Reactivity of the Active-Centre Lysine Residue of Rabbit Muscle Aldolase

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The method of competitive labelling with [^3H]acetic anhydride as the labelling reagent was used to determine the properties of the active-centre lysine residue of rabbit muscle aldolase. This residue is much less reactive than a normal exposed lysine residue towards this reagent, and its reactive properties did not parallel the pH-activity profile for aldolase. At higher pH values it became reactive, but this was shown to be due to disruption of the enzyme structure. The binding of the competitive inhibitor phosphate did not alter the reactive properties. It is concluded that the active-centre lysine has an apparent pK_a greater than 11.5 and probably is made nucleophilic during the catalytic process, perhaps by proton abstraction.

There are two classes of fructose 1,6-diphosphate aldolases. Class II aldolases are found in primitive life forms such as bacteria and yeast and require the presence of bivalent metal ions for activity. Class I aldolases, which do not require metals for activity, are found in higher life forms and are inactivated by NaBH_4 in the presence of the substrate dihydroxyacetone phosphate (Rutter, 1964). This inactivation has been shown to be the result of the reduction of a ketimine Schiff base formed by dihydroxyacetone phosphate and the ϵ -amino group of a single lysine residue of the aldolase polypeptide chain (Grazi *et al.*, 1962). Isotope-exchange studies using H_2^{18}O (Model & Rittenberg, 1968) and the reversal of cyanide inactivation by dihydroxyacetone phosphate (Cash & Wilson, 1966) indicate that Schiff-base formation is an early step in the aldol-cleavage mechanism. It is known that a deprotonated amino group is necessary for Schiff-base formation, and since the pH optimum of class I aldolases occurs in the range pH 6.5–8.0 (Dounce *et al.*, 1950) an abnormally low pK_a may account for the reactivity of the active-site ϵ -amino group. This view has been postulated by Horecker (1972) for aldolase on the basis of the work of Schmidt & Westheimer (1971) on the Schiff-base-forming enzyme acetoacetate decarboxylase. Since this point is crucial to an understanding of the mechanism of action of aldolase, it is necessary to establish experimentally the properties, namely ionization constant and reactivity, of the ϵ -amino group of the active-centre lysine residue.

The reactivity and ionization constant of individual amino groups can be determined by the technique of competitive labelling (Kaplan *et al.*, 1971). Since labelled peptides derived from the functional group being studied are isolated, this approach was used in the present investigation as a means of assigning

these parameters to the ϵ -amino group of the active-centre lysine of aldolase.

Experimental

Materials

Rabbit muscle aldolase was obtained as an $(\text{NH}_4)_2\text{SO}_4$ suspension from Sigma Chemical Co., St. Louis, Mo., U.S.A. and was dialysed against 0.1 M-KCl before use. α -Chymotrypsin was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. [^3H]Acetic anhydride (specific radioactivity 5.9 Ci/mmol) and [^{14}C]acetic anhydride (specific radioactivity 5.1 mCi/mmol) were obtained from Amersham/Searle Corp., Arlington Heights, Ill., U.S.A. All other chemicals were of reagent grade and were used without further purification.

Methods

Trace labelling procedure. Rabbit muscle aldolase (30 mg, 0.75 μmol of subunit) was dialysed against 0.1 M-KCl at 4°C. L-Phenylalanine (1 μmol) was added and the sample was diluted to 2.5 ml with 0.1 M-KCl and placed in a thermostatically controlled water-jacketed N_2 -flushed cell at $10.0 \pm 0.05^\circ\text{C}$ and left for 5 min to reach temperature equilibrium. Some samples were also made 0.1 M in phosphate with potassium phosphate. The pH of each sample was adjusted to the desired value with 2 M-KOH, and 0.3 μmol of [^3H]acetic anhydride (specific radioactivity 5.9 Ci/mmol) in 25 μl of acetonitrile was added with stirring. After 5 min sufficient conc. HCl was added to lower the pH to 2 and urea was added to a final concentration of 8 M. The pH was then adjusted to 8 with 2 M-KOH and maintained at this value during four 25 μl additions of unlabelled acetic anhydride.

Purification of acetylphenylalanine. After acetylation in 8M-urea, the pH of the reaction mixtures was lowered to 2 with conc. HCl and the solutions were extracted with 3×5 ml of ethyl acetate. The combined ethyl acetate extracts were dried under a stream of N_2 and then dissolved in $100\mu\text{l}$ of 0.05M-NH_3 and spotted in a 10cm band on Whatman 3MM paper. Marker [^{14}C]acetylphenylalanine was spotted at each end of the sample band and electrophoresis at pH6.5 was carried out in an apparatus described by Brown & Hartley (1966) for 1 h at a voltage gradient of 60V/cm. The acetylphenylalanine was located by radioautography after electrophoresis, eluted from paper with 0.05M-NH_3 and dried under vacuum.

Preparation of radioactive marker peptide containing the active-site lysine residue. A sample (80mg) of rabbit muscle aldolase was carboxymethylated with iodoacetic acid and treated with CNBr as previously described (Anderson *et al.*, 1969). The resulting peptides were separated on a column ($2.5\text{cm} \times 90\text{cm}$) of Sephadex G-25 (superfine grade) (Gibbons *et al.*, 1970). A peptide, designated as X3 (Anderson *et al.*, 1969), has been sequenced and is known to contain three lysine residues, one of which is the active-site lysine (Lai & Oshima, 1971). A sample (5mg) of this peptide was dissolved in 2.5ml of 0.1M-KCl and $100\mu\text{l}$ of [^{14}C]acetic anhydride was added in $25\mu\text{l}$ portions, the pH being maintained at 8 by using a pH-stat with 5M-KOH as titrant. Since the resulting acetylated peptide was water insoluble, it was purified by dialysis against water and then freeze-dried. This peptide was then used as a source of radioactive marker (Kaplan *et al.*, 1971) in subsequent peptide isolations.

Isolation of a peptide containing the active-site lysine residue from trace-labelled samples. After acetylphenylalanine had been removed by extraction with ethyl acetate, the samples of acetylated aldolase were dialysed against water and freeze-dried. Carboxymethylation with iodoacetic acid and treatment with CNBr were carried out as previously described (Anderson *et al.*, 1969) and the samples were freeze-dried. The samples treated in this way were suspended in 2.5ml of 0.5% NH_4HCO_3 and 1mg of chymotrypsin was added. The samples became clear within a few minutes and were left to digest for 16h at 37°C , and then they were freeze-dried. The digested samples were dissolved in $100\mu\text{l}$ of 0.05M-NH_3 and spotted on Whatman 3MM paper in 10cm bands with [^{14}C]acetylated marker peptide, which had been digested in an identical manner, being applied at each end of the bands. The following purification steps were carried out, by using radioautography to locate the peptides after each step. (1) Electrophoresis at pH6.5 for 1 h at a voltage gradient of 60V/cm. (2) The neutral band at pH6.5 was subjected to chromatography in BAWP (butanol-acetic acid-water-pyridine, 15:3:12:10, by vol.). (3) A band

($m = 0.4$ relative to $\epsilon\text{-Dnp-lysine}$) in BAWP was subjected to electrophoresis at pH3.5. (4) A band ($m = 2.0$ relative to $\epsilon\text{-Dnp-lysine}$) was cut out and the radioactive marker at either end was removed. (5) The ^3H -labelled peptides were eluted from the paper with 0.05M-NH_3 and freeze-dried.

Determination of specific radioactivities of isolated peptides and internal standards. Isolated peptides and internal standards were dissolved in $150\mu\text{l}$ of 0.01M-HCl containing 81.6nmol of norleucine. A sample ($25\mu\text{l}$) of each was added to scintillation vials containing 15ml of Aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.) and radioactivity was determined by scintillation counting in a Beckman LS250 liquid-scintillation counter. For amino acid analysis $50\mu\text{l}$ samples were mixed with equal volumes of 12M-HCl and hydrolysed at 110°C for 22h in evacuated sealed tubes. The amino acids were identified and determined on a Technicon TSM1 amino acid analyser by a single-column procedure.

Ultracentrifugation. Aldolase was dialysed against 0.1M-KCl and adjusted to the desired pH with 2M-KOH immediately before centrifugation. Ultracentrifugation was carried out at 20°C at a protein concentration of 3mg/ml in a Spinco model E Ultracentrifuge equipped with phase-plate schlieren optics. Pictures were taken at 18-min intervals after the maximum speed of 60000rev./min was reached.

Results

The composition of the peptide isolated in the present study was:

Lys	-	Pro	-	Asn	-	Hsr*
1.0	0.90	0.90	0.80			

The amino acid sequence of this peptide, which contains the active-site lysine residue, was determined in a previous study (Gibbons *et al.*, 1970). The *N*-terminal was lysine by the dansyl method (Gray, 1967).

The specific radioactivity of this peptide isolated from each trace-labelled sample was determined. Table 1 shows the various conditions of trace labelling and the specific radioactivities of the subsequently isolated peptides containing the active-site lysine as well as the specific radioactivities of the isolated internal standard. The relative rate constants, αr , shown were calculated from these data and from a previously determined pK_a of 9.50 for phenylalanine under these conditions (Kaplan, 1972) by using the equation:

$$\alpha r = \alpha_s \times \frac{\text{Sp. radioactivity of peptide}}{\text{Sp. radioactivity of acetylphenylalanine}}$$

(Kaplan *et al.*, 1971), where α_s and α are the degrees of dissociation of the amino group of phenylalanine

*Abbreviation: Hsr, homoserine.

Table 1. Summary of competitive labelling data for the active-site lysine residue of rabbit muscle aldolase with acetic anhydride at 10°C

N-Acetylphenylalanine and the active-site lysine peptides were isolated and the specific radioactivities determined as described in the text.

pH	Solvent	$10^{-6} \times$ Specific radioactivity of <i>N</i> -acetylphenylalanine (c.p.m./ μ mol)	α_s	$10^{-6} \times$ Specific radioactivity of active-site lysine peptide (c.p.m./ μ mol)	αr
7.00	0.10M-KCl	3.92	0.00315	0.281	0.00226
7.62	0.10M-KCl	6.47	0.0130	0.595	0.00120
8.02	0.10M-KCl	7.03	0.0320	0.488	0.00222
8.60	0.10M-KCl	15.6	0.112	0.386	0.00277
8.98	0.10M-KCl	20.1	0.232	0.626	0.00722
9.45	0.10M-KCl	21.0	0.471	0.745	0.0167
10.00	0.10M-KCl	17.9	0.760	1.40	0.0594
10.49	0.10M-KCl	10.4	0.907	1.81	0.157
11.06	0.10M-KCl	4.92	0.973	1.98	0.391
11.58	0.10M-KCl	3.85	0.992	2.82	0.727
9.51	0.10M-Phosphate- 0.10M-KCl	15.9	0.506	0.487	0.0154
10.02	0.10M-Phosphate- 0.10M-KCl	10.4	0.767	0.738	0.0544

Fig. 1. Plot of αr versus pH for the data obtained in this study ($\circ$)

The solid line is the curve obtained for a normal lysine group (lysine-224 of elastase) which has pK_a 10.3 and a reactivity, r , 2.5 times that of phenylalanine (Kaplan *et al.*, 1971).

and the ϵ -amino group of the active-site lysine respectively, and r is the ratio of the second-order velocity constant for the reaction of the unprotonated ϵ -amino group of the active-site lysine with acetic anhydride to that of the unprotonated amino group of phenylalanine.

Fig. 1 shows a plot of αr versus pH for the active-site lysine residue of aldolase. The curve for a normal lysine residue (lysine-224 of elastase, pK_a 10.3) is in-

cluded for comparative purposes. Over the pH range examined the active-site lysine is much less reactive than a normal lysine residue. Phosphate is a competitive inhibitor of aldolase (Velick, 1949), but its presence makes no difference to the reactivity of the active-site lysine.

It was apparent that the active-site lysine residue only became reactive at high pH. The possibility that this reactivity resulted from a general loss of structure accompanying alkaline denaturation was examined by observing the effect of pH on the ultracentrifugation pattern of aldolase. The schlieren patterns obtained over the pH range 8–12 showed that at pH values above 11, where the reactivity of the active-site lysine residue is increasing, the enzyme dissociated into subunits.

Discussion

The present study shows that the active-site lysine residue of aldolase has a very low reactivity towards the small uncharged reagent acetic anhydride over the pH range at which the enzyme is active. The residue approaches the reactivity expected of an ϵ -amino group only at pH values where the enzyme is undergoing gross structural changes, as evidenced by a loss of quaternary structure. Since reactivity was accompanied by gross loss of structure, it is only possible to state that the lysine residue has an apparent pK_a greater than 11.5 in native aldolase. It may be argued that the deprotonated form of this lysine residue is not accessible to acetic anhydride. However, this would require that the ϵ -amino group be in a non-polar environment yet accessible to water and a polar substrate and not to acetic anhydride. We consider that this is unlikely, especially in view of the available

X-ray-crystallographic evidence (Blow & Steitz, 1970), which shows that amino groups are only shielded from solvent when they form salt linkages. The possibility that the properties of the lysine residue are altered by a neighbouring positively charged group involved in phosphate binding was examined. No effect on reactivity was observed when trace labelling was carried out in the presence of 0.1M-phosphate. This finding indicates that phosphate binding does not make the active-site lysine residue more reactive by altering its properties.

On the basis of previous arguments about the properties of the active-centre lysine residue of aldolase (Horecker, 1972) it might be expected that this residue would have either an abnormally low apparent acid dissociation constant or an abnormally high reactivity. The present study suggests that the reactivity of this residue towards dihydroxyacetone phosphate does not arise from either of these possibilities and that its reactivity is therefore derived from specific interactions that occur when the enzyme binds a suitable substrate. A conformational change in the enzyme is thought to accompany substrate binding (Mehler & Cusic, 1967). One possible explanation for the observed properties is that the active-centre lysine residue is made nucleophilic by a concerted proton-abstraction process, similar to the process in the serine proteinases (Blow *et al.*, 1969), which makes a previously non-reactive serine residue become reactive. However, since the lysine proton is so easily lost in any case, nothing very elaborate needs to be postulated at this stage.

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