INORGANIC PHOSPHATE BINDING TO APOASPARTATE AMINOTRANSFERASE

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1. Introduction

Anions are known to bind specifically to the active site of aspartate aminotransferase [1]. The binding site of the anions was proposed to be the binding site of the substrate carboxyl groups. The dissociation constant for phosphate bound to the pyridoxal form of the enzyme was 27 mM. However, studies had shown that P_i facilitated the removal of the coenzyme from the holoenzyme [2] and inhibited the reconstitution of the apoenzyme [3,4]. Further, the reconstitution rate depends on the method of resolution [5], and especially on whether or not phosphate has been used to remove the coenzyme. The binding of phosphate to the apoenzyme was studied [5] using a chemical method to determine the phosphate concentration. K_d ~1.6 μM was determined for the two tightly bound phosphates. ^{31}P NMR experiments have confirmed that phosphate binds with high affinity to the apoenzyme and have shown that it is displaced by the coenzyme [6].

P_i concentration in vivo is not negligible (~1 mM); phosphate is therefore likely to inhibit the last step in the formation of the active enzyme structure during its biosynthesis, i.e., the binding of the coenzyme to the protein. It seems important then to know the mode of binding of phosphate to the protein in more detail. The poor accuracy of the data previously obtained, due to the method of determination of phosphate, led us to reinvestigate this question, with two independent dialysis methods, using [^{32}P]-phosphate. The present study shows that two phosphate ions bind to the dimer of apoenzyme with K_d 1.5 μM. No cooperativity was observed.

2. Materials and methods

2.1. *Materials*

Cytoplasmic aspartate aminotransferase (EC 2.6.1.1) from pig heart was prepared as in [7]. Holoenzyme was resolved as in [8]. Enzyme concentrations were determined at 280 nm using $\epsilon = 1.4 \times 10^5$ M^{-1} cm^{-1} for the holoenzyme dimer and $\epsilon = 1.32 \times 10^5$ M^{-1} cm^{-1} for the apoenzyme dimer. Pyridoxal-5'-phosphate (PLP) was purchased from Sigma. PLP concentrations were determined at 388 nm either in 0.1 M NaOH using $\epsilon = 6200$ M^{-1} cm^{-1}, or in 0.05 M triethanolamine (TEA) pH 8.3 using $\epsilon = 5200$ M^{-1} cm^{-1}.

2.2. *Dialysis experiments*

These were performed in 0.05 M TEA–0.1 M NaCl (pH 8.3) buffer at 25°C. $Na_2H^{32}PO_4$ (0.7 Ci.mmol^{-1}) was purchased from CEA (Saclay) and $Na_2H^{31}PO_4$ from Prolabo. Radioactivity countings were performed in a Packard Tricarb scintillation counter, using Instagel (Packard) as the scintillation liquid.

2.2.1. Equilibrium dialysis

Samples (1 ml) of apoenzyme (4–8 μM) and 1 ml check samples of buffer in dialysis bags (Visking membrane, Union Carbide Co.) were put into polyethylene flasks (to avoid adsorption on glass), containing 200 ml buffer, 0.02 μM [^{32}P]phosphate and 0.2–20 μM [^{31}P]phosphate. The flasks were gently shaken during 12 h. Aliquots from each bag and flask were taken for radioactivity counting.

2.2.2. Flow dialysis [9]

These experiments were done as in [10] at

2.2 ml/min flow rate. Enzyme concentration in the upper chamber was 7.5–12.5 μM. Phosphate was varied from 2.7–40 μM.

2.3. *Data analysis*

The binding data were treated to obtain Scatchard plots. The K_d and the no. binding sites/dimer were obtained by fitting the data to the following equation corresponding to a single class of sites:

$$\text{(bound phosphate) / (dimer)} = \bar{\nu} = \frac{n\,(L)}{K_d + (L)}$$

where (L) is the concentration of free phosphate.

3. Results

3.1. *Equilibrium dialysis*

As can be seen from fig.1, the binding isotherm is monophasic. The stoichiometry is 1.93 sites/dimer for the phosphate binding, with K_d ~1 μM.

3.2. *Flow dialsysis*

These experiments lead to a similar conclusion. A typical result is shown in fig.2. The mean stoichiometry is 1.86 sites/dimer and K_d 1.7 μM. Kinetic experiments (fig.3) show that the binding is fast compared to the 'relaxation time' of the system (~30–60 s). We did not observe any slow step.

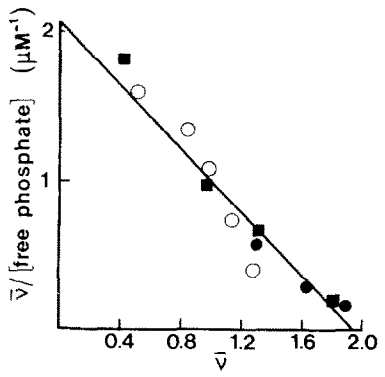

Fig.1. Binding isotherm of phosphate to apoaspartate aminotransferase as determined from equilibrium dialysis experiments (Scatchard plot). The solid line is calculated for a single class of 1.93 sites/dimer with K_d 0.98 μM. Enzyme concentrations (dimer): (■) 2.9 μM; (○) 2.7 μM; (●) 6.6 μM.

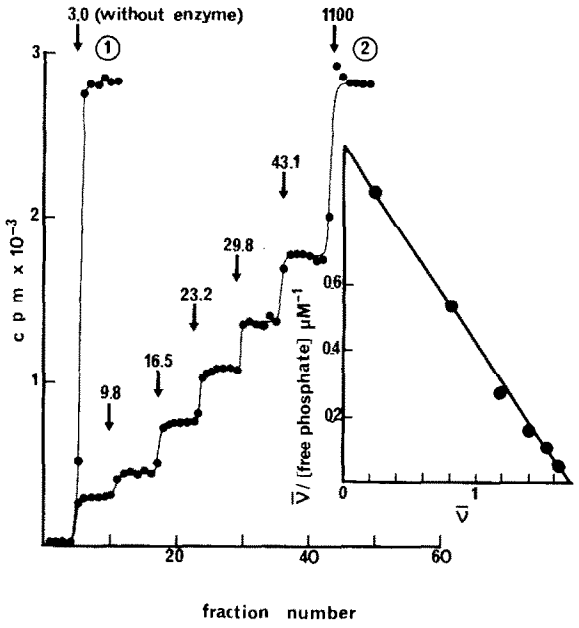

Fig.2. Example of a flow dialysis experiment: ① Experiment with apoenzyme 10.25 μM (dimer). Enzyme solution (0.9 ml) was put in the upper chamber. [^{32}P]phosphate (final conc. 3 μM) 10 μl, was first added. Aliquots (5 μl) of 1.25 mM unlabeled phosphate solution were then added (arrows). The total concentration after each addition is indicated in μM above the arrows. The last addition was of 0.1 M unlabeled phosphate (10 μl). ② Control experiment without enzyme. A single addition of 10 μl labeled phosphate was made to 0.9 ml buffer in the upper chamber. Insert figure: binding isotherm of phosphate to apoenzyme as determined from this experiment (Scatchard plot). The solid line is calculated for a single class of 1.86 sites/dimer with K_d 1.65 μM.

3.3. *Effect of partial reconstitution*

When the apoenzyme is partially reconstituted with PLP, the number of binding sites, as seen either by equilibrium dialysis or by flow dialysis, is reduced (table 1). This number corresponds to the difference between the number of phosphate binding sites in the apoenzyme (2/dimer) and the number of coenzyme binding sites occupied by PLP after reconstitution. The dissociation constant is not significantly modified compared with the value for the apoenzyme. In the holoenzyme, there is no site of high affinity binding for P_i.

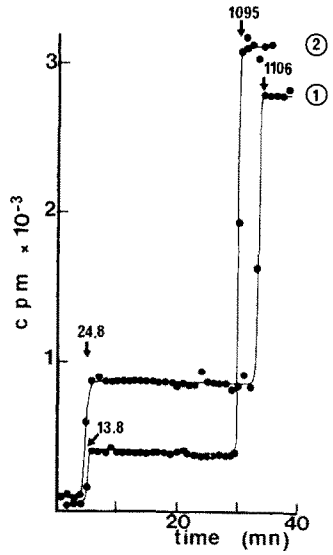

Fig.3. Kinetics of the phosphate binding as seen in flow dialysis experiments. (See fig.2 for experimental details.) Enzyme 11.65 μM (dimer).

4. Discussion

The experimental conditions (high concentrations of Cl^- compared to the phosphate and enzyme concentrations) used in our study did not permit us to observe the non-specific binding of phosphate to the anionic substrate binding site [1]. The results obtained for the high affinity binding of phosphate to the apoenzyme are summarized in table 1. They are very similar for the two methods used: two phosphate ions can bind to the dimer; the two sites are equivalent and independent, and the mean K_d is 1.5 μM. The discrepancy observed with [5] is not really surprising because of the poor accuracy and precision of the chemical method of phosphate determination.

A great advantage of the flow dialysis method is the possibility, in favorable cases, to observe slow binding steps [11]. Our kinetic experiments show that the binding process is fast compared to the time needed to reach the steady-state in the dialysis system, and that no slow relaxation process occurs.

The loss of half of the binding sites when the apoenzyme is half-reconstituted, and the inability of phosphate to bind to the holoenzyme (with a high affinity), clearly show that the binding site of phosphate is inaccessible when the coenzyme is present on the protomer. A similar conclusion was made [6] using the ^{31}P NMR technique. The most likely explanation is that the phosphate ion and the phosphate group of the coenzyme compete for the same site. This hypothesis is reinforced by the known effects of phosphate on both the resolution of holoenzyme [2] and the reconstitution rate of apoenzyme [3,4]. For another pyridoxal phosphate enzyme, glycogen phosphorylase, it has been shown [12] that phosphate, phosphite or fluorophosphate bind at the same site to which the 5′-phosphate of the coenzyme is bound.

Interaction between the subunits of the dimer has been shown [5,13–16]. Our experiments did not allow us to observe any cooperativity in the

Table 1
Site stoichiometry (n) and dissociation constant (K_d) for the binding of P_i to apoenzyme free of coenzyme or partially reconstituted (25°C, pH 8.3)

No. active sites/dimer occupied by PLP	Equilibrium dialysis		Flow dialysis	
	n	K_d (μM)	n	K_d (μM)
0	1.93 ± 0.15[a]	0.98 ± 0.50[a]	1.86 ± 0.10[a]	1.7 ± 0.2[a]
0.94			1.06	3.9[b]
1.00			1.02	2.1
1.10	0.74	0.88		
2.00	0		0	

[a] Mean value from 3 experiments
[b] Experiment at 20°C

binding of phosphate, neither to apoenzyme nor during the reconstitution, when half of the sites were occupied by PLP: in the two cases, binding isotherms were monophasic, and the dissociation constants were very close (table 1). The number of binding sites in the half-reconstituted apoenzyme was not significantly different from 1 site/dimer.

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