

Interaction of AMP with cytosolic apo-aspartate aminotransferase

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Interaction of cytosolic apo-aspartate aminotransferase with AMP has been studied under equilibrium conditions; e.g., equilibrium dialysis and spectrophotometric titration. Results show that a 1:1 stoichiometric complex AMP–apo-aspartate aminotransferase monomer is formed. The calculated dissociation constants with the two different experimental techniques are $40.4 \times 10^{-6} \text{ M}^{-1}$ and $31.4 \times 10^{-6} \text{ M}^{-1}$, respectively. These findings substantiate a previous hypothesis of control of the reconstitution of cytosolic apo-aspartate aminotransferases exerted by AMP.

AMP Aspartate aminotransferase Pyridoxal-5'-phosphate

1. INTRODUCTION

Regulation of enzyme levels in cells may be by metabolic effectors acting on the reconstitution of a holoenzyme from its coenzyme and apoenzyme.

In the case of aspartate aminotransferase, authors in [1] showed that phosphate and sulphate esters of oestrogens inhibit the reconstitution reaction, thus supporting hormonal control of the active enzyme level [2]. We have reported that nucleotides inhibit the rate of binding of pyridoxal-5'-phosphate to cytosolic aspartate aminotransferase [3]. The control of the reconstitution of aspartate aminotransferase by nucleotides seems to be of interest as it could constitute the basis for a malate–aspartate shuttle metabolic regulation system.

Results we obtained [3], even if unambiguously pointing out the interference of 5'-nucleotides on the reconstitution process, did not give any direct information on the nature of the interaction which can be gained only by equilibrium measurements.

The interaction of AMP, which has been shown to be one of the most effective inhibitors of the reconstitution process [4], has been studied under

equilibrium conditions by equilibrium dialysis and absorption spectroscopy.

2. EXPERIMENTAL

2.1. Materials

Cytosolic holo-aspartate aminotransferase (EC 2.6.1.1) from pig heart (α subform) was prepared essentially as in [5]. The pyridoxamine holoenzyme was obtained essentially as in [6]. Enzyme concentration was determined using $E_{280} = 6.58 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the holoenzyme monomer. Pyridoxal-5'-phosphate, cysteine sulphinate and adenosine-5'-phosphate were obtained from Sigma (St Louis MO). $\text{H}_2[^{35}\text{S}]\text{O}_4$ (1.84 Ci/mol) and $[^{14}\text{C}]\text{AMP}$ (538 Ci/mol) were from Radiochemical Centre (Amersham).

2.2. Preparation of the apoenzyme

To 1.0 ml of the pyridoxamine form of the holoenzyme (10–20 mg/ml) in 50 mM Tris–HCl (pH 8.0), 0.4 ml of 2 M sodium acetate (pH 4.8) and 1.5 ml of 4.4 M ammonium sulphate were added. After 1 h of incubation at room temperature, further 3.0 ml of the ammonium sulphate solution were added and the solution was then kept in the refrigerator overnight. The

Abbreviation: PLP, pyridoxal-5'-phosphate

precipitated protein was centrifuged, washed twice with the ammonium sulphate solution and dissolved in the minimum amount of 100 mM NaHCO₃ (pH 8.0). Gel filtration was then performed on a Sephadex G-25 column (1.5 × 20 cm), equilibrated and eluted with 50 mM Tris-HCl (pH 8.0).

At this stage the activity was about 5% of the initial one but if the above procedure was repeated the residual activity became less than 2%. The activity recovery upon addition of an excess of pyridoxal-5'-phosphate was close to 100%.

Resolution with ammonium-[³⁵S]sulphate was performed after neutralization of H₂[³⁵S]O₄ with concentrated ammonia and dilution with (NH₄)₂SO₄ to have a 4.4 M solution (0.2 Ci/mol).

2.3. Equilibrium dialysis

Experiments were performed in micro-dialysis cells (0.6 ml total volume), kept under continuous rotation to minimize aspecific absorption, in a perspex jacketed cylinder connected to a water bath at 25°C. Dialysis membranes were obtained by cutting pieces of Visking tubes previously boiled for 30 min in 5% Na₂CO₃ containing 50 mM EDTA.

Apoenzyme (10–50 × 10⁻⁶ M subunit) in 50 mM Tris-HCl (pH 8.0) was left to reach the equilibrium with the labelled ligand for 12 h. The amount of ligand bound was calculated per mole of apoenzyme subunit, the actual protein concentration being measured by Lowry's method using the holo-enzyme as reference protein.

The binding data were treated as in [7] in terms of ligand bound over total protein concentration (*r*) as a function of the free ligand concentration (*C_F*):

$$r/C_F = -r/K_i + n/K_i$$

where:

K_i = the apparent dissociation constant of the AMP-apoenzyme complex;

n = the number of binding sites/monomer.

2.4. Spectrophotometric titration

Experiments were performed at 25°C using a Cary model 118 spectrophotometer. The sample cell contained apoenzyme (10–20 × 10⁻⁶ M subunit) and variable amounts of AMP in 50 mM Tris-HCl (pH 8.0). To the sample and the reference cell containing the same buffer,

pyridoxal-5'-phosphate (1 × 10⁻³ M solution in Tris-HCl, pH 8.0) was added stepwise. After attaining the equilibrium, the increase of the absorbance at 362 nm was recorded. The coenzyme bound was evaluated by means of an *E*₃₆₂ = 8000 M⁻¹.cm⁻¹. The concentration of the free coenzyme was determined by difference. Under these conditions binding of the coenzyme to the apoenzyme follows a Langmuir isotherm. Hence binding data can be plotted according to the saturation function:

$$\theta = (\text{PLP})_{\text{free}}/K' + (\text{PLP})_{\text{free}}$$

or its linearized form.

K' is the apparent dissociation constant of the reconstituted holoprotein. It is dependent on the AMP concentration and on the dissociation constant, *K_i*, of the AMP-apoenzyme complex according to the relationship:

$$K' = K_a \{ 1 + (\text{AMP})/K_i \}$$

where:

K_a = *K'* when (AMP) = 0, and it is dependent on the buffer medium used [8].

3. RESULTS

3.1. Preparation of the apoenzyme

Care was taken in the apoenzyme preparation to avoid the presence of specific anions which could interfere with the binding of the coenzyme and of the nucleotide to the apoenzyme. For this reason, any procedure using phosphate as resolving agent [1] was avoided because the apoenzyme was contaminated by phosphate ions [9]. The resolution procedure, described in section 2, combines some features of reported methods [10,11]. The apoenzyme obtained resulted free from the resolving anion as shown by the test made with ammonium-[³⁵S]sulphate (fig.1).

3.2. Equilibrium dialysis

The binding of AMP to the apoenzyme was studied by measuring the amount of [¹⁴C]AMP bound to the protein by equilibrium dialysis experiments. A typical Scatchard plot is reported in fig.2. Results (as an average of 5 different experiments) lead us to calculate a 1.02 ± 0.05 ratio of nucleotide bound/protein subunit and a *K_i* = 40.4 ± 0.3 × 10⁻⁶ M. Thus a 1:1 stoichiometry can

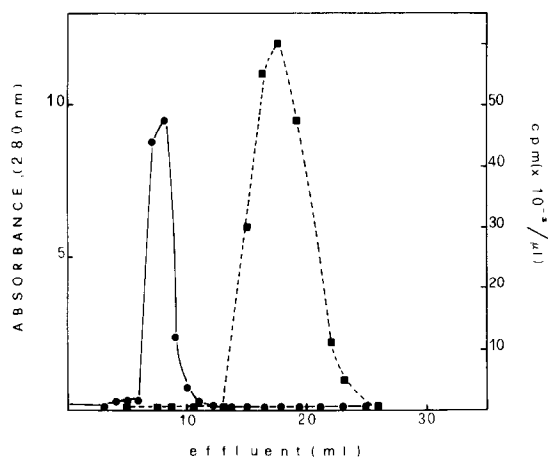


Fig.1. Elution profile of an apoenzyme preparation performed in the presence of $^{35}\text{SO}_4^{2-}$ as described in the experimental section. Absorbance was recorded at 280 nm (●). Radioactivity was assayed on 10- μl aliquots (■). The radioactivity of the pooled protein fraction was assayed on 0.5 ml triplicate aliquots and resulted to be identical to the background.

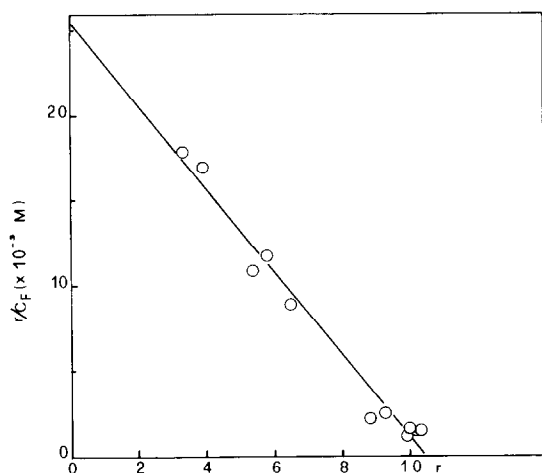


Fig.2. Scatchard plot of equilibrium dialysis experiments with $[^{14}\text{C}]\text{AMP}$ and apo-aspartate aminotransferase ($33.7 \cdot 10^{-6}$ M subunit) performed at 25°C in 50 mM Tris-HCl buffer; r is the molar ratio of AMP bound/enzyme subunit and C_F is the concentration of the free nucleotide. The experimental points were treated by the least square method to obtain the equation of the line.

be demonstrated for the AMP-apo-aspartate aminotransferase interaction and a direct equilibrium constant has been derived.

3.3. Spectrophotometric experiment

The binding of pyridoxal-5'-phosphate to apo-aspartate aminotransferase in the presence of AMP was followed spectrophotometrically by measuring the increase of the absorption at 362 nm due to the formation of the aldimine bond. It should be underlined that experiments were performed in Tris buffer although it is well known that Tris base forms an aldimine with pyridoxal-5'-phosphate [12]. Only in this condition has it been possible to obtain a measurable PLP-apo-aspartate aminotransferase dissociation constant value as shown in the case of the reconstitution of L-serine dehydratase [8]. Saturation curves (fig.3) show that AMP competes with the coenzyme in forming the holoprotein species absorbing at 362 nm. The reconstitution process, also in the presence of AMP, seems to be due to a single site interaction and can be described by the Langmuir equation. Linear plots (fig.4) show that AMP is a competitive inhibitor for

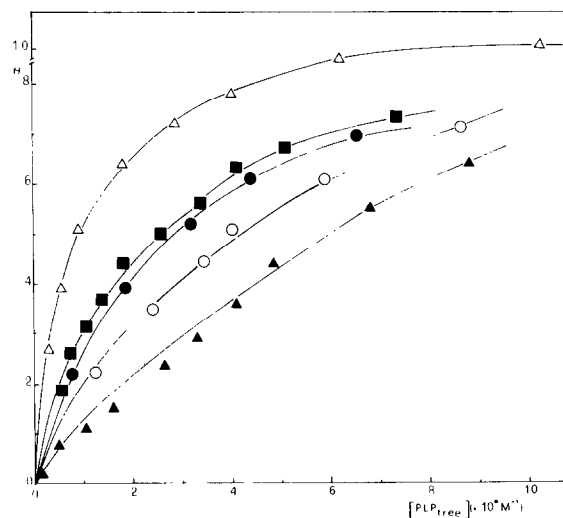


Fig.3. Titration of apo-aspartate aminotransferase ($15 \cdot 10^{-6}$ M subunit) with PLP in the presence of different AMP concentrations: Δ = 0; \blacksquare = 0.051 mM; \bullet = 0.076 mM; \circ = 0.118 mM; \blacktriangle = 0.249 mM. All the experiments were performed at 25°C in 50 mM Tris-HCl buffer.

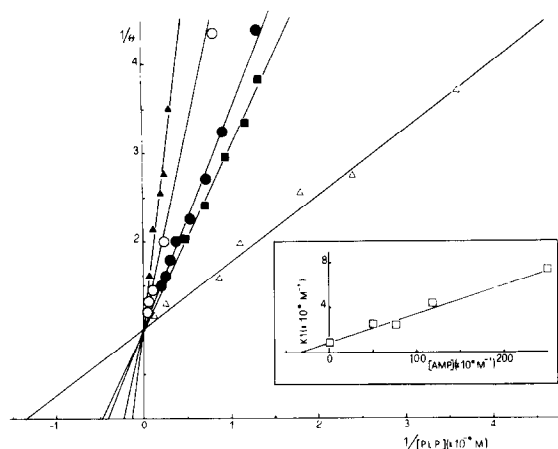


Fig.4. Linear plots of saturation curves of apo-aspartate aminotransferase with PLP in the presence of different AMP concentrations. Symbols were as described in the legend to fig.3. The insert shows the dependence of the apparent dissociation constants (K' , \square) on the concentration of AMP.

pyridoxal-5'-phosphate. In fact, an identical maximum value of the coenzyme bound is always observed independently of the AMP concentration. From the linear plot it is possible to calculate the apparent dissociation constant K' for the PLP–apo-aspartate aminotransferase complex. A derived plot, shown in the insert of fig.4, gives K_i a value of 31.4×10^{-6} M, which is in good agreement with the value obtained by equilibrium dialysis. This experiment clearly shows that the nucleotide is a competitive inhibitor of the coenzyme for the reconstitution reaction, suggesting that pyridoxal-5'-phosphate and AMP could share a common binding domain.

4. DISCUSSION

The results reported above support previous evidence on a specific interaction of AMP with apo-aspartate aminotransferase. It is worthwhile to recall that the values of the dissociation constant of the AMP–apo-aspartate aminotransferase complex found in two different equilibrium conditions, are in very good agreement with the value found by comparing the initial reconstitution rates (table 1). This validates a simple procedure for determining the extent of inhibition of the reconstitution reaction [13].

Table 1

Dissociation constant values found for the AMP–apo-aspartate aminotransferase with different procedures

Procedure	K_i (M^{-1}) ($\times 10^6$)
Comparison of the initial reconstitution rates ^a	37
Equilibrium dialysis	40.4
Competition to PLP binding	31.4

^a Fiore et al. [3]

Present data show that a 1:1 (AMP–apo-aspartate aminotransferase monomer) complex is formed and that AMP competes with pyridoxal-5'-phosphate for the same binding site. Thus the results demonstrate a direct and specific interaction of AMP in the reconstitution process. The effect we have investigated in vitro might constitute the basis of a physiological interaction in vivo.

It has been shown that pyridoxal-5'-phosphate can act as an affinity label for nucleotide binding domains in several enzymes [14]. Present data are suggestive of the use of nucleotides for the recognition of pyridoxal-5'-phosphate binding site. A more detailed picture could arise by investigating such interaction with different pyridoxal-5'-phosphate-dependent enzymes.

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REFERENCES

- [1] Scardi, V., Scotto, P., Iaccarino, M. and Scarano, E. (1963) *Biochem. J.* 88, 172–175.
- [2] Scardi, V., Scotto, P., Iaccarino, M. and Scarano, E. (1963) in: *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E.E. et al. eds) pp.167–174, Pergamon, London.
- [3] Fiore, R., Garzillo, A.M., Marino, G. and Pucci, P. (1980) *Ital. J. Biochem.* 29, 59–60.
- [4] Fiore, R., Garzillo, A.M., Pucci, P. and Marino, G. (1978) *Rend. Accad. Sci. Fis. Mat. (Naples)* 45, 353–358.

- [5] Martinez-Carrion, M., Riva, F., Turano, C. and Fasella, P. (1965) *Biochem. Biophys. Res. Commun.* 20, 206–211.
- [6] Jenkins, W.T. and D'Ari, L. (1966) *Biochem. Biophys. Res. Commun.* 22, 376–382.
- [7] Scatchard, G. (1949) *Ann. NY Acad. Sci.* 51, 660.
- [8] Simon, D. and Kröger, H. (1974) *Biochim. Biophys. Acta* 334, 208–217.
- [9] Vergé, D., Tenn, J.P. and Arrio-Dupont, M. (1979) *FEBS Lett.* 100, 265–268.
- [10] Wada, H. and Snell, E.E. (1962) *J. Biol. Chem.* 237, 127–132.
- [11] Marino, G., Greco, A.M., Scardi, V. and Zito, R. (1966) *Biochem. J.* 99, 589–594.
- [12] Matsuo, J. (1957) *J. Am. Chem. Soc.* 79, 2011–2017.
- [13] Gianfreda, L., Marino, G., Palescandolo, R. and Scardi, V. (1974) *Mol. Cell. Biochem.* 4, 125–129.
- [14] Glazer, A., Delange, R. and Sigman, D. (1975) in: *Chemical Modification of Proteins*, pp.131–134, Elsevier Biomedical, Amsterdam, New York.