Cooperative effects in the binding of pyridoxal 5'-phosphate to mitochondrial apo-aspartate aminotransferase

A.M. Garzillo, G. Marino and B. Pispisa*

Istituto di Chimica Organica e Biologica and Istituto Chimico, Università di Napoli, Via Mezzocannone 16, 80134 Napoli, Italy

Received 31 January 1984; revised version received 26 March 1984

Titrations of mitochondrial apo-aspartate aminotransferase with pyridoxal 5'-phosphate in the presence of AMP, contrary to what has been observed in the case of the cytosolic isoenzyme [(1983) FEBS Lett. 153, 98–102], show sigmoidal isotherms, with Hill coefficients ranging from $n_H = 1.4$, in the absence of AMP, to $n_H = 1.8$, in the presence of 5.9 mM AMP. The experimental data were successfully fitted by the Monod-Wyman-Changeaux model. The best fit, in the absence of AMP, was obtained with $L = 30$, $K_R = 4.72 \times 10^{-7}$ M and $K_T = 1.18 \times 10^{-5}$ M. Binding curves in the presence of AMP fit the model by keeping K_R as a constant. This implies that AMP could bind to the apoenzyme only in the T state. In contrast, binding curves in the presence of phosphate ion (P_i) showed a less pronounced cooperativity, the Hill coefficient dropping to $n_H = 1.0$ in the presence of 0.1 mM P_i. The above results suggest a regulatory role of AMP and P_i in the reconstitution of aspartate aminotransferase.

Aspartate aminotransferase *Pyridoxal 5'-phosphate* *AMP* *Cooperativity*

1. INTRODUCTION

We recently showed that nucleotides inhibit the reconstitution of cytosolic aspartate aminotransferase (EC 2.6.1.1); i.e., the formation of holoenzyme from apoenzyme and pyridoxal 5'-phosphate (PLP) [1].

To extend the knowledge on a putative control of reconstitution of holo-aspartate aminotransferase by nucleotides, we have investigated the interaction of mitochondrial apo-aspartate aminotransferase with AMP under equilibrium conditions.

Saturation curves differ from the hyperbolic ones observed for the cytosolic apoenzyme [1]. The results obtained suggest that the binding of the coenzyme to the mitochondrial apo-enzyme occurs with positive cooperativity, AMP being a negative modulator.

A preliminary communication has already been given at the IUB Symposium 121 on Chemical and Biological Aspects of Vitamin B_6 Catalysis, Athens (1983)

2. EXPERIMENTAL

2.1. *Materials*

Cytosolic and mitochondrial holo-aspartate aminotransferase (EC 2.6.1.1) from pig heart were prepared essentially as in [2,3] and [4], respectively. The pyridoxamine holoenzymes were obtained as in [5]. Enzyme concentration was determined by using $E_{280} = 6.58 \times 10^4\ M^{-1} \cdot cm^{-1}$ and $E_{280} = 6.51 \times 10^4\ M^{-1} \cdot cm^{-1}$ for the cytosolic and the mitochondrial enzyme, respectively.

Pyridoxal 5'-phosphate, cysteine sulfinate and adenosine 5'-phosphate were obtained from Sigma (St. Louis, MO).

All other products were pure grade reagents from various sources.

2.2. *Preparations of the apoenzymes*

The cytosolic and the mitochondrial enzyme were resolved as in [1], but the pH of the resolving buffer, in the last case, had to be increased to pH 5.4. As in [1], care was taken to avoid the presence

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

of phosphate or sulfate anions which could interfere with coenzyme and/or nucleotide binding to the apoenzyme.

The last gel filtration for both apoenzymes was performed on a Sephadex G-25 column (1.5 × 20 cm), equilibrated and eluted with 50 mM Tris–HCl (pH 8.0).

2.3. *Spectrophotometric titration*

Experiments were performed essentially as in [1]. It should be underlined that experiments were performed in Tris buffer although it is well known that Tris base forms an aldimine with PLP [6]. Only in these conditions has it been possible to obtain measurable PLP-apoaspartate aminotransferase dissociation constant values.

The sample cell contained apoenzyme (10–20 × 10^{-6} M subunit) in Tris buffer (pH 8.0) and variable amounts of AMP or sodium phosphate buffer as modulator.

The coenzyme bound was evaluated, after attaining the equilibrium, by measuring the increase in absorption at 362 nm due to the formation of the aldimine bond in the holoenzyme (E_{362} = 8200 $M^{-1} \cdot cm^{-1}$). Obviously, the amount of free coenzyme was evaluated by the difference on the total.

3. RESULTS AND DISCUSSION

The binding isotherm of coenzyme to cytosolic apo-aspartate aminotransferase gives a hyperbolic curve, indicating that the process is not cooperative, and, in such a case, a Langmuir isotherm treatment has been applied successfully [1]. On the contrary, the binding isotherm to mitochondrial apo-aspartate aminotransferase obtained under the same conditions as in [1] exhibits a sigmoidal trend. Fig.1 shows two typical titration experiments for the two apo-enzymes.

Titrations of mitochondrial apo-protein with PLP in the presence of AMP result in more pronounced sigmoidal binding isotherms (fig.2). Hill plots and Scatchard plots (not shown) clearly show that the formation of the holoprotein mitochondrial species occurs with positive cooperativity, the Hill coefficient varying from 1.43 to 1.82. This prompted us to treat the binding isotherm of coenzyme to mitochondrial apo-enzyme by an easy two-state model, taking into account a change in

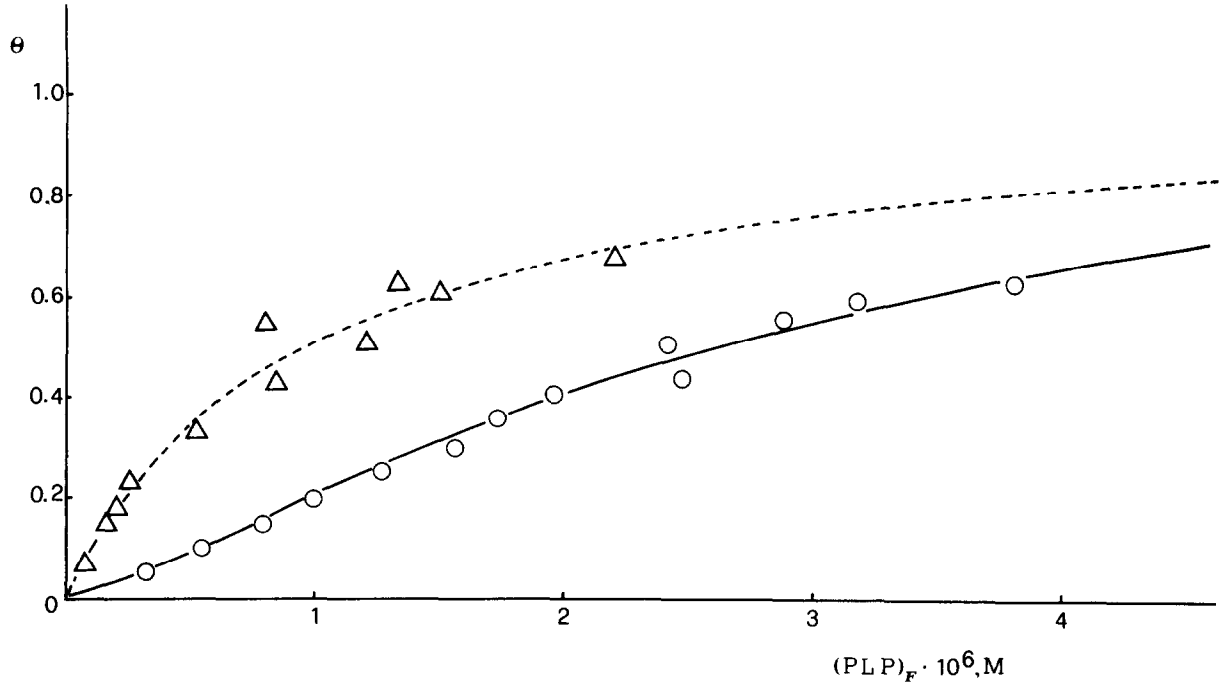

Fig.1. Titration of apo-aspartate aminotransferase with PLP at 25°C in 50 mM Tris–HCl buffer (pH 8). (△) Cytosolic apo-protein (14.58 × 10^{-6} M subunit), (○) mitochondrial apo-protein (20.55 × 10^{-6} M subunit).

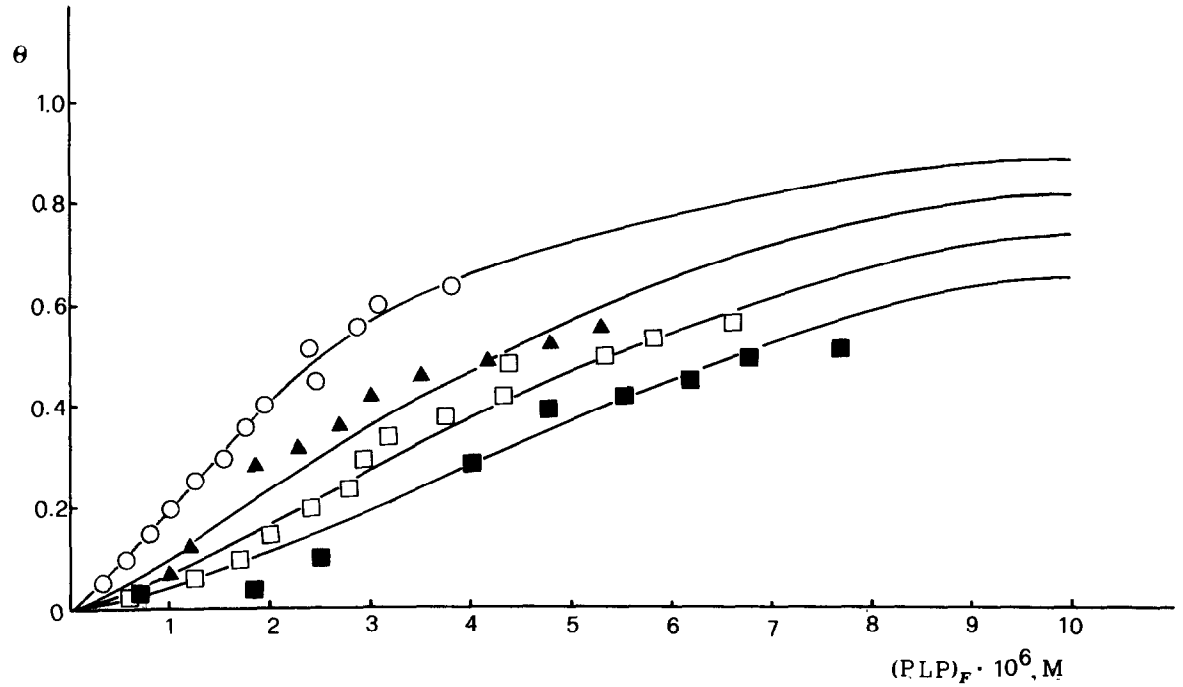

Fig.2. Titration of mitochondrial apo-aspartate aminotransferase with PLP at 25°C in 50 mM Tris–HCl buffer (pH 8) in the presence of different AMP concentrations: (○) [AMP] = 0; (▲) [AMP] = 1.9×10^{-3} M; (□) [AMP] = 3.8×10^{-3} M; (■) [AMP] = 5.9×10^{-3} M.

the association constant with the conformational state of the apo-enzyme. If L° is the equilibrium constant between state R and T of mitochondrial apo-protein when [PLP] = 0 (i.e., $L^\circ = [T]/[R]$), the association curve may be described by the equation derived from the M.W.C. model [7,8]:

$$\theta = \frac{[PLP]_F}{K_R + [PLP]_F} \cdot \frac{1 + L \cdot \frac{K_R}{K_T} \cdot \frac{K_T + [PLP]_F}{K_T} \cdot \frac{K_R}{K_R + [PLP]_F}}{1 + L \cdot \left(\frac{K_T + [PLP]_F}{K_T} \cdot \frac{K_R}{K_R + [PLP]_F}\right)^2} \quad (1)$$

where K_T and K_R are the apparent dissociation constants in the T and R state, respectively, and $[PLP]_F$ is the concentration of the free coenzyme, at equilibrium.

Owing to some paucity of experimental points, no computerized least-square fitting procedure was attempted. Rather a simple trial-and-error graphical procedure was followed. The experimental data were satisfactorily fitted, with an average deviation of 5%. In this connection it is worth noting that, although the experimental data cover only about 60% saturation, all curves show the tendency toward a hyperbolic behaviour at higher concentrations of free PLP. The range of experimental points was thus found to be quite sufficient to test the validity of the model.

Table 1 reports the fitting parameters. It clearly appears that in the absence of AMP the affinity of apo-mitochondrial aspartate aminotransferase in the R state for PLP is much higher than in T state, the former being of the same order of magnitude as that of cytosolic apoprotein for PLP (K_d = 7.63×10^{-7} M) [1]. It must be mentioned that n_H appears to be 1.43 ± 0.04 by plotting the ex-

Table 1

Fitting parameters to saturation curves of apo-mitochondrial aspartate aminotransferase with increasing AMP concentrations and comparison between (n_H^*) calculated and (n_H) experimental Hill coefficients

[AMP] × 10^3 (M)	$K \times 10^5$ (M)	$K_R \times 10^7$ (M)	L	n_H^*	n_H
0	1.18	4.72	30	1.44	1.43
1.90	5.24	4.72	80	1.68	1.70
3.80	8.00	4.72	130	1.73	1.76
5.90	11.76	4.72	210	1.78	1.82

perimental data as compared to $n_H = 1.44$ obtained by using the aforementioned parameters, as reported in [9].

In the presence of increasing AMP concentrations, the fitting to the curves appears to be satisfactory only if K_R is taken as a constant, while K_T and L are increased. Comparison of n_Hs obtained by experimental data and by fitting parameters is rather satisfactory (table 1), thus validating the calculation procedure.

These findings suggest that AMP stabilizes the T conformational state of the apoenzyme, displacing the conformational equilibrium toward this state. AMP inhibits the binding of PLP to apo-mitochondrial aspartate aminotransferase in the T state. In other words, the exclusive binding of AMP to the T state is competitive to the binding of PLP, as shown by plotting K_T values *vs* AMP concentration (fig.3). The value of the inhibition constant, $K_i = 8.2 \times 10^{-4}$ M, is about 20 times higher than that found for cytosolic apoprotein ($K_i = 4.0 \times 10^{-5}$ M [1]). It therefore seems that AMP inhibits the reconstitution of mitochondrial apoenzyme both because it shifts the conformational equilibrium toward the form having less affinity to the coenzyme, and because it hinders the binding of the coenzyme in this conformational state. These results could be explained by the following equilibria:

$$APO_R \rightleftharpoons APO_T + AMP \rightleftharpoons APO_T - AMP$$

We may therefore conclude with some confidence that the positive cooperativity observed in the formation of the mitochondrial holoprotein, particularly enhanced by AMP, is due to concerted conformational changes of the apo-protein within at least two states.

However, authors in [10] showed by fluorescence titration that no cooperativity occurs in the reconstitution of mitochondrial apo-aspartate aminotransferase. These authors prepared the holoenzyme from beef liver and resolved it by the method in [11]. When we tried to titrate an apoenzyme preparation obtained by using phosphate as the resolving ion we did not observe any cooperativity even in the presence of AMP. Thus phosphate which is known to be tightly bound to the apoenzyme [12] could have been responsible for this behaviour. Furthermore, titration of mitochondrial apoprotein obtained by resolving the pig heart holoenzyme by the procedure described in [1] shows Langmuir-like isotherms in the presence of phosphate ($K_d = 5.81 \times 10^{-7}$ M; fig.4). Hill plots show that n_H drops from 1.4 to 1.0.

Although this aspect needs further experimental support, present data could suggest that phosphate (P_i) shifts the conformational equilibrium toward the R state, thus making negligible the amount of the apoenzyme in the T state, according to the following equilibria:

$$P_i - APO_R \rightleftharpoons P_i + APO_R \rightleftharpoons APO_T$$

Finally, to the best of our knowledge, the cooperative binding of PLP to dimeric apoenzyme has been demonstrated previously only for the subunit of tryptophan synthase ([13–15] and references therein).

In conclusion, results obtained by probing the coenzyme site of the cytosolic [1] and mitochondrial (here) pig heart apo-aspartate aminotransferase have shown that in the case of cytosolic isoenzyme the reconstitution occurs with a single site covering process, while in the case of mitochon-

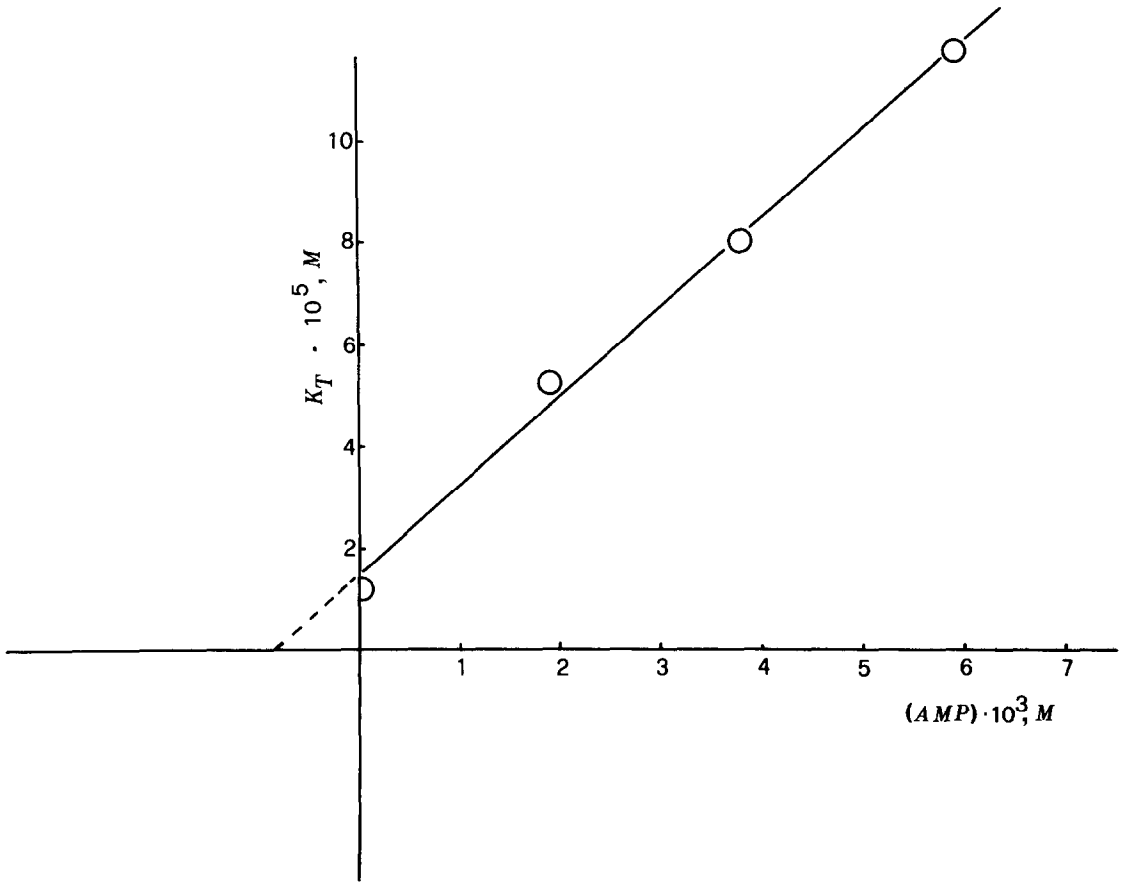

Fig.3. Dependence of dissociation constants of the apo-protein (T form)–PLP complex (K_T) (see table 1) on AMP concentration.

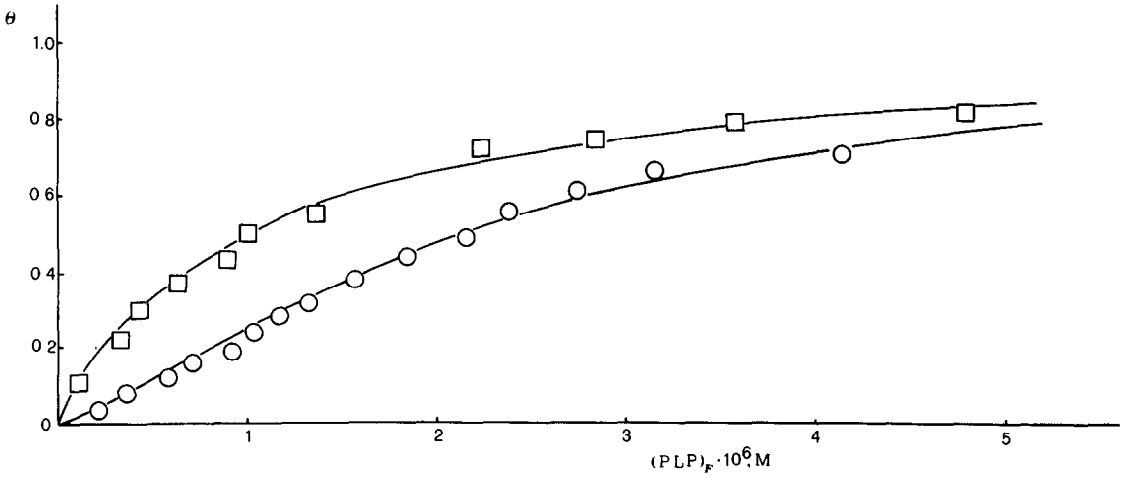

Fig.4. Titration of the mitochondrial apo-protein (14.31×10^{-6} M) with PLP at 25°C in Tris–HCl buffer (pH 8) without effectors (○), and in the presence of 1.07×10^{-4} M phosphate (□).

drial isoenzyme the reconstitution occurs with concerted conformational changes of the apoenzyme subunits by AMP and phosphate.

ACKNOWLEDGEMENT

This work was supported by a grant of the Ministry of Education, University of Naples.

REFERENCES

[1] Di Donato, A., Fiore, R., Garzillo, A.M. and Marino, G. (1983) FEBS Lett. 153, 98–102.
[2] Martinez-Carrion, M., Riva, F., Turano, C. and Fasella, P. (1965) Biochem. Biophys. Res. Commun. 20, 206–211.
[3] Barra, D., Bossa, F., Doonan, S., Fahmy, H.M.A., Martini, F. and Hughes, G.J. (1976) Eur. J. Biochem. 64, 519–526.
[4] Morino, Y., Tanase, S., Watanabe, T., Kaganiyama, H. and Wada, H. (1977) J. Biochem. 82, 847–852.
[5] Jenkins, W.T. and D'Ari, L. (1966) Biochem. Biophys. Res. Commun. 22, 376–382.
[6] Matsuo, Y. (1957) J. Am. Chem. Soc. 79, 2011–2017.
[7] Monod, J., Wyman, J. and Changeaux, J.P. (1965) J. Mol. Biol. 12, 88–118.
[8] Branca, M. and Pispisa, B. (1977) J. Chem. Soc. Farad. Trans. I 73, 213–229.
[9] Levitzki, K.E. (1975) in: Subunit Enzymes (Ebner, K.E. ed) pp.8–26, Marcel Dekker, New York.
[10] Lee, Y.H. and Churchich, J.E. (1975) J. Biol. Chem. 250, 5604–5608.
[11] Scardi, V., Scotto, P., Iaccarino, M. and Scarano, E. (1963) Biochem. J. 88, 172–175.
[12] Verge', D., Tenu, J.P. and Arrio-Dupont, M. (1979) FEBS Lett. 100, 265–268.
[13] Bartholmes, P., Kirschner, K. and Gschwind, H.-P. (1976) Biochemistry 15, 4712–4717.
[14] Tschopp, J. and Kirschner, K. (1980) Biochemistry 19, 4521–4527.
[15] Bartholmes, P., Balk, H. and Kirschner, K. (1980) Biochemistry 19, 4527–4533.