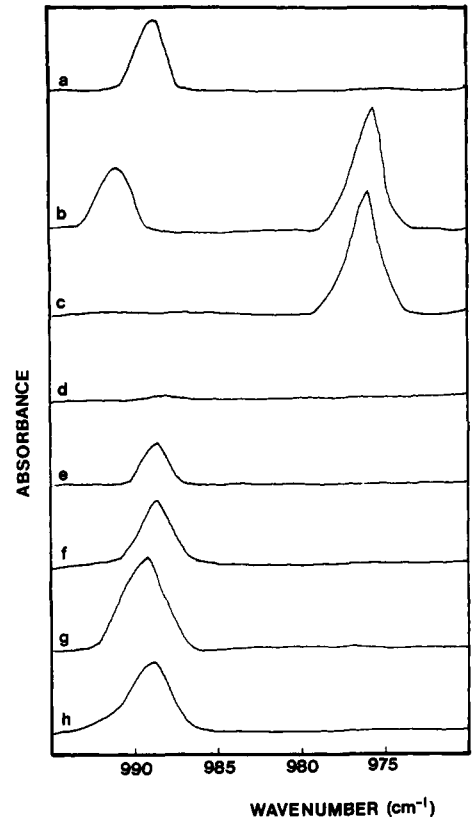

FIGURE 2: Infrared spectra of (a) apoenzyme, (b) apoenzyme after addition of stoichiometric amounts of pyridoxal phosphate, (c) as in (b), after dialysis against 25 mM Tris–acetate buffer pH 7.0, (d) apoenzyme (sample a) after addition of 50 mM sodium arsenate, pH 6.8 (arsenate apoenzyme), (e) arsenate apoenzyme (sample d) after addition of 0.5, (f) 1.0, and (g) 1.5 equiv of phosphate, and (h) as in (g), after dialysis against 25 mM Tris–acetate buffer, pH 7.0.

to 45 μL. The final pH and protein concentration of each sample were determined before and after recording the spectrum to ensure that no change in pH had taken place during data collection. The concentrated samples were analyzed using 50-μm Teflon spaces in all cases. The filtrates from the final concentration steps were used to obtain the solvent background spectra. In this way, the absorbance due to the remaining free phosphate in the sample was substracted, and, assuming equilibration of free phosphate concentrations at both sides of the filtration membrane, the band intensity at 989 cm^{-1} would reflect only the phosphate bound at the active site of the apoenzyme. The apparent p*K* values for these titration curves were calculated by using iterative nonlinear regression methods (Sigma plot 4.1). A single ionizing system gave the best fit to the experimental data.

The reversibility of the pH titration behavior was checked as follows. Forty–five microliters of the sample at pH 5.7 was incubated for 15 min with 2 mL of 25 mM Tris–cacodylate buffer, pH 8.3, containing a given ligand at the same concentration as previously used (100 mM), to minimize possible phosphate rebinding. The final pH for this solution was 8.3. After incubation, the samples were concentrated by ultrafiltration and analyzed as mentioned before. Using this procedure, the increase in the signal at 989 cm^{-1}, if any, when the pH is raised from 5.7 to 8.3 would be mainly due to an effect of pH on the ionization state of phosphate bound at the active site.

Results

Infrared Spectra and pH Titration of Potassium Phosphate. The infrared spectra of the monoanionic and dianionic forms of phosphate in aqueous solution are shown in Figure 1. Phosphate bands corresponding to the stretching mode of vibration appear in the region between 980 and 1200 cm^{-1}, two for the monoanionic ionization state (1077 and 1160 cm^{-1}) and two for the dianionic state (991 and 1079 cm^{-1}). In vibrational spectroscopy such as infrared, the integrated intensity of the bands gives direct information on the distribution of the assigned vibrational modes specified at fixed frequencies. Thus, the pH titration behavior of the band intensities for the dianionic symmetric (991 cm^{-1}) and monoanionic antisymmetric (1160 cm^{-1}) stretching modes shown in Figure 1 unambiguously reflects the amounts of each ionic species at different pH values. For either stretching mode, the p*K* values obtained from these titrations are identical to the well-established p*K* value for the second ionization of phosphate (about 7.0 under the present conditions).

Infrared Spectrum of the Apoenzyme. Assignment of the Phosphate Band. As previously reported, due to the strong absorption of the protein in the region above 1000 cm^{-1} (1000–1250 cm^{-1}) (Sanchez-Ruiz & Martinez-Carrion, 1986), bands in this region cannot be easily used for studying the state of phosphate bound to the apoenzyme. Subtraction of the protein absorption to obtain difference spectra (phosphate–apoenzyme minus arsenate–apoenzyme) gave no clear base lines. On the other hand, using an apoenzyme concentration in the range of 140–180 mg/mL at pH 7.0, a band at 989 cm^{-1} was detected (Figure 2). On the basis of the wavenumber, this band was tentatively assigned to the dianionic symmetric stretching mode of the bound phosphate. Yet, several experiments were carried out in order to ascertain this assignment. These are the following:

(a) PLP binds with higher affinity than phosphate to the active site of the apoenzyme (Martinez-Carrion, 1975).

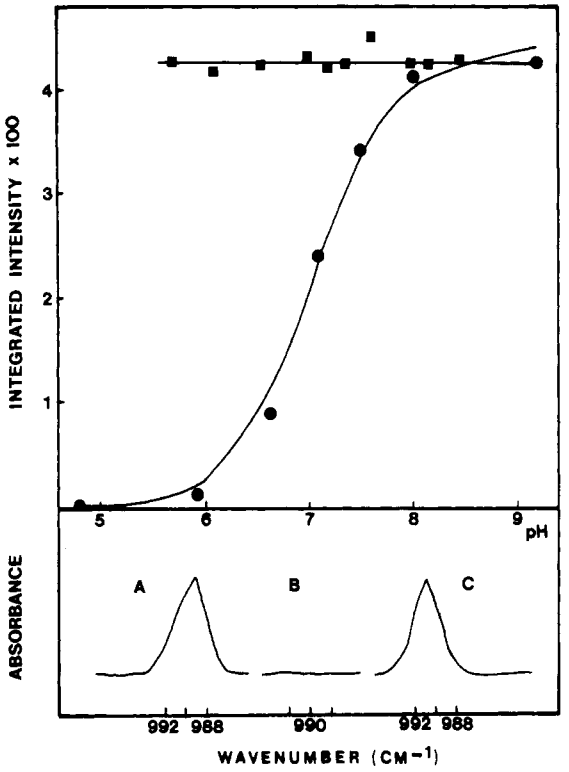

FIGURE 3: (Top) Effect of pH on the integrated intensity of the band due to the symmetric stretching of dianionic phosphate bound to the apoenzyme (■) and free in solution (●). Values refer to 1 mM solution. (Bottom) (A) Band due to the dianionic phosphate bound to apoenzyme at pH 5.7; (B) disappearance of this band by addition of 50 mM sodium arsenate, pH 5.7, to the sample in (A); (C) band due to the dianionic phosphate in solution after the pH of sample B was raised to 8.3.

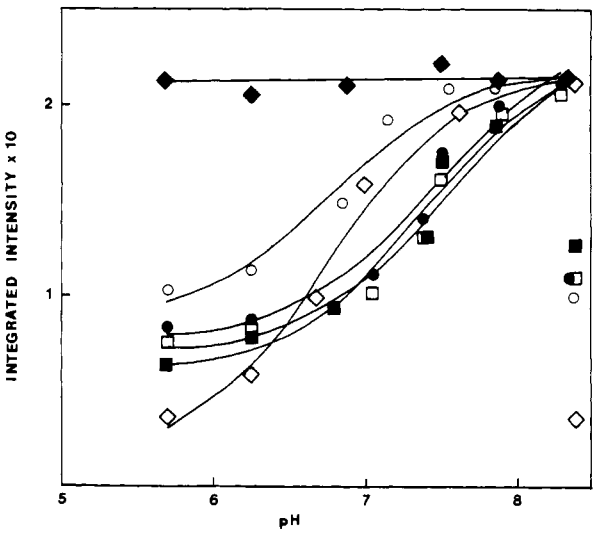

FIGURE 4: Effect of acetate (○), chloride (◇), succinate (●), glutarate (□), aspartate (■), and valine (♦) on the pH dependence of the integrated intensity (in cm^{-1}) of the band due to the apoenzyme-bound dianionic phosphate. Band intensity values are referred to 5 mM enzyme (monomer). The concentration of the anions was 100 mM. The pH titrations were performed starting with the sample at high pH (8.3). Solvent blanks for the infrared measurements contained the same phosphate concentration in solution as the sample (see Experimental Procedures). Values outside the curve were obtained by raising the pH of the respective samples at the end of the titration from pH 5.7 to pH 8.3 in a single step (see Experimental Procedures). The solid lines represent the fitted curves calculated for a single ionization process.

Therefore, addition of PLP to the apoenzyme solution should remove the phosphate from the active site. When PLP is added in stoichiometric amounts, a new band appears at 976 cm^{-1} corresponding to the dianionic symmetric stretching of the bound PLP phosphate group (Sanchez-Ruiz & Martinez-Carrion, 1986). In addition, the 989-cm^{-1} band corresponding to the dianionic symmetric stretching mode for the bound phosphate ion moves to 991 cm^{-1} (free phosphate). Subsequent dialysis against 25 mM Tris–acetate buffer, pH 7.0, eliminates this 991-cm^{-1} band from the infrared spectrum, leaving only the 976-cm^{-1} band (Figure 2) corresponding to bound PLP.

(b) It is well-known (Mattingly et al., 1982; Jansonius et al., 1985) that phosphate at the active site can be displaced by arsenate (see Experimental Procedures) and arsenate apoenzyme does not show absorption in this infrared region (Sanchez-Ruiz & Martinez-Carrion, 1986). After phosphate apoenzyme is transformed into arsenate apoenzyme, followed by dialysis against 25 mM Tris–acetate buffer, pH 7.0, the 989-cm^{-1} band completely disappears (Figure 2c).

(c) Consecutive additions of inorganic phosphate to the arsenate apoenzyme (Figure 2e–g) give rise to a 989-cm^{-1} band, and the increase in the intensity of the band is proportional to the amount of phosphate added. After addition of 1.5 equiv of phosphate, subsequent dialysis produces a decrease in the integrated intensity of the band, yet the size of the remaining band is very similar to the one obtained after the initial addition of 1 equiv of phosphate (Figure 2f,h).

pH Titration of the Apoenzyme. The behavior of inorganic phosphate bound to the apoenzyme was studied throughout the pH range 5.7–8.3. The integrated intensity of the dianionic phosphate infrared band remained almost constant in this pH range, giving values very similar to those obtained for phosphate free in solution at high pH (mostly dianionic) (Figure 3). However, when this same apoenzyme sample at pH 5.7 was incubated with sodium arsenate (50 mM, pH 5.7), the 989-cm^{-1} band completely disappeared. As mentioned before, arsenate ions compete with phosphate for binding to the apoenzyme (Jansonius et al., 1985) and therefore are able to remove phosphate from its binding site. Once free, phosphate would be mostly monoanionic at pH 5.7, showing no absorbance at 989 cm^{-1}. Yet, when the pH is raised again, a new band with an identical intensity value to that obtained prior to the addition of arsenate is obtained. Only the position of the band has changed, having moved slightly to 991 cm^{-1}, the wavenumber corresponding to the position for free dianionic phosphate (Figure 3).

Thus, these results suggest that inorganic phosphate when bound at the apoenzyme active site remains mostly dianionic throughout the studied range of pH.

Effect of Anions on the Apoenzyme-Bound Phosphate. The effect of 100 mM chloride and acetate on phosphate bound at the active site of the apoenzyme at various pH values is shown in Figure 4. In the presence of these small ions, a significant change in the 989-cm^{-1} band size is observed with pH. To prevent absorption of phosphate being removed from the active site by these treatments, the infrared spectra were corrected for the absorption of solvent blanks containing the same free phosphate concentration as the sample and prepared by ultrafiltration of the sample. Thus, the measured infrared signal (difference spectra) should exclusively originate from bound phosphate (see Experimental Procedures). As can be seen in Figure 4, as the pH is lowered, the intensity of the 989-cm^{-1} band decreases.

The apparent pK values obtained from the variation of the intensity of this band with pH in the presence of chloride and acetate were very similar, 6.8 ± 0.05. A more thorough analysis was necessary in order to discern whether this titration

behavior reflects a change in the ionization state of the phosphate in the active site with pH or it is caused by pH-dependent removal of phosphate. The reversibility of the titration curves was analyzed, and the results obtained are also shown in Figure 4. When the pH is raised back from 5.7 to 8.3, no increase in the infrared signal could be detected (point outside curve at pH 8.3 in Figure 4), suggesting that the signal decrease at low pH is due to a pH-dependent phosphate removal from the active site induced by chloride and acetate ions. The presence of these anions prevents its return to its bound state when the pH increases. From these data, it can also be estimated that chloride at the lowest pH studied (5.7) removes about 85% of the phosphate bound at pH 8.3 while acetate produces removal of only about 50%. It should be noted that at pH 8.3, these anions were ineffective in releasing previously bound phosphate. That is, the band intensity for the apoenzyme-bound phosphate did not change when anions were added at this pH.

Effect of Aspartate, Succinate, and Glutarate on the Apoenzyme. Since substrates and inhibitors can bind to the apoenzyme form of this transaminase (Cheng & Martinez-Carrion, 1972), we studied the effects of the substrate aspartate and of the dicarboxylic acid inhibitors succinate and glutarate on the apoenzyme's bound inorganic phosphate. A procedure identical to that described above for the competition by acetate and chloride was used. The titration curves for the FTIR phosphate band in the presence of these reagents (100 mM concentration) are shown in Figure 4. In all cases, a 65–70% maximal decrease in the phosphate band itensity was observed as the pH was lowered, and the apparent pK values calculated from these curves were 7.4 ± 0.05. Again, at the end of the titration, the pH of the sample was raised from 5.7 to 8.3 in a single step in order to compare the remaining band intensity to that obtained initially at high pH. As the pH is increased in the presence of ligands (100 mM), a partial recovery of the FTIR band intensity was observed (approximately 30% for succinate and glutarate and 45% for aspartate) although it never reached its original value at pH 8.3 (Figure 4). These results suggest that the decrease in the FTIR signal as the pH is lowered is due both to pH-dependent phosphate removal and probably to some ionization of the bound phosphate. However, considering the lower affinity of this enzyme for dicarboxylic acids at high pH (Cheng & Martinez-Carrion, 1972; Jenkins & D'Ari, 1966b), some rebinding of phosphate at pH 8.3 cannot be completely discarded at this point as the cause for the increase in the FTIR phosphate band intensity.

To ensure that these changes in the infrared band are not caused by nonspecific effects of these reagents, the same experiment was carried out using the amino acid valine since this amino acid cannot bind to the transaminase and has never been detected to compete with ion or substrate binding (Martinez-Carrion et al., 1967). The addition of 100 mM valine to the apoenzyme solution did not affect the dianionic state of phosphate, as reflected by the absence of any change in the band integrated intensity or position when the pH was changed as it was observed for apoenzyme alone (Figure 4).

Effect of Pyridoxal and Pyridoxamine on the Apoenzyme. Pyridoxal, one of the structural components of the cofactor PLP molecule, was added (20 mM) to a solution of the apoenzyme containing bound phosphate. Solubility reasons hampered the use of higher pyridoxal concentrations, but it has already been shown that at this concentration it can displace about 30% of tightly bound inorganic phosphate (Iriarte et al., 1985). Using 25 mM Tris–acetate buffer as blank to be subtracted from the infrared measurements of the protein

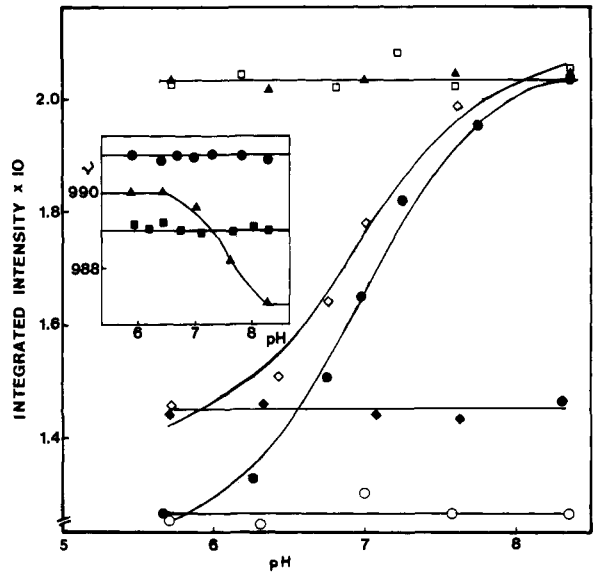

FIGURE 5: Effect of pH on the phosphate band integrated intensity of methylated apoenzyme (▲), of carbamylated apoenzyme before (●) and after subtraction of the appropriate solvent blank (○), and of apoenzyme in the presence of 20 mM pyridoxamine (□) or 20 mM pyridoxal either before (◇) or after correction for solvent absorbance at each individual pH (◆) (see Experimental Procedures). The solid sigmoidal lines are the theoretical curves calculated for a single ionization process. (Inset) Effect of pH in the position of the band due to the dianionic phosphate. Apoenzyme control (■); carbamylated apoenzyme (●); methylated apoenzyme (▲).

solution, the pH profile shown in Figure 5 was obtained. The apparent pK for this phosphate band titration was about 7.0. According to this apparent pK value and taking into account former related reports (Iriarte et al., 1985) based on ^{31}P NMR observations, it seems that phosphate is removed by the binding of pyridoxal to the active site. From the decrease of this band area, it can be calculated that about 30% of the phosphate has been displaced. Nevertheless, in order to determine whether the phosphate is actually being liberated, the same experimental procedure described before for the dicarboxylic acids was utilized. The spectra of the apoenzyme in the presence of pyridoxal and at different pH values were corrected for the contribution of free phosphate by subtraction of a buffer blank that had been equilibrated with the sample by ultrafiltration and therefore contained the same concentration of free phosphate. When this correction was introduced, no pH-dependent titration of the infrared band is detected. Thus, the phosphate removal induced by the presence of pyridoxal is not strongly pH-dependent nor is there any significant ionization of bound phosphate. In conclusion, the pH profile shown in Figure 5 can be completely ascribed to the titration of free (released) inorganic phosphate.

In marked contrast to pyridoxal, the presence of 20 mM pyridoxamine, which is known to bind to apoenzyme (Farach et al., 1983), exerts no effect on the infrared spectrum of apoenzyme-bound phosphate, and no variation in the phosphate band integrated intensity can be seen as the pH changes. Thus, as previously suggested (Iriarte et al., 1985), binding of pyridoxamine to the apoenzyme produces neither changes in ionization of bound phosphate nor its removal from the active site.

Methylation and Carbamylation of the Apoenzyme. Lys-258 forms a Schiff base with PLP in the holoenzyme, yet it is free in the apoenzyme and has been proposed to be involved in the stabilization of the protein phosphate binding pocket (Iriarte et al., 1985). Since binding of phosphate ions by the apoenzyme may involve participation of the active-site Lys-258,

this residue was selectively modified in such a way as to retain its charge but be unable to form a Schiff base with pyridoxal or PLP (reductive methylation; Roberts et al., 1988) or by eliminating its net charge altogether through carbamylation (Slebe & Martinez-Carrion, 1976).

Reductive methylation of Lys-258 (Figure 5) does not affect the state of ionization of the phosphate which remains dianionic over the studied pH range. However, it is noticeable that the position of the FTIR band changes with pH, moving to higher wavenumbers as the pH decreases (Figure 5, inset). Although small (maximum of 3 cm^{-1}), these changes are significant since band positions can be determined with an accuracy of ± 0.4 cm^{-1}. This behavior contrasts that of the apoenzyme with the active-site Lys-258 carbamylated by treatment with potassium cyanate (Slebe & Martinez-Carrion, 1976). In this case, the band position was displaced to slightly higher wavenumbers, but there was no variation with pH. Furthermore, the pH titration profile of the bound phosphate (Figure 5) shows a pH dependence of the phosphate band intensity with a pK value around 7.0. However, when the spectra were corrected for the contribution of free phosphate in the sample as described in the previous two sections, no phosphate ionization could be detected. These results indicate that the titration observed was due to the presence, both at high and at low pH values, of significant amounts of free phosphate in the sample. Thus, a pH-independent phosphate release must be considered as the explanation for the results obtained with the carbamylated apoenzyme.

Discussion

The consequence of specific binding of ions to proteins on their structural and functional properties is a poorly understood aspect of protein behavior. When the ions are selectively bound at the active site, they may have roles related to the protein's mechanism of action, to its thermodynamic stability, and even to its proper folding when first synthesized in the cell.

In aspartate aminotransferase, anions are known to act as competitive inhibitors as well as to participate in product displacement reactions (Jenkins & D'Ari, 1966b; Cheng & Martinez-Carrion, 1972). Yet, little is known regarding the anion binding sites although X-ray evidence and some other spectroscopic studies of the protein in solution (Arnone et al., 1982; Mattingly et al., 1982; Sanchez-Ruiz & Martinez-Carrion, 1986) seem to point to the two arginines at the active site, Arg-386 and Arg-292, as well as the Schiff base between Lys-258 and PLP as likely candidates.

When the anion is phosphate, the binding also takes place at a pocket in the active site of the apoenzyme (Arnone et al., 1982; Jansonius et al., 1985) which will ultimately house the phosphate ester component of the coenzyme PLP. The binding of inorganic phosphate is one of high affinity which contributes to produce a thermal stabilization of the apoenzyme as reflected in its transition temperature of denaturation, T_d = 66 °C, which is half-way between that of "de-ionized" apoenzyme, 61 °C (acetate replacing phosphate), and holoenzyme, 79.5 °C (Iriarte et al., 1985).

Inorganic phosphate gives rise to several bands in the infrared spectrum (Figure 1; Chapman & Thirlwell, 1964). Although interfering absorption, due to the protein moiety, precludes the use of many of the phosphate vibrational modes, dianionic symmetric stretching originates a band which can be employed to follow the behavior of protein-bound phosphate. This band is found at 989 cm^{-1}, whereas for the cofactor PLP, such a phosphate band arises at 976 cm^{-1}. On the other hand, the dianionic symmetric stretching band for the free phosphate dianion in solution appears at 991 cm^{-1}. This creates a special problem since this wavenumber is too close to that for the bound phosphate and hinders the discrimination between the two forms of phosphate if both (free and bound) are present. These facts forced us to utilize different experimental approaches for overcoming this technical problem. Mainly, infrared measurements of blanks containing identical concentrations of free-in-solution phosphate as for bound to the protein have been used to correct from the corresponding enzyme-bound spectra and obtain difference spectra signals which can be solely ascribed to bound phosphate.

Therefore, we utilized the band at 989 cm^{-1} originated by the dianionic phosphate, and estimated the amounts of monoanionic and dianionic phosphate bound to the protein by comparison of intensities of this band between samples of our enzyme and those containing known concentrations of free phosphate in solution. Despite the fact that these kinds of calculations are, essentially, estimates (Sanchez-Ruiz & Martinez-Carrion, 1986), the results obtained are mostly consistent with the interpretation that bound phosphate remains mostly in the dianionic state throughout the pH range investigated (5.7–8.3) (Figure 3).

In former studies of the state of ionization of bound inorganic phosphate of cytosolic aspartate aminotransferase, a slight pH dependence was reported for the ^{31}P NMR signal (Iriarte et al., 1985). These observations suggested that, as it happens with the holoenzyme, the ^{31}P chemical shift of bound inorganic phosphate may sense the ionization of a nearby residue (Sanchez-Ruiz & Martinez-Carrion, 1990; Schnackerz et al., 1989).

When the cofactor is present, a Schiff base is formed between the carbonyl group of the pyridoxal and Lys-258, and there is considerable strain in the O–P–O bond of the bound phosphate ester of PLP (Arnone et al., 1985). In that case, the pH dependence observed for the ^{31}P chemical shift was inferred to be affected by distortion of the O–P–O bond (Mattingly et al., 1982), as induced by protonation of the Schiff base (Martinez-Carrion, 1986; Schnackerz et al., 1989; Sanchez-Ruiz et al., 1990) which causes a change in coenzyme ring orientation and, hence, a perturbation in the O–P–O bond angle (Martinez-Carrion, 1986). On the other hand, in the apoenzyme there is no pyridoxal at the active site, and no direct strain induced by the Schiff base formation or orientation is likely. Therefore, other ionizable residues in the apoenzyme's phosphate ion binding pocket could be responsible for this nonspecific effect detected by NMR. Tyr-70, Arg-266, Thr-109, Ser-255, or even Lys-258, located in the proximity of the phosphate pocket in the apoenzyme, are likely candidates for phosphate hydrogen bonding. The change in the state of ionization of some of these residues, Lys-258 in particular, could produce a slight variation in the alignment of the phosphate, giving rise to a possible geometrical modification affecting the O–P–O angle bond, or produce an electric field effect sensed by the phosphorus.

As previously mentioned, ^{31}P NMR results cannot be unambiguously interpreted as being solely a consequence of phosphorus ionization(s). The complementary technique of FTIR can do so (Sanchez-Ruiz & Martinez-Carrion, 1986). For those reasons, and the fact that for FTIR much smaller amounts of protein (about 6 mg) per sample are required, the latter technique is advantageous for the analysis of the phosphorus ionization state when bound to protein.

Reductive methylation of Lys-258 does not change the dianionic state of the phosphate as judged by the FTIR band intensity. However, a slight pH dependence for the FTIR band position is consistently observed. It would be plausible to think

that the introduction of two methyl groups in the amino group could induce a geometrical strain, or more likely change ion solvation or hydrogen bonding to another proximal active-site residue; i.e., the nature of the counterion can be of consequence. This is consistent with the fact that methylated Lys-258 has a pK value of about 8.0 and may likely swing into the phosphate binding pocket as previously proposed by us for the unmodified lysyl residue (Iriarte et al., 1985). Yet, interpretations of wavelength shifts in vibrational spectrocopy are not as clear as those for intensity changes, and proper modeling of strains, solvation, and hydrogen bonding to ionizable proximal groups are not available, making the present interpretation for wavelength variations in protein systems difficult. Nevertheless, it is interesting to point out that a similar change in band position with pH has been observed for the phosphate band in the reduced form of mitochondrial aspartate aminotransferase and for free PMP in solution (Sanchez-Ruiz et al., 1991).

The dianionic infrared band pattern of bound phosphate is altered by the presence of other anions in the medium and depends on the nature of these anions. Small anions, such as chloride and acetate, produce phosphate removal from the active site, and this removal is dependent on pH. By contrast, dicarboxylic acids, like glutarate and succinate, which are substrate analogues, and the substrate aspartate produce both removal of bound phosphate and an apparent variation in the ionization behavior of the phosphate. Raising the pH back to 8.3 at the end of the titration (pH 5.7) in the presence of dicarboxylates causes a partial increase (around 50%) in the intensity of the band, suggesting that the ionic state of bound phosphate is changing with pH. However, an alternative explanation for this partial increase in the phosphate band as produced by rebinding of phosphate to the enzyme when the pH is raised cannot be completely discarded. Dicarboxylate substrates and inhibitors bind to both the apo- and holoenzyme forms with higher affinity at low pH (Jenkins & D'Ari, 1966b; Chen & Martinez-Carrion, 1972; Martinez-Carrion et al., 1973). It has also been shown that mono- and dicarboxylates interfere with reconstitution of apoenzyme with PMP in a pH-dependent manner (Lambing et al., 1988). This inhibition was much stronger at low pH (6.0) than at high pH (8.5). The inflexion point for the pH dependence of inhibition was around pH 7.4, very similar to the pK obtained by us from the FTIR titration data in the presence of dicarboxylates. In contrast, the binding of phosphate to the apoenzyme shows the opposite pH profile; it binds better at high pH than at low pH (Lambing et al., 1988). Thus, when considering all of the above together with the fact that the apoenzyme binds inorganic phosphate with higher affinity (K_d in the low micromolar range; Arrio-Dupont, 1972; Fonda & Auerbach, 1976; Mattingly et al., 1983) than dicarboxylates (K_d 1–20 mM) (Martinez-Carrion et al., 1973; Mattingly et al., 1983), the increase in the FTIR band intensity when the pH is raised could be due to the apoenzyme binding back some of the small amounts of phosphate remaining in the sample.

All these effects are specific and caused by the binding of small anions or dicarboxylic acids to the active site. Other amino acids, like valine, which do not bind to the active site (Martinez-Carrion, 1967), fail to produce variation in the bound phosphate's spectral properties.

Two places have been proposed for the binding of these organic anions to the holoenzyme: (a) an amino acid residue at the active site would be the binding point for the small anions or for one of the carboxylic groups in a dicarboxylic acid, and the Schiff base could be the binding point for the other carboxylic group at low pH (Jenkins & D'Ari, 1966b; Martinez-Carrion et al., 1973); (b) the dicarboxylic acids form a bridge between the two arginyl residues at the active site as detected for holoenzyme crystal complexes with dicarboxylic acids (Arnone et al., 1982; Jansonius et al., 1985). If the binding of small anions to the apoenzyme occurs in a similar manner as to the holoenzyme, the positively charged active-site residues, arginyls, lysyls, and even histidyls, would be the groups involved (Cheng & Martinez-Carrion, 1972). After binding to these residues, the net positive charge in the phosphate environment would decrease. This modification in the environmental electrostatic charge, and a possible subsequent conformational change that would weaken the binding phosphate hydrogen bonds, could be responsible for the release of phosphate when other anions are present since they also would act as competitors for its pocket in the protein. This release is pH-dependent, as was expected according to the pH dependence for the binding of anions to the active site (Cheng & Martinez-Carrion, 1972) with phosphate release being favored at low pH. The sigmoidal curves obtained (Figure 4) and their inflexion points correspond to the apparent pK values of residues responsible for the respective anion binding. This value is about 6.8 when chloride and acetate are the displacing agents and could fit any one of the three histidines in the active-site region, His-143, -189, and -193 (Arnone et al., 1982); indeed, His-143 is hydrogen-bonded to Asp-222 (Arnone et al., 1985) and may have its pK value raised.

For the dicarboxylic acids, it is less clear how they would bind to the apoenzyme. Yet, they do so with lower affinity than for the holoenzyme (Martinez-Carrion et al., 1973). The inflexion point of the sigmoidal titration curve obtained (Figure 4) corresponds to an apparent pK of about 7.5, significantly higher than that for small anions. This probably reflects the involvement of a different residue with higher pK in binding of dicarboxylates, perhaps as a second anchoring point, in addition to the residue responsible for the binding of small anions. In the apoenzyme, the free Lys-258 could be such a residue. This in turn would cause the removal of this residue from the phosphate binding pocket and a destabilization of the phosphate–protein interactions. In general, the neutralization of positive charges in the active site by introduction of the negatively charged carboxyl groups would decrease the affinity for phosphate and trigger its release.

The introduction in the active site of one of the components of the cofactor, the pyridine moiety, causes no significant modification in the phosphate behavior with pH—witness the lack of effect after the addition of pyridoxamine (Figure 5). By marked contrast, a pH-independent phosphate removal is observed when pyridoxal is added. In this case, there is the capability of interaction between pyridoxal and Lys-258 with formation of a Schiff base, and the 30% phosphate elimination observed reflects the displacement expected for the concentration of pyridoxal used (20 mM), according to the respective affinities of apoenzyme for pyridoxal and phosphate ion (Iriarte et al., 1985). Hence, the resulting apparent titration curve of the phosphate infrared band corresponds to the ionization of free phosphate displaced by pyridoxal (Figure 5).

Similarly, carbamylation of the apoenzyme, which essentially removes the positive charge from this lysyl residue, results in release of phosphate as indicated by the observed pH titration behavior (Figure 5). In contrast, methylation of this same lysine residue, which leaves its charge intact (pK similar to that for the unmodified residue, about 8), does not alter the behavior of phosphate during the pH titration, indicating that the affinity of the protein for phosphate has not been affected.

These results indicate that charge rearrangements at the active site, in particular the removal of the positive charge associated with Lys-258, have a significant effect on the apoenzyme's affinity for phosphate.

In conclusion, FTIR spectroscopy has proven to be a very useful tool to obtain information about the behavior and properties of ionic phosphate bound at the pocket that in the active site of apoaspartate aminotransferase, and probably in many other PLP-dependent enzymes, is occupied by the phosphate ester of the coenzyme PLP when in holoenzyme form. It has been shown that this phosphate remains dianionic over the studied pH range. Electrostatic interactions between dianionic phosphate and amino acid residues in the apoenzyme crystallographically detected pocket consisting of Arg-266, the dipole for the α-helix spanning residues 108–124, and, probably, free Lys-258 can contribute significantly to stabilize the protein structure toward thermal denaturation. Exhaustive removal of phosphate from the apoenzyme or its substitution by another anion with different architecture (acetate) has been reported to induce a pronounced decrease in the thermal stability of the protein. The T_m of the thermal transition for the dephosphorylated enzyme was found to be about 5 °C lower, and the denaturation enthalpy decreased by 2.6 cal g^{-1} (Iriarte et al., 1985). This difference in the denaturation enthalpy for the thermal denaturation of "de-ionized" (acetate buffer) apoenzyme reflects in part contributions of interactions at the single dianionic phosphate binding pocket to the overall protein stability of the apoenzyme. Whether this stabilization by phosphate or other anions has a significant role during the in vivo synthesis of the protein upon liberation from the ribosome and prior to binding of PLP is not known.

Registry No. Lys, 56-87-1; inorganic phosphate, 14265-44-2; aspartate aminotransferase, 9000-97-9; aspartic acid, 56-84-8; succinic acid, 110-15-6; glutaric acid, 110-94-1; pyridoxal, 66-72-8; pyridoxamine, 85-87-0; chloride, 16887-00-6; acetic acid, 64-19-7.

References

Arnone, A., Briley, P. D., Rogers, P. H., Hyde, C. C., Metzler, C. M., & Metzler, D. E. (1982) in *Molecular Structure and Biological Activity* (Griffin, J. F., & Daux, W. L., Eds.) pp 57–77, Elsevier/North-Holland, New York.

Arnone, A., Rogers, P. H., Hyde, C. C., Briley, P. D., Metzler, C. M., & Metzler, D. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 138–155, John Wiley & Sons, New York.

Arrio-Dupont, M. (1972) *Eur. J. Biochem. 30*, 307–317.

Bonsir, S. M., Harruff, R. C., & Jenkins, W. T. (1975) *J. Biol. Chem. 250*, 8635–8641.

Braunstein, A. E. (1973) *Enzymes (3rd Ed.) 9*, 379–481.

Chapman, A. C., & Thirlwell, L. E. (1964) *Spectrochim. Acta 20*, 937–947.

Cheng, S., & Martinez-Carrion, M. (1972) *J. Biol. Chem. 247*, 6597–6602.

Farach, H. A., Mattingly, J. R., Jr., & Martinez-Carrion, M. (1983) *Biochemistry 22*, 1034–1039.

Feliss, N., & Martinez-Carrion, M. (1970) *Biochem. Biophys. Res. Commun. 40*, 932–939.

Fonda, M. L., & Auerbach, S. B. (1976) *Biochim. Biophys. Acta 422*, 38–47.

Gianfreda, L., Marino, G., Palescandolo, R., & Scardi, V. (1974) *Biochem. J. 137*, 199–203.

Gorenstein, D. G., Wyrwicz, A. M., & Bode, J. (1976) *J. Am. Chem. Soc. 98*, 2308–2314.

Griffiths, P. R., & de Haseth, J. A. (1986) in *Fourier Transform Infrared Spectrometry* (Elving, P. J., & Winefordner, J. D., Eds.) Chapter 8, pp 284–311, John Wiley & Sons, New York.

Iriarte, A., Hubert, E., Kraft, K., & Martinez-Carrion, M. (1984) in *Chemical and Biological Aspects of Vitamin B_6 Catalysis* (Evangelopoulos, E. A., Ed.) Part B, pp 269–276, Alan R. Liss, New York.

Iriarte, A., Kraft, K., & Martinez-Carrion, M. (1985) *J. Biol. Chem. 260*, 7457–7463.

Jansonius, J. N., & Vincent, M. G. (1987) in *Biological Macromolecules & Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 3, pp 187–285, John Wiley & Sons, New York.

Jansonius, J. N., Eichele, G., Ford, G. C., Picot, D., Thaller, C., & Vincent, M. C. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 110–138, John Wiley & Sons, New York.

Jenkins, W. T. (1980) *Arch. Biochem. Biophys. 205*, 579–586.

Jenkins, W. T., & D'Ari, L. (1966a) *Biochem. Biophys. Res. Commun. 22*, 376–382.

Jenkins, W. T., & D'Ari, L. (1966b) *J. Biol. Chem. 241*, 5667–5674.

Lambing, J. L., Foster, L. C., Kearney, D. J., Barnes, M. R., Tran, T. T., & Yang, B. I. (1988) *Biochem. Biophys. Res. Commun. 151*, 693–700.

Martinez-Carrion, M. (1975) *Eur. J. Biochem. 54*, 39–43.

Martinez-Carrion, M. (1986) in *Coenzymes and Cofactors: B_6-Pyridoxal Phosphate: Chemical, Biological and Medical Aspects* (Dolphin, D., Poulson, R., & Avramovic, O., Eds.) Part B, pp 1–22, John Wiley & Sons, New York.

Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartorsio, A., Riva, F., & Fasella, P. (1967) *J. Biol. Chem. 242*, 2397–2409.

Martinez-Carrion, M., Cheng, S., & Relimpio, A. (1973) *J. Biol. Chem. 248*, 2153–2160.

Mattingly, M. E., Mattingly, J. R., Jr., & Martinez-Carrion, M. (1982) *J. Biol. Chem. 257*, 8872–8878.

Mattingly, J. R., Jr., Farach, H. A., Jr., & Martinez-Carrion, M. (1983) *J. Biol. Chem. 258*, 6243–6249.

Porubcan, M. A., Westler, W. A., Ibanez, I. B., & Markley, J. L. (1979) *Biochemistry 18*, 4108–4116.

Roberts, W. J., Hubert, E., Iriarte, A., & Martinez-Carrion, M. (1988) *J. Biol. Chem. 263*, 7196–7202.

Sanchez-Ruiz, J. M., & Martinez-Carrion, M. (1986) *Biochemistry 25*, 2915–2920.

Sanchez-Ruiz, J. M., & Martinez-Carrion, M. (1988) *Biochemistry 27*, 3338–3342.

Sanchez-Ruiz, J. M., Iriarte, A., & Martinez-Carrion, M. (1991) *Arch. Biochem. Biophys. 286*, 38–45.

Savitsky, A., & Golay, M. J. E. (1964) *Anal. Chem. 36*, 1627–1638.

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.

Schnackerz, K. D., Wahler, G., Vincent, M. G., & Jansonius, J. N. (1989) *Eur. J. Biochem. 185*, 525–531.

Slebe, J. C., & Martinez-Carrion, M. (1976) *J. Biol. Chem. 251*, 5663–5669.

Stark, G. R., & Smyth, D. G. (1963) *J. Biol. Chem. 242*, 214–226.

Verge, D., Tenu, J. P., & Arrio-Dupont, M. (1979) *FEBS Lett. 9*, 152–156.