Inhibition of Enzymes by Metal Ion-Chelating Reagents

THE ACTION OF COPPER-CHELATING REAGENTS ON DIAMINE OXIDASE

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1. The inhibition of diamine oxidase has been studied by using the following copper-chelating reagents: 1,10-phenanthroline; 2,2'-bipyridyl; 8-hydroxyquinoline (oxine); diethyldithiocarbamate and dithio-oxamide (rubeanic acid). 2. Addition of chelating reagent caused a rapid inhibition of enzyme to a degree dependent solely on the final inhibitor concentration. Addition of substrate gave linear initial rates of reaction showing that under these conditions the inhibition was not being rapidly reversed. 3. The inhibition has been investigated by using new graphical methods and has been found in all cases to involve the chelating agents completely removing two Cu^{2+} ions from the enzyme. An alternative possibility, involving ligand substitution, was eliminated. 4. A value of $K = 8.0 \times 10^{-33} \text{M}^{-2}$ has been found for the enzyme in equilibrium with 2 Cu^{2+} ions (i.e. β_2 , the stability constant for diamine oxidase/two Cu^{2+} , is 32.1).

Diamine oxidase (EC 1.4.3.6) from pig kidney is an enzyme oxidizing diamines to aminoaldehydes according to the stoicheiometry:

The reaction mechanism involves only binary enzyme-substrate complexes, the catalytic sequence being amine on, aminoaldehyde off, O_2 on, followed by ordered release of H_2O_2 then NH_3 (Bardsley *et al.*, 1973). Pyridoxal phosphate is thought to be involved in binding substrate as a Schiff base (Kumagai *et al.*, 1969) and Cu^{2+} ions are required for activity, although the precise mode of involvement remains to be finally elucidated (Mondovi, 1972).

The number of Cu^{2+} ions involved has been given as 2.8 ± 0.7 g-atoms/mol of the enzyme of molecular weight 180000 (Goryachenkova *et al.*, 1968), 2.17 g-atoms/mol of the enzyme of molecular weight 185000 (Yamada *et al.*, 1967) and 1 atom of copper/unit of the enzyme of molecular weight 87000 (Mondovi *et al.*, 1967). An important technique used in this work was a study of the effect of the copper-sequestering agent diethyldithiocarbamate on the Cu^{2+} content and activity of the enzyme. After dialysis against diethyldithiocarbamate (0.05 M) for 12 h, 70% of the Cu^{2+} was removed and enzyme activity, tested against cadaverine, was abolished (Mondovi *et al.*, 1967). The activity could be restored to 40% of the original value by addition of Cu^{2+} after removing the Cu^{2+} -diethyldithiocarbamate complex by centrifugation but activity was restored to 70% of the original value if the Cu^{2+} -diethyl-

thiocarbamate complex was not centrifuged off. Addition of excess of Cu^{2+} (300 nmol/100 μg of enzyme) completely inhibited the enzyme (Mondovi *et al.*, 1967). In another study, Goryachenkova *et al.* (1968) found that when diethyldithiocarbamate was added, an insoluble Cu^{2+} -diethyldithiocarbamate complex was formed which when centrifuged off had removed 90% of the original Cu^{2+} . Activity was 'partially restored' by simultaneous addition of Cu^{2+} (0.1–1 μM) and pyridoxal phosphate to the apoenzyme.

The aim of the present study was to investigate the kinetics of inhibition of diamine oxidase by copper-chelating reagents in order to discover the mechanism of inhibition.

Experimental

The enzyme used in this work had an average specific activity of 1.4 units/mg at 20°C and was assayed by measuring the change in absorbance at 250 nm produced by oxidation of substrate (*p*-dimethylaminomethylbenzylamine) to product (*p*-dimethylaminomethylbenzaldehyde) as described by Bardsley *et al.* (1972). All experiments were conducted in 0.1 M-sodium-potassium phosphate buffer, pH 7.0, at 20°C in air.

Measurement of time-dependent inhibition

Enzyme solution (6 μl containing 0.0054 units $\equiv$ 21 pmol) was placed in a cuvette. At zero time a portion of inhibitor (0.1 ml) was added and mixed manually. After incubation for the required length of

time at 20°C, substrate was added (0.9 ml of 3.0 mM-*p*-dimethylaminomethylbenzylamine) and the change in absorbance read at 250 nm. It was found that 3 s before quenching with substrate was the shortest accurate incubation period possible by this method.

Measurement of active enzyme remaining after incubation with inhibitor

Enzyme solution (6 μ l, as above) was placed in a cuvette with 0.9 ml of metal-chelating reagent and incubated for 10 min. Substrate (30.0 mM) was then added (0.1 ml) and the initial reaction rates obtained. The initial rates were linear for at least 10 min showing that substrate was not appreciably reversing the inhibition caused by Cu^{2+} chelation. In these experiments the final concentrations were enzyme (21 nM), substrate (2.7 mM) and inhibitor as required. A similar technique, but with various substrate concentrations, was employed in obtaining data for double-reciprocal plots.

Fig. 1. Time-dependent inhibition of diamine oxidase by copper-chelating agents

After preincubation of enzyme and inhibitor at 20°C for the time indicated, substrate was added to give a final concentration of 2.7 mM and the percentage of enzyme activity remaining was measured. Results with diethyl-dithiocarbamate at final concentrations (after addition of substrate) of $\circ$, 0.05 mM; Δ , 0.08 mM; $\square$, 0.1 mM; $\bullet$, 0.25 mM and $\blacktriangle$, 0.4 mM. All the reagents tested behaved in the same way giving an immediate loss of activity which was then unchanged for up to 1 h.

Disc electrophoresis

Dissociation into subunits, as metal ions are removed by chelation, has to be taken into account when analysing type II mechanisms and this possibility was studied. Rubanic acid and diethyl-dithiocarbamate somehow prevented adequate gel formation but disc electrophoresis was performed satisfactorily in the presence of 1 mM-phenanthroline, 1 mM-2,2'-bipyridyl and 1 mM-8-hydroxyquinoline. No dissociation into subunits was observed.

Results

Throughout the present study, the initial rates of reaction, as measured by change in absorbance at 250 nm, were linear when substrate was added. Also double-reciprocal plots were linear and addition of substrate did not reverse the inhibition given by copper-chelating reagents. In addition, prolonged dialysis of the enzyme against 0.1 M-sodium-potassium phosphate buffer, pH 7.0, did not cause any decrease in enzyme activity owing to removal of Cu^{2+} ions and addition of Cu^{2+} ions did not increase the initial rate of reaction.

Fig. 1 shows that when diamine oxidase was incubated with copper-chelating reagents the enzyme

Fig. 2. Double-reciprocal plots for diamine oxidase after incubation at 20°C to equilibrium with the inhibitor concentration stated

Final concentration of 1,10-phenanthroline was: $\circ$, no inhibitor; Δ , 0.1 mM of inhibitor; $\square$, 0.16 mM of inhibitor; $\bullet$, 0.2 mM of inhibitor and $\blacktriangle$, 0.26 mM of inhibitor. All other reagents gave similar double-reciprocal plots.

Fig. 3. (a) Slopes and (b) intercepts for double-reciprocal plots as in Fig. 2 replotted against inhibitor concentration for 2,2'-bipyridyl

Replots of slopes and intercepts were similar for all chelating reagents used.

Fig. 4. Plots of λ against different concentrations of various inhibitors

The function $\lambda = (1 - [E]/[E_0])$ against $[I_0]$ for: (a) diethyldithiocarbamate; (b) 8-hydroxyquinoline; (c) 1,10-phenanthroline; (d) 2,2'-bipyridyl; (e) dithio-oxamide (rubeanic acid).

Fig. 5. Plots of a function of λ against $[I_0]$ for several values of y

The function of λ used is: $\lambda[\lambda/(1-\lambda)]^{1/y}$ against $[I_0]$ for 2,2'-bipyridyl, for several values of y (a) $y = 1$; (b) $y = 2$; (c) $y = 3$; (d) $y = 4$.

Fig. 6. Plots of a function of λ against $[I_0]$ for all compounds studied for $y = 2$

Function of λ is: $\lambda[\lambda/(1-\lambda)]^{1/2}$ against $[I_0]$. Experimental points are plotted ($\circ$) and the solid line is the least-squares fit calculated for $k[I_0]^2$ where k is the least-squares regression coefficient as follows: (a) diethyldithiocarbamate $4.74 \times 10^{10} [I]^4$; (b) 8-hydroxyquinoline (oxine) $4.75 \times 10^6 [I]^2$; (c) 1,10-phenanthroline $2.85 \times 10^{11} [I]^3$; (d) 2,2'-bipyridyl $2.99 \times 10^9 [I]^3$; (e) dithio-oxamide (rubeanic acid) $2.96 \times 10^6 [I]^2$.

activity fell immediately to reach an equilibrium position. Further experiments showed that the final position of equilibrium depended only upon the actual final concentration of inhibitor present when substrate was added to measure reaction rate. Excess of inhibitor produced complete inhibition in all cases.

Fig. 2 demonstrates that preincubation with inhibitor followed by addition of various concentrations of substrate caused an immediate effect on the slope of double-reciprocal plots as inhibitor concentration was increased from zero, followed by a gradual increase in the slope and intercept effects as inhibitor concentration was further increased. Suddenly, at more elevated inhibitor concentrations, a dramatic increase in slope and intercept was seen.

Fig. 3 further illustrates this effect by displaying the slopes and intercepts of double-reciprocal plots as a function of inhibitor concentration. From these replots it is obvious that the inhibition is described by some complex function or extremely high-degree polynomial in inhibitor concentration. The inhibition could not possibly be simple S-linear (or parabolic) and/or I-linear (or parabolic) where S and I refer to slopes and intercepts of double-reciprocal plots.

Fig. 4 gives the behaviour of λ as a function of inhibitor concentration. The curves are sigmoid with fairly well defined inflexions. [See Bardsley & Childs (1974) for definitions of symbols and derivations of equations.]

Fig. 5 shows plots of $\lambda[\lambda/(1-\lambda)]^{1/y}$ against inhibitor concentration for various y values.

It is possible to fit polynomials to many of the curves for various y values but in practice $y = 2$ seems to give the best distribution of experimental points. For instance, it is known that $x = 3$ for 1,10-phenanthroline and 2,2'-bipyridyl and by using this value we can see which of the plots derived for several y values give good fits for the cubic expression. Figs. 5(b), 5(c) and 5(f) approximate to cubic equations. Thus for 2,2'-bipyridyl, y could be 2 or possibly 3 but not 1 or 4, and for 1,10-phenanthroline, y is probably 2 but not 1, 3 or 4. We conclude that $y = 2$ is the correct value.

It is of some interest to observe the patterns of behaviour of the function $\lambda[\lambda/(1-\lambda)]^{1/y}$ as plotted against $[I_0]$ for various values of y . When $y = 1$ the curve rises slowly at first but then extremely rapidly as $[I]$ increases as if the function was of a high degree in $[I_0]$. For $y = 2$ the experimental points lie close to a cubic equation in $[I]$ but as y increases beyond 3 the points no longer seem to show any well-defined functional dependence on inhibitor concentration. A similar pattern was shown for the other inhibitors, i.e. in all cases well-defined curves were given for $y = 2$, i.e. 2 Cu^{2+} ions are released from the enzyme in the presence of chelating agent.

Fig. 6 shows graphs of $\lambda[\lambda/(1-\lambda)]^{\frac{1}{2}}$ against $[I_0]$ for all compounds used in the present work together

with curves calculated from least-squares fit to the best polynomial.

Discussion

A recent study of the effect of metal ion-chelating reagents as inhibitors of metalloenzymes (Bardsley & Childs, 1974) describes two mechanisms by which inhibition can be brought about.

Type I mechanisms involve ligand substitution into the co-ordination sphere of the metal at the enzyme active site and are characterized by linear double-reciprocal plots with replots of slope and intercept as simple functions of inhibitor concentration, usually linear. If v_0 is the initial rate with no inhibitor present but at the same substrate concentration, then plots of $(1-v_1/v_0)$ against $[I]$ will usually be smooth curves.

Type II mechanisms involve dissociation of the metal ion to form co-ordination complexes in solution and a characteristic feature of these mechanisms is that the enzyme system approaches an equilibrium position in a time-dependent manner. The final position of equilibrium is dictated by the concentration of inhibitor and plots of the degree of dissociation of enzyme ($\lambda = 1-v_1/v_0$) against inhibitor concentration will usually be sigmoid when multidentate ligands are used.

For type II mechanisms, information concerning the number of metal ions needed for enzyme activity (y) and the stability constant for binding (K_1^{-1}) can be obtained from the following function:

$$\lambda \left(\frac{\lambda}{1-\lambda} \right)^{\frac{1}{y}} = \frac{K_1^{\frac{1}{y}}}{y[E_0]} (1 + K_2[I_0] + K_2K_3[I_0]^2 + K_2K_3K_4[I_0]^3 \dots \dots)$$

where K_2 , K_3 , K_4 etc. are stability constants for successive complexing of metal and chelating agent. Where the metal ion is bound extremely tightly to the enzyme and the cumulative stability constant for metal complexing is well defined this simplifies to:

$$\lambda \left(\frac{\lambda}{1-\lambda} \right)^{\frac{1}{y}} = \frac{K_1^{\frac{1}{y}}}{y[E_0]} \cdot \beta_x [I_0]^x = k[I_0]^x$$

where β_x is the stability constant for $\text{M} + x\text{I} = \text{MI}_x$.

As slope and intercept replots are not simple functions (Figs. 2 and 3), we conclude that a type I mechanism is not operating. Also, since the λ plots are sigmoid and plots of $\lambda[\lambda/(1-\lambda)]^{\frac{1}{2}}$ are well defined functions of $[I_0]$ (Figs. 4, 5 and 6) for $y = 2$, we conclude that a type II mechanism is operating and that two metal ions are released/mol of enzyme. Only K_1 now remains to be determined.

It is only possible to determine K_1 accurately when the stability constant is known for the reaction conditions employed in this work, i.e. aqueous buffer (0.1 M-sodium-potassium phosphate buffer, pH 7.0 at 20°C). Values of stability constants in such a medium

are known (see Sillén & Martell, 1964) for 1,10-phenanthroline ($\beta_3 = 21$) and 2,2'-bipyridyl ($\beta_3 = 18$) and these give values of $1.43 \times 10^{-34} \text{ M}^{-2}$ and $1.59 \times 10^{-32} \text{ M}^{-2}$ respectively for K_1 . Unfortunately, the 8-hydroxyquinoline and diethyldithiocarbamate complexes are not sufficiently soluble in aqueous solution to permit determination of stability constants by the usual methods and, in addition, values of stability constants for rubeanic acid are not available. By using a mean value of $K_1 = 8.0 \times 10^{-33}$ we can give approximate values for these chelating reagents in dilute aqueous solution as follows: 8-hydroxyquinoline ($\beta_2 = 14$); diethyldithiocarbamate ($\beta_4 = 18$) and rubeanic acid ($\beta_2 = 14$). A noteworthy feature of these results is the high degree of complexing found for diethyldithiocarbamate (four instead of the usual value of two in aq. ethanol or dioxan) and rubeanic acid (two usually one) undoubtedly owing to the large ratio of complexing agent to Cu^{2+} ions.

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