Active-site-directed inactivation of wheat-germ aspartate transcarbamoylase by pyridoxal 5'-phosphate

Stephen C. J. COLE and Robert J. YON*

School of Biological Sciences and Environmental Health, Thames Polytechnic, Wellington Street, London SE18 6PF, U.K.

Treatment of 1 μ M wheat-germ aspartate transcarbamoylase with 1 mM-pyridoxal 5'-phosphate caused a rapid loss of activity, concomitant with the formation of a Schiff base. Complete loss of activity occurred within 10 min when the Schiff base was reduced with a 100-fold excess of NaBH₄. Concomitantly, one amino group per chain was modified. No further residues were modified in the ensuing 30 min. The kinetics of inactivation were examined under conditions where the Schiff base was reduced before assay. Inactivation was apparently first-order. The pseudo-first-order rate constant, k_{app} , showed a hyperbolic dependence upon the concentration of pyridoxal 5'-phosphate, suggesting that the enzyme first formed a non-covalent complex with the reagent, modification of a lysine then proceeding within this complex. Inactivation of the enzyme by pyridoxal was 20 times slower than that by pyridoxal 5'-phosphate, indicating that the phosphate group was important in forming the initial complex. Partial protection against pyridoxal phosphate was provided by the leading substrate, carbamoyl phosphate, and nearly complete protection was provided by the bisubstrate analogue, *N*-phosphonoacetyl-L-aspartate, and the ligand-pair carbamoyl phosphate plus succinate. Steady-state kinetic studies, under conditions that minimized inactivation, showed that pyridoxal 5'-phosphate was also a competitive inhibitor with respect to the leading substrate, carbamoyl phosphate. Pyridoxal 5'-phosphate therefore appears to be an active-site-directed reagent. A sample of the enzyme containing one reduced pyridoxyl group per chain was digested with trypsin, and the labelled peptide was isolated and shown to contain a single pyridoxyl-lysine residue. Partial sequencing around the labelled lysine showed little homology with the sequence surrounding lysine-84, an active-centre residue of the catalytic subunit of aspartate transcarbamoylase from *Escherichia coli*, whose reaction with pyridoxal 5'-phosphate shows many similarities to the results described in the present paper. Arguably the reactive lysine is conserved between the two enzymes whereas the residues immediately surrounding the lysine are not. The same conclusion has been drawn in a comparison of reactive histidine residues in the two enzymes [Cole & Yon (1986) *Biochemistry* 25, 7168–7174].

INTRODUCTION

ATCase from *Escherichia coli* is currently one of the most intensively studied regulatory proteins. This is due largely to the fact that the catalytic and regulatory functions are associated with structures that can be dissected from each other experimentally. The regulatory enzyme has the basic structure (C₃)₂(R₂)₃ where C and R are distinct polypeptide chains associated with binding sites for substrates and regulatory effectors respectively. The entity C₃ can be dissected from the complex *in vitro*; it retains full catalytic activity but entirely lacks the regulatory function, and is referred to as the catalytic subunit or catalytic trimer. Reviews of the enzyme include that by Kantrowitz *et al.* (1980*a,b*).

The quaternary structure of the *E. coli* enzyme is not typical of ATCases generally; a wide range of phylogenetic variations in quaternary structure and regulatory behaviour are known. An interesting contrast with the *E. coli* enzyme is the wheat-germ enzyme, a simple trimer of similar size to the *E. coli* catalytic trimer, but which combines a well-developed regulatory function with catalysis (Yon *et al.*, 1982; Yon, 1984). We are attempting to address the following related questions of evolutionary interest. (1) Is the common trimeric structure evidence of

divergent evolution (i.e. are they descended from an archaic trimer)? (2) Can we discern common structural features concerned with catalysis? (3) Can we discern the specific structural differences that account for the presence of a regulatory function in the wheat-germ enzyme and its absence in the isolated catalytic trimer from *E. coli*?

The experiments described below are part of a series using the chemical-modification approach to answer the second question. Several amino acid residues with potential active-centre functions have been identified in the catalytic subunit of the *E. coli* enzyme by chemical reactivity, by site-specific mutagenesis and by protein crystallography (summarized in Krause *et al.*, 1985). The *E. coli* catalytic subunit and the wheat-germ enzyme have similar steady-state kinetic mechanisms, and the stereospecificity of the L-aspartate-binding site is essentially the same (Grayson *et al.*, 1979). Inactivation studies with phenylglyoxal suggested an arginine residue with similar properties in the two enzymes (Kantrowitz & Lipscomb, 1976; Cole *et al.*, 1986). Reactive and essential histidine residues, behaving nearly identically in the two enzymes, were identified by reaction with diethyl pyrocarbonate (Cole & Yon, 1986). However, sequencing around the labelled histidine residue in each case showed a complete

Abbreviations used: ATCase, aspartate transcarbamoylase; PALA, *N*-phosphonoacetyl-L-aspartate; PLP, pyridoxal 5'-phosphate.

* To whom correspondence should be addressed.

lack of homology among the adjoining residues (Cole & Yon, 1986). This work also confirmed the catalytic involvement of histidine-134 of the *E. coli* catalytic subunit, which had been suggested on crystallographic evidence (Krause *et al.*, 1985).

Protein crystallography of the *E. coli* ATCase complexed to PALA (Krause *et al.*, 1985) and hybridization of site-specific mutant ATCases (Wente & Schachman, 1987) implicated lysine-84 of the catalytic chain as an essential residue involved in substrate binding. The catalytic subunit is known to be inactivated by PLP (Greenwell *et al.*, 1973), and it has been shown (Kempe & Stark, 1975) that a lysine residue is specifically labelled by the reagent, the partial sequence around this residue suggesting that it is Lys-84 in the sequence reported by Hoover *et al.* (1983). The pyridoxylated lysine has proved a useful fluorescent probe (Matsumoto & Hammes, 1975; Hahn & Hammes, 1978). In the present paper we describe the effects of PLP on the wheat-germ enzyme and draw some comparisons with its reactivity towards the *E. coli* catalytic subunit.

EXPERIMENTAL

Materials

Wheat-germ ATCase was purified to near homogeneity as described previously (Yon, 1984). It was stored at room temperature in 50% (v/v) glycerol at 1 mg/ml (10 μ M), and used within a few weeks of purification. PLP and NaBH₄ were obtained from Sigma Chemical Co. and BDH Chemicals respectively, and were used without further purification. Substrates, buffers and UMP were from Sigma Chemical Co. or BDH Chemicals. PALA was a gift from Dr. G. R. Stark (Imperial Cancer Research Fund Laboratories, London W.C.2, U.K.). L-Alanyl-L-lysine was from Sigma Chemical Co.

Assay

ATCase activity was assayed as follows. The reaction mixture contained 1 mM-L-aspartate and 1 mM-carbamoyl phosphate and a 0.1 ml portion of the enzyme (diluted if necessary) in a total volume of 1.5 ml. It was buffered in 0.1 M-Tris/acetic acid, pH 8.5, at 25 $\pm$ 0.1 $^{\circ}$ C. The reaction was initiated by the addition of either carbamoyl phosphate or the enzyme, and terminated after 10 min by the addition of 1.5 ml of the strongly acidic reagent mixture of Prescott & Jones (1969). The colorimetric determination of carbamoylaspartate was completed as described by these authors.

Molar enzyme concentrations were calculated from A_{280} measurements, by using a molecular mass of 104 kDa (Yon *et al.*, 1982) and an absorption coefficient of 1.12 ml $\cdot$ mg⁻¹ $\cdot$ cm⁻¹ (Cole & Yon, 1986).

Reaction with PLP

The reaction buffer was freshly prepared 0.1 M-NaHCO₃/Na₂CO₃ buffer, pH 8.5. A portion of the stock enzyme was diluted as required into this buffer by passage through a column (15 cm $\times$ 1.5 cm) of Sephadex G-10. Stock solutions of PLP (50 mM) and NaBH₄ (2 M) were prepared in the same buffer immediately before use. The reaction was normally performed in foil-wrapped tubes to minimize photo-oxidation reactions. The reaction was initiated by adding PLP. At timed intervals thereafter, a sample of the reaction mixture was added to

a molar excess (100-fold over PLP) of NaBH₄. After 5 min the residual enzyme activity was assayed.

Spectroscopy

The formation of a Schiff base (λ_{max} 428 nm) or of its reduced pyridoxamine derivative (λ_{max} 325 nm) was detected spectroscopically. The reaction mixture was placed in the sample cell, while the reference cell contained enzyme only (Schiff base) or buffer plus reagents (reduced Schiff base). The reaction cell was only placed in the light-path when a reading was taken, being otherwise kept in the dark. Timed absorbance readings at a fixed wavelength (428 or 325 nm) were taken in a Perkin-Elmer Lambda 3 spectrophotometer. Schiff-base formation was quantified by using an absorption coefficient at 428 nm of 5600 M⁻¹ $\cdot$ cm⁻¹ (Giovane *et al.*, 1982). The reduced pyridoxamine derivative of the Schiff base was quantified by using an absorption coefficient at 325 nm of 9710 M⁻¹ $\cdot$ cm⁻¹ (Anderson *et al.*, 1966).

Isolation of a pyridoxamine-labelled peptide

The methods were essentially those used by Kempe & Stark (1975) on the enzyme from *E. coli*. Wheat-germ ATCase (0.5 mg) in 1.5 ml of 0.05 M-triethanolamine/HCl buffer, pH 8.0, was treated with 1 mM-PLP until one Schiff base per chain had formed (about 15 min). The Schiff base was reduced with 0.1 M-NaBH₄ for 30 min. The enzyme was then transferred into 0.05 M-ammonium bicarbonate buffer, pH 7.0, and reagents were removed, by filtering through a column of Sephadex G-10 (22 cm $\times$ 0.8 cm). Trypsin was added in a weight ratio of 1:100 and the mixture was incubated at 30 $^{\circ}$ C for 8 h. The mixture of tryptic peptides was loaded on a column (60 cm $\times$ 1.4 cm) of DEAE-cellulose (Whatman DE52) equilibrated in 0.05 M-ammonium bicarbonate buffer, pH 7.0. The column was developed with a gradient (0.05–0.5 M; 300 ml of each) of the same buffer, and 5 ml fractions were collected. Fractions containing pyridoxamine-labelled material (absorbing at 325 nm) were pooled and freeze-dried. The purity of this material was checked by high-voltage paper electrophoresis at pH 1.9 (formic/acetic acid buffer) and at pH 6.5 (pyridine/acetic acid buffer).

N-Terminal sequence determination

N-Terminal sequences of peptides were determined by the indirect dansyl-Edman technique, performed as described by Perham (1978). Dansylation of the peptides and of standard amino acids was performed as described by Weiner *et al.* (1972). Dansyl-amino acids were separated and identified by t.l.c. on double-sided polyamide plates (Gray, 1972). An authentic standard of N²-dansyl-N⁶-pyridoxamine-L-lysine was prepared as follows. A sample (100 μ l) of 40 mM-L-alanyl-L-lysine in 50 mM-triethanolamine/HCl buffer, pH 8, was treated for 30 min at 37 $^{\circ}$ C with 400 mM-PLP. The double Schiff base was reduced by reaction with 200 μ l of 5 M-NaBH₄ for 30 min. Excess borohydride was destroyed by adding 1 drop of acetic acid. The dipyridoxamine-dipeptide was purified by passage through a column of Sephadex G-10 (18 cm $\times$ 0.8 cm) in distilled water. Fractions absorbing at 325 nm were pooled and freeze-dried. The residue was hydrolysed for 15 h at 110 $^{\circ}$ C in 6 M-HCl, and freeze-dried again. After dansylation (Weiner *et al.*, 1972) the mixture contained pyridoxamine-L-alanine and N²-dansyl-N⁶-pyridoxamine-L-lysine. To our knowledge,

Fig. 1. T.l.c. of N^2 -dansyl- N^6 -pyridoxamine-L-lysine and other dansyl-amino acids on polyamide sheets

The origin is O and the modified lysine is X. Neighbouring spots include: 1, Dns-Pro; 2, Dns-Val; 3, Dns-Ile; 4, Dns-Leu; 9, Dns-Gly; 17, Dns- NH_2 . For other details see Perham (1978).

Fig. 2. Time course of enzyme inactivation and chemical modification

Residual activity was measured in the absence (■) and in the presence (●) of $50 \mu\text{M}$ -PLP. Chemical modification (no. of lysine residues per chain, ○) was determined after reduction of the Schiff base with NaBH_4 . The enzyme concentration was $1 \mu\text{M}$.

the position of this compound in relation to the two-dimensional reference map of Gray (1972) has not been shown previously, and is therefore included as Fig. 1.

RESULTS

Preliminary experiments

Wheat-germ ATCase was shown to be inactivated by PLP, concomitant with an increase in A_{428} signifying formation of a Schiff base. However, preliminary experiments showed that a partial re-activation and loss of the Schiff base occurred upon dilution of the modified enzyme into the assay mixture, during the relatively long assay period of 10 min. This complication necessitated the fixing of the equilibrium concentration of Schiff base by its reduction to the stable pyridoxamine derivative, using an excess of NaBH_4 . The addition of borohydride was shown to stop both the forward and the reverse reactions of Schiff-base formation. In subsequent experiments the modified enzyme was always fixed by reduction, the time between reduction and assay being standardized at 5 min. NaBH_4 alone had no apparent effect on the enzyme or on the assay.

Time course of enzyme inactivation and modification

As shown in Fig. 2, the enzyme ($1 \mu\text{M}$) was completely inactivated within 10 min by 1 mM of the reagent. The

Fig. 3. Effect of PLP concentration on the inactivation rate

The enzyme ($0.1 \mu\text{M}$) was treated with PLP at the following concentrations: $50 \mu\text{M}$ (□), $100 \mu\text{M}$ (■), $200 \mu\text{M}$ (△), $250 \mu\text{M}$ (●), $350 \mu\text{M}$ (○) and $500 \mu\text{M}$ (▲). Before assay the Schiff base was reduced with 0.05 M - NaBH_4 for 5 min. Inset: dependence of apparent first-order rate constant on PLP concentration. The curve is the best-fit hyperbola (Cornish-Bowden, 1976), for which the half-saturation concentration of PLP was $290 \pm 80 \mu\text{M}$.

loss of activity took place at the same rate as an increase in absorbance (after reduction) at 325 nm, signifying the formation of a pyridoxamine derivative, presumably at a lysine residue. Complete inactivation correlated closely in time with the incorporation of one pyridoxamine group per chain. The incorporation of subsequent groups, if any, was very slow by comparison. Since the enzyme is known to contain about 25 lysine residues per chain (Cole, 1986) it is concluded that inactivation is the result of Schiff-base formation at a unique, essential and highly reactive lysine residue.

Dependence of inactivation rate on reagent concentration

A semi-logarithmic time plot of the activation in Fig. 2 is linear, i.e. the inactivation follows pseudo-first-order kinetics. The inactivation was measured at a series of concentrations of PLP and at a constant concentration (1 μM) of the enzyme, with the result shown in Fig. 3. The apparent first-order rate constant, k_{app} (the slope of each line), is shown as a function of reagent concentration in the inset to Fig. 3. The relationship is hyperbolic and reminiscent of the Michaelis-Menten relationship of enzyme kinetics. The analogy strongly suggests that inactivation, like catalysis, proceeds within a rapidly formed non-covalent enzyme-reagent complex. Fitting these data to the hyperbolic equation by non-linear regression (Cornish-Bowden, 1976) gave a dissociation constant of $290 \pm 80 \mu\text{M}$.

Inactivation by pyridoxal

Since the carbamoyl phosphate pocket in the active centre binds phosphate, experiments were performed to test whether PLP also bound at this site, i.e. whether the reagent was active-centre-directed. Pyridoxal was found to inactivate the enzyme very slowly compared with PLP; 5 mM-pyridoxal produced only 5% inactivation after 20 min under conditions that were otherwise the same as used for Fig. 2. Benzaldehyde caused no activation. Hence the phosphate moiety of PLP, which is not involved directly in inactivation, is important, presumably in binding to the enzyme.

Competitive inhibition by PLP

The effects of PLP on the initial-rate kinetics of the enzyme were studied. In these experiments the production of carbamoylaspartate was shown to be linear with time for up to 5 min, i.e. no significant inactivation or re-activation was detected in this time. This was due partly to the short time and partly to the relatively low concentrations of PLP used. The concentrations of the inhibitor and carbamoyl phosphate were varied, while that of aspartate was kept constant at 1.0 mM. The Schiff base between aspartate and PLP has a dissociation constant of 14 mM (Greenwell *et al.*, 1973), and therefore the substrate and inhibitor were not significantly depleted by its formation.

With carbamoyl phosphate as the substrate whose concentration was varied, inhibition by PLP gave the classic pattern for competitive inhibition, i.e. different concentrations of PLP gave different slopes but the same intercept in double-reciprocal plots. A replot of slope versus concentration of PLP was linear; regression analysis of this line gave an inhibition (dissociation) constant for PLP of $38 \pm 13 \mu\text{M}$. The disagreement with the apparent dissociation constant ($290 \mu\text{M}$) obtained

from the inactivation study (see above) is discussed below (see the Discussion section).

Effects of ligands on inactivation

The effects of including active-centre ligands and the end-product inhibitor UMP on the rate of inactivation of the enzyme were studied. In each case the ligand was tested at saturation and at an approximately half-saturating concentration based on enzyme kinetics (Grayson *et al.*, 1979; Yon, 1984).

All ligands provided some protection of the reactive lysine. We define the percentage protection by a ligand at saturation as follows:

$$\text{Protection (\%)} = 100 \times (k_0 - k_{\text{sat.}}) / k_0$$

where k_0 and $k_{\text{sat.}}$ are the apparent first-order rate constants for inactivation when the ligand concentration is zero and saturating respectively. The protection given by each ligand at saturation was: carbamoyl phosphate alone, 82%; succinate in the presence of carbamoyl phosphate, 89%; PALA, 96%; UMP, 52%.

The degree of protection provided by half-saturating concentrations of ligands correlated reasonably well with that expected from kinetic studies, i.e. approximately half the saturating level of protection was given by a ligand concentration that was half-saturating by enzyme-kinetic criteria. These findings strongly support the conclusion that the reagent binds at the active centre.

Isolation of a pyridoxamine-containing peptide

A sample of wheat-germ ATCase modified to the extent of one lysine residue per chain was digested with trypsin and subjected to DEAE-cellulose chromatography as described in the Experimental section. The elution profile is given in Fig. 4. Except for the fact that a single prominent peak of labelled material is evident, this profile bears little resemblance to that of the same experiment on the *E. coli* catalytic subunit as reported by Kempe & Stark (1975). The major peak of labelled material in fractions 30–40 was pooled, freeze-dried and shown to consist of a single peptide by high-voltage paper electrophoresis at pH 1.9 and pH 6.5. This peptide is designated T1. One half of it was subjected to *N*-terminal sequencing. The other half was digested with elastase and two subfragments, designated E1 and E2, were purified from the digest by paper electrophoresis. Their sequences were also determined.

Identification of a labelled lysine residue and adjoining sequence

Fig. 5(a) shows the sequence of the first five residues of peptide T1, and the complete sequences of fragments E1 (a tetrapeptide) and E2 (a tripeptide). The pyridoxamine-labelled lysine residue was located in peptides T1 and E2 as indicated; it was identified unambiguously by reference to an authentic standard (see the Experimental section). Peptide E1, which ends in arginine, is clearly the C-terminus of peptide T1. By comparing the sequences of peptides E2 and E1 with the amino acids in the unsequenced part of peptide T1 (shown in parentheses), and assuming no duplication of these residues, peptide T1 may be tentatively denoted by the 11-residue peptide shown in the lower line of Fig. 5(b). The first seven residues are assigned without ambiguity; the modified lysine is residue 5 in the sequence.

For comparison, the upper line of Fig. 5(b) gives the

(a) N-Terminal sequence of peptide T1 and its elastase subfragments E1 and E2. (b) Putative sequence of T1 and comparison with the sequence Ser-80 to Thr-90 of the catalytic chain of *E. coli* ATCase (Hoover *et al.*, 1983). Lys(P) denotes the pyridoxamine-labelled lysine.

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In its reaction with the enzyme, PLP appears to be an active-site-directed reagent. This is not surprising, since it is known that many organic phosphates will bind at the carbamoyl phosphate-binding site of the *E. coli* enzyme. In summary, the evidence that this is so in the present case is as follows: (a) the hyperbolic dependence of the rate constant $k_{app.}$ on reagent concentration suggests a Michaelian intermediate in the reaction; (b) the reagent is a competitive inhibitor of the normal catalysed reaction; (c) protection against inactivation is provided by active-centre ligands; (d) pyridoxal is far less reactive than PLP. An apparent discrepancy in the results is the different dissociation constants for PLP given by inactivation and competitive inhibition (290 μM and 38 μM respectively). A plausible reason for this discrepancy is found on inspection of the following model, which is analogous to the simple Briggs-Haldane model of single-substrate enzyme kinetics:

The two substrates, carbamoyl phosphate and L-aspartate, are bound in an obligatory order with carbamoyl phosphate leading (Grayson *et al.*, 1979). Protection by active-centre ligands against inactivation by PLP agrees with this order. L-Aspartate (or its analogue, succinate) alone provides no protection (result not reported), whereas carbamoyl phosphate alone and in combination with succinate provides 82% and 89% protection respectively. The greatest protection (96%) was provided by the tight-binding bisubstrate analogue PALA. The relatively low protection afforded by the effector compound UMP (52%) is consistent with it binding at a site removed from the active centre, but inducing a conformational change that affects the active centre, as has been proposed elsewhere (Yon, 1972; Cole

& Yon, 1984). However, the location of the UMP-binding site is still debatable (Yon, 1984).

Greenwell *et al.* (1973) showed that PLP is an active-site directed inhibitor of the catalytic subunit of the *E. coli* ATCase, and Kempe & Stark (1975) showed that a unique lysine residue was pyridoxylated in the reaction. In the revised sequence (Hoover *et al.*, 1983) this is Lys-84. Since the wheat-germ enzyme reacts in a very similar fashion with PLP, one might expect some primary sequence homology in the vicinity of the reactive lysine residue. As shown in Fig. 5, very little homology was found. A notable difference between the sequences is the adjacent pair of lysine residues (residues 83 and 84) in the *E. coli* enzyme; since the wheat-germ enzyme lacks a homologous residue, Lys-83 of the *E. coli* enzyme sequence is presumably not an essential lysine residue. The involvement of Lys-84 in substrate binding has been shown in the crystal structure of the dodecameric ATCase complexed to the bisubstrate analogue PALA, i.e. in the R-conformation (Krause *et al.*, 1985; Volz *et al.*, 1986). The ligand is bound near the interface between adjacent C-chains in the same trimer. Most of the binding interactions come from one of these chains, but contributions are also made from Lys-84 and Ser-80 in the adjacent chain. Lys-84 is bound to both the phosphonate and to the α -carboxylate moieties of the ligand. This suggests binding to the phosphate of carbamoyl phosphate and the α -carboxy group of aspartate, and leads to the speculation that Lys-84 may play an important role in drawing the reactive groups together in the carbamoyl-transfer reaction (Volz *et al.*, 1986), a mechanism reminiscent of the compression model for ATCase first suggested by Collins & Stark (1969). The essential lysine residue of the wheat-germ enzyme may play a similar role. However, in the allosteric *E. coli* enzyme, Lys-84 in the adjacent chain is only drawn into position for ligand binding during the conformational transition from the resting state (T-conformation) to the R-conformation of the enzyme (Krause *et al.*, 1985). In view of the different regulatory mechanisms of the two enzymes, it will be of considerable interest to know how the essential lysine residue of the wheat-germ enzyme is located in relation to the bound substrates, and whether it is similarly translocated in an allosteric conformational change.

Finally, this is the second occasion upon which we have identified a residue with functionally similar properties in the wheat-germ and *E. coli* ATCases, which upon sequence analysis turned out to be embedded in non-homologous local primary sequences. In a recent paper (Cole & Yon, 1986) we showed such a relationship between His-134 of the *E. coli* enzyme and an essential histidine residue in the wheat-germ enzyme. Whether these lysine and histidine residues are in similar three-dimensional environments in the two enzymes will only be clear when further progress on the structure of the wheat-germ enzyme has been made.

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