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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND PARTIAL STRUCTURAL CHARACTERIZATION OF CHLOROPLAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE SUBUNITS

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SUMMARY

Chloroplast glyceraldehyde-3-phosphate dehydrogenase (mol. wt. 600 kD) is composed of 80-kD protomers each made up of two subunits (37 and 43 kD). These subunits have been isolated, from S-alkylated protein, in a homogeneous form and in suitable quantities for carrying out structural studies by reversed-phase high-performance liquid chromatography (RP-HPLC) on a large pore size (300 Å) C_{18}-bonded phase. Treatment with dissociating agents necessary to solubilize the S-carboxymethyl enzyme prior to injection, affects the chromatographic behaviour of the enzyme. Urea or guanidine hydrochloride are inadequate for complete dissociation of the protein, which is only partially eluted as distinct subunits, whilst treatment with sodium dodecyl sulphate allows all the injected material to be obtained as separate subunits. Other RP-HPLC supports have been tested: a small pore (100 Å) C_{18}-bonded phase is also suitable for resolution of the subunits. On the contrary, no separation can be achieved using a C_3 alkyl support. When chromatographed with gel permeation HPLC using denaturing media, the subunits were coeluted because of the small differences in their molecular weights.

INTRODUCTION

Structural studies of heteropolymeric proteins may be carried out only after preparative isolation of distinct subunits: conventional separation techniques may often be inadequate, particularly if the monomers are similar in size or in primary structure.

High-performance liquid chromatography (HPLC), which is one of the most versatile fractionation methods for small peptides, has also been successfully employed for the separation of large fragments, *i.e.* membrane-associated polypeptides and proteins[1–7]. The use of reversed-phase HPLC (RP-HPLC) for preparative isolation of proteins, particularly large and closely related proteins, is still limited, although studies regarding the optimization of stationary phases and chromatographic conditions used with these molecules have been accomplished recently[8–10].

This report examines the behaviour of chloroplast glyceraldehyde-3-phosphate

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dehydrogenase (GAPDH, E.C. 1.2.1.13) on gel permeation HPLC (GP-HPLC) and on RP-HPLC supports. GAPDH from spinach leaf chloroplasts is a multimeric protein of 600 kD[11,12] which is thought to result from the association of 80-kD protomers, each made up of two distinct subunits (37 and 43 kD, respectively), in an apparent equimolar ratio[12–15]. Owing to their similarity in molecular weight and, possibly, in primary structure, as previously suggested by indirect evidence[15], preparative isolation of these monomers has proved to be difficult. In addition to this, the presence of hydrophobic interactions, which keep them tightly associated, could explain the failure to achieve their separation using conventional chromatographic techniques: thus, GAPDH subunits have so far only been separated by analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)[12–15].

The first preparative separation of GAPDH subunits was achieved in this present study by a simple RP-HPLC method, which allowed us to obtain each subunit in a suitable quantity for a partial characterization of its structure.

EXPERIMENTAL

High-performance liquid chromatography system

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph was used which included two M 6000 solvent delivery units, a M 680 automated solvent programmer and a U6K sample injector coupled to a Jasco (Japan Spectroscopic, Tokyo, Japan) variable-wavelength detector. Absorbance was recorded with a Houston chart recorder.

The two TSK 4000-SW columns (each 30 cm × 7.5 mm I.D.) used for GP-HPLC were obtained from Altex (Berkeley, CA, U.S.A.); for RP-HPLC the following columns were used: a C_3 column (7.5 cm × 4.6 mm I.D., particle size 10 μm, 300 Å pore size) from Altex, a μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm, 100 Å pore size) from Waters Assoc., a Vydac C_{18} column (25 cm × 4.6 mm I.D., 10 μm, 300 Å pore size) from Separation Group (Hesperia, CA, U.S.A.), an Aquapore RP-300 column (25 cm × 7 mm I.D., 10 μm) from Brownlee Labs. (Santa Clara, CA, U.S.A.) and an Ultrasphere ODS column (25 cm × 4.5 mm I.D., 5 μm) from Beckman (Berkeley, CA, U.S.A.).

Reagents

All reagents were of analytical grade. The acetonitrile supplied by Merck (Darmstadt, F.R.G.) was further filtered through a 0.45-μm Millipore filter (Milford, MA, U.S.A.) and degassed under vacuum prior to use; water was glass-distilled, filtered through a LiChroprep RP-8 column (Merck) and finally degassed. Sequenal grade trifluoroacetic acid (TFA) was obtained from LKB (Bromma, Sweden) and iodoacetic acid was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Carboxypeptidase Y and tosylamino-2-phenylethylchloromethyl ketone (TPCK)-treated trypsin were obtained from Worthington Biochemical Co. All other reagents were from Carlo Erba (Milan, Italy).

Sample preparation

The enzyme was purified from fresh spinach leaves and completely carboxymethylated with $[^{14}C_2]ICH_2COOH$ as previously reported[12].

Unless otherwise stated the protein, at a concentration of 2 mg/ml, was heated to 100°C for 5 min in aqueous solution containing 2% sodium dodecyl sulphate (SDS) and 1% 2-mercaptoethanol and then dialysed for 24 h against the same solution prior to injection. SDS electrophoresis was performed on polyacrylamide gel slabs as previously reported[16].

Carboxypeptidase Y digestion was carried out at room temperature using a protein to substrate ratio of 1:100 (w/w), in 0.1 *M* sodium acetate buffer (pH 5.4) as described by Hayashi[17]. Samples were taken after 30, 60, 120 min and 18 h, respectively, and analysed for free amino acids. Digestion with trypsin was according to Swenson *et al.*[18].

Amino acid and sequence analyses

Amino acid analyses were carried out by ion-exchange chromatography with post-column ninhydrin derivatization according to Moore[19] on a Kontron Cromakon 500 automatic analyser. The amino acid sequence of the whole protein as well as that of the two monomers were determined by liquid-phase sequence degradation in a Beckman System 890 M instrument using the fast-protein Quadrol program with precycled Polybrene. Amino acid derivatives were identified by RP-HPLC on a Beckman Ultrasphere ODS column equilibrated with 74% sodium acetate (pH 5.1) (solvent A) and 26% acetonitrile (solvent B). A linear gradient was run at 40°C and at 1.5 ml/min from 26 to 90% solvent B over a period of 14 min.

RESULTS AND DISCUSSION

Chloroplast GAPDH possesses 14 cysteinyl residues per protomer: to prevent the possible formation of disulphide bonds during chromatographic procedures, which obviously would affect the separation of its subunits, the native enzyme was completely S-alkylated by treatment with $[^{14}C_2]ICH_2COOH$ before HPLC analysis was carried out. As a consequence of this treatment, the protein was insoluble in aqueous solvent and could be redissolved only by the addition of dissociating agents.

Separation of GAPDH subunits has been tentatively approched by size-exclusion HPLC, using two TSK 4000 SW columns connected in series. Samples of $[^{14}C_2]$S-carboxymethylated enzyme, dissolved in 2% SDS buffered solutions, were injected on this chromatographic system and eluted with Tris or phosphate buffers containing different amounts (0.1–1%) of SDS. Despite increasing reports of successful GP-HPLC separation of proteins[20–24], this procedure appeared inadequate to resolve GAPDH monomers, probably because of the small difference in their molecular weights. The enzyme was then submitted to RP-HPLC on a Vydac C_{18} (300 Å pore size) column. This support proved to be effective in separating GAPDH monomers, however, the behaviour of the injected sample is strongly affected by the dissociating agent used to achieve its solubilization.

As shown in Fig. 1, three peaks are obtained when 4 *M* urea was employed: the identification of the eluted material, checked by polyacrylamide gel electrophoresis (inset of Fig. 1), showed that a homogeneous 37-kD subunit is eluted in the first peak, while the second peak contained the 43-kD subunit slightly contamined by the smaller of the two; both the subunits are recovered in a similar amount in the third peak. A substantially identical pattern can be obtained using 6 *M* guanidine hydro-

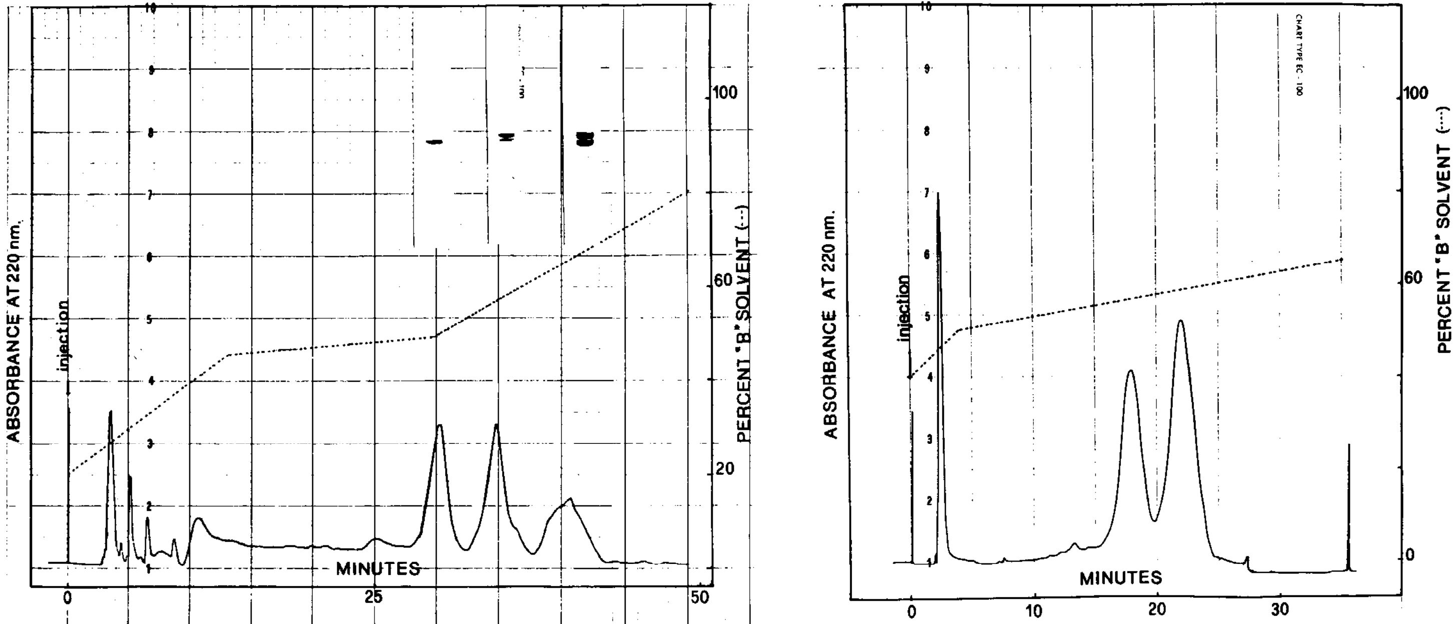

Fig. 1. Elution pattern of urea-treated S-carboxymethylated GAPDH. The protein (2 mg/ml) was treated with 4 *M* urea for 4 h at room temperature. Samples of 100 μl (corresponding to 200 μg of protein), after addition of TFA (final pH 2.3), were injected onto a Vydac C_{18} column (25 cm × 4.6 mm I.D., 300 Å pore size). Solvent A was 0.05% aqueous TFA; solvent B was acetonitrile–2-propanol (2:1, v/v) containing 0.05% TFA. The elution was performed at room temperature and at a flow-rate of 1 ml/min, using the gradient indicated by the dashed line. Absorbance range: 0.64 a.u.f.s. Inset of Fig. 1: SDS-PAGE of the eluted samples.

Fig. 2. Elution pattern of SDS-treated S-carboxymethylated GAPDH. The protein (2 mg/ml) was treated as reported under Experimental. Samples of 100 μl (corresponding to 200 μg of protein) were injected onto a Vydac C_{18} column. Solvent A: 0.05% aqueous TFA; solvent B: acetonitrile–2-propanol (2:1, v/v) containing 0.05% TFA. The elution was performed at room temperature and at a flow-rate of 1.5 ml/min, using the gradient indicated by the dashed line. Absorbance range: 0.64 a.u.f.s.

chloride as dissociating agent. On the contrary, SDS solubilization allowed us to obtain complete separation of the starting sample into two peaks (Fig. 2), each containing one of the two subunits in a homogeneous form (Fig. 3). It is of interest to note that in both cases the injected material was recovered in good yield from the column. Determination of radioactivity showed that each peak in Fig. 1 contained a similar amount of material, and the sum of the three accounted for 75% of the starting sample. Each of the separated subunits was recovered in a good and comparable yield (more than 75%) under the conditions used in Fig. 2. These findings, which excluded the possibility of irreversible interactions with reversed-phase supports, indicated that a water-insoluble sample such as carboxymethylated GAPDH, once solubilized in the presence of dissociating agents, can be eluted by common RP-HPLC solvents.

Separation is not substantially affected by changing the gradient slope, eluent composition (using heptafluorobutyric acid instead of TFA as ion-pairing agent) or

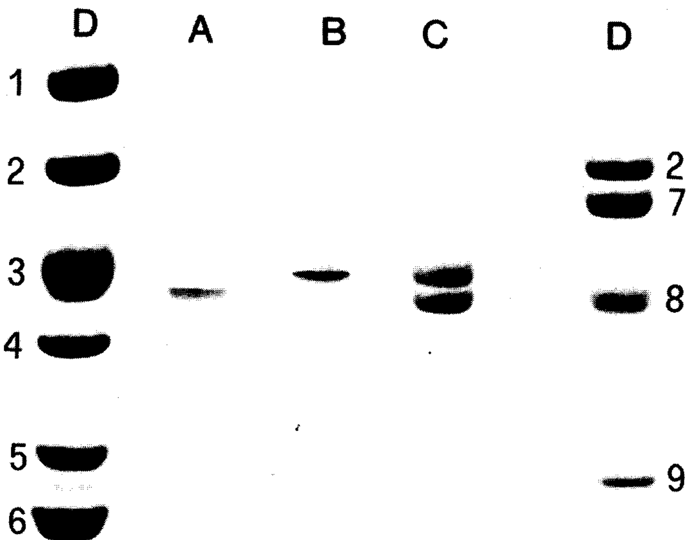

Fig. 3. SDS-polyacrylamide gradient gel electrophoresis of S-carboxymethylated GAPDH and its subunits. (A) Sample from the first peak of Fig. 2, (B) sample from the second peak of Fig. 2, (C) S-carboxymethylated GAPDH, (D) standard proteins: 1 = phosphorylase B (mol.wt. 94 kD); 2 = bovine serum albumin (mol.wt. 67 kD); 3 = ovoalbumin (mol.wt. 43 kD); 4 = carbonic anhydrase (mol.wt. 30 kD); 5 = soybean trypsin inhibitor (mol.wt. 20.1 kD); 6 = α-lactalbumin (mol.wt. 14.4 kD); 7 = catalase (mol.wt. 60 kD); 8 = lactate dehydrogenase (mol.wt. 36 kD); 9 = ferritin (mol.wt. 18.5 kD).

by varying the quantity of the loaded sample (50–200 μg). The failure to completely separate GAPDH subunits after solubilization by urea or guanidine may be due to incomplete dissociation of the sample and/or reaggregation process which occurs during the chromatographic separation as a consequence of removal of the dissociating agent. On the contrary, treatment with SDS, by breaking interchain hydrophobic bonds, causes an irreversible dissociation of the subunits, thus promoting their complete separation. We also examined the chromatographic pattern on a small-pore C_{18} alkyl-bonded support (Fig. 4). Although an adequate separation of the subunits can be obtained from a μBondapak C_{18} 100-Å pore size column, the Vydac C_{18} column, as expected for a large-pore support[25–27], provided a better resolution.

Comparison of the elution profiles in Figs. 2 and 4 shows that the subunits are eluted at the same concentration of solvent B; recoveries from both supports are similar: each of the separated subunits is obtained in a good and comparable yield also from the μBondapak C_{18} column. Several organic modifiers have been tested as solvent B: the best results were obtained with a mixture of acetonitrile–2-propanol in a ratio of 2:1 as suggested by Tarr and Crabb[28].

Unlike the C_{18} support, the C_3 alkyl-bonded support proved to be ineffective in separating GAPDH subunits. As reported in Fig. 5, the enzyme was eluted in a single peak from a large-pore size (300 Å) C_3 alkyl-bonded chain: the identical behaviour of the two subunits on this support is proved by electrophoretic analysis of the eluted material (inset of Fig. 5), showing that the subunits are recovered in the same apparent equimolar ratio as in the starting sample. Several attempts to resolve

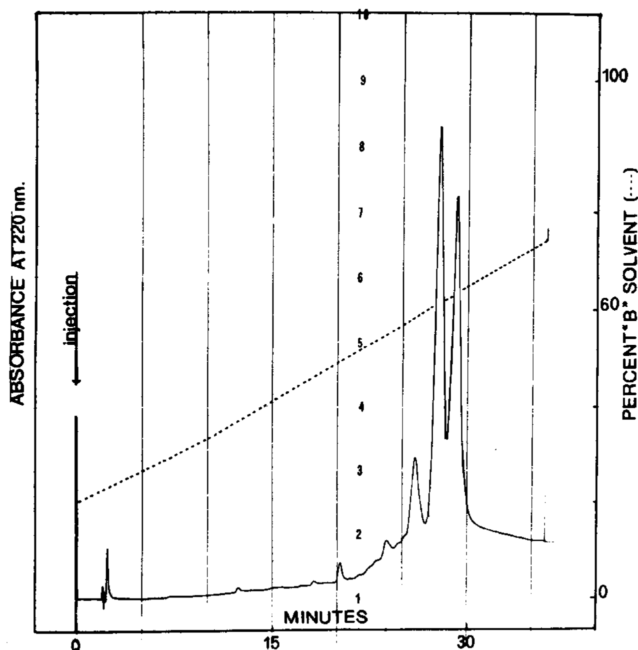

Fig. 4. Elution pattern of S-carboxymethylated GAPDH from a μBondapak C_{18} column (25 cm × 4.6 mm I.D., 100 Å pore size). Experimental conditions as in Fig. 2.

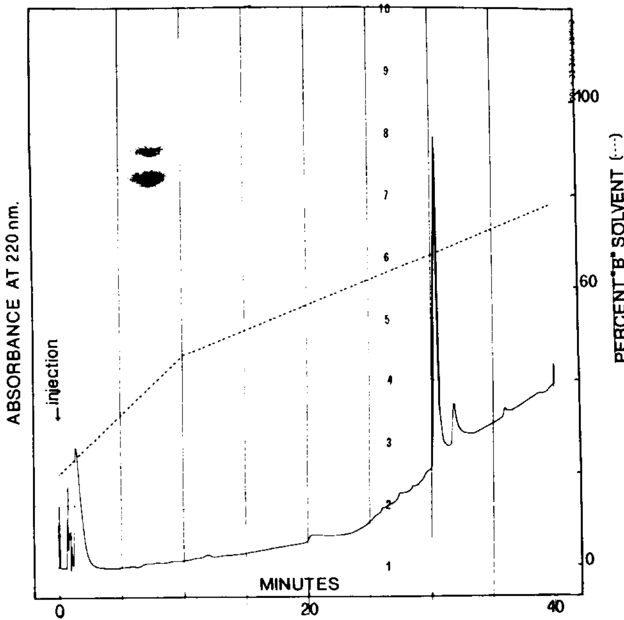

Fig. 5. Elution pattern of S-carboxymethylated GAPDH from an Altex C_3 column (7.5 cm × 4.6 mm I.D., 300 Å pore size). Experimental conditions as in Fig. 2. Inset of Fig. 5: SDS-PAGE of the eluted sample.

them by changing the flow-rate, eluent composition, gradient slope, or by decreasing the amount of the loaded sample were unsuccessful. Because of the well-known successful use of short-chain bonded phases for separating large hydrophobic proteins[8,9,29,30], such results were unexpected. The lack of resolution could be considered a weakness of the C_3 bonded phase with respect to C_{18} supports to establish selective hydrophobic interaction with each of the two subunits. On removal of the SDS coating, interchain interactions appear to be favoured rather than protein-reversed-phase hydrophobic interactions.

As a result of separation of the GAPDH subunits by RP-HPLC, investigation of their structural relationship could be directly approached. Micropreparative purification, carried out by repetitive RP-HPLC on a Vydac C_{18} column under the conditions of Fig. 2, provided a sufficient quantity of GAPDH subunits for some of the initial structural characterization.

The data reported in Table I indicates that GAPDH subunits have a very similar amino acid composition, which is in agreement with that of the whole protein. In addition to this, the total number of peptides isolated by RP-HPLC from a tryptic digest of the whole enzyme (Fig. 6) strongly indicates that the amino acid sequence of the two subunits may be, in a large part, identical. This conclusion is further supported by comparing the chromatographic patterns shown in Figs. 6 and 7. The elution profiles of tryptic peptides from the whole enzyme (Fig. 6) and its smaller subunit (Fig. 7) are similar, although there are certain additional peaks eluting at 20 and 57 min, and between 30 and 40 min in the former, which are not present in the profile of the 37-kD subunit digest.

TABLE I

AMINO ACID COMPOSITION OF THE SUBUNITS ISOLATED FROM SPINACH CHLOROPLAST GAPDH

Values are the mean of five independent determinations.

Amino acid	*Subunits*		*Protomer 80 kD*[12]
	37 kD	*43 kD*	
Lysine	21.6	27.8	50
Histidine	7.1	7.2	14
Arginine	14.9	17.0	31
Cysteine*	5.8	6.5	14
Aspartic acid	48.8	56.6	107
Threonine	22.4	23.9	47
Serine	24.7	26.3	51
Glutamic acid	20.6	21.9	41
Proline	12.6	19.8	33
Glycine	31.9	37.2	69
Alanine	31.5	34.3	66
Valine	36.7	39.2	76
Methionine	4.7	5.9	10
Isoleucine	19.3	20.1	38
Leucine	28.6	33.4	62
Tyrosine	5.8	7.7	14
Phenylalanine	9.7	10.6	21
Tryptophan	n.d.**	n.d.	8

* Determined as S-carboxymethylcysteine.

** Not detected.

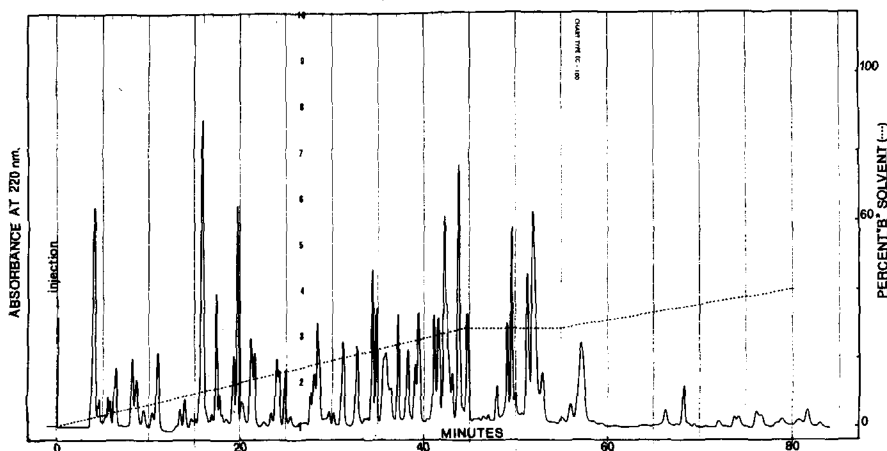

Fig. 6. Elution pattern of S-carboxymethylated GAPDH tryptic digest. After tryptic digestion, TFA was added to the solution (final pH 2.3) and 200 μl (corresponding to about 20 nmol) were injected onto a Aquapore RP-300 column (25 cm × 7 mm I.D.). Solvent A was 0.05% TFA; solvent B was acetonitrile containing 0.05% TFA. The elution was performed at room temperature and at a flow-rate of 2 ml/min, using the gradient indicated by the dashed line. Absorbance range: 1.28 a.u.f.s.

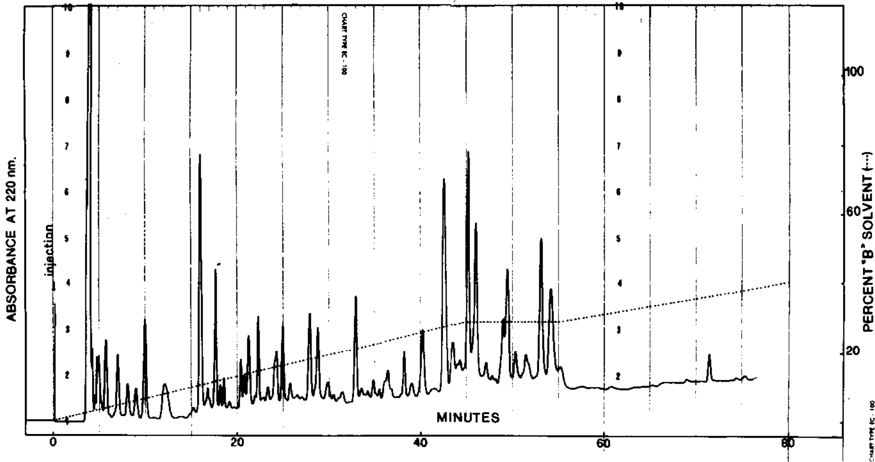

Fig. 7. Elution pattern of a 37-kD subunit tryptic digest. Experimental conditions as in Fig. 6.

It seems likely that the additional peptides come from the 43-kD subunit, particularly from that portion of the primary sequence not present in the 37-kD subunit. In order to localize this different region, N- and C-terminal portions of the sequence have been examined by sequential degradation and carboxypeptidase digestion. N-terminal sequential analysis of the first seven residues carried out on the whole protein gives, at each step, a unique aminoacyl residue: the data obtained, which have been also confirmed by separately sequencing each of the subunits, are the following: Lys-Leu-Lys-Val-Ala-Ile-$AspNH_2$ .....

Determination of the C-terminal residue gives the results reported in Table II, indicating that two different amino acids, alanine and valine, are present at the C-terminal end of the GAPDH protomer: the possibility of attributing each of these to individual chains is prevented by the fact that the isolated subunits are insoluble in aqueous solvents.

Results obtained on GAPDH from *Hordeum vulgaris* and *Sinapis alba* lead Cerff and Chambers[15] to postulate that the structural differences between the sub-

TABLE II

RELEASED AMINO ACIDS AFTER CARBOXYPEPTIDASE Y DIGESTION OF NATIVE GADPH

A sample of 8 mg (100 nmol of protomer, assuming a mol.wt. of 80 kD) was submitted to carboxypeptidase Y digestion in 1 ml of 0.1 *M* sodium acetate buffer (pH 5.4). Aliquots of 0.2 ml were taken at the indicated times and used for amino acid analysis. The listed values represent mol amino acid/mol protomer.

Amino acid	*Incubation time*			
	30 min	*60 min*	*2 h*	*18 h*
Aspartic acid	+	0.07	0.10	0.25
Threonine	–	+	0.06	0.11
Serine	+	0.05	0.20	0.22
Alanine	0.25	0.34	0.42	0.49
Valine	0.18	0.30	0.40	0.47
Leucine	0.07	0.15	0.22	0.33

units are restricted to a short terminal sequence exclusive to the major of the two. In addition to confirming this suggestion, our data strongly indicate that the two subunits differ in the C-terminal portion of the primary structure: but, that this may be the only variable region of the molecule, is still to be proved.

Preparative isolation of GAPDH subunits by RP-HPLC, thus making possible the complete characterization of their primary structure, would facilitate assessment of the role of each of these subunits, in the catalytic activity of the enzyme.

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