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Folding Domains and Intramolecular Ionic Interactions of Lysine Residues in Glyceraldehyde 3-Phosphate Dehydrogenase

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1. Treatment with methyl acetimidate was used to probe the topography of several tetrameric glyceraldehyde 3-phosphate dehydrogenases, in particular the holoenzymes from rabbit muscle and *Bacillus stearothermophilus*. During the course of the reaction with the rabbit muscle enzyme, the number of amino groups fell rapidly from the starting value of 27 per subunit to a value of approx. five per subunit. This number could be lowered further to values between one and two per subunit by a second treatment with methyl acetimidate. The enzyme remained tetrameric throughout and retained 50% of its initial catalytic activity at the end of the experiment. 2. Use of methyl [1-^{14}C]acetimidate and small-scale methods of protein chemistry showed that only one amino group per subunit, that of lysine-306, was completely unavailable for reaction with imido ester in the native enzyme. This result is consistent with the structure of the highly homologous glyceraldehyde 3-phosphate dehydrogenase of lobster muscle deduced from X-ray-crystallographic analysis, since lysine-306 can be seen to form an intrachain ion-pair with aspartic acid-241 in the hydrophobic environment of a subunit–subunit interface. 3. Several other amino groups in the rabbit muscle enzyme that reacted only slowly with the reagent were also identified chemically. These were found to be located entirely in the *C*-terminal half of the polypeptide chain, which comprises a folding domain associated with catalytic activity and subunit contact in the three-dimensional structure. Slow reaction of these 'surface' amino groups with methyl acetimidate is attributed to intramolecular ionic interactions of the amino groups with neighbouring side-chain carboxyl groups, a conclusion that is compatible with the reported three-dimensional structure and with the dependence of the reaction on ionic strength. 4. Very similar results were obtained with the enzymes from *B. stearothermophilus* and from ox muscle and ox liver, supporting the view that the ion-pair involving lysine-306 and aspartic acid-241 will be a common structural feature in glyceraldehyde 3-phosphate dehydrogenases. The *B. stearothermophilus* enzyme was fully active after modification. 5. No differences could be detected between the enzymes from ox muscle and ox liver, in accord with other evidence that points to the identity of these enzymes. 6. The pattern of slowly reacting amino groups in the enzyme from *B. stearothermophilus*, although similar to that of the mammalian enzymes, indicated one or two additional intramolecular ionic interactions of lysine residues that might contribute to the thermal stability of this enzyme.

The chemical reactivity of functional groups has long been used as a probe of protein topography, with groups being designated as 'accessible' or 'buried' by this criterion (Perham & Anderson, 1970). Such experiments have frequently provided valuable information about protein folding, most usefully when evidence from X-ray diffraction is lacking or inadequate (Cohen, 1970; Thomas, 1974). A prerequisite of a good chemical reaction for this purpose is that the modification itself should not disturb the native conformation of the protein. One solution to this problem has been the technique of competitive labelling, whereby the chemical modification of target groups is restricted to a trivial extent (Hartley, 1970; Kaplan *et al.*, 1971), which makes conformational changes consequent on the chemical modification extremely unlikely.

An alternative approach that we have followed is the use of imido esters. These compounds react specifically with protein amino groups (Hunter & Ludwig, 1962, 1972) to yield amidinated proteins that have no change of charge at the modified side chains. Since the side-chain amino groups of lysine

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residues are almost invariably found at the 'surface' of protein molecules, this retention of charge distribution favours the maintenance of local side-chain interactions and the preservation of three-dimensional structure. Experiments with tobacco mosaic virus (Perham & Richards, 1968; Perham, 1973), ribonuclease (Reynolds, 1968) and horse liver alcohol dehydrogenase (Plapp, 1970; Dworschack *et al.*, 1975), in which the modified proteins retain their biological activity, support this conclusion.

We describe here the treatment with methyl acetimidate of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from several sources, taking account of the revisions to the amidination reaction scheme proposed by Browne & Kent (1975*a*,*b*). We are able to classify the lysine side chains in terms of their accessibility to this small reagent and to correlate the results with the three-dimensional structure deduced from X-ray-crystallographic analysis of the lobster muscle enzyme (Moras *et al.*, 1975), in particular with the folding domains ascribed roles in coenzyme binding and subunit interactions in the tetramer (Buehner *et al.*, 1974). Our results provide further evidence for assuming the identity of ox muscle and ox liver glyceraldehyde 3-phosphate dehydrogenase (Lambert & Perham, 1974).

Materials and Methods

Enzymes and reagents

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and NAD^+ (grade II) were purchased from Boehringer und Soehne, Mannheim, Germany. Ox muscle and ox liver enzymes were purified as described elsewhere (Lambert & Perham, 1974) and stored at 4°C in 70%-satd. $(NH_4)_2SO_4$. Freeze-dried *Bacillus stearothermophilus* enzyme was the kind gift of Dr. J. I. Harris and his colleagues at the M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge. Trypsin and chymotrypsin (twice recrystallized) were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Thermolysin (thrice recrystallized) was from Calbiochem, San Diego, CA, U.S.A.

Methyl acetimidate was synthesized by the procedure of Hunter & Ludwig (1962). Methyl [1-^{14}C]-acetimidate, synthesized as described by Bates *et al.* (1975), was kindly given by Mr. D. L. Bates. All other chemicals were of analytical grade.

Enzyme assay

Enzymic activity was determined at 30°C by the method of Allison & Kaplan (1964), except that 6.7mM-fructose 1,6-diphosphate (tetrasodium salt) and 2.08μM-rabbit muscle aldolase (Boehringer; mol.wt. 160000) replaced D-glyceraldehyde 3-phosphate (Lambert & Perham, 1974).

Amidination of native enzymes

Glyceraldehyde 3-phosphate dehydrogenase (approx. 3g/litre) was treated with methyl acetimidate (0.1M) in a buffer consisting of 0.1M-*N*-ethylmorpholine/acetic acid, 2mM-EDTA, 10mM-NAD^+, 2mM-2-mercaptoethanol and 0.02% NaN_3 at pH8.0 and 20°C. The stock solution (1M) of methyl acetimidate was prepared immediately before use as described by Bates *et al.* (1975). The concentration of NAD^+ is sufficient to ensure that all the enzyme is present as holoenzyme during the modification reaction, since it is far in excess of the highest values reported for the dissociation constant of the fourth molecule of bound NAD^+ (de Vijlder & Slater, 1968; Price & Radda, 1971; Bell & Dalziel, 1975). The reaction was stopped when required by adding samples of the reaction mixture to an equal volume of 0.4M-ammonium acetate, pH5.0, which lowered the pH of the mixture to about 6.0. The samples were then dialysed against or gel-filtered into 0.1M-*N*-ethylmorpholine/acetic acid / 2mM-EDTA / 0.02% NaN_3, pH8.0. For the latter method, a column (35cm×1cm) of Sephadex G-25 was used.

Amidination of enzymes in the presence of 5M-*guanidine hydrochloride*

Native or partly amidinated samples of glyceraldehyde 3-phosphate dehydrogenase were reduced and *S*-carboxymethylated with iodoacetic acid in the presence of 5M-guanidine hydrochloride (Gibbons & Perham, 1970). Amidination of the *S*-carboxymethylated proteins with 0.1M-methyl [1-^{14}C]acetimidate was carried out for 4h in the presence of 5M-guanidine hydrochloride as described by Bates *et al.* (1975). The proteins were then dialysed exhaustively against fresh NH_4HCO_3 (5g/litre) to remove non-protein-bound radioactivity.

Complete amidination under these conditions was demonstrated by performing the modification in guanidine hydrochloride first with unlabelled imido ester and then, after 4h, adding a fresh sample of ^{14}C-labelled imido ester under the standard conditions. Protein samples treated this way were subsequently shown to have less than 0.5% of the specific radioactivity of control samples that did not receive the initial treatment with unlabelled imido ester.

Proteolytic digestion

Proteins (2g/litre), suspended in NH_4HCO_3 solution (5g/l), were digested at 37°C for 4h with trypsin (1%, w/w). Chymotrypsin (1%, w/w) was then added and the digestion allowed to continue for a further 4h. The peptides were recovered by freeze-drying. Thermolytic digestion of peptides was carried out as described by Lambert & Perham (1974).

Measurement of extent of amidination

Two methods to measure free amino groups were used. In the first, samples of amidinated protein were assayed by treatment with trinitrobenzenesulphonic acid (Habeeb, 1966; Gibbons & Perham, 1970). In the second, samples of protein treated with unlabelled imido ester were denatured and the amidination was completed by treatment with ^{14}C-labelled imido ester, as described above. The incorporation of radioactivity into the sample was then compared with the incorporation of radioactivity under identical conditions into a sample of protein that had not received the prior treatment with unlabelled imido ester. Since the treatment under denaturing conditions was known to modify all amino groups in the protein, comparison of the specific radioactivities of the proteins provided an accurate estimate of the amino groups left unmodified by the initial amidination of the native protein.

The specific radioactivity of the methyl acetimidate was calculated from a knowledge of the amino acid composition of the protein and the specific radioactivity of the protein sample in which all amino groups had been modified by treatment with the ^{14}C-labelled imido ester in guanidine hydrochloride. The specific radioactivity of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase so treated was 9070c.p.m./nmol of polypeptide chain. By assuming a value of 27 amino groups/chain (Smith & Velick, 1972), the specific radioactivity of the reagent was calculated to be 336c.p.m./nmol.

Measurement of radioactivity

Samples of labelled protein were counted for radioactivity after tryptic/chymotryptic digestion as described above. The freeze-dried digest was dissolved in 20mM-NH_3. Samples of the clear solution were added to 3ml of toluene/Triton (2:1, v/v) containing 2,5-diphenyloxazole (5g/litre) and counted for radioactivity in a Nuclear–Chicago mark II scintillation counter. Duplicate samples of the digest were taken for acid hydrolysis and subsequent amino acid analysis to determine protein concentration.

Amino acid analysis

Peptides and proteins were hydrolysed for 24h at 105°C with 6M-HCl containing 0.05% 2-mercaptoethanol in sealed evacuated tubes. The amino acids were analysed by using a Rank Hilger Chromaspek analyser coupled with a Digico micro 16V computer. To calculate protein concentrations, the amino acid composition of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was taken from Smith & Velick (1972), that of the ox muscle and ox liver enzyme from Lambert & Perham (1974) and that of the *B. stearothermophilus* enzyme from J. I. Harris & J. Walker (personal communication).

For qualitative amino acid analysis of peptides, the amino acids produced by acid hydrolysis were dansylated and the dansyl-amino acids separated and identified by standard procedures (Brown & Perham, 1974).

Separation and characterization of peptides

Peptide 'maps' were prepared from 50μg samples of proteolytic digests by using the micro-scale technique of Bates *et al.* (1975) on silica-gel-coated plastic sheets. 'Maps' were stained with ninhydrin and radioautographs were prepared as described by Bates *et al.* (1975). No material could be detected at the origin of such peptide 'maps' when the proteolytic-digestion procedure described above was used.

Gel filtration on Sephadex G-25 or G-50 (superfine grade), paper electrophoresis, chromatography and dansylation of peptides were carried out by standard techniques (Brown & Perham, 1974). The electrophoretic mobility (m) of a peptide at pH6.5 was measured relative to that of aspartic acid defined as −1.00 (Offord, 1966).

Analytical ultracentrifugation

Protein samples (about 2g/litre) were dialysed against 0.1M-*N*-ethylmorpholine/acetic acid/2mM-EDTA / 10mM-NAD^+ / 2mM-2-mercaptoethanol / 0.02% NaN_3 buffer, pH8.0, overnight at 4°C and then centrifuged in a Beckman model E ultracentrifuge at 20°C. The operating speed was 52640rev./min, and schlieren patterns were photographed at 8min intervals.

Polyacrylamide-gel electrophoresis

Samples of *S*-carboxymethylated protein were run in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Shapiro & Maizel, 1969) as described by Brown & Perham (1974).

Results

Amidination of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, a tetramer of identical polypeptide chains, has a total of 27 amino groups (one *N*-terminal valine residue and 26 lysine residues) per subunit (Smith & Velick, 1972). The effect of treating the holoenzyme with 0.1M-methyl acetimidate in a typical experiment is shown in Fig. 1. The number of unmodified amino groups rapidly fell from the value of 27 per subunit to approx. 5, although in some experiments values as low as 2.5 were recorded (e.g. Fig. 2). After 4h, a further addition (90mM) of fresh reagent decreased the total number of unmodified amino groups to approx. 2 per subunit. The modification of amino groups was accompanied by a rapid fall in the catalytic activity of the enzyme to about half that of the native enzyme. The second addition

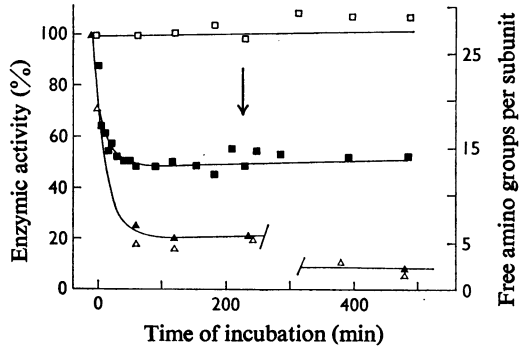


Fig. 1. *Treatment of rabbit muscle glyceraldehyde* 3*-phosphate dehydrogenase* (*holoenzyme*, 2.2*g*/*litre*) *with* 0.1M-*methyl acetimidate at pH*8 *and* 20°*C*

After 4h, a further addition of methyl acetimidate (final concn. 90mM; indicated by the arrow) was made. □, Enzymic activity of untreated enzyme; ■, enzymic activity of treated enzyme; △, number of free amino groups per subunit assayed with trinitrobenzenesulphonic acid; ▲, number of free amino groups per subunit assayed by subsequent treatment with methyl [1-^{14}C]acetimidate. For other details, see the text.

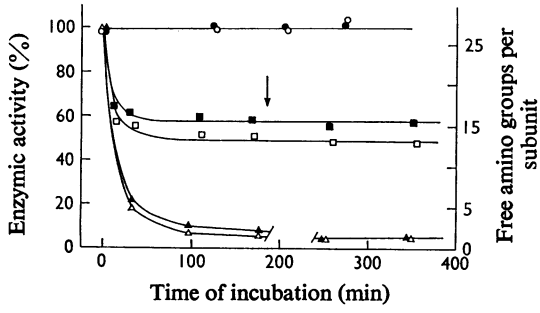


Fig. 2. *Treatment of rabbit muscle glyceraldehyde* 3*-phosphate dehydrogenase* (*holoenzyme*, 2.2*g*/*litre*) *with* 0.1M-*methyl acetimidate at pH*8 *and* 20°*C in the presence or absence of additional* 0.5M-*NaCl*

Enzymic activity of untreated enzyme in the absence (●) and presence (○) of extra salt; enzymic activity of enzyme treated in the absence (■) and presence (□) of extra salt; number of free amino groups per subunit in the absence (▲) and presence (△) of additional salt, assayed by subsequent treatment with methyl [1-^{14}C]acetimidate. For other details, see the text. Arrow indicates second addition of methyl acetimidate (final concn. 0.1M).

of fresh reagent caused no further loss of catalytic activity, despite the increase in the number of amino groups modified.

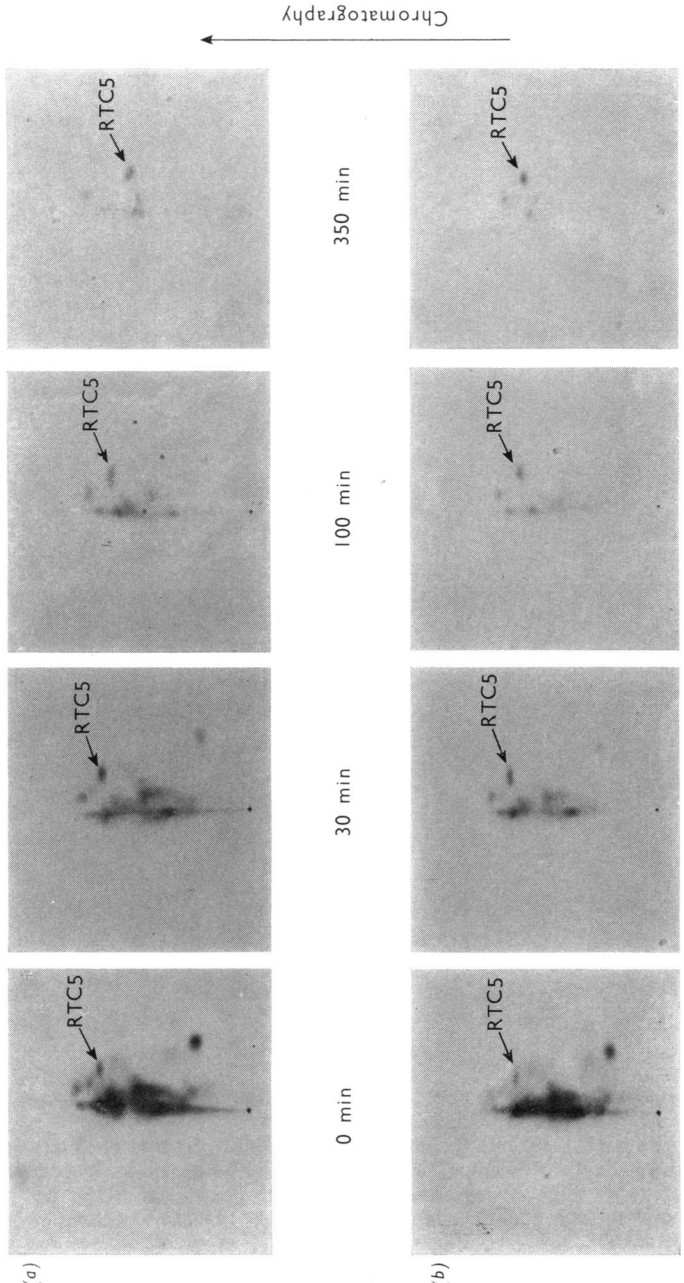
The modified enzyme, after two treatments with methyl acetamidate, showed a single band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, which could not be resolved from that shown by the native enzyme. Similarly, the native and modified enzymes were indistinguishable when sedimented in the anlytical ultracentrifuge. We conclude that no unexpected cross-linking has taken place and that the quaternary structure of the enzyme is unaffected by the amidination of the protein.

It was important to determine the identity of the lysine residues that were in the different classes of reactivity towards methyl acetimidate. This was done by repeating the experiment, as shown in Fig. 2, taking samples of the partly amidinated enzyme at various times during the treatment, terminating the amidination and *S*-carboxymethylating the protein with iodoacetic acid. The *S*-carboxymethylated proteins were then completely amidinated by treatment with 0.1M-methyl [1-^{14}C]acetimidate in 5M-guanidine hydrochloride. The proteins were thus chemically homogeneous, although differing in specific radioactivity. The labelled samples were then digested with trypsin and chymotrypsin, and peptide 'maps' and radioautographs were prepared. It should be noted that in amidinated proteins, tryptic cleavages occur only at the arginine residues (Hunter & Ludwig, 1962). Plate 1(*a*) shows a series of radioautographs of peptide 'maps' prepared from protein samples taken at various time-intervals during the enzyme modification depicted in Fig. 2. The number of labelled peptides rapidly diminished, owing to the prior reaction with unlabelled imido ester, and after 1½h only a few specific radioactive peptides remained. It should be noted that these radioautographs are taken from two-dimensional peptide 'maps' and that, particularly in the neutral band, several peptides can migrate together. Strongly radioactive spots can therefore comprise several weakly labelled peptides, as revealed by further dimensions of electrophoresis and chromatography.

The second treatment of the native enzyme with fresh unlabelled imidoester caused the number of spots to fall still further until only one strongly radioactive spot (RTC5) remained. The intensity of radioactivity in this spot was about the same at each time-point. These results are consistent with the view that most of the lysine residues in the native enzyme tetramer reacted rapidly with the reagent and that some specific residues reacted more slowly. The identities of these latter residues, particularly the lysine residue(s) in peptide RTC5 which appeared to be unmodifiable in the native enzyme, were therefore sought.

Identification of 'protected' lysine residues in rabbit muscle glyceraldehyde 3*-phosphate dehydrogenase*

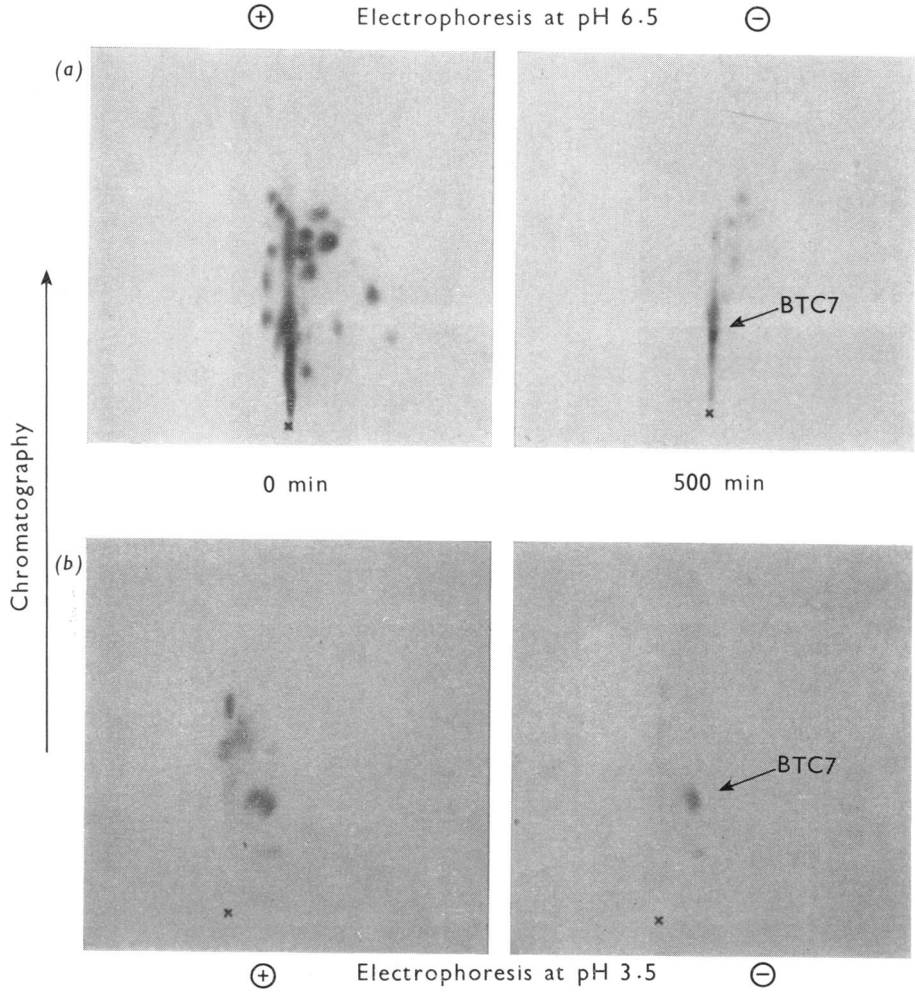
A large sample of native holoenzyme (16mg) was amidinated for 4h with 0.1M-methyl acetimidate and



EXPLANATION OF PLATE 1

Radioautographs of peptide 'maps' of tryptic/chymotryptic digests of amidinated rabbit muscle glyceraldehyde 3-phosphate dehydrogenase

The holoenzyme was treated with methyl acetimidate as shown in Fig. 2. Samples were removed after the time-intervals shown, and these were *S*-carboxymethylated and treated with methyl [1-^{14}C]acetimidate in 5M-guanidine hydrochloride before proteolytic digestion. The amidination of the holoenzyme was carried out in the absence (*a*) and presence (*b*) of 0.5M-NaCl. For further details, see the text.



EXPLANATION OF PLATE 2

Radioautographs of peptide 'maps' of tryptic/chymotryptic digests of amidinated B. stearothermophilus glyceraldehyde 3*-phosphate dehydrogenase*

The holoenzyme was treated twice with methyl acetimidate. Samples were removed after the time-intervals shown and these were *S*-carboxymethylated and treated with methyl [1-^{14}C]acetimidate in 5M-guanidine hydrochloride before proteolytic digestion. (*a*) Radioautographs of two-dimensional 'maps' (electrophoresis at pH6.5 and t.l.c.) of the whole digests; (*b*) radioautographs of two-dimensional 'maps' (electrophoresis at pH3.5 and t.l.c.) of the neutral peptides from (*a*). For other details, see the text.

then, after *S*-carboxymethylation, was completely amidinated by treatment with methyl [1-^{14}C]acetimidate in 5M-guanidine hydrochloride. The specific radioactivity of this protein was measured to be 1.49×10^3 c.p.m./nmol of polypeptide chain. Since the specific radioactivity of the reagent used was 336c.p.m./nmol, the specific radioactivity of the protein indicates that a total of approx. 4.4 amino groups per polypeptide chain were left unmodified after the initial treatment of the native enzyme with unlabelled imido ester.

A sample (12mg) of the labelled protein was digested with trypsin and chymotrypsin and the peptides were fractionated by gel filtration on a column (144cm×0.7cm) of Sephadex G-25 in NH_4HCO_3 buffer (5g/l), pH8.0. The radioactive peaks from the column (located by counting radioactivity of 10μl samples of the 1ml fractions collected) were freeze-dried separately and submitted successively to high-voltage paper electrophoresis at pH6.5, to paper chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent (Waley & Watson, 1953) and to paper electrophoresis at pH3.5. Radioactive peptides were located by radioautography and eluted finally with 50mM-NH_3.

In this way six radioactive peptides that accounted for the principal spots seen in a thin-layer peptide 'map' of the unfractionated digest (cf. Plate 1*a*) were purified. The peptides were characterized by amino acid and dansyl *N*-terminal analysis, by electrophoretic mobility at pH6.5, and by measurement of their specific radioactivities. All were found to be pure by these criteria and the results are listed in Table 1. The amino acid sequence of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase has not been determined, but is known to be highly homologous with that found for the pig muscle enzyme (Harris & Perham, 1968). 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. The homology is borne out by the fact that the six peptides recorded in Table 2, which together contain 47 residues from rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, can be fitted to the amino acid sequence of the pig muscle enzyme with only one change needed, the substitution of alanine (rabbit) for proline (pig) in peptide RTC3 at position 249 of the pig enzyme sequence (Harris & Perham, 1968).

Three of the peptides in Table 2 contained more than one lysine residue. To determine the distribution

Table 1. *Amino acid compositions, mobilities, N-terminal residues and specific radioactivities of tryptic/chymotryptic peptides from amidinated rabbit muscle glyceraldehyde 3-phosphate dehydrogenase*

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

Amino acid	Peptide ...	Amino acid composition (mol/mol of peptide)					
		RTC1	RTC2	RTC3	RTC4	RTC5	RTC6
CmCys		—	—	—	—	—	—
Asp		1.0	1.1	—	2.0	—	—
Thr*		—	0.9	—	—	—	—
Ser*		—	1.1	—	0.8	—	0.9
Glu		—	—	1.1	2.1	—	1.0
Pro		—	0.6	—	0.9	—	—
Gly		—	1.9	—	1.1	—	—
Ala		1.1	—	2.0	1.0	—	1.0
Val		0.7	1.2	—	1.6	1.0	—
Met		—	—	—	—	—	0.7
Ile		0.5	—	—	0.9	—	—
Leu		—	0.9	0.9	1.0	1.0	—
Tyr		—	—	0.9	—	—	—
Phe		0.8	—	—	—	—	—
Lys†		+	+	+	+	+	+
His		2.1	—	—	—	—	—
Arg		—	—	—	—	—	—
Mobility (*m*)		+0.48	+0.28	+0.34	0	+0.57	0
N-Terminal residue		Ala	Lys	Leu (+*O*-Tyr)	Asp	Val	Met
Specific radioactivity (^{14}C c.p.m./nmol of peptide)		72	123	99	89	325	40

* Not corrected for destruction of these amino acids during hydrolysis.

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

Table 2. *Radioactively labelled tryptic/chymotryptic peptides from amidinated rabbit muscle glyceraldehyde 3-phosphate dehydrogenase*

The sequence numbering that is used for the pig muscle enzyme (Harris & Perham, 1968) and the amino acid sequences of the rabbit peptides are deduced by homology with the pig muscle enzymes.

Peptide	Sequence position	Probable sequence	Radioactively modified lysine (mol/mol of peptide)
RTC1	158–165	Ala-Lys-Val-Ile-His-Asp-His-Phe	0.21
RTC2	183–192	Lys-Thr-Val-Asp-Gly-Pro-Ser-Gly-Lys-Leu	0.37
RTC3	246–252	Leu-Glu-Lys-Ala-Ala-Lys-Tyr	0.29
RTC4	253–267	Asp-Asp-Ile-Lys-Lys-Val-Val-Lys-Gln-Ala-Ser-Glu-Gly-Pro-Leu	0.26
RTC5	305–307	Val-Lys-Leu	0.97
RTC6	328–332	Met-Ala-Ser-Lys-Glu	0.12

of radioactive residues in these peptides, further experiments were carried out. Peptide RTC4 (10 nmol) was digested with thermolysin and the products were separated by paper electrophoresis at pH 6.5. Radioactivity was found in peptides corresponding to Ile-Lys-Lys and Val-Val-Lys-Gln, qualitative analysis of the peptides being sufficient to identify them. The radioactivity of peptide RTC4 was therefore distributed between lysine-260 and lysine-256 and/or lysine-257, no distinction being made between the latter two residues. No more precise analysis of the distribution was attempted. An identical experiment was carried out with peptide RTC2 (5 nmol), all the radioactivity being found in a thermolytic peptide corresponding to Lys-Thr. Thus all the radioactivity of peptide RTC2 was associated with lysine-183 and none with lysine-191. Three radioactive peptides were separated by paper electrophoresis from a partial acid hydrolysate (6M-HCl, 100°C, 5 min) of peptide RTC3 (40 nmol). Two of them were basic peptides ($m = +0.33$ and $+0.51$). Analysis by the dansyl method showed them to have *N*-terminal residues of leucine and alanine respectively, and both to contain tyrosine. The third radioactive peptide was neutral ($m = 0$), contained *N*-terminal leucine and no tyrosine. We conclude that the radioactivity of peptide RTC3 is shared between lysine-248 and lysine-251.

The number of mol of radioactively modified lysine per mol of radioactive peptide is also given in Table 2. This information is obtained from the specific radioactivity of the reagent used to complete the amidination under denaturing conditions and therefore reflects the lack of modification of a given lysine residue in the native enzyme tetramer. Lysine-306 (peptide RTC5) appears to be completely unavailable for reaction in the native enzyme, in agreement with the persistence of strong radioactivity in this spot even after a second treatment with unlabelled methyl acetimidate (Plate 1*a*). The number of radioactively modified lysine residues per chain accounted for in the peptides listed in Table 2 is smaller than that calculated simply from the incorporation of radioactivity into the denatured chain. The lysine residues at positions 159, 183, 248, 251, 256, 257, 260, 306 and 331 contribute a total of 2.2 to the value of 4.4 unmodified lysine residues per chain observed in this experiment after 4 h pretreatment of the native enzyme with unlabelled methyl acetimidate. We assume that the remaining total of approx. 2 unmodified amino groups is made up from individually small contributions from the other (approx. 18) lysine residues in the chain, most of which are almost totally modified with unlabelled imido ester at this stage and therefore show up weakly, if at all, in radioautographs of peptide 'maps'. A second pretreatment with unlabelled imido ester will increase the modification of the amino groups other than that of lysine-306, which is left effectively intact, the overall value of approx. 2 mol of radioactively modified lysine residues/mol of peptide chain (Fig. 1) then consisting of 1 mol of lysine-306, with the remaining radioactivity (approx. 1 mol/mol of chain) very weakly distributed over several other lysine residues. This explanation is in accord with the peptide 'maps' shown in Plate 1(*a*) and was supported by further experiments with glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* (see below).

Amidination of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase in the presence of additional 0.5M-NaCl

Lysine-306 is completely protected from amidination in native rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. A possible explanation for the slow reaction observed for several other lysine residues is that their amino groups are involved in weak ionic interactions over the enzyme 'surface'. To test this possibility, the amidination reaction was carried out with 0.1M-methyl acetimidate as before, except that NaCl (final concentration 0.5M) was added to the buffer used. Since the methyl acetimidate was always added as a neutralized solution of the hydrochloride, the final concentration of NaCl in the reaction mixture was 0.6M, compared with 0.1M in the earlier experiments. A control modification experiment, using the

same reagents but omitting the additional NaCl, was carried out simultaneously. The results are shown in Fig. 2.

The number of amino groups remaining unmodified (as measured by subsequent incorporation of radioactivity) fell slightly more quickly in the presence than in the absence of a high salt concentration, but the end point, after a second addition of 0.1M-methyl acetimidate, was the same for both treatments (about 1.3 amino groups per polypeptide chain). The loss of enzymic activity under the two sets of conditions was not very different. Samples of partly amidinated enzyme were taken at various times during the modification reaction, *S*-carboxymethylated and then completely amidinated by treatment with methyl [1-^{14}C]acetimidate in guanidine hydrochloride. Radioautographs were prepared from peptide 'maps' of tryptic/chymotryptic digests of the samples (Plate 1*a* and 1*b*). They indicated that the same slowly reacting amino groups were present, but that they became modified a little more quickly in the presence of strong salt, although lysine-306 remained unmodified in the native enzyme.

Amidination of ox muscle and ox liver glyceraldehyde 3-phosphate dehydrogenase

Samples of ox muscle and ox liver enzyme were treated for 4h with 0.1M-methyl acetimidate under conditions identical with those used for the rabbit muscle enzyme. In all respects, in terms of the modification of amino groups and the loss of catalytic activity, the two ox enzymes were indistinguishable and closely resembled the rabbit muscle enzyme. Peptide 'maps' of tryptic/chymotryptic digests of the *S*-carboxymethylated ox enzymes, whose amidination had been completed with methyl [1-^{14}C]acetimidate, were also indistinguishable, and the radioautographs closely resembled those prepared from the rabbit muscle enzyme (Plate 1*a*). In particular, it was clear that the same set of amino groups, in the same or highly homologous amino acid sequences, were left partly or completely unmodified after the first treatment of the native enzyme with methyl acetimidate.

Amidination of B. stearothermophilus glyceraldehyde 3-phosphate dehydrogenase

A sample (10mg) of the *B. stearothermophilus*

Table 3. *Amino acid compositions, mobilities, N-terminal residues and specific radioactivities of tryptic/chymotryptic peptides from amidinated Bacillus stearothermophilus glyceraldehyde 3-phosphate dehydrogenase*

Abbreviation: CmCys, *S*-carboxymethylcysteine. + Present in analysis, but not quantified. ++, Detected by using the Ehrlich reagent (Dalgliesh, 1952).

	Amino acid composition (mol/mol of peptide)						
Amino acid Peptide ...	BTC1	BTC2	BTC3	BTC4	BTC5	BTC6	BTC7
CmCys	—	—	—	—	—	—	—
Asp	1.1	1.0	—	—	1.0	1.0	2.0
Thr*	—	—	—	—	—	—	1.2
Ser*	—	—	—	—	—	—	1.1
Glu	1.0	0.9	1.7	0.9	1.1	—	1.3
Pro	—	—	—	0.9	+	—	—
Gly	—	—	—	1.1	2.0	1.0	1.7
Ala	2.1	2.0	1.2	—	1.0	—	—
Val	—	—	1.0	0.8	1.0	0.5	2.4
Met	—	—	—	—	1.5	0.9	—
Ile	—	—	—	—	—	0.5	—
Leu	—	1.1	1.3	2.8	2.2	—	—
Tyr	—	—	—	—	—	—	1.9
Phe	—	—	0.9	—	—	—	—
His	1.0	1.0	1.1	—	—	—	0.7
Lys†	+	+	+	+	+	+	+
Arg	—	—	—	—	+	—	+
Trp	—	—	—	—	—	—	++
Mobility (*m*)	0	0	+0.29	+0.25	+0.25	0	0
N-Terminal residue	Glu	Glu	Ala	Val	Val	Val	Val (+*O*-Tyr)
Specific radioactivity (^{14}C c.p.m./nmol of peptide)	106	108	73	89	56	33	260

* Not corrected for destruction of these amino acids during hydrolysis.

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

Val-Lys-Val-Gly-Val-Asp-Gly-Phe-Gly-Arg-Ile-Gly-Arg-Leu-Val-Thr-Arg-Ala-Ala-Phe-Asn-Ser-Gly-
Ac Ser Ile Ile Leu Leu Ser Cys
Lys-Val-Asp-Ile-Val-Ala-Ile-Asn-Asp-Pro-Phe-Ile-Asp-Leu-His-Tyr-Met-Val-Tyr-Met-Phe-Glu-Tyr-Asp-
Ala Gln Val Val Ala Glu Lys
Ser-Thr-His-Gly-Lys-Phe-His-Gly-Thr-Val-Lys-Ala-Glu-Asp-Gly-Lys-Leu-Val-Ile-Asp-Gly-Lys-Ala-Ile-
Val Lys Glu Met Ala Val Lys
Thr-Ile-Phe-Gln-Glu-Arg-Asp-Pro-Ala-Asn-Ile-Lys-Trp-Gly-Asp-Ala-Gly-Thr-Ala-Tyr-Val-Val-Glu-Ser-
Val Asn Met Lys Glu Pro Ser Lys Ala Glu Ile
Thr-Gly-Val-Phe-Thr-Thr-Met-Glu-Lys-Ala-Gly-Ala-His-Leu-Lys-Gly-Gly-Ala-Lys-Arg-Val-Ile-Ile-Ser-
Ile Ser Phe Lys Val
Ala-Pro-Ser-Ala-Asp-Ala-Pro-Met-Phe-Val-Met-Gly-Val-Asn-His-Glu-Lys-Tyr-Asp-Asn-Ser-Leu-Lys-Ile
Cys Leu Ser Lys Asp Met Thr Val
Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys-Val-Ile-His-Asp-His-Phe-Gly-Ile-
Val Leu Glu Asn Glu
RTC 1

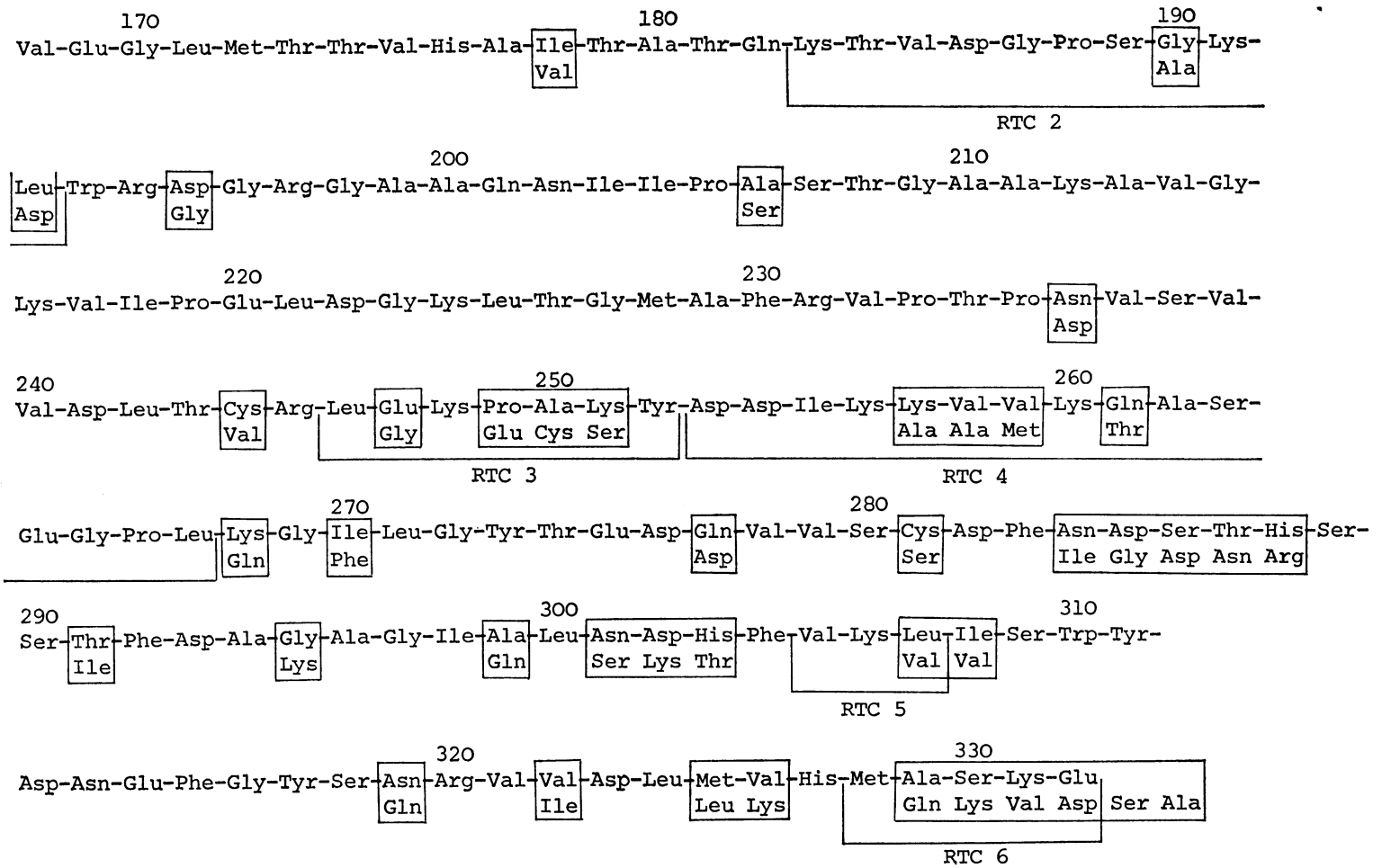


Fig. 3. ***Amino acid sequence of glyceraldehyde 3-phosphate dehydrogenase from pig muscle***

The sequence of the lobster muscle enzyme is identical, except where indicated in the boxed areas below the continuous line of the pig sequence (Harris & Perham, 1968). Peptides RTC1–RTC6 (Table 2) from the homologous rabbit muscle enzyme are underlined.

Table 4. *Radioactively labelled tryptic/chymotryptic peptides from amidinated Bacillus stearothermophilus glyceraldehyde 3-phosphate dehydrogenase*

The sequence numbering is that used for the pig muscle enzyme (Harris & Perham, 1968). The amino acid sequences of the *B. stearothermophilus* peptides are taken from the unpublished work of J. E. Walker & J. I. Harris.

Peptide	Sequence position	Amino acid sequence	Radioactively modified lysine (mol/mol of peptide)
BTC1	103–108	Glu-Asp-Ala-Ala-Lys-His	0.31
BTC2	103–109	Glu-Asp-Ala-Ala-Lys-His-Leu	0.32
BTC3	158–165	Ala-Lys-Val-Leu-His-Gln-Glu-Phe	0.22
BTC4	217–225	Val-Leu-Pro-Glu-Leu-Lys-Gly-Lys-Leu	0.27
BTC5	217–231	Val-Leu-Pro-Glu-Leu-Lys-Gly-Lys-Leu-Asn-Gly-Met-Ala-Met-Arg	0.18
BTC6	299–304	Val-Ile-Asp-Gly-Lys-Met	0.10
BTC7	305–320	Val-Lys-Val-Val-Ser-Trp-Tyr-Asp-Asn-Glu-Thr-Gly-Tyr-Ser-His-Arg	0.77

enzyme was treated for 5h with 0.1M-methyl acetimidate under conditions identical with those used for the rabbit muscle enzyme. After 5h, a further addition of methyl acetimidate (90mM) was made and the treatment continued for 5h. Although the number of unmodified amino groups fell to a final value of approx. 1.8 per polypeptide chain as measured by subsequent incorporation of radioactivity, the catalytic activity remained as high as 90% of that of the native enzyme, in contrast with the 50% loss of activity recorded with the mammalian enzymes (Figs. 1 and 2). In these calculations, the existence of 24 lysine residues per polypeptide chain (mol.wt. 36000) was assumed (J. I. Harris & J. Walker, personal communication).

The identities of the amino groups remaining wholly or partly unmodified after the double treatment of native holoenzyme with unlabelled methyl acetimidate were established, as for rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, by treating them with methyl [1-^{14}C]acetimidate and isolating the labelled peptides from a tryptic/chymotryptic digest of the protein. A radioautograph of a peptide 'map' indicated that there was a principal labelled peptide (BTC7), with zero electrophoretic mobility at pH6.5, although other weaker spots could be seen (Plate 2). The labelled peptides from the tryptic/chymotryptic digest were purified by gel filtration on a column (119cm×1.2cm) of Sephadex G-50 in NH_4HCO_3 buffer (5g/l), pH8.0, and then by paper electrophoresis at pH6.5, followed by paper chromatography and further paper electrophoresis at pH3.5 and pH2.0, as described above for rabbit muscle glyceraldehyde 3-phosphate dehydrogenase.

Seven labelled peptides were isolated in this way; their amino acid compositions are given in Table 3. Peptide BTC7 has a curious composition for a peptide obtained from a tryptic/chymotryptic digest, chymotrypsin having failed to cleave the chain at the aromatic residues. Knowledge of the *N*-terminal amino acids and the compositions was enough, given the amino acid sequences of lobster (Davidson *et al.*, 1967) and pig (Harris & Perham, 1968) muscle enzymes and unpublished sequence information on the *B. stearothermophilus* enzyme (J. Walker & J. I. Harris, personal communication), to identify the probable sequences of the peptides. These sequences are listed in Table 4. From the specific radioactivity of the reagent and the specific radioactivities of the peptides, we can calculate the number of mol of labelled lysine per mol of peptide. Peptide BTC7 has a very high specific radioactivity and its lysine residue was therefore well protected from amidination in the native enzyme. This residue corresponds to lysine-306 of the mammalian enzymes.

Peptides BTC1, BTC2 and BTC3 (Table 4) also contain lysine residues (positions 107 and 159) that are strongly labelled. Peptides BTC4 and BTC5 are derived from overlapping amino acid sequences and contain two modified lysine residues, which were not distinguished with respect to their individual specific radioactivities. The lysine residue of peptide BTC6 is very weakly labelled. As with the mammalian enzymes, therefore, glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* contains one lysine residue (position 306) that is very difficult to amidinate in the native tetrameric enzyme and several other amino groups that are slow to react with methyl acetimidate (particularly at positions 107 and 159).

The number of radioactively modified lysine residues per polypeptide chain accounted for in the peptides listed in Table 4 (1.6mol/mol of chain) is in good agreement with the value of 1.8 calculated from the incorporation of radioactivity into the denatured chain. We ascribe this to the fact that the native *B. stearothermophilus* glyceraldehyde 3-phosphate dehydrogenase was subjected to two pretreatments with unlabelled methyl acetimidate before the amidination was completed by reaction with methyl [1-^{14}C]acetimidate. This would ensure that the weak contributions from amino groups that had almost

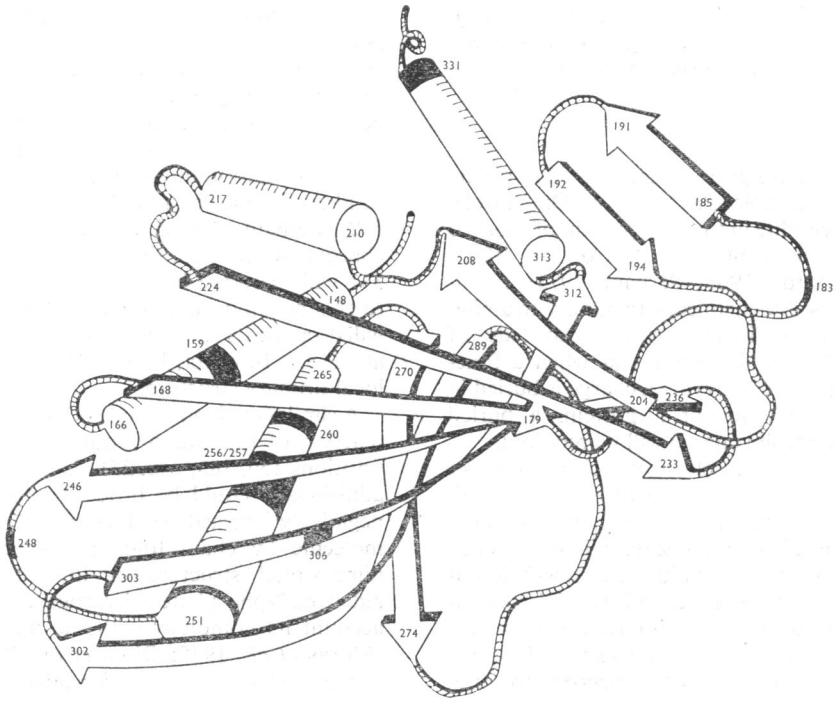


Fig. 4. *Representation of the three-dimensional structure of the catalytic (subunit-interaction) domain of glyceraldehyde 3-phosphate dehydrogenase from lobster muscle (Moras et al., 1975)*

The locations of the lysine residues that react slowly, if at all, with methyl acetimidate (Table 2) are shaded. The sequence numbering is the same as in Fig. 3.

completely reacted with methyl acetimidate in the first pretreatment were blanked out by the second pretreatment, unlike the comparable experiment with the rabbit muscle enzyme described above, where only one pretreatment with unlabelled imido ester was carried out.

The remarkable stability of glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* (Suzuki & Imahori, 1973) allowed the possibility of examining the effect of amidination of the apoenzyme [prepared by charcoal treatment of the enzyme by J. I. Harris by using the method of Suzuki & Harris (1971)]. The modification was therefore carried out in buffers from which the 10mM-NAD^+ had been omitted. By all criteria, retention of activity, loss of amino groups and peptide 'mapping', the apoenzyme behaved identically with the holoenzyme in these experiments.

Location of unreactive amino groups in the three-dimensional structure of glyceraldehyde 3-phosphate dehydrogenase

The work described above has revealed that native rabbit muscle glyceraldehyde 3-phosphate dehydrogenase contains a set of lysine residues whose amino groups react slowly (or not at all) with methyl acetimidate. When these residues are identified in the primary structure of the enzyme [for this purpose we use the highly homologous pig enzyme sequence (Harris & Perham, 1968)], they are strikingly restricted to the *C*-terminal half of the polypeptide chain (Fig. 3). A three-dimensional structure for another highly homologous glyceraldehyde 3-phosphate dehydrogenase, that from lobster muscle (see Fig. 3), has been proposed by Moras *et al.* (1975) on the basis of X-ray-crystallographic studies. It has been shown that the monomer consists of two domains (Buehner *et al.*, 1974): the *N*-terminal half of the chain forms the domain that binds NAD^+ and the *C*-terminal half is the domain that contains residues responsible for catalytic activity and for many of the subunit interactions. The three-dimensional structures of the lobster and pig muscle enzymes are thought to be highly similar (Olsen *et al.*, 1975). All the poorly reactive lysine residues of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase are therefore assigned to the latter domain and they can be located in the three-dimensional

model of this region of the polypeptide chain proposed by Rossmann and his colleagues (Moras *et al.*, 1975), as shown diagramatically in Fig. 4.

Discussion

The amino groups of proteins are thought normally to be located on the molecular 'exterior'. Only rarely, as with a lysine residue in the coat protein of tobacco mosaic virus (Fraenkel-Conrat & Colloms, 1967; Perham & Richards, 1968; Perham, 1973), has it been found impossible chemically to modify an amino group in a native protein. Our growing knowledge of protein structure would suggest that when a functional group capable of carrying a charge is buried in the hydrophobic interior of a protein, a buried counter-ion of opposite charge will be required.

Lysine-306 in rabbit muscle, ox muscle and ox liver glyceraldehyde 3-phosphate dehydrogenase manifests the properties of a buried side chain. Examination of the model of the catalytic domain represented in Fig. 4 shows why this should be. Lysine-306 is at the centre of the large twisted anti-parallel pleated-sheet region that forms a subunit interface across the *P*-axis (Moras *et al.*, 1975). The expected buried negative charge is provided by the carboxyl group of aspartic acid-241 in the same subunit and the residues in the contact region are predominantly hydrophobic. All the conditions for a strong ion-pair within the same subunit are therefore fulfilled.

The conservation, during evolution, of lysine-306 and aspartic acid-241 and of other residues at the subunit interface (Olsen *et al.*, 1975) indicates that this buried intrachain ion-pair at the *P*-axis interface is likely to be a common feature of the three-dimensional structure of even phylogenetically distant glyceraldehyde 3-phosphate dehydrogenases. This is borne out by our observation that the *B. stearothermophilus* enzyme contains a buried lysine residue in a sequence highly homologous with that surrounding lysine-306 of the mammalian enzymes (peptides BTC7 of Table 4 and RTC5 of Table 2). The existence of this lysine residue within a region of structure similar to that described for the lobster muscle enzyme and its participation in an ion-pair with an homologous aspartic acid residue can be clearly seen in the X-ray-crystallographic model of *B. stearothermophilus* glyceraldehyde 3-phosphate dehydrogenase (G. Biesecker, J. C. Thierry & A. J. Wonacott, unpublished work).

Further inspection of the model of the catalytic (subunit interaction) domain of lobster muscle glyceraldehyde 3-phosphate dehydrogenase (Fig. 4) based on the co-ordinates of Moras *et al.* (1975) suggests that there can be no serious restriction of access of methyl acetimidate to the other slowly reacting amino groups of the rabbit muscle enzyme. A clue to the reason for their slow reaction is given by consideration of lysine-331 in peptide RTC6, the penultimate residue at the *C*-terminus of the enzyme (Fig. 3). The X-ray-crystallographic analysis of lobster muscle glyceraldehyde 3-phosphate dehydrogenase shows this region of the primary structure as a helix on the surface of the subunit (Fig. 4) and lysine-331 as sterically unhindered. However, the *C*-terminal residue of the rabbit muscle enzyme is glutamic acid-332 (Perham, 1964), which carries two negative charges. We propose that the amino group of lysine-331 will therefore have its p*K* raised slightly owing to this neighbouring double negative charge, which will decrease its reactivity towards methyl acetimidate at pH8.0. In effect, lysine-331 is 'protected' by the local primary structure.

A similar explanation can be advanced for the slow reaction of the amino groups of lysine residues at positions 159, 183, 248, 251, 256/257 and 260, which additionally could be brought into proximity with negatively charged residues on the 'surface' of the molecule by chain folding. Inspecting the three-dimensional structure of lobster muscle glyceraldehyde 3-phosphate dehydrogenase and taking into account its homology with the pig muscle enzyme (Moras *et al.*, 1975; Olsen *et al.*, 1975) shows that lysine-159 is close to aspartic acid-163, that lysine-251 is close to aspartic acid residues at positions 253 and 254 and that lysine-256 is in close proximity to glutamic acid-175 of the same subunit (making the assumption that the rabbit muscle and pig muscle enzymes have identical residues at these positions). Similarly, lysine-260 is thought to interact with tyrosine-273. Of particular interest is lysine-183, which in the apoenzyme can take part in intramolecular $S \rightarrow N$ acyl migrations of substrate from thioester linkage with cysteine-149 (Harris & Polgar, 1965; Park *et al.*, 1966). Lysine-183 was at one time thought to be closely involved with binding of NAD^+, but this is now disproved (Moras *et al.*, 1975). The slow reaction of lysine-183 with methyl acetimidate in our experiments with the rabbit muscle holoenzyme therefore cannot be explained in simple terms of coenzyme protection and must be put down to the position of this residue as a subunit side-chain contact (Olsen *et al.*, 1975).

In addition to the slowly reacting lysine residues of the catalytic (subunit interaction) domain, glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* was found to have a slowly reacting lysine residue (position 107) in the *N*-terminal half of the sequence (Table 4 and Fig. 3), which comprises the NAD^+-binding domain (Buehner *et al.*, 1974). More than 30% of this amino group remained unchanged after two treatments of the native enzyme with methyl acetimidate. This protection may again be due to the presence of negatively charged carboxyl groups in the vicinity of the amino group, a proposal supported by examination of the three-dimensional

structure of the enzyme (G. Biesecker, J. C. Thierry & A. J. Wonacott, unpublished work). The suggestion that the amidination probe can lead to the identification of lysine amino groups that take part in 'surface' ionic interactions is of further interest in view of the proposal (Perutz & Raidt, 1975) that monomeric ferredoxin from thermophilic bacteria may increase its stability by means of additional ion-pair interactions at the protein surface, without disturbance of the tertiary structure of the protein, a point considered by Hocking & Harris (1976) in reviewing the structures of enzymes from thermophiles. The lysine residues at positions 107 and 159 in *B. stearothermophilus* glyceraldehyde 3-phosphate dehydrogenase, on the basis of their slowness to react with methyl acetimidate (Table 4), might be thought to be particularly significant in this regard.

The enzymic activity of mammalian glyceraldehyde 3-phosphate dehydrogenases quickly fell to about 50% of that of the native enzymes during the amidination reaction. However, even when essentially all the amino groups per subunit other than that of lysine-306 had reacted, the activity remained at about 50% (Figs. 1 and 2). The three-dimensional structure of the enzyme reveals that lysine-306 is not at the active site (Moras *et al.*, 1975). We conclude that no amino group in the enzyme plays an essential part in the catalytic mechanism. In particular, lysine-183, which takes part in $S \rightarrow N$ acyl migrations in the enzyme active site (Harris & Polgar, 1965; Park *et al.*, 1966), can have its amino group modified without causing total inhibition of the enzyme. This is consistent with its replacement by arginine in the enzyme from *B. stearothermophilus* (Olsen *et al.*, 1975), although it should be noted that the side chains of arginine and amidinated lysine residues are still positively charged, which may be significant. It is also worth remarking that, in all these experiments, the glyceraldehyde 3-phosphate dehydrogenases from ox muscle and ox liver were indistinguishable, which is consistent with other evidence that points to their structural identity (Lambert & Perham, 1974).

The accepted view of the mechanism of the amidination reaction has recently been revised and a plausible explanation offered for the cross-linking sometimes seen in the reaction of proteins with monofunctional imido esters (Browne & Kent, 1975*a*,*b*). Cross-linking of troponin (Hitchcock, 1975) and other complex proteins (D. L. Bates, J. R. Coggins & R. N. Perham, unpublished work) during amidination with methyl acetimidate has certainly been seen in other laboratories. However, in the present experiments with glyceraldehyde 3-phosphate dehydrogenase under the conditions we have used, no cross-links were detectable. Secondly, Browne & Kent (1975*b*) describe how treatment of liver alcohol dehydrogenase with ethyl acetimidate at pH 8 produced a modified protein that contained principally *N*-alkyl imidates rather than amidinated lysine residues, of which the former could subsequently be converted into amidines by reaction with NH_3 or be hydrolysed back to regenerate the unmodified amino groups. It is possible that the apparent slowness to react with methyl acetimidate of some lysine residues in glyceraldehyde 3-phosphate dehydrogenase might have a similar explanation; for example, two treatments of the enzyme with 0.1 M-methyl acetimidate were needed to bring the modification to its limit. However, the values for the numbers of poorly reacting amino groups measured by further treatment with methyl [1-^{14}C]acetimidate or with trinitrobenzenesulphonic acid (Fig. 1) agree well and the identification of specific lysine residues in the 'slow' category still implies some unusual property for these particular residues. The possibility that they are involved in 'surface' ion-pairs with negatively charged carboxyl groups must remain the most likely cause.

The amidination technique has proved to be a useful method for studying the topography of glyceraldehyde 3-phosphate dehydrogenase, revealing a buried lysine residue and ionic interactions between lysine residues and neighbouring carboxyl groups at the enzyme 'surface' when interpreted in terms of the published X-ray-crystallographic structure (Moras *et al.*, 1975). Indeed, it is clear that a buried ion-pair involving lysine-306 and aspartic acid-241 at a subunit interface will be a general feature of the subunit. It has to be admitted that the chemical evidence for the ion-pair would not have distinguished an intrachain pair from an interchain pair, but the location of the anomalously reacting amino groups within the subunit-interaction domain is very clear-cut. Our interpretation of the results with glyceraldehyde 3-phosphate dehydrogenase has profited considerably from a knowledge of the three-dimensional structure of the enzyme, as revealed by X-ray crystallography. We have therefore carried out similar experiments with an enzyme, fructose diphosphate aldolase, for which knowledge of the three dimensional structure is lacking. The results of our experiments and our predictions from them can be found in the following paper (Lambert *et al.*, 1977).

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