GENERATION OF THE SUPEROXIDE RADICAL DURING THE PEROXIDATIC OXIDATION OF NADH BY CATALASE AT ACID pH VALUES

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Received 31 May 1977

1. Introduction

Catalase (H_2O_2 : H_2O_2 oxidoreductase, EC 1.11.1.6) catalyses the rapid breakdown of H_2O_2 to O_2 and water: this action is referred to as the 'catalatic' activity of the enzyme. In the presence of H_2O_2, catalase also brings about the 'peroxidatic' oxidation of a small number of compounds, such as ethanol and formic acid [1]. On exposure to acid or alkaline pH, bovine liver catalase dissociates into subunits: this is accompanied by a decrease in catalatic activity and an increase in peroxidatic activity [2–4]. Under these conditions, catalase gains the ability to oxidise NADH in the presence of Mn^{2+} and phenols [3]. Horseradish peroxidase also catalyses oxidation of NADH in the presence of such cofactors at acidic pH values [5] and the mechanism of oxidation seems to involve the superoxide radical, $O_2^{\cdot-}$ [6]. The work reported in the present paper has further characterised the cofactor requirements for NADH oxidation by catalase and has investigated the role of $O_2^{\cdot-}$ in this reaction. The principal tool used was the enzyme superoxide dismutase, which catalyses the breakdown of $O_2^{\cdot-}$ to H_2O_2 and O_2 [7] and is thus a powerful inhibitor of $O_2^{\cdot-}$-dependent reactions [7].

2. Materials and methods

Pure bovine liver catalase was obtained from the Boehringer Corp., London, UK or from the Sigma Chemical Corp., Kingston-upon-Thames, UK. (type C-40; thymol-free). Both preparations gave identical results. Neither contained peroxidase activity as assayed by the guaiacol method [8], nor were they significantly contaminated with superoxide dismutase [9]. Superoxide dismutase ($O_2^{\cdot-}$–$O_2^{\cdot-}$ oxidoreductase, EC 1.15.1.1) prepared according to the method of McCord and Fridovich [10] was purchased from Sigma.

Oxidation of NADH was followed at 20°C by the fall in absorbance at 340 nm. Reaction mixtures, contained, in a final volume of 3 ml, the following reagents at the final concentrations stated: acetic acid–sodium acetate buffer, pH 4.5 (50 mM), NADH (0.15 mM), a phenol (see fig.1 and table legends for the concentration) and $MnCl_2$ (2.25 mM). Reactions were initiated by adding catalase (0.02 ml of a solution of 10 mg/ml of enzyme dissolved in 10 mM KH_2PO_4–KOH buffer, pH 7).

3. Results

The rate of NADH oxidation by catalase was found to be most rapid in acetic acid/acetate buffer at pH 4.5. Both Mn^{2+} and phenols were required as cofactors: Table 1 shows that not all phenols were effective in promoting NADH oxidation. The most effective of those tested was 2,4-dichlorophenol.

Table 2 shows the relationship between the amount of catalase present and the rate of NADH oxidation in the presence of Mn^{2+} and 2,4-dichlorophenol. Addition of 0.2 mg of Sigma catalase (type C-40) gave the maximum reaction rate, but larger amounts were inhibitory.

When superoxide dismutase was present in the reaction mixture, NADH oxidation by catalase in the

Table 1
Requirement of Mn^{2+} and phenols for NADH oxidation by catalase

Phenol present	Concentration (mM)	Rate of NADH oxidation nmol NADH oxidised/min
None	–	0
2,4-dichlorophenol	0.03	43
	0.05	49
	0.07	58
	0.07 (omit $MnCl_2$)	0
4-hydroxycinnamic acid	0.07	27
	0.13	39
	0.2	43
	0.2 (omit $MnCl_2$)	0
p-cresol	0.07	23
	0.13	36
	0.2	39
	0.2 (omit $MnCl_2$)	0
Thymol	0.13	0
salicylic acid	0.13	0
p-hydroxybenzoic acid	0.13	0

Experiments were carried out as described in the Materials and methods section, with phenols present in the reaction mixtures at the final concentrations stated above. The rates presented above have been corrected for the slow non-enzymic breakdown of NADH at pH 4.5

Table 2
Effect of the amount of catalase present on the rate of NADH oxidation

Catalase present (mg)	Rate of NADH oxidation nmol NADH oxidised/min
0	0
0.05	15
0.10	30
0.20	34
0.50	28
1.00	2

Experiments were carried out as described in the Materials and methods section. The phenol present was 2,4-dichlorophenol (0.05 mM). The rates presented above have been corrected for the slow non-enzymic breakdown of NADH at pH 4.5

presence of phenols and Mn^{2+} was completely prevented. Addition of dismutase during the course of the reaction also stopped NADH oxidation (fig.1). An equal amount of bovine serum albumin had no effect, nor did addition of the hydroxyl radical scavenger mannitol, tested up to a final concentration of 100 mM.

4. Discussion

NADH is unstable at pH 4.5 and breaks down slowly to produce H_2O_2 [6]. In these experiments, catalase, acting as a peroxidase, is presumably using the H_2O_2 to catalyse oxidation of NADH of a free-radical NAD $\dot{}$ (eq.1) which can reduce O_2 to $O_2^{\cdot-}$ (eq.2). Although the rate of oxidation of NADH by $O_2^{\cdot-}$ is very slow, $O_2^{\cdot-}$ reacts rapidly with Mn^{2+}, which is present in the reaction mixtures, to give Mn^{3+} (eq.3

[12,13]) which can oxidise NADH (eq. 4 [14,15]).

$$NADH \xrightarrow[\text{catalase}]{H_2O_2} NAD\cdot + H_2O \quad (1)$$

$$NAD\cdot + O_2 \longrightarrow NAD^+ + O_2^{\cdot-}$$
$$(k = 1.9 \cdot 10^9\ M^{-1}\ sec^{-1}\ [11]) \quad (2)$$

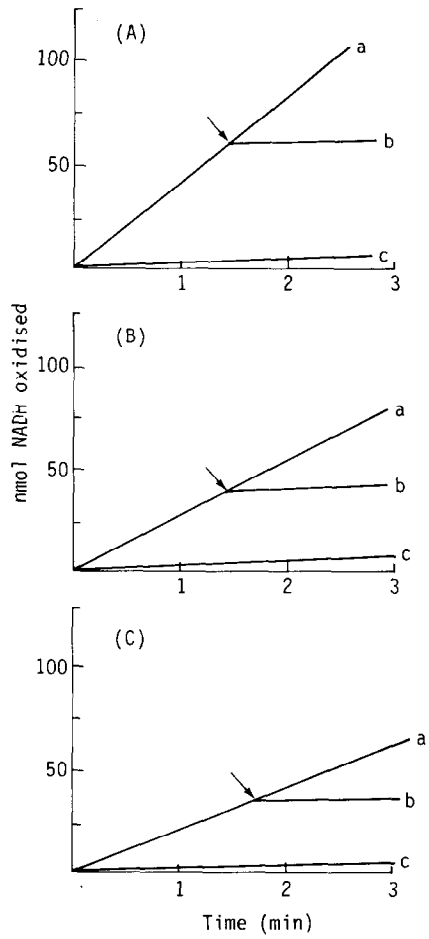

Fig.1. Effect of superoxide dismutase on NADH oxidation by catalase. Assays were carried out as described in the Materials and methods section. Units of superoxide dismutase were as defined using the cytochrome *c* assay [10]. The rates presented have been corrected for the slow non-enzymic breakdown of NADH at pH 4.5. The phenols present were (A) 2,4 dichlorophenol (0.03 mM); (B) 4-hydroxycinnamic acid (0.07 mM); (C) *p*-cresol (0.07 mM). (a) Rate of NADH oxidation in the absence of superoxide dismutase; (b) as a, 10 units dismutase added at the arrow; (c) 10 units dismutase present in the reaction mixture before catalase was added.

$$O_2^{\cdot-} + Mn^{2+} + 2H^+ \longrightarrow H_2O_2 + Mn^{3+} \quad (3)$$

$$Mn^{3+} + NADH \longrightarrow NAD\cdot + Mn^{2+} + H^+ \quad (4)$$

This mechanism would explain why Mn^{2+} is required for NADH oxidation and why superoxide dismutase, by preventing reactions 3 and 4, would severely inhibit NADH oxidation by catalase. A similar mechanism has been proposed to account for NADH oxidation by peroxidase [6,16]. The role of the phenols has yet to be defined.

The H_2O_2 generated in the reaction mixture could also be broken down by the catalatic action of catalase. Presumably the catalatic and peroxidatic activities compete for the available H_2O_2. This might explain why the peroxidatic reaction decreases at high catalase concentrations (table 2).

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