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GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF *SCENEDESMUS OBLIQUUS*

EFFECTS OF DITHIOTHREITOL AND NUCLEOTIDE ON COENZYME SPECIFICITY

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Summary

NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.—) of the photosynthetic alga *Scenedesmus obliquus* is converted to an NADPH specific form by incubation with dithiothreitol. The change in nucleotide specificity is accompanied by a reduction in the molecular weight of the enzyme from 550 000 to 140 000. Prolonged incubation with dithiothreitol results in the further dissociation of the enzyme to an inactive 70 000 dalton species. The 140 000 dalton, NADPH-specific enzyme is stabilized against dissociation and inactivation by the presence of NAD(H) or NADP(H). Optimum stimulation of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activity is achieved on incubation of the NADH-specific enzyme with dithiothreitol and NADPH, or dithiothreitol and a 1,3-diphosphoglycerate generating system.

The relevance of these observations to in vivo light-induced changes in the nucleotide specificity of the enzyme is discussed.

Introduction

A number of plant enzymes involved in photosynthetic carbon assimilation have been shown to undergo rapid activation by light in vivo. NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.—) [1—3], fructose-1,6-diphosphatase [4,5], sedoheptulose-1,7-diphosphatase [5], $NADP^+$-dependent malate dehydrogenase [6] and phosphoribulokinase [7,8] are all subject to such activation. In contrast three catabolic enzymes (NADH-dependent

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glyceraldehyde-3-phosphate dehydrogenase [3], glucose-6-phosphate dehydrogenase [9—11] and phosphofructokinase [12]) are inactivated by light. In almost all of these cases the in vivo effect of light can be mimicked by in vitro incubation with dithiothreitol.

The NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase is probably the most extensively studied of these light-activatable enzymes. The addition of NADPH to broken spinach chloroplasts causes an allosteric activation of the NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activity and dithiothreitol doubles the extent of this activation but cannot be substituted for NADPH [3]. Later work has shown that $NADP^+$ or ATP can also increase the NADPH-dependent activity at the expense of that linked to NADH [13]. The kinetic properties of some activated forms of the NADPH-dependent enzyme have been studied in detail. For example, spinach leaf glyceraldehyde-3-phosphate dehydrogenase has a molecular weight of 600 000 and dissociates into subunits of 140 000 in the presence of nucleotides. This dissociation is accompanied by a hundred fold decrease in the K_m for $NADP^+$, whilst the K_m for NAD^+ and the maximum velocity with each coenzyme remains constant [14,15]. In contrast the enzyme from pea chloroplasts has a molecular weight of 140 000 and there is no evidence of higher molecular weight forms [16]. This enzyme is activated by dithiothreitol and the activation probably involves a simple conformational change [17] resulting in an increase in the V, values with NADP(H) as coenzymes. However, the V values for the NAD(H)-dependent reactions and the K_m values for all substrates remain unaffected [18].

We have isolated two distinct glyceraldehyde-3-phosphate dehydrogenases from the green alga, *Scenedesmus obliquus* [19,20]. One enzyme, the T-enzyme, has a molecular weight of 140 000 and the ratio of V with NADH as coenzyme to that with NADPH is 0.15. By contrast the D-enzyme has a molecular weight of 550 000 and a V ratio (NADH : NADPH) of 16.2. Incubation of the D-enzyme with a 1,3-diphosphoglycerate generating system causes dissociation into a species similar to the T-enzyme with respect to molecular weight and V ratio [21]. We now report a detailed study of the effect of dithiothreitol and nucleotides on the glyceraldehyde-3-phosphate dehydrogenase of *S. obliquus*.

Methods

Enzyme isolation and estimation

NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (D-enzyme) was isolated from *Scenedesmus obliquus* and estimated in the photosynthetic direction (i.e. with 1,3-diphosphoglycerate and reduced coenzyme as substrates) as described previously [21].

Enzyme activation

(a) For kinetic measurements. 0.3 unit of the purified D-enzyme in 20 ml of 85 mM Tris · HCl, pH 7.5, was incubated at 27° C with the compounds specified. At various times during the activation 0.5 ml aliquots were removed from the activation mixture and the remaining components necessary for enzyme assay were added. The activity was measured after starting the reaction with

reduced pyridine nucleotide. The concentration of activators used were: dithiothreitol (18 mM) and pyridine nucleotide (1.3 mM). When a 1,3-diphosphoglycerate generating system was used for activation [21] either cysteine · HCl (4.5 mM) or dithiothreitol (18 mM) was also present.

The 1,3-diphosphoglycerate generating system comprised [21]: ATP (1.6 mM), $MgCl_2$ (1.6 mM), 3-phosphoglycerate (3.5 mM) and phosphoglycerate kinase (10 units/ml) in the Tris · HCl buffer.

An enzyme unit is defined as the quantity of enzyme transforming 1 μmol of substrate per minute at 25° C.

(b) For ultracentrifugation. D-enzyme (2 mg/ml) in 0.1 ionic strength (120 mM) Tris · HCl, pH 7.5, was dialysed for 2 h at 27° C against the same buffer containing NADPH (1.3 mM) and dithiothreitol (18 mM). Following this treatment the sedimentation behaviour of the enzyme was examined in the analytical ultracentrifuge. The NADH and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activities of the enzyme in a parallel incubation were also measured. These properties of the enzyme were re-examined after storage overnight at 4° C and also following dialysis of the stored preparation against the Tris · HCl buffer to remove NADPH and dithiothreitol.

When dithiothreitol alone was the activator, D-enzyme (3.8 mg/ml) in 0.1 ionic strength (120 mM) Tris · HCl pH 7.5 was incubated with 18 mM dithiothreitol at 4° C. These conditions were chosen to cause a slow activation of the enzyme. At specified times a portion of the incubation mixture was taken for examination of the sedimentation behaviour of the enzyme and for determination of the coenzyme dependence of its activity.

Analytical ultracentrifugation

Analytical ultracentrifugation was conducted in a Beckman-Spinco Model E analytical ultracentrifuge equipped with monochromator and photoelectric scanner on the absorption optical system. All experiments were performed using an AN-D rotor and double sector cells with Epon-aluminium centrepieces and sapphire windows. The temperature was always 10° C. Sedimentation velocity experiments were performed at 60 000 rev./min and the schlieren optical system was employed. Low speed sedimentation equilibrium experiments were performed according to the method of Van Holde and Baldwin [22] using a 3 mm solution column. High speed meniscus depletion experiments were carried out according to Yphantis [23].

A value of 0.74 ml/g was assumed for the partial specific volume of the enzyme and data on the densities and viscocities of the solutions were obtained from International Critical Tables.

Results

(1) Ligand-induced changes in the coenzyme-dependence of the glyceraldehyde-3-phosphate dehydrogenase of S. obliquus.

Incubation of the D-enzyme with 18 mM dithiothreitol resulted in an increase in the NADPH-dependent activity over the first 20 min and a subsequent slow decline in activity on prolonged incubation (Fig. 1). The stimulation of activity produced by dithiothreitol was enhanced and stabilized by

NAD(H) or NADP(H) and no decline in activity accompanied prolonged incubation (Fig. 1). Moreover, NADP(H) were more effective than NAD(H) and the reduced forms were more effective than the corresponding oxidised nucleotides in stimulating activity (Fig. 1). A 1,3-diphosphoglycerate generating system and cysteine also promoted an increase in NADPH-dependent activity which, by contrast with the dithiothreitol dependent stimulation, resulted in maximum activation after 50 min. The activity then remained constant and showed no tendency to decline (Fig. 1). Activation by 1,3-diphosphoglycerate was inhib-

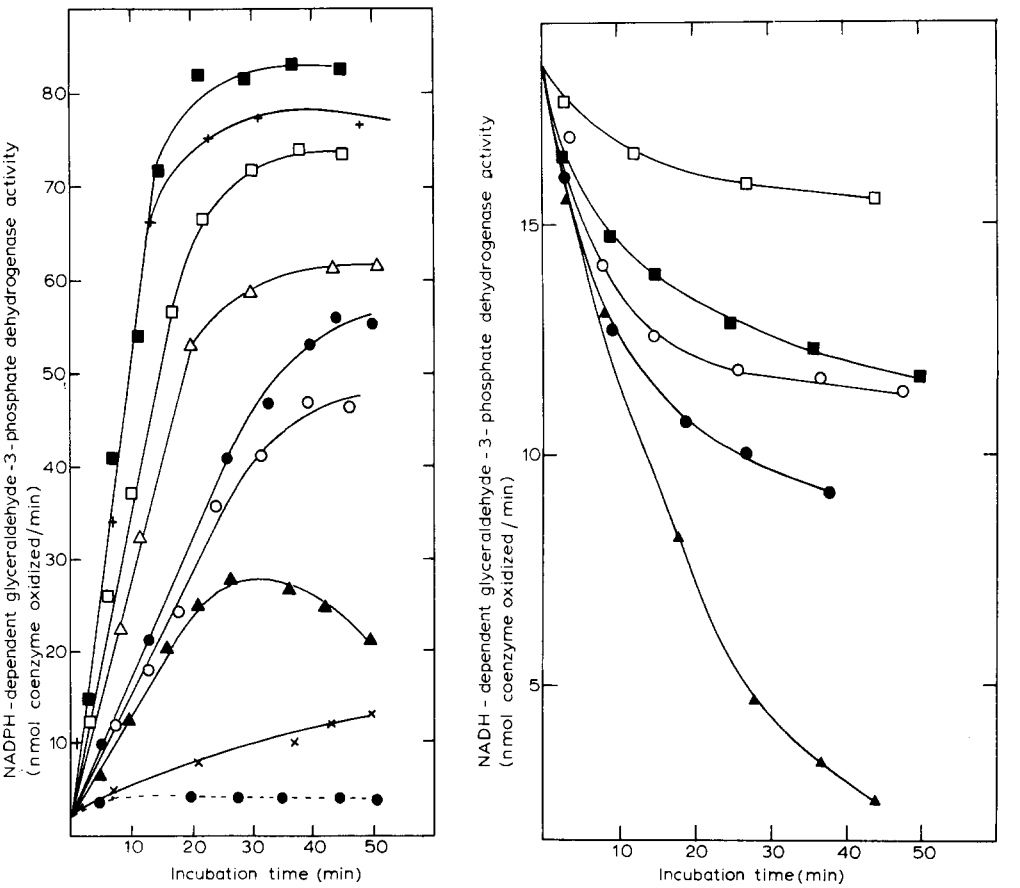

Fig. 1. The effects of thiols, nucleotides and a 1,3-diphosphoglycerate generating system on the NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activity of the D-enzyme of *S. obliquus*. D-enzyme was incubated with dithiothreitol (18 mM) or cysteine (4.5 mM) and the nucleotides specified (1.3 mM) as described under methods. The time-course of changes in the NADPH-dependent activity was determined. The activators were: dithiothreitol alone (▲———▲); dithiothreitol and NAD^+ (○———○); dithiothreitol and $NADP^+$ (□———□); dithiothreitol and NADH (●———●); dithiothreitol and NADPH (■———■); cysteine and NADPH (X———X); 1,3-diphosphoglycerate generating system and cysteine in the presence of (●- - - -●) and absence of NAD^+ (△———△); 1,3-diphosphoglycerate generating system and dithiothreitol (+———+).

Fig. 2. The effect of ligands on the NADH-dependent glyceraldehyde-3-phosphate dehydrogenase activity of the D-enzyme of *S. obliquus*. Dithiothreitol alone (▲———▲); dithiothreitol and NADPH (■———■); cysteine and NADPH (□———□); 1,3-diphosphoglycerate generating system and cysteine (○———○); 1,3-diphosphoglycerate generating system and dithiothreitol (●———●).

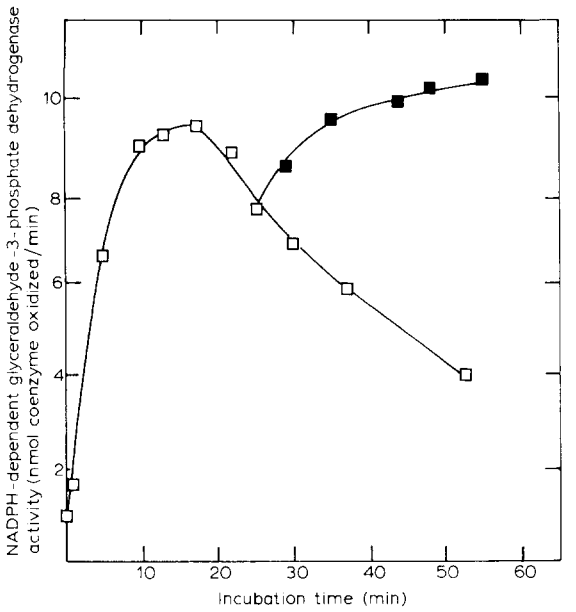

Fig. 3. NADPH-induced stimulation of the dithiothreitol-promoted activation of the NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activity of the D-enzyme of *S. obliquus*. Enzyme was incubated with dithiothreitol (18 mM) as described in methods. After 25 min incubation, NADPH (1.3 mM) was added to a portion of the incubation mixture. NADPH-dependent activity in the presence of dithiothreitol (□——□); and in the presence of dithiothreitol and added NADPH (■——■).

ited rather than stimulated by NAD (Fig. 1) and a similar effect was noted for NADP.

The effect of thiols on the stimulation of the NADPH-dependent activity of the enzyme by either NADPH or the 1,3-diphosphoglycerate generating system is also shown in Fig. 1. Incubation of the enzyme with NADPH in the presence of cysteine only resulted in a slight stimulation of NADPH-dependent enzyme activity and this contrasted with the massive stimulation occasioned by NADPH and dithiothreitol. When the 1,3-diphosphoglycerate generating system was used to promote the NADPH-dependent activity, dithiothreitol was again the more effective accompanying thiol. However in this case the difference in effect of the two thiols was much less marked. In all cases when there was stimulation of the NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activity there was also a decrease in the NADH-dependent activity (Fig. 2). This decrease in activity was most pronounced when the enzyme was incubated with dithiothreitol alone. The decline in NADPH-specific activity occasioned by prolonged incubation with dithiothreitol could be reversed by the addition of NADPH (Fig. 3).

(2) Changes in the sedimentation characteristics accompanying ligand-induced changes in coenzyme dependence of glyceraldehyde-3-phosphate dehydrogenase activity.

(A) Activation by incubation with NADPH and dithiothreitol. In order to examine the effects of activation on the molecular properties of the enzyme

the rates of oxidation of NADPH and NADH by the native D-enzyme measured and the sedimentation behaviour of the enzyme preparation was determined by ultracentrifugation. The same parameters were re-determined immediately after activation with NADPH and dithiothreitol and again after standing overnight at 4° C. The untreated D-enzyme had an $s_{20,w}$ of 14.2 S and its enzyme activity was predominantly linked to NADH. After the activation there was no boundary corresponding to the 14.2 S species seen previously, but two boundaries of $s_{20,w}$ = 7.4 S and $s_{20,w}$ = 4.4 S were now present, these changes were in fact

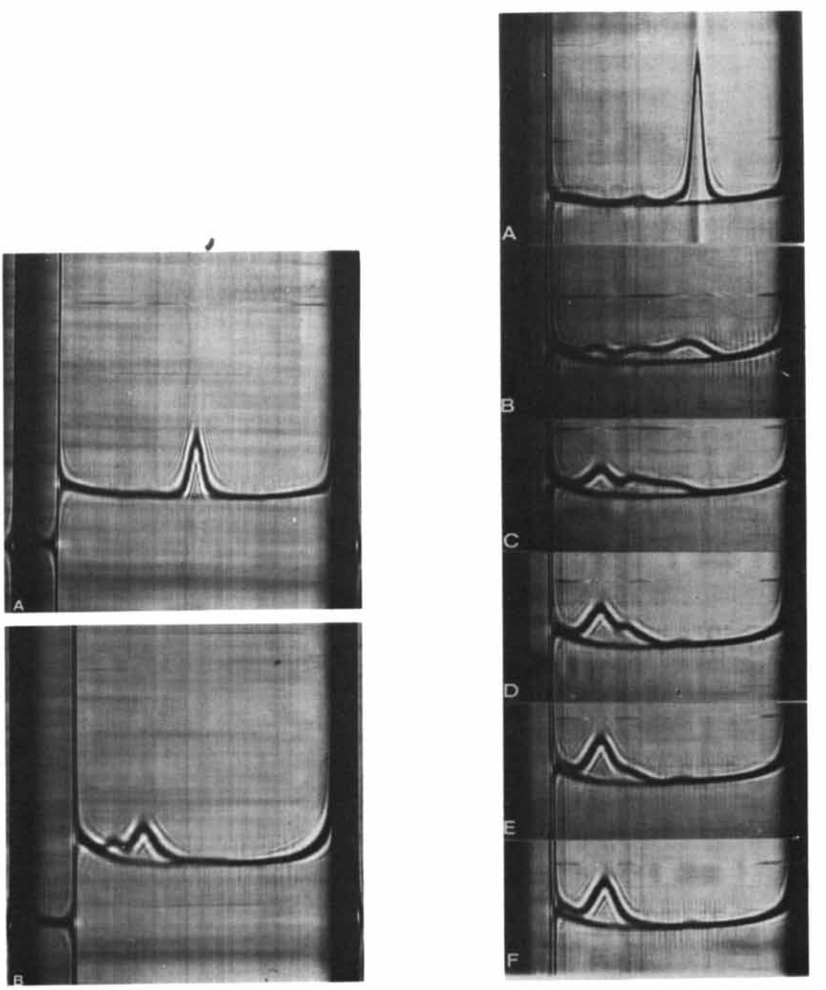

Fig. 4. The effect of dithiothreitol and NADPH on the sedimentation behaviour of the D-enzyme of *S. obliquus*. Photographs were all taken 40 min after reaching a speed of 60 000 rev./min at a schlieren analyser angle of 60°. A Native D-enzyme B. After 2 h dialysis against dithiothreitol (18 mM) and NADPH (1.3 mM) at 27° C followed by 18 h storage at 4° C.

Fig. 5. The effect of dithiothreitol on the sedimentation behaviour of the D-enzyme of *S. obliquus*. Photographs were all taken 46 min after reaching a speed of 60 000 rev./min at a schlieren analyser angle of 60°. Preincubation in the absence of dithiothreitol (18 mM) (A) and at the following times during incubation, 1.33 h (B), 4.5 h (C), 22 h (D), 74 h (E), 290 h (F).

TABLE I

Effect of incubation with NADPH (1.3 mM) and dithiothreitol (18 mM) on the coenzyme dependence of the glyceraldehyde-3-phosphate dehydrogenase activity of the D-enzyme of *S. obliquus*.

Stage	Glyceraldehyde-3-phosphate dehydrogenase activity (units/ml)	
	NADPH-linked	NADH-linked
Native D-enzyme	7	70
After 2 h dialysis against NADPH and dithiothreitol	192	40
After storage of dialysed extract overnight at 4°C	180	39
After dialysis of stored extract against Tris · HCl, pH 7.5	109	23

complete after 2 h (Fig. 4). The enzyme activity was now predominantly linked to NADPH, which is typical of the T-enzyme (Table I). No reversal of the activity or sedimentation changes occurred either on storage of the enzyme or on dialysis to remove NADPH and dithiothreitol. The weight-average molecular weight of the NADPH and dithiothreitol treated sample was calculated to be 144 000 by low speed sedimentation equilibrium and when determined by the Yphantis high speed equilibrium technique [21], which ensures that the meniscus is depleted of all but the lightest component, the weight-average molecular weight was calculated to be 75 000. This probably implies that the 7.4 S and 4.4 S species correspond to a 140 000 dalton tetramer and 75 000 dalton dimer, respectively. However, the molecular weight of the dimer is probably overestimated owing to the presence of a substantial amount of tetramer.

(B) Activation by incubation with dithiothreitol. Prolonged incubation of the D-enzyme with dithiothreitol in the absence of nucleotides eventually resulted in the loss of the glyceraldehyde-3-phosphate dehydrogenase activity (see above). It was possible that this treatment was producing merely the 4.4 S species which was thought to be an inactive form of the enzyme.

The effect of incubation of the D-enzyme with 18 mM dithiothreitol at 4°C

TABLE II

Effect of incubation with dithiothreitol (18 mM) on the coenzyme dependence of glyceraldehyde-3-phosphate dehydrogenase activity of the D-enzyme of *S. obliquus*.

Time of incubation with dithiothreitol (h)	Glyceraldehyde-3-phosphate dehydrogenase activity (units/ml)	
	NADPH-linked	NADH-linked
0	10	115
1.3	166	74
4.5	108	74
22	38	64
74	7	52
290	5	8

on the sedimentation characteristics and coenzyme dependence are shown in Fig. 5 and Table II. Following incubation with dithiothreitol for 1.3 hours, sedimentation velocity determination indicated the presence of three boundaries of $s_{20,w}$ 13.4 S, 8.2 S and 4.6 S. Apart from the slowest sedimenting boundary (4.6 S) these do not correspond to any seen previously. This may be due to the complexity of the situation which undoubtedly involves several dissociation equilibria. At this time the enzyme activity was predominantly linked to NADPH. Further incubation with dithiothreitol resulted in a progessive decrease in activities and a decrease in the concentrations of the 13.4 S and 8.2 S species in favour of the 4.6 S species (Fig. 5, Table II). Eventually the enzyme was present almost exclusively as the 4.6 S form and was inactive. At this stage the molecular weight obtained by high speed equilibrium centrifugation was 70 000. During the period of inactivation of the enzyme (1.3 h onwards) the NADPH-dependent activity declined more rapidly than that linked to NADH.

Discussion

The present investigation demonstrates that the activity of the D form of glyceraldehyde-3-phosphate dehydrogenase with NADPH as coenzyme is transiently stimulated by incubation with dithiothreitol, followed by a decline in both the activities with NADH and NADPH (Figs. 1 and 2, Table II). This stimulation of NADPH-dependent activity is promoted by pyridine nucleotides while the long term decline in activity is blocked by these substances (Fig. 3, Table I). This is similar to the situation seen with the spinach chloroplast enzyme [3], which is optimally stimulated by NADPH in the presence of dithiothreitol. The essential difference between the D-enzyme of *S. obliquus* and that of spinach chloroplast is the fact that the NADPH-dependent activity of the latter is promoted additionally by ATP while that of the former is stimulated by 1,3-diphosphoglycerate [3,20,21].

The time-course of the changes in the NADPH-dependent activity promoted by dithiothreitol in the absence of nucleotides probably results from the sum of two effects. Initially the D-enzyme is converted to a form resembling the native T-enzyme (T′) and this conversion is accompanied by the decline in NADH- and increase in NADPH-dependent activities (Figs. 1 and 2). On prolonged incubation, the reduction in specific activity with either nucleotide probably results from long term inactivation of the T′-form when exposed to dithiothreitol. The role of nucleotide in this process is probably to stabilise the activated form (T′) against this further reduction and inactivation.

The D-enzyme has a molecular weight of 550 000, and consists of sixteen subunits of 35 000, whilst the T-enzyme is made up of four similar subunits [19]. The present data therefore can be most readily explained if the promotion of NADPH-dependent activity is accompanied by dissociation of the hexadecamer to a tetramer (T′) similar in properties to the T-enzyme. The eventual inactivation of the enzyme might result from irreversible formation of dimer by further dithiothreitol-dependent reduction of the protein. These ideas are consistent with the changes in sedimentation coefficient and molecular weight observed (Fig. 5) and with previous observations on the stimulation of

NADPH-dependent activity by 1,3-diphosphoglycerate [21]. However, when 1,3-diphosphoglycerate produced activation the changes were reversible [21]. The role of nucleotide in the activation process would be to stabilise the tetramer. At no stage during this investigation has a sedimentation boundary corresponding to a discrete octamer, intermediate in size between the D- and T-enzymes, been observed. However, the sedimentation coefficients observed on treatment of the D-enzyme with dithiothreitol (Fig. 5) imply that other dissociation equilibria may be involved in the activation process. It must be concluded that if octamer is formed as an intermediate between the D- and T'-enzymes, its dissociation to the T'-form of the enzyme is extremely rapid. The foregoing views are summarized in Fig. 6.

The detailed mechanism of the reductive activation of the D-enzyme by dithiothreitol is not yet established. However, it is notable that both 1,3-diphosphoglycerate and glyceraldehyde-3-phosphate may be covalently bound to the enzyme through a thiolester linkage [24]. Possibly treatment of the enzyme with powerful thiol-based reducing agents such as dithiothreitol removes bound substrate or nucleotide and thereby promotes a conformational transition within the protein. This process might involve the reduction of either inter- or intrasubunit disulphide bonds or disulphide exchange. The suggestion that the D- to T'-enzyme transition might be accompanied by displacement of bound nucleotide is supported by observations on the 1,3-diphosphoglycerate-promoted D- to T'-enzyme conversion [21]. This process is blocked by either free nucleotide ($NAD(P)^+$) or inorganic phosphate [21] and is compatible with the sequential mechanism reported by Harrigan and Trentham for the muscle enzyme [25]. According to this scheme acylation of the enzyme by 1,3-diphosphoglycerate is followed sequentially by release of phosphate and the bound NAD^+, thus permitting nucleotide exchange through the acyl-enzyme intermediate. This is supported by the recent finding of Seydoux et al. [26] working with the sturgeon enzyme that NAD^+ is more loosely bound to the acyl-enzyme than to the free enzyme and that site-site anti-cooperative effects are abolished by acylation. Thus in the sturgeon enzyme acylation is accompanied

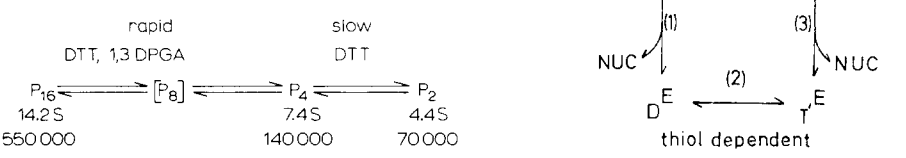

Fig. 6. Scheme for the depolymerization of *S. obliquus* glyceraldehyde-3-phosphate dehydrogenase accompanying changes in nucleotide specificity.

Fig. 7. Scheme for the thiol-dependent interconversion of the D- and T'-forms of *S. obliquus* glyceraldehyde-3-phosphate dehydrogenase. The generation of the active, NADPH-dependent enzyme ($_T$'E) from the NADH-dependent enzyme ($_D E^{Nuc}$) can be achieved by loss of bound nucleotide (reaction 1) followed by thiol-dependent conformation change involving depolymerisation (reaction 2). Alternatively in the presence of a dithiol (e.g. dithiothreitol) depolymerisation (reaction 4) might occur prior to loss of nucleotide (reaction 3).

by decreased affinity for oxidized nucleotide and significant alteration of subunit interactions. The D- to T′-enzyme conversion promoted by 1,3-diphosphoglycerate requires a thiol containing reducing agent such as cysteine [21]. The present study demonstrates that an effective means of promoting a D- to T′-enzyme transition is by incubation of the enzyme with a 1,3-diphosphoglycerate generating system and dithiothreitol. This may imply that in the presence of a mild reducing agent such as cysteine the conversion involves a thiol-dependent conformation change in the enzyme following acylation and release of bound nucleotide. An alternative route to the T′-enzyme form would be through a direct promotion of the conformational transition by dithiothreitol. This is summarized in Fig. 7. Release of bound nucleotide on acylation (reaction 1) would be followed by a thiol-dependent conformation change (reaction 2). Normally reaction 4 would be regarded as a kinetically unimportant route for the transition but in the presence of dithiothreitol the rate of this reaction would become significant. On the basis of the present data no comment on the relative stabilities of apo- and holoenzyme in the D- and T′-configurations can be made. However, it should be pointed out that the proposed scheme does not require preferential binding of nucleotide to either conformation of the protein. It merely implies that the conformational change is more rapid in the apoenzyme.

It is well documented that changes in the activity of some photosynthetic enzymes promoted by dithiothreitol resemble those produced in vivo by light (e.g. ref. 27). In higher plants the action spectrum for the light-induced stimulation of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase activity resembles the absorption spectrum of chlorophyll [3] and, since this activation is inhibited by dichloromethylurea, photosynthetic electron flow has been considered necessary for activation [3]. Therefore it is probable that a chloroplast component produced by photosynthetic electron flow acts as a reducing agent in vivo to produce the changes which we have observed in vitro with dithiothreitol. The most likely candidates for such a role are reduced ferredoxin, which has been shown to be necessary for the stimulation of fructose-1,6-diphosphatase activity [4], and photoproduced vicinal dithiols [28]. Dithiols could be produced directly by the photoreduction of a protein disulphide bond and such a photoreducible chloroplast protein has been isolated [29]. However, it is also possible that light-induced conformational changes of the chloroplast membrane result in exposure of chloroplast protein thiols [30] which could act as the in vivo modulators of enzyme activity.

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