Inhibition of catalase by 3,3'-diaminobenzidine

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3,3'-Diaminobenzidine strongly inhibits bovine liver catalase in two distinct ways. One of these was competitive with respect to H_2O_2 , approached a limit of 100% inhibition and was rapidly reversed by dilution or by dialysis. The other was dependent upon H_2O_2 , approached a limit of 60% and was not reversed by dilution or dialysis. Exposure to diaminobenzidine followed by dialysis did not modify the electrophoretic mobility of the enzyme, its K_m for H_2O_2 or its optical spectrum, although $V_{\max}$ was halved. Diaminobenzidine protects catalase against the irreversible inactivation imposed by 3-amino-1,2,4-triazole plus H_2O_2 . CN^- -protected catalase against the apparently irreversible inhibition by diaminobenzidine, and dialysis against CN^- reversed this inhibition. Ethanol was similarly protective, and ethanol plus H_2O_2 reversed the inhibition by diaminobenzidine. Several other aromatic diamines inhibited in a fashion similar to that of diaminobenzidine, but were less effective. A scheme of reactions that accounts for these observations is proposed.

3,3'-Diaminobenzidine (DAB) has repeatedly been used as a chromogenic substrate for peroxidases and for the peroxidatic activity of glutaraldehyde-treated catalase (Graham & Karnovsky, 1966; Fahimi, 1968; Novikoff & Goldfischer, 1969; Herzog & Fahimi, 1974). DAB plus horseradish peroxidase has also been used to provide a negative stain for catalase activity on polyacrylamide gels (Gregory & Fridovich, 1974). In this method, gel electrophoretograms were soaked first in DAB plus horseradish peroxidase and then in H_2O_2 . Zones containing catalase would become depleted of H_2O_2 and thus not show the chromogenic peroxidation of DAB by horseradish peroxidase, leaving achromatic zones against a uniformly stained background. It was subsequently noticed that changing the order of application of these reagents, such that DAB was the last reagent applied, markedly increased the sensitivity of this negative stain for catalase activity (Clare *et al.*, 1984). This result was explained on the basis of the inhibition of catalase by DAB. Since the inhibition of catalase by DAB had not previously been reported, we undertook an investigation of this inhibition and also examined the effects of related compounds.

Abbreviations used: DAB, 3,3'-diaminobenzidine.

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Materials and methods

Catalase (bovine liver, $2 \times$ crystallized, 31 000 units/mg of protein) and DAB, as the tetrahydrochloride, were purchased from Sigma Chemical Co. 3-Amino-1,2,4-triazole was from Aldrich Chemical Co. All other compounds were purchased as the reagent grade.

Catalase activity was measured, in terms of O_2 evolution, with a Clark oxygen electrode, in medium containing 50 mM-potassium phosphate, 0.1 mM-EDTA and 20 mM- H_2O_2 , at pH 7.8 at 22°C. Optical absorption spectra were recorded with a Shimadzu model U.V. 240 recording spectrophotometer. Irreversible effects of DAB on catalase were examined by lowering the concentration of DAB by dilution or by eliminating unbound DAB by dialysis.

Electrophoresis on 7.5%-polyacrylamide gel cylinders was performed by the procedure of Davis (1964). Gel electrophoretograms were stained for protein with Coomassie Blue G-250 or with the silver staining method of Morrissey (1981). Gels were stained for catalase activity as previously described (Clare *et al.*, 1984).

Ethanol peroxidation experiments were done with a Conway micro-diffusion apparatus with (final concentrations) 0.001 μM -catalase, 15 mM-ethanol, 50 mM-potassium phosphate and 6 mM-

semicarbazide hydrochloride all at pH 7.8 in the centre well and 1M-H₂O₂ in the outer well. The acetaldehyde semicarbazone formed in the centre well was measured spectrophotometrically at 224nm by the method of Burbridge *et al.* (1950).

Results

Inhibitions by DAB: reversible and irreversible

DAB, when present in the catalase assay mixture, inhibited the decomposition of H₂O₂, to a limit of 100% inhibition at high concentrations of DAB (line 1 in Fig. 1). As shown by the inset in Fig. 1, plotting these data on reciprocal co-ordinates demonstrated that inhibition was a biphasic function of the concentration of DAB.

Fig. 1. *Inhibition of catalase by DAB*

Line 1 (●), inhibition of catalase in the presence of DAB. Catalase (0.02 nM) was incubated for 2 min at 22°C at pH 7.8 in medium containing 50 mM-potassium phosphate and 0.1 mM-EDTA plus the indicated concentration of DAB in a final volume of 2.0 ml. H₂O₂ (10 μl of 4.0 M stock solution) was then added to give 20 mM, and the rate of O₂ evolution was measured with the Clark electrode. Line 2 (○), irreversible inhibition of catalase by DAB. Catalase (0.01 μM) was incubated for 2 min with an increasing range of concentrations of DAB as described above. Samples (4.0 μl) were then diluted 500-fold into 2.0 ml of 20 mM-H₂O₂ in the phosphate/EDTA buffer and the rate of O₂ evolution was measured. The inset shows percentage inhibition (line 1, ●) or percentage irreversible inhibition (line 2, ○) of catalase activity as a function of DAB concentration, on reciprocal co-ordinates.

This suggested that DAB was inhibiting the activity of catalase by two distinct routes. The dual action of DAB was made evident by examining inhibition that was not reversed by dilution. As shown by line 2 in Fig. 1, DAB caused an inhibition that was not rapidly reversible, and this approached a limit of 60% inhibition at high concentrations of DAB. The inhibition of catalase by DAB present in the assay mixture appeared to be competitive with [H₂O₂].

The DAB-treated catalase was examined, and Fig. 2 demonstrates that DAB treatment did not change the electrophoretic mobility of the catalase. This was the case whether the gels were stained for protein (gels a, b, e and f) or for catalatic activity (gels c, d, g and h) and held true over a 100-fold range of protein concentration and over a 10-fold range of catalatic activity. Moreover, the apparent *K_m* for H₂O₂ of both the native and the DAB-treated enzymes was 50 mM under our conditions. *V_{max}* of the DAB-treated catalase was only half that of the native enzyme. Finally, the rate of thermal inactivation at 60°C was only marginally slower for the DAB-treated than for the native enzyme. The residual catalytic activity of the DAB-treated and then dialysed catalase was thus not distinguishable from that of the native enzyme.

Effect of H₂O₂ on DAB inhibition

Catalase (0.01 μM) was treated with DAB (5 or 200 μM) for 1 min at 25°C and was then dialysed against buffer or against 5.0 mM-H₂O₂ in buffer, both at approx. 10°C. At intervals samples were taken from the dialysis sac and were diluted 500-fold before assay. The results demonstrated that H₂O₂ increased the irreversible aspect of DAB inhibition. Thus 5 μM-DAB gave 25% irreversible inhibition, and this decreased to 0% inhibition as the DAB was removed by dialysis. This result indicates that the inhibition by DAB, which appeared irreversible on the minutes time scale of dilution, was slowly reversible on the hours time scale of dialysis. In contrast, dialysis against 5 mM-H₂O₂ showed very little reversal of the dilution-insensitive inhibition. When the initial concentration of DAB was 200 μM the dilution-insensitive inhibition was 60% and was diminished to only 50% during 20 h of dialysis against buffer. At such high initial concentrations of DAB, dialysis against 5 mM-H₂O₂ produced only marginally less reversal of the dilution-insensitive inhibition than did dialysis against buffer. It appears likely that H₂O₂ is essential for the truly irreversible aspect of the inhibition by DAB and that this H₂O₂ effect is apparent only at low concentrations of DAB, because autooxidation allows accumulation of significant amounts of H₂O₂ in more concentrated solutions of DAB.

Fig. 2. Electrophoretic mobility of native and DAB-treated catalase

Catalase ($0.01 \mu\text{M}$) with or without $200 \mu\text{M}$ -DAB in the phosphate/EDTA buffer was dialysed against this buffer and then subjected to electrophoresis on 7.5% polyacrylamide gels. Gel electrophoretograms were then stained for protein (gels a, b, e and f) or for catalase activity (gels c, d, g and h). (a) $5.0 \mu\text{g}$ of native enzyme; (b) $5.0 \mu\text{g}$ of DAB-treated enzyme; (c) 1.0 unit of native catalase; (d) 1.0 unit of DAB-treated catalase; (e) 50 ng of native catalase; (f) 50 ng of DAB-treated catalase; (g) 0.1 unit of native catalase; (h) 0.1 unit of DAB-treated catalase.

DAB protects against inhibition by 3-aminotriazole

The effects of DAB on catalase were reminiscent of the effects of 3-amino-1,2,4-triazole. Thus 3-aminotriazole inhibits catalase reversibly in the absence of H_2O_2 , but irreversibly in its presence (Margoliash & Novogrodsky, 1958; Margoliash *et al.*, 1960). This suggested that DAB might protect catalase against the effects of 3-aminotriazole. When $0.01 \mu\text{M}$ -catalase was dialysed against 5.0 mM - H_2O_2 plus 20 mM -3-aminotriazole there was a progressive and ultimately total loss of activity. When the catalase was treated with $200 \mu\text{M}$ -DAB and then dialysed against either buffer or more DAB, there was the approx. 60% dilution-irreversible inhibition seen previously. The remaining activity, however, was much less susceptible to inactivation during subsequent exposure to 3-aminotriazole and H_2O_2 as compared with native enzyme. This result suggests that DAB, in exerting a partial irreversible inhibition, binds to catalase in a way that precludes the binding of 3-aminotriazole.

Cyanide protects against inhibition by DAB

Margoliash *et al.* (1960) demonstrated that catalase that had been inactivated by exposure to 3-aminotriazole plus H_2O_2 was no longer able to bind CN^- . This result suggested that CN^- might protect catalase against inactivation by DAB, and it did so. Thus $0.02 \mu\text{M}$ -catalase exposed to $200 \mu\text{M}$ -

DAB suffered a very rapid dilution-irreversible inhibition, which remained constant at approx. 55% over 3 h of incubation with the DAB. When $100 \mu\text{M}$ - CN^- was added before the DAB it protected somewhat, and $200 \mu\text{M}$ - or $400 \mu\text{M}$ - CN^- protected markedly. When $400 \mu\text{M}$ - CN^- was added 2 min after the DAB it caused a gradual and partial reversal of the DAB inhibition. Moreover, dialysis of catalase plus $20 \mu\text{M}$ -DAB against $200 \mu\text{M}$ - CN^- caused a virtually complete reversal of the dilution-irreversible inhibition over several hours.

Effect of DAB on the optical spectrum

DAB diminished the Soret band of catalase, while shifting it to longer wavelengths. There was a concomitant increase in absorbance in the 500–600 nm region. These changes are shown in Fig. 3. Since DAB can inhibit catalase completely, when present in the assay mixture, but inhibits to a maximum of approx. 60% when the DAB is removed by dilution or dialysis, it appeared important to examine the irreversible effects of DAB on the spectrum of catalase. The enzyme, at $0.4 \mu\text{M}$, was treated with DAB and then separated from the DAB by exhaustive dialysis. Optical spectra were then recorded. The results, shown in Fig. 4, demonstrate that no changes occur in the spectrum of DAB-inhibited catalase when the free DAB is removed, even though the enzyme remains up to approx. 50% inhibited. Moreover, addition of

Fig. 3. Effect of DAB on the optical spectrum of catalase

The spectrum of $0.2\text{ }\mu\text{M}$ -catalase was recorded in the absence and in the presence of DAB. In all cases DAB was present at the same concentration in blank and sample cuvettes, so that its contribution to the spectrum, which would have been insignificant in any case, was eliminated. The concentrations of DAB were: line 1, none; line 2, $40\text{ }\mu\text{M}$; line 3, $80\text{ }\mu\text{M}$; line 4, $160\text{ }\mu\text{M}$; line 5, $320\text{ }\mu\text{M}$. In the inset we record changes in absorbance on an expanded scale in the $500\text{--}700\text{ nm}$ region in the presence of the following concentrations of DAB: line 1, none; line 2, $100\text{ }\mu\text{M}$; line 3, $200\text{ }\mu\text{M}$; line 4, 300 or $400\text{ }\mu\text{M}$.

Fig. 4. Effect of exposure to DAB followed by dialysis on the optical spectrum of catalase

Catalase ($0.4\text{ }\mu\text{M}$) was treated with the following concentrations of DAB in the phosphate/EDTA buffer: line 1, none; line 2, $50\text{ }\mu\text{M}$; line 3, $100\text{ }\mu\text{M}$; line 4, $250\text{ }\mu\text{M}$; line 5, $500\text{ }\mu\text{M}$. The enzyme was then dialysed against the buffer to remove free DAB; it was diluted to $0.2\text{ }\mu\text{M}$ with the buffer and spectra were recorded. The inset shows the effect of DAB on the optical spectrum of irreversibly inhibited catalase. For this, catalase that had been exposed to $250\text{ }\mu\text{M}$ -DAB and then dialysed (as in line 3 above) was placed in a cuvette and spectra were recorded in the presence of the following concentrations of DAB: line 1, none; line 2, $100\text{ }\mu\text{M}$; line 3, $200\text{ }\mu\text{M}$; line 4, $400\text{ }\mu\text{M}$.

DAB to the irreversibly inhibited and dialysed enzyme then caused a diminution and red-shift of the Soret band identical with that seen with the native and fully active enzyme (see inset in Fig. 4).

Inhibition of catalase by a variety of aromatic amines

A number of compounds, more or less related to DAB, were examined for their ability to inhibit catalase. As shown in Table 1, *o*-dianisidine was an effective inhibitor, but *p*-phenylenediamine, tetramethylbenzidine and diaminoazobenzene were somewhat less effective. These compounds resembled DAB in showing both reversible and irreversible effects. Thus, as shown in Table 2, exposure of catalase to these compounds, followed by 500-fold dilution before assay, left the enzyme approx. 50% inhibited. The similarity to DAB inhibition extended to the competition with H_2O_2 . Thus catalase was 80% inhibited by $200\text{ }\mu\text{M}$ -*p*-phenylenediamine when assayed at $20\text{ mM-H}_2\text{O}_2$, but was only 5% inhibited when the H_2O_2 was at 400 mM .

Effect of ethanol on DAB inhibition

Margoliash *et al.* (1960) observed that ethanol, and other substrates for the peroxidatic action of catalase, protected against the irreversible inactivation of catalase by 3-aminotriazole plus H_2O_2 . If the inactivation of catalase by 3-aminotriazole depended upon oxidation of 3-aminotriazole by catalase Compound I, then any substrate capable of oxidation by Compound I should compete with 3-aminotriazole and thus protect the enzyme. This led to the conclusion that a product of the oxidation of 3-aminotriazole by Compound I was responsible for coupling with and thus inactivating the catalase. Since H_2O_2 was seen to increase the irreversible inhibition of catalase by DAB, it seemed likely that DAB, like 3-aminotriazole, was being oxidized by Compound I to a reactive intermediate, and that ethanol might therefore protect.

Ethanol, present in a 1000-fold molar excess over DAB, prevented the irreversible inhibition of catalase by DAB, but only weakly re-activated

Table 1. *Inhibition of catalase by aromatic amines*

$[I]_{50\%}$ denotes the concentration needed for 50% inhibition. Inhibition (200 μM) (%) denotes the percentage inhibition achieved at 200 μM . In all cases the inhibitors were present in the phosphate/EDTA-buffered assay mixture containing 20 mM- H_2O_2 , at pH 7.8 at 22°C.

Inhibitor	Structure	$[I]_{50\%}$	Inhibition (200 μM) (%)
Diaminobenzidine		20 μM	92
<i>o</i> -Dianisidine		25 μM	73
Benzidine		200 μM	50
Aniline		> 1.0 mM	22
Diaminoazobenzene		75 μM	48
Tetramethylbenzidine		62 μM	55
<i>o</i> -Phenylenediamine		100 μM	53
<i>m</i> -Phenylenediamine		> 1.0 M	18
<i>p</i> -Phenylenediamine		41 μM	83

enzyme that had been inactivated by exposure to DAB and then freed of this compound by dialysis. In contrast, exposure of DAB-inactivated catalase to ethanol, in the presence of a vapour-diffusion source of H_2O_2 achieved in a Conway micro-diffusion dish as described in the Materials and methods section, resulted in 95% re-activation. Moreover, native and DAB-inhibited catalase exhibited identical ethanol peroxidase activity.

Repeated exposures to DAB

Catalase that had been exposed to 200 μM -DAB and then dialysed, and that exhibited 50% of the native catalatic activity, was precisely as susceptible to inhibition of this residual activity, by DAB added to the assay mixture, as was native enzyme (results not shown). However, when such DAB-treated and dialysed enzyme was exposed to

Table 2. *Reversibility of the inhibition of catalase by aryl amines*

Catalase (0.01 μM) was incubated with each of the compounds listed at 500 μM in the phosphate/EDTA buffer at pH 7.8 at 22°C for 2 min. Activity recovered is given as percentages of the native activity recovered after 500-fold dilution into 20 mM- H_2O_2 in the phosphate/EDTA buffer.

Inhibitor	Concentration (μM)	Activity recovered (%)
Diaminobenzidine	500	47
<i>o</i> -Dianisidine	500	50
<i>p</i> -Phenylenediamine	500	52

200 μM -DAB and then diluted, the activity was only slightly diminished. The enzyme suffered 50% irreversible inhibition at the first exposure to DAB and only approx. 15% at the second exposure.

Discussion

The following scheme of reactions provides a basis for explaining the observations reported above:

Reactions (1) plus (2) represent the normal catalytic cycle of the enzyme, wherein successive encounters with H_2O_2 result in bivalent oxidation and then reduction of the active-site ferrihaem. Reaction (3) denotes a rapid and reversible association between the resting enzyme and DAB, which yields an inactive complex. This accounts for the reversible inhibition by DAB, which was competitive with H_2O_2 . Reactions (1), (4) and (5) suggest that catalase might catalyse the peroxidation of DAB. However, the slowness of reaction (5) makes this difficult to detect, in accord with the conclusion of Chance *et al.* (1979). If reaction (5) were much accelerated by glutaraldehyde treatment of the catalase, these reactions could explain the reported peroxidation of DAB by glutaraldehyde-treated catalase (Graham & Karnovsky, 1966; Fahimi, 1968; Novikoff & Goldfischer, 1969; Herzog & Fahimi, 1974). It is of interest that the other amines that we found most effective in inhibiting catalase, namely *o*-dianisidine and *p*-phenylenediamine, were also reported to be perox-

idized by the glutaraldehyde-treated enzyme (Herzog & Fahimi, 1974).

DAB, like so many other reductants, autoxidizes and so generates H_2O_2 . Aerobic solutions of DAB contain low concentrations of H_2O_2 , and adding DAB to catalase will therefore allow the formation of catalase Compound I, which may also react with DAB to yield a complex of the enzyme with the oxidized di-imino form of DAB (reaction 4). Reaction (4) thus accounts for the irreversible inhibition caused by DAB. Exogenous H_2O_2 did augment the irreversible inhibition of catalase by DAB.

It was noted that the higher the concentration of catalase being used the greater was the concentration of DAB needed to impose a given degree of irreversible inhibition. This too bespeaks a role for H_2O_2 in the irreversible inhibition. Thus at higher concentrations of catalase more H_2O_2 would be catalytically decomposed before the enzyme could be trapped by reaction (4) and more H_2O_2 would therefore be needed. Insofar as the irreversible inhibition by DAB is concerned, the limiting factor was not DAB as such, since it was present in great excess over the enzyme over the entire range of enzyme concentrations used. Rather, the limiting factor was H_2O_2 and raising the DAB concentration provided the additional H_2O_2 needed when dealing with higher concentrations of the enzyme.

Enzyme treated with DAB and then diluted or dialysed was 50–60% inactivated, but exhibited an optical spectrum indistinguishable from that of the native enzyme. We must conclude that DAB_{ox} binds to the protein in the active site, rather than to the haem. The seemingly irreversible inhibition caused by exposure to DAB could be prevented by ethanol and reversed by ethanol plus H_2O_2 . We therefore write reaction (5) as a slow dissociation of the $[\text{E-DAB}_{\text{ox}}]$ complex. The data suggest that ethanol can bind to $[\text{E-DAB}_{\text{ox}}]$ and speed the dissociation of this complex (reaction 5). Cyanide appeared to act in a similar way.

H_2O_2 plus ethanol was much more effective in reversing the DAB inhibition of catalase than was ethanol added on its own. This suggests the following additional reactions:

We here suppose that $[\text{E-DAB}_{\text{ox}}]$ can be oxidized by H_2O_2 (reaction 6) to $[\text{I-DAB}_{\text{ox}}]$, which is approximately half as reactive towards H_2O_2 as is free I, but which can be rapidly reduced by ethanol to active enzyme, acetaldehyde and free DAB_{ox} .

(reaction 7). Reaction (7) is analogous to the reaction of catalase Compound I with ethanol (Kremer, 1970).

Addition of DAB to catalase produced changes in its optical spectrum that match those caused by adding H_2O_2 as such (Fig. 3 and inset in Fig. 4) (Chance & Schonbaum, 1962). This is an indication that the aerobic DAB solutions did indeed contain H_2O_2 . Inhibition of catalase by DAB added to the reaction mixture was not a simple function of DAB concentration, as shown by the inset in Fig. 1. The data indicated more inhibition at high [DAB] than would have been predicted from that seen at low [DAB]. This is probably due to the circumstance that raising the concentration of DAB was, in effect, also raising the concentration of H_2O_2 and thus providing an additional route of inhibition (reactions 2 and 4).

One aspect of the inhibition by DAB remains puzzling, and this is the limit of 50–60% on the irreversible inhibition. Treatment of catalase with DAB, followed by dilution or dialysis, did not produce more than the 50–60% inactivation, and this was the case even when DAB was present in great excess. Moreover, repeated treatments with DAB did not result in much more than 60% total inactivation. Reactions (1)–(7) do not explain this limit. It may be that tight binding of DAB to two of the subunits of this tetrameric enzyme, as in reaction (4), may preclude tight binding at the remaining subunits, while allowing reversible binding. Alternatively, as put forth in reactions (6) and (7) and the accompanying discussion, the $[\text{E-DAB}_{\text{ox}}]$ complex may retain approx. 50% of the catalytic activity of E. This would make sense in view of the failure of irreversibly inactivated catalase to show any deviation in spectrum from that of the native enzyme. It thus seems likely that the DAB_{ox} is bound close enough to the haem to impose some steric hindrance to the reaction, but not close enough to perturb the haem spectrum.

The inhibition of catalase by DAB is intriguing both because of the peculiarities described above and because DAB has long been the reagent of choice for the histochemical staining and for activity-staining of this enzyme.

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