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SUBUNIT STRUCTURE AND ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM SPINACH CHLOROPLASTS

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Summary

Glyceraldehyde-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : $NADP^+$ oxidoreductase (phosphorylating), EC 1.2.1.13) from spinach chloroplasts is a polymeric protein of approx. 600 000 daltons and sodium dodecyl sulphate gel electrophoresis shows that it consists of two subunits of molecular weight 43 000 and 37 000. Comparison of amino acid analyses and tryptic peptide maps indicates that the two subunits have a different primary structure. The native enzyme contains 0.5 mol of $NADP^+$ and 0.5 mol of NAD^+ per protomer of 80 000 daltons, no reduced pyridine nucleotides have been detected.

Almost complete inactivation is obtained by reaction of two cysteinyl residues per 80 000 daltons with tetrathionate or iodo[$^{14}C_2$]acetic acid; since the same amount of radioactivity is incorporated in the two subunits it is likely that they are both essential for the catalytic activity.

Charcoal stripping of native glyceraldehyde-phosphate dehydrogenase produces an apoprotein which still retains most of the enzymatic activity but, unlike the holoenzyme, is gradually inactivated by storage at 4°C and does not react with iodoacetate under the same conditions in which the holoenzyme is completely inactivated.

Introduction

Like the analogous enzymes from other sources [1] NAD^+-linked glyceraldehyde-phosphate dehydrogenases (D-glyceraldehyde-3-phosphate : NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from the cytoplasm of higher plants are tetramers of molecular weight 140 000 [2,3] and are thought to play their catalytic role in glycolysis [2—6]. The high degree of homology found in mammalian, yeast and bacterial glyceraldehyde-phosphate dehydrogenases, however,

does not apply to NAD^+-specific glyceraldehyde-phosphate dehydrogenase from green plants; isoelectrofocusing data of McGowan and Gibbs [2] indicated that several molecular forms of this enzyme occur in different parts of the pea plant.

Glyceraldehyde-phosphate dehydrogenase linked with both $NADP^+$ and NAD^+ (EC 1.2.1.13) is located in the chloroplasts and is thought to function mainly in the reductive CO_2 fixation of photosynthesis [7—9]: in particular the spinach chloroplast enzyme purified in several laboratories [10—13] has been found to be substantially different in catalytic and structural properties from any previously studied glyceraldehyde-phosphate dehydrogenase. Different values (from 1.5 million to 79 000 daltons) have been reported for its molecular weight [10—13] according to the various aggregation states of the multimeric protein, which are apparently affected by the addition of $NADP^+$ or dithiothreitol [11,12]. Although a change in affinity toward $NADP^+$ and NAD^+, as a consequence of the reversible depolymerization of the enzyme has been reported, the relationship between state of aggregation and kinetic properties has not been completely elucidated.

The homogeneous glyceraldehyde-phosphate dehydrogenase (molecular weight 600 000) purified by Yonushot et al. [10] is more active with $NADP^+$, while the same molecular weight enzyme has been recently reported to be mainly active with NAD^+ [11,13]. In addition, Pawlizki et al. [12] have shown that the NAD^+ and $NADP^+$ activities are principally associated with molecular forms of molecular weight 240 000 and 79 000, respectively.

From these conflicting reports it appears that a real understanding of the role played by the different molecular forms of glyceraldehyde-phosphate dehydrogenase in higher plants will not be possible unless their structural relationship is known.

In consideration of the singular properties described for the chloroplast glyceraldehyde-phosphate dehydrogenase, we have undertaken a detailed study of this enzyme.

Materials and Methods

Glyceraldehyde 3-phosphate, diethylacetal barium salt; glyceric acid 3-phosphate, sodium salt; β-$NADP^+$, β-NAD^+, β-NADPH and β-NADH, ATP and phenylmethylsulfonylfluoride were crystalline preparations from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Iodoacetic acid and sodium tetrathionate was obtained from Fluka A.G. (Buchs, Switzerland) and iodo[$^{14}C_2$]acetic acid from The Radiochemical Center (Amersham).

Tos-Phe CH_2Cl-treated trypsin was supplied by Worthington Biochemical Co. (N.J., U.S.A.). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), alcohol dehydrogenase (EC 1.1.1.1), lactate dehydrogenase (EC 1.1.1.27) and glutathione reductase (EC 1.6.4.2) were obtained from C.F. Boehringer and Soehne (Mannheim, Germany). Phosphoglycerate kinase from yeast (EC 2.7.2.3) was purchased from Sigma Chemical Co. All other chemicals were of analytical grade and were purchased from C. Erba (Milan, Italy).

Enzyme purification. The enzyme was prepared from fresh spinach leaves by the procedure of Yonushot et al. [10] with the following modifications: (1)

10 μM phenylmethylsulfonylfluoride was added to the extraction buffer to prevent proteolytic degradation of the enzyme; (2) the precipitate from 60% $(NH_4)_2SO_4$ saturation was dissolved in 25 mM potassium phosphate/2.5 mM EDTA/1 mM β-mercaptoethanol buffer, pH 7.5 and the same buffer was used throughout the subsequent steps; (3) the pooled active fractions from DEAE-cellulose were precipitated by adding solid $(NH_4)_2SO_4$ up to 70% saturation. After centrifugation, the precipitate was redissolved in 25 mM potassium phosphate/2.5 mM EDTA/1 mM mercaptoethanol buffer, pH 7.5 and applied to a Biogel A-5 m column (3.4 × 100 cm). Elution was carried out with the same buffer at a flow rate of 10 ml/h. The fractions containing glyceraldehyde-phosphate dehydrogenase activity were pooled, concentrated by ultrafiltration up to 10 mg protein/ml and stored at 4° C.

Protein determination. Protein concentration was determined according to Lowry et al. [14] using crystalline bovine serum albumin as a standard.

Assay procedures. The oxidative assay for glyceraldehyde-phosphate dehydrogenase was carried out by measuring the increasing absorbance at 340 nm and 25° C, in 30 mM sodium pyrophosphate buffer (pH 8.5) containing 5 mM EDTA, 6.6 mM sodium arsenate, 1.0 mM β-$NADP^+$ or NAD^+, 1.5 mM glyceraldehyde-phosphate and 0.4—0.6 μg of enzyme (final volume 1.5 ml).

The reductive assay was carried out by measuring the decreasing absorbance at 340 nm and 25° C, in 30 mM Tris · HCl buffer (pH 8.5), 2 mM ATP, 5 mM $MgCl_2$, 0.2 mM NADPH or NADH, 1 mM 3-phosphoglycerate and 6.4 units of phosphoglycerate kinase.

After 1 min of incubation the reaction was started by adding 0.8 μg of glyceraldehyde-phosphate dehydrogenase.

Polyacrylamide gel electrophoresis. Electrophoresis on 3.75 and 5% polyacrylamide separating gel was performed at pH 9.5 [15] and pH 8.0 [16]; sodium dodecyl sulphate gel electrophoresis was carried out according to Weber et al. [17]. For scintillation counting, solubilized gel was obtained by using *N,N'*-diallyltartardiamide as cross-linking agent [18].

Molecular weight determination. The molecular weight of native glyceraldehyde-phosphate dehydrogenase was determined on a Biogel A 1.5m column (2 × 90 cm) using 25 mM potassium phosphate/2.5 mM EDTA/1 mM β-mercaptoethanol buffer, pH 7.5, as eluant, and the following reference proteins: ferritin, β-galactosidase, catalase, rabbit muscle glyceraldehyde-phosphate dehydrogenase and bovine serum albumin. The void volume was determined using Dextran Blue 2000.

Molecular weight determinations of sodium dodecyl sulphate-treated enzyme were carried out according to Weber et al. [17] using as standards the following proteins: catalase, aldolase, bovine serum albumin, chymotrypsinogen and cytochrome *c*.

Amino acid analyis, tryptic digestion, peptide maps. Amino acid analysis, tryptic digestion were performed and peptide maps prepared as already reported [19]. Performic acid oxidation of the protein was carried out according to Hirs [20]. The tryptophan content of the protein was determined according to Liu et al. [21].

Coenzyme determination and preparation of apoprotein. The total amount of oxidized pyridine coenzymes bound to the glyceraldehyde-phosphate dehy-

drogenase was estimated by direct spectrophotometric analysis of the protein-free supernatant obtained from 12 mg of enzyme treated with 6% $HClO_4$ [22], using an absorbance coefficient of $18 \cdot 10^6$ $cm^2 \cdot mol^{-1}$ at 260 nm, pH 7.0 [23].

NAD^+ content was determined on protein-free supernatant using alcohol dehydrogenase according to Ciotti et al. [24]; $NADP^+$ content was estimated by glucose-6-phosphate dehydrogenase according to Klingenberg [25].

In order to check for the presence of reduced pyridine nucleotides bound to the enzyme, alkaline protein-free extracts were assayed by spectrophotometric and enzymatic analysis using glutathione reductase [25] and lactate dehydrogenase [26].

Pyridine coenzymes were removed from the protein by charcoal treatment as described by Cseke and Boross [27].

Treatment of the enzyme with sodium tetrathionate and iodo[$^{14}C_2$]acetic acid. Before all the experiments, aliquots of the stock solution of glyceraldehyde-phosphate dehydrogenase were filtered on Sephadex G 25 using 45 mM pyrophosphate/5 mM EDTA buffer, pH 8.5, as eluant. 2 mg of the enzyme (0.025 μmol, assuming a molecular weight of 80 000) were incubated with sodium tetrathionate (molar ratio enzyme : sodium tetrathionate from 1 : 0.25 to 1 : 2.5) in 1 ml of 45 mM sodium pyrophosphate buffer/5 mM EDTA, pH 8.5, at 0° C for 2 min. Two samples of 0.05 ml were diluted to 1 ml respectively with sodium pyrophosphate buffer alone and with sodium pyrophosphate buffer containing 10 mM dithiothreitol and assayed for the activity.

The reactivity of native glyceraldehyde-phosphate dehydrogenase towards iodo[$^{14}C_2$]acetic acid was studied by incubating 2 mg of protein (0.025 μmol) with increasing amounts of iodo[$^{14}C_2$]acetic acid for 10 min at 0° C in 1 ml of 45 mM sodium pyrophosphate buffer/5 mM EDTA, pH 8.5 (molar ratio enzyme: iodo[$^{14}C_2$]acetic acid from 1 : 2 to 1 : 40). After assay of the enzymatic activity, the protein was precipitated with 4 vols. of cold acetone containing 5% of 1 M HCl, and the precipitate was washed with cold acetone/HCl to remove excess of radioactivity.

The alkylated cysteinyl residues were determined by radioactivity measurement and by standard amino acid analysis.

Results

Enzyme purification and molecular weight determinations

Chloroplast glyceraldehyde-phosphate dehydrogenase was purified as described in the methods section: both $NADP^+$- and NAD^+-linked activities were assayed through all purification steps (Table I).

Under our experimental conditions, glyceraldehyde-phosphate dehydrogenase activity was eluted in two peaks from Biogel: in a typical preparation from 5 kg of fresh spinach leaves about 30% of the initial $NADP^+$ activity was recovered into the first peak, with about 6% in the second.

The ratio of activity with $NADP^+$ to that with NAD^+ was respectively 2 for the glyceraldehyde-phosphate dehydrogenase eluted in the first peak and 1 for the enzyme eluted in the second peak; the elution profile reported in Fig. 1 shows that this ratio was constant for each fraction during the agarose column chromatography.

TABLE I

PURIFICATION OF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE FROM 5 kg of SPINACH LEAVES

Specific activity is referred as μmol of $NADP^+$ or NAD^+ reduced/min per mg of protein.

	Total protein (mg)	Total activity		Specific activity		$NADP^+$/ NAD^+
		$NADP^+$	NAD^+	$NADP^+$	NAD^+	
Leaf extract	47 380	22 962	33 611	0.48	0.71	0.68
Heat fraction	37 190	32 076	40 385	0.86	1.08	0.79
$(NH_4)_2SO_4$ 45% saturation fraction	24 750	26 705	27 450	1.08	1.11	0.97
$(NH_4)_2SO_4$ 60% saturation fraction	7 800	16 210	11 700	2.07	1.50	1.38
Acetone fraction	2 652	16 151	11 680	6.09	4.40	1.38
DEAE cellulose step	501	11 986	8 628	23.86	17.22	1.38
$(NH_4)_2SO_4$ 70% saturation fraction	268	10 710	7 711	39.96	28.77	1.39
Biogel step:						
First peak	103	7 210	3 433	70.00	33.33	2.10
Second peak	93	1 432	1 290	15.40	13.92	1.11

The enzyme obtained from the first peak proved to be homogeneous by electrophoresis carried out at different pH on polyacrylamide gel, while the pooled fractions from the second peak gave several bands of protein; the electrophoretic patterns in polyacrylamide gel, run at pH 9.5, are shown in Figs. 2A and B.

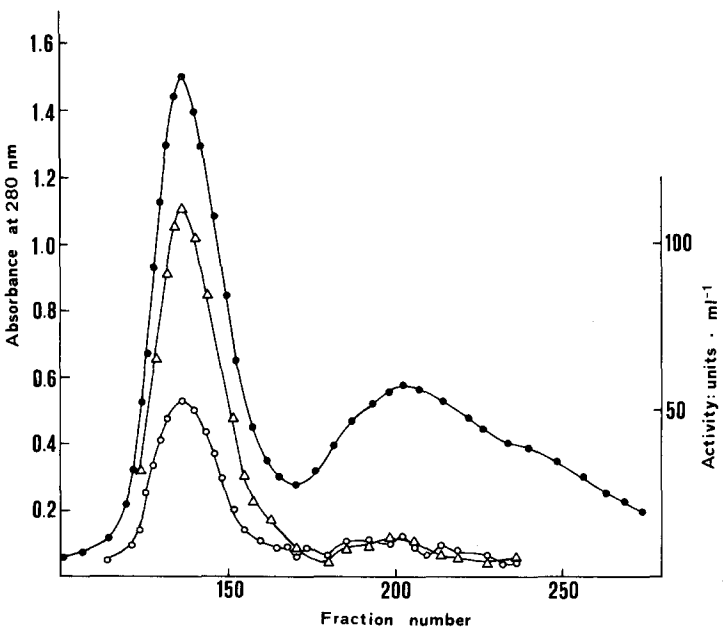

Fig. 1. Elution profile of glyceraldehyde-phosphate dehydrogenase from Biogel A-5 m (3.4 × 100 cm column; fraction volume: 3.1 ml). •, Absorbance at 280 nm; △, $NADP^+$ activity as μmol of $NADP^+$ reduced/min per ml; ○, NAD^+ activity as μmol of NAD^+ reduced/min per ml.

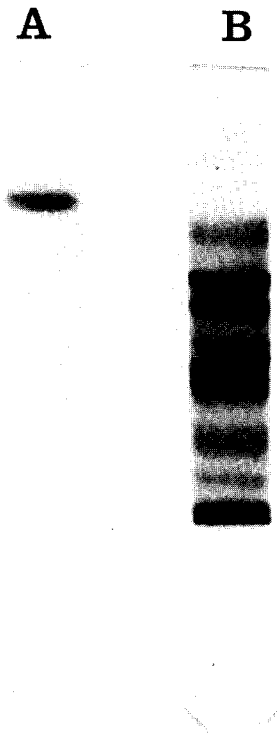

Fig. 2. Electrophoresis in 5% polyacrylamide gel according to Davis [15]. Pooled fractions were from the first (A) and second (B) Biogel A-5 m peak.

Under standard purification and assay procedures, the activity recovered in the two peaks, as well as the ratio of $NADP^+$ to NAD^+ activities, were found to be constant in different preparations.

The homogeneous glyceraldehyde-phosphate dehydrogenase retained its full activity for several months; on the contrary, a spontaneous loss of activity occurred at 4° C in the enzyme from the second peak. The molecular weight of homogeneous enzyme, as determined by Biogel A-1.5m chromatography, was found to be 600 000 (±10%), in good agreement with the data of Yonushot et al. [10].

On sodium dodecyl sulphate electrophoresis the homogeneous glyceraldehyde-phosphate dehydrogenase was resolved into two protein bands with molecular weights of 43 000 and 37 000, respectively (Fig. 3). The stained bands had equal heights on photometric scanning, thus it may be assumed that the monomeric unit of native glyceraldehyde-phosphate dehydrogenase has a molecular weight of 80 000 and is made up of two non-identical subunits, with molecular weights of 43 000 and 37 000, respectively.

Amino acid analysis and peptide maps

The amino acid composition of the enzyme, expressed as the nearest whole

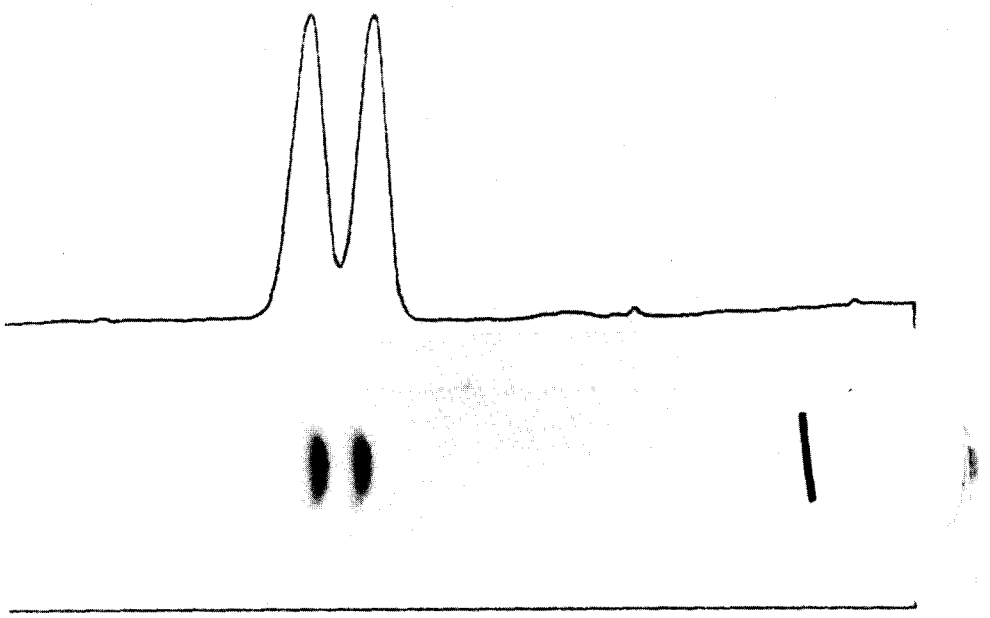

Fig. 3. Electrophoresis in sodium dodecyl sulphate of glyceraldehyde-phosphate dehydrogenase according to Weber and Osborn [17].

number of individual amino acids/80 000 daltons is given in Table II: the values of serine and threonine are corrected for losses during acid hydrolysis, while valine and isoleucine values are derived from 72 h hydrolysis. The total number of arginine and lysine residues is 81; the number of cysteine residues, estimated both as carboxymethyl-cysteine and cysteic acid gave a mean value of 14; a total of 8 tryptophan residues were found.

Samples of *S*-[^{14}C]carboxymethylated enzyme were submitted to tryptic digestion and then fingerprinted: a typical peptide map, after autoradiography and staining with ninhydrin followed by Ehrlich reagent, is shown in Fig. 4A.

The paper strip containing the neutral peptides, which appear to be very poorly resolved, was cut out and stitched to a fresh sheet of Whatman 3 MM paper for further electrophoresis at pH 2.2 (Fig. 4B).

About 80 spots were revealed, in good agreement with the 82 expected from the total number of arginine and lysine residues.

The autoradiography of the map showed 11 labelled peptides, three of which appeared to contain about twice the radioactivity of the others (spots a, b, c, in Fig. 4A). Five tryptophan-positive spots were detected after treatment with acidic Ehrlich reagent: one of these (spot a in Fig. 4A) appeared to be more stained than the others.

These findings demonstrate that the two bands obtained by sodium dodecyl sulphate gel electrophoresis are indeed subunits with a different primary structure and exclude the possibility that they belong to an unique amino acid sequence and differ only for a fragment of 6000 daltons.

TABLE II

AMINO ACID COMPOSITION OF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE

The values are the mean of ten independent determinations.

Amino acid	Residues per 80 000-dalton monomer
Lysine	50
Histidine	14
Arginine	31
Cysteine [a]	14
Aspartic acid	107
Threonine [b]	47
Serine [b]	51
Glutamic acid	41
Proline	33
Glycine	69
Alanine	66
Valine [c]	76
Methionine	10
Isoleucine [c]	38
Leucine	62
Tyrosine	14
Phenylalanine	21
Tryptophan [d]	8
Total residues	752

[a] Determined both as S-carboxymethylcysteine and cysteic acid.
[b] Corrected for destruction during acid hydrolysis.
[c] Corrected for slow release during acid hydrolysis.
[d] Determined according to Liu and Chang [21].

Pyridine nucleotides content and kinetic properties

The presence of bound pyridine nucleotides in native glyceraldehyde-phosphate dehydrogenase was indicated by absorbance ratio of 1.2 (±0.1) at 280/260 nm increasing to 2.0 (±0.1) after charcoal treatment. Table III shows that 0.5 mol of NAD^+ and 0.5 mol of $NADP^+$ were found per 80 000-dalton protomer, while no reduced coenzymes were observed in any of the preparations assayed.

Removal of the bound coenzymes by charcoal treatment did not alter appreciably the enzymatic activity; Table IV shows that the same apparent K_m for oxidized nucleotides was found when an apoprotein with an absorbance ratio of 2.0 at 280/260 nm was used. However, the apoprotein was much more unstable than holo-glyceraldehyde-phosphate dehydrogenase: complete loss of activity occurred in about 48 h at 4° C in pyrophosphate buffer, pH 8.5, even at protein concentration (2.5 mg/ml) at which native glyceraldehyde-phosphate dehydrogenase was stable for several weeks.

The pH optimum for activity is in the range 8.0—8.8 in the forward, as well as in the reverse, direction and a pH of 8.5 was chosen for the assay.

Michaelis-Menten kinetics were observed for all substrates tested with K_m for NADP(H) lower than for NAD(H) but with no difference in the K_m of the reduced and oxidized forms of either coenzymes (Table IV).

SH dependence of the activity

Samples of holo-glyceraldehyde-phosphate dehydrogenase (2 mg of protein

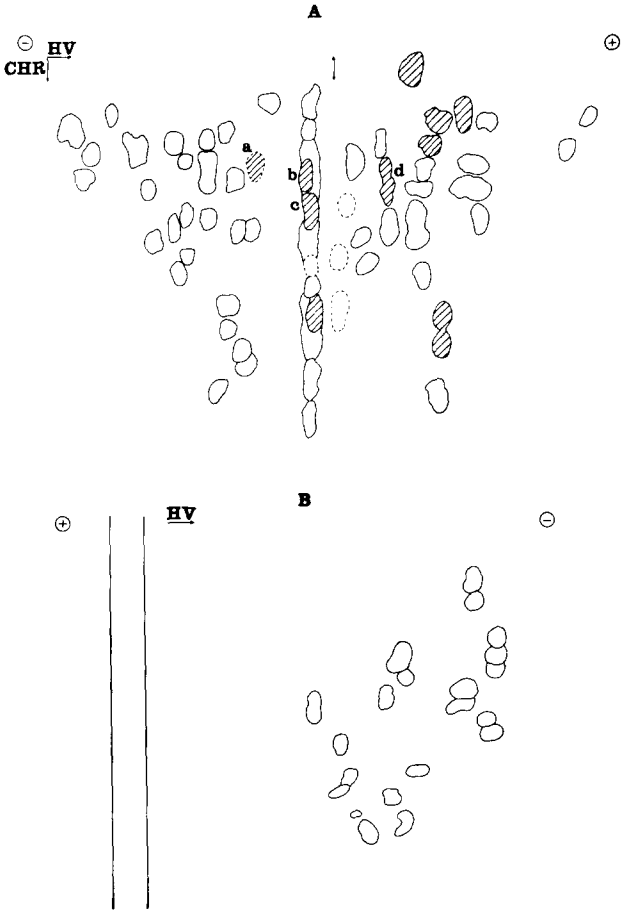

Fig. 4. Peptide maps of a tryptic digest of *S*-[^{14}C]carboxymethylated glyceraldehyde-phosphate dehydrogenase. A. Electrophoresis (HV): pH 6.5; 60 V/cm; 40 min. Chromatography (CHR): acetic acid/*n*-butanol/H_2O/pyridine (6 : 30 : 24 : 20). Radioactive peptides are shown as hatched spots. Tryptophan-containing peptides are shown as dotted spots. B. The strip corresponding to the neutral peptides electrophoresed at pH 2.2, 80 V/cm, 20 min.

corresponding to 0.025 μmol, assuming a molecular weight of 80 000) were incubated at 0°C in 1 ml of pyrophosphate/EDTA buffer with increasing amounts of tetrathionate. Fig. 5A shows that the enzyme is stoichiometrically inactivated by the addition of a 2-fold molar excess of tetrathionate. The inactivation is complete within 2 min at 0°C and can be reversed by the addition of dithiothreitol.

When the same amount of protein was reacted with iodo[$^{14}C_2$]acetic acid (from 2- to 40-fold molar excess on 80 000 daltons) in pyrophosphate buffer, pH 8.5, at 0°C for 10 min, a loss of oxidative activity paralelled by an increase of the number of *S*-alkylated cysteinyl residues was observed (Fig. 5B). Complete inhibition occurred when 2 mol of cysteine per 80 000 daltons were alkylated at a 25-fold molar excess of iodo[$^{14}C_2$]acetic acid. At this extent of labelling, two radioactive spots are present in the tryptic peptide map of the inactivated enzyme (spots c and d in Fig. 4A).

TABLE III

PYRIDINE NUCLEOTIDES CONTENT OF DIFFERENT PREPARATIONS OF SPINACH LEAF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE

The pyridine nucleotide content was determined on protein-free supernatant: a, by direct spectrophotometric analysis at 260 nm [23]; b, with glucose-6-phosphate dehydrogenase according to Klingenberg [25]; c, with alcohol dehydrogenase according to Ciotti et al. [24].

Preparation No.	(a) $NADP^+ + NAD^+$ (mol/80 000 daltons)	(b) $NADP^+$ (mol/80 000 daltons)	(c) NAD^+ (mol/80 000 daltons)
1	0.97	0.45	—
2	1.04	0.49	0.44
3	0.98	0.50	0.47
4	1.03	0.49	0.50
5	0.99	0.50	0.53
6	0.97	0.49	0.54

In order to detect any difference in the reactivity of the subunits and, possibly, to establish a dependence of the inactivation on one of the two, samples of inactivated glyceraldehyde-phosphate dehydrogenase, with 2 mol of iodo-[$^{14}C_2$]acetic acid bound per monomer (80 000 daltons) were electrophoresed in sodium dodecyl sulphate gel. The stained bands were cut out and, after solubilization of the gel, submitted to scintillation counting. The same amount of radioactivity was found in the two bands, indicating that the reaction with iodoacetate takes place at each of the two subunits.

The inactivation by iodoacetate could be completely prevented by preincubating the enzyme for 5 min with glyceraldehyde-phosphate (10-fold molar excess over iodoacetate) before the addition of iodoacetate. No loss of activity was observed when apo-glyceraldehyde-phosphate dehydrogenase was treated with iodoacetate under the same conditions as holo-glyceraldehyde-phosphate dehydrogenase.

TABLE IV

APPARENT K_m VALUES OF HOLO- AND APO-GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE

The concentrations of the unvaried substrates were: 1 mM $NADP^+$ or NAD^+; 2 mM glyceraldehyde 3-phosphate; 1 mM 3-phosphoglycerate; 0.2 mM NADPH or NADH. The concentration of the single substrate was varied as following: glyceraldehyde 3-phosphate from 0.01 to 2.5 mM; $NADP^+$ or NAD^+ from 0.01 to 2 mM; 3-phosphoglycerate from 0.02 to 1 mM; NADPH from 0.01 to 0.4 mM and NADH from 0.01 to 0.2 mM. The K_m values are expressed in mol · l^{-1}.

	Glyceraldehyde 3-phosphate		$NADP^+$ ($\times 10^5$)	NAD^+ ($\times 10^4$)	3-Phosphoglycerate		NADPH ($\times 10^5$)	NADH ($\times 10^4$)
					NADPH ($\times 10^4$)	NADH ($\times 10^4$)		
	$NADP^+$ ($\times 10^4$)	NAD^+ ($\times 10^4$)						
Holoenzyme	2.3	9.4	4.1	2.0	2.0	2.6	3.9	1.7
Apoenzyme	18	11	5.5	3.1	—	—	—	—

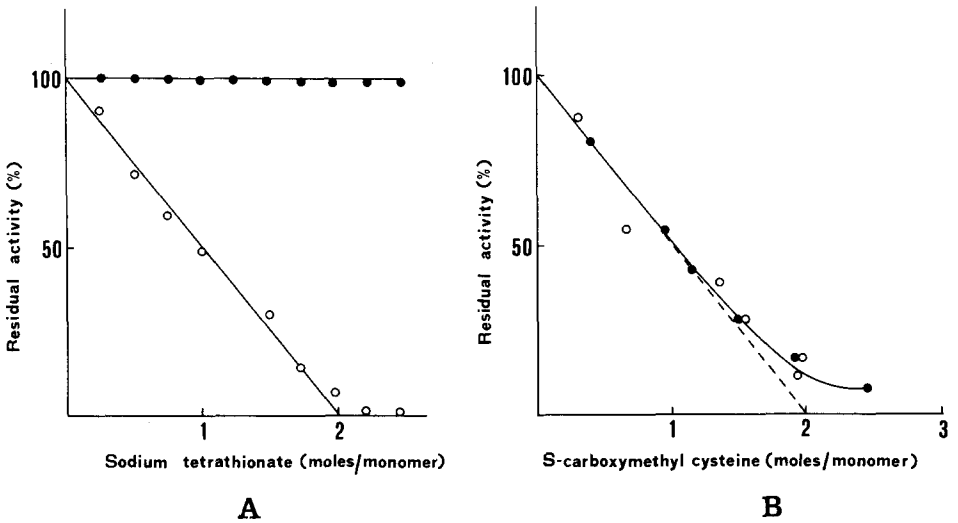

Fig. 5. Effect of increasing concentrations of tetrathionate and iodo[$^{14}C_2$]acetic acid on oxidative activity of glyceraldehyde-phosphate dehydrogenase. A. Glyceraldehyde-phosphate dehydrogenase, 0.025 μmoles monomer (molecular weight 80 000) were incubated in 1 ml of 45 mM sodium pyrophosphate, buffer/5 mM EDTA, pH 8.5, with increasing amounts of sodium tetrathionate (0.25—2.5 molar excess). After 2 min at 0°C, two samples of 0.05 ml were diluted to 1 ml respectively with sodium pyrophosphate buffer alone and with the same buffer containing 10 mM dithiothreitol. After 20 min 0.5 μg of enzyme were assayed for activity. •, dithiothreitol-diluted enzyme; ○, no dithiothreitol. B. Glyceraldehyde-phosphate dehydrogenase 0.025 μmol monomer (molecular weight 80 000) were incubated in 1 ml of 45 mM sodium pyrophosphate buffer/5 mM EDTA, pH 8.5, with increasing amounts of iodo[$^{14}C_2$]-acetic acid (2—40 molar excess) for 10 min at 0°C. After the activity had been assayed on 0.5 μg of enzyme, the protein was precipitated with 4 vols. of cold acetone containing 5% 1 M HCl, and washed to eliminate the excess of radioactivity. The alkylated cysteinyl residues were determined by scintillation counting and as *S*-carboxymethylcysteine after 6 M HCl hydrolysis. •, *S*-carboxymethylcysteine by amino acid analysis; ○, *S*-carboxymethylcysteine by scintillation counting.

Discussion

Glyceraldehyde-phosphate dehydrogenase from spinach chloroplasts was purified as a protein of 600 000 daltons, being mainly active with $NADP^+$, in good agreement with the results of Yonushot et al. [10].

The previously reported higher molecular weight form of glyceraldehyde-phosphate dehydrogenase [11,13] was not detected in our experimental conditions; however, the existence of several forms of the enzyme was confirmed by the finding that a minor fraction, equally active with $NADP^+$ and NAD^+, was eluted from Biogel after the 600 000-dalton enzyme (Table I, Fig. 1). That this may be a mixture of lower molecular weight forms of glyceraldehyde-phosphate dehydrogenase is indeed suggested by the broad elution pattern.

Pupillo and Piccari [11] have reported an interconversion of the 600 000-dalton form (in their study mainly active with NAD^+) to a low molecular weight form (mainly active with $NADP^+$) effected by low concentrations of $NADP^+$; Pawlizki et al. [12] found that dithiothreitol was required to promote the interconversion between high and low molecular weight forms, while $NADP^+$ had no effect.

It is difficult to reconcile these contrasting results unless the subunit struc-

ture of glyceraldehyde-phosphate dehydrogenase is studied and the contribution of the single monomers, with their coenzyme content, to the various aggregational states of the protein is known.

In addition to the coenzyme requirement, our preparations of spinach glyceraldehyde-phosphate dehydrogenase show kinetic properties which differ from that previously reported for the same molecular weight (600 000) enzyme [11,13]: hyperbolic kinetics were observed for all substrates and almost the same K_m values for the oxidized and reduced form of both pyridine coenzymes were found. It is likely that these findings may be explained by the different isolation and assay procedures. Glyceraldehyde-phosphate dehydrogenase appears to be affected by the ionic composition of the medium, since a loss of activity in Tris buffer has been reported [13], while in our experience the enzyme retains its full activity in phosphate or pyrophosphate buffers. It seems possible that the protein may be stabilized in a specific conformation by bound anions such as phosphate or pyrophosphate; this would explain the different kinetic behaviour and, possibly, any difference in molecular properties.

Two different subunits with an apparent ratio of 1 : 1 were obtained from the homogeneous high molecular weight enzyme (peak I, Fig. 1) by sodium dodecyl sulphate gel electrophoresis: their molecular weights of 43 000 and 37 000 are in good agreement with the results of Pawlizki et al. [12]. The total number of peptides observed on tryptic peptide maps and, in particular, that of cysteine and tryptophan containing peptides, is not different from the number predicted by amino acid analysis, assuming the enzyme to consist of non identical subunits. It may be concluded that the two subunits have a different primary structure.

It may well be shown by further amino acid sequence studies that the two subunits show a high degree of homology and may have originated by the usual evolutionary process of gene duplication. Since 0.5 mol of bound NAD^+ and 0.5 mol of bound $NADP^+$ were found per 80 000 daltons (which is the sum of the subunits molecular weights) it is tempting to speculate that the two subunits may have specialized in the binding of NAD^+ and $NADP^+$, respectively, and that their various aggregations, according to coenzyme availability within the chloroplast or dark-light transition, may give rise to the reported differences in molecular weight and coenzyme specificity. The stoichiometry of the bound pyridine nucleotides can be accounted for by the presence of non-equivalent binding sites; this would explain the loss of loosely-bound coenzyme during purification steps. Negative cooperativity is reported for some glyceraldehyde-phosphate dehydrogenases [28—30] and it is therefore possible that chloroplast enzyme does not differ, in this respect, from analogous proteins from other sources.

It seems clear, however, that the main form of glyceraldehyde-phosphate dehydrogenase prevailing in the chloroplasts (molecular weight 600 000) results from the assembly of 8 protomers, each made up of two non-identical subunits and contains 4 mol of bound NAD^+ and 4 mol of bound $NADP^+$.

It is of interest to note that, like its counterparts from other sources, chloroplast glyceraldehyde-phosphate dehydrogenase is a sulphydryl-containing enzyme and that both subunits are endowed with catalytic activity since the binding of iodo[$^{14}C_2$]acetic acid occurs equally on each, with complete inacti-

vation of the enzyme. Experiments are in progress to identify the amino acid sequence around the two reactive cysteine residues.

Acknowledgments

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