The Reaction of Aldolase with 2-Methylmaleic Anhydride

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(Received 24 September 1969)

1. The reaction of rabbit muscle aldolase with 2-methylmaleic anhydride is described. All the protein amino groups can be reversibly blocked. 2. As the reaction proceeds, the enzyme activity decreases until, at about 50% citraconylation of amino groups, the enzyme is completely inhibited. At this stage, little or no dissociation of the enzyme tetramer is observed and 75% of the activity is recoverable on unblocking the amino groups. 3. At 80% blocking, the enzyme is completely dissociated but little enzymic activity is recoverable after unblocking. Inability to recover activity after citraconylation and unblocking correlates with the onset of dissociation of the citraconyl-aldolase seen on ultracentrifugation. 4. The only irreversible modification of the enzyme primary structure detectable after the citraconylation and unblocking reactions is the partial loss of thiol groups. It is probable that this is responsible for the inability to reform active enzyme from the citraconylated subunit. 5. Other reversible side reactions of maleic anhydride and citraconic anhydride that may occur with proteins are discussed.

One of the interesting questions about oligomeric enzymes is whether the active site exists within or between protein subunits. In the absence of crystallographic evidence, one possible approach would be to dissociate the subunits and see whether enzyme activity exists in a separated subunit. The standard methods of dissociation generally involve rather drastic treatment: extremes of pH, use of guanidine hydrochloride etc. Another alternative is to induce protein subunits to stay apart as a result of some chemical modification and then to look at the enzyme activity.

It is well known that proteins can be dissociated by reaction of the amino groups with acid anhydrides, e.g. succinic anhydride (Habeeb, Cassidy & Singer, 1958). However, succinylation has been shown to be relatively non-specific (Gounaris & Perlmann, 1967) and suffers from the disadvantage of being irreversible. This lack of reversibility means that the desirable control of trying to regenerate the native protein structure after chemical modification cannot be carried out (Perham & Richards, 1968). Fortunately, several reversible reactions with anhydrides have been described, namely maleic anhydride (Butler, Harris, Hartley & Leberman, 1969), 2-methylmaleic (citraconic) anhydride (Dixon & Perham, 1968) and tetrafluorosuccinic anhydride (Braunitzer, Beyreuther, Fujiki & Schrank, 1968).

We chose to use citraconic anhydride for the purpose since the conditions necessary for unblocking are mild (Dixon & Perham, 1968) and more

experience of its use seemed desirable. Fructose diphosphate aldolase (EC 4.1.2.13) is a very suitable test protein because it is known to be tetrameric (Penhoet, Kochman, Valentine & Rutter, 1967) and to be capable of withstanding the mildly acidic conditions required to remove the citraconyl group. Thus, although the enzyme is dissociated into the constituent four subunits at pH 2, these can subsequently be associated to re-form active enzyme in high yield (Schachman, 1963).

Some of the results of this study have been presented briefly elsewhere (Perham, 1969a).

MATERIALS AND METHODS

Enzymes and reagents. Aldolase was prepared from rabbit skeletal muscle by a modification of the method of Taylor, Green & Cori (1948). White skeletal muscle from a mature female rabbit was minced and extracted, first with 2 vol. of 5 mM-EDTA, pH 6.0, and then with 1 vol. of 5 mM-EDTA, pH 6.0, containing 0.15 M-NaCl. This second extraction was necessary to secure quantitative release of the aldolase from the structural protein of the muscle. All extractions were carried out with mechanical stirring for 30 min. After each extraction the muscle was squeezed through muslin to separate it from the extract. This and all subsequent steps were carried out at 4°C. The extracts were then pooled and protein precipitating between 40 and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ was collected by centrifugation at 12000 rev./min in an M.S.E. 18 centrifuge. This material was dissolved in 5 mM-EDTA, pH 6.0, and $(\text{NH}_4)_2\text{SO}_4$ removed by dialysis against the same buffer. The protein was

adsorbed on a CM-cellulose column (15 cm $\times$ 10 cm) previously equilibrated with 5 mM-EDTA, pH 6.0. The column was washed with several column volumes of the same buffer, then with several volumes of 5 mM-EDTA, pH 6.0, containing 20 mM-NaCl, until the protein concentration in the effluent became negligible. Aldolase was eluted with 5 mM-EDTA, pH 6.0, containing 50 mM-NaCl and then precipitated by adding 600 g of $(\text{NH}_4)_2\text{SO}_4$ /l of solution. The precipitate was dissolved in 5 mM-EDTA, pH 6.0, at a concentration of approx. 10 mg/ml and recrystallized twice by addition of solid $(\text{NH}_4)_2\text{SO}_4$.

The enzyme was assayed as described by Blostein & Rutter (1963) with a Beckman DK2 recording spectrophotometer at 30°C. A specific activity of approx. 16 units/mg was commonly found. Enzyme concentration was measured spectrophotometrically at 280 nm assuming an extinction of 0.91 for a solution of 1 mg/ml in a 1 cm light-path (Baranowski & Niederland, 1949).

Trypsin (twice recrystallized) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Citraconic anhydride and *N*-acetyl-L-cysteine were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.; *N*-ethylmorpholine was from Kodak Ltd., Kirkby, Liverpool, U.K.; and NADH disodium salt and fructose 1,6-diphosphate trisodium salt were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The citraconic anhydride and *N*-ethylmorpholine were redistilled before use. All other reagents were of A.R. grade. Iodo-[2- ^{14}C]acetic acid (15.5 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and diluted with carrier iodoacetic acid to a specific radioactivity of 1.5 mCi/mmol before use.

Reaction of aldolase with citraconic anhydride. Rabbit muscle aldolase (20 mg) dissolved in 4 ml of 50 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.2, containing 5 mM-EDTA was treated at 0°C with an excess of citraconic anhydride, with continuous stirring. The reagent dissolved as the reaction proceeded, the pH being maintained at 8.2 by addition of 1 M-NaOH. In experiments where complete reaction of the amino groups was sought, a 30-fold molar excess of reagent over total amino groups was employed. If only partial reaction was required, less anhydride was used, a threefold molar excess being sufficient to cause about 50% blocking of the protein amino groups. When the reaction was complete, the citraconylated aldolase was purified by dialysis against 20 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.2, at 2°C.

Unblocking of citraconyl-aldolase. Samples of citraconyl-aldolase (4 mg/ml) were dialysed against 10 mM-HCl, pH 2.0, at room temperature (20°C) for 6 h. After dialysis for a few minutes against HCl a precipitate of the citraconylated protein formed, but during the course of the next hour this went back into solution. To regenerate enzymic activity from protein that had been exposed to the pH 2 treatment, the samples were then dialysed against 20 mM-ammonium acetate buffer, pH 5.5, at 4°C to anneal the subunits and re-form the quaternary structure (Schachman, 1963).

Reaction with iodo[2- ^{14}C]acetic acid. Samples of protein were dissolved in 0.1 M-tris-HCl buffer, pH 8.2, containing 8 M-urea, to give a final concentration of 10 mg/ml. Dithiothreitol (1 μmol /ml of protein solution) was added and the tube flushed with N_2 . After 30 min at room

temperature, iodo[2- ^{14}C]acetic acid (5 μmol /ml of protein solution) dissolved in water and neutralized with 1 M-NaOH was also added and the tube flushed with N_2 . The mixture was kept at room temperature for 1 h and the carboxymethylated protein was then purified by dialysis against 0.5% (w/v) NH_4HCO_3 , pH 8.0, at 2°C. Under these conditions, all the incorporated radioactivity was found associated with *S*-carboxymethylcysteine after acid hydrolysis and paper electrophoresis at various pH values.

Peptide 'maps' and radioautographs. Proteins were digested with 1% (w/v) trypsin in 0.5% (w/v) NH_4HCO_3 , pH 8.0, at 37°C for 6 h, and the buffer was removed by freeze-drying. Peptide 'maps' and radioautographs were then prepared as described by Harris & Perham (1965). Details of the conditions used for paper electrophoresis and chromatography were given by Perham (1967). Peptides were detected by staining with the ninhydrin-cadmium reagent of Heilmann, Barrolier & Watzke (1957).

Assay of free amino and thiol groups. Protein solutions were dialysed exhaustively against 20 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.2. Free amino groups were then determined with TNBS* as described by Habeeb (1966). An extinction coefficient of $1.10 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$ was assumed for the TNP-amino group. For the assay of free thiol groups, proteins were dissolved in 0.1 M-*N*-ethylmorpholine-acetic acid buffer, pH 8.2, containing 8 M-urea, and treated with DTNB by the method of Ellman (1959). In the determination of both free amino and thiol groups, samples of control aldolase subjected to the same conditions as the citraconylated samples were always assayed at the same time. All results are expressed relative to such controls.

Ultracentrifugation. Samples of protein at about 3 mg/ml were dialysed against 20 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.0, containing 0.5 M-NaCl. Centrifugation was carried out in a Spinco model E ultracentrifuge at 48000 rev./min and 20°C. Under these conditions, the sedimentation coefficient of citraconylated protein increased with increasing salt concentration, reaching a plateau value at about 0.4 M-NaCl. All runs were therefore done in the presence of 0.5 M-NaCl, at which salt concentration the sedimentation coefficient was independent of protein concentration. Photographs of the schlieren patterns produced were taken every 16 min. The fraction of protein dissociated was estimated by enlarging the negatives, tracing the areas under the peaks, and cutting out and weighing the tracings.

Amino acid analysis. Peptides and proteins were hydrolysed and analysed for amino acids by using a Beckman 120C automatic analyser as described by Perham (1967). Protein concentration was estimated by amino acid analysis, assuming a subunit molecular weight of 40000 (Kawahara & Tanford, 1966).

Radioactivity measurements. For scintillation counting, triplicate samples were added to 3 ml of toluene-triton (2:1, v/v) containing 5 g of 2,5-diphenyloxazole/l and counted in a Nuclear-Chicago Unilux counter. In other experiments, triplicate samples dissolved in 0.01 M-NaOH

* Abbreviations: TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNP, trinitrophenyl; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Cmc, *S*-carboxymethylcysteine.

were plated on to planchets, dried *in vacuo* and counted in a Nuclear-Chicago gas-flow counter. Proteins were always digested with trypsin before sampling for counting, to promote solubility. Protein concentration was estimated by means of amino acid analysis.

RESULTS

Citraconylation of aldolase. Assuming a subunit molecular weight of 40000 (Kawahara & Tanford, 1966; Morse & Horecker, 1968), aldolase has 27 amino groups and seven thiol groups, shown by amino acid analysis (Table 1). The number of amino groups per subunit as assayed with TNBS was 30.4. However, in addition to amino groups, TNBS also reacts with thiol groups (Freedman & Radda, 1968). In a separate experiment under conditions identical with those used for aldolase, the reaction of cysteine, *N*-acetylcysteine and *S*-carboxymethylcysteine with TNBS was studied. It was found that, under these conditions, the presence of a free thiol group contributed an additional 50% approximately to the E_{335} observed for a substrate with only a free amino group. With aldolase, therefore, a protein with seven free thiol groups per monomer, a correction of some 3.5 groups has to be subtracted from the value for the free amino groups in the TNBS assay. Since this correction assumes all the protein thiol groups react with TNBS, it represents an upper limit.

Applying the correction, the number of free amino groups in the aldolase monomer assayable with TNBS drops to 27, in excellent agreement with the analytical results. The excellence of this agreement, because of the slight inaccuracies inherent in the TNBS assay with respect to the molar extinction coefficient chosen for the TNP-amino group (Habeeb, 1966), may well be fortuitous. Nonetheless, it is evident that the TNBS assay gives a close estimate of the free amino groups in aldolase, and the correction was subsequently neglected since it was small.

Samples of aldolase citraconylated to different extents were examined for enzymic activity and for sedimentation properties in the ultracentrifuge. With increasing citraconylation of the protein, the number of free amino groups diminished and the enzymic activity also fell, until at about 50% citraconylation of amino groups the enzymic activity was destroyed completely (Fig. 1). On this basis it seems likely that activity is lost by a general modification of structure, in addition to specific reaction with catalytically or structurally important lysine residues.

There was little change in the sedimentation coefficient of the protein with up to 50% citraconylation. However, as the citraconylation of the amino groups was increased from 60 to 80%, there was a dramatic change in the sedimentation behaviour of the protein. In addition to the peak with an uncorrected sedimentation coefficient of approx. 7.5S corresponding to the undissociated enzyme, there

Table 1. *Amino acid composition of rabbit muscle aldolase*

The amino acid composition of rabbit muscle aldolase (Anderson, Gibbons & Perham, 1969) is based on a subunit molecular weight of 40000 (Kawahara & Tanford, 1966).

Amino acid	Amino acid composition (mol/40000g)
Lys	26.6
His	10.1
Arg	14.9
Cmc	7.5
Asp	28.7
Thr	21.4
Ser	20.3
Glu	40.9
Pro	18.6
Gly	29.6
Ala	40.5
Val	21.1
Met	3.3
Ile	19.5
Leu	33.0
Tyr	10.9
Phe	7.7
Trp	3.7

Fig. 1. Aldolase activity (expressed as a percentage of sp. activity of control enzyme) (○) and dissociation (expressed as percentage dissociation into slowly sedimenting material) (●) plotted as a function of percentage citraconylation of the protein amino groups as measured by the TNBS assay.

Fig. 2. Sedimentation of citraconylated aldolase in 20 mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.0, containing 0.5 M-NaCl. Sedimentation was at 48000 rev./min and 20°C. Percentage citraconylation of protein amino groups was measured by the TNBS assay: (a) 0%; (b) 51%; (c) 75%; (d) 82%; (e) 90%. Photographs were taken about 80 min after maximum speed was reached. Sedimentation is from the right to left.

now appeared a more slowly sedimenting peak that grew in size with increasing citraconylation (Fig. 2). This peak was the sole component seen on ultracentrifugation above about 80% citraconylation and had an uncorrected sedimentation coefficient that approached 2.8S (Fig. 3). It is taken to be the monomeric citraconylated subunit of aldolase. It cannot yet be ruled out that the two peaks seen in the ultracentrifugal examination of partly dissociated protein are due to a slow equilibrium between monomer and tetramer.

Similar experiments were carried out in which aldolase was treated with maleic anhydride by the method of Butler *et al.* (1969). The results were

Fig. 3. Sedimentation coefficients of citraconylated aldolase plotted as a function of percentage citraconylation of protein amino groups as measured by the TNBS assay: ○, more rapidly sedimenting peak; ●, more slowly sedimenting peak.

entirely comparable with those obtained with citraconic anhydride except that the onset of protein dissociation occurred a little earlier, at about 40% maleylation, becoming complete at about 70% maleylation. Protein in which the amino groups were totally maleylated had an uncorrected sedimentation coefficient of 2.4S. This small difference in the sedimentation coefficients of the totally maleylated and totally citraconylated proteins is reproducible and appears to be genuine, perhaps reflecting a slightly greater unfolding of the maleylated subunit. Aldolase dissociated into its subunits at pH 2 has $s_{20,w}$ 2.0S (Schachman, 1963).

Unblocking of citraconyl-aldolase. Samples of citraconylated protein were dialysed against 10 mM-hydrochloric acid, pH 2, at room temperature (20°C). After a few minutes the citraconyl-protein precipitated but during the course of the next hour it came back into solution. Dialysis for 6 h was found to be sufficient to regenerate more than 95% of the protein amino groups, as indicated by the TNBS assay. In attempts to regenerate enzymic activity from protein that had been through the pH 2 treatment, the samples were next dialysed against 20 mM-ammonium acetate buffer, pH 5.5, containing 20 mM-mercaptoethanol. This treatment was included to anneal the subunits (Schachman, 1963). After dialysis for 5–10 h at 4°C, about 80% of the activity of control samples reproducibly returned. The yield of activity from citraconylated samples compared with a parallel control is shown in Table 2.

It is clear that dissociation of the subunits during

citraconylation is accompanied by a sharply falling recovery of activity after unblocking. To test whether this was due to incomplete regeneration of the aldolase primary structure, several experiments were undertaken. It is unlikely that recovery of amino groups is incomplete since tests with model compounds (Dixon & Perham, 1968) and, indeed, the TNBS assay of unblocked aldolase are not in accord with this. However, other side reactions are less easily eliminated. It has been pointed out (Perham, 1969b) that, since maleic anhydride and citraconic anhydride contain double bonds, alkylation of protein thiol groups is a distinct possibility. However, when *N*-acetylcysteine (2mM) was stirred at 0°C with a 50-fold molar excess of citraconic anhydride and the pH maintained at 8 by addition of 5M-sodium hydroxide, little loss of thiol group could be detected by assay with DTNB over a period of 1h, suggesting that this reaction, if it occurs at all during citraconylation under these conditions, is slow.

Perhaps the best test for regeneration of primary structure is identity of the tryptic peptide 'maps' before and after reaction. Samples of aldolase and of completely citraconylated aldolase were *S*-carboxymethylated with iodo[2-¹⁴C]acetic acid after the unblocking treatment at pH 2. Peptide 'maps' and radioautographs of their tryptic digests were then compared. Little or no difference was apparent

with the exception of the general intensity of the spots in the radioautographs locating ¹⁴C-containing peptides, all of which seemed appreciably weaker in the sample of protein that had been citraconylated. This apparent loss of thiol groups in aldolase after citraconylation was confirmed in a number of ways.

Aldolase that had been completely citraconylated was *S*-carboxymethylated either before or after the unblocking treatment at pH 2 and then submitted to hydrolysis and amino acid analysis. Instead of the seven residues of *S*-carboxymethylcysteine per monomer observed in control aldolase, the *S*-carboxymethylcysteine now amounted to only 3.6 and 4.0 residues in each case (Table 3), showing that the unblocking treatment gave little improvement in the yield. Moreover, in the protein samples that had been citraconylated there were three small additional peaks eluted close together, well before the carboxymethylcysteine, although after the position that cysteic acid would occupy. Assuming for them an analyser colour constant the same as that of aspartic acid, they accounted together for approx. 3 residues. The same three peaks in the same relative proportions were also found in the amino acid analysis of citraconyl-aldolase that had not been carboxymethylated. If *N*-acetylcysteine (10mM) is incubated with a large excess (80-fold) of citraconic acid at pH 8 and room temperature for 15h and then subjected to acid hydrolysis as for proteins, an appreciable proportion (up to 40%) of the *N*-acetylcysteine is converted into a form that is eluted immediately in front of aspartic acid on the amino acid analyser. This, presumably, is the alkylated derivative of cysteine referred to above. However, because of its elution position on the analyser, it is difficult to identify it with any one of the three additional peaks observed in the analysis of citraconyl-aldolase.

In other experiments, the thiol content of citraconyl-aldolase before and after unblocking was measured by means of the DTNB method and also by counting the radioactivity of the ¹⁴C-labelled

Table 2. *Recovery of aldolase activity after citraconylation of the enzyme protein*

Activity is expressed relative to a sample of non-citraconylated aldolase put through the same treatment for unblocking and annealing.

Citraconylation of amino groups (%)	Recovery of enzymic activity (%)
0	100
50	75
80	9
90	3

Table 3. *Thiol group content of aldolase before and after complete citraconylation*

In the DTNB assay (b), the proteins were reduced in 20mM-*N*-ethylmorpholine-acetic acid buffer, pH 8.0, containing 8M-urea and 20mM-dithiothreitol for 1½h at 20°C. Excess of dithiothreitol was removed by dialysis against 8M-urea before assay.

Protein	Amino acid analysis for <i>S</i> -carboxymethyl- cysteine (mol/40000g)	Thiol group content (mol/40000g)		
		DTNB assay		¹⁴ C-labelled <i>S</i> -carboxymethyl- cysteine
		(a)	(b)	
Control	7.1	6.0	5.3	7.0
Citraconyl-aldolase before unblocking	3.6	3.7	—	3.4
Citraconyl-aldolase after unblocking	4.0	—	2.7	4.2

S-carboxymethylcysteine produced by reaction with iodo[2-¹⁴C]acetic acid. These results are shown in Table 3. For the DTNB assay after unblocking, the protein samples (dissolved in 20 mM - *N*-ethylmorpholine-acetic acid buffer, pH 8.0, containing 8 M-urea) were reduced with 20 mM-dithiothreitol at room temperature for 1½ h, and the excess of dithiothreitol was then removed by dialysis against 8 M-urea for 11 h. This lengthy dialysis against urea probably explains why the thiol-group content is low in the subsequent assay (Table 3), some oxidation of the protein thiol groups having occurred. Apart from this, the results of thiol group analysis by various methods agree reasonably well and show always that the thiol-group content of aldolase is diminished after citraconylation, both before and after unblocking. Oxidation of these thiol groups to form disulphide bridges cannot be the simple explanation of this fall, since reduction with dithiothreitol does not regenerate them and anomalous peaks are seen in the amino acid analysis of the citraconyl-protein that together account approximately for the loss of thiol groups.

The inability to recover enzymic activity after complete citraconylation of the aldolase subunit (Table 2) also correlated with ultracentrifugal analysis of the annealed unblocked protein. Thus, whereas protein that had been 50% citraconylated (and therefore little dissociated; Fig. 1) gave 75% recovery of enzymic activity and an uncorrected sedimentation coefficient of 6.7S, little or no material with a sedimentation coefficient approaching that of the native enzyme could be obtained from aldolase that had been completely citraconylated. Indeed, after the annealing treatment at pH 5.5, such protein frequently formed a precipitate.

DISCUSSION

Citraconic anhydride can react with all the amino groups of aldolase at pH 8. Presumably as the reaction proceeds, dissociation and unfolding of the protein subunits exposes any amino groups not immediately available for modification. In this reaction, maleic anhydride and citraconic anhydride are entirely comparable and the peptide 'maps' of maleyl- and citraconyl-aldolase are identical. If the extent of citraconylation is compared with the dissociation of the enzyme tetramer, it appears that little dissociation occurs with less than 50% reaction of the protein amino groups and that it is not complete until about 80% citraconylation is reached. Similar results are noted with maleic anhydride, except that dissociation occurs at somewhat smaller extents of modification of the protein. These results do not agree entirely with the similar study of maleyl-aldolase by Sia & Horecker (1968),

where 15% modification of the protein amino groups was reported to cause complete dissociation of the tetramer.

The inability to recover appreciable enzymic activity from completely citraconylated aldolase is disappointing. With up to 50% citraconylation, good recovery of enzymic activity was possible after unblocking (Table 2). However, after complete citraconylation and the concomitant dissociation of the enzyme tetramer, little activity was recoverable. This cannot be ascribed to lack of regeneration of protein amino groups, and the only other modification of the primary structure detectable after the unblocking treatment is partial loss of thiol groups. It is difficult at the moment to identify the modification of these thiol groups. It seems unlikely that it is merely oxidation to disulphide bridges, since the modification appears to survive reduction with dithiothreitol and acid hydrolysis in evacuated tubes. Neither can it be easily reconciled with alkylation of the thiol groups with citraconic acid or anhydride. However, Turano, Giartosio, Riva & Fasella (1964) have shown that incubation of aspartate aminotransferase (EC 2.6.1.1) at 70°C in maleate buffers causes production of *S*-($\alpha\beta$ -dicarboxyethyl) derivatives of the enzyme.

The radioautographs of peptide 'maps' indicate that there is not a specific loss of individual thiol groups but rather a partial loss of radioactivity in all the peptides containing ¹⁴C-labelled carboxymethylcysteine. Unfortunately, this cannot readily be accounted for by new spots appearing in the peptide 'maps', perhaps because the peptides were diluted out in the 'maps' by heterogeneity. It is, however, probably more than coincidence that the inability to recover enzymic activity after unblocking correlates with the dissociation of the enzyme seen on ultracentrifugation.

In addition to the effect on thiol groups, residues other than lysine may react with citraconic anhydride to give products that are stable at pH 8 yet labile at acidic pH, thereby remaining undetected in the experiments described. It is known that succinic anhydride can react with the hydroxyl groups of serine, threonine and tyrosine residues (Gounaris & Perlmann, 1967). Similarly, although maleic anhydride is reported to be uniquely specific for amino groups in several proteins examined (Butler *et al.* 1969), with tobacco-mosaic-virus protein appreciable side reaction of ¹⁴C-labelled maleic anhydride with groups other than amino and thiol groups can be demonstrated (L. King & R. N. Perham, unpublished work). These products, presumably *O*-maleyl esters, are stable on prolonged exposure to alkaline pH (pH 9), but, unlike the maleyl amide bond, may be degraded at pH 8.5 with the strong nucleophile hydroxylamine. Like

the maleyl amide bond, however, they are also broken down by exposure to mildly acidic conditions. Thus, in this particular case at least, and contrary to previous speculation (Butler *et al.* 1969; Dixon & Perham, 1968), it appears that hydrolysis of the maleyl ester bond involves the protonated form of the free carboxyl group. This mechanism would be in accord with that proposed for the intramolecular catalysis of the hydrolysis of maleyl and citraconyl ester and amide bonds (Bruce & Benkovic, 1966) and the corresponding phthalyl esters (Thanassi & Bruce, 1966). In interpreting the dissociation of proteins caused by reaction with citraconic (and maleic) anhydride, some care must therefore be used unless the possibility of side reaction with groups other than amino groups is specifically eliminated. This clearly calls for the use of ^{14}C -labelled citraconic anhydride. In the present experiments, treatment of the citraconyl-protein with 0.5M-hydroxylamine at pH 8.5 (sufficient to degrade citraconyl esters in tobacco-mosaic-virus protein) failed to cause improvement in the recovery of enzyme activity after unblocking. (This precaution was to some extent unnecessary since it was known that exposure to acid pH was also sufficient to break down such esters.)

Thus, in these experiments, the only modification of the primary structure of aldolase detectable after citraconylation and unblocking is that of thiol groups. For the moment, therefore, it is to this that we must ascribe the inability to recover substantial enzymic activity after reaction. This supposition can be tested by repeating the experiments on aldolase in which the thiol groups have been reversibly protected. It seems advisable always to protect thiol groups in proteins before treatment with maleic anhydride or citraconic anhydride, if only to prevent the risk of irreversible alkylation. With *S*-carboxymethylated aldolase, it is clear that the citraconylation can be completely reversed at mildly acidic pH to regenerate exactly the original primary structure, as shown by tryptic peptide 'maps'. The usefulness of the reagent in studies of protein primary structure (Dixon & Perham, 1968) is therefore, fortunately, not in question.

We are grateful to Mr M. Riley and Dr H. B. F. Dixon for valuable discussion and to Mrs Mary Richards and Mrs Susan Hall for help with the amino acid analysis. I. G. is happy to acknowledge the receipt of a Scholarship from the Science Research Council.

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