Pyridoxal arsenate as a prosthetic group for aspartate aminotransferase

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The apoenzyme of aspartate aminotransferase formed a stable, active holoenzyme on treatment with pyridoxal in the presence of arsenate.

INTRODUCTION

Lagunas & Sols (1968) showed that several enzymes whose natural substrates are phosphoric monoesters can act on unphosphorylated substrates in the presence of arsenate. It is likely that the esters of arsenate form in the active sites of the enzymes, because of the ease with which such esters form and hydrolyse (Braunstein, 1931):

The high rate of esterification and de-esterification of arsenate in comparison with phosphate is due to the easier access of a fifth ligand to the larger central atom. Several examples of such enzyme-catalysed transformations of alkyl arsenates are now known, e.g. of glucose 6-arsenate (Long & Ray, 1973; Lagunas, 1970, 1980) and of adenosine 5'-arsenate (Lagunas *et al.*, 1984).

If a solution of preformed ester of arsenic acid is used, the enzyme-catalysed reactions may be rapid (Lagunas, 1970; Long & Ray, 1973), but such esters are unstable, and hydrolyse with a half-life of less than 1 h under physiological conditions (Lagunas, 1980; Lagunas *et al.*, 1984). (Acylation or phosphorylation of arsenates are much less stable, with half-lives of about 1 s; Gresser, 1981; Moore *et al.*, 1983; Slooten & Nuyten, 1983.) If, however, instead of a preformed ester of arsenic acid the enzymes are supplied with unphosphorylated substrates in the presence of arsenate, their reactions are slow, probably because the formation of the ester is slow and is required for each turnover of the enzyme. We therefore thought that an apoenzyme might be satisfactorily activated by the dephosphorylated form of its prosthetic group in the presence of arsenate, since a single event of esterification should be enough for many turnovers. The present paper shows that this is true for aspartate aminotransferase (EC 2.6.1.1) from pig heart cytosol. Indeed the apoenzyme forms a stable holoenzyme when treated with arsenate and pyridoxal. This holoenzyme analogue evidently contains pyridoxal arsenate in place of pyridoxal phosphate.

MATERIALS AND METHODS

Aspartate aminotransferase

The enzyme was partially purified from pig heart by a modification of the method of Jenkins *et al.* (1959) and Martinez-Carrion *et al.* (1967). Their first four steps, extraction, heat treatment, precipitation with $(\text{NH}_4)_2\text{SO}_4$, and dialysis, gave a product suitable for most of the work. In the first step we used 3 mM-succinic acid/47 mM-disodium succinate buffer instead of the maleate/EDTA buffer originally proposed in order to avoid damage caused by heating with maleate (Turano *et al.*, 1964). The preparation obtained showed a specific activity in the assay of $17 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. For spectroscopy, the α -subform of the

enzyme was separated from this by a modification of the method of Martinez-Carrion *et al.* (1967). A gradient from 40 mM-sodium acetate, pH 5.2 (i.e. 9 mM-acetic acid/31 mM-sodium acetate) to 100 mM-sodium acetate, pH 5.4 (i.e. 15 mM-acetic acid/85 mM-sodium acetate) was used to elute the α -subform separately from other forms from CM-Sephadex. This preparation exhibited a specific activity of $170 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and appeared almost pure on gel electrophoresis by the method of Laemmli (1970).

Apoenzyme

The apoenzyme was prepared (Dixon & Severin, 1968) by gel-filtering the holoenzyme into water through a solution containing 1 M- NaH_2PO_4 , 50 mM-glutamic acid and 0.2 M-sodium glutamate, pH 4.7. The apoenzyme usually was used within 24 h of preparation.

Aspartate aminotransferase assay

The assay was based on monitoring the production of oxaloacetate by its A_{260} (Cammarata & Cohen, 1951; see also Jenkins *et al.*, 1959; Velick & Vavra, 1962); at this wavelength its molar absorption coefficient is $850 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Gelles & Hay, 1958). A solution of 8 mM-2-oxoglutaric acid, 0.2 M-Tris and 0.1 M-HCl was mixed with an equal volume of 20 mM-L-aspartic acid at 25 °C. The reaction was initiated by the addition of enzyme. For measuring specific activities, protein was determined by staining with bicinchoninic acid (Smith *et al.*, 1985).

Preparation of the holoenzyme analogue

The apoenzyme solution was mixed with a solution containing pyridoxal and arsenate of suitable pH. The mixture was then incubated at room temperature. Regeneration of enzyme activity was monitored by withdrawing samples from the mixture for assay. Since there is low activity of apoenzyme in the presence of pyridoxal alone (Wada & Snell, 1962), the activity was corrected by subtracting that of a control with pyridoxal under the same conditions. This was under 4% of the activity regenerated under optimal conditions. The activity was expressed as a percentage of that given by pyridoxal phosphate under optimal conditions (Severin & Dixon, 1968).

Preparation of the pyridoxamine form of the holoenzyme and its analogue

The method of Jenkins & D'Ari (1966) was used as follows. 3-Sulphinoalanine was added to the holoenzyme or its analogue in 40 mM-sodium acetate buffer, pH 5.4, to a concentration of 2 mM. After a few minutes the sample was gel-filtered through Sephadex G-25 into 100 mM-Tris/HCl buffer, pH 8.3. The active fractions were concentrated by ultrafiltration through a 10 μm -pore-size Amicon membrane.

RESULTS

Conditions for forming the holoenzyme analogue

Effect of arsenate and pyridoxal concentrations. Fig. 1 shows that 100 mM-arsenate was optimum, and regeneration was less above and below this concentration. Fig. 2 shows that increasing pyridoxal concentration increases the rate and extent of the regeneration of transaminase activity. Higher concentrations of pyridoxal than those shown interfere with the assay for activity by their high absorbance.

Effect of pH. The progress curves differed as the pH was varied over the range 6–9, and the degree of activity regenerated varied by less than 20 %; pH 8.5 seemed to give a slightly better increase of activity than the lower values.

Degree of regeneration. When the best conditions from the previous tests were combined, i.e. 100 mM-arsenate, 10 mM-pyridoxal and a pH of 8.5, 64 % of the activity obtained with pyridoxal phosphate was reached (Fig. 3), and this level was achieved in 72 h. It also appears that Tris buffer accelerated regeneration of activity in comparison with merely adjusting the pH of the arsenate solution with NaOH.

Fig. 1. Effect of arsenate concentration on the regeneration of enzyme activity from apoenzyme in the presence of pyridoxal and arsenate

The apoenzyme was incubated in the presence of 5 mM-pyridoxal in Tris/HCl buffer, pH 7.5, at room temperature (about 20 °C). Activities are expressed as percentages of that obtained with 40 μ M-pyridoxal phosphate in 15 min under the same conditions.

Fig. 2. Effect of pyridoxal concentration on the regeneration of enzyme activity from apoenzyme in the presence of pyridoxal and arsenate

The apoenzyme was incubated in the presence of 50 mM-arsenate in 50 mM-Tris/HCl, pH 7.5, and at room temperature (about 20 °C). Activities are expressed as percentages of that obtained with 40 μ M-pyridoxal phosphate in 15 min under the same conditions.

Fig. 3. Progress of the regeneration of enzyme activity from apoenzyme in the presence of pyridoxal and arsenate under optimized conditions

The apoenzyme was incubated in the presence of 10 mM-pyridoxal and 0.1 M-arsenate in 100 mM-Tris/HCl, pH 8.5, and at room temperature (about 20 °C). This is compared with incubations with 50 mM-arsenate or 0.1 M-phosphate under the same conditions and with 0.1 M-arsenate in water at pH 8.5 (the pH was adjusted with NaOH in this case).

Fig. 4. Stability of the holoenzyme analogue after gel filtration

The analogue formed by incubating the apoenzyme with pyridoxal and arsenate was gel-filtered and incubated at neutral pH and 4 °C in water or in the presence of the reagents specified.

Stability of the holoenzyme analogue formed

The holoenzyme formed was stable in the arsenate/pyridoxal mixture for weeks. It was removed from this mixture by gel filtration into water on a column of Sephadex G-25. Over 96 % of the activity loaded on to the column was recovered in the effluent. The transaminase activity in this solution and in the same enzyme solution after the addition of phosphate, arsenate, methylarsonate or Tris/HCl was monitored (Fig. 4) for 4 days at 4 °C. More than 90 % of the activity was retained by the holoenzyme analogue in water or Tris/HCl solutions. The presence of phosphate, arsenate or methylarsonate, however, resulted in a loss of about 25 % of the activity in 4 days. The pyridoxamine form of the analogue also retained over 90 % of its activity for 4 days in 100 mM-Tris/HCl buffer, pH 8.3, at 4 °C.

Absorption spectrum of the holoenzyme analogue

In the light of this stability we decided to study the spectra of the holoenzyme analogue. For this the α -subform was isolated and the holoenzyme analogue was prepared from its apoenzyme. Similar regeneration was obtained to that in Fig. 3 (100 mM-arsenate, 10 mM-pyridoxal and Tris/HCl, pH 8.5). The product was gel-filtered into 40 mM-sodium acetate buffer, pH 5.4. About 99 % of the activity was recovered in the effluent. It was concentrated by ultrafiltration by using an Amicon 10 μ m-pore-size membrane. The absorption spectra of holoenzyme analogue

Fig. 5. Absorption spectrum of the holoenzyme analogue in comparison with those of the apoenzyme and holoenzyme

Spectra were recorded in sodium acetate buffer, pH 5.4. The concentrations were: holoenzyme (α -subform), 3.2 mg/ml; apoenzyme, 1.3 mg/ml; holoenzyme analogue, 3.5 mg/ml. Path length: 1 cm.

Fig. 6. Absorption spectra of the pyridoxamine forms of the holoenzyme and the holoenzyme analogue

The spectra were recorded in 0.1 M-Tris/HCl buffer, pH 8.3. The concentrations were 1 mg/ml. The path length of the spectrophotometric cell was 1 cm.

along with those of the other forms (natural holoenzyme and apoenzyme) are shown in Fig. 5. Although its spectrum differs markedly from that of the natural holoenzyme, it also shows bands centred on 430 nm and 360 nm. The absorption spectra of the pyridoxamine forms are shown in Fig. 6.

DISCUSSION

Binding of pyridoxal and arsenate

The apoenzyme is known to bind free pyridoxal; indeed, it actually catalyses transamination between pyridoxal and amino acids to form pyridoxamine and oxo acids (Wada & Snell, 1962). Evidently the pyridoxal and pyridoxamine can bind strongly enough to participate in the reaction, but, unlike their phosphates, weakly enough to be able to dissociate from the enzyme at

neutral pH. Phosphate also binds to the apoenzyme, since it greatly slows the recombination of pyridoxal phosphate (Banks *et al.*, 1963, pp. 212–213; Severin & Dixon, 1968). The latter authors omitted to say how much the re-activation by 10 μ M-pyridoxal phosphate in a Tris/HCl buffer was slowed by 0.1 M-phosphate; it changed the half-time of re-activation from about 10 min to about 2 h. Arrio-Dupont (1972), Fonda & Auerbach (1976) and Vergé *et al.* (1979) have since made much more detailed studies of phosphate binding. We have found (results not shown) that arsenate similarly slows the recombination of apoenzyme with pyridoxal phosphate.

Possible esterification at the active site

Since the apoenzyme binds both components of pyridoxal arsenate and is likely to place them close to each other, this probably leads to formation of the ester, in view of the fact that esters of arsenic acid form and hydrolyse so easily. Such ester formation, in the light of the tight binding of apoenzyme with pyridoxal phosphate, would explain the stability of the holoenzyme analogue in contrast with the freely reversible dissociation of both pyridoxal and phosphate.

Lagunas & Sols (1968) and Lagunas (1970) suggested esterification or a similar reaction to explain why enzymes acted on the unphosphorylated parent compounds of their substrates in the presence of arsenate. Lagunas (1980), however, doubted whether the action of glucose-6-phosphate dehydrogenase on glucose and arsenate could be so explained (rather than by reaction of the enzyme with the small amounts of glucose 6-arsenate formed in solution), because she found that this dehydrogenase did not accelerate equilibration of glucose and arsenate with glucose 6-arsenate. It is conceivable, however, that the enzyme could accelerate esterification at the active site to give enzyme-bound glucose 6-arsenate and yet be unable to catalyse formation of free glucose 6-arsenate, provided that the dissociation of the ester is slow.

Dissociation and hydrolysis of pyridoxal arsenate

De-esterification of pyridoxal arsenate should be rapid in solution, since nucleoside 5'-arsenates show half-lives of 30–45 min at pH 7 and 25 °C (Lagunas *et al.*, 1984), and glucose 6-arsenate shows one of about 6 min (Lagunas, 1980). The stability of the holoenzyme analogue shows that the strong binding by the enzyme is enough to prevent the hydrolysis of pyridoxal arsenate. Part of the mechanism of this may be exclusion of water from the neighbourhood of the labile bond (Martinez-Carrion, 1986). The binding is expected to be tight, since pyridoxal phosphate does not dissociate from the enzyme appreciably at neutral pH; it appears to be bound with the phospho group predominantly doubly dissociated (Sanchez-Ruiz *et al.*, 1991, and references cited therein; Korpela *et al.*, 1987).

The fact that arsenate, phosphate and methylarsonate all aid dissociation of the holoenzyme analogue suggests that, in this analogue, the arsono-oxy group occasionally dissociates from the protein without being hydrolysed from the pyridoxal part of the molecule. Equilibrium must greatly favour its rebinding to the protein in order to explain the great increase in stability of bound pyridoxal arsenate in comparison with free compound. But if there is phosphate, arsenate or methylarsonate in the solution, this may bind instead of the temporarily displaced arsono-oxy group of pyridoxal arsenate, leaving this group in an aqueous environment where it is susceptible to hydrolysis. This may also be the explanation of the slightly surprising observation that high arsenate concentrations lower the rate and extent of formation of holoenzyme analogue from pyridoxal and arsenate. Clearly the filling of the phosphate-binding site with arsenate while the pyridoxal site is either empty or filled with pyridoxal

should not do this. It might however, by the mechanism described, assist the hydrolysis of bound pyridoxal arsenate.

Enzymic activity of the arsenical analogue

We can only place a limit on the degree of activity possessed by enzyme that contains pyridoxal arsenate in place of pyridoxal phosphate. We recover 64% of the initial activity by this replacement, but we have no direct evidence of the degree to which the apoenzyme has formed holoenzyme analogue in this treatment. This degree is likely to be high, because addition of pyridoxal phosphate gave only a slight further increase in activity. The somewhat confused absorption spectra of the analogue in both pyridoxal and pyridoxamine forms suggest that we do not have a homogeneous product. Hence the 64% represents a lower limit on the activity of this analogue. This is remarkable because other replacements of the $-O-PO_3^{2-}$ group, reviewed by Metzler & Fonda (1985), e.g. by $-CH_2-PO_3^{2-}$ (Hullar, 1969), lead to quite low levels of enzyme activity.

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