The Inhibition of Caeruloplasmin by Cyanide

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1. The reversible inhibition of the oxidase activity of caeruloplasmin by cyanide was investigated. 2. The kinetics are unusual, being competitive but with the inhibited complex formed only during cycling. 3. Inhibitory concentrations of cyanide are comparable with that of caeruloplasmin. 4. One azide group completely inhibits a caeruloplasmin molecule but two cyanide groups are required. 5. The results suggest that azide binds to a half-reduced or fully reduced conformational isomer of the enzyme whereas cyanide binds to completely reoxidized isomers, and that inhibited complexes contain ligand bridges between copper atoms.

Many substances have been claimed to inhibit the oxidase activity of the plasma copper-containing protein caeruloplasmin against phenylenediamines or other substrates (for references see Curzon & Cumings, 1966; Curzon & Speyer, 1967). A number of these substances were reinvestigated under standard conditions (Curzon & Speyer, 1967) and shown to cause apparent inhibition by reaction with the product of enzyme action or with contaminant activating metal assumed to be present (Curzon, 1961; Levine & Peisach, 1963). A number of true inhibitors, i.e. those acting on the enzyme, were distinguished from the above group. The most powerful were cyanide and azide, which probably inhibit by interacting with caeruloplasmin copper.

Inhibition occurred at azide concentrations similar to that of the enzyme and the binding of a single azide group/enzyme molecule was shown to cause complete inhibition (Curzon, 1966, 1967). This effect was interpreted as being due to the copper atoms that change their oxidation state during enzyme action being close together when in the cuprous state so that the binding of a single azide group is sufficient to prevent their simultaneous reoxidation to cupric copper by an oxygen molecule. Because of these findings, and because cyanide inhibition also occurred at inhibitor concentrations similar to that of the enzyme, it was thought that the study of inhibition by cyanide might be of interest.

MATERIALS AND METHODS

Caeruloplasmin. Human caeruloplasmin (American Red Cross, batch no. 1995) was used. Treatment and specifications were as described by Curzon & Speyer (1967).

Substrate. DPD* dihydrochloride was dissolved in water and made alkaline, and the free base was extracted by ether, crystallized twice, converted into a 0.1M solution of the sulphate by addition of the calculated amount of dil. H₂SO₄ to give bis-DPD sulphate and stored at -25°. Specially purified bis-DPD sulphate was used in some experiments, indicated in the text. This was prepared as above but the alkaline hydrochloride was freed from possible metal contamination by passage through Chelex 100 chelating resin (BioRad Laboratories, Richmond, Calif., U.S.A.), and EDTA was added to a final concentration of 0.1 mM before extraction with ether purified by distillation. The H₂SO₄ used was also distilled. Working solutions of substrates were brought to pH 5.5 with NaOH previously passed through Chelex 100.

Inhibitors. NaN₃ (laboratory reagent grade) was recrystallized as described by Curzon (1966). KCN (A.R.) was 97% pure (AgNO₃ titration). K¹⁴CN (The Radiochemical Centre, Amersham, Bucks.) had a specific radioactivity of 0.67 mc/mg.

Chelating agents. EDTA, as the A.R. disodium salt, A.R. 1,10-phenanthroline hydrate and desferrioxamin (Ciba Laboratories, Horsham, Sussex) were used.

Caeruloplasmin oxidase activity method. Bis-DPD sulphate was oxidized by caeruloplasmin and *E*₅₅₀ of the red reaction product was measured with a Unicam SP.800 spectrophotometer. Determinations were made in the presence of EDTA to avoid enhancement of activity by traces of iron (Curzon & O'Reilly, 1960; Curzon, 1961; Levine & Peisach, 1963). Except where stated otherwise the reaction mixture was held in a 1 cm-path cell in a constant-temperature housing at 25° and contained substrate, 10 mM-sodium acetate-acetic acid buffer, pH 5.5, 4 μM-EDTA, inhibitor and 0.38 μM-caeruloplasmin, which was added last (as soon as possible after the inhibitor), to give a total volume of 2.5 ml. Caeruloplasmin concentration was calculated assuming that it contains 8 atoms of

* Abbreviations: DPD, *NN*-dimethyl-*p*-phenylenediamine.

Fig. 1. Attachment to cuvette for anaerobic experiments. The two narrow polythene tubes by means of which nitrogen was passed through the contents of the side arm and cuvette were fixed in the right hand tubulus by an Araldite plug. The polythene tube to the cuvette terminated in a glass capillary. A polythene outlet tube (not shown) was attached to the left hand tubulus and terminated in a capillary to minimize back-diffusion of air. For details of use see the text.

copper/molecule. E_{550} was measured at intervals or continuously against a blank containing all the reagents except the enzyme. $\Delta E/\text{min.}$ during the period of linear increase of E_{550} was measured in the presence of and absence of inhibitors, and the inhibition calculated. In certain experiments, indicated in the text, the acetate buffer was specially purified before use by passage through Chelex 100.

Anaerobic incubation of DPD-reduced caeruloplasmin with cyanide or azide. Incubation mixtures contained 1 mM-DPD (as sulphate), 10 mM-sodium acetate-acetic acid buffer, pH 5.5, 4 μM -EDTA and 1.2 μM -cyanide or 4 μM -azide. Also, 0.38 μM -caeruloplasmin was present in experiments at 25° and 2.28 μM -caeruloplasmin in those at 7.5°. The total volume was 3 ml. The apparatus used is shown in Fig. 1. Caeruloplasmin and acetate buffer were pipetted into a cuvette connected to the apparatus by the ground-glass joint. Substrate was pipetted into the side arm. In azide experiments the inhibitor was also pipetted into the side arm. A stream of N_2 was freed from traces of O_2 by passing it over MnO [prepared by passing $\text{N}_2 + \text{H}_2$ (95:5) over red-hot > 20 mesh MnO_2] and bubbled through the cell and side-arm solutions. The inlet and outlet tubes were then closed. After rapid mixing by inversion, the mixture was incubated for 10 min. E_{550} remained constant, showing that the apparatus was air-tight. The inlet and outlet tubes were opened and a stream of air (cooled for the 7.5° experiments) was passed through the cell for about 10 sec. E_{550} was read continuously over the next 10 min., and rose linearly in the absence of inhibitor. As cyanide was volatile in the stream of N_2 it was added after the apparatus had been closed by

injecting 0.1 ml. of cyanide solution in water through which N_2 had been bubbled, through the wall of the polythene outlet tube.

Modification of the oxidase activity method for the determination of the number of cyanide molecules bound/enzyme molecule. With the standard conditions for determination of oxidase activity, enzyme concentration was too low for accurate determination of cyanide bound to the enzyme. Higher enzyme concentrations resulted in extinctions due to product that were too high to be measured. Depression of the rate of product formation by incubating at 7.5°, as used with azide (Curzon, 1966), was impracticable as cyanide equilibrated very slowly with the enzyme at this temperature. Therefore the activity method was modified and a higher enzyme concentration and a shorter light-path (0.2 cm.) at 25° were used. The cell contained substrate, buffer and EDTA at concentrations as in the standard method, various concentrations of cyanide and 1.52 μM -caeruloplasmin, mixed by inversion, in a total volume of 0.6 ml. In the absence of cyanide, the rate of increase of $E_{550}^{0.2\text{cm.}}$ was constant up to $E \approx 0.9$. Equilibrium with cyanide occurred after 5 min. of enzyme action.

Cyanide is undissociated at pH 5.5 and may be appreciably volatile (Curzon & Speyer, 1967). It was found with [^{14}C]cyanide that under the above conditions cyanide was lost at 0.3%/min. As inhibitions were calculated from extinctions obtained between 5 and 10 min. incubation time, cyanide concentrations, which are critical in this experiment, have been corrected for 2% loss and for the 3% impurity, but have been left uncorrected in other experiments.

RESULTS

Effect of time on cyanide inhibitions. In general, the inhibitors studied by Curzon & Speyer (1967) reached equilibrium with caeruloplasmin at 25° within the first 2 min. of enzyme action. Cyanide, however, required 10–15 min. to attain inhibitory equilibrium (Fig. 2) under these conditions with 1 mM-substrate. E_{550} then increased linearly with time for at least 15 min. Equilibration was more rapid at higher substrate concentrations.

The rate of formation of the inhibited complex increased with temperature, equilibrium being attained in 3 min. at 37° but requiring > 30 min. at 7.5°. At 25° the slow equilibrium after addition of DPD was unchanged by 60 min. preincubation of the inhibitor with the enzyme and buffer in the absence of DPD. The slow formation of the inhibited complex was not due to a gradual increase in susceptibility of the enzyme during cycling nor to an effect of product on the enzyme-cyanide equilibrium, since a similar course of development of inhibition was observed when cyanide was added after 15 min. incubation of enzyme with buffer and substrate.

The effect of incubation of cyanide with the reduced enzyme was then investigated. Caeruloplasmin, substrate, buffer and cyanide were mixed anaerobically at 25° as described in the Materials and Methods section. E_{550} immediately rose to

Fig. 2. Effect of time on the inhibition of caeruloplasmin oxidase activity by cyanide at 25°. The following concentrations of cyanide were used: O, none; Δ , $1.2 \mu\text{M}$. Other conditions were as described in the Materials and Methods section.

0.1–0.2, indicating an oxidation–reduction reaction resulting in the formation of oxidized DPD and reduced enzyme, and then remained stationary. After incubation for 10 min., air was admitted to permit enzyme cycling. The subsequent time-course of inhibition was as in Fig. 2. Qualitatively similar results were obtained with azide at 7.5°. Thus neither the oxidized nor the reduced enzyme in their resting forms, but an intermediate form or forms of the enzyme occurring only during cycling, react with azide or cyanide in the formation of inhibited complexes.

Effect of substrate concentration on inhibition by cyanide. Study of the effect of substrate concentration on caeruloplasmin activity is complicated, as the Lineweaver–Burk plots are curved downwards (Osaki, 1966; Curzon, 1967; Løvstad & Walaas, 1967). The above authors interpreted this effect as being due to two types of substrate-binding site with different K_m values. Azide gave parallel Lineweaver–Burk plots (Curzon, 1966), showing anti-competitive inhibition, but plots obtained with cyanide showed competitive kinetics (Fig. 3a). This is also illustrated (Fig. 3b) by a plot of inhibition against $-\log$ (substrate concentration). The negative slope indicates competition (Webb, 1963). Parallelism of the azide Lineweaver–Burk plots was confirmed over the substrate concentration range used for the cyanide experiments.

At substrate concentrations below 0.1 mM only substrate-binding sites with the lower K_m values are involved and therefore Lineweaver–Burk plots

Fig. 3. Effect of cyanide on caeruloplasmin oxidase activity at various substrate concentrations at 25°. The concentration of SO_4^{2-} was constant at 17.5 mM, attained by addition of Na_2SO_4 solution. Other conditions were as described in the Materials and Methods Section. (a) Lineweaver–Burk plots at the following concentrations of cyanide: O, none; Δ , $1 \mu\text{M}$; $\square$, $2 \mu\text{M}$; $\bullet$, $3 \mu\text{M}$. (b) Inhibitions by $1 \mu\text{M}$ -cyanide plotted against $\log[S]$.

become straight lines. Unfortunately, it was not possible to study cyanide inhibition kinetics under these conditions, as equilibrium could not be

demonstrated in the short period before the substrate was significantly depleted.

Competitive kinetics may result from combination of cyanide with the substrate or with impurities in it, resulting in formation of non-inhibitory material. The former mechanism is unlikely but the latter could occur by formation of complexes of cyanide with trace metal impurities. This requires consideration even though activity determinations were done in the presence of EDTA, as the cumulative stability constants of many metals with cyanide are much greater than with EDTA (Sillén & Martell, 1964). However, inhibitions were not significantly altered when the specially purified reagents were used, nor when Fe^{2+} , Ni^{2+} or Cu^{2+} at a final concentration of $1\text{ }\mu\text{M}$ was added to the incubation mixture. Also, 10mM -1,10-phenanthroline or 0.2mM -desferrioxamin did not affect inhibition by cyanide. Desferrioxamin is a powerful specific iron chelator (Schwarzenbach & Schwarzenbach, 1963). The above observations indicate that the competitive kinetics are unlikely to be due to metal ion impurities.

The inhibition was previously shown to be

reversible on Sephadex (Curzon & Speyer, 1967), indicating that a caeruloplasmin-cyanide complex is formed. Also, when the cycling enzyme was equilibrated with cyanide and the substrate concentration then increased from 0.5mM to 12.5mM , inhibition decreased sharply, which is further evidence of reversibility and competitive kinetics. Though cyanide inhibition was slow, reversal was rapid, the new equilibrium after an increase in the substrate concentration being attained within 2min. The inability of cyanide to equilibrate with caeruloplasmin except when the latter is cycling indicates that though the kinetics of inhibition by cyanide are competitive the mechanism is not simple.

Determination of the number of cyanide groups bound to each caeruloplasmin molecule. At 25° , inhibition occurred at concentrations of cyanide similar to that of the caeruloplasmin. Therefore an appreciable fraction of the cyanide present is bound to the enzyme and mutual-depletion kinetics occur (Webb, 1963), the concentrations of free enzyme and free cyanide being mutually decreased. The graphical method of Easson & Stedman (1936)

Fig. 4. Determination of number of cyanide- and azide-binding sites/caeruloplasmin molecule. Inhibition was determined with various cyanide and azide concentrations at 25° . Cyanide experiments: $\circ$, 10mM -DPD (as sulphate); $\bullet$, 1mM -DPD (as sulphate). Azide experiments: $\triangle$, 10mM -DPD (as sulphate); $\blacktriangle$, 1mM -DPD (as sulphate). Other conditions were as described in the Materials and Methods Section. Results were plotted as discussed in the text by the method of Easson & Stedman (1936). $[I_t]$ is the total concentration of cyanide or azide and α is the fractional activity. Intercepts were calculated by conventional statistical methods. In the cyanide-inhibition experiments intercepts were $2.70 (\pm 0.26)$ and $1.86 (\pm 0.34)$ at 10mM and 1mM substrate concentrations respectively. In the azide experiments the intercept was $1.86 (\pm 0.59)$. Caeruloplasmin concentration was $1.52\text{ }\mu\text{M}$.

may be used to determine the number of cyanide groups bound/caeruloplasmin molecule. The method was originally applied by these workers to non-competitive inhibition, in which case the enzyme is all available to inhibitor. It was modified for application to the cyanide inhibition as follows. Inhibition results were plotted by the method of Easson & Stedman at 10mM and 1mM substrate concentrations (Fig. 4). Inhibition was not significantly affected when the specially purified substrate and buffer were used (see the Materials and Methods section). The apparent numbers of cyanide groups bound/enzyme molecule at these substrate concentrations are given by the relevant intercepts of Fig. 4 divided by the enzyme concentration, i.e. 1.78 and 1.23 at 10mM and 1mM substrate concentration respectively. The above substrate concentrations correspond to 88% and 60% saturation of the enzyme with substrate (Curzon, 1967). If the fraction of enzyme molecules available to cyanide is proportional to the degree of saturation, then the number of cyanide groups bound/available enzyme molecule is 2.02 (± 0.19) and 2.03 (± 0.38) (calculated from the results at 10mM and 1mM substrate concentration respectively). The 95% confidence limits are given in parentheses.

Fig. 4 also shows some results with azide under the general conditions of the cyanide experiments. Here the points do not differ significantly at 10 mM and 1 mM substrate concentration, indicating that, unlike the behaviour with cyanide, essentially all the enzyme molecules are available to azide at 1 mM substrate concentration. Under these conditions, although the enzyme is only 60% saturated, the low- K_m sites are almost completely saturated (94%) (Curzon, 1967). The number of azide groups bound/enzyme molecule is $1.22 (\pm 0.39)$, which is in reasonable agreement with the previous finding of 0.94 under different conditions (Curzon, 1966).

DISCUSSION

The reaction between caeruloplasmin and its substrate may be considered in simplified form as:

where E^{2+} and E^+ represent oxidized and reduced forms of the enzyme with the four copper atoms that can change their oxidation state in the cupric and cuprous states respectively, S represents the substrate and S^+ the monoradical oxidation product (Broman, Malmström, Aasa & Vänngård,

1963; Peisach & Levine, 1963). Caeruloplasmin also contains four permanently cuprous copper atoms/molecule. The rate of reoxidation of the reduced enzyme is dependent on oxygen concentration only when it is much lower than in the aerobic experiments described in this paper (Broman *et al.* 1963).

As the inhibition of caeruloplasmin by cyanide is reversible on Sephadex (Curzon & Speyer, 1967) it appears to be due to caeruloplasmin-copper-cyanide complex-formation and not to the reversible removal of copper from caeruloplasmin as a cyanide complex. As enzyme-substrate interaction is necessary for formation of the cyanide-inhibited complex, the kinetics of competitive inhibition are not due to a simple competitive mechanism.

An enzyme mechanism that would account for the cyanide and azide inhibitions is suggested (Scheme 1). Here the resting oxidized form of the enzyme, E_a^{2+} , forms a complex with substrate, $E_a^{2+}S_4$, in equilibrium with a conformational isomer, $E_b^{2+}S_4$. The reverse isomerization reaction is slow. Complex $E_b^{2+}S_4$ breaks down to product and a reduced form of the enzyme, E_b^+ , which can slowly isomerize to the reduced resting form, E_a^+ . These reduced forms are reoxidized by oxygen to the forms E_b^{2+} and E_a^{2+} respectively and the b isomer forms the enzyme-substrate complex $E_b^{2+}S_4$. Isomer E_b^{2+} may also slowly isomerize to isomer E_a^{2+} . The above is an extension of the mechanism suggested by Rabin (1967) as an explanation of enzyme-substrate co-operative effects. Now if all reverse isomerizations from b forms to a forms are slow, the reaction path after the initial reduction of the caeruloplasmin cupric copper to the cuprous state is essentially completely via b forms of the enzyme. Form E_b^{2+} is envisaged as the form to which cyanide binds, competing with substrate, whereas azide binds to the form E_b^+ , preventing its oxidation to the form E_b^{2+} . As form E_b^+ is an intermediate complex, the Lineweaver-Burk plots with different concentrations of azide are parallel. In the absence of substrate, forms E_b^+ and E_2^{2+} isomerize to forms E_a^+ and E_a^{2+} , which are unavailable to azide and cyanide respectively. Hence the inhibited complexes are both only formed in the presence of substrate, i.e. when the enzyme is cycling. The slow formation of the inhibited complexes implies that further conformational change of the forms E_b^+ and E_b^{2+} may be necessary for azide and cyanide binding.

At high substrate concentration one azide group/caeruloplasmin molecule is bound in the inhibited complex but two cyanide groups are bound. Caeruloplasmin has two kinds of substrate-binding site with different K_m values (Curzon, 1967). The maximum enzyme velocity when both kinds of site are saturated with substrate is twice the

Scheme 1. Proposed mechanism for the caeruloplasmin-substrate reaction and for cyanide and azide inhibitions.

maximum velocity when only the low- K_m sites are saturated. This suggests that complete saturation with substrate results in simultaneous reduction of all the four copper atoms that change their oxidation state, but that saturation of the low- K_m sites causes the reduction of only two copper atoms. However, even when only the low- K_m sites are saturated the blue colour of caeruloplasmin disappears (Curzon, 1967), which indicates reduction of all the cupric copper atoms (Blumberg, Eisinger, Aisen, Morell & Scheinberg, 1963). Therefore at low substrate concentrations, when only low- K_m sites are involved, two partial cycles each involving two copper atoms that change their oxidation state must occur before reoxidation by oxygen. Thus the reaction scheme at low substrate concentration is of the following type:

where A and B are low- and high- K_m substrate-binding sites respectively. At high substrate concentration both the low- and high- K_m sites are available and:

Whether the low- K_m sites or all the sites are involved, reoxidation of the completely reduced enzyme occurs (Broman *et al.* 1963) by:

Now inhibition is complete with one azide group/caeruloplasmin molecule whether all the substrate-binding sites or only the low- K_m sites are saturated. This suggests that azide can inhibit by binding with a half-reduced form of the enzyme.

Although two cyanide groups are bound/caeruloplasmin molecule when both the low- K_m and the high- K_m sites are saturated, less cyanide is bound when only the low- K_m sites are saturated.

The simplest explanation of these results is that cyanide binds the permanently cuprous copper atoms of the enzyme form E_b^{2+} . At lower substrate concentrations conformational change is suggested to be restricted to the regions of the copper atoms that change their oxidation state and of cuprous copper atoms at or near the low- K_m sites, whereas at higher substrate concentrations conformational change also occurs in the regions of the other permanently cuprous copper atoms at or near the high- K_m sites. It is assumed that binding of an azide group to the half-reduced enzyme prevents access of a second azide group to the completely reduced enzyme. The finding that only one azide or two cyanide groups are bound in the inhibited complexes, although the caeruloplasmin molecule contains eight copper atoms, suggests that these ligands bridge copper atoms that are close together in inhibitable forms of the enzyme. Bridging is well known in crystalline metal complexes and occurs in cuprous azide (Wilsdorf, 1948), and cyanide is particularly prone to form bridged complexes (Shriver, 1966). The necessity to bridge sites would impose a considerable restriction upon the dimensions of inhibiting ligands, and it is noteworthy that cyanide is the only inhibitor so far found that gives competitive kinetics with caeruloplasmin (Curzon & Speyer, 1967). These kinetics show that enzyme-cyanide complex-formation excludes enzyme-substrate complex-formation and vice versa. This mutual exclusion may be due to competition between cyanide and substrate for the same sites. Alternatively it may be due to cyanide and substrate binding at different sites but each preventing binding of the other. Blumberg (1966) has suggested a kinetic model for caeruloplasmin in which all the

copper atoms change their oxidation state but only half may be cupric at any time. In these circumstances consideration of any particular group of caeruloplasmin copper atoms as 'permanently cuprous' or 'copper atoms that change their oxidation state' may be an oversimplification. It is noteworthy that for pairs of copper atoms to be bridged by azide or cyanide they must be 3.5 Å or 3.0 Å apart respectively and that such arrangements are close to optimum for bridging by oxygen, so that simultaneous electron transfers may occur from cuprous copper atoms to oxygen.

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