Further Characterization of Aspartate Aminotransferase from *Haloferax mediterranei:* Pyridoxal Phosphate as Coenzyme and Inhibitor

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The enzyme aspartate aminotransferase has been isolated from the halophilic bacterium *Haloferax mediterranei* in its apoenzyme form. The interaction with its coenzyme (pyridoxal phosphate) has been investigated. For concentrations up to 0.05 mM, the incubation with pyridoxal phosphate reconstituted the active complex (holoenzyme) following a second order kinetic with a k_2 of 5.2 $min^{-1}mM^{-1}$. This active complex showed a dissociation constant (K_d) of 7.8×10^{-6} M. For concentrations higher than 0.1 mM, pyridoxal phosphate produced an inactivation process with a complex second order kinetic. This inactivation is partially reverted by dialysis or by lysine treatment. Thus, after 80% of inactivation, 55% of the original activity is recovered by a long-time dialysis, and with 50 mM lysine also a partial reactivation (among 20-33%) is observed. The enzyme treated with 1 mM pyridoxal phosphate has a different behavior in Sepharose chromatography indicating that the modified enzyme presents a smaller size due to a conformational change.

Introduction

Aspartate aminotransferase (AspAT, E.C. 2.6.1.1) is a dimer with two identical subunits, being inactive in the monomeric state. For catalysis, the enzyme needs the coenzyme pyridoxal 5′-phosphate (PLP), which is covalently bound to the apoenzyme forming a Schiff base with Lys-258. During the transfer of the amino group, PLP is converted to pyridoxamine 5′-phosphate (PMP), which is bound noncovalently (see reviews: Braunstein, 1973 and Hayashi *et al.,* 1990). AspAT is usually obtained in the holoenzyme form although the dissociation of the coenzyme takes place when it is as pyridoxamine phosphate form and the medium contains a high amount of salt (Wada and Snell, 1962; Furbish *et al.,* 1969). It is known that both PLP and PMP stabilize the dimeric structure against thermal denaturation (Relimpio *et al.,* 1981; Iriarte *et al.,* 1984; Lain-Guelbenzu *et al.,* 1990) or against dissociation by urea (Ivanov *et al.,* 1973) or by guanidine hydrochloride (Herold and Kirschner, 1990).

AspAT from halophilic bacteria, as most of halophilic enzymes, has been unknown until now. No one had paid attention to this enzyme until we characterized the AspAT from *Haloferax mediterranei* (García-Muriana *et al.,* 1991; García-Muriana *et al.,* 1994). This enzyme has also two identical subunits but presents different features. One of these, a usual one for halophilic proteins, is the necessity of a high ionic strength in order to maintain the structure and activity of the protein (García-Muriana *et al.,* 1992). Another feature is the existence of the enzyme in the cell in the apoenzyme form in equilibrium with the holoenzyme. This equilibrium is defined by a high dissociation constant, causing the purification to yield only the apoenzyme.

There are aminotransferases, as GABA-AT from brain, that are obtained in holoenzyme form, but a simple dialysis produces the dissociation of the coenzyme (Jenkins and Fonda, 1985). In the same way, glutamate decarboxylase (GAD) is

Abbreviations: AspAT, aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; GABA-AT, γ-aminobutyrate aminotransferase; GAD, glutamate decarboxylase.

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present in the brain cells mainly as apoenzyme despite the fact that PLP is in a high concentration (Susz *et al.*, 1966). Taking into account these results, Cooper *et al.* (1978) have suggested a regulation mechanism for the synthesis of GABA based in the equilibrium holoenzyme = apoenzyme + PLP. A review on PLP-dependent enzymes that lose the coenzyme by different methods has been made by Yang and Metzler (1979).

Taking into account the extreme conditions in which the halophilic bacteria usually have to live, and the high stability of the apoenzyme form (García-Muriana *et al.*, 1991; García-Muriana *et al.*, 1994), a mechanism for the adaptation could take place, in which the cell should maintain the AspAT in the apo form when the growing conditions, such as salinity or temperature, are unfavorable.

In this paper, we have studied the PLP interaction with the AspAT from *Haloferax mediterranei*. At first, a positive effect on the enzymatic activity was observed, but for higher concentrations of PLP, the inhibition of enzyme occurs, which shows a saturation kinetic and a partial reversion by dialysis.

Materials and Methods

Chemicals

NADH, PLP, L-aspartate, α-ketoglutarate, malate dehydrogenase (pig heart), lysine and cysteine were from Sigma Chemical Co. All other chemicals used were of analytical grade or the finest grade available.

Bacterial strains and growth

The strain ATCC 33500 of *Haloferax mediterranei* used in this work was kindly provided by Dr. Ruíz-Berraquero from the University of Sevilla. The halophilic bacteria was grown aerobically in the medium described by Rodriguez-Valera *et al.*(1980). After three days of growing, the cells were collected by centrifugation and resuspended in a medium containing 0.01 M sodium phosphate (pH 7.2) and 3 M NaCl.

Enzyme activity

AspAT was obtained following the method described by García-Muriana (1991). The activity was measured by coupling the oxalate production with malate dehydrogenase and following the NADH oxidation at 340 nm. The assay was carried out in buffer solutions with 3 M KCl.

Kinetic constants

The apparent pseudo-first order constants (k_{app}) were calculated from the semilogarithmic plot of reaction time-courses and the second order constant (k_2) by plotting the consequent apparent pseudo-first order constant versus PLP concentration. The dissociation constants for the active complex (K_d) was determined by the Eadie plot following the method of Fersht (1985).

Results

Activation of the apoenzyme by PLP

The effect of incubation with different concentrations of PLP on the activity of AspAT from *H. mediterranei* is shown in Fig. 1. In the absence of the coenzyme, the enzyme shows a very low activity that increases in the presence of PLP. This effect follows a normal distribution with the maximum activity at 0.05 mM PLP.

The time-courses of PLP effect for concentrations between 0.05 and 0.5 mM have been analyzed. The maximum activation is obtained with 0.05 mM PLP, showing, in this case, the time-course

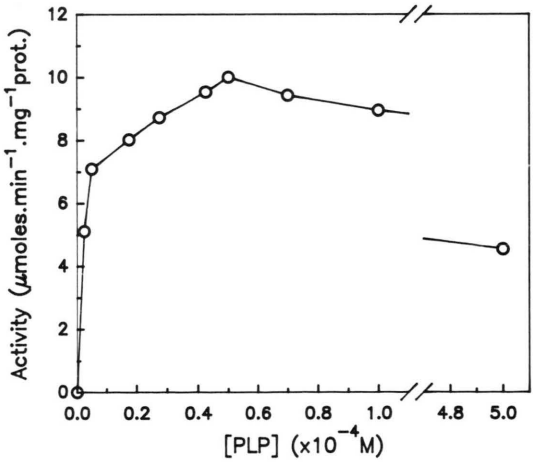

Fig 1. Effect of pyridoxal phosphate concentration on the activity of halophilic aspartate aminotransferase. AspAT was incubated at 20 °C in the presence of 0.01 M phosphate (pH 7.8), 3.5 M KCl and different amounts of PLP. After 15 min of incubation, the activity was measured as indicated in Materials and Methods.

a hyperbola form. For concentrations higher than 0.05 mM, the reactivation occurs faster but after several minutes of incubation a fall of the activity occurs. The maximum activity reached in every case depends on the concentration of PLP, thus, for 0.5 mM PLP, the maximum activity is only about 60% of that obtained with 0.05 mM.

The time-courses for the activation with different concentrations of PLP show a pseudo-first order kinetic with a good linear fit for semilogarithmic plot (Fig. 2). Then, the dependence of apparent pseudo-first order constant (k_{app}) on the concentration of PLP was analyzed by the direct linear plot (Fig. 2, Inset) and the second order constant obtained from its slope (k_2 = 5.2 min^{-1} mM^{-1}). The straight line of this plot indicates that only one residue of lysine is involved in the activation process such as in other transaminases (Inoue *et al.*, 1991; Goldberg *et al.*, 1993).

In order to know the affinity of AspAT by PLP, the dissociation constant (K_d) was determined as indicated by Fersht (1985). The concentration of E-PLP complex, assumed as the enzyme activity, in terms of the total enzyme concentration, is given by the equation:

$$[\text{E-PLP}] = E_o \frac{[\text{PLP}]}{K_d + [\text{PLP}]} .$$

The double reciprocal plot of the activity versus PLP concentration is shown in Fig. 3, and the dissociation constant (K_d = 7.8x10^{-6} M) was obtained from the slope . This constant is higher than that found in most of the enzymes using PLP as coenzyme (Jenkins and Fonda, 1985), although, there are exceptions, such as the GAD, with a dissociation constant of 5x10^{-4} M (Susz *et al.*, 1966).

Inactivation of the holoenzyme by PLP

As was indicated above, the inactivation occurs when the enzyme is incubated with PLP at concentrations higher than 0.05 mM. As can be seen in Fig. 4, the inactivation follows a pseudo-first order kinetic. The direct plot of the apparent first-order constants versus [PLP] (Fig. 4, Inset) gives a curve (at least two slopes). This curve suggests the existence of several sites in the enzyme for the interaction of PLP.

On the other hand, the inactivation is partially reverted by dilution or by treatment with lysine. Twenty-fold dilution of the inactivated sample produces a recovery of 55% of the original activity

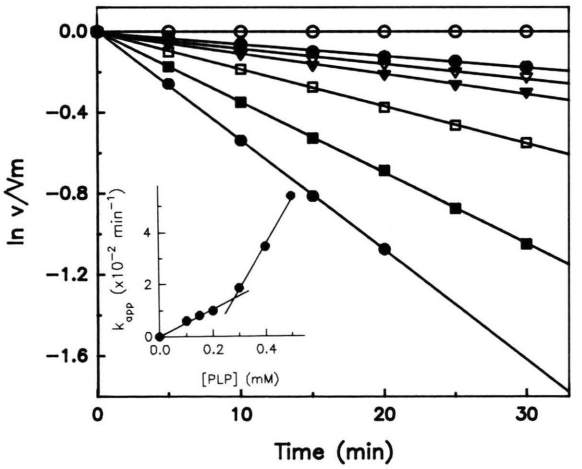

Fig 2. Kinetic rates for the formation of the active complex apoenzyme-pyridoxal phosphate of halophilic aspartate aminotransferase. Preparations of AspAT were incubated at 20 °C in the presence of 0.01 M phosphate (pH 7.8), 3.5 M KCl and 0.01 (●), 0.02 (▽), 0.03 (▲), 0.04 (□) and 0.05 (■) mM of PLP. The activity was measured as in Materials and Methods at the indicated times. The ratio Vm/v in logarithms scale was plotted and the apparent first-order constant (k_{app}) was determined from the slope for every concentration of PLP. Inset, it is shown the plotting of k_{app} *versus* PLP concentration for k_2 determination.

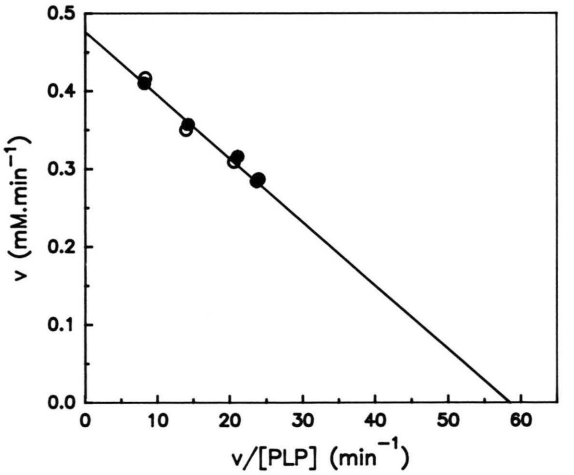

Fig 3. Eadie plot for the analysis of dissociation constant (K_d) of the active AspAT-PLP complex. AspAT was incubated during 15 (○) or 30 (●) min with PLP at the indicated concentrations. The AspAT activity was measured and *v versus v*/[PLP] plotted. Others experimental conditions as in Fig. 1.

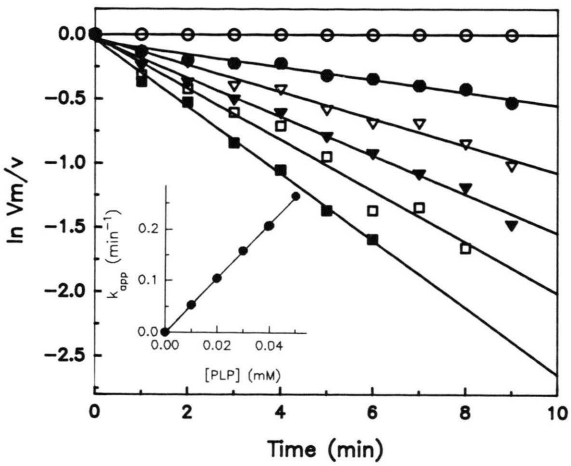

Fig 4. Inactivation of halophilic aspartate aminotransferase by high concentration of pyridoxal phosphate. AspAT was incubated at 20 °C in the presence of 0.01 M phosphate (pH 7.8), 3.5 M KCl and PLP at the following concentrations: 0.05 (○), 0.1 (●), 0.15 (▽), 0.2 (▼), 0.3 (□), 0.4 (■) and 0.6 (●) mM. The AspAT activity was measured at different times and the values in logarithm scale plotted *versus* time. Inset, plot of k_{app} *versus* PLP, the k_2 determined from the slope.

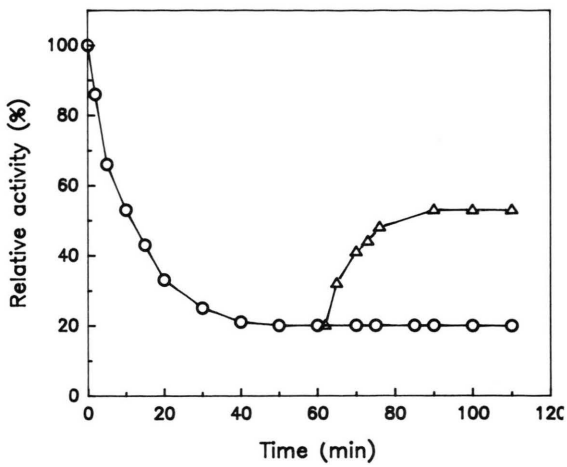

Fig 5. Partial reversion by dilution of the inactivation by pyridoxal phosphate of the halophilic aspartate aminotransferase. The apoenzyme was incubated in the presence of buffer solution containing: 0.01 M phosphate (pH 7.8), 3.5 M KCl and 1 mM PLP in dark at 30 °C. After 60 min half of the volume was diluted in the same buffer 20 times. The activity was measured as indicated in Materials and Methods at the indicated times in both diluted (△) and non-diluted (○) samples.

(Fig. 5). A similar result is obtained with dialysis (data not shown). In the same way, the inactivation is reverted by the treatment with lysine (Table I). This effect is not observed when lysine is substituted by cysteine.

Finally, it is interesting to note that a change in the size of the inactivated enzyme is observed by filtration through Sepharose. This change, which has been observed in other enzymes treated with PLP (Tagaya *et al.*, 1985; Tokushige *et al.*, 1985), should be produced by a conformational change in the modified subunits or in the assembly of the subunits or even by dissociation in the monomers.

With the idea in mind of a possible conformational change in the inactivated complex, as Tagaya found in glycogen synthase inhibited by UDP-pyridoxal (Tagaya *et al.*, 1985) or Tokushige in the malic enzyme inhibited by PLP (Tokushige *et al.*, 1985), we have made a similar analysis doing the double reciprocal plot of k_{app} and [PLP]. A positive intercept on the *y* axis must be observed if an intermediate complex with a different conformation is produced by the inactivation.

The negative interception observed in our case suggests that the inactivation is not due to a simple conformational change of the protein.

Table I. Reversion by lysine of the inactivated halophilic aspartate aminotransferase by pyridoxal 5′-phosphate. AspAT was inactivated as in Fig. 1 with 1 mM PLP. At the indicated times, the activity was determined before (residual activity) and after (activity recovered) the incubation with 50 mM lysine at 20 °C by 10 min.

Time of inactivation [min]	Residual activity [%]	Activity recovered [%]	Difference [%]
15	40	73	33
30	25	52	27
60	20	35	15

Discussion

Activation by PLP of the apoenzyme

In the halophilic AspAT, the catalytic effect by PLP is observed when the enzyme is incubated in the presence of small amounts of PLP (Fig. 1). Micromolar concentrations are enough to produce

the activation of the enzyme, showing the maximum activity at 0.05 mM.

The PLP-dependent enzymes, among them the transaminases, usually present a high affinity for the coenzyme (Jenkins and Fonda, 1985), although there are exceptions, such as GAD and GABA-AT, which bind the coenzyme weakly and lose it by dialysis (Jenkins and Fonda, 1985; Churchich and Moses, 1981). In GABA-AT from pig, two catalytic binding sites with different affinity (k_d = 10^{-9} M and $3x10^{-6}$ M) have been reported (Chrchich and Moses, 1981). These observations have been used to support the hypothesis of a regulation mechanism, based in the equilibrium between the apo and holo forms, for these enzymes *in vivo* (Cooper *et al.*, 1978).

The halophilic AspAT presents a dissociation constant (K_d of 7.8 x 10^{-6} M) lower than the usual in transaminases (10^{-7} M) (Jenkins and Fonda, 1985). This low affinity by the coenzyme determines the isolation of the AspAT from the halophilic cells in its apo form.

In addition to the low affinity by PLP, the AspAt shows a slow kinetic with a k_2 of 5.2 min^{-1} mM^{-1} for the activation, which coincides with that described by Vergé and Arrio-Dupont (1981) for the nonhalophilic transaminases.

The studies carried out by differential scanning calorimetry on halophilic AspAT (García-Muriana *et al.*, 1991; García-Muriana *et al.*, 1994) have also demonstrated a higher stability for the apoenzyme than for the holoenzyme under conditions of hyposalinity.

These results suggest a mechanism of adaptation for the halophilic cells living in unfavorable conditions, similar to the GAD and GABA-AT, in which the apoenzyme should be the resistant form.

Inactivation of the holoenzyme by PLP

When the halophilic AspAT is incubated with high concentrations of PLP the inactivation of the enzyme take place. This inactivation depends on, besides the coenzyme concentration, the time of incubation. Thus, after 30 min with 1 mM PLP, the activity is 80 % of the original.

The inactivation follows a complex kinetic, showing a double slope on the plot of k_{app} against [PLP], which indicates the existence of two groups in the protein that bind the coenzyme with different affinity.

The PLP is, in addition to a coenzyme, a reagent used for modification of proteins due to its covalent union with the amine group or with a distal-lysine residue. For this reason many enzymes are inactivated by this compound. The reactivity of those groups has been related with the polar environment. Thus, in RNA-polymerase, which is inactivated by the bond of PLP with a lysine residue, Bull *et al.* (1975) have suggested that the reactivity of the lysine is caused by the low pKa of the distal-amino group that is located in a nonpolar environment. Also, Tagaya *et al.* (1985) have demonstrated in glycogen synthase that the peptide labeled by UDP-pyridoxal have several hydrophobic residues, these being related with the reactivity of the protein. In the halophilic AspAT, hydrophobic effects seem to have no relation with the PLP modification since a change of the salt concentration do not affect significantly the inactivation process (results not shown).

The partial reversion of the inactivation by dialysis (Fig. 5) or by treatment with lysine (Table I) indicates a non-homogeneous modification of the halophilic AspAT by PLP. Similar results have been found by Podesta *et al.* (1986) in PEP carboxylase from maize. This enzyme is inactivated by PLP with the formation of a noncovalent complex prior to the formation of a Schiff base with some amino groups of the protein, the inactivation being partially reversible. These observations indicate that other groups, besides to the amino distal, are responsible of the interaction with PLP. This has been demonstrated in halophilic dihydrolipoamide dehydrogenase (Danson *et al.*, 1984). Also, in AspAT from pig, several sulfhydryl groups are modified by PLP, but only when the substrate is linked to the protein (Christen and Fiordan, 1970; Birchmeier *et al.*, 1973a; Birchmeier *et al.*, 1973b).

On the other hand, the irregular behavior of the modified halophilic AspAT in the Sepharose chromatography is similar to that found by Tagaya and Fukui (1986) in the lactate dehydrogenase from rabbit. This result suggests that the modification by PLP produces a change in the structure of the AspAT, which can be a conformational change followed by a contraction of the molecular structure determining the smaller size observed in the chromatography.

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