A COMPARISON OF THE GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE FROM OX MUSCLE AND LIVER

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1. Introduction

Several enzymes from mammalian brain, liver, heart and skeletal muscle exist in multiple molecular forms [1]. For example, one glycolytic enzyme, fructose diphosphate aldolase [EC 4.1.2.13] has been found to occur as distinct forms in the muscle, liver and brain of rabbit [2]. The muscle and liver forms have been shown by means of amino acid sequence analysis to be highly homologous, the products of divergent evolution from a common ancestor [3, 4]. The structural differences are accompanied by differences in enzymic specificity, which in turn reflect the differences in metabolism of the two tissues [3, 5].

It has been reported that the enzyme that follows aldolase in the glycolytic pathway, glyceraldehyde 3-phosphate dehydrogenase [EC 1.2.1.12]*, showed several structural variations when the enzymes from ox liver and muscle were compared [6]. Thus the liver enzyme was reported to contain only 2 cysteine residues per subunit, like yeast GAPDH [7, 8], as opposed to four cysteine residues per subunit in ox and pig muscle GAPDH [9]. A study of liver and muscle GAPDH from rabbit indicated that the enzymes exhibited quantitative differences in their catalytic properties apparently suited to the metabolic requirements of each tissue [10]. However, structural differences could not be identified, in conflict with the evidence obtained with ox GAPDH.

* *Abbreviations*: GAPDH: glyceraldehyde 3-phosphate dehydrogenase; SDS: sodium dodecyl sulphate; Trien: triethylenetetramine.

In this paper a simple method of purifying GAPDH from ox liver and muscle is described. The isolated enzymes have been compared using a variety of protein chemical techniques but no structural differences between the ox liver and muscle enzymes were detected. In particular, the thiol groups of the two enzymes are identical in number and there is no reason to believe that the two enzymes differ in structure.

2. Materials and methods

GAPDH activity was assayed at 30°C by a modification of the method of Allison and Kaplan [11] using 6.7 mM fructose diphosphate (tetra sodium salt) and 2.08 μM aldolase (Boehringer, rabbit muscle, mol. wt. 160000) in place of D-glyceraldehyde 3-phosphate. Protein concentration was estimated by the absorbance at 280 nm [12]. Electrophoresis of proteins was carried out in polyacrylamide gels in the presence of SDS [13] or urea [14], and on cellulose acetate strips in barbital buffer (ionic strength 0.075 mol/l), pH 8.6, containing 0.07% (v/v) 2-mercaptoethanol at 250 V for 90 min using a Beckman R-101 Microzone apparatus.

Crystalline GAPDH purified from ox liver or muscle was *S*-carboxymethylated with iodo-[2-^{14}C]acetic acid in 8 M-urea [14]. Amino acid analysis of the protein was carried out as described previously [15], and the N-terminal residue of the protein was identified by the dansyl technique [16]. Samples of *S*-carboxymethylated

protein were digested with trypsin and chymotrypsin under standard conditions [14], and with thermolysin (Calbiochem, containing 13% calcium) in 0.5% NH_4HCO_3 at 55°C for 5 hr. Peptide maps and autoradiographs were prepared by the methods of Harris and Perham [17] and tryptophan-containing peptides were made visible by the Ehrlich stain [18]. The tryptophan content of the protein was estimated by amino acid analysis after hydrolysis of the protein at 105°C for 48 hr with 4 M methane sulphonic acid containing tryptamine [19].

3. Results

GAPDH was prepared from 1.7 kg of fresh ox liver which was homogenized in 4 litres of 10 mM EDTA, 20 mM 2-mercaptoethanol, pH 7.0 at 0°C, and cell debris removed by centrifugation at 23000 *g*. All subsequent steps were carried out at 4°C. The protein that precipitated between 65% and 100% saturation with ammonium sulphate was dissolved in 184 ml of 5 mM Tris, 5 mM trien 2 HCl, 20 mM 2-mercaptoethanol buffer, pH 8.6, dialysed against the same buffer and then applied to a column of DEAE-cellulose (15 cm × 6.7 cm). Elution was carried out with the same buffer and fractions containing GAPDH activity, which was not retained by the column, were pooled. This protein, after concentration by ultrafiltration, and dialysis into 20 mM sodium phosphate buffer, pH 6.3, containing 2 mM EDTA and 20 mM 2-mercaptoethanol, was applied to a column of cellulose phosphate (11.7 cm × 6.7 cm) equilibrated with the same buffer. After washing with the buffer until no further protein appeared in the eluate, the column was developed with a linear gradient (total volume 5 litres) consisting of a 10-fold increase in sodium phosphate concentration in the buffer. The fractions containing GAPDH activity were pooled, concentrated by ultrafiltration, and the protein was then applied (in two batches) to a column of Sephadex G-150 (136 xm × 3 cm) in 5 mM Tris, 10 mM EDTA, 20 mM 2-mercaptoethanol, pH 7.5. The material of highest specific activity, and showing no contaminating protein bands on SDS-gel electrophoresis, was pooled and stored at 4°C in 70% saturated ammonium sulphate. The purification is summarized in table 1. Ox muscle GAPDH was prepared in a similar fashion, except that the cellulose-phosphate step was unnecessary.

Table 1
Summary of GAPDH preparation from 1.7 kg of ox liver

Step	Total activity[b] (Units)	Specific activity (Units per mg protein)
Extraction	55 480	0.19
65–100% $(NH_4)_2SO_4$	48 576	2.9
DEAE-cellulose step	43 934	23.1
Cellulose phosphate step	37 000	52.6
Sephadex G-150[a]	14 000	72.0

[a] Only material of highest specific activity and showing no contaminating bands on SDS-gel electrophoresis, was pooled.
[b] Units of activity = μmole NAD^+ reduced per minute.

SDS-gel electrophoresis of ox liver and muscle GAPDH showed one band of molecular weight 36 000, and N-terminal analysis of both proteins gave valine as the only N-terminal amino acid. The enzyme from both tissues also behaved identically during electrophoresis on polyacrylamide gels in the presence and absence of urea and on cellulose acetate strips. The urea-acrylamide gels showed several narrowly separated protein bands which are probably attributable to varying degrees of amine loss from susceptible amide residues in the protein: both enzymes showed the same pattern and were not resolved when mixed before electrophoresis.

The results of the amino acid analysis are listed in table 2. The compositions of the enzyme from ox liver and muscle can be seen to be identical within the limits of experimental error although the existence of one or two amino acid replacements cannot be totally excluded. Radioactivity in the acid hydrolysates was found to be confined to *S*-carboxymethylcysteine.

Tryptic peptide maps of the *S*-carboxymethylated ox liver and muscle enzymes appeared to be identical, and autoradiography of the maps to determine the positions of [^{14}C]*S*-carboxymethylcysteine-containing peptides gave the characteristic pattern of three labelled peptides, one

Table 2
Amino acid composition of GAPDH from ox liver and muscle[a]

Amino acid	Residues per subunit mol. wt. 36 000		
	Ox muscle GAPDH	Ox liver GAPDH	Pig Muscle GAPDH[9]
LYS	27.3	27.0	26
HIS	11.1	11.1	11
ARG	11.2	11.4	10
CYS[b]	4.0	4.1	4
ASP	39.3	38.8	38
THR[c]	22.0	21.9	22
SER[c]	18.5	18.3	19
GLU	21.4	21.5	18
PRO	11.6	11.7	12
GLY	33.4	33.1	32
ALA	32.5	32.7	32
VAL[c]	34.2	34.8	34
MET	8.8	8.9	9
ILE[c]	21.2	21.1	21
LEU	19.1	19.3	18
TYR	9.3	9.1	9
PHE	14.2	14.3	14
TRP[d]	2.0	2.3	3

[a] All analysis figures are the mean of six hydrolysates.
[b] Cysteine was determined as *S*-carboxymethylcysteine.
[c] Serine and threonine values are corrected for destruction of these residues, and valine and isoleucine values are corrected for slow release of these residues during acid hydrolysis.
[d] Tryptophan was estimated from duplicate analysis of the protein hydrolysed with methane sulphonic acid.

of which appeared to contain twice the radioactivity of each of the other two peptides, previously found for pig muscle GAPDH (see ref. [17]). The *S*-carboxymethylated ox liver and muscle enzymes were also subjected to peptide mapping after tryptic followed by chymotryptic digestion and tryptic followed by thermolytic digestion, and again they proved indistinguishable. Immediately after the latter maps had been developed with ninhydrin, which showed that there was no staining material at the origin, they were further stained for tryptophan. In both the liver and muscle GAPDH maps, three tryptophan-containing peptides were detected. Analysis of duplicate protein samples hydrolysed with methane sulphonic acid gave average values for their tryptophan content per subunit of mol. wt 36 000 of 2.0 residues for the muscle GAPDH and 2.3 residues for the liver GAPDH.

4. Discussion

There is no difference in structure detectable between GAPDH from ox liver and muscle by the standard techniques of protein chemistry short of complete sequence analysis of both proteins. In particular, there are clearly four cysteine residues in each subunit of the liver enzyme and not two as previously reported [6]. Replacements among the neutral amino acids are possible, but the likelihood of such differences is small in view of the results of peptide mapping using tryptic–chymotryptic digests, and tryptic–thermolytic digests of the proteins. The question of whether there are quantitative kinetic differences between the ox liver and muscle enzymes remains to be evaluated. Such differences have been reported for rabbit GAPDH [10], but it may be that these differences are simply an artefact of the isolation procedure.

The amino acid sequences of GAPDH isolated from skeletal muscle of a wide variety of mammals [9, 20], from lobster muscle [21] and from yeast [7, 8] are highly homologous. This together with the apparent identity of the GAPDH from ox muscle and liver, suggests that all GAPDHs have arisen by slow divergent evolution from a common ancestor. In contrast, the two classes of fructose diphosphate aldolases [22] function by clearly different mechanisms and it is probable that there is no homology between the two classes [22, 23]. Even within class 1, to which class belong the isoenzymic forms of aldolase found in various mammalian tissues [3], it is possible that the newly discovered bacterial form [24] is a consequence of a convergent rather than a divergent pattern of evolution.

On the basis of the structural analysis of the enzymes, it therefore seems reasonable to propose that in mammals the multiple forms of aldolase are the products of separate but homologous structural genes that have arisen by a process of gene duplication and evolution whereas GAPDH is coded for by a single structural gene, at least in liver and muscle. This proposal is difficult to reconcile with any theory of biosynthesis of glycolytic enzymes that places the structural genes

of GAPDH and aldolase in the same operon [25], in keeping with the results of other experiments [26]. Several other glycolytic enzymes do have isoenzymic forms in liver and muscle [1, 27] and it may well be that GAPDH is unusual in this respect. Further structural and kinetic analysis of glycolytic enzymes isolated from various tissues should throw more light on the evolution of glycolytic enzymes and the control of the pathway.

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