GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM THE RED KANGAROO (*MEGALEIA RUFA*): PURIFICATION AND THE AMINO ACID SEQUENCE AROUND A REACTIVE CYSTEINE

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase from leg muscle of *M. rufa* has been extracted and purified. The reaction of the enzyme with iodoacetate, the amino acid composition, tryptic fingerprint, and some amino acid sequences (including that around the reactive cysteine) indicate that kangaroo glyceraldehyde-3-phosphate dehydrogenase is almost identical with pig glyceraldehyde-3-phosphate dehydrogenase.

These results are in accord with previous findings that, in contrast to other proteins that have been investigated, the structure of glyceraldehyde-3-phosphate dehydrogenase has changed little during the divergent evolution of the various species examined.

I. INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD oxidoreductase, E.C. 1.2.1.12), which catalyses the oxidation and phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, is a key enzyme in the metabolism of carbohydrates. The molecular weight of the native enzyme is 145,000 (Harrington and Karr 1965) and it is composed of four identical subunits, each containing one reactive cysteine residue (Harris, Meriwether, and Park 1963; Harris *et al.* 1970). The amino acid sequences of glyceraldehyde-3-phosphate dehydrogenases from lobster *Homarus vulgaris* (Davidson *et al.* 1967) and pig muscle (Harris and Perham 1968) have been determined and that of the yeast enzyme is almost fully known (Jones and Harris 1968).

Present in most living organisms, glyceraldehyde-3-phosphate dehydrogenase is usually easy to purify in a high yield and as a consequence it could be a fruitful subject for comparative sequence studies aimed at a better understanding of taxonomic relationships. In this connection, Allison and Harris (1965) have examined the amino acid sequence around the reactive cysteine of the enzyme isolated from a variety of plant and animal sources. They found that the sequence in this region generally was conserved, but some substitutions occur at a number of sites. Therefore it seemed of interest to extend this knowledge to a hitherto unexamined source, the order Marsupialia, by purifying and investigating glyceraldehyde-3-phosphate dehydrogenase from the red kangaroo (*Megaleia rufa*).

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II. Materials and Methods

(a) *Materials*

NAD and the diethyl acetal of barium glyceraldehyde 3-phosphate were obtained from the Sigma Chemical Company, St. Louis. Sodium glyceraldehyde 3-phosphate was prepared as previously described (Davidson 1970). [2-^{14}C]Iodoacetic acid (15·5 μCi/μmole) was obtained from the Radiochemical Centre, Amersham, and diluted 10-fold with unlabelled iodoacetic acid. Its specific activity was determined as *S*-carboxymethylcysteine after reaction with cysteine. Carboxymethyl(CM)-cellulose (CM 52) was obtained from Whatman & Co., London, and prepared for use according to the manufacturer's instructions (technical bulletin 1E2). Trypsin was treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone by the method of Kostka and Carpenter (1964). All other reagents were commercial products of high purity and glass-distilled water was used throughout.

(b) *Assay of Glyceraldehyde-3-phosphate Dehydrogenase Activity*

Enzyme activity was measured spectrophotometrically according to the method described by Velick (1955) with certain modifications. The following were made up to 2·9 ml in a cuvette of 1 cm light path: sodium pyrophosphate buffer, pH 8·5 (140 μmoles); NAD (0·75 μmole); glyceraldehyde-3-phosphate (0·75 μmole); and 5–10 μg of enzyme. The rate of change of absorbance at 340 nm at 25°C was recorded—there was little or no increase in absorbancy at this stage unless inorganic phosphate was present. Arsenate was then added (15 μmoles in 50 μl) to start the reaction. Enzyme units were defined by multiplying the increment in absorbance that occurred between 15 and 45 sec by 2000.

(c) *Estimation of Protein*

Throughout the purification of the enzyme, protein was estimated by a microBiuret method (Itzhaki and Gill 1964). Concentrations of pure solutions of enzyme were estimated from the absorbance at 280 nm by assuming that $E^{1\%}_{1cm} = 10$ at that wavelength (Allison and Kaplan 1964).

(d) *Radioautography and Counting of ^{14}C-labelled Material*

Ionograms were autoradiographed by contact for a suitable period (18–60 hr) with Kodak blue brand X-ray film (BB34). The ^{14}C contents of samples were determined by counting dried aliquots on planchets in a Nuclear Chicago gas flow counter.

(e) *Purification of Kangaroo Glyceraldehyde-3-phosphate Dehydrogenase*

All steps of the following procedure were carried out at 0–5°C and the following buffers were used: buffer A, 5 mM Tris–chloride, pH 7·3, 5 mM EDTA, and 5 mM β-mercaptoethanol; buffer B, 10 mM Na^+ phosphate, pH 7·1, 1 mM EDTA, and 1 mM β-mercaptoethanol; buffer C, 10 mM Na^+ phosphate, pH 7·4, 5 mM EDTA, and 5 mM β-mercaptoethanol.

Step 1: Extraction.—Leg muscle (0·55 kg) from a freshly killed red kangaroo was frozen, minced through an electric meat grinder, and then stirred with 750 ml of buffer A for 2 hr before centrifuging at 900 *g* for 30 min. The pellet was washed with a further 250 ml of buffer, recentrifuged, and the combined supernatant solutions were treated with ammonium sulphate as in step 2.

Step 2: Ammonium Sulphate Fractionation.—Solid ammonium sulphate (43 g per 100 ml of solution; 65% saturation) was added with stirring and after standing for 1 hr the solution was centrifuged at 6000 *g* for 30 min. The supernatant solution was then taken to 85% saturation with solid ammonium sulphate (14 g per 100 ml of solution) and allowed to stand for 18 hr. The precipitate was collected by centrifugation at 5000 *g* for 25 min and redissolved in 200 ml of buffer B. The ammonium sulphate was removed by dialysis for 24 hr against the same buffer.

Step 3: CM-cellulose Chromatography.—The dialysed solution of proteins was applied to a column of CM-cellulose (2·5 by 30 cm) that had been equilibrated with buffer B. The sample was

washed on with 70 ml of the same buffer before a linear gradient of sodium chloride to 0·16M (700 ml) was applied. The effluent was collected in 10-ml fractions which were monitored for absorbance at 280 nm and assayed for glyceraldehyde-3-phosphate dehydrogenase activity. The active fractions were pooled and the protein was concentrated by ammonium sulphate precipitation (85%) with subsequent dialysis against buffer C. At this stage, the enzyme still contained small quantities (approximately 5%) of haemoglobin.

Step 4: Gel Filtration on Sephadex G100.—The dialysed protein obtained from step 3 was loaded on to a column (2 by 130 cm) of Sephadex G100 equilibrated with buffer C. It was eluted with the same buffer at a flow rate of 18 ml per hour. Fractions of 5 ml were collected and monitored for absorbance at 280 nm and assayed for glyceraldehyde-3-phosphate dehydrogenase activity. The fractions that contained enzyme were pooled, taken to 85% saturation with ammonium sulphate, and centrifuged.

Step 5: Crystallization.—The protein pellet from step 4 was dissolved in the minimum volume of buffer C and solid ammonium sulphate was added until the solution was 65% saturated. It was then adjusted to pH 8·0 with 30% (v/v) ammonia solution and centrifuged to remove traces of denatured protein. Saturated ammonium sulphate solution (adjusted to pH 8·0) was added slowly with stirring until the first sign of persistent turbidity and the solution was then allowed to stand. After 2 days microscopic crystals could be seen—they appeared as rosette-like clusters of plates. The enzyme was stored in this form.

(*f*) *Starch-gel Electrophoresis*

Starch gels (pH 8·9) were prepared and used for electrophoresis as described by Allison and Kaplan (1964). After electrophoresis the gel was sliced in two horizontally; protein was detected in one half with the amido black stain and enzyme activity was detected by incubating the other half for 10 min at 20°C with the enzyme assay mixture [see Section II(*b*)]. $NADH_2$, produced by the enzyme, was seen by its ultraviolet fluorescence.

(*g*) *Amino Acid Analyses*

Samples of *S*-carboxymethyl-glyceraldehyde-3-phosphate dehydrogenase were hydrolysed in duplicate with 6N HCl *in vacuo* at 110°C for 23, 48, and 72 hr. After removing HCl *in vacuo* over NaOH the amino acid compositions of the hydrolysates were determined on a Beckmann 120B amino acid analyser by the method of Spackman (1963). Cysteine and methionine were estimated as cysteic acid and methionine sulphone, respectively, according to the method of Moore (1963).

Peptides were hydrolysed for 24 hr at 110°C *in vacuo*.

(*h*) *Large-scale Carboxymethylation of Glyceraldehyde-3-phosphate Dehydrogenase*

Whilst the reactive thiol of glyceraldehyde-3-phosphate dehydrogenase is not readily oxidized during storage of the enzyme in the crystalline form, the following procedure ensures a maximum content of sulphydryl groups for carboxymethylation by adding thiol to the crystalline enzyme.

Dithioerythritol (5 μmoles) was added to 10 ml of a suspension of enzyme (80 mg; 2·2 μmoles of subunit) in 85% ammonium sulphate and the mixture was centrifuged at 2000 *g* for 20 min at 3°C. The pellet was dissolved in 4·0 ml of 10 mM Tris–chloride buffer (pH 7·4) and 1 mM in EDTA and then passed through a column (1 by 15 cm) of Sephadex G25 equilibrated with the same buffer. Oxidation of the thiol groups at this stage is not a problem if solutions are kept at 3°C and there is no delay before carboxymethylation. The enzyme was then treated with [2-^{14}C]iodoacetate (4 μmoles per μmole of enzyme subunit; specific activity $1·2 \times 10^6$ counts of ^{14}C per minute) at 3°C for 4 hr (under these conditions the reaction of iodoacetate is specific for the reactive thiol—see Section III). The reaction was terminated by the addition of a small excess of β-mercaptoethanol, the pH was then adjusted to 8·3 with 1·0M Tris–chloride, pH 8·3, and the remaining thiol groups on the enzyme were reacted with unlabelled iodoacetate (10 μmoles per μmole of enzyme subunit) for 2 hr at 30°C in 8M urea. An excess of β-mercaptoethanol was then added and the solution was dialysed against 1 mM HCl for 16 hr.

(i) Tryptic Digestion and Gel Filtration

The *S*-carboxymethyl-glyceraldehyde-3-phosphate dehydrogenase (approx. 0·75 mg/ml in 1 mM HCl) was digested with trypsin (1·2 mg; approximately 1 : 50 on a weight basis) for 4 hr at 37°C after the addition of solid ammonium bicarbonate (2 mg/ml). The protein precipitated from solution on the addition of the ammonium bicarbonate, but the suspension cleared during the first 30 min of digestion. The digest was freeze-dried, dissolved in 3·0 ml of 0·05M NH_3 solution, and passed through a column (2 by 140 cm) of Sephadex G50 at 10–15 ml/hr. Fractions of 5 ml were collected and monitored for radioactivity and absorbance at 225 nm.

(j) Sequence Analysis

The various conditions that were used for paper electrophoresis and chromatography, end group determination, and sequence determination have been described previously (Davidson 1970).

III. Results

(a) Purification of Kangaroo Glyceraldehyde-3-phosphate Dehydrogenase

The progress of the purification of red kangaroo muscle glyceraldehyde-3-phosphate dehydrogenase is indicated by the data in Table 1. The purification of this enzyme from other mammalian sources has usually been achieved by repeated recrystallizations from concentrated ammonium sulphate solution (Elödi and

TABLE 1

PURIFICATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM RED KANGAROO MUSCLE

The procedure outlined in Section II was used on 550 g of kangaroo muscle

Step	Volume (ml)	Total Protein (g)	Total Units	Enzyme (mg)*	Specific Activity (units/mg)
1. Extract	920	28·6	n.d.†	n.d.	n.d.
2. Ammonium sulphate fraction (soluble in 65%, insoluble in 85%)	151	5·5	60×10^6	600	11,000
3. CM-cellulose active fractions	84	0·24	10×10^6	97	40,000
4. Sephadex G100 active fractions	32	0·085	$8·5 \times 10^6$	85	100,000

* Calculated from 100,000 units per milligram.

† Glyceraldehyde-3-phosphate dehydrogenase activity cannot be measured in the tissue extract because of the rapid re-oxidation of $NADH_2$ by α-glycerophosphate dehydrogenase. This latter enzyme is removed in the 0–65% ammonium sulphate precipitate.

Szorényi 1956; Allison and Kaplan 1964); however, in this case the use of CM-cellulose and Sephadex G100 was found to be more satisfactory in yielding pure enzyme in a short time. We were unable to achieve a reproducible binding of the enzyme to CM-cellulose, probably as a result of variable binding of phosphate ions to the enzyme (Velick and Hayes 1953). The use of buffers not containing phosphate would be preferred. Column profiles obtained with CM-cellulose and Sephadex G100 are shown in Figures 1(*a*) and 1(*b*).

The likely homogeneity of the enzyme was indicated by two criteria. Firstly, the specific activity was constant across the peak of enzyme that was eluted from the final, Sephadex G100, column [Fig. 1(*b*)]. Secondly, a single band of protein, containing glyceraldehyde-3-phosphate dehydrogenase activity, was observed to migrate towards the cathode on starch-gel electrophoresis at pH 8·9.

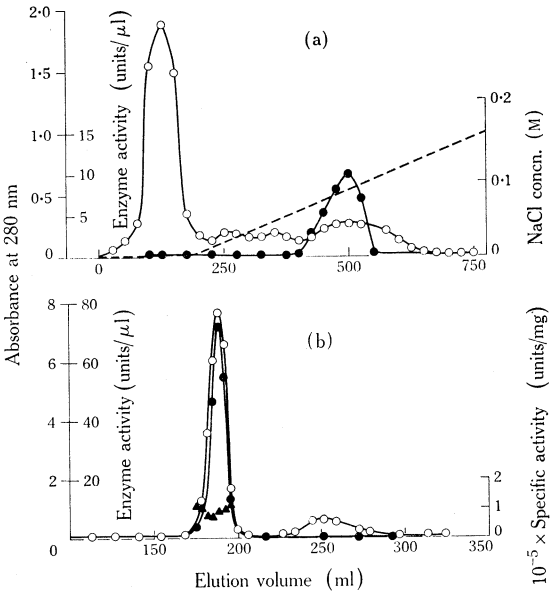

Fig. 1.—Chromatography on CM-cellulose (*a*) and gel filtration on Sephadex G100 (*b*) of kangaroo glyceraldehyde-3-phosphate dehydrogenase. For experimental details see Section II(*e*). ○ Absorbance at 280 nm. ● Enzyme activity. ▲ Enzyme specific activity. - - - - Salt concentration gradient.

(*b*) *Amino Acid Analysis*

The amino acid composition of the purified enzyme is shown in Table 2. The absolute values of individual amino acid residues have been calculated to give a total of 332, since this is the number found in each subunit of pig glyceraldehyde-3-phosphate dehydrogenase. Cysteine and methionine were estimated after performic acid oxidation and hydrolysis for 24 hr. By analogy with other glyceraldehyde-3-phosphate dehydrogenases (Harris *et al.* 1970), the cysteic acid residues were probably derived from cysteine. Tryptophan was not determined since previous work by one of the authors (B.D.) has indicated that its estimation in lobster glyceraldehyde-3-phosphate dehydrogenase by the standard techniques yields an incorrect value compared with that found by sequence analysis.

(*c*) *Inhibition by Iodoacetate*

Kangaroo glyceraldehyde-3-phosphate dehydrogenase is rapidly inactivated by iodoacetate at neutral pH and low temperature (see Table 3). In this experiment the enzyme was inactivated at 4°C with [2-^{14}C]iodoacetate for 3 hr, the excess reagent

TABLE 2

AMINO ACID COMPOSITION OF RED KANGAROO, PIG, AND LOBSTER GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Values given are moles per mole of glyceraldehyde-3-phosphate dehydrogenase. Those for the red kangaroo have been calculated from the quantities recovered by amino acid analysis after normalization to 152 residues of the stable amino acids (Asp, Glu, Gly, Ala, Leu, and Phe)

Residue	Red Kangaroo					Pig*	Lobster†
	23 hr	48 hr	72 hr	Average	Nearest Integer		
Lysine	28·7	n.d.	26·0	27·8	28	26	28
Histidine	9·9	n.d.	9·9	9·9	10	11	5
Arginine	9·9	n.d.	11·8	10·9	11	10	9
Cysteic acid	4·1‡	n.d.	n.d.	4·1	4	4	5
Aspartic acid	37·1	35·9	36·5	36·5	36	38	32
Threonine	22·1	20·9	21·3	22·3§	22	22	20
Serine	17·1	14·5	15·2	17·5§	18	19	25
Glutamic acid	19·8	19·0	20·5	19·8	20	18	24
Proline	11·6	n.d.	9·8	10·7	11	12	12
Glycine	31·6	33·6	31·2	32·1	32	32	30
Alanine	31·4	31·6	31·5	31·5	31	32	32
Valine	26·0	28·9	33·3	35·1‖	35	34	38
Methionine	7·3‡	n.d.	n.d.	7·3	7	9	10
Isoleucine	17·0	19·0	19·9	21·2‖	21	21	18
Leucine	19·6	19·6	19·3	19·5	19	18	18
Tyrosine	10·1	10·0	10·2	10·1	10	9	9
Phenylalanine	14·2	14·1	14·6	14·3	14	14	15
Tryptophan	n.d.	n.d.	n.d.	n.d.	n.d.	3	3
Total	—	—	—	—	332¶	332	333

* Harris and Perham (1968).

† Davidson *et al.* (1967).

‡ Estimated as cysteic acid and methionine sulphone after performic acid oxidation (Moore 1963).

§ Estimated by linear extrapolation to zero time of hydrolysis.

‖ Estimated by linear extrapolation to infinite time of hydrolysis (i.e. $1/t = 0$).

¶ Assuming the presence of 3 tryptophan residues.

TABLE 3

INHIBITION OF KANGAROO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE BY IODOACETATE

Assay Conditions	Iodoacetate Added*	Time (hr)	Enzyme Activity (%)
Crystalline glyceraldehyde-3-phosphate dehydrogenase (2·4 mg in 0·35 ml of 85% saturated ammonium sulphate solution) was centrifuged and then dissolved in 1·0 ml of cold 5 mM Tris–chloride buffer, pH 7·4, containing 1 mM EDTA and 5 mM dithioerythritol. After 10 min at 3°C the enzyme was passed through a column of Sephadex G25 (1·5 by 18·5 cm) equilibrated with the same buffer, less dithioerythritol. A sample of the eluted enzyme (66 nmoles of subunit) was incubated at 4°C with a threefold excess of iodoacetate (200 nmoles) in 3·0 ml and samples (2 μl) were assayed at appropriate times	3	0	100
	3	2	24
	3	3	<10
	0	3	91

* Mole per mole of enzyme subunit.

was removed (by reaction with β-mercaptoethanol), and the remaining thiol groups were reacted with *unlabelled* iodoacetate in 8M urea. The sample was digested with trypsin and subjected to electrophoresis firstly at pH 6·5 and then at right angles at pH 3·5. Autoradiography revealed one major radioactive peptide, which indicated that iodoacetate reacts specifically with one amino acid of the enzyme under the conditions used. The inactivation of kangaroo glyceraldehyde-3-phosphate dehydrogenase by iodoacetate therefore closely resembles, both in its kinetics and specificity the inactivation of pig glyceraldehyde-3-phosphate dehydrogenase by the same reagent (Perham and Harris 1963).

Further similarities with the pig enzyme were revealed when the above fingerprint was stained with cadmium ninhydrin. A total of 35–40 ninhydrin-positive spots were detected and at least 80% of these appeared to occupy identical positions with peptides in the corresponding fingerprint of pig glyceraldehyde-3-phosphate dehydrogenase (Harris and Perham 1965).

(*d*) *Amino Acid Sequence around the Reactive Cysteine*

In order to determine the amino acid sequence around the reactive cysteine of kangaroo glyceraldehyde-3-phosphate dehydrogenase, 80 mg of enzyme was labelled differentially with [2-^{14}C]iodoacetate and unlabelled iodoacetate (see Section II) so that the radioactive label was on the reactive cysteine. The incorporation of radioactivity was 1·1 μmole of label per μmole of enzyme subunit. The material was digested with trypsin and passed through a column of Sephadex G50.

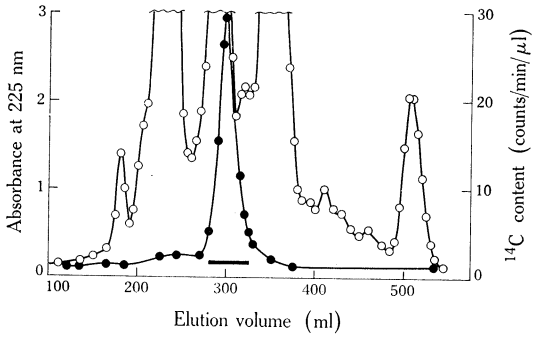

Fig. 2.—Fractionation of a tryptic digest of *S*-[2-^{14}C]carboxymethylglyceraldehyde-3-phosphate dehydrogenase on Sephadex G50. For experimental details see Section II(*i*). ○ Absorbance at 225 nm. ● Counts of ^{14}C per millilitre. ——— Fractions pooled for purification of radioactive peptides.

The column profile (Fig. 2) indicates that the ^{14}C was eluted in a single peak. The relevant fractions were pooled and freeze-dried and, after paper electrophoresis at pH 6·5 and pH 3·5, two pure radioactive peptides were obtained. No other radioactive peptides were present in significant quantities.

Amino acid compositions, net charge, and end group analyses of these peptides are presented in Table 4 and their amino acid sequences, determined by the dansyl–Edman method, are shown in Table 5. Since the only significant difference between

T1 and T2 is their net negative charge and since T2 is present only in low yield it is most likely that T2 represents a doubly de-amidated form of T1 and the two peptides represent a unique amino acid sequence.

TABLE 4

PROPERTIES OF RADIOACTIVE PEPTIDES ISOLATED FROM *S*-[2-^{14}C] CARBOXYMETHYL-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Purification procedures for both peptides as follows: gel filtration on Sephadex G50, electrophoresis at pH 6·5, performic acid oxidation, and finally electrophoresis at pH 3·5. Yields, based on recovery of material applied to the Sephadex G50 column, were 22% for T_1 and 4% for T_2. Numbers in parentheses indicate the number found by sequence determination

Property	Peptide T_1	Peptide T_2
Amino acid		
Lysine	0·9 (1)	1·1 (1)
Aspartic acid	1·9 (2)	1·9 (2)
Threonine	1·9 (2)	2·4 (2)
Serine	2·1 (2)	1·8 (2)
Glutamic acid	—	1·0‖
Proline	1·4 (1)	0·9 (1)
Glycine	0·5 §	1·0 §
Alanine	2·8 (3)	3·0 (3)
Valine	0·5 (1)	1·0 (1)
Isoleucine	0·55 (1)	0·30 (1)
Leucine	1·80 (2)	1·5 (2)
Tyrosine	—	0·2 ‖
Phenylalanine	—	0·3 ‖
Carboxymethylcysteine sulphone (total)*	2·1 (2)	1·1 (2)
[2-^{14}C]Carboxymethylcysteine sulphone†	0·75 (1)	0·50 (1)
Molecular weight	1880	1880
Mobility at pH 6·5 (with respect to aspartic acid)	0·22	0·40
Predicted charge‡	−1	−3
N-terminus	Ile.Val....	Ile.Val....
C-terminus	....Ala.Lys	....Ala.Lys

* Determined by amino acid analysis.

† Determined by ^{14}C content.

‡ After Offord (1966).

§ Although amino acid analysis indicated the presence of some glycine, this was not found by sequence determination (see Table 5). This is not unusual since glycine is present as a minor contaminant in the paper used for electrophoresis.

‖ These amino acids are thought to be contaminants since peptides T_1 and T_2 have the same *N*- and *C*-terminal sequences. The level of glutamic acid is relatively high since T_2 and glutamic acid have the same electrophoretic mobility at pH 3·5. There was insufficient quantity of T_2 to allow a further purification step.

IV. Discussion

The results presented above indicate that there are few differences between kangaroo and pig glyceraldehyde-3-phosphate dehydrogenases.

Firstly, a comparison of the amino acid compositions of the two enzymes (Table 2) indicates that there is no significant difference between the content of any amino acid residue, with the possible exception of methionine. It must be stressed that this comparison reflects the *minimum* difference between the two sequences since it is the *net* sum of any mutations that have occurred since the separate evolution of the two species. Thus the difference of 12% between the amino acid compositions of lobster and pig glyceraldehyde-3-phosphate dehydrogenases is in fact a manifestation of a difference of 28% in their amino acid sequences (Harris *et al.* 1970).

Table 5

Amino acid sequence around a reactive cysteine of kangaroo glyceraldehyde-3-phosphate dehydrogenase

The following symbols are used to indicate the method of sequence determination: —— amino acid analysis; —→ dansyl and dansyl–Edman; ←— carboxypeptidase A plus B

T_1 :	Ile	Val	Ser	Asn	Ala	Ser	Cys	Thr	Thr	Asn	Cys	Leu	Ala	Pro	Leu	Ala	Lys
	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	↔	←
T_2 :	Ile	Val	Ser	Asp	Ala	Ser	Cys	Thr	Thr	Asp	Cys	Leu	Ala	Pro	Leu	Ala	Lys
	→	→	—	—	—	—	—	—	—	—	—	—	—	—	—	←	←
	1				5					10					15		

Secondly, a comparison of tryptic fingerprints indicates a difference of not more than 20% between the two enzymes. In contrast to the first comparison this gives a *maximum* value for the difference between the two enzymes because of the high incidence of mutation of basic amino acid residues in glyceraldehyde-3-phosphate dehydrogenase (Harris *et al.* 1970). A single mutation of a basic to a non-basic residue will markedly alter the properties of two tryptic peptides. Consequently, only about 20% of peptides appear identical in tryptic fingerprints of pig and lobster glyceraldehyde-3-phosphate dehydrogenases, although their amino acid sequences show 72% identity.

Thirdly, the amino acid sequence around the reactive cysteine of kangaroo glyceraldehyde-3-phosphate dehydrogenase was found to be identical with that of the pig enzyme (see Table 6). Because of the relatively invariant nature of the amino acid sequence in this part of the molecule over a wide range of species, this result does not, in itself, provide convincing evidence for the similarity of the complete molecule. However, in the course of purifying peptides T1 and T2 a number of other peptides were purified and their amino acid sequences determined (Table 7). The sequence of amino acids from residue 246 to residue 257 is of particular interest since this is a region of the protein where there is an unusually large number of differences between the pig and lobster enzymes. Nevertheless, the amino acid sequences of the pig and kangaroo enzymes in this region are identical. Thus, 11% of the total amino acid sequence of kangaroo glyceraldehyde-3-phosphate dehydro-

genase has been determined and found to be identical with the corresponding area of the pig enzyme.

TABLE 6

COMPARISON OF AMINO ACID SEQUENCES AROUND THE REACTIVE CYSTEINE OF VARIOUS GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES

Source	Sequence
	*
Various†	..Ile.Val.Ser.Asn.Ala.Ser.Cys.Thr.Thr.Asn.Cys.Leu.Ala.Pro.Leu.Ala.Lys
Man‡	.....Ile.....
Halibut‡	..Val.....
Lobster‡	Asp.Met.Thr.Val.....Val.....
Blue crab‡	..Val.....Val.....

* Reactive cysteine residue.

† Red kangaroo; pig, yeast (both Perham and Harris 1963); rabbit (Harris, Meriwether, and Park 1963); chicken, honey bee, ostrich, sturgeon (all Allison 1968).

‡ Allison (1968).

The last common ancestor of the pig and kangaroo existed some 135 million years ago (Romer 1962) so that the large extent of similarity of the enzyme from these two species is a further indication of its slow rate of evolution. By comparing the amino acid sequences of pig and lobster glyceraldehyde-3-phosphate dehydro-

TABLE 7

AMINO ACID SEQUENCES OF SOME TRYPTIC PEPTIDES FROM KANGAROO, LOBSTER, AND PIG GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES

The residue numbers are those of the pig enzyme

Lobster*:	Leu.	Gly	.Lys.	Glu	.Cys.	Ser	.Tyr.	Asp.	Asp.	Ile.	Lys.	Ala	.		
Pig† and kangaroo‡:	Leu.	Glu	.Lys.	Pro	.Ala.	Lys	.Tyr.	Asp.	Asp.	Ile.	Lys.	Lys	.		
	246			250								257			

Lobster, pig: Val.Ile.Pro.Glu.Leu.Asp.Gly.Lys.

Kangaroo: Val.Ile.Pro.Glu.Leu.Asp.Gly.Lys.
217 224

* Davidson *et al.* (1967).

† Harris and Perham (1968).

‡ E. H. Blackburn and M. J. Gething, unpublished data.

genases, Dayhoff (1969) has calculated that the number of accepted point mutations per 100 amino acid residues per 10^6 years is 2. The values of the corresponding parameter for the cytochromes *c* and the β-chain of haemoglobin are 3 and 13 respectively. Thus, glyceraldehyde-3-phosphate dehydrogenase is mutating more slowly

than either of these proteins and only histones (0·6 accepted point mutation per 100 residues per 10^6 years) appear to mutate at a slower rate. The significance of this is uncertain because of the absence of sufficient information about the rate of evolution of other enzymes. It may indicate, however, that glyceraldehyde-3-phosphate dehydrogenase evolved a structure quite suitable for its function before the separate evolution of the various species that have so far been examined.

V. Acknowledgments

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