Intramolecular Ionic Interactions of Lysine Residues and a Possible Folding Domain in Fructose Diphosphate Aldolase

By JOHN M. LAMBERT,* RICHARD N. PERHAM and JOHN R. COGGINS†
*Department of Biochemistry, University of Cambridge, Tennis Court Road,
Cambridge CB2 1QW, U.K.*

(Received 17 June 1976)

1. Treatment with methyl acetimidate was used to probe the topography of the tetrameric fructose 1,6-diphosphate aldolase from ox liver. A single treatment with imido ester in the presence or absence of 20 mM-fructose 1,6-diphosphate caused the number of amino groups in the enzyme to fall to approx. 30% of the starting number (assumed to be 30 per subunit). The catalytic activity of the aldolase modified in the presence of fructose 1,6-diphosphate was unaffected, whereas that of the enzyme modified in the absence of substrate fell by about 20%. 2. Use of methyl [1-¹⁴C]acetimidate and small-scale methods of protein chemistry showed that the amino group of lysine-27 (the numbering is that of the highly homologous rabbit muscle enzyme) is essentially unavailable for amidination in the native enzyme and is therefore predicted to be buried in a hydrophobic environment, probably in the form of an ion-pair with a negatively charged side-chain carboxyl group. All the other lysine residues that reacted poorly with methyl acetimidate in the native enzyme (a total of 7) were found to be within the primary structure bounded by lysine-107 and lysine-227. An important member of this group of lysine residues displaying aberrant reactivity is lysine-227, which is known to form an imine with the substrate as part of the catalytic mechanism of the enzyme. 3. The results of the amidination experiments can be correlated in an interesting way with previous studies of thiol-group modification in the aldolases. Taken together, and arguing in part by analogy with the results of identical experiments with glyceraldehyde 3-phosphate dehydrogenases where the three-dimensional structure is known [Lambert & Perham (1977) *Biochem. J.* 161, 49–62], they suggest that the region of primary structure from residues 107–227 may form the whole or part of a three-dimensional structural feature, perhaps a folding domain. A three-dimensional structure deduced from X-ray-crystallographic analysis will be needed to interpret these findings more closely. 4. The amino groups of lysine residues are commonly thought to reside at the 'surface' of protein structures. The patterns of specific lysine residues in glyceraldehyde 3-phosphate dehydrogenases and in aldolases that have been found to react poorly with methyl acetimidate in the native enzymes can be attributed to intramolecular ionic interactions deep in hydrophobic pockets and at the protein 'surface'. Such ionic interactions may contribute significantly to the stability of a given protein.

Fructose 1,6-diphosphate aldolase (EC 4.1.2.13) has been the subject of many experiments involving the modification of amino acid side chains designed to test the role of various functional groups in catalysis [for a review, see Horecker *et al.* (1972)]. Aldolases of Class I, to which class belong the mammalian aldolases (Rutter, 1964), form an imine intermediate between a lysine amino group of the protein and the substrate. For this reaction, the amino group needs to be unprotonated. However, competitive labelling experiments with acetic anhydride show that this amino group is anomalous in its reactivity, behaving

as though it had an apparent $pK \geq 11.5$ (Anderson & Kaplan, 1974). It thus appears that the amino group only becomes reactive enough or, more likely, accessible enough, to form the imine during or after substrate binding. The involvement of an additional lysine residue in substrate binding has been suggested by studies of the inhibition caused by pyridoxal phosphate (Shapiro *et al.*, 1968; Anai *et al.*, 1973).

Of the eight thiol groups per subunit (mol.wt. 40000) in the rabbit muscle enzyme, experiments with the reagents *p*-mercuribenzoate (Swenson & Boyer, 1957), 5,5'-dithiobis-(2-nitrobenzoic acid) (Eagles *et al.*, 1969), bromoacetate (Szajani *et al.*, 1970), iodoacetamide and *N*-ethylmaleimide (Anderson & Perham, 1970) and disulphide monosulphoxides (Steinman & Richards, 1970) showed that four

* Present address: Department of Biological Chemistry, University of California, Davis, CA 95616, U.S.A.

† Present address: Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

groups are 'exposed' and four are 'buried'. The exposed thiols could be further classified as those that react rapidly and those that react slowly with the various reagents, but the number of thiol groups in each class was found to vary with the reagent used (Perham & Anderson, 1970). Two thiol groups can be protected from reaction by substrates (Steinman & Richards, 1970) and a thiol group has been implicated in the catalytic mechanism (Horecker *et al.*, 1972; Lai *et al.*, 1974), although the evidence is not wholly convincing (Perham & Anderson, 1970). It is probable that the loss of enzymic activity is due to the presence of the modifying group, rather than to modification of a catalytically essential cysteine residue (Anderson, 1972).

A complete three-dimensional structure is not yet available for aldolase, although preliminary X-ray-diffraction studies have provided evidence that the tetrameric rabbit muscle protein has pseudo-222 symmetry (Eagles *et al.*, 1969; Heidner *et al.*, 1971). Studies of the primary structure of rabbit muscle aldolase have yielded two conflicting amino acid sequences (Lai *et al.*, 1974; Sajgo & Hajos, 1974) and re-examination of some regions of the primary structure is therefore needed. The experiments of Saunders & Weber (1975), which involved guanidination of native rabbit muscle aldolase with accompanying loss of catalytic activity, were interpreted as suggesting that lysine amino groups are buried at the subunit-subunit interfaces. The non-covalent forces binding the four subunits together appear to be strong, since more than 50% citraconylation of lysine residues, which introduces a net charge change of -2 for each lysine residue modified, has been shown to be necessary to effect dissociation of the tetramer to monomer (Gibbons & Perham, 1970).

In the present paper we describe the treatment of ox liver aldolase with methyl acetimidate as a probe of amino-group reactivity and protein topography, by using the method described in detail for glyceraldehyde 3-phosphate dehydrogenase (Lambert & Perham, 1977). Unlike the glyceraldehyde 3-phosphate dehydrogenases (Lambert & Perham, 1974), liver aldolase is demonstrably different from the muscle form of the enzyme within the same species (Blostein & Rutter, 1963; Penhoet *et al.*, 1969), although the amino acid sequences of the muscle and liver enzymes are highly homologous (Forcina & Perham, 1971). We chose to use ox liver aldolase for these experiments, since a large part of the amino acid sequence has been determined in this laboratory (Forcina & Perham, 1971; B. G. Forcina, unpublished work), and it was conceivable that useful peptides, particularly overlap sequences, would be isolated during the course of the work. The known sequence homology with rabbit muscle aldolase (Forcina & Perham, 1971) could be used to locate pertinent lysine residues in the primary structures reported for the

rabbit muscle enzyme (Lai *et al.*, 1974; Sajgo & Hajos, 1974), and we hoped further that our results might help resolve the conflicts in these structures.

Materials and Methods

Enzymes and reagents

Ox liver aldolase was purified by the method of Penhoet *et al.* (1969) and stored at 4°C in 70%-satd. $(\text{NH}_4)_2\text{SO}_4$. Trypsin and chymotrypsin (twice recrystallized) were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. NAD⁺ (free acid, grade II), NADH and a triose phosphate isomerase/ α -glycerol phosphate dehydrogenase suspension were purchased from Boehringer und Soehne, Mannheim, Germany. Fructose 1,6-diphosphate (tetrasodium salt) was obtained from BDH Chemicals, Poole, Dorset, U.K.

Methyl acetimidate and methyl $[1-^{14}\text{C}]$ acetimidate were synthesized by the methods described previously (Bates *et al.*, 1975; Lambert & Perham, 1977) and all other chemicals were of analytical grade.

Enzyme assay

Enzymic activity was determined at 30°C by a modification (Gibbons & Perham, 1974) of the method of Blostein & Rutter (1963), adding the test sample to a cuvette containing Tris/HCl buffer (50 mM, pH 7.5), NADH (0.1 mM), fructose 1,6-diphosphate (1.3 mM) and the triose phosphate isomerase/ α -glycerol phosphate dehydrogenase coupling enzyme (4 μg), all in a final volume of 3.0 ml.

Amidination of native enzyme

Aldolase (2–3 g/litre) was treated with methyl acetimidate (final concentration 0.1 M) in a buffer consisting of 0.1 M-N-ethylmorpholine/acetic acid, 2 mM-EDTA, 2 mM-2-mercaptoethanol and 0.02% NaN₃ at pH 8.0 and 20°C. The stock solution of methyl acetimidate (1.0 M) was prepared immediately before use as described by Bates *et al.* (1975) and the reaction stopped when required as described in the preceding paper (Lambert & Perham, 1977).

Amidination of enzyme in the presence of 5 M-guanidine hydrochloride

Native or partly amidinated samples of aldolase were reduced, S-carboxymethylated and amidinated with methyl $[1-^{14}\text{C}]$ acetimidate under conditions identical with those previously described (Lambert & Perham, 1977; Bates *et al.*, 1975).

Characterization of amidinated enzyme

Proteolytic digestion of amidinated aldolase with trypsin and chymotrypsin, measurement of radio-

activity and of extent of amidination, and quantitative amino acid analysis to determine protein concentration, were carried out by procedures identical with those previously adopted for glyceraldehyde 3-phosphate dehydrogenase (Lambert & Perham, 1977). The amino acid composition used in these experiments was that calculated for a subunit molecular weight of 40000 (Forcina & Perham, 1971).

The specific radioactivity of the methyl [$1-^{14}\text{C}$]acetimidate was calculated from a knowledge of the amino acid composition of the protein and the specific radioactivity of an aldolase sample in which all the amino groups had been modified by treatment with the ^{14}C -labelled imido ester in guanidine hydrochloride. There are 30 lysine residues per polypeptide chain by amino acid analysis, and the *N*-terminal residue was found to be blocked when examined by the dansyl method of Gray & Hartley (1963), both of which results are in agreement with previous observations in this laboratory (B. G. Forcina, unpublished work). Thus we have assumed a value of 30 free amino groups per subunit in the calculation of the specific radioactivity of the reagent.

Separation and characterization of peptides

Thin-layer peptide 'maps' were prepared from 50 μg samples of proteolytic digest by using the techniques of Bates *et al.* (1975), as described by Lambert & Perham (1977).

Gel filtration on Sephadex G-50 (superfine grade), paper electrophoresis, chromatography and dansyl-Edman degradation of peptides were carried out by standard techniques (Brown & Perham, 1974).

Results

Amidination of ox liver aldolase in the presence and absence of fructose 1,6-diphosphate

Methyl acetimidate (final concentration 0.1 M) was added to each of two identical samples of aldolase (2.1 g/litre) in the *N*-ethylmorpholine/acetic acid buffer, pH 8.0, one of which contained fructose 1,6-diphosphate (20 mM). The effect of the imido ester treatment on these samples is shown in Fig. 1. The number of unmodified amino groups quickly fell from the value of 30 per subunit in the native enzyme, to around 10 amino groups per subunit, in the presence and the absence of fructose diphosphate. The enzymic activity of the aldolase modified in the presence of fructose diphosphate showed no decrease during the course of the experiment, whereas under the same conditions, enzyme modified in the absence of fructose diphosphate suffered an approx. 20% loss of activity. A further addition of methyl acetimidate (0.1 M) caused the number of free amino groups to fall

to a value as low as four per subunit in some experiments, and the catalytic activity of the unprotected enzyme again diminished by about 20%, measured 4 h after the second addition of reagent.

Samples of partly amidinated protein were taken at various times during the treatment of the native aldolase in the presence and the absence of fructose diphosphate shown in Fig. 1. The amidination was terminated and these samples were then *S*-carboxymethylated with iodoacetic acid and completely amidinated by treatment with 0.1 M-methyl [$1-^{14}\text{C}$]acetimidate in 5 M-guanidine hydrochloride (Lambert & Perham, 1977). The chemically homogeneous protein samples were digested with trypsin and chymotrypsin and submitted to peptide 'mapping' on silica-gel thin-layer sheets, from which radioautographs were prepared (Plate 1).

The number of labelled peptides rapidly diminished owing to the prior reaction of amino groups with the unlabelled imido ester. However, even after 8 h incubation and two treatments with unlabelled reagent, several radioactive peptides were present in the 'maps', in contrast with the results of similar experiments with glyceraldehyde 3-phosphate dehydrogenase, where only one peptide was strongly labelled at this stage (Lambert & Perham, 1977). These results are consistent with the observation that about ten amino groups remained unmodified in native aldolase after a single treatment with imido ester, whereas with glyceraldehyde 3-phosphate dehydrogenase only about four amino groups remained

Fig. 1. Treatment of ox liver aldolase (2.1 g/litre) with 0.1 M-methyl acetimidate at pH 8 and 20°C in the presence (open symbols) and absence (solid symbols) of 20 mM-fructose 1,6-diphosphate

Catalytic activity of untreated enzyme (○, ●), catalytic activity of the treated enzyme (□, ■), number of free amino groups per subunit assayed by subsequent treatment with methyl [$1-^{14}\text{C}$]acetimidate (Δ, ▲). For other details, see the text.

unmodified under similar conditions (Lambert & Perham, 1977).

Radioautographs of aldolase 'maps', where the modification of native enzyme was carried out in the presence of fructose diphosphate, showed no difference in radioactive peptide pattern from radioautographs of 'maps' of aldolase where the prior modification was performed without fructose diphosphate present in the buffer (Plate 1). Identical amino groups were labelled in each case, in keeping with the similarity of the free amino-group determinations with respect to time and the fact that the enzyme unprotected by substrate still had 64% of the native enzymic activity after two treatments with methyl acetimidate. Therefore a peptide containing the essential lysine residue that carries the substrate in imine linkage in the enzyme (Gibbons *et al.*, 1970; Horecker *et al.*, 1972) would be expected to be among the labelled peptides detected in thin-layer 'maps' of protein modified in the presence or the absence of fructose diphosphate. With these considerations in mind, we decided to restrict the identification of radioactive peptides to aldolase that was amidinated in the absence of fructose diphosphate.

Identification of 'protected' lysine residues in ox liver aldolase

A sample of aldolase (10mg) was treated with 0.1M-methyl acetimidate for 4h, as shown in Fig. 1, and was *S*-carboxymethylated before completing the amidination with methyl [^{14}C]acetimidate in 5M-guanidine hydrochloride. The specific radioactivity of this protein was 4.1×10^3 c.p.m./nmol of polypeptide chain, whereas the specific radioactivity of aldolase fully amidinated only with labelled imido ester was measured to be 12.3×10^3 c.p.m./nmol of polypeptide chain. Since there are 30 amino groups per subunit in ox liver aldolase, the specific radioactivity of the reagent used was calculated to be 410 c.p.m./nmol, indicating a total of approx. 10 amino groups per polypeptide chain that must have been left unmodified after the initial treatment of the native enzyme with unlabelled imido ester.

The sample (10mg) of amidinated aldolase was digested with trypsin and chymotrypsin and the peptides were fractionated by gel filtration on a column of Sephadex G-50 (119cm $\times$ 1.2cm) in NH_4HCO_3 buffer (5g/litre), pH 8.0. The radioactive peaks from the column (located by counting radioactivity of 5 μl samples of the 1.2ml fractions) were freeze-dried separately and submitted successively to high-voltage paper electrophoresis at pH 6.5, to paper chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent and to paper electrophoresis at pH 3.5 and 2.0. In this way eight radioactive peptides were purified from the digest, whereas several very weakly radioactive pep-

tides could not be followed through all of the purification stages.

The peptides were characterized by amino acid and dansyl *N*-terminal analysis, and by measurement of their specific radioactivities. The results are listed in Table 1. The amino acid sequence of ox liver aldolase has not been completely determined, but it is known to be highly homologous with that found for the rabbit muscle enzyme (Forcina & Perham, 1971; B. G. Forcina, unpublished work). Our thanks are due to Dr. B. G. Forcina for providing unpublished sequence information on ox liver aldolase, to confirm the fit of the peptides to the homologous rabbit muscle sequence of Lai *et al.* (1974). This homology, given the information listed in Table 1, is sufficient to enable us to write down the probable sequences of the peptides, as shown in Table 2.

Peptides ATC2 (yield 8nmol) and ATC4 (yield 8nmol) were judged to have some glycine impurity in their analyses and peptide ATC7 (yield 10nmol) to have glutamic acid and glycine as impurities (Table 1). These amino acids were detected as impurities during dansyl *N*-terminal analysis of these peptides (all of which were isolated in low yield) and are likely to be contaminants from paper. Dansyl-Edman degradation was used to confirm their identities. To demonstrate that peptides ATC4 and ATC5 were from overlapping sequences produced by chymotryptic digestion, two rounds of dansyl-Edman degradation were performed, yielding the common sequence Lys-Ile-Asx-.

Peptide ATC2 proved difficult to place by reference to the homologous rabbit muscle aldolase sequence (Lai *et al.*, 1974), and four rounds of dansyl-Edman degradation were performed with this peptide (Table 2). One confusing problem was that the dansylated form of the dipeptide Ile-Lys, in which the lysine *N*⁶-amino group is amidinated and therefore positively charged, is slow to hydrolyse and migrates very similarly to Dns-aspartic acid on polyamide layer sheets. Longer hydrolysis times (40h) showed the *N*-terminal amino acid of the peptide to be isoleucine.

The number of mol of labelled lysine per mol of radioactive peptide is also given in Table 2. This information is calculated from the specific radioactivity of the reagent used to complete the amidination of the polypeptide chain under denaturing conditions. It therefore reflects the lack of modification of a given lysine residue in the native enzyme tetramer. One amino group (peptide ATC1) reacted very poorly with methyl acetimidate when in the native tetramer. This amino group belongs to a lysine residue that corresponds to lysine-27 of the homologous rabbit muscle aldolase sequence (Lai *et al.*, 1974). A further group of three lysine residues showed very high degrees of amino-group 'protection' when in the native tetrameric enzyme: they are those of lysine-152, with

EXPLANATION OF PLATE I

Radioautographs of peptide 'maps' of tryptic/chymotryptic digests of amidinated ox liver aldolase

The native enzyme was treated with methyl acetimidate as shown in Fig. 1. After 4 h, the treatment with 0.1 M-methyl acetimidate was repeated. Samples were removed after various time-intervals from the beginning of the experiment, as shown, and these were *S*-carboxymethylated and treated with methyl [1-¹⁴C]acetimidate in 5 M-guanidine hydrochloride before proteolytic digestion. The amidination of the native enzyme was carried out in the presence (a) and absence (b) of 20 mM-fructose 1,6-diphosphate.

Table 1. *Amino acid compositions, N-terminal residues and specific radioactivities of tryptic/chymotryptic peptides from amidinated ox liver aldolase*

Abbreviation: CmCys, S-carboxymethylcysteine. +, indicates present in analysis, but not quantified.

Amino acid	Peptide	...	Amino acid composition (mol/mol of peptide)							
			ATC1	ATC2	ATC3	ATC4	ATC5	ATC6	ATC7	ATC8
CmCys						+	+			
Asp			2.0	1.0	1.8	4.0	3.8		1.9	1.2
Thr*			1.0			1.3	1.0	1.1		1.0
Ser*			0.9			1.0	1.0			
Glu			1.0	1.2	0.1†	2.8	3.2	1.0	0.7‡	1.2
Pro				+		+	+			+
Gly			3.0	1.7	1.1	1.1‡	0.3‡		0.3‡	1.2
Ala			3.1	0.8	1.2	2.3	3.0		0.8	0.2‡
Val			1.5	1.2	0.1‡			1.8	0.8	0.2‡
Met			0.9							
Ile			1.5	1.1		1.8	2.0			
Leu			1.0	1.6		1.9	2.0	0.9	1.1	2.5
Tyr									1.1	
Phe					0.6					
Lys†			+	+	+	+	+	+	+	+
His						0.7	0.9		1.4	
Arg							1.0			
N-Terminal residue			Ile	Ile	Lys	Lys	Lys	Val	Lys (+O-Tyr)	Leu
Specific radioactivity (¹⁴ C c.p.m./nmol of peptide)			384	206	284	330	343	145	361	347

* Not corrected for destruction of these residues.

† Not quantified owing to incomplete hydrolysis of amidine (Reynolds, 1968).

‡ Assumed to be impurity.

Table 2. *Radioactively labelled tryptic/chymotryptic peptides from amidinated ox liver aldolase*The numbering in the sequence is according to Lai *et al.* (1974). → denotes a residue established by dansyl-Edman degradation in the present study.

Peptide	Sequence position	Probable sequence	Radioactively modified lysine (mol/mol of peptide)
ATC1	22-39*	Ile-Val-Ala-Asx-Gly-Lys-Gly-Ile-Leu-Ala-Ala-Asx-Glx-Ser-Val-Gly-Thr-Met	0.94
ATC2	106-115*	Ile-Lys-Leu-Asx-Glx-Gly-Val-Ala-Pro-Leu	0.50
ATC3	138-144*	Lys-Lys-Asp-Gly-Ala-Asp-Phe	0.69
ATC4	152-170*	Lys-Ile-Asx-Asx-Glx-Cys-Pro-Ser-His-Leu-Ala-Ile-Glx-Glx-Asx-Ala-Asx-Thr-Leu	0.80
ATC5	152-172*	Lys-Ile-Asx-Asx-Glx-Cys-Pro-Ser-His-Leu-Ala-Ile-Glx-Glx-Asx-Ala-Asx-Thr-Leu-Ala-Arg	0.84
ATC6	202-207*	Val-Thr-Glu-Lys-Val-Leu	0.35
ATC7	212-220†	Lys-Ala-Leu-Asn-Asp-His-His-Val-Tyr	0.88
ATC8	221-229†	Leu-Glu-Gly-Thr-Leu-Leu-Lys-Pro-Asn	0.85

* Sequence deduced from homology with rabbit muscle aldolase (Lai *et al.*, 1974; B. G. Forcina, unpublished work).

† Deduced by comparison with ox liver aldolase sequence information (Forcina & Perham, 1971).

0.82 mol of labelled lysine/mol of peptide (mean of peptides ATC4 and ATC5), lysine-227, with 0.85 mol of labelled lysine/mol of peptide (peptide ATC8) and lysine-212, with 0.88 mol of labelled lysine/mol of peptide (peptide ATC7). Within this group of lysine residues, as expected, was found the essential lysine residue, lysine-227 (Lai *et al.*, 1974), which forms an imine with the substrate in the catalytic mechanism of the enzyme.

The radioactivity of peptide ATC3 may be distributed between lysine residues 138 and 139, since these were not distinguished. A further two peptides contained lysine residues only moderately labelled. These are lysine-107 (peptide ATC2) and lysine-205 (peptide ATC6).

The sum of the specific radioactivities of the purified peptides (5.0 mol of radioactively modified lysine residues/mol of protein chain; Table 2) was found to account for about 50% of the radioactivity of the modified protein before peptide fractionation. This value corresponds to the similar value of about 50% of the radioactivity of the modified protein accounted for by the labelled peptides purified from rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Lambert & Perham, 1977). As before, the most likely explanation is that there is a low background of incomplete amidination at all of the lysine residues in the initial modification of the native protein, which allows all these amino groups subsequently to become weakly radioactively labelled. Only one treatment of the native enzyme with unlabelled imido ester was used in these experiments in an attempt to show up the partial reaction of slowly reacting amino groups. Very weakly labelled peptides would not subsequently appear in the peptide 'maps', despite their contribution to the overall specific radioactivity of the protein that was measured. Similarly, lack of specificity in the tryptic/chymotryptic digestion would lead to overlapping sequences that could dilute labelled peptides in the 'maps'.

Location of unreactive amino groups in the primary sequence of aldolase

The work described above has revealed that native ox liver aldolase contains a set of lysine residues that react slowly (or not at all) with methyl acetimidate. These residues can be identified in the primary structure of the enzyme by using the homologous sequence of the rabbit muscle enzyme determined by Lai *et al.* (1974), shown in Fig. 2. All but one of the poorly reacting amino groups are restricted to the central region of the polypeptide chain, bounded by lysine residues at positions 107 and 227. The exception, lysine-27, bears the amino group that is most highly radioactively labelled and which can be considered to be completely inaccessible to imido ester in the native tetrameric enzyme.

Discussion

The aldolases from rabbit muscle and ox liver are highly homologous and are likely to have very similar three-dimensional structures (Forcina & Perham, 1971). There are two similar, though different, published sequences for the rabbit muscle enzyme (Lai *et al.*, 1974; Sajgo & Hajos, 1974), but, as yet, there is no X-ray structure for the protein from any source.

Eight different lysine residues were found to be 'protected' from amidination in the native tetrameric enzyme from ox liver (Table 2, Fig. 2). The amino group of lysine-27 (peptide ATC1 of Table 2 and Fig. 2) is completely protected from reaction with imido ester until the protein is denatured and in this property closely resembles lysine-306 of glyceraldehyde 3-phosphate dehydrogenase (Lambert & Perham, 1977). We would therefore predict that this amino group is buried in a hydrophobic environment in the protein, probably in the form of an ion-pair with a negatively charged side-chain carboxyl group. With glyceraldehyde 3-phosphate dehydrogenase we can locate the ion-pair at a subunit interface (Lambert & Perham, 1977), but, in the absence of X-ray-crystallographic evidence, we can make no firm prediction about its location for ox liver aldolase.

All the remaining 'inaccessible' lysine residues were found to be in one region of the polypeptide chain (Fig. 2) within the residues bounded by lysine-107 (peptide ATC2), which is thought to be involved in the secondary binding of substrate (Shapiro *et al.*, 1968; Lai *et al.*, 1974), and lysine-227 (peptide ATC8), which forms an imine with the substrate (Horecker *et al.*, 1972). Some of these lysine residues become very highly labelled after the single treatment of the native protein with unlabelled imido ester (Table 2). Such residues must therefore have reacted poorly with imido ester in the native aldolase tetramer. Their number contrasts interestingly with that found for glyceraldehyde 3-phosphate dehydrogenase where, apart from lysine-306, no lysine residue showed comparable lack of modification after a single treatment of the native enzyme with unlabelled imido ester (Lambert & Perham, 1977).

An explanation advanced for poorly reacting amino groups in glyceraldehyde 3-phosphate dehydrogenase (Lambert & Perham, 1977), that of ionic interaction of surface lysine amino groups with neighbouring carboxyl groups, which lowers the reactivity of an amino group towards the imido ester, can be advanced again for ox liver aldolase. This would be a likely explanation for the slow reaction of lysine-107 (peptide ATC2), lysine-205 (peptide ATC6) and lysine-138/lysine-139 (peptide ATC3, no distinction being made between these residues) with methyl acetimidate in the native enzyme. Lysine-107 is thought to have a role in catalysis, since modification at this site with pyridoxal phosphate causes loss of

Fig. 2. Amino acid sequence of rabbit muscle aldolase (Lai et al., 1974)

The inferred positions of peptides ATC1-ATC8 (Table 2) from the homologous ox liver enzyme are underlined.

activity in the rabbit muscle enzyme (Shapiro *et al.*, 1968). However, simple ionic interactions may not be sufficient to account for the very slow reaction of the amino groups of lysine-152, lysine-212 and lysine-227 (Table 2, Fig. 2). It is possible that these groups are involved in ion-pair interactions near the protein surface (interactions that would be strengthened if they occurred in hydrophobic pockets), but the groups may also be poorly accessible to reagents for steric reasons. Thus the amino group of the catalytically essential lysine-227 has previously been shown to react only very slowly with acetic anhydride, exhibiting an apparent $pK \geq 11.5$ (Anderson & Kaplan, 1974). In effect, the protein has to become denatured before the amino group reacts. The amino group must be unprotonated before the imine can form with the substrate and, if the pK of the group was to be perturbed, it might be expected to be perturbed in the acidic direction, as with the lysine residue at the active site of acetoacetate carboxylase that has a pK of 5.9 (Schmidt & Westheimer, 1971). However, a protein conformational change accompanies substrate binding to rabbit muscle aldolase (Mehler & Cusic, 1967) and the properties of functional groups in the active site are likely to undergo significant changes when the substrate binds. Certainly, our results agree with those of Anderson & Kaplan (1974) in showing that lysine-227 exhibits unusual properties in the native enzyme. The lack of modification of lysine-227 in the native enzyme after a single treatment with unlabelled imido ester (85% of the N^6 -amino group was still unmodified) was reflected in the enzymic activity at this point of the time-course (Fig. 1), which was still 77% of that of the untreated control enzyme.

Except for lysine-27, all the lysine residues that react unusually slowly (or not at all) with methyl acetimidate are found in the central portion of the primary structure bounded by residues 107 and 227 (Fig. 2). We may speculate that this sequence, which includes some of the residues important for catalysis, could comprise a folding domain (or a region sandwiched between folding domains), the structure of which affords protection against modification to some of the amino groups of lysine residues within the domain. Support for this idea may be found by consideration of the reactivity of cysteine residues in the enzyme. In native rabbit muscle aldolase there are eight such residues per chain at positions 72, 134, 149, 177, 199, 237, 287 and 336. Four of these residues, at positions 134, 149, 177 and 199, are unreactive towards various reagents (Steinman & Richards, 1970; Perham & Anderson, 1970; Lai *et al.*, 1974). All four residues lie within the central portion of the sequence marked out by the lysine residues that react unusually slowly with methyl acetimidate (positions 107–227).

Our interpretation of the topography of aldolase must await further consideration until a detailed

X-ray-crystallographic model of the enzyme can be built. We have obtained very similar results with fructose diphosphate aldolase from rabbit muscle (J. M. Lambert, R. N. Perham & J. R. Coggins, unpublished work), although we have not thought it worth while at this stage to go further with the identification of the amino groups that react slowly with methyl acetimidate for this enzyme. However, the pattern of such lysine residues, as revealed by peptide 'maps' and amino-group assay, was very similar to that observed for ox liver aldolase, which strengthens the prediction that the muscle and liver iso-enzymes have closely related three-dimensional structures (Forcina & Perham, 1971).

In conclusion, on the basis of the two oligomeric proteins examined in these studies, glyceraldehyde 3-phosphate dehydrogenase and aldolase, it seems safe to predict that patterns of specific lysine residues will show aberrant reactivities in all oligomeric proteins and that the most likely explanation is ionic interactions at the protein 'surface' or in more inaccessible hydrophobic environments. The contributions of such interactions to the stability of proteins should not be overlooked. Lowering the free energy of a protein molecule by $6 \text{ kJ} \cdot \text{mol}^{-1}$ would be expected to decrease the rate of thermal denaturation 10-fold at 30°C , and such free energies are well within the range expected for ionic interactions, even in aqueous solution.

We are grateful to the Medical Research Council for the award of a Scholarship to J. M. L., and to the Science Research Council (grant B/SR/9480) and the Wellcome Trust for financial support. We are indebted to Dr. B. G. Forcina for making available to us unpublished amino acid-sequence analysis of the ox liver aldolase.

References

- Anai, M., Lai, C. Y. & Horecker, B. L. (1973) *Arch. Biochem. Biophys.* **156**, 712–719
- Anderson, P. J. (1972) *Can. J. Biochem.* **50**, 111–119
- Anderson, P. J. & Kaplan, H. (1974) *Biochem. J.* **137**, 181–184
- Anderson, P. J. & Perham, R. N. (1970) *Biochem. J.* **117**, 291–298
- Bates, D. L., Perham, R. N. & Coggins, J. (1975) *Anal. Biochem.* **68**, 175–184
- Blostein, R. & Rutter, W. J. (1963) *J. Biol. Chem.* **238**, 3280–3285
- Brown, J. P. & Perham, R. N. (1974) *Biochem. J.* **137**, 505–512
- Eagles, P. A. M., Johnson, L. N., Joynson, M. A., McMurray, C. H. & Gutfreund, H. (1969) *J. Mol. Biol.* **45**, 533–544
- Forcina, B. G. & Perham, R. N. (1971) *FEBS Lett.* **18**, 59–63
- Gibbons, I. & Perham, R. N. (1970) *Biochem. J.* **116**, 843–849

- Gibbons, I. & Perham, R. N. (1974) *Biochem. J.* **139**, 331–334
- Gibbons, I., Anderson, P. J. & Perham, R. N. (1970) *FEBS Lett.* **10**, 49–53
- Gray, W. R. & Hartley, B. S. (1963) *Biochem. J.* **89**, 59P
- Heidner, E. G., Weber, B. H. & Eisenberg, D. (1971) *Science* **171**, 677–680
- Horecker, B. L., Tsolas, O. & Lai, C. Y. (1972) *Enzymes 3rd Ed.* **7**, 213–258
- Lai, C. Y., Nakai, N. & Chang, D. (1974) *Science* **183**, 1204–1206
- Lambert, J. M. & Perham, R. N. (1974) *FEBS Lett.* **40**, 305–308
- Lambert, J. M. & Perham, R. N. (1977) *Biochem. J.* **161**, 49–62
- Mehler, A. H. & Cusic, M. E., Jr. (1967) *Science* **155**, 1101–1103
- Penhoet, E. E., Kochman, M. & Rutter, W. J. (1969) *Biochemistry* **8**, 4391–4395
- Perham, R. N. & Anderson, P. J. (1970) *Biochem. Soc. Symp.* **31**, 49–58
- Reynolds, J. H. (1968) *Biochemistry* **7**, 3131–3135
- Rutter, W. J. (1964) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 1248–1257
- Sajgo, M. & Hajos, G. (1974) *Acta Biochim. Biophys. Acad. Sci. Hung.* **9**, 239–241
- Saunders, G. C. & Weber, B. H. (1975) *Arch. Biochem. Biophys.* **168**, 525–530
- Schmidt, D. E., Jr. & Westheimer, F. H. (1971) *Biochemistry* **10**, 1249–1253
- Shapiro, S., Enser, M., Pugh, E. & Horecker, B. L. (1968) *Arch. Biochem. Biophys.* **128**, 554–562
- Steinman, H. M. & Richards, F. M. (1970) *Biochemistry* **9**, 4360–4372
- Swenson, A. D. & Boyer, P. D. (1957) *J. Am. Chem. Soc.* **79**, 2175–2179
- Szajani, B., Sajgo, M., Biszku, E., Friedrich, P. & Szabolcsi, G. (1970) *Eur. J. Biochem.* **15**, 171–178