Kinetic and Molecular Properties of Citraconyl-aldolase

THE REVERSIBLE DENATURATION AND HYBRIDIZATION OF THE NATIVE AND MODIFIED ENZYMES

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1. The preparation of enzymically active *N*-citraconyl derivatives of fructose diphosphate aldolase from rabbit muscle is described. Reaction is restricted to amino groups and the derivatives are not very heterogeneous with respect to the number of substituents. 2. Linear double-reciprocal plots of enzyme velocity against substrate concentration are found up to about 15% blocking of amino groups. With more than 15% blocking, there is a marked downward curvature in the double-reciprocal plots at high substrate concentrations. 3. Over the range 0–25% blocking of amino groups the apparent $V_{\max}$ for fructose diphosphate falls to 10% that of the native enzyme, and the apparent K_m rises from 1 to 400 μM . 4. Various pieces of evidence suggest that citraconyl-aldolase is slightly distorted in structure compared with the native enzyme. However, the kinetic properties and tetrameric structure of citraconyl-aldolase can be completely recovered after denaturation in 4M-guanidine hydrochloride. 5. After removal of the citraconyl groups in acid conditions the kinetic and molecular properties of native enzyme are restored. 6. Hybrid forms of aldolase can be constructed containing native and citraconylated subunits and the suitability of these derivatives for the study of subunit interactions in the enzyme is discussed. 7. The kinetic properties of hybridized aldolase containing native and citraconylated subunits are not exactly those predicted from the kinetic properties of the two parental forms. This result is interpreted in terms of conformational changes induced in the native and modified subunits when both are present in a hybrid molecule, evidently as a result of interactions in the tetramer.

The hybrids formed between variants of the class-I fructose 1,6-diphosphate aldolases (EC 4.1.2.13) when mixtures of two aldolases are denatured and then annealed have been of importance in establishing their tetrameric subunit structure. Natural isoenzymes (Penhoet *et al.*, 1969; Penhoet & Rutter, 1971) and chemical derivatives of aldolase (Meighen & Schachman, 1970; Brenner-Holzach & Leuthardt, 1972) have been used to make hybrids. Kinetic studies of the hybrids suggest that no functional interactions occur between the subunits. However, for the hybrids of tissue-specific isoenzymes of aldolase (Penhoet & Rutter, 1971) the differences in the kinetic parameters of the parental enzymes are not great, so that it would have been difficult to detect small changes in the kinetic behaviour of the hybridized subunits. The succinylated (3-carboxypropionylated) derivative of aldolase used by Meighen & Schachman (1970) and the active-site-blocked derivative of Brenner-Holzach & Leuthardt (1972) were enzymically inactive, yet the specific activities observed for the hybrids formed between native and inactivated enzyme were close to the values expected if each native subunit behaved independently of its neighbours in the tetramer.

However, a full analysis of the kinetic behaviour of the hybrids was not attempted and so small changes in the kinetic parameters of the native subunits could have been overlooked.

By combining the approaches of Meighen & Schachman (1970) and Penhoet & Rutter (1971) it should be possible to conduct a rigorous test for subunit interactions in aldolase. Ideally, one would like to have a chemically unique derivative with kinetic properties very different from those of the native enzyme. This derivative should be tetrameric and should be able to be reversibly dissociated and annealed to yield tetramers that retain their enzymic activity. Further, the affinity of the native subunits for each other and for the modified subunits should be similar under the conditions of annealing if efficient hybridization of native and modified subunits is to be possible. If the parental species and their hybrids are also to be separable from one another, the modified enzyme should be clearly resolved from the native enzyme in some fractionation system. Finally, it would be an advantage as a control to be able to reverse the chemical modification and regenerate native enzyme (cf. Perham & Richards, 1968).

Meighen & Schachman (1970) have shown that partial *N*-succinylation yields a derivative of aldolase that satisfies most of the above criteria. However, the acylation of amino groups with succinic anhydride is not reversible and the derivative they chose to work with had no enzymic activity. 2-Methylmaleic anhydride (citraconic anhydride) is a reversible acylating agent for protein amino groups (Dixon & Perham, 1968; Perham, 1971). If the excess of reagent over protein is kept small, reaction is almost entirely restricted to amino groups and the reaction can be reversed quantitatively at low pH in conditions that allow essentially complete regeneration of the structure and enzymic activity of aldolase (Gibbons & Perham, 1970). An added advantage of citraconylation is that citraconyl amides have a strong absorbance in the u.v. region, which enables quantification of the extent of reaction by simple spectral measurements. Because citraconic anhydride is asymmetric, two isomeric products result from reaction with amino groups. Fortunately, the presence of these isomers does not seem to complicate the interpretation of results, since the same charge change and a similar shape change is introduced at each site of reaction. The kinetics of deacylation of the two isomeric products are identical under the conditions used to deblock citraconyl-aldolase (Gibbons, 1970).

Complete *N*-citraconylation of aldolase induces dissociation of the tetramer to monomers, but a tetrameric structure and a gradually diminishing enzymic activity are retained up to about 50% citraconylation of amino groups (Gibbons & Perham, 1970). In the present paper we describe the effect of partial citraconylation on the kinetic properties of aldolase, we demonstrate that partially citraconylated aldolase can be reversibly denatured in solutions of guanidine hydrochloride and that the modified enzyme can be hybridized with native enzyme. We also produce evidence that the native and citraconylated subunits in hybrids show slightly different kinetic properties in comparison with the same subunits in the parental forms. In the accompanying paper (Gibbons, 1974) there is a detailed account of the kinetic properties of isolated hybrids that confirms and extends these conclusions.

Materials and Methods

Enzymes and reagents

Rabbit muscle aldolase, the mixture of triose phosphate isomerase and L-1-glycerol phosphate dehydrogenase used in the aldolase assay, and NADH (disodium salt), fructose 1,6-diphosphate (trisodium salt) and fructose 1-phosphate (disodium salt) were the products of C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The

concentrations of the fructose phosphate solutions were determined by titration against NADH by using the usual aldolase assay.

Carboxypeptidase A was obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Citraconic anhydride (British Drug Houses, Poole, Dorset, U.K.) was distilled under decreased pressure. Guanidine hydrochloride was recrystallized from ethanol. Tren*–3HCl, prepared as described by Dixon *et al.* (1972), was the gift of Dr. H. B. F. Dixon. DEAE-cellulose was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K. All other reagents were of A.R. grade.

Enzyme assay

Aldolase was assayed by the method of Blostein & Rutter (1963). A final volume of 3 ml of 50 mM-Tris–HCl, pH 7.5 (30°C), containing 0.1 mM-NADH and 20 µg of the triose phosphate isomerase–L-1-glycerol phosphate dehydrogenase mixture was incubated at 30°C and the assay started by addition of fructose 1,6-diphosphate to a final concentration of 0.67 mM. The native enzyme had a specific activity of 13 units/mg. Activity with fructose 1-phosphate was assayed in the same way except the buffer strength was 100 mM.

Determination of protein concentration

Protein concentration was determined spectrophotometrically at 280 nm. Specific extinction coefficients of aldolase samples citraconylated to different extents were determined, the protein being measured by total hydrolysis and amino acid analysis (Perham, 1967). Defining the specific extinction coefficient of citraconyl-aldolase as the extinction of a solution containing 1 mg of protein/ml (i.e. not allowing for the increase in molecular weight caused by the substituents) in a 1 cm light-path, a constant value of 0.96 was obtained over the range 0–50% reaction of amino groups. This value is 5% higher than that determined gravimetrically by Baranowski & Niederland (1949).

Citraconyl-aldolase

Aldolase was made to react with citraconic anhydride as described by Gibbons & Perham (1970). After all added reagent had dissolved, the protein was dialysed at 4°C against 50 mM-Tris–HCl containing 2 mM-Tren–3HCl (as a heavy-metal-chelating agent) and 2 mM-2-mercaptoethanol, pH 8.5 (20°C). When stored in this buffer at 4°C, the half-life of the citraconyl-amide group is about 100 days (Gibbons, 1970).

*Abbreviation: Tren, triaminotriethylamine.

Determination of the extent of citraconylation of protein amino groups

The E_{252}/E_{280} ratio was chosen as a measure of the extent of reaction because the extinction value of the protein at 280 nm is virtually unchanged by citraconylation up to about 50% reaction of amino groups, and at 252 nm the spectrum of the native enzyme exhibits a minimum. The ϵ_{252} of the citraconylamide group is 1.9×10^3 . This assay was calibrated against the trinitrobenzenesulphonic acid assay for free amino groups (Habeeb, 1966) as described by Gibbons & Perham (1970).

Determination of total thiol groups

Thiol groups were measured by the method of Ellman (1959). Protein solutions (1 mg/ml) dissolved in 20 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.0, were mixed with 1.5 vol. of a solution of 0.6 mM-5,5'-dithiobis-(2-nitrobenzoic acid) in 50 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.0, containing 7.4 M-guanidine hydrochloride, giving a final concentration of 5.5 M-guanidine hydrochloride. The E_{412} was read immediately against an appropriate blank from which protein had been omitted.

Reversible denaturation with guanidine hydrochloride

All solutions were at 0°C. Aldolase solution (1 vol.; 2 mg/ml) was rapidly diluted into 3 vol. of a solution containing 17 mM- K_2HPO_4 , 11 mM- KH_2PO_4 , 1 mM-EDTA, 100 mM-dithiothreitol and 6 M-guanidine hydrochloride to give a final concentration of 4.3 M-guanidine hydrochloride. The mixture was immediately dialysed against sufficient 30 mM- K_2HPO_4 -20 mM- KH_2PO_4 -2 mM-EDTA-2 mM-2-mercaptoethanol, pH 6.9, to lower the concentration of guanidine hydrochloride to less than 40 mM, over at least 24 h. The annealed protein was then dialysed against 50 mM-Tris-HCl-2 mM-Tren-3 HCl-2 mM-2-mercaptoethanol, pH 8.5 (measured at 20°C), at 0°C for a further 24 h.

Concentration of protein solutions

When necessary, protein solutions were concentrated by ultrafiltration in an Amicon cell fitted with a PM-10 membrane. Before electrophoresis on cellulose acetate, samples were dialysed at 0°C against 4 mM-2-mercaptoethanol adjusted to pH 8.5 with 1 M- NH_3 , then concentrated by vacuum evaporation without freezing.

Cellulose acetate-strip electrophoresis

Electrophoresis and staining were carried out in a Beckman R-101 Microzone apparatus as described by Meighen & Schachman (1970). In the acid stain

the citraconyl groups will be essentially quantitatively removed after 2 h, so the intensity of staining should not be affected by the extent of reaction.

Ultracentrifugation

Usually, protein samples (1–3 mg/ml) were dialysed overnight at 4°C against the specified buffer and then ultracentrifuged at 52 000 rev./min at 20°C. Schlieren patterns were photographed at 16 min intervals. Sedimentation coefficients are given uncorrected for the effects of solvent viscosity or of protein concentration.

Digestion with Carboxypeptidase A

Samples of aldolase (1.5 mg/ml) dissolved in 50 mM-Tris-HCl-5 mM-EDTA-5 mM-2-mercaptoethanol, pH 7.5, were digested at 24°C with Carboxypeptidase A (15 μ g/ml) solubilized by the method of Ambler (1967). The digestion was stopped by addition of 1 M-HCl to bring the pH to about 2.

Results

Specificity of citraconylation

Although acylation with citraconic anhydride is quite specific for amino groups in proteins, it is known that under certain conditions esterification of serine and threonine hydroxyl groups (King & Perham, 1971) and alkylation of cysteine thiol groups (Gibbons & Perham, 1970) can also occur [see Perham (1971) for a review]. Spectra of citraconylated aldolase provided no evidence for the presence of *O*-citraconyl groups, since the observed spectra agree with those calculated for the observed number of blocked amino groups measured by trinitrobenzenesulphonic acid assay and the known spectral properties of citraconyl-amides (Gibbons, 1970). When a sample of citraconyl-aldolase with 35% of its amino groups modified was assayed for total thiol groups in 5.5 M-guanidine hydrochloride, 7.5 thiol groups per subunit (mol.wt. 40 000) were found in comparison with the value of 7.7 found for the native enzyme. So, at least up to 35% reaction of amino groups, citraconylation is restricted almost entirely to amino groups. Consequently, the extent of reaction will be defined as the percentage blocking of amino groups. The native enzyme has approx. 28 amino groups/subunit (Anderson *et al.*, 1969). Above 50% citraconylation of amino groups, irreversible loss of protein thiol groups has been observed (Gibbons & Perham, 1970).

Stability of the citraconylated and native tetramer

Gibbons & Perham (1970) have demonstrated that at pH 8 citraconyl-aldolase is tetrameric up to about 50% reaction of amino groups. Obviously, if

Fig. 1. Proportion of aldolase tetramer as a function of pH

Samples of native (●) and 35%-citraconylated aldolase (○) were dialysed against the buffers described in the text and then centrifuged at 20°C and 52 000 rev./min. The fraction associated was measured by comparing the area under the faster-moving peak with the total area under both peaks in the schlieren trace.

citraconyl-aldolase is to be useful in hybridization studies it should retain its quaternary structure in all conditions to which it is exposed except those deliberately aimed at dissociating it into subunits. An investigation of the stability of the citraconylated tetramer was therefore undertaken. Samples of native and 35%-citraconylated aldolase (3 mg/ml) were dialysed overnight at 4°C against the following buffers, all of which contained 2 mM-EDTA (disodium salt) and 3 mM-2-mercaptoethanol: 100 mM-glycine-NaOH, pH 9.9; 100 mM-butylamine-HCl, pH 10.3; 100 mM-butylamine-HCl, pH 11.0; 100 mM-butylamine-HCl, pH 11.5; all at 20°C. Ultracentrifugation of these samples showed a fast-sedimenting peak and a slowly moving peak, taken to be tetramer and monomer respectively (cf. Gibbons & Perham, 1970). The sedimentation coefficient of the faster peak fell as the pH rose. The fraction of protein associated in the tetrameric form was calculated from the relative areas of the peaks in the schlieren pattern and the pH-dependency of the breakdown of the tetramer is shown in Fig. 1. Although the native enzyme is slightly more stable than the citraconyl-enzyme, both are stable up to pH 10, a pH value higher than any used in subsequent experiments.

It is essential if valid inferences are to be drawn from a study of the kinetic properties of enzyme hybrids that no dissociation of the enzyme or its derivatives should occur under the conditions of the enzyme assay. Direct proof of the stability

Fig. 2. Double-reciprocal plot for the fructose 1,6-diphosphate aldolase activity of 8% citraconyl-aldolase

The reciprocal of the enzymic rate normalized to the maximum rate is plotted against the reciprocal of the fructose 1,6-diphosphate concentration. $V_{\max}$ was 90% that of the native enzyme.

of aldolase to dilution is difficult to obtain, but the proportionality of rate to enzyme added for both native and citraconylated enzymes over the range of enzyme concentration used (0.004–0.024 unit/ml in the actual assay) supports the idea. A similar conclusion has been reached by Lebherz (1972) on the basis of gel-filtration and subunit-exchange experiments with rabbit and chicken aldolases. In contrast, at very low ionic strength (less than 1 mM-Tris-HCl, pH 7.5) where dissociation might be expected to occur, there is a fourfold increase in the apparent specific activity of native aldolase as the enzyme concentration is increased from 0.1 to 1 $\mu\text{g/ml}$. The apparent change in specific activity is completely abolished by raising the ionic strength. Binding of the enzyme to the assay cuvette is not responsible for the loss of activity at low enzyme concentration. These observations reassure one that if dissociation of aldolase did occur in the normal assay, it would be detected because the specific activities of the associated and dissociated species would probably be different. Indeed, isolated 'matrix-bound' subunits of rabbit muscle aldolase are reported to be enzymically active, but their specific activity is approximately one-quarter that of the matrix-bound tetramer (Chan, 1970; Chan & Mawer, 1972).

Enzymic properties of citraconyl-aldolase

The kinetic behaviour of a series of aldolase derivatives made to react to different extents with citraconic anhydride was examined. For up to about 10% reaction of amino groups, double-reciprocal plots of rate against fructose 1,6-diphosphate concentration were linear. For example, in Fig. 2 there is no curvature even when the fructose 1,6-

Fig. 3. Kinetics of fructose 1,6-diphosphate aldolase activity of 30%-citraconylated aldolase

The rate (v) was normalized to the rate in the standard assay (V^*). This derivative had an apparent $V_{\max}$, (obtained by extrapolation of the linear part of the curve) of 15% that of the native enzyme.

Table 1. Comparison of the apparent $V_{\max}$ for fructose 1,6-diphosphate cleavage, obtained by extrapolation of the linear parts of double-reciprocal plots, with the activity in an assay containing 5.1 mM-fructose 1,6-diphosphate. Results are expressed as a percentage of the $V_{\max}$ determined for the native enzyme.

Percentage of amino groups citraconylated	$V_{\max}$	Activity with 5.1 mM-fructose 1,6-diphosphate (%)
0	100	94
8	92	91
11	70	71
20	58	72
25	37	62
29	17	41

diphosphate concentration exceeds 100 times K_m . Above 15% modification, however, there was pronounced downward curvature of double-reciprocal plots at high concentrations of fructose 1,6-diphosphate, as shown in Fig. 3. In contrast, with fructose 1-phosphate as substrate, all the double-reciprocal plots were straight, but since the K_m values with fructose 1-phosphate are greater than 1 mM, it was not possible to make measurements at concentrations greatly in excess of K_m .

To examine the divergence from linearity with fructose 1,6-diphosphate as substrate, rate measurements were made at a very high substrate concentration. Table 1 compares the values of $V_{\max}$.

Fig. 4. $V_{\max}$ values for fructose 1,6-diphosphate (●) and fructose 1-phosphate (○) aldolase activity

The values were found by extrapolation of the linear parts of double-reciprocal plots as a function of the extent of citraconylation of protein amino groups.

Fig. 5. K_m values for fructose 1,6-diphosphate (●) and fructose 1-phosphate (○) aldolase activity

Values were obtained by extrapolation of the linear parts of double-reciprocal plots as a function of the extent of citraconylation of protein amino groups.

obtained by extrapolation of the linear parts of the double-reciprocal plots with the enzymic activities measured at 5.1 mM-fructose 1,6-diphosphate. As shown in Fig. 12, where the data of Fig. 3 are replotted, at 5.1 mM-fructose 1,6-diphosphate 30%-citraconylated aldolase (the most extensively modified

derivative that was examined) is essentially saturated with substrate. When 30% modification is reached, the $V_{\max}$ obtained by extrapolation of the linear parts of the double-reciprocal plots underestimates the maximum rate obtainable by a factor of at least 2.5.

$V_{\max}$ and K_m values found by extrapolation of the linear parts of double-reciprocal plots are presented in Figs. 4 and 5 as functions of the extent of citraconylation. Since the $V_{\max}$ with fructose 1-phosphate is hardly altered, it is clear that reaction of catalytically essential residues of aldolase with citraconic anhydride can only be slight. Rabbit muscle aldolase remains tetrameric from 0 to 50% citraconylation of amino groups (Gibbons & Perham, 1970), so that dissociation of the enzyme is not responsible for the changes in the kinetic parameters. The fall in $V_{\max}$ with fructose 1,6-diphosphate and the increase in K_m for both substrates with increasing reaction of amino groups must therefore be largely due to the distortion of the active site caused by modification of non-essential residues, as predicted by Gibbons & Perham (1970).

Citraconylation of aldolase alters the pH and ionic-strength optima of the enzyme (Figs. 6 and 7). The pH-activity profile of 30%-citraconylated

aldolase shows pH-dependence above pH7, something that is not found with the native enzyme (Penhoet *et al.*, 1969). Whereas the native enzyme is almost isoelectric at pH7, the citraconyl-enzyme is highly negatively charged (cf. Plate 1). Increasing the pH will cause an increase in the negative charge on each subunit. Extra negative charge would be expected to have a greater effect on citraconyl-aldolase than on the native enzyme in strengthening intra- and inter-subunit charge repulsion and thus distorting the subunits.

Raising the ionic strength enhances the activity of both native and citraconyl-aldolase (Fig. 7), but the modified enzyme reaches its optimum activity at a much higher ionic strength than that required for the native enzyme. The effect is primarily due to ionic strength, since salts other than KCl (e.g. sodium acetate, NH_4Cl and NaCl) produce similar results. Presumably the electrostatic repulsion within and between the negatively charged subunits of citraconyl-aldolase is decreased as the ionic strength of the solvent rises.

To account for the fact that rabbit muscle aldolase exhibits a much greater $V_{\max}$ for fructose 1,6-diphosphate cleavage than for fructose 1-phosphate

Fig. 6. pH-activity profile of 29%-citraconylated aldolase

The activity was measured at 30°C with a substrate concentration of 4.7mM-fructose 1,6-diphosphate by using the normal assay components buffered by 47mM-imidazole and different concentrations of HCl. KCl was added to give a constant chloride concentration of 80mM. At the substrate concentration used, any change of K_m with pH should not affect the rate significantly. Activity is expressed as a percentage of the maximum. The dotted line shows the profile for the native enzyme (Penhoet *et al.*, 1969) and the arrow ($\downarrow$) indicates the pH in the standard assay.

Fig. 7. Variation of activity of native aldolase (○) and 29%-citraconyl-aldolase (●) with ionic strength

The native enzyme was assayed in the usual way except that the buffer concentration was decreased to 6.7mM. The fructose 1,6-diphosphate concentration (0.67mM) is enough essentially to saturate the native enzyme. The citraconyl-enzyme was assayed at its optimum pH (6.8) buffered with 6.7mM-imidazole-HCl; fructose 1,6-diphosphate concentration (4.7mM) sufficient virtually to saturate the aldolase derivative was used. In both cases KCl was added to produce the specified ionic strength. The arrow ($\downarrow$) indicates the ionic strength in the standard assay.

EXPLANATION OF PLATE I

Electrophoresis of proteins on cellulose acetate strips at pH6.5

N, native enzyme; C, 40%-citraconylated aldolase before (A) and after (D) denaturation and annealing in optimal conditions. The dashed line indicates the origin. Protein bands were made visible by staining with nigrosin.

Fig. 8. Ratio of fructose 1,6-diphosphate aldolase activity after limited Carboxypeptidase-A digestion to the activity before digestion as a function of the percentage citraconylation

Assays were carried out in the presence of 4.7 mM-fructose 1,6-diphosphate.

cleavage, Mehler & Cusic (1967) proposed that the muscle enzyme undergoes a conformational change on binding fructose 1,6-diphosphate (but not fructose 1-phosphate) that stimulates the aldolase activity. These authors demonstrated that the C-terminal tyrosine residue is essential for this enhancement of activity, since its removal decreases the V_{max} for fructose 1,6-diphosphate to the almost unchanged value for fructose 1-phosphate without altering the affinity of the enzyme for either substrate. If citraconylation distorts the tertiary structure of aldolase, one would expect that the activity with fructose 1,6-diphosphate would be more sensitive to acylation of the enzyme with citraconic anhydride than the residual fructose 1,6-diphosphate aldolase activity that is left after removal of the C-terminal tyrosine residue. In an experiment designed to test this idea, citraconyl-aldolase derivatives were treated with Carboxypeptidase A until their specific activities had fallen to a constant value; this required only a few minutes (Mehler & Cusic, 1967). Aldolase activities with fructose 1,6-diphosphate as substrate were compared before and after digestion by using a high concentration of substrate (4.7 mM) to ensure that the most extensively citraconylated sample was essentially saturated. All the samples released 0.67 ± 0.05 tyrosine residue per subunit, which is the usual extent of digestion unless prolonged incubation or denaturing conditions are used (Morse *et al.*, 1967). As expected, the ratio of activities

after and before removal of the C-terminal tyrosine increased with the percentage reaction of amino groups, as shown in Fig. 8. When assayed in the presence of 29 mM-fructose 1-phosphate, the native enzyme retained 70% of its activity after treatment with carboxypeptidase and the activity of the most citraconylated derivative did not change.

Reversible denaturation and annealing

Trial experiments indicated that higher recoveries of activity could be obtained after removal of the denaturant by dialysis than by rapid dilution, and by using guanidine hydrochloride rather than urea as denaturant. Dialysis in the cold (0°C) gave better results than dialysis at room temperature (20°C).

To determine the concentration of guanidine hydrochloride necessary to dissociate aldolase into its subunits, native aldolase samples (2 mg/ml) dissolved in 20 mM-N-ethylmorpholine-acetic acid buffer, pH 8.2, containing 2 mM-EDTA (disodium salt) and 2 mM-2-mercaptoethanol were adjusted to various concentrations of guanidine hydrochloride by adding the solid salt. From 0 to 1 M-guanidine hydrochloride, ultracentrifugation at 52000 rev./min showed a normal schlieren peak for the protein, the sedimentation coefficient falling to 5.0S. When concentrations of guanidine hydrochloride in the range 1.5–2 M were used, the protein sedimented much more slowly and the schlieren peak became so diffuse that accurate s values could no longer be measured. A concentration of 4 M-guanidine hydrochloride should thus be more than sufficient to dissociate aldolase into its subunits.

In the denaturation and annealing procedure as finally developed, both native enzyme and citraconyl-aldolase with up to 40% of the amino groups modified gave recoveries of respective specific activity better than 95%. There was no loss of protein by precipitation. Slight variations in the protocol result in significant losses in the specific activity of the citraconyl derivatives. In particular, a high concentration of dithiothreitol in the denaturing buffer was essential to ensure a high recovery of activity; 100 mM-2-mercaptoethanol was not an effective substitute. The yield of activity was lower if the pH of the annealing buffer was above 7, or the protein concentration in the annealing step was higher than 0.5 mg/ml. Addition of 0.25 M-NaCl to the annealing buffer had no effect when optimal conditions were used.

Electrophoresis of denatured and annealed preparations of citraconyl-aldolase on cellulose acetate strips showed essentially complete recovery of the starting material together with a trace component that had about half the electrophoretic mobility of the citraconyl-aldolase. Plate 1 shows that citraconyl-aldolase separates well from native enzyme and is

Fig. 9. Schlieren patterns of 30%-citraconylated aldolase after denaturation in guanidine hydrochloride and annealing in suboptimal conditions

(a) The whole sample; (b) and (c) fractions 1 and 2 respectively obtained by chromatography of sample (a) on DEAE-cellulose as shown in Fig. 10. The samples were centrifuged in 20mM-*N*-ethylmorpholine-acetate buffer, pH 8.0, at 20°C and 52000 rev./min. The photographs were taken about 1 h after the rotor reached full speed. Sedimentation is from right to left.

not very heterogeneous, producing a band only some three times that of the native enzyme in width. Denatured, annealed preparations of both native and citraconyl-aldolase revealed no differences when compared with control samples by ultracentrifugation. However, if suboptimal conditions were used for denaturing and annealing citraconyl-aldolase a more slowly sedimenting peak was seen. Fig. 9(a) shows the schlieren pattern obtained after denaturing and annealing 37%-citraconylated aldolase (specific activity 2 units/mg in the standard assay) at ten times the optimal protein concentration. The recovery of specific activity was 70% and the loss in activity correlated with the proportion of protein in the slower peak, suggesting that the faster peak was fully active and that the slower peak was inactive. It is likely that

Fig. 10. Chromatography on DEAE-cellulose of 30%-citraconylated aldolase after denaturation and annealing in suboptimal conditions

The sample (20mg) in 50mM-Tris-HCl-2mM-Tren-3HCl-2mM-2-mercaptoethanol, pH 8.5, was applied to a column (2cm×6cm) of DEAE-cellulose equilibrated with the same buffer at 24°C. All the protein was retained by the column and was subsequently eluted with a gradient from 0 to 0.50M-NaCl in the same buffer over a volume of 200ml. The column was pumped at 80ml/h and fractions (2.4ml) were collected. Specific activity is expressed relative to that of the citraconyl-aldolase before denaturation and annealing.

the trace component sometimes detected by electrophoresis after denaturation and annealing of citraconyl-aldolase in optimal conditions corresponds to this slowly sedimenting inactive material (cf. Meighen & Schachman, 1970).

The two components in the suboptimally annealed sample of citraconyl-aldolase could be partially separated by chromatography on DEAE-cellulose (Figs. 9 and 10). Fraction 2 (Fig. 10) had almost the same specific activity as the starting sample of citraconyl-aldolase and could be identified with the faster-sedimenting peak in the original centrifugation (Figs. 9a and 9c). Fraction 1 had a specific activity half that of the starting sample of citraconyl-aldolase and contained approximately equal proportions of the faster- and slower-sedimenting material in the ultracentrifuge (Fig. 9b). It is therefore possible to infer that the faster-sedimenting protein is tetrameric citraconyl-aldolase with the same specific activity

Fig. 11. Kinetics of fructose 1,6-diphosphate aldolase activity of native aldolase before (●) and after (○) denaturation and annealing in optimal conditions

The activity is normalized to that found (V^*) in the standard assay (0.67 mM-fructose 1,6-diphosphate).

Fig. 12. Kinetics of fructose 1,6-diphosphate aldolase activity of 30%-citraconylated aldolase before (●) and after (○) denaturation and annealing in optimum conditions

The activity is normalized to that found (V^*) in the standard assay (0.67 mM-fructose 1,6-diphosphate).

as the original sample and that the protein that sediments more slowly is enzymically inactive. Repeating the denaturation and annealing process on fraction 1 (Fig. 10), this time in optimal conditions, increased the specific activity from 50 to 70% of that of the original citraconyl-aldolase, indicating that activity can be recovered from the inactive species. The properties of the inactive species suggest that it is composed of subunits folded up into a less compact structure than in the tetramer, or possibly is an aggregate of such subunits. There is no evidence for two differing populations in the citraconyl-aldolase preparation, since variation of the annealing conditions changes the yield of the inactive species and activity can be recovered from it. One might speculate that the inactive species represents an intermediate between denatured citraconyl subunits and the active citraconyl tetramer. During refolding in suboptimal conditions some of the citraconyl-aldolase may become trapped in this intermediate form by a kinetic barrier. Ikai & Tanford (1971) have proposed that metastable intermediates can exist which are not on the direct pathway between native and denatured states of proteins.

Meighen & Schachman (1970) have observed a similar phenomenon on reconstituting succinyl-aldolase after denaturation in urea. In their experiments, as much as 20% of the succinyl-aldolase failed to recover tetrameric structure after dissociation and annealing.

A kinetic analysis of aldolase activity before and after denaturation and annealing was carried out with native enzyme and 30%-citraconylated aldolase. Because of the divergence away from Michaelis-Menten kinetics found with the citraconyl enzyme at high fructose 1,6-diphosphate concentrations, the best way to plot the results is as a function of the logarithm of the substrate concentration. For the

experiment described, the activities have been normalized to the rate in the standard assay (Figs. 11 and 12). Since rabbit muscle aldolase obeys Michaelis-Menten kinetics, it would be expected to give a sigmoidal plot of activity against log (substrate concentration). Because of the low K_m of the native enzyme for fructose 1,6-diphosphate (approx. 1 μM) most of the sigmoid curve corresponding to substrate concentrations lower than K_m is experimentally inaccessible. Denaturation and annealing obviously have no detectable effect on the kinetic properties of aldolase and its citraconyl derivative. If the same comparison is made with citraconylated or native aldolase under suboptimal annealing conditions where the recovery of activity is lower, there is still no difference in the way the rate changes as a function of substrate concentration.

Regeneration of native structure and activity on removal of the citraconyl groups

After acid treatment (pH 2) and annealing by the method of Gibbons & Perham (1970), the native enzyme retained about 70% of its initial specific activity. If citraconyl-aldolase (40% modified, having a specific activity of 7% that of the native enzyme in the standard assay) was treated with acid and annealed, it recovered virtually the same specific activity as a control sample of the native enzyme given the same treatment.

Samples of native enzyme, and of native and 45%-citraconylated aldolase which had been treated at pH 2, were compared by electrophoresis on 4% (w/v) polyacrylamide gels containing 9.5M-urea (Davis, 1964). In this system, which dissociates and unfolds the enzyme subunits, rabbit muscle aldolase has two closely spaced bands corresponding to two chain types which are thought to differ by a single

charge (Chan *et al.*, 1967; Anderson *et al.*, 1969). Any covalent modification that alters the charge on a subunit would therefore be expected to be detectable. There was no difference between the banding patterns of the native enzyme and of acid-treated native or citraconyl-aldolase, indicating that the blocking groups had all been removed (cf. Gibbons & Perham, 1970). The citraconyl-aldolase used in these experiments has been stored at pH 8.5 and 4°C in the presence of 2 mM-2-mercaptoethanol. The fact that all the blocking groups could be removed by acid treatment at pH 2 is good evidence that reaction of mercaptoethanol with the conjugated double bond of the citraconyl blocking groups had not occurred, since such reaction would render the citraconyl-amide bond stable at pH 2 (cf. succinyl-amide bonds).

Hybridization of citraconyl and native aldolase

When native and succinyl-aldolase are mixed and subjected to denaturation and annealing procedures, some of the native and modified subunits combine to form hybrid tetramers (Meighen & Schachman, 1970). Experiments in which native and 30%-citraconylated aldolase were mixed together and then denatured in 4M-guanidine hydrochloride and annealed under the optimal conditions described above indicated that the expected hybrid forms were produced, since protein and associated enzymic activity were found with chromatographic and electrophoretic mobilities intermediate between those of the parental forms. However, the resolution of hybrids that could be obtained was poor despite the limited heterogeneity of the citraconyl-aldolase used. Thus it was evident that in order to make a detailed kinetic study of the hybrids, the citraconyl-aldolase would itself have to be subjected to a preliminary fractionation. This is described in the accompanying paper (Gibbons, 1974).

However, some experiments were carried out with unfractionated citraconyl-aldolase. There were small but significant losses of activity (up to 10%) measured in the standard assay when mixtures of native and 30%-citraconylated aldolase were denatured and annealed in conditions where the unmixed controls gave better than 95% recovery of activity in the standard assay. For one molar ratio of native to citraconyl-aldolase (1:3), a detailed comparison was made of the kinetic properties of the unfractionated product obtained by denaturing and annealing a mixture of the parental forms and a control produced by mixing the same ratio of parental forms. The results are shown in Fig. 13 where the activities are plotted normalized to the activity observed in the standard assay, thereby avoiding any errors that might arise from inaccuracies in the measurement of protein concentration. At low substrate concentration, the normalized rate

Fig. 13. Kinetics of fructose 1,6-diphosphate aldolase activity of a mixture of native and 30%-citraconylated aldolase (1:3, w/w) before (●) and after (○) denaturation and annealing

The materials used were identical with those described in Figs. 11 and 12, and the activities have been normalized in the same way. The denatured annealed sample recovered 92% of the activity of the control mixture in the standard assay.

for the preparation in which hybrids are present is slightly lower than expected, whereas at very high substrate concentrations it rises slightly above the control value. For all substrate concentrations the rate of change of normalized activity with increasing substrate concentration is greater in the denatured annealed mixture than in the mixture of parental forms.

Discussion

Citraconylation of aldolase undoubtedly produces a somewhat heterogeneous mixture of *N*-acylated enzyme species, so that citraconyl-aldolase would be expected to exhibit divergence from Michaelis-Menten kinetics because of the range of K_m values of differently modified molecules. Such kinetic heterogeneity begins to appear at about 15% blocking of amino groups and becomes progressively more pronounced as the extent of citraconylation rises above this value. A similar kinetic pattern is observed for the mixture of isoenzymes of purine nucleoside phosphorylase from erythrocytes (Turner *et al.*, 1971). These authors propose that the apparent high substrate activation they observe is caused by the different K_m values of the isoenzymes. These isoenzymes are thought to be derived from a parental form that exhibits Michaelis-Menten kinetics by a series of chemical modifications *in vivo* that progressively increases the negative charge on the enzyme molecule. However, the apparent high substrate activation of citraconyl-aldolase (Fig. 12) may not be entirely due to heterogeneity of the modified enzyme. Increasing the ionic strength raises the

activity of citraconyl-aldolase quite markedly. Presumably, the high concentrations of the multiply charged substrate fructose 1,6-diphosphate needed to saturate citraconyl-aldolase could also cause a non-specific activation.

As the extent of citraconylation increases from 0 to 30%, the rate of increase in the K_m values for both substrates becomes progressively larger. This may be partly attributed to increasing distortion of the enzyme structure from the native conformation, owing to the disturbance of the charge balance on the subunits. Charge repulsion would be expected to increase with the square of the net charge on the subunits and eventually, of course, it overcomes the secondary forces holding the subunits together. The greater sensitivity of fructose 1,6-diphosphate aldolase activity to citraconylation in comparison with fructose 1-phosphate aldolase activity and 'carboxypeptidase-resistant' fructose 1,6-diphosphate aldolase activity (Fig. 4) shows that modification of catalytically essential residues in aldolase during the early stages of citraconylation is only slight and reflects the extra stringency of the structural requirements for full activity with fructose 1,6-diphosphate. Whether citraconylation hinders a conformational change in the enzyme on binding substrate or merely disrupts the precise positioning of active-site groups, including the C-terminal tyrosine residue, these results are compatible with the increasing distortion of aldolase as the extent of reaction is raised (Gibbons & Perham, 1970).

From the complexity of the changes in the enzymic properties of aldolase after reaction with citraconic anhydride, it is clear that the interpretation of the results of experiments in which proteins are chemically modified at several sites should be approached with caution. For example, a derivative with a vastly increased K_m may show no signs of activity in a normal assay.

The ability of citraconylated derivatives to undergo denaturation and annealing and to recover kinetic properties identical with the starting material proves that all the amino groups of aldolase that had reacted with citraconic anhydride (up to 40% overall reaction) are of little consequence in the folding-up and association of aldolase subunits, although the available evidence suggests that citraconyl-aldolase has a somewhat distorted less compact structure compared with native aldolase. However, under the right conditions, the folding process leads to this characteristic structure being faithfully recovered. When citraconyl-aldolase is denatured and annealed, differently modified subunits are offered an opportunity to redistribute themselves among the tetrameric products. For example, less heavily substituted subunits might tend to anneal more readily with each other than with more extensively modified subunits because of the

lower intersubunit charge repulsion. However, if redistribution of subunits does occur it evidently has no kinetic consequence. This would in turn imply that the subunits of citraconyl-aldolase catalyse the enzymic reaction independently of the nature of the neighbouring subunits in the tetramer. On the other hand, the small but significant changes in the kinetics observed for denatured and annealed mixtures of native and citraconyl-aldolase in comparison with the equivalent mixture of parental forms (Fig. 13) show that native and citraconylated subunits can modify the activity of other subunits in a hybrid tetramer. In view of the substantially increased K_m of the citraconyl-aldolase used, it is reasonable to suppose that at very low substrate concentrations the catalytic activity observed is almost entirely due to native subunits, whereas at very high substrate concentrations native and citraconylated subunits are both contributing to the catalysis. Since the activity of the hybridized mixture is lower at low substrate concentrations and higher at high substrate concentrations than the equivalent mixture of parental forms, the obvious inference is that in hybrid tetramers the activity of native subunits is lower than in native tetramers whereas that of the citraconylated subunit is higher than in citraconylated tetramers. The form of the curves in Fig. 13 indicates that this must be due in part to decrease and increase in the values of V_{max} for the native and citraconyl subunits respectively when they find themselves in hybrid tetramers. Effects on K_m are not easy to rule out, but it seems likely that they are small, and this is borne out by a detailed study of purified hybrids (Gibbons, 1974). These changes in the catalytic activity of the native and citraconylated subunits can be explained by assuming that in the hybrid tetramer the presence of native subunits induces a more 'native-like' folding of the citraconylated subunit and conversely that the presence of citraconylated subunits distorts the conformation of native subunits away from that found in the native tetramer. The limited chemical heterogeneity of even 30%-citraconylated aldolase thus explains why comparable effects are not seen when citraconyl-aldolase is denatured and annealed in the absence of native enzyme. These experiments with chemically modified enzymes therefore have something in common with the phenomenon of complementation (Crick & Orgel, 1964).

Meighen & Schachman (1970) and Brenner-Holzach & Leuthardt (1972) showed that in hybrids of native and chemically modified aldolase the catalytic activity of a native subunit was expressed despite the presence of one or more inactive subunits in the tetramer. The experiments with citraconyl-aldolase described in the present paper support this conclusion but also indicate that the folding of native and citraconylated subunits is in fact slightly altered in the hybrids, presumably by interaction in the

tetramer. It is difficult to be certain that the same effect does not occur in hybrids of the natural isoenzymes of aldolase (Penhoet & Rutter, 1971), since the kinetic properties of the pairs of parental forms studied (aldolase A/aldolase C and aldolase B/aldolase C) are not greatly different. However, since aldolases A, B and C are closely homologous it is likely that they will have closely similar three-dimensional structures (Penhoet *et al.*, 1969; Forcina & Perham, 1971). In particular, the regions of contact between the subunits are probably highly conserved because they contribute to the interior region of the molecule, and it is now generally recognized that most substitutions in homologous proteins occur at the surface of the molecule without general distortion of the three-dimensional structure. Therefore any distortion of the polypeptide chain caused by interaction between subunits in hybrid tetramers composed of aldolases A, B and C is likely to be small in comparison with the changes we detect in hybrids of aldolase A and citraconyl-aldolase A.

It has been suggested that a number of oligomeric enzymes function by a 'flip-flop' mechanism (see Lazdunski *et al.*, 1971). For example, for malate dehydrogenase (Harada & Wolfe, 1968) and alkaline phosphatase (Halford *et al.*, 1969; Halford, 1972) it is proposed that each subunit in the dimer is active in turn and that catalytic steps and conformational change occur alternately in the two subunits. The interactions between the subunits are thus of fundamental importance to the catalytic mechanism, and the prediction would be that the monomer cannot therefore ordinarily be active. Although such a mechanism has never been suggested for aldolase, the kinetic properties of the hybrids containing citraconylated or succinylated subunits must exclude it in all our conditions of assay. Indeed, it seems likely that a study of comparable hybrids would be of value in the investigation of enzymes that appear to operate by this unusual mechanism.

Citraconylation provides a method of progressively increasing the K_m of aldolase, while retaining substantial catalytic activity. The citraconylated derivatives, although slightly distorted, are tetrameric and can be denatured reversibly in conditions that allow hybrids of native and citraconylated enzyme to form. Deacylation of citraconyl-aldolase followed by dialysis to neutral pH yields a product indistinguishable from the native enzyme. Thus citraconyl-aldolase provides an excellent parental form for further work involving enzyme hybrids, and such studies are described in the accompanying paper (Gibbons, 1974).

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