The dark (oxidized) form of the light-activatable NADP-malate dehydrogenase from pea chloroplasts is catalytically active in the presence of guanidine-HCl

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The dark form of NADP-malate dehydrogenase from pea leaves which has been shown to contain one disulfide bridge per subunit does not exhibit any catalytic activity in the absence of thiol reducing agents. Upon reduction of these disulfide bridges the enzyme becomes catalytically active. In this presentation, however, it is shown that the oxidized dark form of NADP-malate dehydrogenase becames catalytically competent when assayed in the presence of 200–250 mM guanidine-HCl. This guanidine-dependent activity of the oxidized enzyme is characterized by higher apparent K_m values for the substratres as compared to the reduced enzyme, but is still specific for NADPH. Up to 25% of the V_{max} of the reduced enzyme was obtained for the oxidized guanidine-activated NADP-malate dehydrogenase. The results suggest that the reduction of the regulatory disulfide is not essential for catalytic activity.

NADP-malate dehydrogenase *Guanidine hydrochloride* *Pea chloroplast* *Light-activatable enzyme* *Oxidized-enzyme catalytic activity*

1. INTRODUCTION

Chloroplast NADP-malate dehydrogenase (as well as some other chloroplast enzymes, e.g., fructose-1,6-bisphosphatase) is activated when leaves or isolated chloroplasts are illuminated [1] in a process dependent upon the photosynthetic electron flow [2]. A similar activation can also be achieved by incubation with dithiothreitol or reduced thioredoxin [3,4].

It has been shown that the appearance of catalytic activity in the enzyme (pea) is linked to the reduction of a single cystine per subunit [5]. Purified oxidized NADP-malate dehydrogenase (maize) has no detectable catalytic activity (less than 0.01% of the activity of reduced enzyme) [6]. Therefore, it seems plausible that one or both thiol groups are essential for catalytic activity of NADP-malate dehydrogenase. However, it is not yet known whether these regulatory thiols are required to participate in catalysis or whether they merely enable the enzyme to adapt a conformation capable of catalysis. The reduced NADP-malate dehydrogenase is apparently completely inhibited by a variety of thiol reagents including alkylating agents, mercurials and arsenicals [6]. Notably in the case of the homologous NAD-malate dehydrogenase from *B. subtilis* [7] and from bovine heart [8] thiol groups are clearly not required for catalytic activity. By contrast to NADP-malate dehydrogenase oxidized chloroplast fructose-1,6-bisphosphatase, another light-activatable enzyme, has significant catalytic activity, but reduction further increases that activity [9]. The problem as to whether the thiols of NADP-malate dehydrogenase are involved in catalysis could be resolved if it were possible to show that the oxidized enzyme possessed catalytic activity in some circumstances.

Here we present data which show that in the presence of guanidine-HCl, but with no thiol reducing agent, the oxidized NADP-malate dehydrogenase from pea has about a quarter of the catalytic activity of that of the reduced enzyme.

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2. MATERIALS AND METHODS

NADP-malate dehydrogenase was purifed from pea (*Pisum sativum* L., cv. Kleine Rheinländerin) plants as described in [10]. Fully reduced NADP-malate dehydrogenase was obtained by incubation with partially purified pea thioredoxin and 10 mM DTT in 0.1 M Tris-HCl buffer (pH 8.0) at room temperature under nitrogen atmosphere. From the time course of activation maximum activity could be determined. The standard assay for NADP-malate dehydrogenase activity contained (if not indicated otherwise): 0.1 M Tris-HCl buffer, 1 mM EDTA, 0.2 mM NADPH and 2 mM oxaloacetate. Guanidine-HCl was purchased from Fluka and solutions were prepared in 0.1 M Tris-HCl and adjusted to pH 8.0.

3. RESULTS AND DISCUSSION

Oxidized NADP-malate dehydrogenase purified from pea leaves does not exhibit any detectable catalytic activity when assayed under standard conditions. However, inclusion of the denaturant guanidine in the assay mixture could elicit some catalytic activity, even in the absence of a thiol reducing agent. This activity is dependent upon both oxaloacetate and NADPH and, moreover, as might be expected from the Haldane relationship [11], guanidine also stimulated the reaction in the reverse direction. The optimal stimulation was obtained in the presence of 200–250 mM guanidine (fig.1). Higher concentrations of guanidine inhibited the activity of the oxidized enzyme, presumably due to successive unfolding of the polypeptide chain. The maximum catalytic activity (assayed with the standard assay for the reduced enzyme) represented about 13% of the activity of the enzyme which had been completely reduced by DTT-reduced thioredoxin and assayed in the absence of any guanidine. When elevated concentrations of substrates (0.5 mM NADPH, 5 mM oxaloacetate were included in the assay, the activity obtained was 23% of the reduced enzyme. The V_{max} of the oxidized guanidine-activated enzyme was about one quarter of that of the reduced enzyme in the absence of guanidine.

Although the reduced enzyme is maximally active in the standard assay, there is a stimulation of the guanidine-activated oxidized NADP-malate dehydrogenase when assayed at elevated substrate concentrations (fig.1), suggesting that the apparent k_m values for the substrates are higher than those of the reduced enzyme. Table 1 shows the K_m values obtained for various forms of the enzyme. K_m values for both NADPH and oxaloacetate of the guanidine-activated oxidized enzyme were in-

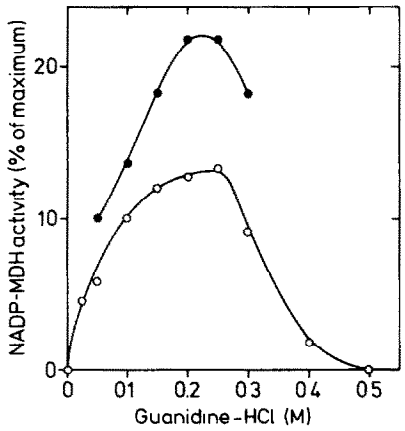

Fig.1. Purified NADP-malate dehydrogenase from pea was assayed in the presence of increasing amounts of guanidine at pH 8.0. No reducing agent was present. The concentration of the substrates was either 0.2 mM NADPH and 2 mM oxaloacetate (○) or 0.5 mM NADPH and 5 mM oxaloacetate (●). The maximum activity (100%) refers to the activity of an equal amount of NADP-malate dehydrogenase activated by DTT-reduced thioredoxin and assayed in the absence of guanidine-HCl.

Table 1

Apparent K_m values for the substrates as determined for oxidized and reduced NADP-malate dehydrogenase from pea.

	K_m (mM)	
	Oxaloacetate	NADPH
Oxidized + 250 mM guanidine	0.32	0.22
Reduced	0.048	0.039
Reduced + 200 mM guanidine	0.077	0.095

The standard conditions were used, but either the oxaloacetate concentration (0.01–2.0 mM, at 0.2 mM NADPH) or the NADPH concentration (0.01–0.3 mM, at 2 mM oxaloacetate) was varied

creased about 6-fold when compared to the reduced enzyme in the absence of guanidine, or 3-fold when compared to the reduced enzyme in the presence of guanidine. The pH optima for the catalytic activity of both, the guanidine-activated oxidized and the reduced enzyme occurred at pH 8.

Since the oxidized NADP-malate dehydrogenase contains one thiol group per subunit which is not accessible under non-denaturing conditions [5], we wanted to avoid the possibility that guanidine caused exposure of this thiol and that two such subunits reduced the regulatory disulfide of a third subunit. Therefore the inhibition of activity by $HgCl_2$ and by *N*-ethylmaleimide was investigated. The activity of the oxidized NADP-malate-dehydrogenase in the presence of guanidine was unaffected by 0.1 mM $HgCl_2$ in the presence of 1 mM EDTA and by 5 μM $HgCl_2$ in the absence of EDTA, as well as by 1 mM *N*-ethylmaleimide at pH 8.0, conditions which completely abolish catalytic activity of the reduced form of NADP-malate dehydrogenase [6]. Thus it can be concluded that the reductive formation of the regulatory dithiol did not occur in the presence of guanidine and is therefore not essential for catalytic activity.

When the oxidized NADP-malate dehydrogenase was exposed to 250 mM guanidine in the assay mixture, catalytic activity appeared instantaneously. No lag phase of activity was detected in the assay, nor was there any decline in activity for at least 15 min. Preincubation (10 min, room temperature) of the enzyme with 250 mM guanidine and 50-fold dilution into the assay mixture containing no added guanidine resulted in little or no activation of the enzyme. It appears that this activation, which is stable in the continued presence of guanidine, is rapidly reversible in the absence of guanidine. This activation process seems to be specific for guanidine, since NaCl and urea (25–300 mM) did not activate the oxidized NADP-malate dehydrogenase. The guanidine-treated enzyme can, however, obtain the full activity of the reduced enzyme when it is incubated with DTT.

Upon gel filtration the oxidized NADP-malate-dehydrogenase appears to migrate as a monomer in the presence of 200 mM guanidine-HCl, pH 8.0. This indicates that the formation of a tetramer is not required under those conditions. Presumably guanidine activated the oxidized enzyme by some partial deformation of the enzyme active site. Such a deformation might also alter the coenzyme specificity. However, guanidine treatment did not stimulate the NAD-dependent activity indicating that the guanidine-activated oxidized enzyme retains the specificity found in the reduced enzyme.

It should be mentioned that among 4 enzyme preparations one occurred which was not activated by guanidine. This fact seems to provide another example of the heterogeneity of the purified enzyme concerning its catalytic and regulatory properties. It has already been described that some preparations of NADP-malate dehydrogenase did not exhibit the usual property [12,13] of the inhibition of activation by NADP [13]. On the other hand, the activation by guanidine did not seem to be an artifact occurring during the purification procedure, because the activity of the oxidized NADP-malate dehydrogenase in the presence of 200 mM guanidine could be obtained with the crude extract as well and was copurified with the activity of the DTT-activatable NADP-malate dehydrogenase.

It appears that guanidine can activate oxidized NADP-malate dehydrogenase by a relatively specific, but apparently subtle conformational change.

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