Plant & Cell Physiol. 25(4): 601–609 (1984)

Purification and Immunochemical Characterization of NADP-Dependent Glyceraldehyde 3-Phosphate Dehydrogenase of *Euglena gracilis*[1]

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NADP-dependent glyceraldehyde 3-phosphate dehydrogenase from *Euglena gracilis* (EC 1.2.1.13) was purified about 170-fold by a two-step procedure involving DEAE-SH cellulose chromatography and affinity chromatography on ADP-Sepharose. The homogeneous enzyme from mildly sonicated cells contained equal amounts of two types of subunits with mol wts of 34,000 (A) and 38,000 (B). The active enzyme had a mol wt 144,000 and is therefore an A_2B_2 tetramer. Enzyme from strongly sonicated *Euglena* cells contained, in addition, a second allomer with a probable A_4 structure. NAD-dependent glyceraldehyde 3-phosphate dehydrogenase, a tetramer with 36,000 mol wt subunits, was unrelated immunologically to the NADP-dependent enzyme although the latter also showed minor NAD-dependent activity. Both isoenzymes of the NADP-linked glyceraldehyde 3-phosphate dehydrogenase, however, were immunologically identical.

Key words: *Euglena gracilis* — Glyceraldehyde 3-phosphate dehydrogenase — Subunits.

The photosynthetic NADP-dependent GAPDH of *Euglena gracilis* appears to be markedly different from similar enzymes of higher plants and green algae (Cerff and Chambers 1979, Ferri et al. 1978, O'Brien et al. 1976, 1979, Goto 1979, Pawlitzki and Latzko 1974, Pupillo et al. 1972, Pupillo and Piccari 1973, Pupillo and Faggiani 1979). The euglenoid NADP-GAPDH shows little or no NAD-linked activity and has a constant mol wt of 136,000 or 150,000 with no tendency to combine with second order tetramers (Grissom and Kahn 1975, Theiss-Seuberling 1980, 1981, Vacchi et al. 1973).

The enzyme of *Euglena gracilis* has been characterized in detail by Grissom and Kahn (1975) and compared with purified NAD-dependent GAPDH from the same species. These authors, however, isolated NADP-GAPDH from chloroplast fragments, a finding that does not agree with previous reports of the localization of this activity in a soluble plastid phase (Smillie 1963, Vacchi et al. 1973). In the procedure reported by me, *Euglena* NADP-GAPDH was purified to homogeneity from a 'soluble' protein fraction and from a particulate fraction whose enzyme was made soluble by prolonged sonication. The electrophoretic and immunological properties of

Abbreviations: G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DTE, dithioerythritol; Servacel DEAE-23-SH, diethyl amino ethyl cellulose, high capacity; BSA, bovine serum albumin; PMSF, phenyl methyl sulfonyle fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; MTT, 3(4,5-dimethylthiazol-2yl) 2,5 diphenyltetrazolium bromide; PMS, phenazine methosulfate; PGA, 3-phosphoglyceric acid.

[1] Dedicated to Prof. Dr. O. H. Volk on his 80th birthday.

both preparations were investigated and compared with those of NAD-dependent GAPDH that had been purified simultaneously from the same cultures. Part of these results have been presented in shortened form (Theiss-Seuberling 1981).

Materials and Methods

Cultures—Euglena gracilis strain Z was grown autotrophically (Cramer and Myers 1952) in 20 liter flasks maintained in a water bath at 21°C. The cultures were aerated with an air–5% CO_2 mixture, which was stirred magnetically and illuminated by two TL 20 W/29 and one TL 20 W/33 Philips fluorescent lamps from both sides (1,000 lux at flask level). About 10^6 cells/ml of algae were harvested by centrifugation. The harvested cells were washed twice by centrifugation (5,000 rpm, 10 min, 4°C) then lyophilized and stored as a dry powder at −20°C until use. (Lyophilizing and storing (or using) the cells immediately after harvest had no effect on the results.)

Assays—Enzyme activities were measured and recorded in the reductive (A) and oxidative (B) directions at 366 nm. Assay A contained 75 μmol Tris-HCl, pH 7.50, 5 μmol PGA, 10 μmol $MgSO_4$, 1 μmol ATP, 0.4 U 3-phosphoglycerate kinase, 1 μmol DTE and 0.24 μmol NADH or NADPH in 1.0 ml. Assay B contained 75 μmol Tris-HCl, pH 8.5, 20 μmol sodium arsenate, 5 μmol EDTA, 3 μmol $NADP^+$ or NAD^+, and 1.5 μmol G3P. The reaction was started with enzyme (plus G3P in reaction B). Protein was determined by A_{280} or by the Coomassie Blue method with BSA as the standard (Bradford 1976).

Enzyme purification—Fifteen gram batches of lyophilized cells were suspended in 20 mM Tris-HCl buffer, pH 8.0 that contained 2 mM EDTA, 2 mM DTE, 0.2 mM PMSF and 10% (v/v) glycerine, after which the suspensions were soincated in the presence of 0.2 mm glass beads. The temperature in each case was maintained below 10°C by dipping the vessels in a salt-ice bath, or the cooling time period was shortened by dipping the vessels periodically and very quickly in liquid nitrogen. Sonication was effected by bath of the two modes: A) in such a way that most plastids remained microscopically intact until osmotically burst ('mild' sonication); B) up to complete disintegration of all membranes ('harsh' sonication). In each experiment light miscroscopy was used to check the rate of disintegration. Sonication for A was ca. 45 s and for B about 20 min. The slurry was centrifuged ($46{,}000 \times g$, 2×10 min) and its supernatant saved. Glycerine was added to 50% (100 ml total volume).

The fluid was applied to a 7-cm diameter column packed with 70 g of Servacel DEAE-23-SH cellulose which had been previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM Na_2EDTA, 1 mM DTE, 0.2 mM PMSF, and 50% glycerine. Although time consuming, the use of glycerine is necessary, as will be shown, to stabilize the enzyme. Fractions of 10 ml were collected. The column was washed with the buffer used for the equilibration until the A_{280} dropped below 0.1, and NADP-GPD subsequently was eluted by starting a linear KCl gradient (0–80 mM). Fractions with the highest activities were pooled and batches were mixed with 2',5' ADP-Sepharose 4B that had been equilibrated with the above buffer. The slurry produced was applied to the column (2×15 cm) and the column washed exhaustively with the same buffer. Pure NADPH-GAPDH was obtained by the use of an $NADP^+$ gradient, 0 to 0.5 mM. Peak fractions were concentrated with a Millipore immersible CX filter (molecular cut off, 10,000 daltons) by vacuum aspiration with a water pump then stored at −20°C.

Electrophoresis—Disc PAGE was performed as described by Davis (1964) with 7.2% acrylamide (2.8% in the concentrating gel). The method of Laemmli (1970) was used for SDS-PAGE. All gels were stained for protein with Coomassie Brilliant Blue R-250. The following mixture was used (25 ml total volume) for substrate staining of the active enzyme: 22.5 ml of 0.05 M Tris-HCl buffer, pH 8.5; 12.5 mg MTT; 24 mg $NADP^+$; 6.2 mg arsenate;

7 mg cystein; 2.5 mg PMS; and 25 μmol of free DL-G3P at pH 7 (prepared from the diethylacetal form by the sulfuric acid method and added at the moment of use). This method was derived from Thurmann's substrate-staining procedure of glutamate dehydrogenase (1965). After 30 min of staining, the gels were fixed in 10% acetic acid and photographed or scanned without delay. Appropriate controls omitting enzmye or the substrate were routinely used.

Immunological techniques—Immunization of the rabbits was by injection of 0.1 mg aliquots (in 0.5 ml water) or purified NADP-GAPDH preparations from 'mild' or 'harsh' sonications that had previously been mixed with the same volume of Freund's adjuvant. This was followed by two similar booster injections 2 and 6 weeks later. Two weeks after the last injection 30 ml of blood was withdrawn, and the serum treated with 18% (w:v) Na_2SO_4 at room temperature. After centrifugation (26,000×*g*, 15 min room temperature) the precipitate was resuspended in 0.15 mM NaCl with 0.001% NaN_3 then stored at −20°C.

Double diffusion experiments were done according to Ouchterlony (1965) with purified NADP-GAPDH, NAD-GAPDH, or clarified cell extracts as the antigen.

For the immunosubtraction experiments, increasing amounts of antiserum against purified NADP-GAPDH (obtained by 'mild' sonication) were mixed with NADP-GAPDH obtained by 'harsh' sonication. Alternatively, antiserum against NADP-GAPDH from 'harsh' sonications was mixed with purified NADP-GAPDH from 'mild' sonication. After 10 min of mixing, the samples were applied to PAGE.

Results

Enzyme stability—NADP-dependent GAPDH of *Euglena* is known to be a very unstable enzyme. Previous attempts to stabilize its activity include the use of $NADP^+$, glycerine and dioxane (Vacchi et al. 1973, Seuberling 1969, Grissom and Kahn 1975). The protective effects of these compounds are given in Table 1; 50% glycerine was nearly as effective as glycerine plus NADPH and was far more effective than either dioxane or NADPH. Consequently, 50% glycerine (without NADPH) was added to all the media. In this way the purified protein or the crude extracts, could be stored for months at −20°C with little loss of activity.

Table 1 Protective effects of some compounds depending on time on NADP-GAPDH activity in the supernatant (46,000×*g*/10 min) of *Euglena* cell extract in 20 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 2 mM DTE

Additions	Activity after	
	2 days	3 days
None	0	0
+Glycerine 50%	3.76	3.94
+Glycerine 20%	3.15	0.61
+Dioxane 10%	1.45	0.48
+NADPH 14 μM	0	0.06
+NADPH 14 μM+glycerine 20%	5.45	4.55
+NADPH 14 μM+glycerine 50%	4.42	4.24

Immediate addition of water, dioxane, glycerine, NADPH, or their combinations, did not affect the activity of the cell extract. The mean value of activity immediately after the addition of a compound (4.24 μmol NADPH/min/10 mg protein) was used as the control value. For comparisons, the enzyme activity was always related to the same protein concentration in the control cell extract (10 mg protein/ml cell extract; this activity was estimated four times with 5 μl of each sample).

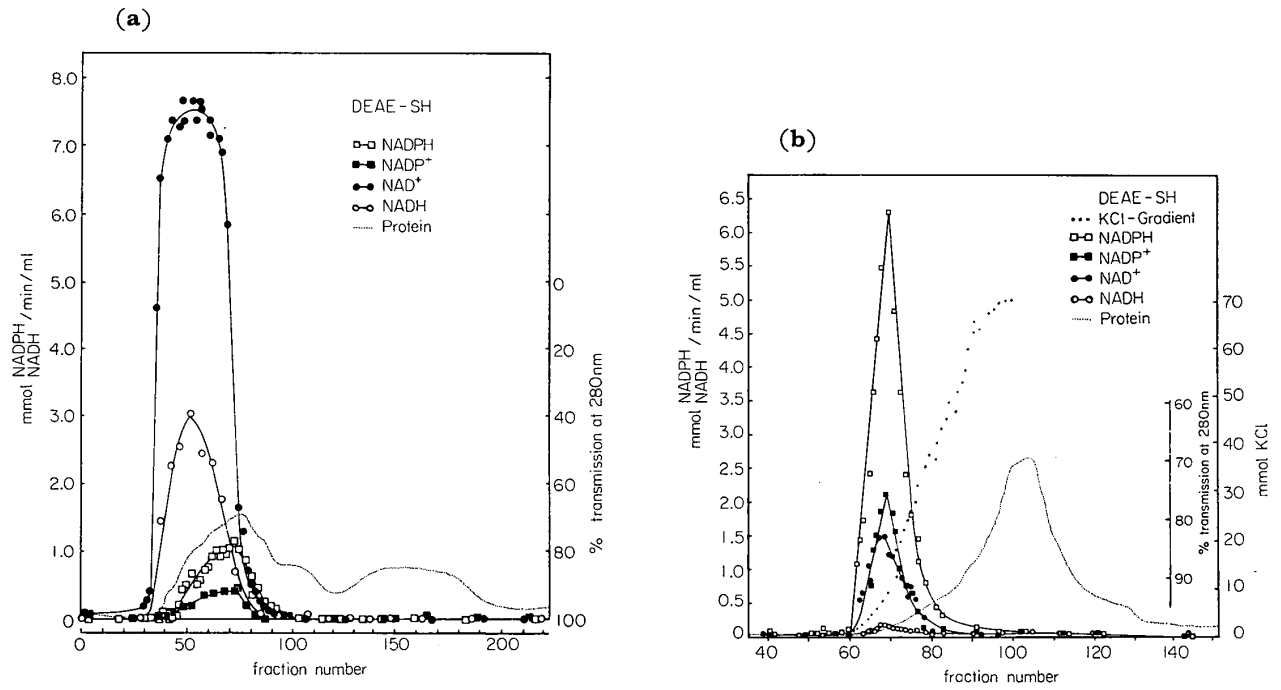

Fig. 1 Elution pattern of a DEAE-SH cellulose column after the addition of clarified *Euglena gracilis* cell extract (a). The same column was washed until neither enzyme activity nor protein was eluted. Then the NADP-GAPDH was eluted with low concentrations of KCl (b).

Purification procedures—Preliminary experiments indicated that Servacel DEAE-23-SH cellulose is a suitable support for the binding of NADP-GAPDH, better than Servacel DEAE-SS or DEAE-SN. This enzyme could be separated from NAD-GAPDH in one step and did not interact with the resin under the conditions described in **Materials and Methods**, being eluted in advance of the bulk of the slightly retarded proteins (Fig. 1a) which usually contained traces of NADP-GAPDH (not due to overloading). The major NADP-GAPDH activity, loosely adsorbed, was eluted by low KCl concentrations (about 20 mM, at the peakcenter, Fig. 1b), together with the lower NAD-dependent activity.

The final purification of NADP-GAPDH was by single affinity chromatography on ADP-Sepharose. The strongly bound activity eluted by a $NADP^+$ gradient, which again was coincident with a minor NAD-dependent peak (Fig. 2). The specific activity was increased about 170-fold. This protocol has been followed successfully many times with both 'mildly'

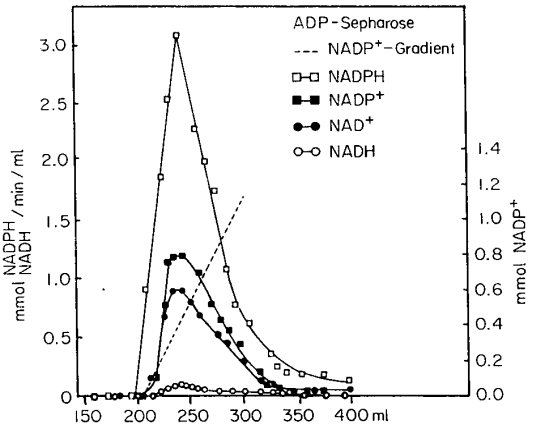

Fig. 2 Affinity chromatography of NADP-GAPDH with ADP-Sepharose. The enzyme was applied to 2′,5′ ADP-Sepharose 4B and, after extensive washing, was eluted with an NADP-gradient.

and 'harshly' sonicated material. Results were the same after additions of PMSF to all the purification steps. PMSF is believed to prevent bacterial destruction of NADP-GAPDH during purification. As purification in the presence of PMSF gives two NADP-GAPDHs with different molecular weights, then the GAPDH with the larger molecular weight (isoenzyme 1) could not, due to bacterial destruction, be a precursor of that with the smaller molecular weight (isoenzyme 2).

Properties of NADP-GAPDH—Enzyme purity and molecular weight were investigated by disc PAGE, SDS-PAGE and Sephadex exclusion chromatography. The results depended partially on the mode of cell breakage. When cells were mildly sonicated, avoiding extensive destruction of their organelles, the purified enzyme yielded one protein band on PAGE (Fig. 3a) which coincided with activity staining (Fig. 3b). Two bands of similar intensity were produced by SDS-PAGE (Fig. 4a) that had mol wts of 34,000 (band A) and 38,000 (band B) as determined by the method of Weber et al. (1972). The standard markers used were a mixture of cytochrome c, hemoglobin, trypsin inhibitor from soybean, lactate dehydrogenase and pyruvate kinase as well as a mixture of lysozyme, myoglobin, chymotrypsinogen, ovalbumin and bovine serum albumin. The measured size of the active isoenzyme 1 protein was about 144,000, which corresponds to an A_2B_2 tetramer. The molecular weight was estimated by Sepharose 6B

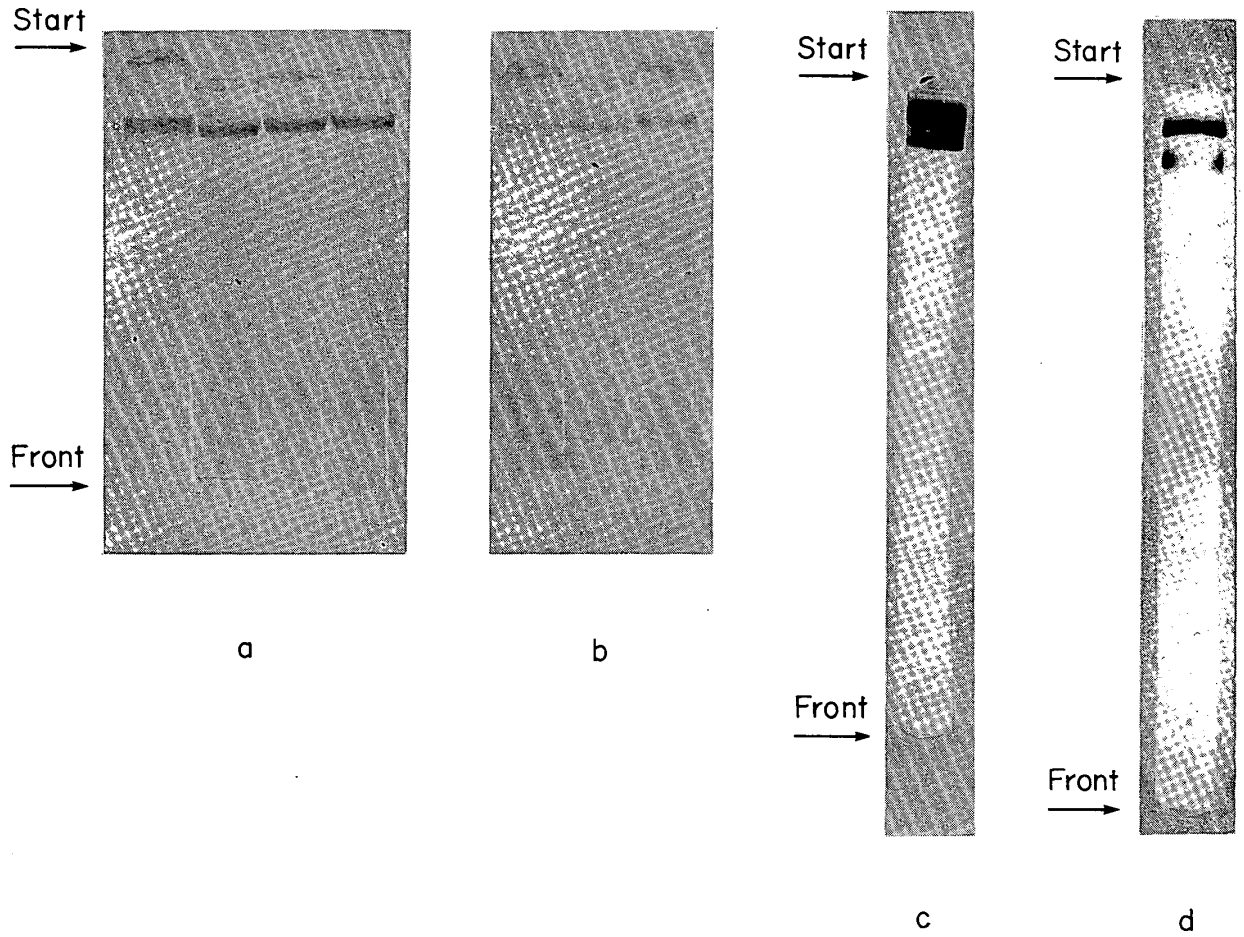

Fig. 3 PAGE pattern of purified NADP-GAPDH. (a) Purified enzyme from mildly sonicated cells. Protein band stained by Coomassie Blue. (b) Activity staining coincides with the protein band. (c) Isoenzymes of NADP-GAPDH from cells ruptured in the 'harsh' mode. Isoenzyme 2 is somewhat retarded in comparison to isoenzyme 1. Both bands have NADP-activity (d). a and b, as well as c and d, were coelectrophorized. The origin on the PAGE plates is indicated by an arrow.

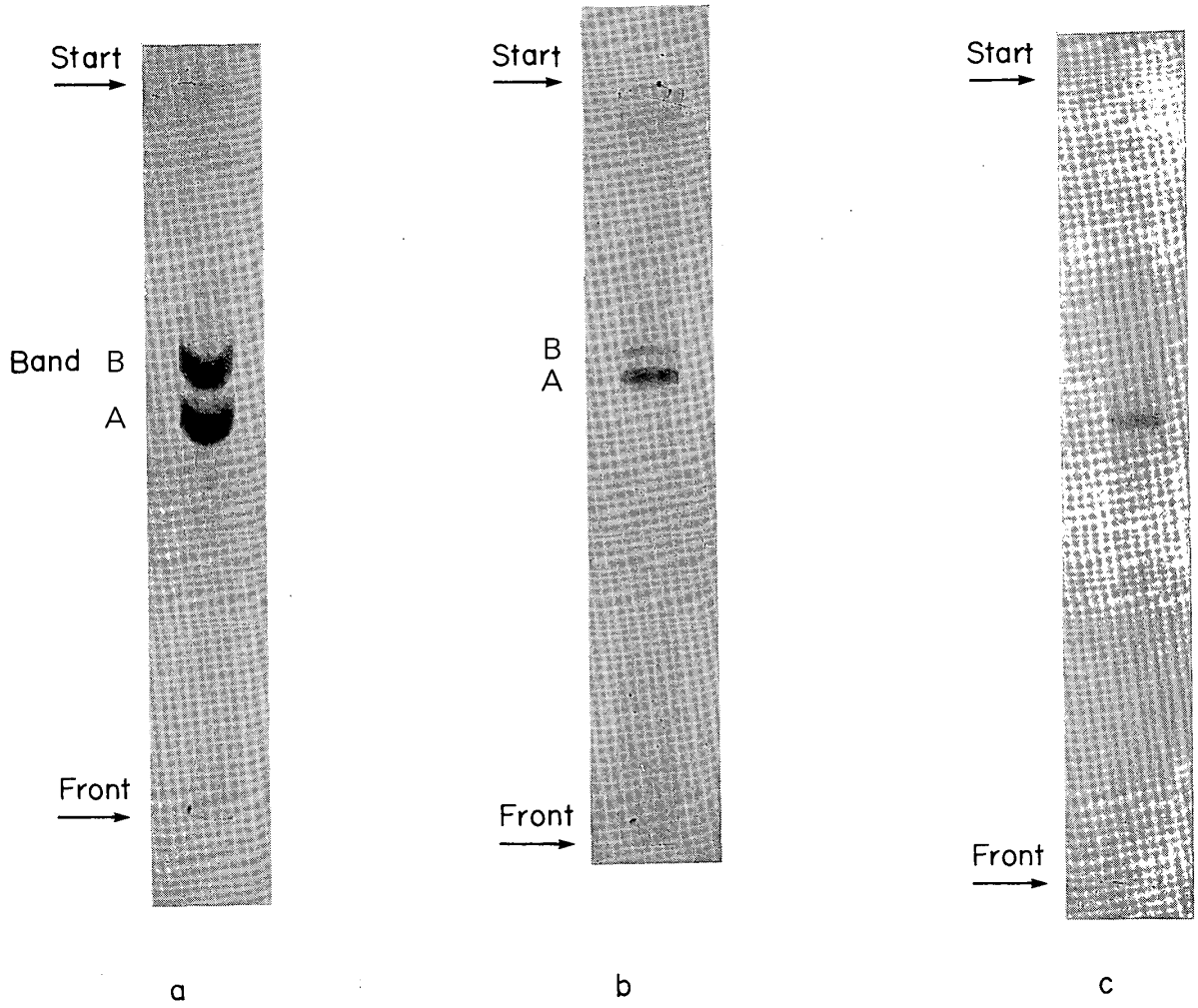

Fig. 4 Subunit patterns of purified NADP-GAPDH and NAD-GAPDH, separated by Dodecylsulfate polyacrylamide gel electrophoresis. (a): NADPH-GAPDH isoenzyme 1. The two bands have similar intensities. (b): NADP-GAPDH isoenzyme 1 and isoenzyme 2. The stain intensity of band A exceeds that of band B. (c): With NAD-GAPDH only a single electrophoretic band is visible. Proteins were stained with Coomassie Brilliant Blue.

chromatography (protein markers; cytochrome c, peroxidase, yeast glyceraldehyde 3-phosphate dehydrogenase and ferritin).

A second isoenzyme was found with PAGE after rupturing *Euglena* cells in the 'harsh' mode (see **Materials and Methods**) and following the above procedure. Isoenzyme 2 was somewhat retarded in PAGE in comparison to isoenzyme 1. Both bands contained enzyme activity (Fig. 3, c and d). SDS-gel electrophoresis showed the same two bands for isoenzyme 1 alone; but, the intensity of the band A stain exceeded that of band B (Fig. 4b). Therefore, isoenzyme 2 may have an A_4 (or possibly an A_3B_1) structure.

NAD-GAPDH—As testified to by PAGE separation, this cytosolic enzyme was essentially pure after the Servacel DEAE-SH cellulose step (Fig. 1a) rendering further purification unnecessary. The single electrophoretic band was not identical to that of NADP-GAPDH as ascertained by coelectrophoresis (Fig. 5), although the mol wt values were similar (140,000, determined by Sepharose 6B and Sephadex G200 chromatography). A single subunit with a mol wt of 36,000 was found by SDS gel electrophoresis (Fig. 4c).

Immunochemical characterization—NADP-GAPDH isoenzyme 1 produced a sharp precipitate line with its antibody (Fig. 6). Serum from non-immunized rabbits formed no band. Extracts

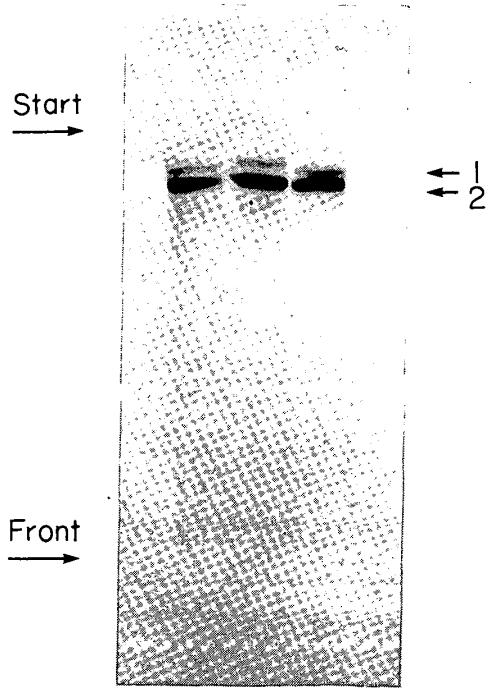

Fig. 5

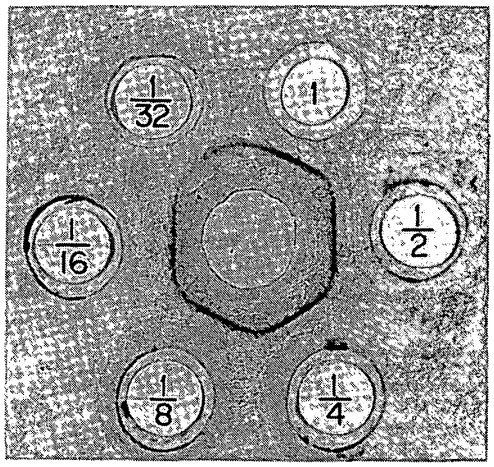

Fig. 6

Fig. 5 PAGE of NADP-dependent GAPDH and of NAD-dependent GAPDH. Band 1, NADP-GAPDH; band 2, NAD-GAPDH; stained for protein.

Fig. 6 Immunological characterization of NADP-GAPDH from *Euglena gracilis* on Ouchterlony double diffusion plates. The center contained 1 mg of purified NADP-GAPDH isoenzyme 1, the outer wells antiserum raised against the enzyme. The well numbered 1 contained 20 μl of antiserum, produced as described (cf. **Materials and Methods**: immunological techniques), the other wells the given dilutions of antiserum.

of green cells also yielded one band, regardless of the type of sonication. Similar behavior was shown by the antiserum against NADP-GAPDH from 'harsh' sonication (a mixture of isoenzymes 1 and 2). Therefore, isoenzymes 1 and 2 are immunologically identical. The same conclusion was drawn from results of immunosubtraction experiments with PAGE. When antiserum against isoenzyme 1 was allowed to react with both isoenzyme 1 and 2, the increase in antiserum was reflected directly in the formation of a single antigen-antibody complex band in the gel. Of course, serum immunized against both isoenzymes also gave one band at a sufficiently high titer. Hence, it was confirmed that isoenzyme 2 could not be a contaminant because that would result in a second complex band with its own antibody. Also, this enzyme shared common determinants with isoenzyme 1 (or, isoenzyme 2 is simply another form of isoenzyme 1) that had been formed by protease action or association with lipid from the 'membrane'.

Discussion

Euglena NADP-GAPDH has relatively few properties in common with the extensively characterized enzyme from higher plants and Chlorophyta (Cerff and Chambers 1979, Ferri et al. 1978, Goto 1979, O'Brien et al. 1976, 1979, Pawlitzki and Latzko 1974, Pupillo et al. 1972, Pupillo and Piccari 1973, Pupillo and Faggiani 1979, Theiss-Seuberling 1980, 1981). Major differences include: (1) the *Euglena* enzyme's inability to assemble a specific high molecular weight form that in higher plants seems to be associated with a reversible decrease in NADP-dependent activity; (2) the extreme lability of its activity in air; and (3) the presence of very

low NAD-dependent activity (Theiss-Seuberling 1981, Vacchi et al. 1973), or of no NAD-activity (Grissom and Kahn 1975), which contrasts with the substantial NAD-activity of the chloroplast dehydrogenase from green tissues and green algae. I have shown, however, that minor NAD-dependent activity is a property of the homogeneous *Euglena gracilis* protein.

The structure of the *Euglena* enzyme resembles that of higher plant chloroplast GAPDH (Cerff and Chambers 1979) in that two isoenzymes are present. Isoenzyme 1 has an A_2B_2 structure, and isoenzyme 2 a probable A_4 structure, based on their respective calculated mol wts of 144,000 and 136,000. Isoenzyme 2 was obtained only after the thorough breakdown of the membranes. This is interpretable either in terms of strong binding to the thylakoids or of selective denaturation of the B-subunits and the reassociation of A monomers to the 136,000 mol wt tetramers. The former view appears more likely and is supported by the finding (Grissom and Kahn 1975) that purified *Euglena* NADP-GAPDH, isolated from chloroplast fragments had an A_4 structure of mol wt 133,000.

The NADP-GAPDH of green unicells such as *Scenedesmus* (O'Brien et al. 1976, 1979) and *Chlorella* (Ahrenhöfer 1979) may consist of a single type of subunit. This may sound like evolutionary nonsense, but the selective isolation of either A_4 or A_2B_2 structures cannot be excluded on the basis of present results.

Isoenzymes 1 and 2 of NADP-GAPDH are related immunologically and are sharply distinct from the soluble NAD-GAPDH of the same organism. This, again, is consistent with the situation in higher plants, making it improbable that NADP-GAPDH is derived from cytosolic GAPDH simply by post-translational modification as was concluded earlier from physiological and inhibition experiments (Hudock and Fuller 1975, Cerff 1974). The two-step purification of NADP-GAPDH containing one or both variants and its stabilization, as well as the one-step procedure for NAD-GAPDH, show promise of aiding in the further biochemical and developmental characterization of this class of enzyme in *Euglena*.

I thank Professor, Dr. K. Zetsche in whose department this research was done and Professor Dr. P. Pupillo (Modena) for his collaboration and critical readings of the manuscript. I also am grateful to Professor Dr. K. Altland for instructing me in the method of immunosubstraction. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Habilitandenstipendium).

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(Received October 13, 1982; Accepted March 21, 1984)