Studies on the Sub-units of Triose Phosphate Isomerase

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The sub-unit structure of rabbit muscle triose phosphate isomerase was studied by determination of the number of unique cysteine peptides. Alkylation of the thiol groups with radioactive iodoacetate in the presence of guanidine hydrochloride gave the *S*-carboxy[¹⁴C]methyl derivative of the protein. This was digested with trypsin, and the radioactive peptides were fractionated by ion-exchange chromatography; four main radioactive peaks were obtained, one of which contained two radioactive peptides. Peptide 'maps' of the tryptic digest showed five main spots. The relationship between the members of both sets of five peptides was established. The radioactive peptides were characterized, and the results indicated the presence of five unique cysteine residues in the protein. Since there are approximately ten thiol groups/molecule, there are two closely related or identical sub-units. Studies of the terminal residues bear out this suggestion; only one kind of *N*-terminal residue (alanine) and one kind of *C*-terminal residue (glutamine) were detected. These results are in accord with the evidence from crystallography.

Most intracellular enzymes are built up from several sub-units, i.e. the molecule contains more than one polypeptide chain. Peptide 'maps' suggested that triose phosphate isomerase (EC 5.3.1.1) (Czok & Bücher, 1960) might contain two similar sub-units (Burton & Waley, 1966), and the present work was undertaken to test this suggestion by more rigorous methods. Investigation of the number of unique cysteine peptides is probably the best method of determining the number of structurally identical units in the molecule, since the thiol group can be labelled with a radioactive agent, and this greatly facilitates the separation and study of the peptides containing cysteine. Comparison of the number of unique cysteine peptides with the total number of cysteine residues in the protein then provides information on the number of related sub-units. The molecular weight of TIM† was previously estimated as 43 000, based on exclusion chromatography (Burton & Waley, 1966); however, equilibrium ultracentrifugation, a more reliable method, gives a value of 60 000 (M. P. Esnouf & S. G. Waley, unpublished work), and the corresponding value for the number of cysteine residues/mol. is 8–10. Hence we expect to find 8–10 cysteine peptides if there are no sub-units, 4–5 cysteine

peptides if there are two sub-units, and 2–3 cysteine peptides if there are three or four sub-units. The application of this method, supplemented with a study of terminal residues, is described below.

METHODS

Paper chromatography. Paper chromatography of peptides was carried out in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953); when used preparatively for peptides containing Cmc, the solvent contained 0.2% of thiodiglycol. Paper chromatography of amino acids was usually carried out in 2-methylpropan-2-ol-butan-2-one-aq. NH₃ (sp.gr. 0.88)-water (5:3:1:1, by vol.) on Whatman no. 52 paper (Jones & Heathcote, 1966), with twofold development; other solvents used were: butan-1-ol-acetic acid-water (4:1:5, by vol.), phenol-sodium borate (Wade, Matheson & Hanes, 1961) and, as a second solvent (after butan-1-ol-acetic acid-water) in two-way chromatography, pyridine-water (4:1, v/v).

Paper electrophoresis. This was carried out on 75 cm. strips of Whatman no. 3MM paper in a cooled-plate apparatus at 64 v/cm. for 60–80 min. at pH 4.4 (20 ml. of acetic acid and 11 ml. of pyridine/2.5 l.); the origin was 25 cm. from the anode.

Peptide 'maps'. The protein was digested with trypsin (treated with L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one; from Seravac Laboratories, Maidenhead, Berks.), as described by Burton & Waley (1966). The digest was fractionated by paper electrophoresis at pH 4.4, followed by paper chromatography in butan-1-ol-acetic acid-water-pyridine for 16 hr. at right angles. Peptides

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† Abbreviations: TIM, triose phosphate isomerase; Cmc, *S*-carboxymethylcysteine.

were detected with ninhydrin (Heilmann, Barollier & Watzke, 1957).

Amino acid analysis. This was carried out after oxidation of 4.6 mg. of protein as described by Moore (1963); the amounts of amino acids in the hydrolysate of the oxidized protein, expressed as μ moles/60 mg. of protein, were: aspartic acid 42, glutamic acid 49, alanine 44, leucine 26 and cysteine acid 8.9. For comparison, the amounts of the stable amino acids in the hydrolysate of unoxidized protein were: aspartic acid 37, glutamic acid 45, alanine 43, leucine 29 (Burton & Waley, 1966). Thus the number of half-cystine residues (and also of thiol groups) is in the range 8–10, whether based on the content of stable amino acids (Moore, 1963) or on the weight of protein taken. The radioactive peptides (about 0.1 μ mole) were hydrolysed with 0.05 ml. of redistilled 6 N-HCl in an evacuated tube, and the hydrolysates were analysed on a Technicon amino acid analyser.

1-Dimethylaminonaphthalene-5-sulphonyl derivatives. The procedure of Gray & Hartley (1963) was followed; the derivatives were identified by electrophoresis at pH 4.4 and by thin-layer chromatography (Waley, 1965).

Detection and measurement of radioactivity. This was carried out as described by Burton & Waley, 1966.

N-Terminal residue. This was investigated by two methods: (1) Edman degradation; (2) the cyanate method of Stark & Smyth (1963).

(1) The enzyme was oxidized by performic acid (prepared by adding 0.5 ml. of 30% (w/v) H_2O_2 to 9.5 ml. of 99% formic acid and incubating in a stoppered flask for 2 hr. at room temperature). Formic acid (0.5 ml., 99%) was added to 12.6 mg. of freeze-dried protein, followed by 1 ml. of chilled performic acid. After 2.5 hr. the oxidized protein was precipitated with 5 ml. of 25% (w/v) trichloroacetic acid and the precipitate was washed with 10% (w/v) trichloroacetic acid, ethanol and ether. The oxidized protein was treated with phenyl isothiocyanate, and the thiazolinone was split off as described by Doolittle (1965), and converted into the phenylthiohydantoin in 0.3 ml. of aq. HCl in 30% (v/v) ethanol, pH 1, by the method of Ilse & Edman (1963). The phenylthiohydantoin was isolated by extraction with ethyl acetate (3 $\times$ 1 ml.), the extracts were evaporated and the residue was dissolved in 20 μ l. of ethylene dichloride. The spectrum between 230 and 300 $m\mu$ was determined and the products were identified by thin-layer chromatography on fluorescent silica gel (Kieselgel GF.254; Camlab Glass Ltd., Cambridge) in the solvent systems: chloroform–formic acid (20:1, v/v), chloroform–methanol (19:1, v/v) and methanol–formic acid (70:3:2, by vol.).

(2) The stock suspension (containing 12 mg. of TIM) was dialysed against water. One-half of the solution was added to 1 ml. of *N*-ethylmorpholine acetate buffer, pH 8.0, containing 4 M-guanidine hydrochloride; 25 mg. of potassium cyanate was then added and the solution was incubated at 50° overnight. Acetic acid (2 ml.) was added slowly and the solution was dialysed against 50% (v/v) acetic acid overnight. The acetic acid was removed by evaporation and the residue dissolved in 0.3 ml. of 50% (v/v) acetic acid and 0.3 ml. of conc. HCl. The solution was cooled in solid CO_2 and the tube was evacuated, sealed and incubated for 1 hr. in a boiling-water bath. The contents were evaporated to dryness and dissolved in 0.2 ml. of water. The solution was passed through a column (3 cm. $\times$ 0.5 cm. diam.) of Dowex 50 (H^+ form; X2; 200–400 mesh). The hydantoin was eluted

in the first 3 ml. of aqueous eluate, which was evaporated to dryness. Any pyrrolidonecarboxylic acid that could have formed was hydrolysed by the addition of 0.5 ml. of 3 N-HCl to the dry sample and heating at 100° for 30 min. The HCl was removed by evaporation and the residue dissolved in 0.2 ml. of water. The solution was again passed through a similar column of Dowex 50 and the hydantoin was eluted in 3 ml. of water and evaporated to dryness. Hydrolysis was carried out by the addition of 0.4 ml. of 0.2 N-NaOH and incubation in a sealed evacuated tube at 109° overnight. The solution was neutralized by the addition of 0.4 ml. of 0.2 N-HCl and put through a column (4 cm. $\times$ 0.5 cm. diam.) of Dowex 50 (H^+ form). The column was washed with 3 ml. of water and the amino acids were eluted with 4 ml. of aq. 1 N- NH_3 soln.

The other half of the dialysed enzyme solution was incubated at 50° in the presence of the *N*-ethylmorpholine acetate buffer and treated subsequently as above, to detect any artifacts that may have arisen in the absence of guanidine and cyanate.

C-Terminal residue. Native TIM did not react satisfactorily with carboxypeptidase, so digestions were carried out in urea. A suspension of TIM (10 mg.) was dialysed against water and then against 0.5% NH_4HCO_3 in 6 M-urea. Carboxypeptidase (A and B; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was dissolved in 0.1 N-NaOH and immediately neutralized with 0.1 N-HCl; it was added to the TIM solution to give a weight ratio of 1:20. The mixture was incubated at 37°. Portions were removed after 1 hr. and 6 hr. and added to 150 mg. portions of sulphonated polystyrene beads (Zeo-Karb 225, 15–30 mesh, water regain 0.75 g./g.). The suspension was shaken mechanically for 1 hr. The resin was then washed with two 2 ml. portions of water followed by two 2 ml. portions of acetone and finally with two 2 ml. portions of water, to remove the urea. The amino acids were eluted with two 1 ml. portions of aq. 5 N- NH_3 soln. Controls were also carried out, containing carboxypeptidase but no TIM, and treated in a similar way.

RESULTS

Homogeneity of TIM. The enzyme from rabbit muscle [Boehringer Corp. (London) Ltd., London, W. 5] showed one band (Fig. 1) on disc electrophoresis in polyacrylamide gel in tris-EDTA-borate, pH 9 (60.5 g. of tris, 7.65 g. of disodium salt of EDTA and 416 g. of boric acid/l.), but starch-gel electrophoresis showed one major band and two minor ones (Burton & Waley, 1966). Application of differing amounts of protein showed that the main band represented about 85% of the protein (Fig. 2). The main band also contained most of the enzymic activity, but the minor bands were also active (Burton & Waley, 1966). Homogeneity of size has been shown by equilibrium ultracentrifugation (M. P. Esnouf & S. G. Waley, unpublished work). This evidence suggests that the preparation of TIM is mostly composed of one component; the nature of the minor components is discussed below.

Disc electrophoresis in 8 M-urea (in the tris-EDTA-boric acid buffer) was carried out under the conditions that have been used for aldolase (Chan,

Fig. 1. Disc electrophoresis of TIM in (a) the presence and (b) the absence of urea, at pH 9. The origin is at the top, and the anode at the bottom.

Fig. 2. Starch-gel electrophoresis of TIM at pH 8 (discontinuous system), for 3.7 hr. at 17 v/cm. Concentrations of protein (left to right) were: 5 mg./ml., 1 mg./ml., 0.5 mg./ml. and 0.25 mg./ml. The line is the origin slot; the anode is at the top.

Morse & Horecker, 1967); only one band was seen (Fig. 1).

Terminal amino acid residues. The Edman degradation gave the phenylthiohydantoin of alanine. The amount obtained (on the basis of a molar extinction coefficient of 16 000 at 270 m μ) was 0.2 μ mole from 0.2 μ mole (12 mg.) of protein. Since our yield was only 50% with γ -crystallin, a single-chain protein (Björk, 1964), the yield of 100% with

TIM is consistent with the presence of more than one chain. The cyanate method gave a major component identified as alanine, and two minor components. The two minor components were both present in the control experiment lacking guanidine and cyanate. The amount of alanine obtained (after allowing for losses observed when the hydantoin from alanine was hydrolysed and desalted) was 0.05 μ mole from 0.1 μ mole of protein. The determination was based on the method of Bode (1955). The only amino acid liberated in appreciable amounts by carboxypeptidase was glutamine; the amount was not determined.

Reaction of TIM with sodium iodo[2- 14 C]acetate. The reaction was carried out in the presence of guanidine hydrochloride, as the thiol groups are unreactive in the native protein. The stock solution of radioactive reagent was prepared by adding 22.2 mg. of iodoacetic acid in 1 ml. of water to 2.66 mg. of iodo[2- 14 C]acetic acid containing 100 μ C; the specific radioactivity of the solution was 2.54×10^3 counts/sec./ μ mole. The TIM was dialysed against 4 mM-tris-1 mM-EDTA, pH 7.2; to 20 ml. of the dialysed solution, containing about 80 mg. of enzyme, were added 1.5 ml. of M-tris, pH 8.3, 0.08 ml. of dithiothreitol (1 mg./ml.), 0.7 ml. of the stock solution of iodo[2- 14 C]acetic acid and 9 g. of recrystallized guanidine hydrochloride. After 1 hr., the solution was treated with 0.2 ml. of 2-mercaptoethanol and the protein was separated on a column (15 cm. high $\times$ 4.5 cm. diam.) of Sephadex G-25 in 0.05 M-ammonium hydrogen carbonate. The radioactivity of the protein (about 6 moles of 14 C/mole of protein) and the presence of thiol (about 1 mole/mole of protein) suggested that alkylation was incomplete, and so the freeze-dried protein (49 mg.) was treated with 0.66 mg. of dithiothreitol in 4 M-guanidine hydrochloride containing 0.2 M-tris, pH 8.3, and 1 mM-EDTA for 1.5 hr. to reduce any disulphide bonds, and then alkylated with 0.17 ml. of the stock solution of iodo[2- 14 C]acetic acid for 2 hr. at room temperature in the dark. 2-Mercaptoethanol (5 μ l.) was then added, and the solution was dialysed against mM-hydrochloric acid until diffusible radioactive compounds had been removed. The radioactivity of the protein (CM-TIM) corresponded to 7.8 moles of 14 C/mole of enzyme. Acid hydrolysis of a portion of CM-TIM, followed by paper electrophoresis and radioautography, gave only one radioactive spot, corresponding to Cmc.

Hydrolysis of CM-TIM with trypsin. Ammonium hydrogen carbonate (48 mg.) was added to 4 ml. of CM-TIM (1%) and then 0.02 ml. of a 1% solution of trypsin (treated with L-1-chloro-4-phenyl-3-toluene-p-sulphonamidobutan-2-one) in mM-hydrochloric acid was added, and the mixture was kept at 37° for 4.5 hr. with the further addition of 0.01 ml. of the solution of trypsin after incubation for 1.5 hr.

Radioautography of a peptide 'map' showed a 'core' spot remaining at the origin, four other spots of comparable intensity, and one fainter spot (Fig. 3).

Fractionation of peptides by ion-exchange chromatography. The digest was brought to pH 2 and added to a column (80 cm. high $\times$ 0.9 cm. diam.) of Bio-Rad

Aminex 50W (X2) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), prepared as described by Schroeder, Jones, Cormick & McCalla (1962) and equilibrated with 0.2M-pyridine acetate, pH 3.1. Elution was carried out at 40°, with a linear gradient from 0.2M-pyridine acetate, pH 3.1, to 2M-pyridine acetate, pH 5; 600 ml. of each buffer was used, and the buffers contained 0.2% of thiodiglycol. The molarity of the buffers refers to the pyridine, and the pH was measured at room temperature. Fractions (about 2 ml.) were collected every 20 min.; portions (0.02 ml.) were taken for counting, and further portions (0.175 ml.) were taken (from alternate tubes) for detection of peptides by the Lowry reaction. Even simple peptides can be detected in this reaction (Chou & Goldstein, 1960) and the failure to detect free amino acids is not important in the present context. The reaction was carried out as described by Bailey (1967), except that solution A contained N-sodium hydroxide (rather than 0.1N-sodium hydroxide), to neutralize the buffer in the sample. The results (Fig. 4) show that good resolution was achieved, and that there were four main radioactive peaks (peaks 8, 9, 17 and 20) and one minor one (peak 23).

The tubes corresponding to peaks were pooled (tubes 75 and 76 were pooled for fraction 8 and tubes 79 and 80 for fraction 9, to avoid cross-contamination), and portions (about 3% of the pooled fractions) were analysed by paper chromatography and radioautography. The results (Fig. 5) showed that most of the fractions contained one to three peptides, and that the radioactive peaks could be related to the radioactive spots in the peptide 'map' (Fig. 3) as follows. Peaks 8 and 9 corresponded to the spots numbered 35 and 33 respectively, from their R_F values; this was confirmed by the presence of tryptophan in peak 9 and spot 33 (Ehrlich reaction) and by the fact that the radio-

Fig. 3. Radioactive peptides formed on digestion of carboxy-[^{14}C]methyl-TIM with trypsin. The peptides (about 2.5 mg.) were separated by electrophoresis at 64 v/cm. for 65 min. at pH 4.4 (horizontal direction, anode at the left), followed by chromatography (vertical direction, downwards) in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) on Whatman no. 3 paper for 16 hr. Radioautography was carried out with Kodak Royal Blue X-ray film for 5 days.

Fig. 4. Ion-exchange chromatography of peptides (37 mg.) from digestion of carboxy-[^{14}C]methyl-TIM with trypsin. Chromatography was carried out on a column (80 cm. high $\times$ 0.9 cm. diam.) of Dowex 50 (X2) at 40°; elution was effected with a gradient from 0.2M-pyridine-acetate, pH 3.1, to 2M-pyridine-acetate, pH 5. (a) Peptides detected by the Folin-Lowry reaction; (b) radioactivity. ----, pH gradient.

active spot in peak 8 was ninhydrin-negative, as was spot 35. Peak 17 contained two radioactive peptides (peptides 17B and 17C); these corre-

sponded to spots 19 and 36. Peak 20 contained radioactive material that did not travel on paper and this corresponded to the spot at the origin in Fig. 3. Finally the minor peak 23 gave only a very weak colour with ninhydrin and probably corresponded to spot 34, which was not detected by ninhydrin on the peptide 'map'.

Fig. 5. Paper chromatography of portions of the peaks from ion-exchange chromatography. The peak numbers given refer to the ion-exchange chromatography (Fig. 4). Paper chromatography was carried out in butan-1-ol-acetic acid-water-pyridine on Whatman no. 1 paper for 16 hr.; ninhydrin-positive and radioactive spots are shown. The cross-hatching shows ninhydrin colour (////) and radioactivity (\\\\).

Characterization of radioactive peptides

Peptide 8. This gave one radioactive spot and no ninhydrin-positive spots, both on chromatography (Fig. 5) and on electrophoresis at pH 4.4. The anionic mobility at pH 6.5, measured as described by Offord (1966), was 0.39. The absence of an amino group suggests that the *N*-terminal residue was derived from glutamine or from Cmc (cf. Smyth & Utsumi, 1967) by cyclization. The amino acid composition (Table 1) suggests that the peptide was homogeneous.

Peptide 9. This peptide also gave one radioactive spot (coinciding with the ninhydrin-positive one) on chromatography and electrophoresis, and it contained tryptophan (Ehrlich reaction). The anionic mobility at pH 6.5 was 0.59, which suggests (Offord, 1966) that aspartic acid rather than asparagine was present. If one residue of tryptophan was present, peptide 9 was a hexapeptide; it lacked basic amino acids (Table 1), and this and the presence of

Table 1. Amino acid composition of radioactive tryptic peptides from S-carboxy[^{14}C]methyl triose phosphate isomerase

The values are given in μmoles found; between one-third and two-thirds of each peptide was taken for hydrolysis.

Peptide ...	8		9		17C1		20A		17B
Amino acid	μmoles found	Molar ratios	μmoles found	Molar ratios	μmoles found	Molar ratios	μmoles found	Molar ratios	μmoles found
Asp	273	2.01	139	0.94					121
Thr	261	1.92	130	0.88	181	1.93			29
Ser					95	1.01	67	1.05	23
Glu	157	1.16					124	1.95	72
Pro	369	2.72							
Gly			149	1.00	287	3.06	246	3.87	39
Ala	578	4.26	156	1.05	114	1.22	265	4.17	224
Val	300	2.21			105	1.12	107	1.68	92
Met									
Ile	154	1.14			78	0.83	111	1.74	94
Leu							154	2.42	
Tyr	115	0.85			66	0.70			32
Phe	128	0.94							
His							67	1.05	25
Lys					125	1.33	71	1.12	77
Arg	141	1.04							
Cmc	101 (100*)	0.74	105 (100*)	0.71	73 (90*)	0.67	60 (90*)	0.94	75 (80*)
Trp			Present						

* Approximate value, from radioactivity.

Cmc account for its migration towards the anode at pH 4.4. The *N*-terminal amino acid was aspartic acid; possibly the *C*-terminal amino acid was tryptophan, and the peptide arose by a 'chymotryptic' cleavage. The peptide cannot have been derived from the *C*-terminus of the chain, because it lacked glutamic acid and the *C*-terminal residue of the protein was glutamine. Thus the partial sequence of peptide 9 was:

Asp-(Thr,Gly,Ala,Cmc)-Trp

Peptide 17C1. Fraction 17 was further fractionated by paper chromatography in butan-1-ol-acetic acid-water-pyridine containing 0.2% of thiodiglycol, and the two radioactive bands (compare Fig. 5) were eluted. The material from the slower band (peptide 17B) was recovered in small amount (0.14 μ mole) and so was not further purified. The material from the faster band (peptide 17C) was fractionated by electrophoresis at pH 4.4, and the peptide in the radioactive band (peptide 17C1) was eluted. Amino acid analysis (Table 1) showed that peptide 17C1 was homogeneous and contained 12 residues; the peptide was neutral at pH 6.5. Treatment with leucine aminopeptidase released isoleucine, possibly followed by tyrosine, and the 'dansyl' method confirmed that the *N*-terminal residue was isoleucine. Further information about the sequence around the Cmc residue was provided by digestion of about 0.02 μ mole of peptide 17C1 in 0.04 ml. of 1% ammonium acetate, pH 8.5, with 1 μ g. of subtilisin (Novo Terapeutisk Laboratorium, Copenhagen, Denmark) at 37° for 6 hr. The digest was fractionated by paper electrophoresis at pH 4.4, and the radioactive spot was cut out and sewn into Whatman no. 3 paper for chromatography in butan-1-ol-acetic acid-water-pyridine (both the buffer for electrophoresis and the solvent for chromatography contained 0.2% of thiodiglycol). The radioactive spot was eluted into a capillary tube with 6*N*-hydrochloric acid and hydrolysed, and the hydrolysate was analysed by paper chromatography. The results indicated that the composition of the peptide was: (Cmc,Lys,Gly,Ala,Ser,Thr) (probably in equimolar proportions), and hence the partial sequence of the peptide 17C1 was:

Ile-(Thr,Gly₂,Val,Tyr)-(Gly,Ala,Ser,Thr,Cmc)-Lys

Peptide 17B. The amino acid analysis (Table 1) showed that this peptide had not been obtained pure, but there was insufficient material for further purification and analysis. The values in Table 1 suggest the presence of a peptide containing Cmc and lysine (major peptide 17B1) contaminated with about 30% of another peptide (minor peptide 17B2). The major peptide lacked glycine, and we may

Fig. 6. Action of trypsin (Tr) and chymotrypsin (Ch) on peptide 17C1. Radioautography was carried out after paper chromatography in butan-1-ol-acetic acid-water-pyridine. The origin is at the top, and movement is downwards.

tentatively conclude that it represented a unique cysteine peptide.

Digestion with subtilisin, followed by electrophoresis and chromatography, gave several weak radioactive spots, and the peptides could not be further characterized.

Attempts to relate peptides 17B1 and 17C1. Portions (about 5 μ moles) of peptide 17C1 in 10 μ l. of 1% ammonium hydrogen carbonate were treated with 3 μ g. of trypsin or chymotrypsin for 28 hr. at room temperature. Paper chromatography in butan-1-ol-acetic acid-water-pyridine (Fig. 6) showed that the reaction with trypsin was incomplete, but appeared to give the same radioactive products as chymotrypsin did; the peptide with the lowest R_F (peptide 17C1, Ch1), which was the main product of the action of chymotrypsin, may have been formed from the peptide that moved just in front of it. Neither enzyme split peptide 17C1 to peptide 17B1. If peptide 17B1 had been related to 17C1, the most likely reason for the occurrence of the former in a tryptic digest would have been either incomplete tryptic cleavage or chymotryptic cleavage of the latter. This experiment showed that peptide 17B1 did not appear to be related to peptide 17C1, and so makes it more

likely that peptide 17B1 was a unique cysteine peptide.

Peptide 20A. Fraction 20 from the ion-exchange chromatography contained the radioactive 'core' spot that did not travel in butan-1-ol-acetic acid-water-pyridine and several other spots that did travel in this system (Fig. 5). This fraction, which was difficult to handle as after evaporation it was not readily redissolved, was purified by paper chromatography (in butan-1-ol-acetic acid-water-pyridine containing 0.2% of thiodiglycol); the recovery of the radioactive peptide (peptide 20A) was about 50%, and the amino acid analysis (Table 1) suggested that peptide 20A could be homogeneous.

A portion (about 20 μ moles) of peptide 20A was digested with subtilisin, and the main radioactive peptide (peptide 20AS1) was purified by electrophoresis and hydrolysed. The results indicated that peptide 20AS1 was:

It may be concluded that peptide 20A was, or contained, a unique cysteine peptide.

Peptide 23. This was a minor radioactive peptide (Fig. 4), corresponding to the smallest radioactive spot in the peptide 'map' (Fig. 3), and it gave only a weak reaction with ninhydrin (Fig. 5), but contained tryptophan. On storage, the peptide partly decomposed and no longer travelled in butan-1-ol-acetic acid-water-pyridine. No further work has been done on this peptide, but it may have been the precursor of peptide 9; thus peptide 9 may have arisen from peptide 23 by splitting at the tryptophan bond, and peptide 23 cannot be regarded as a unique cysteine peptide.

Yields of Cmc peptides. The four peptides 8, 9, 17C1 and 20A were finally obtained in amounts of 0.25–0.27 μ mole, and the amount of the fifth peptide, 17B, was about half as much. The losses on purification were quite large and about 0.4–0.6 μ mole of the four more abundant peptides was obtained from the ion-exchange column (after allowing for the fact that peak 17 contains two Cmc peptides). These amounts were obtained from 0.6 μ mole of protein, and, since a quantitative yield of a radioactive peptide is unlikely owing to incomplete digestion, these yields were consistent with the presence of two similar or identical sub-units in the proteins (see the Discussion section).

DISCUSSION

Characterization of cysteine peptides. Some idea of the number of structurally identical sub-units in a protein molecule can be gained from the peptide 'maps', and we have previously suggested (Burton & Waley, 1966) that this number might be two for TIM. This method, however, should be regarded as

providing only a provisional estimate (Harris & Hindley, 1965), and in particular it fails to take into account immobile material remaining at the origin on the peptide 'map' (Fig. 3). Characterization of the cysteine peptides, a much more reliable method (Harris & Hindley, 1965; Harris & Perham, 1965; Weeds & Hartley, 1967), has been used here.

The radioactive Cmc peptides were fractionated by ion-exchange chromatography. Comparison of the isolated peptides with a radioautograph of the peptide 'map' (Fig. 3) showed that none of the Cmc peptides had been lost by irreversible adsorption on the column, and that the immobile radioactive peptide corresponding to the origin spot could be partially purified by ion-exchange chromatography, an unexpected but fortunate finding. There were five main radioactive peptides, and four of these were probably obtained pure. The amino acid compositions (Table 1) show that these represented unique peptides, each containing one residue of Cmc. The fifth peptide (peptide 17B1) was not obtained pure, but the evidence suggests that this, too, was a unique peptide. Of these five peptides, one contained arginine, three contained lysine and one (peptide 9) contained neither, but did contain tryptophan. Trypsin usually produces some chymotryptic cleavages, and tryptophyl bonds are the most readily cleaved by chymotrypsin, so that the presence of such a peptide in a tryptic digest is not unexpected. None of the other main Cmc peptides contained tryptophan, so that peptide 9 was not derived from any of them, but the minor peptide 23 did contain tryptophan, so that these two may well be related. The evidence thus suggests the presence of five unique peptides containing cysteine in TIM.

The value for the amount of cysteine acid in hydrolysates of oxidized TIM was about 10 residues/molecule, and this was in reasonable agreement with the number (8–9) of thiol groups estimated colorimetrically, and the radioactivity (equivalent to about 8 moles of ^{14}C /mole of protein) of the labelled protein; both these methods may easily give somewhat low results. Thus disulphide bonds are probably absent. These values are based on the revised value of 60 000 for the molecular weight of TIM, based on equilibrium ultracentrifugation (M. P. Esnouf & S. G. Waley, unpublished work). If there are 10 cysteine residues and 5 unique cysteine peptides, the 'chemical sub-unit' probably corresponds to a molecular weight of 30 000. It seems a reasonable working hypothesis that the sub-units of molecular weight 30 000 are closely related, and possibly identical, and the presence of only one *N*-terminal amino acid (alanine) and one *C*-terminal amino acid (glutamine) is consistent with this hypothesis. Moreover, the crystallographic evidence leads to the same conclusion (Johnson & Waley, 1967). However, it should be

borne in mind that horse liver alcohol dehydrogenase contains two 'chemical sub-units' of molecular weight about 40000 (Harris, 1964), but it has recently been reported that smaller sub-units (mol. wt. 20000) can be obtained (Drum, Harrison, Li, Bethune & Vallee, 1967), thus suggesting an $\alpha_2\beta_2$ type of molecule. It is, of course, well known that the molecules of lactate dehydrogenase and of glyceraldehyde 3-phosphate dehydrogenase contain four chains; the same, probably, applies to aldolase.

Nature of minor components. The presence of sub-units may be connected with the minor components in rabbit muscle TIM. Although minor components are often attributed to hybrids, an alternative explanation is perhaps more likely here. Ox lens TIM also contains a major and two minor components (Burton & Waley, 1968) and the electrophoretic pattern is similar to that shown in Fig. 2, except that the mobilities of all the components are greater. Extracts of pig muscle, too, show three bands with TIM activity (Scopes, 1964). The general features of the patterns resemble those observed for malate dehydrogenase and attributed to conformational isomers (Kitto, Wassarman & Kaplan, 1966) rather than those usually observed with hybrids.

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REFERENCES

- Bailey, J. L. (1967). *Techniques in Protein Chemistry*, p. 340. London: Elsevier Publishing Co.
 Björk, I. (1964). *Exp. Eye Res.* **3**, 254.

- Bode, F. (1955). *Biochem. Z.* **326**, 433.
 Burton, P. M. & Waley, S. G. (1966). *Biochem. J.* **100**, 702.
 Burton, P. M. & Waley, S. G. (1968). *Exp. Eye Res.* (in the Press).
 Chan, W., Morse, D. E. & Horecker, B. L. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1013.
 Chou, S.-C. & Goldstein, A. (1960). *Biochem. J.* **75**, 109.
 Czok, R. & Bücher, Th. (1960). *Advanc. Protein Chem.* **15**, 315.
 Doolittle, R. F. (1965). *Biochem. J.* **94**, 742.
 Drum, D. E., Harrison, J. H., Li, T.-K., Bethune, J. L. & Vallee, B. L. (1967). *Proc. nat. Acad. Sci., Wash.*, **57**, 1434.
 Gray, W. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 59p.
 Harris, J. I. (1964). *Nature, Lond.*, **203**, 30.
 Harris, J. I. & Hindley, J. (1965). *J. molec. Biol.* **13**, 894.
 Harris, J. I. & Perham, R. N. (1965). *J. molec. Biol.* **13**, 876.
 Heilmann, J., Barollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
 Ilse, D. & Edman, P. (1963). *Aust. J. Chem.* **16**, 411.
 Johnson, L. N. & Waley, S. G. (1967). *J. molec. Biol.* **29**, 321.
 Jones, K. & Heathcote, J. G. (1966). *J. Chromat.* **24**, 106.
 Kitto, G. B., Wassarman, P. M. & Kaplan, N. O. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 578.
 Moore, S. (1963). *J. biol. Chem.* **238**, 235.
 Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
 Schroeder, W. A., Jones, R. T., Cormick, J. & McCalla, K. (1962). *Analyt. Chem.* **34**, 1570.
 Scopes, R. K. (1964). *Nature, Lond.*, **201**, 924.
 Smyth, D. S. & Utsumi, S. (1967). *Nature, Lond.*, **216**, 332.
 Stark, G. R. & Smyth, D. G. (1963). *J. biol. Chem.* **238**, 214.
 Wade, E. H. M., Matheson, A. T. & Hanes, C. S. (1961). *Canad. J. Biochem. Physiol.* **39**, 141.
 Waley, S. G. (1965). *Biochem. J.* **96**, 722.
 Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.
 Weeds, A. G. & Hartley, B. S. (1967). *J. molec. Biol.* **24**, 307.