DPNH PEROXIDASE: EFFECTOR ACTIVITIES OF DPN^+

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SUMMARY

At suboptimal H_2O_2 concentrations, DPNH inhibits the peroxidase activity of the flavoprotein, DPNH peroxidase, by converting the enzyme to an unstable intermediate that decays slowly to inactive enzyme. It is postulated that at concentrations of DPNH that are saturating for peroxidase activity, this unstable intermediate is responsible for most of the DPNH oxidation that is supported by alternate electron acceptors, such as O_2 and menadione. DPN^+ behaves as an activator by reversing the equilibria that lead to the unstable intermediate, thus converting the enzyme to the kinetically active complex that reduces H_2O_2. The data show that DPN^+ binding will stimulate the peroxidase activity (by lowering the K_m for H_2O_2) and simultaneously lead to strong inhibition of both the rate of enzyme inactivation and the rate at which DPNH is oxidized by alternate electron acceptors.

INTRODUCTION

DPNH peroxidase of Streptococcus faecalis (1) is a dimer (2) containing 2 moles of FAD and one mole of a non-flavin electron acceptor per mole of enzyme (3). Evidence that the non-flavin acceptor is a disulfide group has been presented (3). Neutron activation analysis has shown that the enzyme does not contain selenium (2). In contrast, glutathione peroxidase, which has no heme or flavin prosthetic groups, is reported to contain one atom of Se per subunit (4).

Previous work with DPNH peroxidase (3) suggested that the site at which H_2O_2 is reduced consists of a complex between the reduced non-flavin acceptor and enzyme bound FAD and DPN^+. The present paper deals with the role of DPN^+ as an activator of DPNH peroxidase activity and an inhibitor of several competing reactions. It is postulated that these multiple effects are brought

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about when DPN^+ binds to an enzyme species that has lost DPN^+ from the peroxide site.

MATERIALS AND METHODS

Enzyme was prepared as described previously (1,3). The minimum molecular weight per flavin is ~ 60,000. According to anaerobic titration experiments (3) the two FAD prosthetic groups of the enzyme are non-equivalent.

Peroxidase assays were carried out as already described (1,3) in 0.033 M sodium acetate buffer, pH 5.4, at 25°. Kinetic parameters, as calculated from the coefficients of the reciprocal rate equation (2,5) are: turnover (per flavin), 4000 min^{-1}; K_m for DPNH, 6.8 x 10-6 M; K_m for H_2O_2, 2.7 x 10^{-5} M. If H_2O_2 is omitted from the standard assay system (DPNH, 0.1 mM), the enzyme loses 85% of its activity in 30 min.

DPNH oxidation in the presence of acceptors other than H_2O_2 was measured in the same way as peroxidase activity, except that the alternate electron acceptors were substituted for H_2O_2. Menadione and potassium ferricyanide were used at concentrations of 0.06 and 0.21 mM respectively. DPNH oxidase activity was determined in air-saturated buffer. Under these conditions, the observed turnovers per flavin with air, menadione or ferricyanide are 130, 1530 and 2040 min^{-1} respectively.

RESULTS AND DISCUSSION

In air, DPNH can be oxidized by DPNH peroxidase at about 3-3.5% of the rate obtained at saturating H_2O_2 concentration. Fig. 1 shows that DPN^+ strongly inhibits this DPNH oxidase activity, but activates the DPNH peroxidase reaction. Under the conditions used, the ratio of oxidase to peroxidase decreases by a factor of 16 as the concentration of added DPN^+ increases from zero to 1.1 mM. This stimulatory effect of DPN^+ is obtained only at suboptimal concentrations of peroxide (see later). The inset of Fig. 1 is a plot from which the apparent dissociation constant for DPN^+ as activator (given in the legend) can be calculated.

The left side of Fig. 2 shows the effect of DPN^+ on several non-specific DPNH oxidations; also shown is the effect of DPN^+ on the rate at which enzyme is inactivated in the absence of H_2O_2. (Previous work (6) showed that when DPNH peroxidase, at a concentration of 1 to 10 nM, is incubated with DPNH in the absence of H_2O_2, peroxidase activity slowly decays; the inactivation can be stopped, but not reversed, by the addition of H_2O_2.) As indicated in Fig. 2, DPN^+ inhibits the DPNH oxidation reactions supported by alternate electron acceptors, and also inhibits the rate of enzyme inactivation. Maxi-

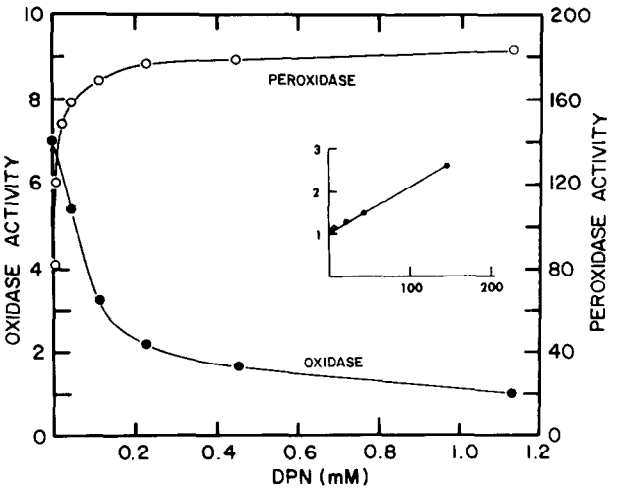

Fig. 1. Effect of DPN^+ on the DPNH oxidase and DPNH peroxidase activities of DPNH peroxidase. The H_2O_2 concentration in the peroxidase assay was 0.13 mM; the DPNH concentration was 0.1 mM for both assays. Activities are expressed as Δ absorbance at 340 nm per min per ml enzyme. Inset: Reciprocal of fractional activation of peroxidase activity (V/v) on the ordinate, vs. $1/DPN^+$ (mM^{-1}) on the abscissa, where V = activity in the presence of a saturating concentration of DPN^+ and v = activity at a given concentration of DPN^+. The velocities are corrected for enzyme activity observed in the absence of added DPN^+. The apparent dissociation constant for DPN^+, given by the slope of the reciprocal plot, is 0.011 mM.

mal inhibition varies from 30% for ferricyanide reduction to 80-90% for the other reactions. This suggests that the enzyme species present at saturating DPN^+ concentration is responsible for 70% of the ferricyanide reductase activity and 10-20% of the other activities shown. On the right side of Fig. 2 is a reciprocal plot of the data for the strongly inhibited reactions. Apparent dissociation constants for DPN^+ as inhibitor are given in the legend of the figure.

A mechanism that can explain the effector properties of DPN^+ is shown in Fig. 3. Species I-III are those that have been detected in anaerobic titration experiments (3). The first molecule of DPNH to react converts oxidized enzyme (I) to species II; II is stable in air for at least 24 hr, but is stoichiometrically oxidized by H_2O_2 to I. Although II is air-stable, dialysis results in its conversion to I under aerobic conditions (2). Pro-

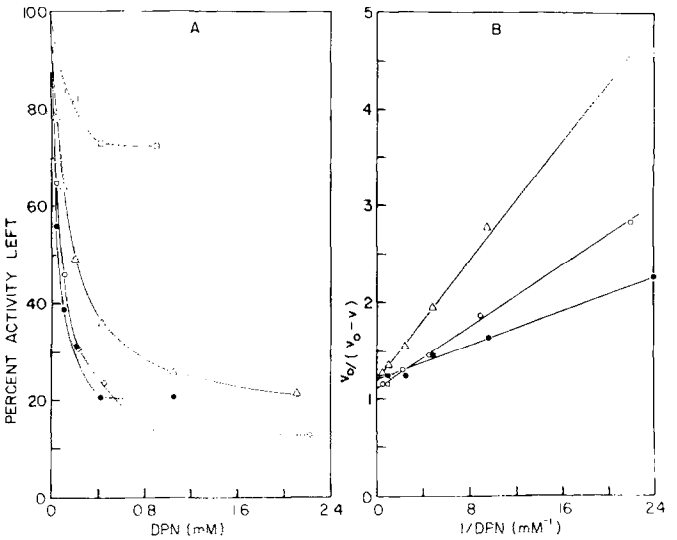

Fig. 2. A. Inhibition by DPN^+ of the rate of DPNH peroxidase inactivation, compared with the inhibitory effect of DPN^+ on several alternate electron transport reactions of DPNH peroxidase. ● , rate of inactivation of peroxidase activity; ○ , DPNH oxidase activity; △ , DPNH-menadione reductase activity; □ , DPNH-ferricyanide reductase activity. Rates of the reactions are normalized to 100 at $DPN^+ = 0$. B. Plot of reciprocal of fractional inhibition vs. reciprocal of DPN^+ concentration. Symbols are same as for A. The variables v and v_o stand respectively for the velocity in the presence and absence of DPN^+. The slope of the plot gives the apparent dissociation constant for DPN^+. The theoretical intercept for complete inhibition is 1. Apparent dissociation constants for DPN^+ in the inactivation, DPNH oxidase and menadione reductase reactions are respectively 0.043, 0.071 and 0.14 mM.

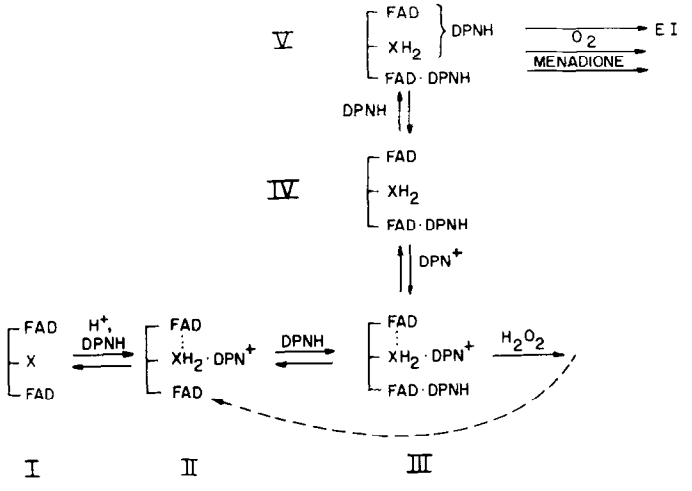

Fig. 3. A mechanism consistent with the effector properties of DPN^+. Enzyme species involved in the oxidation of III to II are not shown. X represents the non-flavin acceptor. EI stands for inactive enzyme.

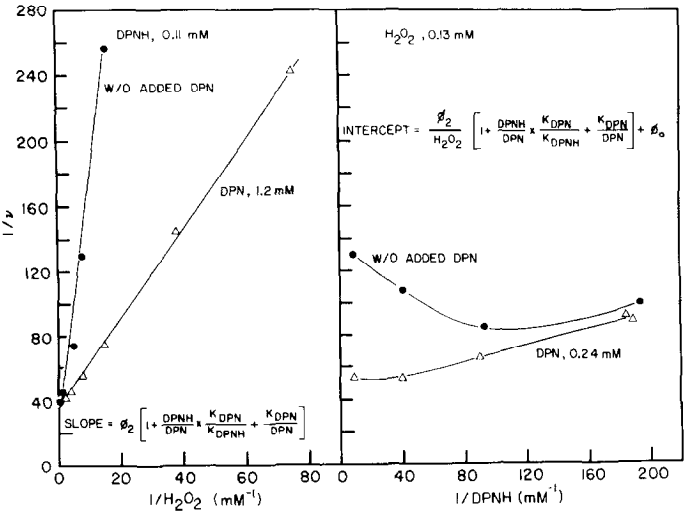

Fig. 4. Reciprocal of peroxidase activity vs. reciprocal of variable substrate concentration at fixed concentrations of DPNH (left) and H_2O_2 (right). Units of 1/v are minutes x Δ absorbance^{-1}.

cedures that cause inactivation of peroxidase activity lead also to parallel loss in the ability of enzyme to form species II (3). Because of these observations, the upper site of II is considered to be the locus at which H_2O_2 is reduced.

A second molecule of DPNH converts II to III; III is slowly oxidized in air to II. It was suggested (3) that the complex at the lower binding site of III transfers reducing equivalents to the upper site, after H_2O_2 is reduced at the peroxide locus. Such a mechanism would account for the nonlinearity (concave upward) of reciprocal plots when DPNH, at low concentration, is the variable substrate (3). Under the conditions of Figs. 1, 2, and 4 (left side), the concentration of DPNH is high enough so that the mole fractions of I and II are negligible. The effector role of DPN^+ is attributed to the remaining species, III to V.

It is assumed that at the low enzyme concentrations used for the experiments reported here, DPN^+ can dissociate from III. The resultant species then binds DPNH to form V. This species is considered to be the unstable

intermediate that decays slowly to inactive enzyme; it is also regarded as the intermediate that accounts for most of the DPNH oxidase and menadione reductase activity, and for a portion of the ferricyanide reductase activity. Since DPN^+ drives the equilibria towards III, an active peroxidase species, an explanation is available for the ability of DPN^+ to activate the peroxidase reaction and simultaneously to inhibit the non-specific DPNH oxidation reactions and enzyme decay. The low DPNH oxidase activity detected at saturating DPN^+ concentration may be attributable to the very weak oxidase activity of III.

Dissociation of DPN^+ from the active site is in itself an inhibitory step for the peroxidase reaction. The reason for postulating a subsequent binding with another molecule of DPNH is that the apparent dissociation constant for DPN^+ (K_{app}) decreases with decreasing DPNH concentration (2). The reactions as written will account for this dependence of K_{app} on the DPNH concentration. (K_{app} as evaluated from the reciprocal plots in Figs. 1 and 2B is equal to the concentration of DPN^+ that causes one-half the maximum effect). Derivation of the expressions for K_{app} shows that the oxidant concentration must also be taken into account. Just by inspection, it can be seen that the presence of H_2O_2 will cause a reduction in the equilibrium concentration of species III to V and thus favor binding of DPN^+ to the enzyme intermediates of the main catalytic pathway. In theory, at high enough H_2O_2 concentration, DPN^+ binding can become stoichiometric ($K_{app} = 0$). This would explain why the activation of peroxidase activity by DPN^+ (Fig. 1) is observed only at low peroxide concentration; at high peroxide concentration even small amounts of DPN^+ will be efficiently bound to the active peroxidase species. The decrease of K_{app} in the presence of H_2O_2 also explains the difference in one-half saturation values for DPN^+ as activator of peroxidase activity and inhibitor of oxidase activity (Fig. 1).

On the other hand, alternate oxidants may cause an increase in K_{app} by favoring dissociation of DPN^+ from III. Since K_{app} will depend in part on

oxidant concentration and on the mechanism and turnover for oxidation of species V, it is understandable that the K_{app} values given in Fig. 2 are not identical.

If the mechanism of Fig. 3 is valid, the equilibrium between III, IV and V should affect the steady-state kinetics observed in the presence of H_2O_2, because IV and V are abortive complexes for the peroxidase reaction. As predicted by the mechanism, DPN^+ causes a decrease in the apparent K_m for H_2O_2 (Fig. 4, left side); at saturating DPN^+ concentration, the apparent K_m decreases 5-fold. The previously reported K_m for H_2O_2 (1) is high because DPN^+ was not added to the assay system, therefore the inhibitory slope terms, shown in the equation in Fig. 4, were not cancelled. The right side of Fig. 4 again illustrates the competition between DPNH and DPN^+. Equations given in this figure are consistent with the observed slope and intercept effects. These equations were derived from the mechanism of Fig. 3.

Although the reaction is not shown in Fig. 3, DPN^+, at high concentration, becomes an inhibitor of DPNH peroxidase (7) by competing with DPNH for species II (K_I for DPN^+ = 1.5 mM). This inhibition did not occur under the conditions of Fig. 1, because the DPNH concentration was high enough to reverse the effect.

The scheme shown in Fig. 3 is a simplified version of the mechanism. It has been found, for example, that subsequent to DPN^+ binding, the initial complex formed is converted to III in a slow step ($k = \sim 2\ min^{-1}$, at saturating DPN^+ concentration). This slow relaxation is not detectable when H_2O_2 is present, a phenomenon that may explain the previously noted requirement for an effector molecule of H_2O_2 in the steady-state rate equation (7). These features can be accomodated in an expanded mechanism, but the simple version shown here illustrates most of the effector reactions of DPN^+.

Regardless of the mechanism, it is clear that DPN^+ binding to the enzyme can stimulate peroxidase activity, stabilize the enzyme against DPNH-dependent inactivation and simultaneously make the enzyme more specific for its physiological oxidant.

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