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PURIFICATION AND PROPERTIES OF NAD$^+$-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM SPINACH LEAVES

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Summary

An NAD$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase (phosphorylating), EC. 1.2.1.12) has been purified from spinach leaves as a homogeneous protein of 150 000 daltons.

Kinetic constants of $2.5 \cdot 10^{-4}$ M and $4 \cdot 10^{-4}$ M have been calculated for NAD$^+$ and glyceraldehyde 3-phosphate, respectively.

The amino acid composition is characterized by a cysteine content higher than that found in analogous enzymes.

On sodium dodecyl sulphate gel electrophoresis, the native enzyme dissociates into two subunits of 37 000 and 14 000 daltons. The two subunits have been isolated in equimolar amounts by gel filtration; end-group analysis shows that alanine is the *N*-terminal residue of the large subunit, while serine is found at the *N*-terminus of the small subunit.

Comparison of amino acid analyses and peptide maps shows that the two subunits have a different amino acid sequence. These results indicate that the NAD$^+$-dependent glyceraldehyde-3-phosphate dehydrogenase, isolated from spinach leaves has an atypical oligomeric structure, the protomer being formed by two different subunits.

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12) from green plants has been intensively studied for its central role in glycolysis (as in the process of the photosynthetic carbon fixation). Catalytic activities, NAD$^+$ and NADP$^+$ dependent, have been found in green tissues [1,2], the NADP$^+$-linked activity being associated with photosynthetic production of hexoses whereas the NAD$^+$-

linked activity is associated with the glycolytic process.

In the green leaf, $NADP^+$-dependent dehydrogenase is localized exclusively in the chloroplast, while the NAD^+-activity is found both in the cytoplasm and the chloroplast [3,4]. The $NADP^+$- and the NAD^+-dependent activities found in the chloroplast are associated with the same enzyme [5—7], which is a high polymer of two different subunits (8).

The data are consistent with the presence of two different enzymes catalyzing the dehydrogenation of glyceraldehyde 3-phosphate: a chloroplast enzyme which is active both in the presence of NAD^+ and $NADP^+$, and a cytoplasmatic enzyme which is specific for NAD^+. Evidence for the latter enzyme in the spinach leaf was obtained by Yonushot et al. [6], who observed high levels of NAD^+-dependent activity in the supernatant after ammonium sulphate precipitation of the chloroplast enzyme.

Our studies have been focused on the extraction and purification of the supernatant enzyme of Yonushot et al. [6]. An NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase has been isolated from spinach leaves and some of its properties have been elucidated.

Materials and Methods

Fresh spinach obtained from the local market has been used as the enzyme source. D,L-glyceraldehyde 3-phosphate (barium salt), NAD^+ and phenylmethylsulphonylfluoride were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); the proteins used as standard markers for molecular weight determination were supplied by Boehringer (Mannheim, Germany). Iodoacetic acid was purchased from Fluka AG (Buchs, Switzerland), (Tos-Phe-CH_2Cl)-treated trypsin was a product of the Worthington Biochemical Co. (New Jersey, U.S.A.). All the other chemicals used were analytical grade from C. Erba (Milan, Italy).

Activity assay. Enzyme activity was routinely assayed at 25°C measuring the absorbance increase at 340 nm. The reaction mixture contained, in a final volume of 1.5 ml: 30 mM pyrophosphate buffer (pH 8.4); 3.3 mM EDTA; 6.6 mM arsenate; 0.66 mM NAD^+; 1 mM D-glyceraldehyde 3-phosphate and 5—10 μl of enzyme solution. Enzyme activities are expressed in International Units.

Protein determination, polyacrylamide gel electrophoresis and molecular weight studies. The protein content was determined by a modification of the biuret method [9]. Polyacrylamide gel electrophoresis was performed according to the method of Davis [10], whereas gel electrophoresis in sodium dodecyl sulphate was performed according to the procedure of Weber and Osborne [11].

The molecular weight determination of the native enzyme was carried out by thin layer gel chromatography on Sephadex G-200 Superfine using the "sandwich" technique of Determan and Maettner [12].

Amino acid analyses. The amino acid analyses were carreid out on a Beckman 120 B autoanalyzer, equipped with long path-length cells, according to the method of Spackman [13]. Hydrolysis of the protein samples, in sealed evacuated tubes, was performed in 6 M HCl for 24 h at 106°C. Cysteine plus

cystine content was determined as cysteic acid, following oxidation with dimethylsulphoxide, according to the method of Spencer and Wold [14]. Tryptophan was determined according to the method of Liu and Chang [15].

The N-terminal amino acid was identified as the dansyl derivative by the procedure of Gray [16].

Tryptic digestion and peptide maps. After extensive dialysis against water, the reduced carboxymethylated enzyme was adjusted at pH 8.4 with 5% NaOH and trypsin (1% w/w) was added. Digestion was carried out for 4 h at 37°C; the reaction was stopped by addition of glacial acetic acid (5% final concentration).

Peptide maps were obtained by submitting the material from tryptic digestion to high voltage paper electrophoresis at pH 6.5 (60 V/cm, 40 min), using the immersed strip method, with Varsol as coolant. The electrophoretic step was followed by a chromatographic run (acetic acid/*N*-butanol/pyridine, water 6 : 30 : 20 : 24) at right angle to the direction of electrophoresis. Resolution of the neutral peptides was achieved by a further electrophoresis at pH 2.2 (80 V/cm, 20 min).

Results

Enzyme purification. About 4 kg of depetiolated spinach leaves were extracted and heat-treated, as described by Yonushot et al. [6]. The supernatant remaining after precipitation of the $NADP^+$-dependent enzyme with 60% ammonium sulphate saturation, was brought to 90% saturation and the precipitate was redissolved in a minimal volume of 20 mM phosphate buffer (pH 7.5), containing 1 mM EDTA and 1 mM 2-mercaptoethanol; the same buffer has been used through all the purification procedure. The solution was then precipitated, in an ice/salt bath, with 0.8 volumes of cold acetone; the precipitate, redissolved in phosphate buffer, was chromatographed on a Sephadex G 100 column (5 × 100 cm) at a flow rate of 24 ml/h.

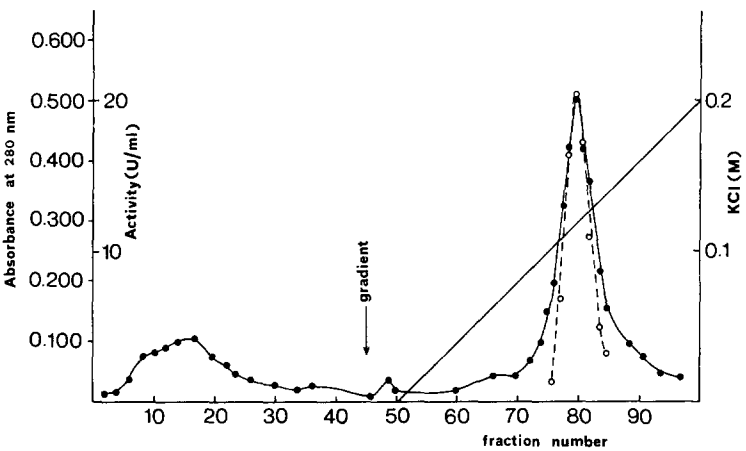

Fig. 1. Chromatography of glyceraldehyde-3-phosphate dehydrogenase on DEAE-Sephadex A-50 ●——●, A_{280nm}; ○——○, enzyme activity.

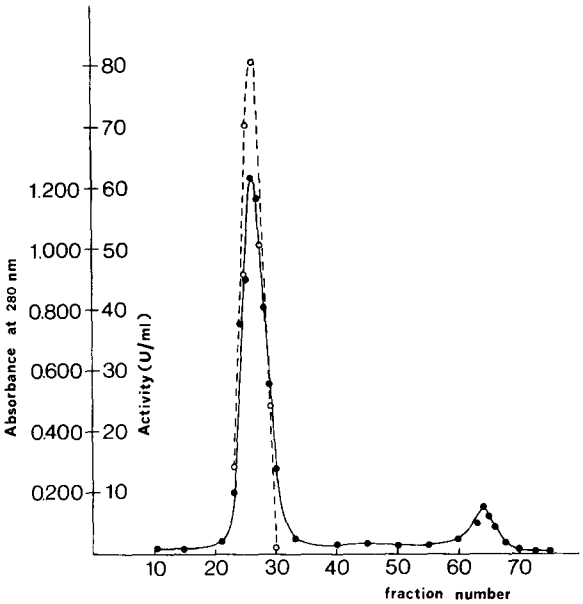

Fig. 2. Chromatography of glyceraldehyde-3-phosphate dehydrogenase on Sephadex G-100 Superfine. ●——●, A_{280nm}; ○——○, enzyme activity.

The collected active fractions were applied to a DEAE-Sephadex A 50 column (2.4 × 40 cm): elution was performed through a 500 ml linear gradient formed by a phosphate buffer 20 mM (pH 7.5) containing KCl from 0 to 0.2 M (Fig. 1); fractions of 10 ml were collected at 30 min intervals. The active fractions were concentrated by ultrafiltration and a final gel chromatography on a Sephadex G 100 Superfine column (1.8 × 34 cm) was carried out (Fig. 2): fractions of 1 ml were collected at 24-min intervals. The fractions showing the maximum and constant specific activity were pooled. The purification steps are summarized in Table I.

Optical properties and NAD⁺ content. The purified enzyme shows a peak at 278 nm. The ratio A_{280}/A_{260} is 1.46: by interpolation of the data reported by Seydoux et al. [17], for the sturgeon muscle enzyme, this figure would indicate a NAD^+ content of about 1.8 mol per mol enzyme.

Kinetic parameters. The K_m for glyceraldehyde 3-phosphate was determined while maintaining the NAD^+ concentration at the fixed value of 2.66 mM and varying glyceraldehyde 3-phosphate from 0.06 to 2.4 mM. The K_m was determined for NAD^+ at 4.82 mM glyceraldehyde 3-phosphate and varying the nucleotide concentration from 0.01 to 2 mM.

The enzyme showed K_m values for NAD^+ and glyceraldehyde 3-phosphate of $2.5 \cdot 10^{-4}$ M and $4 \cdot 10^{-4}$ M respectively. No activity has been found with $NADP^+$.

Molecular weight. A value of about 150 000 daltons for the native enzyme was obtained by thin layer gel filtration on Sephadex G 200 Superfine.

Subunit heterogeneity. The native purified enzyme, which gave on polyacrylamide gel electrophoresis a single band, both at pH 9.5 and 8.0 (Fig. 3a and b),

TABLE I

PURIFICATION OF NAD^+-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM SPINACH LEAVES

The starting material was 4 kg of spinach leaves. To avoid misinterpretation, the table starts from the step after which most of the activity due to the NAD^+-specific cytoplasmic glyceraldehyde-3-phosphate dehydrogenase is detected. The previous steps include Waring Blendor homogenisation followed by heat treatment and 45—60% saturated ammonium sulphate precipitation of the chloroplast enzyme as reported by Yonushot et al. [6]; up to this stage the chloroplast enzyme largely contributes to the observed NAD^+-dependent activity.

Fraction	Volume (ml)	Protein (mg)	Activity (total units)	Specific activity (units/mg protein)
Ammonium sulphate 60—90% precipitate	200	3800	3500	0.92
Acetone fraction	100	800	3400	4.25
Sephadex G-100	350	264	1880	7.12
DEAE Sephadex A-50	118	21	900	42.8
Sephadex G-100 Superfine	7	7	438	62.5

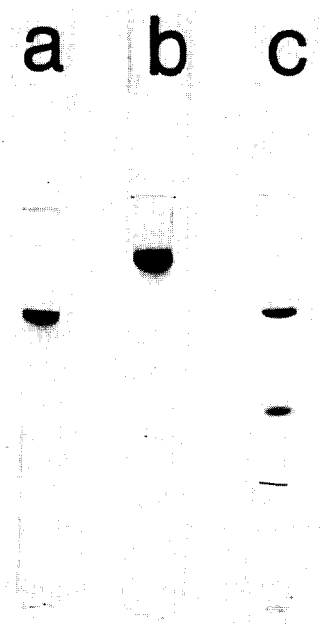

Fig. 3. Polyacrylamide gel electrophoresis of purified glyceraldehyde-3-phosphate dehydrogenase. (a) Analytical electrophoresis on 7.5% polyacrylamide gel in Tris/glycine buffer (pH 8.3), separation at pH 9.5; 30 μg sample; Amido Black staining. (b) Analytical electrophoresis on 7.5% polyacrylamide gel, in diethylbarbituric acid/Tris buffer (pH 7.0), separation at pH 8.0; 80 μg sample; Amido Black staining. (c) Electrophoresis in sodium dodecyl suphate on 10% polyacrylamide gel 10 μg sample; Coomassie Brilliant Blue staining.

showed, after denaturation in 1% sodium dodecyl sulphate in the presence of 1% 2-mercaptoethanol, two different bands (Fig. 3c), corresponding to 37 000 and 14 000 daltons.

This was a surprising finding, since all other known NAD^+-dependent glyceraldehyde 3-phosphate dehydrogenases are composed of chemically identical subunits, ranging in molecular weight from 32 000 to 36 000.

The possibility that the smaller subunit was a proteolysis product of the larger one or that the two subunits derived by the proteolytic splitting of a higher molecular weight precursor was therefore considered. Isolation of the enzyme in the presence of 10 μM phenylmethylsulphonylfluoride, a protease inhibitor, was carried out, but the enzyme showed again, on sodium dodecyl sulphate electrophoresis, only the two bands, in the usual ratio. To verify if the low molecular weight material could arise by some non enzymatic hydrolysis of peptide bonds, preincubation of the enzyme in sodium dodecyl sulphate was also performed by omitting the heating at 100°C for 2 min or by prolonging it to several minutes: in both cases no alteration of the electrophoretic pattern was observed. Also the omission of the reducing agent, 2-mercaptoethanol, in the incubation mixture, failed to produce a different pattern.

Another possibility was that the low molecular weight material could arise from a contaminant protein, but attempts to purify the enzyme further by gel chromatography on a calibrated Sephadex G 200 Superfine column (1.75 × 93 cm, fractions of 2.5 ml collected at 30-min intervals, 5 mg of enzyme chromatographed) resulted only in enzyme losses, without increase in specific activity and without changes in the relative amounts of the two bands, which

TABLE II

AMINO ACID COMPOSITION OF NAD^+-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM SPINACH LEAVES

The residues were calculated for a molecular weight of 150 000, and per 1000 residues. The data for the enzyme from other sources were taken from the literature and reported by Duggleby and Dennis [18].

Amino acid	Residues/ 150 000 daltons	Residues/1000	Residues/1000 (range of 20 species)
Lysine	120	85	76—91
Hystidine	34	24	13—39
Arginine	50	35	25—34
Cysteine *	27	19	6—15
Aspartic acid	144	102	94—134
Threonine	93	66	49—84
Serine	93	66	45—80
Glutamic acid	116	82	49—76
Proline	62	44	28—46
Glycine	136	96	81—106
Alanine	124	88	94—116
Valine	125	88	86—112
Methionine	24	17	12—31
Isoleucine	73	52	51—82
Leucine	90	64	55—66
Tyrosine	30	21	24—36
Phenylalanine	56	39	30—48
Tryptophan **	16	11	
Total residues	1413	1000	

* Determined as cysteic acid after dimethylsulphoxide oxidation [14].

** Determined according to the method of Liu et al. [15].

were still present on sodium dodecyl sulphate electrophoresis.

The molecular weight of the enzyme, calculated from the elution profile of the G 200 column, was again 150 000.

Amino acid analysis, N-terminal end-group analysis and peptide maps. The amino acid composition of the native enzyme is reported in Table II, where the data are compared with amino acid composition of the enzyme extracted from other sources. Unusually high figures have been obtained for the cysteine content, this being three times higher than the amount present in the enzyme from pea seeds [18].

The N-terminal end group analysis revealed two dansyl derivatives of amino acids, namely alanine and serine.

The tryptic peptide map is shown in Fig. 4. Fig. 4A shows the acidic and basic peptides, while Fig. 4B shows the neutral peptides, after they have been cut off from the sheet depicted in A and have been submitted to a further electrophoresis at pH 2.2.

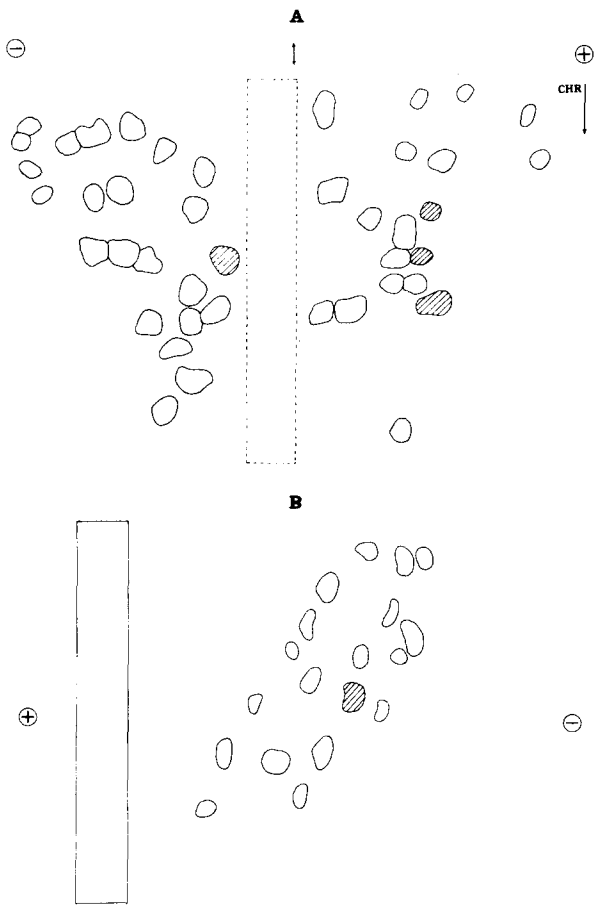

Fig. 4. Tryptic peptide map of glyceraldehyde-3-phosphate dehydrogenase. A, Acidic and basic peptides. Electrophoresis: pH 6.5, 60 V/cm, 40 min. Chromatography: acetic acid/*n*butanol/pyridine/water (6 : 30 : 20 : 24). B, Neutral peptides. Electrophoresis: pH 2.2, 80 v/cm, 20 min. Ninhydrin staining followed by Ehrlich reagent staining. Tryptophan-containing peptides are shown as hatched spots.

The total number of peptide spots is 61.

If the enzyme were composed of four identical subunits, as all other glyceraldehyde-3-phosphate dehydrogenases are, then the number of tryptic peptides expected on the basis of the arginine and lysine content of the enzyme would be 44; a similar result would be obtained if the smaller subunit were a hydrolysis product of the larger one.

Isolation and amino acid analysis of the two subunits. An aliquot of the purified enzyme solution, dialyzed against 0.01 M sodium phosphate buffer (pH 7), was boiled for 2 min in the presence of 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol. The treated enzyme solution (2.5 ml approx. 8 mg of protein) was applied to a column of Biogel A-5 m (2.4 × 85 cm), equilibrated with 0.01 M sodium phosphate buffer (pH 7), containing 0.1% sodium dodecyl sulphate. The same buffer was used for the elution and fraction of 3 ml were collected at 1 h intervals. The elution profile is reported in Fig. 5.

A further check was carried on by submitting the more concentrated fractions of the two peaks from the Biogel chromatography to sodium dodecyl sulphate electrophoresis: a protein band with a mobility of 0.42 was obtained from the first elution peak, whereas a band with a mobility of 0.75 was observed for the second elution peak. Identical values have been obtained for

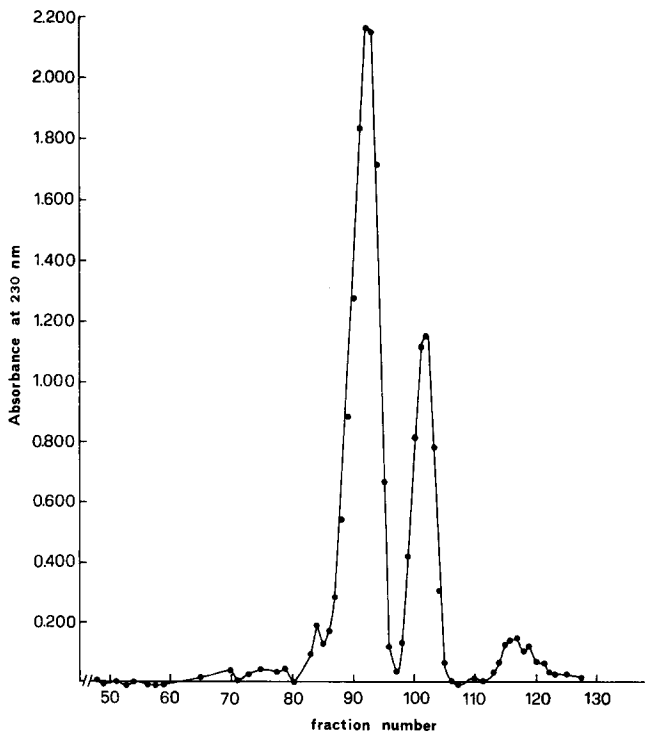

Fig. 5. Elution profile from Biogel A-5 m of glyceraldehyde-3-phosphate dehydrogenase, after denaturation with sodium dodecyl sulphate.

the native enzyme, when submitted to sodium dodecyl sulphate electrophoresis.

The elution profile reported in Fig. 5, gives information on the relative amounts of the two subunits.

The elution profile was constructed by measuring the absorbance at 230 nm, due to peptide bonds, whose number is roughly constant per protein unit weight, therefore the area of each peak is proportional to the amount of the polypeptide chain in the peak.

The area of the first peak is about 2.5 times (2.31) the area of the second peak; on the other hand also the molecular weight of the material in the first peak is approximately 2.5 times the molecular weight of the material in the second peak (37 000/14 000 = 2.64).

From the above data the two polypeptide chains seem to be present in equimolecular amounts.

The material from the two peaks was separately collected, lyophilized, resuspended in water (one tenth of the original volume), precipitated by 90% cold acetone, repeatedly washed and dried.

The N-terminal amino acid was found to be alanine for the 37 000-dalton subunit and serine for the 14 000-dalton subunit.

The amino acid composition of the two subunits is reported in Table III. The sum of arginine and lysine residues of the two polypeptide chains being 56, the number of expected tryptic peptides is 58, in good agreement with the value of 61 found on the tryptic peptide map of the native enzyme.

TABLE III

AMINO ACID COMPOSITION OF THE TWO SUBUNITS OF NAD^+-DEPENDENT GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM SPINACH LEAVES

The residues were calculated for molecular weights of 37 000 and 14 000.

Amino acid	Large subunit residues/ 37 000 daltons	Small subunit residues/ 14 000 daltons
Lysine	28	11
Hystidine	8	3
Arginine	12	5
Cysteine *	6	3
Aspartic acid	38	10
Threonine	25	6
Serine	22	8
Glutamic acid	25	15
Proline	13	8
Glycine	33	14
Alanine	29	9
Valine	36	8
Methionine	5	3
Isoleucine	18	8
Leucine	22	9
Tyrosine	8	3
Phenylalanine	15	5
Tryptophan **	4	1
Total residues	347	129

* Determined as cysteic acid after dimethylsulphoxide oxidation [14].

** Determined according to the method of Liu et al. [15].

Discussion

Unlike all other known NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenases, which are composed of identical subunits, the enzyme from spinach leaves can be dissociated, in sodium dodecyl sulphate, in two non-identical subunits of 37 000 and 14 000 daltons, respectively.

The homogeneity of the purified enzyme, checked by chromatographic and electrophoretic procedures, is also supported by constant amino acid composition and constant ratio in the amounts of the two subunits in different enzyme preparations: both subunits must therefore belong to the enzyme molecule.

The possibility that the small subunit arises from a proteolytic breakdown of the larger subunit can be excluded, since the same relative amount of the two chains is found, when the purification is carried out in the presence of a protease inhibitor; in addition the number of tryptic peptides is in good agreement with the value expected in the case of two subunits differing in sequence.

On the other hand, the enzyme purified in the presence of protease inhibitor showed, on sodium dodecyl sulphate electrophoresis, no trace of a subunit of roughly 50 000 daltons, whose proteolytic splitting might give rise to the two observed subunits.

The two subunits have been isolated in equimolar amounts and a molecular weight of 150 000 has been found for the native enzyme, thus, on the assumption that any anomalous behaviour of the protein on gel filtration is absent, the conclusion to be drawn is that the enzyme is made up by the association of three dimers, each composed of two different subunits. This conclusion is supported by the amino acid composition of the two isolated subunits, which gives the best fit with the amino acid composition of the native enzyme on the assumption of a three large and three small subunits association.

Why the enzyme extracted from spinach leaves is different from the other known glyceraldehyde-3-phosphate dehydrogenases is difficult to argue. Such a diversity could be an artifact however, only on the assumption that the 37 000-dalton subunit is the 'true' enzyme subunit, while the 14 000-dalton subunit is a 'spurious' polypeptide chain which, during the extraction process, aggregates through rather strong molecule-to-molecule interaction, with the 'true' subunit.

Whatever is the structure of the enzyme within the cell, our results indicate that we have extracted from spinach leaves a catalytically active NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase, which is composed of two different subunits.

Acknowledgments

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References

1 Fuller, R.C. and Gibbs, M. (1959) Plant Physiol. 34, 324—329
2 Hageman, R.H. and Arnon, D.I. (1955) Arch. Biochem. Biophys. 57, 421—436

3 Heber, U.W., Pon, G. and Heber, M. (1963) Plant Physiol. 38, 355—360
4 Ogren, W.L. and Krogmann, D.W. (1965) J. Biol. Chem. 240, 4003—4008
5 Schulman, M.D. and Gibbs, M. (1968) Plant Physiol. 43, 1805—1812
6 Yonushot, G.R., Ortwerth, B.J. and Koeppe, O.J. (1970) J. Biol. Chem. 245, 4193—4198
7 Melandri, B.A., Pupillo, P. and Baccarini, A. (1970) Biochim. Biophys. Acta 220, 178—189
8 Pawlizki, K. and Latzko, E. (1974) FEBS Lett. 42, 285—288
9 Brumby, P.E. and Massey, V. (1968) in Biochemical Preparations (Lands, W.E.M., ed.), Vol. 12, pp. 29—41, Wiley, New York
10 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
11 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4404—4412
12 Determan, H. and Maettner, U. (1969) FEBS Lett. 2, 163—165
13 Spackman, D.H. (1967) in Methods in Enzymology (Hirs, C.H.W. ed.), Vol. XI, pp. 3—15, Academic Press, New York
14 Spencer, R.L. and Wold, F. (1969) Anal. Biochem. 32, 185—190
15 Liu, T.Y. and Chang, Y.H. (1971) J. Biol. Chem. 246, 2842—2848
16 Gray, W.R. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, M. eds.), Vol. XXV, pp. 121—138, Academic Press, New York
17 Seydoux, F., Bernhard, S., Pfenninger, O., Payne, M. and Malhotra, O.P. (1973) Biochemistry 12, 4290—4300
18 Duggleby, R.G. and Dennis, D.T. (1974) J. Biol. Chem. 249, 162—166