ACTA CHEMICA SCANDINAVICA 27 (1973) 121–126

The Effect of Anions on the Oxidase Activity of Rat Ceruloplasmin

ROLF A. LØVSTAD* and EARL FRIEDEN

Department of Chemistry, Florida State University, Tallahassee, Florida 32306, USA, and Institute for Medical Biochemistry, University of Oslo, Oslo, Norway

The oxidation of DPD and PPD by rat ceruloplasmin was studied. With DPD as substrate the double reciprocal plots ($1/V$ *vs.* $1/[S]$) were non-linear, indicating two different, active sites. Recent investigations, however, suggest that the non-linearity is due to the complex interaction between ceruloplasmin, DPD and oxidation products of DPD.

When PPD acted as substrate in the presence of NADH, straight lines were obtained in double reciprocal plots, indicating only one type of active site.

The kinetics of inhibition of the PPD oxidase activity by some monovalent anions were studied, and the order of inhibition was found to be $F^- > I^- > NO_3^- > Br^- > Cl^-$. The inhibitory power of Cl^-, Br^-, NO^-_3 and I^- followed the lyotropic series, the effect being inversely proportional to the radius of the hydrated anion. Kinetic studies revealed a non-competitive inhibition mechanism in the case of Cl^-, Br^-, NO_3^- and I^-, while F^- gave a "mixed type" inhibition pattern. The results suggest that F^- and the other anions investigated bind to different sites on the enzyme molecule. The effect of anions on rat ceruloplasmin was compared with their effect on human ceruloplasmin.

Ceruloplasmin (E.C.1.12.3) is a blue serum protein, containing copper atoms, and having oxidase activity against several aromatic diamines and diphenols.[1–4] The blue color and oxidase activity can be attributed to protein bound cupric ions.[5] During reaction electrons are transferred from substrate to cupric ions, reducing them to cuprous. The latter are reoxidized by molecular oxygen.

Holmberg and Laurell [6] found that human ceruloplasmin was strikingly sensitive to monovalent anion inhibition. The kinetics of anion inhibition were further investigated by Curzon and Speyer,[7,8] who reported that the order of inhibitory effect of halide ions was $F^- > I^- = Cl^- > Br^-$.

Although the kinetics of human ceruloplasmin have been extensively studied,[1–8,10–12] little work has been done on ceruloplasmin from other sources.

* Present address: Institute for Medical Biochemistry, University of Oslo, Oslo, Norway.

This paper describes the kinetic properties of rat ceruloplasmin and the effect of some monovalent anions on the oxidase activity of the enzyme. The results have been compared with those reported for human ceruloplasmin.

EXPERIMENTAL

Materials. DPD.HCl,* PPD, and NADH were purchased from Sigma Chemical Company and Desferal from Ciba Pharmaceutical Company. All other reagents were of the purest grade available commercially. Aqueous solutions were prepared in deionized, glass distilled water.

Ceruloplasmin was purified from serum obtained from female Wistar rats (250 – 300 g). Rat serum (50 ml) was diluted ten times with 0.05 M potassium phosphate buffer, pH 6.8, containing 0.1 M KCl, and placed on a DEAE-Sephadex A-50 column (1.6 cm × 15 cm), equilibrated with 0.05 M potassium phosphate buffer, pH 6.8, in 0.1 M KCl. The purification was carried out in the cold room (4°). Ceruloplasmin formed a blue band on the top of the column, which was thoroughly washed with 0.05 M potassium phosphate buffer, pH 6.8, in 0.1 M KCl. The enzyme was eluted with 0.5 M potassium phosphate buffer, pH 6.8, in 0.1 M KCl. The ratio between the absorbances at 610 mμ and 280 mμ was 0.011.

The enzyme solution was 40 % saturated with ammonium sulfate and centrifuged for 20 min at 12 300 *g* and 0°. The supernatant, containing ceruloplasmin, was 50 % saturated with ammonium sulfate, and 70 % of the total ceruloplasmin content was found in the precipitate after centrifugation for 20 min at 12 300 *g* and 0°. The rest of the ceruloplasmin precipitated at 60 % saturation with ammonium sulfate. The ceruloplasmin-containing precipitates were dissolved in 0.6 M sodium acetate buffer, pH 6.0. The ratio between the absorbances at 610 mμ and 280 mμ was 0.015 for both samples.

Oxidase activity measurements. The activity of rat ceruloplasmin was determined as the rate of formation of the red colored radical, DPD^+, an oxidation product of DPD. DPD^+ has an absorption maximum at 552 mμ ($\varepsilon = 9800\ M^{-1}\ cm^{-1}$). [1,3] A lag period occurred when the absorbance at 552 mμ was recorded, the rate increasing during the first 1 – 2 min. After the lag period the 552 mμ absorption increased linearly and the activity was determined from the linear part of the curve.[1] An iron chelating agent, Desferal, was added to all reaction mixtures in order to prevent the activating effect of iron ions on the ceruloplasmin catalyzed oxidation.[1] DPD was dissolved in 25 μM EDTA immediately before use, and stored in an ice bath. The Cl^- concentration in the reaction mixtures was kept at 2.0 mM by adding NaCl, since DPD in the monohydrochloride form was used.

The ceruloplasmin catalyzed oxidation of PPD in the presence of NADH was also investigated. In this case the oxidation product of PPD was reduced to PPD by NADH, and the activity was determined spectrophotometrically at 340 mμ as the rate of NADH oxidation ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). No initial lag period was observed with this system. It was necessary to correct for a slight non-enzymic oxidation of NADH.

Anion inhibitors were incubated with rat ceruloplasmin for 10 min at 30° before adding substrate. Incubation of enzyme with inhibitor for 2 h resulted in the same degree of inhibition as noted when the rates were measured after 10 min incubation.

A Beckman DK-1 recording spectrophotometer, equipped with a thermocell (1 cm light path), was used and the temperature was kept at 30 ± 0.3° in all experiments except one.

RESULTS AND DISCUSSION

The effect of enzyme concentration on the initial rate of PPD and DPD oxidation was investigated, and as shown in Fig. 1, the activity increased

* Abbreviations: PPD, *p*-phenylenediamine; DPD, *N,N*-dimethyl-*p*-phenylenediamine, DPD^+, *N,N*-dimethyl-*p*-phenylenediamine radical, Desferal, deferoxamine B-methane sulfonate, NADH, dihydronicotinamide adenine dinucleotide, EDTA, ethylenediaminetetraacetic acid.

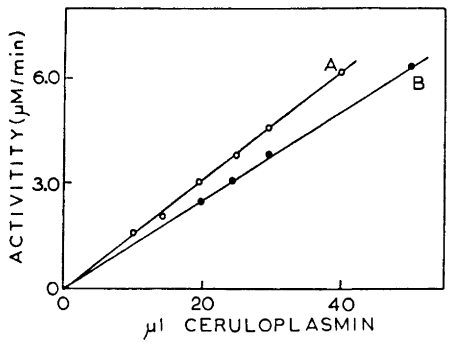

Fig. 1. Effect of rat ceruloplasmin concentration on the oxidase activity at 30°. When PPD (A) acted as substrate the reaction mixture contained 10 – 40 μl ceruloplasmin ($E_{610}=0.10$) (total volume 1.0 ml), 0.5 mM Desferal, 0.24 mM NADH, and 4.0 mM PPD in 0.10 M sodium acetate buffer, pH 5.5. In the presence of DPD (B) the reaction mixture contained 20 – 50 μl ceruloplasmin ($E_{610}=0.03$) (total volume 1.0 ml), 0.5 mM Desferal and 1.0 mM DPD in 0.10 M sodium acetate buffer, pH 5.3.

linearly with enzyme concentration when substrate concentration was kept constant. The pH optimum of rat ceruloplasmin was 5.6 with PPD as substrate and 5.0 with DPD at 30°.

Fig. 2a shows a non-linear relationship between rat ceruloplasmin activity and DPD concentration in a double reciprocal plot ($1/V$ *vs.* 1/[S]) at 30° and

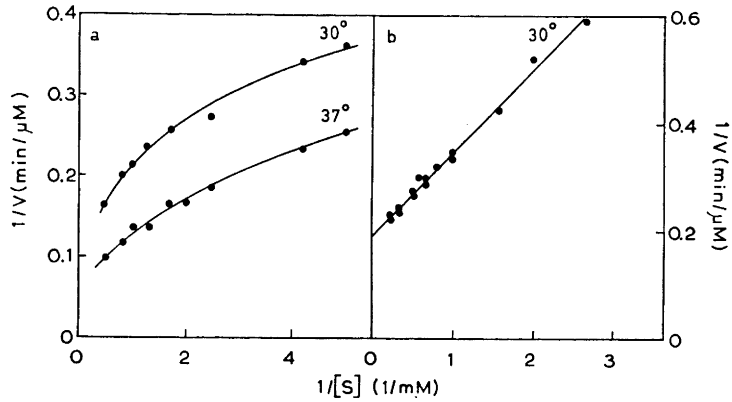

Fig. 2. a. The reciprocal rate of DPD oxidation plotted against the reciprocal DPD concentration. The reaction mixture contained 25 μl rat ceruloplasmin ($E_{610}=0.07$) (total volume 1.0 ml), 0.5 mM Desferal, 2.0 mM Cl^- and DPD (0.2 mM – 2.0 mM) in 0.1 M sodium acetate buffer, pH 5.5. b. The reciprocal rate of PPD oxidation in the presence of NADH plotted against the reciprocal PPD concentration. The reaction mixture contained 25 μl rat ceruloplasmin ($E_{610}=0.10$) (total volume 1.0 ml), 0.5 mM Desferal, 0.24 mM NADH and PPD (0.38 mM – 5.0 mM) in 0.1 M sodium acetate buffer, pH 5.5.

37°. A similar result has been reported for human [1,3] and porcine [9] ceruloplasmin. Curzon [1] and Walaas *et al.*[3] interpreted the non-linear plots in terms of two different active sites acting on the same substrate. However, kinetic studies by Pettersson and Pettersson [10,11] demonstrated that the kinetics observed could be explained by the fact that the first oxidation product of DPD, DPD^+, interacts with ceruloplasmin as reported by Walaas *et al.*,[3] and

that there exists a non-enzymic equilibrium between DPD and its oxidation products, DPD^+ and DPD^{2+}. Kinetic experiments by Curzon,[12] using a DPD-ascorbate coupled system, confirmed the results of Pettersson and Pettersson.

When PPD acted as substrate for rat ceruloplasmin in the presence of NADH, a straight line was obtained in a double reciprocal plot (Fig. 2b), indicating that PPD reacts with only one type of active site. This is in accordance with the observation of Curzon.[12] The apparent Michaelis constant (K_m) for PPD was estimated to 1.0 mM. In the following kinetic studies the PPD-NADH coupled system was used, since the complex mechanism of DPD-ceruloplasmin interaction gives rise to the non-linearity shown in the double reciprocal plots in Fig. 2a.

Monovalent anions of the Hofmeister or lyotropic series [13] had an inhibitory effect on the rat ceruloplasmin catalyzed oxidation of PPD (Fig. 3). The order

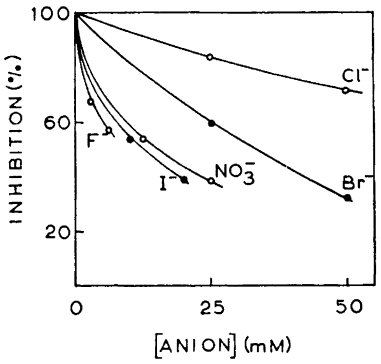

Fig. 3. Inhibitory effect of anions on the maximum rate (V_{max}) of the ceruloplasmin catalyzed oxidation of PPD, as calculated from Fig. 4.

of effectiveness was $F^- > I^- > NO_3^- > Br^- > Cl^-$. The same order of inhibition was observed when DPD acted as substrate. Except for F^- the order of inhibitory power conforms to the lyotropic series as shown for several enzymes ($I^- > Br^- > Cl^- > F^-$).[14–16] The unusually strong binding of F^- to copper enzymes like tyrosinase,[17] laccase,[18] and human ceruloplasmin [7] suggests that F^- binds to protein copper. Studies by Andréasson and Vänngård,[19] made with the electron paramagnetic resonance technique, demonstrated that F^- binds to the type 2 Cu(II) on the human ceruloplasmin molecule.

The order of inhibition of rat ceruloplasmin by halide ions differs somewhat from the order reported by Curzon and Speyer [7] for human ceruloplasmin ($F^- > I^- = Cl^- > Br^-$). I^- is a much better inhibitor than Cl^- and Cl^- inhibits less than Br^- compared to human ceruloplasmin.

The order of inhibitory power of the anions could not be attributed to differences in anionic size in the case of rat ceruloplasmin. However, the degree of inhibition was found to decrease with increasing radius of the hydrated anion (r_s),[20] indicating that the size of the hydrated anion may strongly affect the anion-protein interaction (Fig. 5). F^- did not fit into the plot in Fig. 5 being heavily hydrated ($r_s = 3.06$ Å) and would be expected to be the weakest inhibitor among the anions investigated, as is usually the case.[14–16] It is therefore probable that F^- binds to another site on the enzyme molecule.

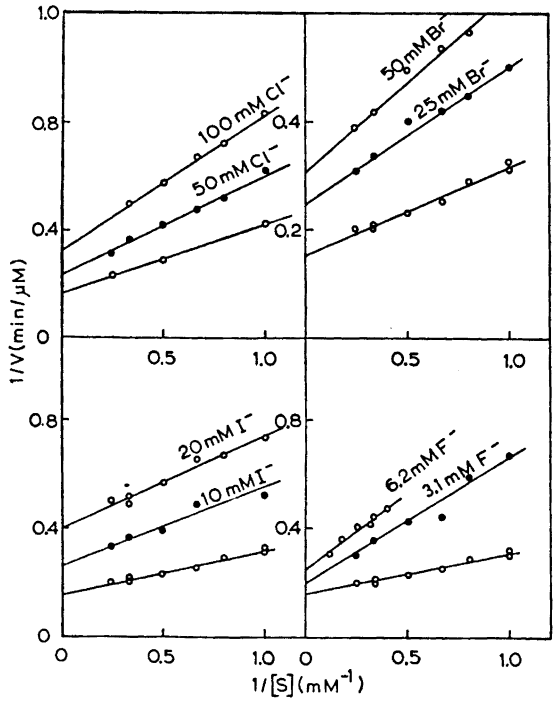

Fig. 4. Effect of anions on the PPD oxidase activity of rat ceruloplasmin at 30°. The reaction mixture contained 25 μl rat ceruloplasmin ($E_{610}=0.10$) (total volume 1.0 ml), 0.5 mM Desferal, 0.24 mM NADH and PPD (1.0 mM – 4.0 mM) in 0.1 M sodium acetate buffer, pH 5.5.

Fig. 4 shows the effect of several inhibitor and substrate concentrations on the oxidase activity of rat ceruloplasmin. The straight lines obtained in the double reciprocal plots intersect very close to the abscissa in the case of Cl^-,

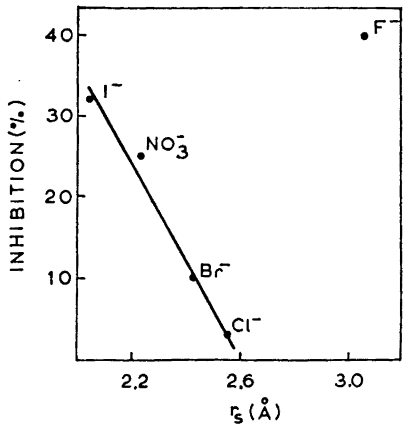

Fig. 5. The inhibitory effect of anions (5.0 mM) on the maximum PPD oxidase activity (V_{max}) of rat ceruloplasmin plotted against the radius of the hydrated anions. Experimental conditions as described in the legend to Fig. 4.

Br^-, NO_3^-, and I^-. This non-competitive mechanism of inhibition indicates that these anions bind to another site on the enzyme than the substrate, and may affect the reaction by inducing a change at the active site or by preventing a possible electron transport on the protein molecule. Ceruloplasmin is reoxidized by molecular oxygen after reduction by substrate and has to store four electrons in order to reduce one molecule of oxygen to water. A transport of electrons from the active site to other parts of the enzyme may therefore take place.

Cl^-, Br^-, NO_3^-, and I^- probably bind to the same site on the enzyme, since the inhibitory mechanism is the same for all of them (Fig. 4), and the order of inhibition follows the lyotropic series.

The mechanism of F^- inhibition is somewhat different as demonstrated in Fig. 4. The lines obtained intersect much closer to the ordinate, suggesting a "mixed type" of inhibition,[21] the effect of F^- being reduced by increasing substrate concentration. This mechanism of inhibition is generally explained by assuming that the inhibitor and substrate bind to different sites on the protein molecule, the inhibitor affecting both the substrate affinity of the enzyme and the rate of product formation.[21]

Acknowledgements. We thank Professor O. Walaas, Dosent E. Walaas and Dr. S. Osaki for their help and interest and Mrs. A. Horn for technical assistance. This work was supported in part by the *U.S. Public Health*, Grant HE 08344.

R. Løvstad was a recipient of U.S. Int. Public Health Service Research Fellowship 1-FO-5-TW-1430-01.

Paper No. 36 in a series from Department of Chemistry, Florida State University, Tallahassee, Florida 32306, USA, on copper biosystems.

REFERENCES

1. Curzon, G. *Biochem. J.* **103** (1967) 289.
2. Peisach, J. and Levine, W. G. *Biochim. Biophys. Acta* **77** (1963) 615.
3. Walaas, E., Løvstad, R. A. and Walaas, O. *Arch. Biochem. Biophys.* **121** (1967) 480.
4. Løvstad, R. A. *Acta Chem. Scand.* **25** (1971) 3144.
5. Broman, L., Malmström, B. G., Aasa, R. and Vänngård, T. *J. Mol. Biol.* **5** (1963) 301.
6. Holmberg, C. G. and Laurell, C. B. *Acta Chem. Scand.* **5** (1951) 921.
7. Curzon, G. and Speyer, B. E. *Biochem. J.* **105** (1967) 243.
8. Curzon, G. and Speyer, B. E. *Biochem. J.* **109** (1968) 25.
9. Sekiguchi, T. and Nosoh, Y. *Arch. Biochem. Biophys.* **138** (1970) 319.
10. Pettersson, G. and Pettersson, I. *Acta Chem. Scand.* **23** (1969) 3235.
11. Pettersson, G. *Acta Chem. Scand.* **23** (1969) 2317.
12. Curzon, G. *Abstract, 8th International Congress of Biochem.*, Switzerland 1970.
13. Alexander, J. *Colloid Chemistry*, Van Nostrand, New York 1937, p. 143.
14. Pocker, Y. and Stone, J. T. *Biochemistry* **6** (1967) 668.
15. Fridovich, I. *J. Biol. Chem.* **238** (1963) 592.
16. Massey, V. *Biochem. J.* **53** (1953) 67.
17. Krueger, R. C. *Arch. Biochem. Biophys.* **57** (1955) 52.
18. Peisach, J. and Levine, W. G. *J. Biol. Chem.* **240** (1965) 2284.
19. Andréasson, L.-E. and Vänngård, T. *Biochim. Biophys. Acta* **200** (1970) 247.
20. Padova, J. *J. Chem. Phys.* **40** (1964) 691.
21. Dixon, M. and Webb, E. C. *Enzymes*, Longmans, London 1964, p. 324.

Received June 14, 1972.