Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 829-836

OXIDIZED NADP AS A POTENTIAL ACTIVE-SITE-DIRECTED REAGENT OF PIGEON LIVER MALIC ENZYME

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Received December 1, 1978

Summary: Pigeon liver malic enzyme (malate dehydrogenase (decarboxylating), EC 1.1.1.40) was reversibly inactivated by periodate-oxidized NADP in a biphasic manner. The reversibility could be made irreversible by treating the modified enzyme with sodium borohydride. The inactivation showed saturation kinetics and could be prevented by nucleotide (NADP or NADPH). Fully protection was afforded by the combination of NADP, Mn^{2+} and L-malate. Oxidized NADP was also found to be a coenzyme and noncompetitive inhibitor of L-malate in the oxidative decarboxylase reaction catalyzed by malic enzyme.

Introduction

Pigeon liver malic enzyme catalyzes the NADP-dependent oxidative decarboxylation of L-malate to give bicarbonate and pyruvate (1,2). This enzyme plays a major role in lipogenesis by providing reducing equivalents for hepatic biosynthesis of fatty acids (3,4). In our previous studies, the affinity label, bromopyruvate, proved to be a useful reagent in the study of the reaction mechanism of malic enzyme (5,6). However, bromopyruvate actually modifies many nonessential groups outside the active center of malic enzyme (5). Thus a more specific affinity label would be a better instrument for an exploration on the geometry of the active center. In this communication, we wish to show that periodate-oxidized NADP is a potential candidate for this purpose. Oxidized NADP has been found to be a powerful inactivator specific for the NADP binding site of 6-phosphogluconate dehydrogenase (7,8).

Material and Methods

All chemicals used were of reagent grade. Distilled and deionized water was used throughout this work.

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Pigeon liver malic enzyme was purified according to Hsu and Lardy (9). Each preparation was routinely checked for high specific activity, and analyzed for purity by the acrylamide gel electrophoresis. The staining for protein by Coomassie blue always coincides with the staining for enzyme activity by phenazine methosulfate (10). The purified enzyme was then dialyzed exhaustively against 50 mM Tris-Cl buffer (pH 7.0 at 25°C) containing 10% glycerol. Protein concentration was determined at 278 nm, using an extinction coefficient of 0.86 for a 0.1% (w/v) protein solution (9).

Oxidation of NADP was performed essentially according to Easterbrook-Smith et al. (11), except that ATP was replaced by NADP. This treatment results in the cleavage of the bond between carbon 2' and 3' of the ribose ring bound to nicotinamide and formation of two aldehyde groups at these carbons. The concentration of oxidized NADP was determined by measuring the absorbance at 259 nm, using a value of 18,000 $cm^{-1}M^{-1}$ for the extinction coefficient (12). The purity of oxidized NADP was confirmed by thin layer chromatography performed on polyetheneimine sheet, using 0.8 M ammonium bicarbonate as the developing solvent and ultraviolet light to locate the position of the nucleotide. Chromatography of the purified sample revealed only one spot with a R_F value of 0.79. No NADP (R_F 0.94) was detectable.

Malic enzyme activity was assayed according to Hsu and Lardy (9). The assays were carried out in a Beckman 24 spectrophotometer equipped with a recorder. The cell compartment was thermoregulated at 30°C. All reagents were brought to 30°C before mixing. Enzyme was added to start the reaction. The formation of NADPH was monitored at 340 nm and was always linear with time. The definition of enzyme unit and specific activity was as described before (9).

Modification experiments were performed at 25°C by addition of oxidized NADP to an enzyme solution at pH 7.5. The progress of reaction was monitored by assaying the enzyme activity on small aliquots withdrawn at the designated time intervals.

Results and Discussion

Reversible inactivation of malic enzyme by oxidized NADP.

As shown in Fig 1A, oxidized NADP, an analog of NADP, caused an inactivation of malic enzyme activity very rapidly. The pseudo-first-order plot for the inactivation followed a biphasic kinetics (Fig. 1A inset). There was first a rapid reaction, until about 90% of inactivation was reached; the remaining activity then lost at a much slower rate. Under our experimental conditions, the observed inactivation rate constant (k_{obs}) calculated from the slopes of these two lines were 0.138 min^{-1} and 0.018 min^{-1}, respectively. The inactivation could be reversed by dilution (Fig. 1B, line a), but no reactivation was observed when the modified enzyme was reduced by sodium borohydride (Fig. 1B,

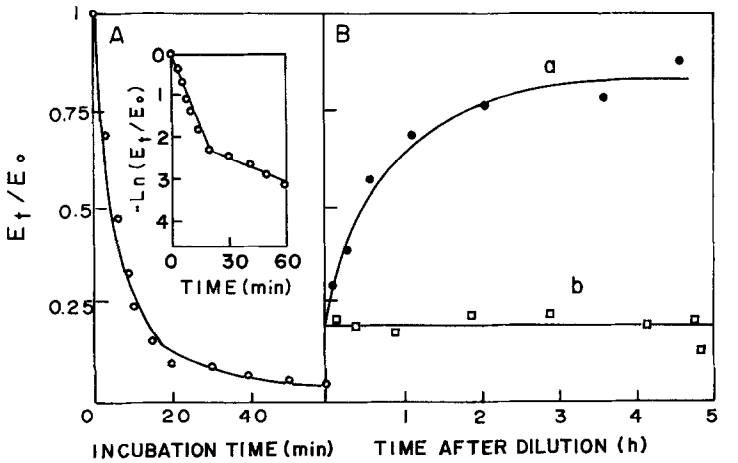

Fig. 1. Reversible inactivation of pigeon liver malic enzyme by oxidized NADP. A. Residual enzyme activity as a function of time. The reaction mixture contained 1.1 μM malic enzyme in 30 mM borate buffer (pH 7.5) and 2.25 mM oxidized NADP. Incubation was at 25°C. E_o, Original enzyme activity; E_t, enzyme activity at time indicated. Inset, semilog plot of the inactivation data. B. Effect of dilution on the activity of oxidized-NADP treated enzyme. Malic enzyme was incubated with oxidized NADP for 60 min as in A and then diluted 200 x with 50 mM borate buffer (pH 7.5, containing 10 mg/ml bovine serum albumin) with (□—□) or without (●—●) a pretreatment with 50 mM sodium borohydride.

line b). These results strongly suggest that in the binding of oxidized NADP to malic enzyme, a Schiff base is formed between the aldehyde groups of oxidized NADP and lysyl amino group(s) of the enzyme.

Dependence of the rate of inactivation on oxidized NADP concentrations. Kinetics of the activity loss was examined over a range of oxidized NADP concentrations. When the observed inactivation rate constants of the first phase modification were plotted against oxidized NADP concentrations, a hyperbolic curve resembles the Michaelis-Menten kinetic behavior of a substrate was obtained (Fig. 2A), suggesting the formation of an enzyme-oxidized NADP complex before inactivation taking place. The double reciprocal

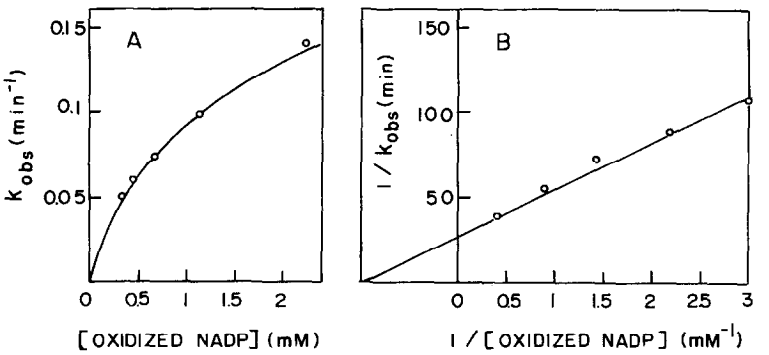

Fig. 2. Effect of oxidized-NADP concentration on the rate of inactivation of malic enzyme. Conditions for incubation were as described in Fig. 1A, except that oxidized-NADP concentration was varied as indicated. Pseudo-first-order plots at all concentrations were linear. The observed inactivation rate constants (k_{obs}) were obtained from the slopes of these plots. Fig. 2B shows a linear double reciprocal plot of these data.

plot of $1/k_{obs}$ against 1/oxidized NADP concentration was linear (Fig. 2B). The values of maximum inactivation rate constant (k_{max}) and dissociation constant of the presumed enzyme-oxidized NADP complex calculated from this plot were 0.19 min^{-1} and 1 mM, respectively (cf. 13).

Protection of malic enzyme by substrates against oxidized-NADP modification. Full protection against the inactivation was afforded by the coenzyme NADP together with Mn^{2+} and L-malate. NADP alone also gave a substantial protection (Fig. 3). Table 1 shows the effects of other reaction components on the rate of inactivation. NADPH was also effective. Metal ion (Mn^{2+} or Mg^{2+}) gave some protection. The protection by nucleotide (both NADP and NADPH) strongly suggests that modification was at the nucleotide binding site.

Oxidized NADP as a coenzyme and an inhibitor. The above results suggests that oxidized NADP acts as an active-site-directed reagent. Therefore, it is not surprising to find that this compound also

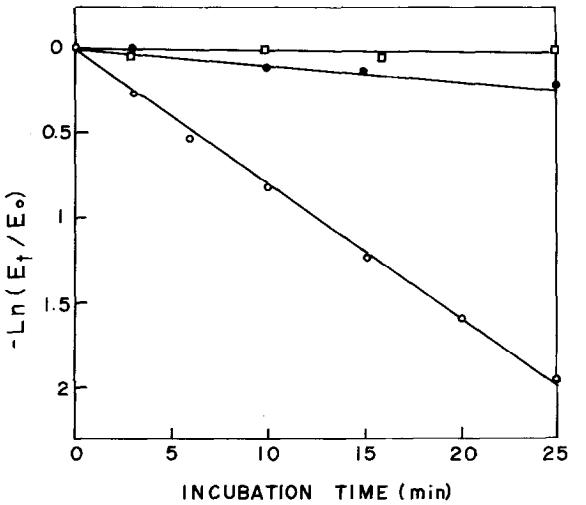

Fig. 3. Protection of the malic enzyme against oxidized-NADP inactivation. Conditions were as described in Fig. 1A, except that 1.13 mM oxidized NADP was used with the following additions: o, none; ●, 4.45 mM NADP; □, 4.45 mM NADP plus 0.39 mM L-malate and 0.78 mM $MnCl_2$.

Table 1. Effect of reaction components on inactivation rate

Experiment	Additions	% Protection
I	NADP (4.45 mM) + Mn^{2+} (0.78 mM) + L-malate (0.39 mM)	98
	NADP (4.45 mM)	89
	none	0
II	NADPH (3.69 mM)	86
	NADP (3.72 mM)	79
	Mg^{2+} (2.62 mM)	46
	Mn^{2+} (6.6 mM)	36
	pyruvate (22 mM)	0
	L-malate (0.8 mM)	0
	none	0

Conditions were as described in Fig. 1A, except that 1.13 mM and 0.79 mM of oxidized NADP was used in experiment I and II, respectively. The k_{obs} of the inactivation were obtained from each experimental set. The percent protection was calculated according to the equation:

$$\left\{\left[k_{obs}(\text{unprotected}) - k_{obs}(\text{protected})\right] / k_{obs}(\text{unprotected})\right\} \times 100$$

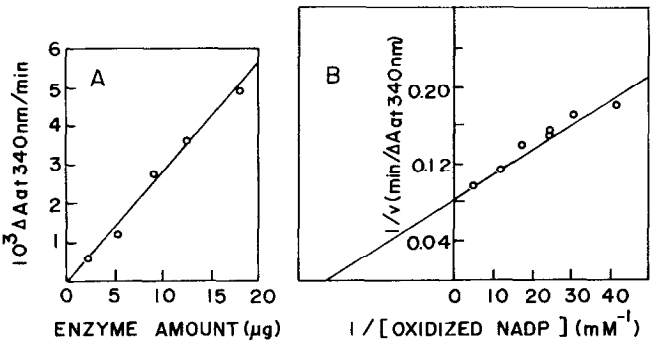

Fig. 4. Reduction of oxidized NADP in the malic enzyme catalyzed oxidative decarboxylation. A. Reduction of oxidized NADP as a function of enzyme concentration. The reaction mixture contained triethanolamine-Cl buffer (pH 7.4), 200 μmol; L-malate at pH 7.4, 1.5 μmol; $MnCl_2$, 12 μmol; oxidized NADP, 0.575 μmol and water and enzyme to make a final volume of 3.0 ml. B. Effect of oxidized NADP concentration on the enzyme activity. Conditions were the same as in A, except that oxidized NADP was used as the variable substrate and the amount of enzyme used in each assay was 18 μg.

functions as a coenzyme and an inhibitor of malic enzyme. Fig. 4A shows the correlation between the reduction rate of oxidized NADP and enzyme concentration. The effect of oxidized NADP concentration on reaction rate is shown in Fig. 4B. It should be noted that the binding between malic enzyme and oxidized NADP must be much less efficient, since the apparent Michaelis constant (K_m) for oxidized NADP (30 ± 2 μM) or the dissociation constant of the enzyme-oxidized NADP complex (K = 1 mM) is much higher than the Michaelis constant for NADP (1-3 μM)(14). Oxidized NADP was also found to be an inhibitor of malic enzyme (Fig. 5A), and functions as a noncompetitive inhibitor against L-malate in the malic enzyme-catalyzed oxidative decarboxylase reaction (Fig. 5B).

Results presented in this communication indicate that oxidized NADP acts as an affinity label which reversibly combines with malic enzyme, followed by a reaction with the proximal lysyl amino group(s) to yield a Schiff base. Further kinetic experiments are

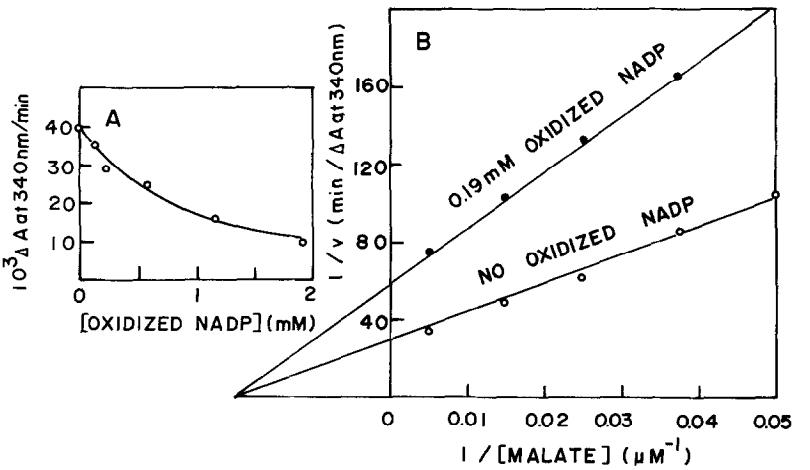

Fig. 5. Inhibition of the malic enzyme activity by oxidized NADP. A. Enzyme were assayed as described in the text with different concentrations of oxidized NADP. B. Oxidized NADP and L-malate concentrations were varied as indicated. The amount of enzyme used in each assay was 1.47 μg.

underway to delineate the unique properties of this compound as an inhibitor, a modifying agent. and a coenzyme of malic enzyme. The exact nature of the amino acid residue involved in inactivation and its functional role are also under investigation.

Acknowledgements

We wish to express our deep gratitude to Professor Foo Pan for helpful suggestions and stimulating discussion. This work was supported in part by the National Science Council, Republic of China (project No. NSC-66B-0412-06(04)).

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