Plant Physiol. (1978) *61*, 369–372

Glyceraldehyde-3-Phosphate Dehydrogenase (NADP) from *Sinapis alba* L.

REVERSIBLE ASSOCIATION OF THE ENZYME WITH A PROTEIN FACTOR AS CONTROLLED BY PYRIDINE NUCLEOTIDES *IN VITRO*[1]

Received for publication October 5, 1977 and in revised form November 21, 1977

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ABSTRACT

Aggregation of glyceraldehyde-3-P dehydrogenase (NADP) (EC 1.2.1.13) from *Sinapis alba* seedlings during gel filtration on Sepharose 6B is dependent on the presence of a fraction ("binding fraction") which can be separated from the enzyme by precipitation with 55% ammonium sulfate. Association of the enzyme with this binding fraction is NAD-dependent whereas $NADP^+$ causes release. Dithioerythritol (2 mM) has no influence on these reversible processes.

Binding fractions, partially purified by ammonium sulfate and acetone fractionation, were submitted to dodecylsulfate-polyacrylamide gel electrophoresis. They always contain one or two dominant polypeptides with apparent molecular weights 42,000 and 58,000. The 42,000 polypeptide co-migrates during dodecylsulfate electrophoresis with the corresponding subunit of the enzyme. It comprises up to 70% of the total protein in partially purified binding fractions and can be regarded as a major protein in seedling extracts.

The differential transport behavior of glyceraldehyde-3-P dehydrogenase (NADP) on Sephadex G-200 in the presence of NAD^+ and $NADP^+$ can be used as a simple and effective purification procedure. The enzyme isolated in this way has an isoelectric point of about 4.5 and maintains under all tested conditions a heterogeneous subunit composition of at least three different polypeptide chains (apparent molecular weights, 39,000, 42,000, 43,000).

The present data suggest that NAD(P)-controlled aggregation of glyceraldehyde-3-P dehydrogenase (NADP) from *Sinapis alba* L. is due primarily to enzyme association with a separate binding fraction rather than to enzyme polymerization. It is possible that a major component of this binding fraction, the 42,000 polypeptide, consists of "surplus" nonactive enzyme subunits, which self-associate and interact with the NAD-conformer of the enzyme.

Pupillo and Giuliani-Piccari (10, 11) have reported that purified glyceraldehyde-3-P dehydrogenase (NADP) from spinach polymerizes in the presence of NAD^+, whereas $NADP^+$ causes dissociation into "protomers." The results of a preceding investigation (1) are compatible with this concept inasmuch as they indicate that the enzyme from *Sinapis alba* seedlings exists in two distinct conformations in the presence of NAD(H) and NADP(H), respectively. The NAD-conformer was found to interact with Sepharose 6B, but it could not be shown to polymerize after the enzyme had been purified by means of transport retardation on Sepharose 6B (fraction S). In crude or partially purified extracts, however, association was also readily demonstrated for the *Sinapis* enzyme. This indicates the possibility that in the *Sinapis* system NAD-conformers associate with a separate "binding fraction" which is lost during purification. The present investigation is a first attempt to characterize the composition of this binding fraction.

MATERIALS AND METHODS

Column Isoelectric Focusing. Electrofocusing of the enzyme was performed at 300 v for 72 hr at 10 C in a 110-ml LKB column (LKB 8101) as described by the manufacturer. The column contained 2% carrier ampholytes (pH 3–10), 2 mM dithioerythritol, 1 mM EDTA, and 0.20 mM $NADP^+$ in a 5 to 50% sucrose gradient. The enzyme sample was applied in 2 ml of electrofocusing medium during the filling of the column. Two mg of $NADP^+$ was added twice to the cathode solution (10 ml of 0.25 N NaOH) at the beginning of the experiment and after 24 hr.

Densitometric Readings. These were conducted with a Joyce Loebl Chromoscan (MK II) from photographs of the polyacrylamide gels using the reflectance technique (as described in the instruction manual of the manufacturer). All further details are described under "Materials and Methods" in reference 1.

RESULTS

Experiment 1 (Fig. 1) demonstrates that the association of glyceraldehyde-3-P dehydrogenase (NADP) from far red light-grown mustard seedlings is dependent on a fraction which can be separated from the enzyme by precipitation with 55% ammonium sulfate (crude binding fraction). After removal of this fraction the enzyme activity travels considerably slower during NAD filtration on Sepharose 6B (compare profiles A and B with C in Fig. 1). The enzyme fractions in Figures 1, B and C (enclosed by the vertical dashed lines) were pooled, concentrated, and refiltered under the same conditions as before. For B this treatment leads to a strong concentration of the enzyme in the heavy fraction eluting ahead of the bulk protein (Fig. 2A), whereas for C co-migration of the enzyme with the bulk protein is observed (profile not shown).

Figure 2 shows the reversibility of enzyme association with the crude binding fraction as affected by pyridine nucleotides. Binding fraction and enzyme were dissociated by NADP filtration and then recovered separately (FI and FII in Fig. 2B). NAD filtration of a mixture of FI and FII gives a profile (Fig. 2C) which is essentially identical to Figure 2A, whereas NAD filtration of FII alone leads to retarded enzyme elution, indicating interaction of dissociated enzyme molecules with Sepharose 6B (1).

In Figure 3 a dilute 40 to 85% ammonium sulfate fraction (10 mg/column instead of 100 to 200 mg, as in the filtrations of Fig. 1) was submitted to NAD filtration on Sepharose 6B together with

[1] This work was supported by the Deutsche Forschungsgemeinschaft (Habilitandenstipendium and SFB 46).

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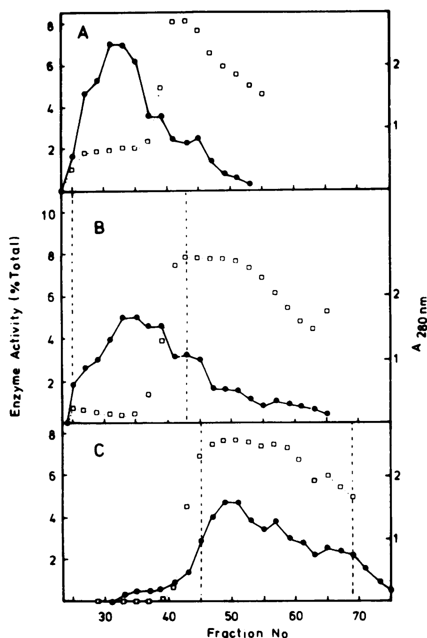

FIG. 1. Ammonium sulfate precipitation of a crude binding fraction. To three separate seedling extracts solid ammonium sulfate was added to give 40% (A), 50% (B), and 55% (C) saturation, respectively. The precipitate was removed by centrifugation (30 min at 20,000*g* for A and B, 2 hr at 20,000*g* for C) and the supernatant enzyme was recovered by a second precipitation with 85% ammonium sulfate. This fraction (100–200 mg of protein) was dissolved in filtration buffer (0.1 M Tris-Cl, pH 7.4, containing 0.20 mM NAD^+, 2 mM dithioerythritol, and 1 mM EDTA) and filtered with 0.20 mM NAD^+ on Sepharose 6B (1.6 × 70 cm). The enzyme fractions enclosed by the vertical dashed lines in B and C were pooled (for each profile separately), concentrated, and refiltered under the same conditions (see Fig. 2A and text). Fraction volume: 1.8 ml. Activity recoveries (A–C): 54, 70, 59%. (●——●): NADP-activity; (□ · · · □): UV profile (280 nm).

four marker proteins. Under these conditions 65% of the total recovered NADP activity eluted as a homogeneous aggregate of mol wt ≈800,000 and about 25% traveled as a void volume aggregate (mol wt ≥4 million). All peaks also have NAD activity (= NAD-GPD II[2] [1, 2]).

In an attempt to characterize the molecules responsible for enzyme association, the enzyme was partially purified by ammonium sulfate (55–85% saturation) and subsequent acetone fractionation (0–50%, v/v [1]). Enzyme purified in this way contains enough binding material to permit NAD aggregation on Sephadex G-200. Such an aggregate was recovered and separated into a binding fraction (partially purified binding fraction) and dissociated enzyme by NADP filtration on Sephadex G-200 as shown in Figure 2, A and B (for Sepharose 6B). In a subsequent binding test it was ascertained that the partially purified binding fraction fully restores the aggregation capacity of the enzyme on Sephadex G-200, which is lost after NADP filtration. All three species (associated enzyme, dissociated enzyme, and partially purified binding fraction) were submitted to dodecylsulfate electrophoresis on gradient polyacrylamide slab gels (1, 12). As shown in Figure 4 the associated enzyme (channel 2 in A and profile B) and the dissociated enzyme (channel 3 in A and profile C) both contain primarily polypeptides of apparent mol wt 39,000, 42,000, and 43,000. The 39,000 and 42,000 bands both seem to be divided into two individual polypeptides with very similar mol wt, so that altogether five separate polypeptides can be distinguished. The major differences between the two patterns is a quantitative one, in that the associated enzyme contains more 42,000 protein than

[2] Abbreviations: GPD: glyceraldehyde-3-P dehydrogenase; LDH: lactate dehydrogenase.

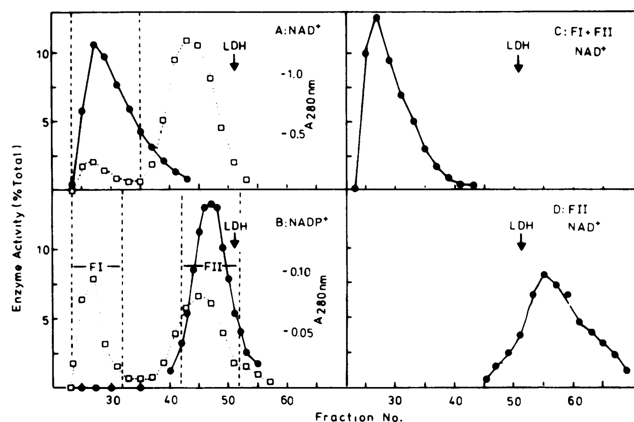

FIG. 2. Association and release of the enzyme with the crude binding fraction as effected by pyridine nucleotides. Profile A: refiltration of the pooled enzyme fractions from Figure 1B. The enzyme fractions in A (enclosed by the vertical dashed lines) were again pooled, concentrated, and filtered with 0.05 mM $NADP^+$ (B). The binding fraction (FI) and the enzyme (FII) were recovered separately and the $NADP^+$ present in the eluate was exchanged for NAD^+ (0.20 mM) by filtration on a small Sephadex G-25 column. C: NAD filtration of a mixture of FI and FII. D: NAD filtration of FII alone containing an equal amount of protein. Filtration medium, column size, and fraction volume as in Figure 1. Recoveries of NADP activity (A–D): 86, 102, 94, 97%.

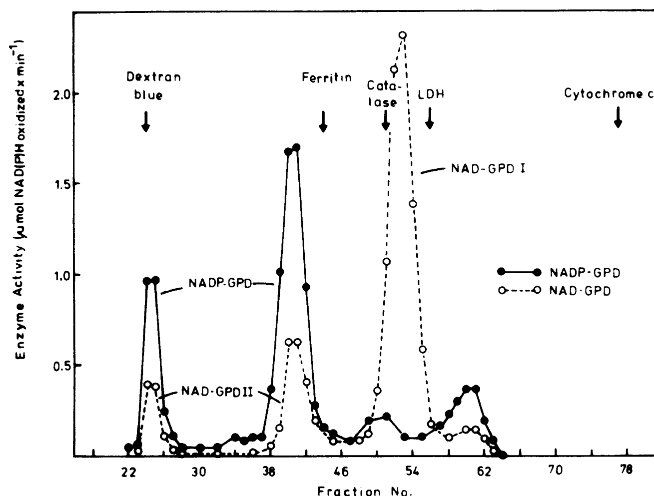

FIG. 3. Aggregation of the enzyme in dilute crude preparations. A dilute 40 to 85% ammonium sulfate fraction (10 mg of protein) was filtered on a Sepharose 6B column (2.5 × 100 cm) in the presence of 0.20 mM NAD^+ together with the four marker proteins Cyt *c* (12,500), lactate dehydrogenase (145,000), catalase (240,000), and ferritin (540,000). The void volume was determined with dextran blue in a separate filtration. Filtration medium: as in Figure 1 but without dithioerythritol. Fraction volume: 6 ml. The recoveries were 47 and 62% for NADP and NAD activity, respectively.

the dissociated enzyme (compare densitometric profiles of Fig. 4, B and C). As expected, this protein is *the* major component of the partially purified binding fraction (channel 4), comprising about 60 to 70% of the total protein content. The binding fraction also includes a minor band which comigrates with the 43,000 subunit of the enzyme. Purification of the associated enzyme by affinity chromatography on NAD-Sepharose (1) removes the surplus 42,000 protein but not the residual part, which can only be eluted together with the enzyme activity (channel 5, Fig. 4A). For the polypeptide pattern in channel 1 a crude binding fraction (40–55% ammonium sulfate fraction) was filtered on Sephadex G-200 and the protein traveling in the void volume (the peak fraction of the

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UV profile) was submitted to dodecylsulfate electrophoresis. There is a major component of apparent mol wt 42,000 in this 40 to 55% ammonium sulfate fraction, the latter containing at least 20% of the total soluble protein in extracts.

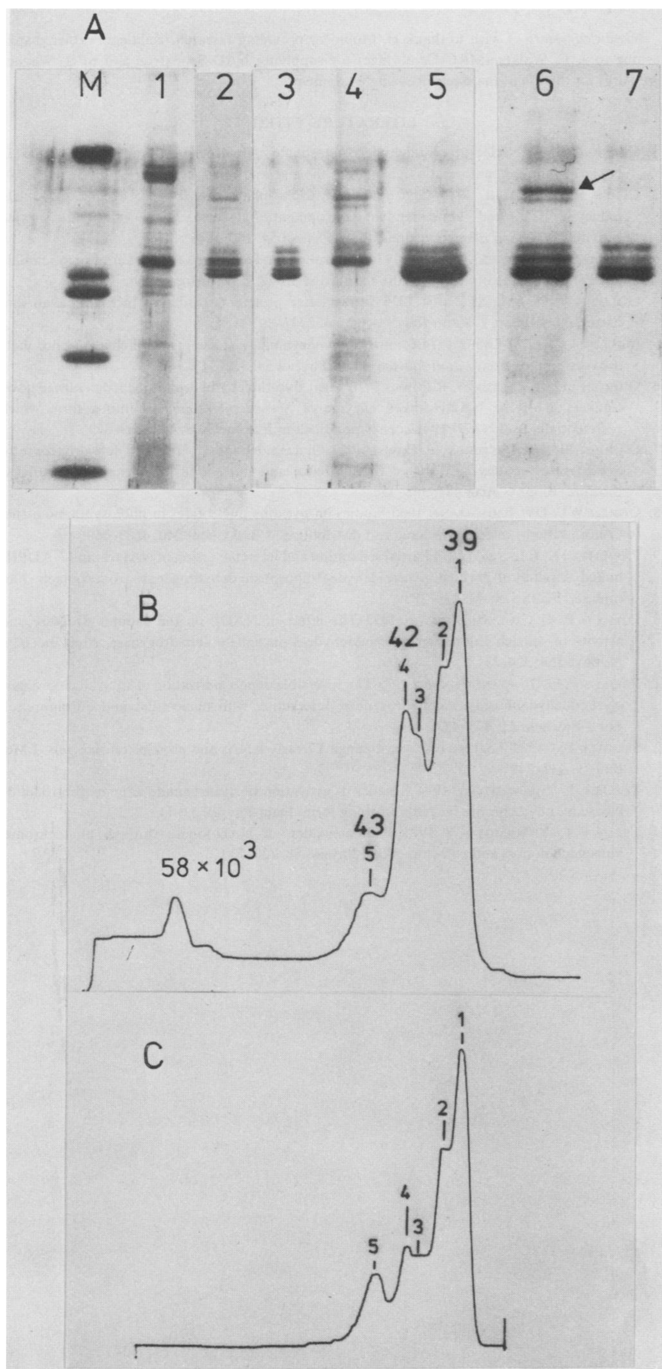

FIG. 4. Polypeptide composition of the partially purified binding fraction as compared to the enzyme subunit composition. A: dodecylsulfate gradient polyacrylamide gel electrophoresis of associated enzyme (channel 2), dissociated enzyme (channel 3), and partially purified binding fraction (channel 4). Channel 5: associated enzyme after affinity chromatography on NAD-Sepharose. Channel 1: crude binding fraction (see text). Channels 1 to 5: far red light-grown seedlings. Channels 6 and 7: associated and dissociated enzyme from white light-grown seedlings. Channel M: marker proteins (from top to bottom): BSA, 68,000; aldolase, 40,000; glyceraldehyde-3-P dehydrogenase (NAD) (muscle), 36,000; chymotrypsinogen A, 25,000; Cyt *c*, 12,500. B and C: densitometric readings of patterns 2 and 3 in A, respectively (see under "Materials and Methods").

All preceding experiments were performed with preparations derived from far red light-grown seedlings. To exclude far red light artifacts the experiments leading to the findings of Figure 4 (channels 1–5) were repeated for seedlings grown under continuous fluorescent white light for 10 days. The results (channels 6 and 7 of Fig. 4A) show that the subunit pattern of the enzyme is independent of the light quality and that the associated enzyme from white light grown seedlings (channel 6) contains surplus 42,000 protein and another major polypeptide of about 58,000 daltons (channel 6, arrow) which are lost together with the aggregation capacity after NADP filtration (channel 7). The 58,000 polypeptide is also present (although in smaller amounts) in enzyme aggregates and in the partially purified binding fraction from far red light-grown seedlings (channels 2 and 4 in A and profile B).

Figure 5 contains the result of a column electrofocusing experiment with the dissociated enzyme from white light-grown seedlings. The enzyme migrates as a single peak with an isoelectric point of about 4.5. The polypeptide pattern of the recovered peak fractions from this experiment (not presented) is identical to that shown in channel 7 of Figure 4A.

DISCUSSION

The present results indicate that NAD-dependent aggregation of glyceraldehyde-3-P dehydrogenase (NADP) from *S. alba* reflects primarily association of the enzyme with a separate binding fraction rather than effector-controlled enzyme polymerization, as reported for the enzyme from spinach (10, 11) and *Scenedesmus obliquus* (6, 7). The present findings should not be confused with the associative phenomena described for the spinach enzyme by Pawlitzki and Latzko (9). These were observed in the absence of pyridine nucleotides and were accompanied by an 80% loss in enzyme activity.

The electrophoretic analysis of partially purified binding fractions suggests that enzyme association is due to a specific protein-protein interaction rather than to unspecific interactions of the enzyme with various macromolecules and particles. The formation of aggregates of a distinct mol wt (800,000, Fig. 3) in dilute crude preparations also supports this view. It has been repeatedly shown that binding fractions from extracts, partially purified by ammonium sulfate and acetone fractionation, contain only one or two dominant polypeptides with apparent mol wt of 42,000 and 58,000. These polypeptides have no NAD(P) activity and they seem to self-associate in their native form, since they always travel as large aggregates during gel filtration, even after they have been purified

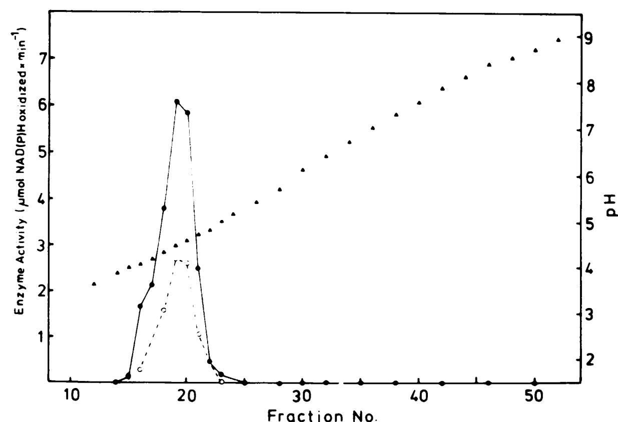

FIG. 5. Column isoelectric focusing of dissociated enzyme (white light-grown seedlings). Electrofocusing of 400 μg of enzyme was performed with pH 3 to 10 ampholytes as described under "Materials and Methods." (●——●); NADP activity; (○---○): NAD activity; (▲▲▲▲): pH gradient. Recovery of NADP activity: 58%.

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to about 80% (channel 4 of Fig. 4A). The 42,000 polypeptide is a major protein of seedling extracts (channel 1 of Fig. 4A). Since it co-migrates during dodecylsulfate electrophoresis with the corresponding subunit of the enzyme, it is even possible that it represents surplus nonactive subunits of glyceraldehyde-3-P dehydrogenase NADP).

The present *in vitro* findings may be related to the light (phytochrome)-mediated regulation of the enzyme *in vivo*. A light (phytochrome)-triggered conversion of NAD(H) to NADP(H) is well documented (3, 8, 13, 14) and it is also probable that this conversion takes place inside the chloroplast (8), the microenvironment of the enzyme. Furthermore, there is increasing evidence from studies with mammalian cells and bacteria (4) that proteins susceptible to intracellular degradation are characterized by abnormal conformations and also by their tendency to aggregate or interact with certain surfaces *in vitro*. In view of the structural analogies with the present enzyme it is tempting to speculate that proteolytic degradation of the enzyme occurs mainly in the dark with the abnormal NAD conformation, whereas the NADP conformation (in the light) is largely protected against intracellular proteolysis. The findings of Pupillo and Giuliani-Piccari (10, 11) that aggregates of the spinach enzyme show a decrease in NADP relative to the NAD activity could not be confirmed for the *Sinapis* enzyme. This may be due to immediate dissociation of the associated *Sinapis* enzyme in assay mixtures.

The differential transport behavior of the enzyme during gel filtration in the presence of NAD^+ and $NADP^+$ can be used as a simple and effective purification procedure. The enzyme isolated in this way contains at least three different polypeptide chains (with apparent mol wt 39,000, 42,000, and 43,000) which cannot be removed by affinity chromatography or isoelectric focusing. This argues against the possibility that the observed subunit heterogeneity is in whole or in part an isolation artifact.

Pawlitzki and Latzko (9) found two different subunits for the spinach enzyme with apparent mol wt 39,000 and 42,000. Recent investigations in this laboratory by de Looze (unpublished data) showed two separate subunits for the enzyme from *Chenopodium rubrum*, which co-migrate on dodecylsulfate gels with the 39,000 and the 43,000 subunits of the mustard seedling enzyme. According to McGowan and Gibbs (5) the enzyme from *Pisum sativum* migrates as a single band during dodecylsulfate electrophoresis. Together these data indicate that the subunit composition of glyceraldehyde-3-P dehydrogenase (NADP) varies considerably among higher plants.

Acknowledgments—I wish to thank H. Mohn for providing research facilities. Further thanks are due to J. E. Walker (MRC Cambridge) for supplying NAD-Sepharose and to E. Wagner (Freiburg) for the use of the electrofocusing equipment.

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