Inactivation of wheat-germ aspartate transcarbamoylase by the arginine-specific reagent phenylglyoxal

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Wheat-germ aspartate transcarbamoylase (EC 2.1.3.2) was inactivated by phenylglyoxal in a first-order process, provided that the inactivation time did not exceed 10 min. Apparent first-order rate constants were linearly dependent on phenylglyoxal concentration, indicating a bimolecular reaction between a single active-centre residue and phenylglyoxal, with second-order constant of $0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$. A plot of apparent first-order rate constant versus pH showed a steep rise above pH 9.5, indicating that the essential residue has a pK_a value of 10.5 or higher, consistent with an arginine residue. Saturating concentrations of the following ligands provided a degree of protection (percentages in parentheses) against 1 mM-phenylglyoxal: *N*-phosphonoacetyl-L-aspartate, a bisubstrate analogue (94%); carbamoyl phosphate (75%); UMP, an end-product inhibitor (53%). Succinate (an analogue of L-aspartate) alone gave no protection, but in combination with carbamoyl phosphate raised the protection to 92%, in agreement with the known binding order of the two substrates. These results indicate that the essential arginine residue is close to the carbamoyl phosphate site, probably oriented towards the aspartate site. Attempts to desensitize the UMP-binding site by reaction with phenylglyoxal, while protecting the active centre, were unsuccessful. The essential active-centre arginine residue is compared with a similar residue in the *Escherichia coli* enzyme.

INTRODUCTION

Aspartate transcarbamoylase (EC 2.1.3.2) has been purified from wheat germ and shown to be a trimer of 37000 Da chains (Yon *et al.*, 1982). In gross quaternary structure the enzyme resembles the catalytic subunit of the well-studied aspartate transcarbamoylase from *Escherichia coli*, a trimer of 33000 Da chains (reviewed by Kantrowitz *et al.*, 1980*a,b*). However, whereas the allosteric features of the *E. coli* enzyme are only expressed in a 300000 Da complex of distinct catalytic and regulatory subunits, the wheat-germ trimeric enzyme shows all the features of an allosteric enzyme without the addition of regulatory subunits (Yon, 1972, 1984). It would be of interest to understand the structural and functional basis of this divergence in regulatory properties. A relevant question is: to what extent have active-centre residues been conserved?

Functionally important residues in the active centre of the *E. coli* enzyme catalytic subunit have been studied by chemical modification, crystallography and site-specific mutagenesis (reviewed in Krause *et al.*, 1985). We are engaged in preliminary studies of the wheat-germ enzyme that will allow comparison with this published work. Structural conservation in the catalytic-centre region is expected on the basis of similar catalytic mechanisms and stereospecificity in the binding pocket for L-aspartate (Grayson *et al.*, 1979). Since both substrates, carbamoyl phosphate and L-aspartate, are anionic, their binding pockets are likely to contain lysine and/or arginine residues. Phenylglyoxal has been used to modify arginine residues in many proteins, usually with high specificity (Takahashi, 1968; Riordan, 1979). Kantrowitz &

Lipscomb (1976) showed that the *E. coli* enzyme catalytic subunit is completely inactivated by the reaction with phenylglyoxal of one arginine residue per polypeptide chain, and that this residue appears to be in the binding pocket for carbamoyl phosphate. A similarly situated arginine residue was found in the related enzyme ornithine transcarbamoylase (Marshall & Cohen, 1980). We report in the present paper the effects of phenylglyoxal on the activity of wheat-germ aspartate transcarbamoylase.

EXPERIMENTAL

Materials

Aspartate transcarbamoylase was purified from wheat germ as described by Yon (1981), with the exception that the two organic-solvent fractionation steps were combined. The enzyme was more than 96% pure, as indicated by polyacrylamide-gel electrophoresis of the native enzyme and of the SDS-denatured enzyme. The enzyme was stored at 1 mg/ml (approx. $10 \mu\text{M}$) in 50% (v/v) glycerol at room temperature.

Phenylglyoxal was obtained from Sigma Chemical Co. and was used without further purification. Substrates, buffers and UMP were from sources described previously (Grayson *et al.*, 1979). *N*-Phosphonoacetyl-L-aspartate was a gift from Dr. G. R. Stark (Imperial Cancer Research Fund Laboratories, London W.C.2, U.K.).

Methods

Except when the pH was varied (see Fig. 2 legend) reaction of enzyme and phenylglyoxal was performed in

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0.1 M- NaHCO_3 adjusted to pH 9.5 by addition of Na_2CO_3 . The final concentration of the enzyme was 0.1 μM ; concentrations of phenylglyoxal and ligands are given in the Figure legends. The reaction mixture, without phenylglyoxal, was equilibrated at 25 °C. Reaction was initiated by adding phenylglyoxal, and at timed intervals 0.1 ml samples were removed for enzyme assay as described by Cole & Yon (1984). Trial experiments showed that, under the protection of substrates (see the Results section) and with phenylglyoxal diluted 15-fold, there was negligible further inactivation during the assay period of 10 min. In the absence of phenylglyoxal, no loss in enzyme activity was detected for at least 30 min.

In experiments aimed to detect desensitization at the UMP-binding site the active centre was protected by an excess of the bisubstrate analogue *N*-phosphonoacetyl-L-aspartate. In these experiments both the inhibitor and phenylglyoxal were removed before the assay, by gel filtration on columns (10 ml) of Sephadex G-10 equilibrated with the assay buffer.

RESULTS

Trial experiments in several buffer systems and phenylglyoxal concentrations showed that the enzyme was inactivated in a process that was apparently first-order for the first 10 min at least, but thereafter became more complex. The results reported in the present paper were all taken in the early phase.

Kinetics of inactivation

Fig. 1 shows the effect of various phenylglyoxal concentrations on the early phase of inactivation at pH 9.5. The constant enzyme concentration was 0.1 μM . At each concentration of phenylglyoxal, the logarithm of the residual activity was a linear function of time, showing that inactivation was apparently first-order. The slope (apparent first-order rate constant) increased linearly over the whole range of tested phenylglyoxal concentrations (Fig. 1 inset), indicating a bimolecular reaction between phenylglyoxal and a single active-centre residue. Since there was a large molar excess of phenylglyoxal over enzyme, the second-order rate constant is the slope of the inset to Fig. 1, i.e. 0.023 $\text{mM}^{-1}\cdot\text{min}^{-1}$. Concentrations of phenylglyoxal higher than 10 mM could not be tested, since they inactivated the enzyme too rapidly to be timed by our methods.

pH-dependence of apparent first-order rate constant

Phenylglyoxal usually reacts with high specificity towards arginine residues in proteins (Takahashi, 1968; Riordan, 1979). To confirm that this is the case for wheat-germ aspartate transcarbamoylase, we investigated the dependence of the inactivation rate constant on pH in the range pH 7–10.5, the upper limit at which the enzyme activity is stable for more than 30 min. The result is shown in Fig. 2. The sharp rise in k_{app} above pH 9.5 indicates an increase in the concentration of the reactive (deprotonated) form of a group having a $\text{p}K_a$ of 10.5 or greater. Since the curve shows no sign of approaching a plateau, precise estimation of a $\text{p}K_a$ value is not feasible. Nevertheless, the evidence is clearly consistent with a reactive arginine residue.

Fig. 1. Effect of phenylglyoxal concentration on rate of inactivation of wheat-germ aspartate transcarbamoylase

Phenylglyoxal concentrations were 0 mM (○), 1 mM (●), 3 mM (△), 5 mM (▲) and 10 mM (□). The enzyme was at 0.1 μM . Measurements were in 0.1 M- $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer, pH 9.5. Inset: dependence of apparent first-order rate constant on phenylglyoxal concentration.

Fig. 2. Effect of pH on apparent first-order rate constant for the inactivation of wheat-germ aspartate transcarbamoylase by phenylglyoxal

Buffers were as follows: pH 7–9, 0.1 M-Tris/HCl containing 10 mM- NaHCO_3 ; pH 9–10.5, 0.1 M- $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$. Inactivation was by 1 mM-phenylglyoxal in each case. The enzyme was at 0.1 μM .

Effects of enzyme ligands on the apparent first-order rate constant

If the essential arginine residue(s) has a functional role in the binding of an anionic substrate, inhibitor or other ligand, then one may expect this ligand to protect against

Fig. 3. Effects of wheat-germ aspartate transcarbamoylase ligands on inactivation by 1 mM-phenylglyoxal

Inactivation was in 0.1 M- $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer, pH 9.5. The enzyme was at 0.1 μM . (a) Effect of carbamoyl phosphate at 0 μM (●), 20 μM (○), 50 μM (▲) and 100 μM (△). (b) Effect of carbamoyl phosphate at 20 μM plus succinate at 0 mM (●), 1 mM (○), 2 mM (▲) and 5 mM (△). (c) Effect of *N*-phosphonacetyl-L-aspartate at 0 μM (●), 0.001 μM (○), 0.01 μM (▲), 0.1 μM (△) and 1.0 μM (■). (d) Effect of UMP at 0 μM (●), 5 μM (○), 10 μM (▲) and 20 μM (△).

inactivation by phenylglyoxal. Aspartate transcarbamoylase has several such ligands. Fig. 3 shows the inactivation of a constant concentration of the enzyme by 1 mM-phenylglyoxal at pH 9.5, in the presence of each of these ligands. Each ligand was tested at several different concentrations, up to and including saturation.

In every case k_{app} was decreased in a saturable manner by increasing ligand concentration. However, in no case was k_{app} reduced to zero, i.e. no ligand gave complete protection against phenylglyoxal, even when saturating. From the trend towards saturation, it was possible to make a rough estimate of the ligand concentration providing half-maximal protection. For each ligand this was in good agreement with earlier, independent, estimates of the dissociation constant (Grayson *et al.*,

1979; Yon, 1984; Cole & Yon, 1984), confirming that the protective effects were indeed due to ligand binding.

In order to compare the protective effects of ligands, protection (%) is defined as $100 \times (k_0 - k_{\text{sat}})/k_0$, where k_0 and k_{sat} are the values of k_{app} at zero and saturating ligand concentrations respectively. Carbamoyl phosphate is the first substrate to bind in an obligatory-order mechanism (Grayson *et al.*, 1979). Saturation by this ligand provided a moderate degree of protection (75%). Succinate (an analogue of the second substrate, L-aspartate) alone had no effect, as expected from the binding order. However, in combination with carbamoyl phosphate it raised the degree of protection to 92%. This is very close to the protection afforded by the tight-binding bisubstrate analogue *N*-phosphonacetyl-L-aspartate

(94%). These results suggest that the essential arginine residue is located near the periphery of the carbamoyl phosphate-binding site, probably oriented towards the aspartate-binding site.

UMP is a potent end-product inhibitor of wheat-germ aspartate transcarbamoylase. At saturation UMP provided considerably less protection (53%) than either carbamoyl phosphate or *N*-phosphonoacetyl-L-aspartate, possibly indicating that, for this ligand, protection of an active-centre arginine residue is transmitted by a conformational (allosteric) effect (however, see the Discussion section).

If phenylglyoxal reacts with arginine residue(s) in the UMP-binding site, one would expect a progressive decrease in the inhibitory ability of UMP with time. To test for this, phenylglyoxal was allowed to react with the enzyme in the presence of saturating *N*-phosphonoacetyl-L-aspartate to provide maximal protection of the active centre. The residual activity and the percentage inhibition of this activity by 0–1 mM-UMP were measured after various times. After 10 min, i.e. when over 80% of unprotected active centres would have reacted, there was negligible loss in sensitivity towards UMP. Although this does not disprove the presence of arginine residues in the UMP-binding site, any such residues must be modified at a low rate by comparison with active-centre arginine residues.

DISCUSSION

Phenylglyoxal shows a high degree of specificity in its reaction with arginine residues, both in model compounds and in proteins (Takahashi, 1968; Riordan, 1979; Cheung & Fonda, 1979). We assume here that inactivation of wheat-germ aspartate transcarbamoylase is the result solely of arginine-residue modification. The pH profile of inactivation is consistent with this assumption, although the exact pK_a value of the residue could not be estimated.

The present results indicate that inactivation is the result of a bimolecular encounter between a single active-centre arginine residue and phenylglyoxal, with a second-order rate constant of $0.023 \text{ mM}^{-1} \cdot \text{min}^{-1}$. Inactivation of intact *E. coli* aspartate transcarbamoylase by phenylglyoxal was studied by Kantrowitz & Lipscomb (1976). Although the result is not explicitly discussed in their paper, measurements taken from their Fig. 1 confirm that the inactivation rate constants were also linearly dependent on phenylglyoxal concentration, suggesting bimolecularity. Allowing for the different pH values (8.3 and 9.5) of the two sets of measurements, and the pH-dependence of the *E. coli* enzyme (Kantrowitz & Lipscomb, 1976), the second-order rate constant of the wheat-germ enzyme is the smaller by a factor of about 3-fold. The two constants are therefore remarkably close. In the case of the *E. coli* enzyme it was shown that the arginine residue in question was uniquely reactive, and was the first to be attacked by phenylglyoxal. On the present evidence we cannot reach the same conclusion for the wheat-germ enzyme, since we have not studied the chemical modification of arginine residues directly, e.g. by incorporating radiolabelled phenylglyoxal.

Comparison of the protective effects of active-centre

ligands suggests that, although no ligand protects the residue completely, the essential arginine residue is partially shielded by both substrates, the contribution of carbamoyl phosphate being the greater. It is proposed that the residue occupies a relatively exposed location at the periphery of the carbamoyl phosphate-binding site oriented towards the aspartate-binding site. This conclusion is qualitatively similar to that reached by Kantrowitz & Lipscomb (1976), who studied the catalytic subunit of the *E. coli* enzyme; as has already been noted, the second-order rate constants for the two enzymes are within an order of magnitude. Marshall & Cohen (1980) similarly found an essential arginine residue in the carbamoyl phosphate-binding site of the related enzyme ornithine transcarbamoylase. One is tempted to speculate that these are examples of a conserved arginine residue with a vital function in all carbamoyltransferases.

Although they do not rule out the possibility, our experiments give no clear evidence of arginine residues in the regulatory UMP-binding site of the wheat-germ enzyme. The binding of UMP does, however, provide protection of active-centre arginine residues, since saturation with UMP results in 53% protection against inactivation. This protection could conceivably arise in two ways, as follows. (1) The UMP-binding site is spatially remote from the active centre and exerts protection by a conformational change; we have independent evidence that UMP binding produces an extensive conformational change in the enzyme (Cole & Yon, 1984). (2) The UMP-binding site partially overlaps the active centre, thereby shielding the essential residue, albeit less effectively than substrates do; this explanation would be consistent with a tentative suggestion by one of us (Yon, 1984) that UMP inhibits competitively. The location of the UMP-binding site remains an unresolved question at present.

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