Short Communications

Molecular forms of chloroplast glyceraldehyde-3-P-dehydrogenase

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Summary. Different molecular forms of NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.13) have been purified from spinach leaves: the major form (GAPDH I) corresponding to the enzyme of 600,000 daltons molecular weight, and 2 minor forms (GAPDH II and III) with apparent molecular weights of 300,000 and 150,000 daltons respectively. The 3 forms result from a different arrangement of 2 distinct subunits (43,000 and 37,000 daltons). All the forms show an higher affinity for NADP(H) than for NAD(H).

Chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP-linked GAPDH, E.C. 1.2.1.13) has been isolated by several workers as a multimeric protein of mol.wt 600,000 daltons[1-4] which undergoes aggregational changes in the presence of pyridine coenzymes; NADP has been found to induce dissociation of the enzyme, while NAD counteracts this effect[3,5,6]. These properties have been exploited in a purification procedure[6,7] which permitted the isolation, from seedlings of higher plants, of 2 separate tetrameric forms[7-9] having the same mol.wt (approximately 170,000 daltons) but a different subunit composition (A_2B_2 and A_4 isoenzymes). These were postulated to be the native forms of chloroplast GAPDH, while the previously reported higher molecular weight form, which was still present even in the conditions used for isolation of A_2B_2 isoenzyme[8], was suggested to be an isolation artifact. Even more recently it has been shown that in crude extracts from spinach leaf chloroplasts, the only detectable GAPDH activity is associated with a protein of mol.wt 600,000 daltons[10].

In the present study 3 different molecular forms of NADP-dependent GAPDH have been obtained from spinach leaves by a single separation procedure; the relative amounts of each of them as well as their molecular properties strongly suggest that the 600,000-dalton enzyme is the most important form of chloroplast GAPDH.

Methods. The different molecular forms of GAPDH were obtained from 4.5 kg of fresh spinach leaves, using buffer with a physiological concentration of phosphate ions[11], essentially as previously reported[4]; after heat treatment, the $(NH_4)_2SO_4$ fraction at 45-65% saturation was collected, and chromatographed on DEAE cellulose. The active fractions were brought to 70% saturation in $(NH_4)_2SO_4$, and examined by gel filtration: Sephacryl S300 (3.4 × 120 cm column) instead of Biogel A5m was used. 3 peaks of GAPDH activity were obtained: a major one contained the 600,000-dalton enzyme (GAPDH I) as an homogeneous protein; the other 2 (GAPDH II and III) were separately purified by an additional gel filtration on Sephacryl S300 (2.5 × 90 cm column) followed by affinity chromatography on agarose-hexane NAD (1 × 2 cm column).

A 25 mM K phosphate/2.5 mM EDTA/1 mM β-mercaptoethanol/100 mM NaCl buffer, pH 7.5, was used through-

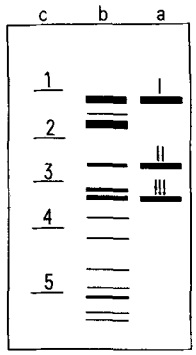

Figure 1. Electrophoretic pattern on polyacrylamide gradient gel of GAPDH at 45-65% $(NH_4)_2SO_4$ purification step. *a* 20 µl of sample containing 900 U/ml of GAPDH NADP activity stained for activity. *b* 10 µl of sample stained with Coomassie Blue. *c* Standard proteins: 1, thyroglobulin (669,000); 2, ferritin (440,000); 3, catalase (232,000); 4, lactate dehydrogenase (140,000); 5, bovine serum albumin (67,000).

Table 1. Kinetic properties and relative yield of chloroplast GAPDH activities

	GAPDH activity eluted from sephacryl S 300 (Fig. 2)					Purified forms		
	Total activity		Relative yield	Specific activity		Total protein	Specific activity	
	NADP	NAD	(100%)	NADP	NAD	(mg)	NADP	NAD
Peak I	9000	4600	71	94	48	96.0	94	48
Peak II	1050	843	8	22	17	0.4	84	65
Peak III	2500	2560	20	33	36	2.5	88	86

	Apparent K_m-values of the purified forms							
	Glyceraldehyde-3-phosphate				3-Phosphoglycerate			
	NADP (10^4)	NAD (10^4)	NADP (10^5)	NAD (10^4)	NADH (10^4)	NADPH (10^4)	NADH (10^5)	NADPH (10^4)
GAPDH I	2.3	9.4	4.1	2.0	2.0	2.6	3.9	1.7
GAPDH II	9.2	-	8.0	2.3	-	-	-	-
GAPDH III	3.8	2.0	3.7	1.9	2.7	4.4	3.0	6.2

Activity was determined at 25 °C in 30 mM pyrophosphate buffer, 5 mM EDTA, 6.6 mM Na arsenate, 1 mM NADP or NAD and 2 mM glyceraldehyde 3-phosphate, pH 8.5. For K_m determination the concentration of the single substrate was varied: NADP or NAD from 0.01 to 2 mM; glyceraldehyde-3-phosphate from 0.01 to 2.5 mM; NADPH or NADH from 0.01 to 0.4 mM, and 3-phosphoglycerate from 0.02 to 1 mM. Specific activity is referred as moles of reduced coenzyme · min^{-1} · mg of protein. The K_m-values are expressed in moles · l^{-1}.

out this step: 10 mM NAD was added to the same buffer to elute the enzyme from the affinity chromatography column. The use of NADP as affinity ligand was not successful, since other proteins, devoided of GAPDH activity, eluted with both forms. Protein determination, enzyme assay and aminoacid analysis were carried out as previously reported[4].

Molecular weights were determined both by gel filtration on Sephacryl S300, and by electrophoresis on polyacrylamide gradient gel slabs as previously described[10]. Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out on Pharmacia PAA4/30 gradient gel slabs in 40 mM TRIS, 20 mM Na acetate, 2 mM EDTA and 0.2% SDS pH 7.4, for 2.5 h at 150 V. Samples of protein (4–6 µg) were treated with 2.5% SDS, 5% β-mercaptoethanol, at 100 °C for 5 min.

Results and conclusions. Under our experimental conditions, the NADP-linked GAPDH activity of the crude extract was almost completely recovered (more than 95%) in the $(NH_4)_2SO_4$ fraction between 45 and 65% saturation. Our observation differs from previous results on seedlings[6,7]; this might be due to the different tissue or to the different media used in the extraction and purification procedures. Electrophoretic analysis carried out on the 45–65% $(NH_4)_2SO_4$ fraction showed that NADP-dependent GAPDH activity was associated with 3 protein bands differing in molecular weight (fig. 1). Three active peaks (I, II, III) were also resolved by Sephacryl S300 chromatography (fig. 2); these were significantly different in relative amount, specific activity and in NADP/NAD activity ratio (table 1). Peak I contained an homogeneous enzyme of mol.wt 600,000 daltons (GAPDH I), as judged by its elution volume on Sephacryl S300 and by electrophoretic behaviour (fig. 3, A and B). The activity eluted in peaks II and III (GAPDH II and III) moved as proteins of mol.wt 300,000 and 150,000 daltons, respectively when further purified on a Sephacryl S300 column calibrated with standard proteins (fig. 3, A). An additional affinity chromatography failed to give GAPDH II as a completely homogeneous species. The material so obtained showed, on overloaded gel electrophoresis, together with the main band of the enzyme (300,000 daltons ± 10%) a faster moving component, the amount of which can be estimated to be less than 10% (fig. 3, B). The spurious protein is devoided of GAPDH activity; in addition to this, its electrophoretic behavior seems to exclude any contamination of GAPDH II by the other forms of the enzyme. GAPDH III was purified to homogeneity (fig. 3, B); its mol.wt was confirmed to be 150,000 daltons ± 10% by electrophoresis. Both forms were obtained in a low yield (table 1). Unlike GAPDH I which is stable for several months at 4 °C, GAPDH II and III loose spontaneously activity; this instability is particularly marked in the former.

Kinetic parameters of the 3 separated forms of GAPDH are shown in table 1: all are active with both pyridine coen-

Table 2. Aminoacid composition of GAPDH III (homotetramer mol. wt 150,000 daltons) from spinach leaves and from white mustard. The data for the white mustard enzyme were taken from Cerff and Chamber 9

Aminoacid	Residues per monomer (37,000 daltons) spinach leaves	Residues (%) Spinach leaves	White mustard
Lysine	22	6.2	7.2
Histidine	7	2.0	1.7
Arginine	15	4.2	4.2
Cysteine[a]	7	2.0	3.0
Aspartic acid	50	14.0	13.7
Threonine[b]	23	6.5	5.4
Serine[b]	25	7.0	7.3
Glutamic acid	21	5.9	6.3
Proline	13	3.7	4.4
Glycine	32	9.0	9.1
Alanine	31	8.7	9.0
Valine[c]	41	11.5	8.0
Methionine	5	1.4	1.0
Isoleucine[c]	21	5.9	5.1
Leucine[c]	28	7.9	8.3
Tyrosine	6	1.7	1.7
Phenylalanine	9	2.5	3.1
Tryptophan	-	-	1.7
Total residues	356		

[a] Determined as cysteic acid. [b] Corrected for destruction during acid hydrolysis. [c] Corrected for slow release during acid hydrolysis.

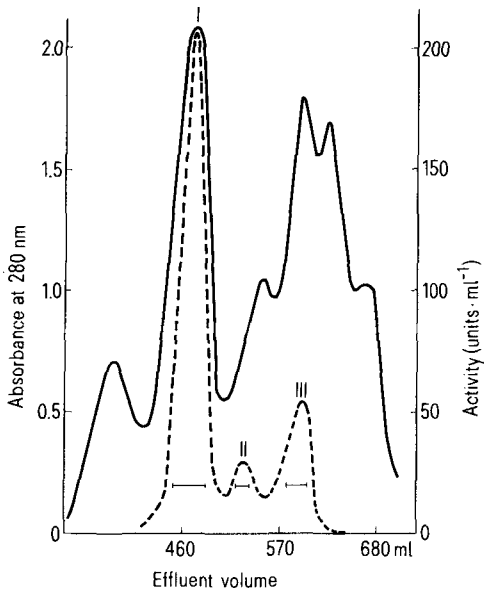

Figure 2. Elution profile of chloroplast GAPDH activities from Sephacryl S300 (3.4 × 120 cm column). Elution buffer: 25 mM K phosphate, 2.5 mM EDTA, 1 mM β-mercaptoethanol pH 7.5. Flow rate: 12 ml/h. Pooled fractions are indicated by solid bars. ——, Absorbance at 280 nm; – – –, NADP activity as µmoles of NADP reduced $min^{-1} \cdot ml$.

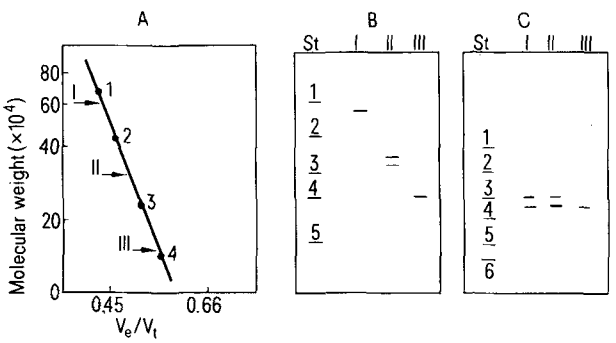

Figure 3. Molecular weight determination and subunit composition of the purified forms of chloroplast GAPDH. *A* Semilogarithmic plot of mol.wt against V_e/V_t as determined by gel filtration on Sephacryl S300 (2.5 × 90 cm column). Elution buffer: as in figure 2. Flow rate: 10 ml/h. Standard proteins: 1–4 as in figure 1. Arrows indicate V_e/V_t of GAPDH I, II and III. *B* Electrophoretic pattern on polyacrylamide gradient gel electrophoresis of GAPDH I, II and III. Standard proteins: as in figure 1. *C* Electrophoretic pattern on SDS polyacrylamide gradient gel electrophoresis of GAPDH I, II and III. Standard proteins: 1, phosphorylase B (94,000); 2, bovine serum albumin (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000); 5, trypsin inhibitor (20,100); 6, α-lactalbumin (14,400).

zymes and their affinity, as judged by apparent K_m values, is higher for NADP(H) than for NAD(H).

GAPDH II gives, on SDS electrophoresis, 2 bands of 43,000 and 37,000 daltons: their mobility is the same as that of the subunits which constitute the 600,000-dalton enzyme, but their relative proportions are different. Although the presence of a minor contaminating band of protein prevents the unambigous definition of the subunit composition of GAPDH II (mol.wt 300,000 daltons), it is likely that both this, and the 600,000-dalton species, derive from a different assembly of the same subunits: their molar ratio is 1:1 in GAPDH I, while an excess of the lighter subunit is present in GAPDH II (fig. 3,C). Similar results have been also reported by Pawlizki and Latzko[12].

The 150,000-dalton enzyme shows, on SDS electrophoresis, a single band of 37,000 daltons which migrates similarly to one of the corresponding subunits of the two forms of GAPDH reported above (fig. 3,C); therefore this form corresponds to the previously reported A_4 homotetramer[6-9], as suggested also by its amino acid composition (table 2) which does not significantly differ from that of the analogous enzyme from mustard seedlings[9].

The same subunits (43,000 and 37,000, daltons) which constitute, in equimolar amounts, the 600,000-dalton enzyme, can originate, in a different arrangement, 2 minor forms of active GAPDH; a homotetramer, made up of mol.wt 37,000 daltons subunit and the 300,000-dalton enzyme which might arise from an asymmetric assembly of both of them.

From the reported data it seems reasonable to conclude that the most stable arrangement of the 2 subunits occurs in the 600,000-dalton enzyme; consequently, in agreement with the observation on crude chloroplast extracts[10], this should be the main form of native GAPDH. The other 2 species, rather than true isoenzymes, might originate either from an unbalanced production of the 2 subunits, or from a dissociation and reassociation process of GAPDH I taking place within the chloroplast, or during the purification procedure.

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Exchange of water between the harbor porpoise, *Phocoena phocoena*, and the environment

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Summary. During determination of total body water and net water turnover in the harbor porpoise, *Phocoena phocoena*, it was demonstrated that the porpoise exchanged water with an isosmotic environment by way of free diffusion and with hypo- or hyperosmotic environment by way of osmosis.

We have determined the total water turnover rate in 1 subadult female harbor porpoise *(Phocoena phocoena)* kept in isosmotic salt water (320 mosmol kg^{-1}) by measuring the rate of disappearance from the plasma over a period of 5 days of i.v. injected tritiated water[1]. In 2 experiments, the relative rate was quite constant with a turnover rate of 12.0 l and 13.1 l per 24 h. The total body water, obtained from the degree of dilution of the tritiated water, was found to be 14.5 and 14.6 kg, respectively, or 51.8 and 47.7% of the body weight. These low figures for body water are in accordance with a 45% blubber mass as given by Slijper[2]. Thus, the total rate of water turnover was 13.5 and 12.0 kg per 24 h. The high turnover rate of water was inconsistent with the mean daily water intake from the food during the experiment. This was calculated as 1.5 kg of preformed water to which should be added the water of oxidation, totalling 1.9 kg.

Similar data with respect to water turnover, body water content and water intake were found in 17 experiments with 5 animals including the experimental animal.

The measured turnover rates were also inconsistent with data on oral water consumption measured by 2 other methods in 2 other delphinid species[3,4], which report ingestion of saltwater of the order of 5-10 ml/kg b.wt per 24 h corresponding to 150-300 ml per 24 h in our experimental animal.

A high oral intake of water is also in contrast with the way in which the harbor porpoise swallows its prey. When the

Table 1. Recovered activity and losses of radioactivity in 2 experiments in 1 harbor porpoise sprinkled for 1 h with isosmotic saline. Fecal output was negligible

Experiment	Activity recovered in urine (dpm $h^{-1} \cdot 10^7$)	Activity recovered in tub water (dpm $h^{-1} \cdot 10^7$)	Total activity recovered (dpm $h^{-1} \cdot 10^7$)	Loss of activity from body water (dpm $h^{-1} \cdot 10^7$)
1	0.139	4.10	4.23	4.21
2	0.0273	3.67	3.70	4.11