Iodination of Glyceraldehyde 3-Phosphate Dehydrogenase from *Bacillus stearothermophilus*

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The reaction of iodine with glyceraldehyde 3-phosphate dehydrogenase from *Bacillus stearothermophilus* was investigated. The active-site thiol group of the cysteine residue homologous with cysteine-149 in the pig muscle enzyme was protected by reaction with tetrathionate. The apoenzyme was readily inhibited by KI₃ solution at pH 8, but the coenzyme, NAD⁺, protected the enzyme against inhibition and decreased the extent of iodination. At pH 9.5, ready inhibition of both apo- and holo-enzyme was observed. Tryptic peptides containing residues iodinated at pH 8 were isolated and characterized. One of the most reactive residues in both holo- and apo-enzymes was a tyrosine homologous with tyrosine-46 in the pig muscle enzyme, and this residue was iodinated without loss of enzymic activity. Other reactive tyrosine residues in the apoenzyme were in positions homologous with residues 178, 273, 283 and 311 in the pig muscle enzyme, but they were not readily iodinated in the holoenzyme. Histidine residues in both holo- and apo-enzymes were iodinated at pH 8 in sequence positions homologous with residues 50, 162 and 190 in the pig muscle enzyme. The inhibition of the enzyme was not correlated with the iodination of a particular residue. The results are discussed in relation to a three-dimensional model based on the structure of the lobster muscle enzyme and demonstrate that conformational changes affecting the reactivity of several tyrosine residues most probably occur on binding of the coenzyme.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from the thermophilic bacterium *Bacillus stearothermophilus* (Amelunxen, 1966; Suzuki & Harris, 1971) has a primary structure homologous with those of the enzyme from other sources (Bridgen *et al.*, 1972; Bridgen & Harris, 1973), including lobster muscle (Davidson *et al.*, 1967), pig muscle (Harris & Perham, 1968) and yeast (Jones & Harris, 1968; Jones, 1969). X-ray-crystallographic investigations (A. J. Wonacott, R. M. Sweet & G. Biesecker, personal communication) have shown that the tertiary and quaternary structure of this enzyme is broadly similar to that of the lobster muscle enzyme (Buehner *et al.*, 1973, 1974). In view of the unusually high thermal stability of the enzyme from *B. stearothermophilus* (Amelunxen, 1966; Suzuki & Harris, 1971), it was decided to make a comparison of the detailed structure of this enzyme with that of the muscle enzymes.

One method for studying similarities in the distribution of residues between the exterior and the interior of the enzymes is to compare the reactivities of homologous residues. Homologous tyrosine residues in the lobster muscle, pig muscle and yeast enzymes have similar reactivities towards iodine

(Thomas & Harris, 1970), and iodination was chosen as a tool for studying the distribution of tyrosine residues in the enzyme from *B. stearothermophilus*.

Iodination may be performed under mild conditions, but oxidation of thiol groups, which occurs rapidly (Hughes & Straessle, 1950), is a side reaction which could lead to conformational changes affecting the reactivity of tyrosine residues. Carboxymethylation of the active-site thiol group was used in the earlier work (Thomas & Harris, 1970) to minimize such problems. The enzyme from *B. stearothermophilus* has only two cysteine residues per subunit, compared with five in the lobster muscle enzyme, so the problem of oxidation of thiol groups is much less serious. In the experiments described here, the active-site thiol group was protected from oxidation by the formation of a mixed disulphide bond with sodium tetrathionate (Pihl & Lange, 1962; Parker & Allison, 1969). This method of protection, which is reversible (K. Suzuki & J. I. Harris, personal communication), enables studies to be made on the inhibition of the enzyme during iodination, whereas carboxymethylation causes irreversible inhibition (Harris *et al.*, 1963). Some indication of tyrosine residues essential to the catalytic activity of the enzyme might thus be forthcoming.

The apoenzyme from *B. stearothermophilus* is also stable, and iodination of both holo- and apo-enzymes

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was performed, in order to investigate differences in the reactivity of the nine tyrosine residues per subunit in the absence and presence of NAD^+ , which would indicate that these residues were in the binding site for the coenzyme, or that conformational changes which occur on binding of the coenzyme (Listowsky *et al.*, 1965; Durchschlag *et al.*, 1971; Fenselau, 1972) alter the reactivity of these residues.

Materials and Methods

Glyceraldehyde 3-phosphate dehydrogenase was purified from extracts of *B. stearothermophilus*, strain NCA 1503 (prepared at the Microbiological Research Establishment, Porton, U.K.), by the method of Suzuki & Harris (1971). The apoenzyme, with E_{280}/E_{260} 1.8–1.9, was prepared by charcoal treatment (Suzuki & Harris, 1971). The enzyme was assayed spectrophotometrically in 50 mM- $\text{Na}_4\text{P}_2\text{O}_7$ /1 mM-EDTA, pH 8.5 (adjusted with HCl), containing 5 mM- Na_2HAsO_4 , 0.25 mM- NAD^+ and 0.5 mM-DL-glyceraldehyde 3-phosphate, at 25°C. DL-Glyceraldehyde 3-phosphate was prepared from the barium salt of the diethyl acetal (Boehringer, Mannheim, W. Germany), and NAD^+ (Boehringer grade II) was purified by the method of Dalziel (1963). The specific activity of the enzyme used in these experiments was 30–38 units/mg of protein, according to the age of the preparation, where 1 unit of enzyme activity is 1 μmol of substrate transferred/min. The concentration of enzyme was determined spectrophotometrically, by using $E_{280}^{0.1\%}$ 1.0 for the holoenzyme (K. Suzuki & J. I. Harris, unpublished work, cited by Suzuki & Imahori, 1973).

Sodium tetrathionate was prepared by the method of Gilman *et al.* (1946). Guanidinium chloride and urea were Aristar grade from BDH, Poole, Dorset, U.K. Tris, 3,5-di-iodotyrosine and L-thyroxine were from Sigma (London) Chemical Co., London S.W.6, U.K. 3-Iodotyrosine and *N*-acetyl-L-tyrosine ethyl ester were from Calbiochem, San Diego, CA, U.S.A. *N*-Acetyl-DL-tryptophan (puriss.) was from Koch-Light, Colnbrook, Bucks., U.K. A solution of Na^{125}I (carrier-free) in NaOH was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Trypsin (twice crystallized) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and thermolysin was from Calbiochem. Other reagents were of analytical grade from BDH.

The iodinating reagent generally used was 25 mM- I_2 in 0.1 M-KI, with the addition of Na^{125}I where appropriate to give 0.2 mCi/ml of reagent. It was assumed that ^{125}I was randomly distributed among I^- , I_3^- and I_2 in the solution, which was stored at 4°C in the dark. ^{125}I was measured by liquid-scintillation counting on a Nuclear-Chicago Unilux II counter, and the activity was compared with that of a sample of ^{125}I in KI solution counted under identical con-

ditions at the same time, thus allowing correction for decay of the radioisotope. The scintillant used was toluene/2-methoxyethanol (3:1, v/v) containing 4.9 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen/litre.

Paper electrophoresis was performed as described previously (Thomas & Harris, 1970), and paper chromatography in butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) was by the method of Waley & Watson (1953). Radioautographs were prepared by exposure of dried papers to Kodak Kodirex Auto Process X-ray film. Ion-exchange chromatography was performed on Whatman DEAE-celluloses DE-52 and CM-52 (W. and R. Balston, Maidstone, Kent, U.K.) with gradients of NH_4HCO_3 and acetic acid/ammonium acetate buffers respectively. Peptide sequencing and *N*-terminal determinations were by standard methods (Hartley, 1970; Gray, 1972a,b).

The content of tyrosine and iodotyrosine residues in iodinated protein samples was determined spectrophotometrically in 6 M-guanidinium chloride and 50 mM-HCl or 50 mM-NaOH, by a method similar to that of Edelhoch (1962). Model spectra were calculated by using the observed spectra of *N*-acetyl-tyrosine ethyl ester, mono- and di-iodotyrosine and thyroxine in the same solvents. A Gilford spectrophotometer with a Unicam monochromator was used.

Iodination of the enzyme

Several experiments were performed, with either non-radioactive iodine or ^{125}I , in studies on the inhibition of the enzyme under various conditions, analysis for the oxidation of thiol groups, iodination of tyrosine and histidine residues, and the identification of the position of iodinated residues in the primary structure of the protein. Details of the experiments are recorded in the Results section.

Results

Inhibition of glyceraldehyde 3-phosphate dehydrogenase by iodine

Apoenzyme (8.06 mg) in 50 mM-Tris/1 mM-EDTA, adjusted to pH 8.0 with HCl ('pH 8 Tris buffer') (2 ml) was treated with 0.06 ml of 7 mM- $\text{Na}_2\text{S}_4\text{O}_6$. After 5 min, all activity was lost, and the reversibly inhibited enzyme was divided into four portions (each of 0.5 ml), to each of which was added 0.5 ml of pH 8 Tris buffer. Additions to the four solutions (A–D) were as follows: (A) water (0.1 ml); (B) 0.5 M- Na_2HPO_4 +HCl, pH 8 (0.1 ml); (C) 15 mM- NAD^+ (0.007 ml) and water (0.09 ml); (D) 15 mM- NAD^+ (0.007 ml) and 0.5 M- Na_2HPO_4 +HCl, pH 8 (0.09 ml). To each solution were added portions of

Fig. 1. Inhibition of glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* by KI_3

The apoenzyme (1.8 mg/ml; $12.5 \mu M$) was treated with tetrathionate to protect the active-site thiol group, and was iodinated as described in the text, in 45 mM-Tris/HCl buffer, pH 8, containing 0.9 mM-EDTA and: (O) no additions; (Δ) 45 mM- Na_2HPO_4 adjusted to pH 8 with HCl; (Δ) 0.96 mM- NAD^+ ; (●) 45 mM- Na_2HPO_4 adjusted to pH 8 with HCl, and 0.96 mM- NAD^+ . The enzymic activity was determined after treatment with mercaptoethanol and is expressed as a percentage of the specific activity of the native enzyme.

18.1 mM- I_2 in 181 mM-KI solution, up to 112 mol of I_2 /mol of tetrameric enzyme (28 mol/mol of subunits; 3.1 mol/mol of tyrosine residues) at $23 \pm 2^\circ C$. Samples (0.05 ml) were withdrawn after 10 min reaction after each addition and diluted into 50 mM- $Na_2P_2O_7$ /1 mM-EDTA, adjusted to pH 8.5 with HCl (pH 8.5 pyrophosphate buffer), and containing 0.1 M-2-mercaptoethanol. The enzymic activity of the diluted, re-activated samples was determined.

The results are shown in Fig. 1. Apoenzyme (solution A) was readily inhibited, but the holoenzyme (solution C) was not inhibited. Sodium phosphate (45 mM) partially protected the apoenzyme from inhibition (solution B). Other experiments were performed in a similar way, and the activity of some preparations of apoenzyme was decreased to less than 5% of the initial activity after the addition of 60 mol of I_2 /mol (15 mol of I_2 /mol of enzyme subunit). The residual 20% enzymic activity shown in Fig. 1

Fig. 2. Inhibition of apo-(glyceraldehyde 3-phosphate dehydrogenase) from *B. stearothermophilus* by $K^{125}I_3$

The apoenzyme (4.17 mg/ml) was treated with tetrathionate to protect the active-site thiol groups, and was iodinated in 50 mM-Tris/HCl buffer, pH 8, with $K^{125}I_3$, as described in the text. ●, Mole ratio of KI_3 added to enzyme subunits; O, number of atoms of iodine incorporated per enzyme subunit. The enzymic activity was determined after treatment with 2-mercaptoethanol, and is expressed as a percentage of the specific activity of the native enzyme.

may be due to incomplete removal of NAD^+ with charcoal in this preparation, which had E_{280}/E_{260} 1.85.

The inhibition of the apoenzyme in pH 8 Tris buffer at $20 \pm 3^\circ C$ was also studied by using $K^{125}I_3$. The incorporation of iodine into the protein was estimated by precipitation of the ^{125}I -labelled protein with an equal volume of 10% (w/v) trichloroacetic acid and counting the radioactivity of the acetone-washed precipitate on glass-fibre filter discs (Whatman, type GF/C) by liquid-scintillation counting. The results are shown in Fig. 2. Only about one-half of the theoretical amount of iodine was incorporated into the enzyme, the remainder being consumed in oxidative side reactions, since the yellow iodine colour completely disappeared within a few minutes.

The iodination of apo- and holo-enzymes was also performed, in a similar way, in 50 mM-Tris/1 mM-EDTA adjusted to pH 9.5 with HCl, at $0-5^\circ C$. The ionic strength of this buffer is low. The results are shown in Fig. 3. Both apo- and holo-enzymes were inhibited by KI_3 under these conditions.

Fig. 3. Inhibition of glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* by iodination at pH 9.5

The enzyme (2.4 mg/ml) was treated with tetrathionate to protect the active-site thiol groups and was iodinated in 50 mM-Tris/1 mM-EDTA, pH 9.5, at 0–5°C, as described in the text. The enzymic activity was determined after treatment with 2-mercaptoethanol and is expressed as a percentage of the specific activity of the native enzyme. ○, Apoenzyme; ●, holoenzyme, with additional NAD⁺ (0.1 mM).

Determination of thiol groups after iodination and analysis of iodinated protein

Holo-(glyceraldehyde 3-phosphate dehydrogenase) (35 mg/ml) with excess of NAD⁺ (1 mM) and 10 mM-Na₂HPO₄ in pH 8 Tris buffer (4.0 ml) was reversibly inhibited with Na₂S₄O₆ (4.4 mol/mol of enzyme) and treated with 60 mol of I₂/mol of enzyme (15 mol/mol of subunit; 1.67 mol/mol of tyrosine residues) at about 22°C. Some iodine remained after 30 min, and the reaction was quenched with 2-mercaptoethanol (0.01 ml). The iodinated enzyme was isolated by gel filtration on a column (18 mm × 180 mm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) in 40 mM-Na₂HPO₄ / 1 mM-EDTA / 1 mM-2-mercaptoethanol, adjusted to pH 8.0 with HCl. A sample of the iodinated holoenzyme was freed from NAD⁺ by gel filtration on a column (30 mm × 380 mm) of Sephadex G-150 in the presence of sodium dodecyl sulphate (4.5 mg/ml) in the pH 8 phosphate buffer (S. Libor, personal communication). The apoenzyme derivative was concentrated by pressure dialysis and freed from phosphate and mercaptoethanol by gel filtration on a column (15 mm × 220 mm) of Sephadex

G-25 in the pH 8 Tris buffer. Sodium dodecyl sulphate is removed from the active enzyme by this procedure (G. Allen, unpublished work). The enzymically active partially iodinated apoenzyme was again reversibly inhibited with Na₂S₄O₆ and treated with 22.8 mol of I₂/mol (5.7 mol/mol of subunit). The product was treated with 2-mercaptoethanol and freed from low-molecular-weight compounds by gel filtration on a column (15 mm × 220 mm) of Sephadex G-25 in pH 8 Tris buffer.

The enzymic activity of iodinated holo- and apoenzymes was determined and the number of reactive thiol groups measured by titration with 5,5'-dithiobis-(2-nitrobenzoate) (Ellman, 1959). Samples were denatured with 6 M-guanidinium chloride and the total number of thiol groups was measured in this solvent, containing 50 mM-Tris/HCl buffer, pH 8.

Samples of these protein derivatives were exhaustively dialysed against water, dried and hydrolysed for 18 h at 105°C with 6 M-HCl containing 1% (w/v) phenol. The hydrolysates were analysed on a Locarte amino acid analyser.

The iodinated holoenzyme was fully active (38.5 units/mg) and contained 1.99 thiol groups per subunit after denaturation, the same value as for the native enzyme, which had specific activity 38 units/mg. Each subunit of the native enzyme possesses a single reactive thiol group, which is essential for the enzymic activity, and a second thiol group, which only becomes reactive towards 5,5'-dithiobis-(2-nitrobenzoate) on denaturation (K. Suzuki & J. I. Harris, personal communication). The retention of full enzymic activity shows that the reactive active-site thiol group had not been oxidized.

After further iodination in the absence of NAD⁺, the specific activity decreased to 8.5 units/mg. There were 0.89 reactive thiol groups per subunit, and 1.69 thiol groups per subunit were reactive towards 5,5'-dithiobis-(2-nitrobenzoate) after denaturation. Thus the 78% loss of enzymic activity is unlikely to be due to the loss of thiol groups.

Amino acid analysis after acid hydrolysis of glyceraldehyde 3-phosphate dehydrogenase showed that only histidine residues were lost on iodination. About 1.0 histidine residue per subunit was lost from holoenzyme iodinated with 15 mol of I₂/mol of subunit, and about 1.5 histidine residues were lost from apoenzyme treated with 8 or 20 mol of I₂/mol of subunit. Tyrosine and methionine were unchanged. However, during hydrolysis in 6 M-HCl with 1% phenol, iodinated tyrosine residues are converted into tyrosine (Roche *et al.*, 1951), and methionine sulfoxide reverts, at least partly, to methionine (Ray & Koshland, 1960).

The formation of iodotyrosine derivatives was studied by a spectral method similar to that of Edelhoch (1962). The spectral analysis used the absorbances at 280 nm–340 nm in 6 M-guanidinium

chloride and 50mM-HCl or 50mM-NaOH of the iodinated protein, and comparison with experimentally determined spectra of *N*-acetyltryptophan, *N*-acetyltyrosine ethyl ester, mono- and di-iodotyrosine and thyroxine in the same solvents. The ratios of the chromophoric derivatives were found by this method, and the results could be expressed in terms of residues per subunit by normalizing the sum of tyrosine derivatives to nine. Iodinated holoenzyme was freed from NAD^+ before the spectral analysis by gel filtration on a column (10mm $\times$ 100mm) of Sephadex G-25 in 6M-guanidinium chloride and pH8 Tris buffer.

A preparation of holoenzyme iodinated at 10.7 mg/ml at pH8 with 15 mol of I_2 /mol of subunit and with 7.4 atoms of iodine incorporated per mol of subunit (determined with ^{125}I) contained 6.1 tyrosine, 0.7 monoiodotyrosine and 2.2 di-iodotyrosine residues/subunit, indicating that the remaining 2.3 atoms of iodine/subunit were incorporated into histidine residues. This preparation of iodinated holoenzyme was freed from NAD^+ by gel filtration in sodium dodecyl sulphate solution, as described above. Further iodination with 5.7 mol of KI_3 /mol of subunit resulted in the loss of 68% of the enzymic

activity and incorporation of 2.7 atoms of iodine/mol of subunit. Spectral studies indicated that there were 5 tyrosine, 1.5 monoiodotyrosine and 2.5 di-iodotyrosine residues per subunit, suggesting that in the further iodination about 1.3 iodine atoms/subunit were incorporated into histidine residues.

Other experiments performed under similar conditions gave similar results apart from variation in the amount of iodine incorporated into histidine residues, which is obtained by difference and is accurate only to within about 1 atom/subunit. Typical spectra and model spectra are shown in Fig. 4. The observed spectra were consistent with the lack of oxidation of tryptophan residues during iodination at pH8, although this spectral analysis is not very sensitive for the determination of this residue, since the absorbance is little altered by acid or alkali.

Investigation of iodinated residues in tryptic peptides

Tryptic peptides containing radioactive iodine were isolated from iodinated apoenzyme, iodinated holoenzyme and from the enzyme initially iodinated in the presence of NAD^+ with non-radioactive iodine, to block reactive residues not involved in the enzymic

Fig. 4. Spectra of samples of iodinated glyceraldehyde 3-phosphate dehydrogenase (*B. stearothermophilus*) in 6M-guanidinium chloride and (i) 50mM-HCl or (ii) 50mM-NaOH

(a) —, Holoenzyme iodinated with 15 mol of KI_3 /mol of enzyme subunit. NAD^+ was removed from the iodinated holoenzyme by gel filtration in 6M-guanidinium chloride before determination of the spectra. ----, Model spectrum calculated to give the best fit, for 3 tryptophan, 6.5 tyrosine, 0.5 monoiodotyrosine and 2.0 di-iodotyrosine residues per subunit. (b) —, Apoenzyme iodinated with 20 mol of KI_3 /mol of subunit; ----, model spectra calculated for 3.0 tryptophan, 3.2 tyrosine, 1.6 monoiodotyrosine and 4.2 di-iodotyrosine residues per subunit.

activity, then separated from NAD^+ and further iodinated with $^{125}\text{I}_2$ in order to identify residues that become reactive in the absence of the coenzyme and which may be involved in the enzymic activity.

The apoenzyme (107mg in 10ml) in pH8 Tris buffer was treated with $\text{Na}_2\text{S}_4\text{O}_6$ as described above and iodinated with 20mol of $^{125}\text{I}_2$ /mol of subunit (2.2mol/mol of tyrosine residues), added in three batches over 45min at 26°C , with rapid stirring; 1M-Tris base was added during the reaction to maintain pH8.0. The yellow colour had almost disappeared at the end of the reaction, but this ratio of iodine to protein was chosen because with higher ratios the reagent was not completely consumed within 1h. 2-Mercaptoethanol solution (25%, v/v, in the pH8.5 pyrophosphate buffer; 0.2ml) was added and the solution was dialysed against $2 \times 500\text{ml}$ of 50mM-Tris/HCl buffer, pH8, containing 10mM-KI, and then against 500ml of 0.1% (w/v) NH_4HCO_3 solution, for at least 3h each, and concentrated by dialysis against dry Sephadex G-200. All operations were performed in the dark as far as was convenient, to avoid further photochemical oxidation or iodination.

The holoenzyme (140mg) was iodinated similarly, but with excess of NAD^+ (0.1mM) and with only 15mol of I_2 /mol of enzyme subunit, since some iodine colour remained at the end of the reaction.

Another sample of holoenzyme (140mg) was iodinated in the same way, but with 15mol of non-radioactive I_2 /mol of subunit. The coenzyme was removed by charcoal treatment and the enzyme was further iodinated with 5mol of $^{125}\text{I}_2$ /mol of enzyme subunit. This amount of iodine was chosen since it was sufficient to cause loss of most of the enzymic activity without incorporating many atoms of ^{125}I , and was therefore suitable for the study of any particularly reactive residues in the apoenzyme which might be involved in the activity of the enzyme.

The products in each case were analysed for incorporation of iodine and for the content of tyrosine derivatives.

Each sample was digested with 1% (w/w) trypsin, at pH8.3, in a pH-stat (Radiometer, Copenhagen, Denmark), at 38°C , under N_2 , for 1.5h. The solution was heated to 100°C for 5min, to ensure denaturation of undigested protein, and after cooling to 38°C a further 1% (w/w) of trypsin was added. Digestion was continued for a further 2h and was stopped by freeze-drying. The peptide mixture was fractionated on a column (18mm $\times$ 1490mm) of Sephadex G-50 (superfine) in 50mM- NH_4HCO_3 , at 18ml/h, at $20 \pm 3^\circ\text{C}$, and fractions were analysed for ^{125}I by liquid-scintillation counting. Fractions containing radioactive peptides were combined and freeze-dried. Further purification of radioactive peptides was by ion-exchange chromatography, paper electrophoresis and paper chromatography.

Peptides from the iodinated apoenzyme

The apoenzyme, iodinated with 20mol of KI_3 /mol of subunit, contained 1.6 monoiodotyrosine and 4.2 di-iodotyrosine residues, and 12.5 atoms of iodine, per subunit. The tryptic digest of this material (2.7 μmol of subunits) was soluble in 50mM- NH_4HCO_3 and was fractionated on Sephadex G-50 as shown in Fig. 5. Fractions were combined as shown and freeze-dried. The combined fractions were analysed by paper electrophoresis at pH6.5 and radioautography (Plate 1).

Fraction 1 (950nmol of I) was mainly insoluble in pH6.5 electrophoresis buffer (Thomas & Harris, 1970). It was dissolved in 8M-urea and chromatographed on a column (10mm $\times$ 80mm) of DEAE-cellulose DE-52 in 5–500mM- NH_4HCO_3 (a linear gradient prepared from 100ml of each buffer). Several minor components were observed, but most of the radioactivity was not eluted. A later experiment showed that three major peptides were present in this fraction in similar, and low, yields.

Fraction 2 was resolved into fractions 2A (1300nmol of I) and 2B (1500nmol of I) by chromatography on DE-52 DEAE-cellulose as for Fraction 1. Fraction 2A was further purified by chromatography on a column (7mm $\times$ 60mm) of DEAE-cellulose DE-52 in 8M-urea and 25–500mM- NH_4HCO_3 (a linear gradient prepared from 50ml of each buffer) followed by paper electrophoresis at pH8.9 [1% $(\text{NH}_4)_2\text{CO}_3$] for 75min at 1kV. The radioactivity was

Fig. 5. Fractionation of the labelled tryptic peptides from iodinated apo-(glyceraldehyde 3-phosphate dehydrogenase)

The apoenzyme was iodinated with 20mol of K^{125}I_3 /mol of subunit and digested with trypsin, as described in the text. The tryptic digest was applied to a Sephadex G-50 (superfine) column (18mm $\times$ 1490mm) and the column was eluted with 50mM- NH_4HCO_3 , at 18ml/h, at $20 \pm 3^\circ\text{C}$. The fraction size was about 4ml.

EXPLANATION OF PLATE I

Radioautograph of electrophoretogram (pH 6.5) of fractions from the tryptic digest of iodinated apo-(glyceraldehyde 3-phosphate dehydrogenase) from B. stearothermophilus

Samples of combined fractions 1-7 (Fig. 5) were subjected to paper electrophoresis at pH 6.5 as described by Thomas & Harris (1970), and the radioautograph was prepared by overnight exposure.

EXPLANATION OF PLATE 2

Radioautograph after paper electrophoresis at pH6.5 of tryptic peptides from iodinated apo- and holo-(glyceraldehyde 3-phosphate dehydrogenase)

Glyceraldehyde 3-phosphate dehydrogenase (6.14mg/ml) from *B. stearothermophilus*, in 50mM-Tris/HCl buffer/1mM-EDTA, pH8.0, at 20°C, was treated with various amounts of $K^{125}I_3$. (1)–(6), apoenzyme; mole ratios of $K^{125}I_3$ to enzyme subunits: (1) 1.84, (2) 3.68, (3) 5.5, (4) 7.34, (5) 11.0, (6) 19.6. (7)–(12), holoenzyme; mole ratios of $K^{125}I_3$ to enzyme subunits: (7) 1.84, (8) 3.68, (9) 5.5, (10) 7.34, (11) 11.0, (12) 18.4. Bands A, B and D are derived from peptides containing 'tyrosine-46' and 'histidine-50' (peptides 5C, 6A and 7A). Band C is the neutral band, containing several peptides, particularly those from fraction 4 (Fig. 5). Band E is derived from peptides containing 'tyrosine-173' (peptide 3), 'tyrosine-311' (peptides 5A and 6B), 'tyrosine-46' and 'histidine-50' (peptide 6B1). Band F is derived from fraction 1 (Fig. 5). Band G is derived from peptides containing 'tyrosine-283' (peptides 2A1 and 2B). Band O is material at the origin, which is insoluble or absorbed to the paper.

Fig. 6. Determination of the partial sequence of peptide 2B

Thermolytic peptides were isolated and characterized as described in the text. Neutral peptides are labelled N and acidic peptides A. Sequences determined by the dansyl-Edman method are shown thus: —

distributed in a broad band, staining yellow and turning pink with ninhydrin/cadmium reagent (Heilmann *et al.*, 1957), and the band was divided into two parts, 2A1 and 2A2. *N*-Terminal determination and amino acid analyses showed that two peptides were present, each contaminated by a small amount of the other. Peptide 2A1 had the corrected analysis: Asx(Asx₂, Thr₃, Ser₃, Pro₀₋₁, Gly₂, Ala, Val₂, Met, Ile₂, Leu, Tyr, Lys), with 1 atom of I. Peptide 2B was purified by chromatography on DE-52 DEAE-cellulose in 8M-urea and 25–500mM-NH₄HCO₃ as for fraction 2A, and had the analysis: Asx(Asx₂₋₃, Thr₃, Ser₃₋₄, Gly₂, Ala, Val₂, Met, Ile₂, Leu, Tyr, Lys), with 2 atoms of I. Thus peptides 2B and 2A1 have almost identical analyses, but peptide 2B probably contains di-iodotyrosine.

A sample (90nmol) of peptide 2B was digested with thermolysin (3μg) for 15min at 60°C, in 0.04ml of 0.5M-NH₄HCO₃. Peptides were purified by paper electrophoresis and paper chromatography and the partial sequence of peptide 2B was deduced, assuming that the *C*-terminus was lysine and the *N*-terminus asparagine (from the ninhydrin colour) rather than aspartic acid, as in Fig. 6. This sequence is homologous with the pig muscle enzyme residues 282–303 (Harris & Perham, 1968). Thus 'tyrosine-283' (residues in the enzyme from *B. stearothermophilus* are numbered as for the homologous residues in the pig muscle enzyme, but with quotation marks; these are not necessarily the actual numbers of the residues in the sequence) is extensively iodinated in the apoenzyme from *B. stearothermophilus*.

Fraction 3 contained mainly a single slightly acidic radioactive peptide, which was purified by paper electrophoresis and chromatography, staining yellow with ninhydrin/cadmium, and with *N*-terminal glycine and the analysis: Gly(Ser₂, Glx₂, Pro, Ala, Val, Ile, Leu₂, Tyr, Arg), with 2 I atoms. The sequence was determined: Gly-Ile-Leu-Ala-I₂Tyr-Ser-Glx-Glx-Pro-Leu-Val-Ser-Arg. The tyrosine residue is in a sequence homologous with that around tyrosine-273 in the pig muscle enzyme.

Fraction 4 was partly insoluble in pH 6.5 electrophoresis buffer. The mixture was divided into a soluble and an insoluble part (4 and 4' respectively) in 1.4ml of 5mM-(NH₄)₂CO₃. Subfraction 4 (2200nmol of I) was fractionated on a column (10mm×80mm) of DE-52 DEAE-cellulose in 5–500mM-NH₄HCO₃ (100ml of each buffer) into two major components, 4A (760nmol of I) and 4B (720nmol of I), only the latter absorbing significantly at 280nm. Peptide 4A was separated on a column (7mm×40mm) of CM-cellulose CM-52 in 8M-urea/25–1000mM-acetic acid/NH₃, pH 4.5 (40ml of each buffer), into peptide 4A1 (253nmol of I) and 4A2 (212nmol of I). Peptide 4A1 had *N*-terminal isoleucine and the analysis: Ile(Asx, Pro, Leu₂, X, Lys), where X is an unknown residue presumably derived from an iodinated histidine (cf. peptide 7A). The sequence was Ile-Leu-Asx-Leu-Pro-X-Lys, which is homologous with the sequence residues 185–191 in the pig muscle enzyme. Peptide 4A2 had the analysis Val(Glx₂, Gly, Val, Ile, Leu, Phe, X, Arg), and the sequence Val-Leu-IHis-Glx-Glx-Phe-Gly-Ile-Val-

Arg, which is homologous with the sequence residues 160–169 in the pig muscle enzyme. Peptide 4B was purified similarly, giving peptides 4B1 (163 nmol of I) and 4B2 (200 nmol of I). Peptide 4B1 had the analysis Val(Asx, Ser₁₋₂, Glx₂, Gly, Ala₂₋₃, Val, Ile, Leu₂, Tyr₁₋₂, Lys) (42 nmol) and the partial sequence Val-Leu-Asx-Glx-I-Tyr-Ala-Glx-. Peptide 4B2 had the analysis Gly(Asx₂, Thr₃, Ser, Glx, Val, Met₂, Tyr, His, Arg) with 2 atoms of I. The sequence was determined, but the results were not definite after the fourth residue: Gly-Met-Met-Thr-?-Val-His-Ser-I₂Tyr-Thr-Asx-Asx-Gly-Arg.

The insoluble part, 4', was dissolved in 8M-urea, and a single radioactive peptide, 4C (940 nmol of I) was isolated by chromatography on a column (7 mm × 47 mm) of DEAE-cellulose DE-52 in 8M-urea and 5–500 mM-NH₄HCO₃ (40 ml of each buffer). The analysis was Gly(Asx₂, Thr₃, Ser, Glx, Val, Met₂, Tyr, His, Arg) (275 nmol), and 3 atoms of I, suggesting that both tyrosine and histidine were iodinated. The analysis and *N*-terminus were identical with those for peptide 4B2, and the peptide is homologous with the sequence residues 170–183 in the pig muscle enzyme, showing that 'tyrosine-178' is readily iodinated in the apoenzyme from *B. stearothermophilus*. Residue 178 in the pig muscle enzyme is isoleucine (Harris & Perham, 1968).

Fraction 5 was resolved by paper electrophoresis at pH 6.5 and paper chromatography into peptides 5A, 5B and 5C.

Peptide 5A was Ehrlich-positive (Smith, 1953) and acidic, with *N*-terminal valine (+Val-Val) and the analysis: Val(Asx₂, Thr, Ser₂, Glx, Gly, Tyr₂, His?, Arg) + Trp (50 nmol of peptide containing 100 nmol of I). This corresponds to the sequence residues 307–320 in the pig muscle enzyme; the low analysis for valine was due to slow cleavage of the Val-Val bond.

Peptide 5B was almost neutral, Ehrlich-positive, with *N*-terminal valine (+Val-Val) and the analysis: Val(Asx₂, Thr, Ser₂, Glx, Gly₂, Val, Met, Tyr₂, His, Arg) + Trp (70 nmol of peptide containing about 50 nmol of I); it was not completely pure. The partial sequence was Val-Val-Ser-Trp?-I-Tyr-Asx?-Asx?-Glx?-, which is homologous with the sequence after residue 307 in the pig muscle enzyme.

Peptide 5C was basic, with the analysis: Tyr(Asx, Ser, Gly, Val, His, Arg) (87 nmol, with about 50 nmol of I).

Fraction 6 was resolved into two radioactive components by paper electrophoresis at pH 6.5: 6A and 6B. Peptide 6A (basic, 880 nmol of I) had the analysis: Tyr(Asx, Ser, Gly, Val, His, Arg) (300 nmol), and peptide 6B (slightly acidic, Ehrlich-positive, 970 nmol of I) had *N*-terminal valine (+Val-Val) and tyrosine. Peptide 6B was purified on a column (7 mm × 50 mm) of DEAE-cellulose DE-52 in 25–500 mM-NH₄HCO₃ (40 ml of each buffer). Less than

half of the applied radioactivity was recovered; the major peak (peptide 6B1, 280 nmol of I) had the analysis: Tyr(Asx, Ser, Val, His) (93 nmol). The sequence was I₂Tyr-Asx-Ser-Val-His?, homologous with the sequence residues 46–50 in the pig muscle enzyme.

Fraction 7 gave, after paper electrophoresis at pH 6.5, peptides 7A and 7B. Peptide 7A (207 nmol of I) was slightly acidic and had the analysis Tyr(Asx, Ser, Gly, Val, X, Arg), where X is some iodinated derivative of histidine. Peptide 7B (52 nmol of I) was acidic, with the analysis: Tyr(Asx, Ser, Val, X) (9 nmol).

In summary, the following tyrosine residues were iodinated in the apoenzyme, and peptides containing the tyrosine residues were isolated in the total yields shown: 'tyrosine-46' (520 nmol, in peptides 5C, 6A, 6B1, 7A, 7B), 'tyrosine-178' (360 nmol in peptides 4B2 and 4C), 'tyrosine-273' (250 nmol, in peptide 3), 'tyrosine-283' (360 nmol in peptide 2A1 and 2B), 'tyrosine-311' (120 nmol in peptides 5A and 5B, and more in 6B), and possibly the other tyrosine residue in the tryptic peptide containing 'tyrosine-311'. Some histidine residues were also iodinated: 'histidine-50' (133 nmol in peptides 6B1, 7A and 7B), 'histidine-162' (250 nmol in peptide 4A2) and 'histidine-190' (140 nmol, in peptide 4A1).

The total yield of iodine in purified peptides was 4800 nmol or 14% of the iodine incorporated into the protein. Although peptides were isolated in different molar yields, this suggests that a yield of 14% (380 nmol) of peptides containing a particular iodinated residue represents extensive iodination of that residue.

The spectral method (see above) indicated that 5.8 tyrosine residues per subunit were iodinated, but only 5 labelled tyrosine residues were clearly identified in peptides. The 2.5 iodine atoms per subunit in excess of those bound to tyrosine residues agrees well with the estimated 3 labelled histidine residues observed. Peptides containing more than 1 tyrosine or histidine residue capable of being iodinated may be isolated in lower than average yields, since multiple products, differing in their extent of iodination, may be formed from the same peptide sequence, each in low yields, which may have been discarded as minor products.

It may be noted that iodotyrosine residues adsorb to Sephadex G-50. The three peptides 5C, 6A and 7A, comprising the sequence 'Tyr₍₄₆₎-Arg₍₅₂₎' were eluted from the Sephadex G-50 column in the order of increasing extents of iodination, and peptides comprising the sequence around 'Tyr-311' (which also includes a tryptophan residue) were eluted from the column later than would have been expected from their molecular weights. Such adsorption effects have been observed with halogenated phenols (Bræk & Housley, 1969).

Peptides from the iodinated holoenzyme

This preparation of holoenzyme treated with 15 mol of KI_3 /mol of subunit contained 7.4 atoms of I/subunit, and contained 1–2 di-iodotyrosine, 0–1 monoiodotyrosine and 6.5 tyrosine residues, indicating that about 3 atoms of I were in iodinated histidine residues. The coenzyme was still firmly bound, but was removed by gel filtration in 6M guanidinium chloride or sodium dodecyl sulphate solutions or by charcoal treatment. The iodinated holoenzyme had 80% of the initial specific activity of the enzyme.

The tryptic digest (140 mg; 3.9 μ mol of subunits) of iodinated holoenzyme was fractionated on the Sephadex G-50 column as shown in Fig. 7, and the fractions were analysed by paper electrophoresis at pH 6.5. The clearest differences from the apoenzyme preparation were that radioactive peptides were absent from fractions 1, 2 and 3. A peptide giving a yellow stain with ninhydrin/cadmium and in a position similar to peptide 3 after paper electrophoresis and chromatography, but containing no iodine, was isolated in a yield of 1.35 μ mol, with the analysis: Gly(Ser₂, Glx₂, Pro, Ala, Val, Ile, Leu₂, Tyr, Arg). Thus 'tyrosine-273' is well protected by NAD^+ .

The following iodinated peptides were purified and characterized as for those from the apoenzyme derivative: Val(Glx₂, Gly, Val, Ile, Leu, Phe, IHis, Arg) (300 nmol of I) (cf. peptide 4A2), Val(Glx₂, Leu, Phe, IHis) (170 nmol of I; probably a 'chymotryptic' cleavage product from peptide 4A2), Gly(Asx₂, Thr₃, Ser, Glx, Val, Met₂, ITyr, His, Arg) (140 nmol of I, in 85 nmol of peptide; cf. peptides 4B2 and 4C).

Fig. 7. Fractionation of the labelled tryptic peptides from iodinated holo-(glyceraldehyde 3-phosphate dehydrogenase)

The holoenzyme was iodinated with 15 mol of $K^{125}I_3$ /mol of subunit and digested with trypsin, as described in the text. The tryptic digest was fractionated on the Sephadex G-50 column (18 mm $\times$ 1490 mm) as described in the legend to Fig. 5.

The following peptides were characterized by their electrophoretic and chromatographic properties and *N*-termini: 4A1 (120 nmol of I), 5C (605 nmol of I), 6A (420 nmol of I), 7A (120 nmol of I). The total yield of iodine in peptides containing 'tyrosine-46' and 'histidine-50' was 1800 nmol.

In addition, a non-radioactive peptide, absorbing strongly at 280 nm, was isolated in relatively high (740 nmol) yield with the analysis: Val(Asx₂, Thr, Ser₂, Glx, Gly, Val, Tyr₂, His, Arg), which, with the addition of tryptophan, corresponds to the sequence homologous with that around tyrosine-311 in the pig muscle enzyme.

These results show that 'tyrosine-46', 'histidine-50', 'histidine-162' and 'histidine-190' are iodinated in the holoenzyme as much as in the apoenzyme, and are thus not essential for enzymic activity. 'Tyrosine-273' and 'tyrosine-283' were well protected by NAD^+ . 'Tyrosine-311' (and possibly also the other tyrosine residue in the same tryptic peptide) was also protected by NAD^+ . 'Tyrosine-178' was partially protected by NAD^+ ; only 85 nmol of an iodinated peptide containing this residue was isolated (with 140 nmol of I) from the digest of the holoenzyme, whereas 360 nmol of peptides, containing 1140 nmol of I, were isolated from the digest of the apoenzyme.

Peptides from iodinated apoenzyme previously treated with non-radioactive iodine in the presence of NAD^+

The second reaction with KI_3 (see legend to Fig. 8) led to the loss of 68% of the enzymic activity and the incorporation of 2.7 of atoms of I/subunit. The tryptic digest was fractionated on the Sephadex G-50 column as shown in Fig. 8, and fractions were combined as shown. No peptides were purified in high yield, but low yields of peptides containing 'tyrosine-283', 'tyrosine-273', 'tyrosine-46' and 'histidine-50' were observed, as characterized by their electrophoretic and chromatographic properties and *N*-termini. Also fraction D contained at least four radioactive peptides, which were not fully characterized.

Thus the inhibition of the enzyme during the second iodination reaction was the result of either partial iodination at several sites in the molecule or oxidative reactions in which iodine was not incorporated.

An estimate of the relative rates of iodination of the different residues at pH 8 was made by digesting with trypsin samples of holo- and apo-enzyme iodinated to different extents, and analysing the tryptic peptides by electrophoresis at pH 6.5 (Plate 2). Although the results are not clear-cut, owing to the relatively large amount of radioactivity in insoluble peptides at the origin (band O) and in several neutral peptides (band C), the differences between holo- and apo-enzymes are apparent. Bands derived from 'tyrosines-173' and '-283' are absent from the

Fig. 8. Fractionation of the labelled tryptic peptides from apo-(glyceraldehyde 3-phosphate dehydrogenase) iodinated with $K^{125}I_3$ after previous partial iodination with KI_3 in the presence of NAD^+

Holo-(glyceraldehyde 3-phosphate dehydrogenase) was treated in the presence of excess of NAD^+ (0.1 mM) with 15 mol of KI_3 /mol of subunit, as described in the text. The coenzyme was removed and the enzyme was further iodinated with 5 mol of $K^{125}I_3$ /mol of subunit. The iodinated protein was digested with trypsin and the tryptic digest was fractionated on the column (18 mm $\times$ 1490 mm) of Sephadex G-50 as described in the legend to Fig. 5.

iodinated holoenzyme, whereas the bands derived from 'tyrosine-46' are clearly observed even at the lowest extents of iodination.

No single tyrosine residue is sufficiently more reactive than others to be easily identified by this method, although 'tyrosine-46' is among the most reactive residues, as shown by the samples 1-3 and 8-10 in Plate 2. Attempts at semi-quantitative peptide 'mapping', by using paper chromatography or electrophoresis at pH 3.5 as a second dimension, were unsuccessful, owing to the low solubility of several of the radioactive peptides.

As shown in Fig. 3, both holo- and apo-enzymes were inhibited by KI_3 at pH 9.5. Samples of the preparations iodinated to different extents were digested with trypsin and the digests were analysed by paper electrophoresis at pH 6.5. Digests of both apo- and holo-enzymes have electrophoretograms similar to those for the apoenzyme iodinated at pH 8 (Plate 2, samples 1-6). Possibly the low ionic strength of the pH 9.5 buffer solution was a factor contributing to the ready inhibition of the holoenzyme, since this enzyme is sensitive to salt concentration (G. Allen, unpublished work). 'Tyrosine-46' was readily iodinated at pH 9.5 as well as at pH 8.

Discussion

One of the most reactive tyrosine residues in both apo- and holo-(glyceraldehyde 3-phosphate dehydro-

genase) from *B. stearrowthermophilus* is 'tyrosine-46', which is also the most reactive residue in the enzyme from pig muscle, lobster muscle and yeast (Thomas & Harris, 1970). However, iodination of this residue does not lead to inhibition of the enzyme: this residue is presumably on the surface of the enzyme and not involved in the enzymic activity.

'Tyrosine-273' is well protected by NAD^+ from iodination. 'Tyrosine-283' and 'tyrosine-178' are also protected by NAD^+ , the latter only partially, but these tyrosine residues are not present in homologous positions in the primary sequences of other glyceraldehyde 3-phosphate dehydrogenases, where residue 178 is valine, isoleucine or leucine, and residue 283 is phenylalanine. These residues are thus unlikely to be intimately concerned in the catalytic mechanism, which is assumed to be common to the enzymes from different sources. 'Tyrosine-311' (and possibly 'tyrosine-317' in the same peptide) is protected by NAD^+ . No tyrosine residues in positions homologous with residues 39, 42, 91, 137 and 252 in the lobster muscle enzyme were observed to be iodinated. Sequence studies (J. Walker & J. Bridgen, personal communication) have already shown that tyrosine residues are not present in positions 39, 42 or 252, and there may be no tyrosine residue in position 91, but a tyrosine residue is present in position 137.

Three histidine residues, 'histidine-50', 'histidine-162' and 'histidine-190' were iodinated in both holo- and apo-enzymes, and are presumably on the surface of the enzyme and not involved in the enzymic activity.

The inhibition of the apoenzyme from *B. stearrowthermophilus* is not correlated with the iodination of a particular residue essential to the activity. It is possible that the inhibition is due to oxidation of one or more residues or to small extents of iodination and oxidation at several sites in the molecule. Since the molecule is a tetramer it is possible that iodination of only one-quarter of the total amount of a particular residue could lead to conformational changes of the whole enzyme such that all activity is lost. The oxidation of tryptophan residues may be an important side reaction with some proteins, such as lysozyme (Hartdegen & Rupley, 1967), but no reaction was expected in the glyceraldehyde 3-phosphate dehydrogenase from *B. stearrowthermophilus*, since no tryptophan residue in either holo- or apo-enzymes is reactive towards *N*-bromosuccinimide (G. Allen, unpublished work).

The simplest interpretation of the protection of 'tyrosine-273', 'tyrosine-283' and 'tyrosine-178' by NAD^+ would be that these residues are in the binding site for the coenzyme. However, it is known that binding of the coenzyme to other glyceraldehyde 3-phosphate dehydrogenases causes conformational changes (Listowsky *et al.*, 1965; Kirschner *et al.*, 1966; Durchschlag *et al.*, 1971; Fenselau, 1972), so tyrosine

residues outside the active sites may have different reactivity when the coenzyme is bound, and crystallographic data indicate that the different reactivities observed are probably related to such conformational changes.

When most of the experimental work was completed, the solution of the crystallographic structure of the lobster muscle holoenzyme was announced (Buehner *et al.*, 1973, 1974). The three-dimensional structure of the enzyme from *B. stearotherophilus* is similar to that of the lobster enzyme (A. J. Wonacott & R. M. Sweet, unpublished work, cited by Buehner *et al.*, 1974) and high-resolution crystallographic studies show that this similarity extends to the secondary structure (A. J. Wonacott, personal communication).

A model of the enzyme built by using co-ordinates as for the lobster muscle holoenzyme shows that all of the reactive tyrosine and histidine residues are on the surface of the enzyme. 'Tyrosine-46' is close to an intersubunit boundary, but outside the active site, as expected. 'Tyrosine-273' and 'tyrosine-283' are well removed from intersubunit boundaries and on the opposite side of the subunit from the active site. In the model, 'tyrosine-273' is surrounded by several carboxylate groups, which may suppress its ionization and hinder the approach of the negatively charged reagent, I_3^- . Probably conformational changes occur in this region of the enzyme on binding of NAD^+ , perhaps best described as a 'tightening' of the protein structure, as suggested for the yeast enzyme (Thomas & Harris, 1970).

Two of the tyrosine residues which can be iodinated in the apoenzyme, but are less reactive in the holoenzyme, 'tyrosine-178' and 'tyrosine-311', are close to, or part of, the coenzyme-binding site in the model. The protection of these residues by NAD^+ may be a direct steric effect. A histidine residue, 'histidine-176' is in the same tryptic peptide as 'tyrosine-178', and this histidine residue was also iodinated to some extent. This residue is directly adjacent to the active-site 'cysteine-149' in the three-dimensional structure of the model and its partial iodination may also contribute to the loss of activity of the enzyme.

A possible explanation for the differing reactivities in holo- and apo-enzymes of residues remote from the coenzyme-binding site is that iodination of residues close to this site induces conformational changes which, in turn, increase the reactivity of the remaining tyrosine residues. The lack of precipitation after extensive iodination of the apoenzyme from *B. stearotherophilus* and its stability to denaturing reagents such as urea (Amelunxen *et al.*, 1970) or sodium dodecyl sulphate (K. Suzuki & J. I. Harris, unpublished work, cited in Suzuki & Imahori, 1973) and its stability after extensive succinylation (3-carboxypropionylation) (Allen & Harris, 1976)

indicate that extensive unfolding of the polypeptide chain is unlikely to occur during iodination. However, smaller conformational changes cannot be excluded.

Elödi & Libor (1969) and Libor & Elödi (1970) found that in the pig muscle apoenzyme the most reactive residues were tyrosine-137 and tyrosine-252, whereas Thomas & Harris (1970) found that in the holoenzyme tyrosine-137 had low reactivity and that tyrosine-46 was more reactive than tyrosine-252. Although the results presented here do not clarify this particular problem, since iodinated residues homologous with tyrosine-137 and tyrosine-252 were not observed in the enzyme from *B. stearotherophilus*, they do demonstrate that the earlier results are not incompatible, as there are significant differences in the extent and pattern of iodination of holo- and apo-enzymes.

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