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The influence of Catecholamines on the Visible Absorption and on the Electron Spin Resonance Absorption Spectrum of Ceruloplasmin

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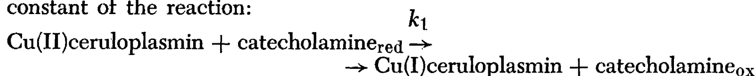
The interaction of catecholamines with ceruloplasmin has been studied by measurement of the visible absorption spectrum as well as by electron spin resonance spectrometry. By aid of a stopped flow technique the rate constant of the reaction:



$$\text{Cu(II)ceruloplasmin} + \text{catecholamine}_{red} \xrightarrow{k_1} \text{Cu(I)ceruloplasmin} + \text{catecholamine}_{ox}$$

has been determined spectrophotometrically, recording the decrease of the absorbancy at 605 mμ. Decreasing values of the rate constant k_1 were observed : Dopamine 〉 Noradrenaline 〉 Adrenaline 〉 Isopropylnoradrenaline 〉 DOPA. At steady state equilibrium, in the presence of substrate, the absorption band at 605 mμ of ceruloplasmin was decreased by 50—60 %. The interaction of N,N-dimethyl-ppd with ceruloplasmin was somewhat faster and the visible absorption was lower at equilibrium. It has been shown that oxygen accelerates the interaction of catecholamines with ceruloplasmin, k_1 in O_2 being greater than k_1 in N_2.

By ESR spectrometry it has been confirmed that the decrease of the visible absorption band is directly related to the reaction Cu(II) → Cu(I) in the enzyme when substrates are added. In nitrogen the intensity of the ESR signal is reduced to a greater extent by catecholamines than in oxygen atmosphere.

It is concluded that the bonding of catecholamines to ceruloplasmin involves the amine group in the side chain. N-alkyl substitution decreases the velocity of the interaction of the substrate with the enzyme, and the formation of the initial oxidation product of catecholamines during activity occurs at a slower rate.

A variety of polyamines and polyphenolic compounds can serve as substrates for ceruloplasmin. These include p-phenylenediamine, hydroquinone, catechol, adrenaline, DOPA[*1], noradrenaline[2], dopamine, isopropylnoradrenaline[3], 5-hydroxytryptamine[4], and several hydroxyindoles[5]. Ceruloplasmin can also

* Abbreviations used: Cu(II)CP, oxidized ceruloplasmin; Cu(I)CP, reduced ceruloplasmin; $NADH_2$, reduced nicotinamide adenine dinucleotide; DOPA, L-β-3,4-dihydroxyphenylalanine; Dopamine, 3-hydroxytyramine; N,N-dimethyl-ppd, N,N-dimethyl-p-phenylenediamine; ESR, electron spin resonance.

oxidize $NADH_2$ in the presence of *p*-phenylendiamines and catecholamines[2]. Oxidation of the substrates by ceruloplasmin is associated with a reversible reduction oxidation of the copper; $Cu(II)CP \rightleftharpoons Cu(I)CP$[6]. The importance of copper for the catalytic activity has been illustrated in studies on the binding and removal of copper[7–9]. Electron spin resonance spectroscopy has indicated that resting ceruloplasmin contains 4 Cu(II) and 4 Cu(I) atoms[10]; further confirmation of this was obtained by magnetic susceptibility data[11]. The very interesting ESR studies of Malmström and Vänngård[12] show that copper in resting ceruloplasmin is coordinated in a unique manner.

The present work is concerned with the properties of ceruloplasmin during activity in the presence of catecholamine substrates. Kinetic measurements on the visible absorption spectrum after addition of substrates have been made by a technique of rapid spectrophotometric recording. The valence state of copper in the enzyme during activity has been further illustrated by electron spin resonance absorption spectra.

EXPERIMENTAL

Material

Ceruloplasmin was obtained from AB Kabi, Stockholm, and purified as described previously[2], on a DEAE cellulose column. After concentration by ultrafiltration (Metal-Drucksfiltrationsgeräte MD, 70–15, Membranfiltergesellschaft GMBH, Göttingen), ceruloplasmin was dialyzed against 0.01 M NaCl. In some experiments the native Kabi enzyme was used. Most of the substrates used were those reported earlier[2]. In addition experiments were made with the following compounds: L-β-3,4-dihydroxyphenylalanine (DOPA), Sigma Chemical Comp; 3-hydroxytyramine · HCl (Dopamine), Sigma Chemical Comp; D,L-N-isopropylarterenol · HCl, California Corporation Biochemical Research.

Spectrophotometric technique

The kinetic measurements were done on a DK Beckman spectrophotometer by aid of a stopped flow technique. The flow apparatus consisted of two 2 ml injection syringes connected with a mixing chamber. The plungers of the syringes were connected to each other by a plexiglass pushing block. The mixing chamber was connected by Tygon tubing to a circulating system in a plexiglass cuvette. The light beam passed through a bore of 1.5 mm diameter in the plexiglass, covered by quartz plates on each side. In front of the cuvette a diaphragm was placed fixed to the front of the cuvette holder. A small hole in the diaphragm confined the light beam to a cross-section of less that 1.5×1.5 mm. This slender light beam could pass through the liquid without touching the cuvette walls. A similar arrangement was used for the reference cuvette. The light path through the cuvette was 10 mm.

In flow experiments the plungers were rapidly pushed down and simultaneously the liquid was discharged from the mixing chamber into the circulation system of the cuvette. The solutions were previously saturated with the gas phases used, air, oxygen or nitrogen, according to a procedure previously described[6]. The temperature was kept constant at +5°C by circulating water from a thermostat. The filled syringes, as well as the mixing chamber, and the circulation system in the cuvette, were surrounded by plexiglass water jackets. To obtain a temperature equilibrium the whole system was cooled for one hour at +5°C before mixing. Immediately before the experiment the condensed vapor on the surface of the cuvette was blown away with an air-blower.

Electron spin resonance (ESR) spectroscopy

The spectrometer used has been described in detail elsewhere[13]. It operates at a frequency of 9200 Mc/second. It has a rectangular transmission cavity (H_{102}mode), crystal detection, and a modulation frequency of 110 kc/sec. Phase sensitive detection is used, and the spectra recorded represent the first derivative of the actual absorption curves. The microwave frequency was measured with a wavemeter, and the magnetic field was measured with a proton resonance

field meter. The accuracy of these measurements is about 0.1 %. The samples were placed in quartz tubes of 4 mm diameter, frozen immediately and kept in liquid air during the experiment.

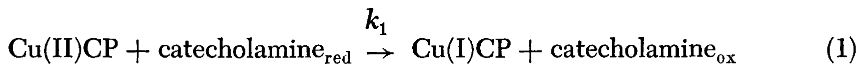
RESULTS

Catecholamines as substrates for ceruloplasmin

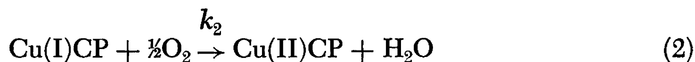
It has previously been reported that N-alkyl substitution in the side chain increases the rate of oxidation of catecholamines to the corresponding "chromes"[3]. Oxidation of dopamine and noradrenaline was very slow compared to adrenaline and isopropylnoradrenaline. However, when the activity of ceruloplasmin against these substrates was studied by the indirect oxidation of $NADH_2$, the results were quite different. As shown in Fig. 1 the oxidation of $NADH_2$ by dopamine and noradrenaline in the presence of ceruloplasmin occurs rapidly. The rate is nearly of the same order of magnitude as mediated by N,N-dimethyl-ppd. On the other hand, in the above system the oxidation of $NADH_2$ was very slow in the presence of adrenaline or isopropylnoradrenaline. The activity of ceruloplasmin against the non-decarboxylated DOPA was very slow by the direct as well as by the indirect method. The importance of characterizing ceruloplasmin activity against the different substrates by the direct as well as the indirect method is obvious.

Visible absorption spectrum of ceruloplasmin

The interaction of catecholamines with ceruloplasmin has been studied in experiments involving measurements of rapid reaction kinetics. At room temperature the blue colored ceruloplasmin is bleached in a few seconds after addition of the substrates. In order to study the interaction of catecholamines with ceruloplasmin, it was therefore necessary to perform the experiments at low temperature (+5°C). Under these conditions the rate of alteration of the blue absorption band of ceruloplasmin could be followed by a recording spectrophotometer. With all substrates studied the intensity of the absorption was decreased but without shift of the maximum at 605 mμ. A typical experiment is presented in Fig. 2. The rate of alteration occurs more rapidly with dopamine and noradrenaline than with adrenaline and isopropylnoradrenaline, while DOPA interacts very slowly with the enzyme. However, the interaction of N,N-dimethyl-ppd with ceruloplasmin was the most rapid reaction observed. One can assume that the interaction of catecholamines with ceruloplasmin involves the reactions:



$$\mathrm{Cu(II)CP} + \mathrm{catecholamine_{red}} \xrightarrow{k_1} \mathrm{Cu(I)CP} + \mathrm{catecholamine_{ox}} \quad (1)$$



$$\mathrm{Cu(I)CP} + \tfrac{1}{2}O_2 \xrightarrow{k_2} \mathrm{Cu(II)CP} + H_2O \quad (2)$$

The $\mathrm{catecholamine_{ox}}$ may be a free radical or a quinone, which in the presence of oxygen is further dehydrogenated to the red colored "chrome". Reaction (1) could be expected to be of the second order. However, deviations might occur since the association of the catecholamine with the catalytic center may involve coordination with the copper as well as bonding to the enzyme protein. It was therefore of interest to calculate the rate constant k_1 from different points on the experimental curves to see if they came out as true constants. In these experi-

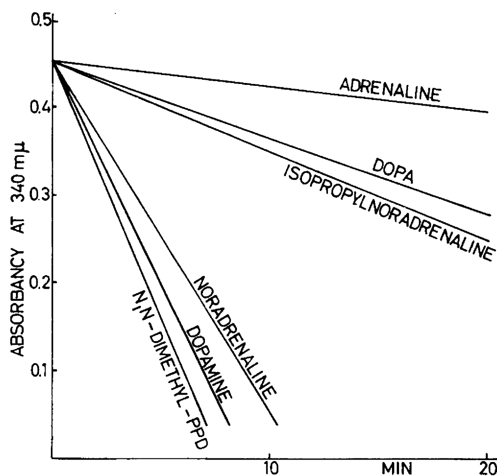


Fig. 1. The rate of oxidation of $NADH_2$ by ceruloplasmin in the presence of different catecholamines. Incubation system: 3.8×10^{-7} M ceruloplasmin, 1.55×10^{-4} M substrate, 1×10^{-4} M $NADH_2$, 0.05 M acetate buffer pH 5.9. Temp. 38°C.

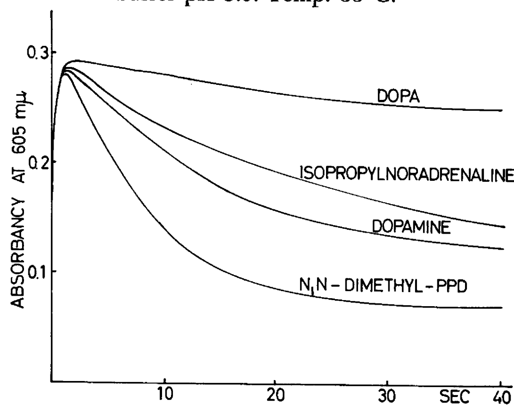


Fig. 2. The change of absorbancy at 605 mμ of ceruloplasmin by addition of catecholamines. The curves for noradrenaline and adrenaline (not recorded on the figure) are intermediate between dopamine and isopropylnoradrenaline. System (initial concentrations): 3.15×10^{-5} M ceruloplasmin, 5×10^{-4} M substrate, 0.03 M acetate buffer pH 5.9. Temp. 5°C. Satured with air.

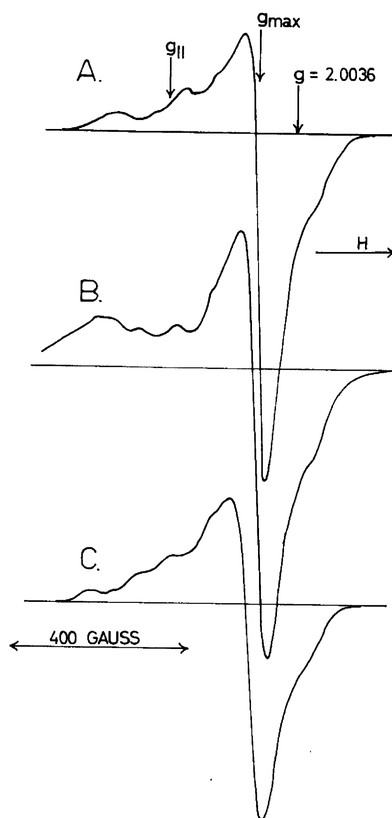


Fig. 3. Electron spin resonance absorption spectra (derivatives) of ceruloplasmin in the absence and in the presence of dopamine. Conditions: 8.2×10^{-5} M ceruloplasmin, 1.67×10^{-4} M Dopamine, 0.01 M acetate buffer pH 5.9. Liquid air temp. A. Resting ceruloplasmin, saturated with 100 % oxygen. B. Ceruloplasmin + Dopamine saturated with 100 % oxygen. C. Ceruloplasmin + Dopamine saturated with 100 % nitrogen. g = 2.0036, resonance field of diphenylpicrylhydrazyl (DPPH).

ments where the substrates had been added in excess, the calculations were made under the assumption that reaction (2) could be neglected during the initial phase of the curve.

Table 1. Values of k_1 for the interaction of substrates with ceruloplasmin calculated at different times from the time course of the absorbancy at 605 mμ (Fig. 2).

Isopropylnor-adrenaline		Dopamine		N,N-dimethyl-ppd	
Time sec	k_1 $M^{-1} \times sec^{-1}$	Time sec	k_1 $M^{-1} \times sec^{-1}$	Time sec	k_1 $M^{-1} \times sec^{-1}$
1.7	89	1.4	124	0.8	250
4.0	95	2.3	145	2.5	314
6.5	83	3.4	148	4.0	407

According to Broman *et al.*[10] it was assumed that ceruloplasmin contained 4 catalytic copper centers. From Table 1 it is seen that the values of k_1 for dopamine and isopropyl noradrenaline were nearly constant within the experimental error, during the initial period of the reaction ($1 \rightarrow 6$ sec). The same has been observed for the other catecholamine substrates investigated. However, with N,N-dimethyl-ppd, the values of k_1 varied considerable after a reaction time above 1.5 sec. In Table 2 the mean values of k_1 for the different catecholamines are presented. The initial part of the curves (Fig. 2), where constant values of k_1 are obtained, have been used. It is seen that k_1 for these substrates is decreased by N-alkyl substitution in the side chain, while k_1 DOPA is extremely low. However, the reaction velocity for N,N-dimethyl-ppd was greater than for any catecholamine investigated.

At steady state equilibrium the visible absorbancy at 605 mμ of ceruloplasmin was decreased by 50–60 % in the presence of catecholamines. The alteration in absorbancy by addition of N,N-dimethyl-ppd was even more pronounced (75 %).

Table 2. The influence of catecholamines on the absorbancy of ceruloplasmin at 605 mμ. k_1 = second order rate constant. Average values calculated from different points on the initial part ($1 \rightarrow 5$ sec) of the curves in Fig. 2.

Substrate	Decrease of absorbancy at 605 mμ at equilibrium %	Average rate constant k_1 $M^{-1} \times sec^{-1}$
N,N-dimethyl-ppd	74	265*
Dopamine	60	143
Noradrenaline	56	120
Adrenaline	52	99
Isopropylnoradrenaline	50	85
DOPA	23	7

* Average of approximate values calculated from $0.5 \rightarrow 1.5$ sec on the curve. At reaction times ≥ 2 sec deviation of k_1 from the initial value did occur.

In addition, the importance of oxygen for the interaction of catecholamines with ceruloplasmin was studied. The k_1 for dopamine was studied in nitrogen atmosphere after rigorous deoxygenation of the reaction system, and compared with k_1 in 100 % oxygen. In several experiments it was demonstrated that the interaction of dopamine with ceruloplasmin was accelerated by oxygen, k_{1O_2} being 20–30 % higher than k_{1N_2}.

Electron spin resonance absorption spectrum of ceruloplasmin

Malmström and Vänngård[12] observed exceptionally low hyperfine splitting constants in the ESR absorption spectrum of ceruloplasmin. The ESR absorption spectra of frozen ceruloplasmin recorded in this work exhibited the same characteristics for the resting enzyme (Fig. 3 A). They were characterized by $g_{max} = 2.057$, $g_{\parallel} = 2.196$ and a hyperfine splitting constant $A = 0.008$ cm^{-1}.

The addition of substrates decreased the intensity of the ESR signal of ceruloplasmin. By using the same enzyme preparation as in Fig. 2 under same conditions, dopamine decreased the ESR signal by 30 % and N,N-dimethyl-ppd by 45 %. By addition of $Na_2S_2O_4$ the resting enzyme was completely reduced. An extension of these studies will be reported later. Under steady state condition, in the presence of substrates, the intensity of the ESR signal was influenced by oxygen tension. As demonstrated in Fig. 3 B and C dopamine decreased the ESR signal of this enzyme preparation by 6 % in oxygen compared with 30 % in 100 % nitrogen. In some experiments it was observed that the hyperfine structure of the ESR absorption spectrum was somewhat changed after addition of substrates in the presence of oxygen (Fig. 3 B). This effect is now under investigation.

DISCUSSION

The high intensity of the visible blue absorption band in resting ceruloplasmin with molar extinction 1200[1] indicates an unusual type of bonding of copper in the enzyme. It should also be pointed out that the exceptional low hyperfine splitting constants in the ESR signal have not been observed in simple Cu(II)-complexes[12]. However, a similar low hyperfine structure has been observed in laccase[12], pseudomonas copper proteins[14] and in cytochrome oxidase[15]. This suggests that copper is coordinated in a unique manner in ceruloplasmin as well as in other copper oxidases.

In experiments with urea-denaturation of ceruloplasmin Broman *et al.*[10] obtained indication that the strong visible absorption as well as the low hyperfine structure was associated with the oxidase activity. Williams[16] has pointed out that oxime- and hydrazine-copper complexes show visible absorption bands of similar high molar extinctions as the copper oxidases. He has suggested that these complexes as well as ceruloplasmin arise through a high degree of charge transfer where the electrons are associated with the metal ligand complex as a whole, rather than with the copper atom alone.

As shown in the present work the interaction of ceruloplasmin with its substrates greatly change the properties of the enzyme. This can be attributed to reduction of Cu(II) → Cu(I), demonstrated by a decrease in the intensity of the

ESR signal. Simultaneously a decrease of the visible absorbancy by 50 to 75 % takes place.

Orgel[17] has suggested that copper oxidases may be oxygen carriers similar to hemocyanin. In such copper oxygen complexes resonance between different structures of similar energy levels is possible. In the present work it has been shown that at increased oxygen tension the rate constant k_1 for the reaction of ceruloplasmin with catecholamines is increased. In addition, increased oxygen tension influences the steady state level Cu(II)CP $\rightleftharpoons$ Cu(I) CP in favour of Cu(II)CP. However, it has been shown that the catalytic activity of ceruloplasmin as determined by the $NADH_2$ system was inhibited by increased oxygen tension.

The importance of the side chain of catecholamines for the interaction with ceruloplasmin has clearly been demonstrated here. By alkylation at the nitrogen atom (adrenaline, isopropylnoradrenaline) the interaction with ceruloplasmin is retarded and the formation of the initial oxidation product is inhibited. The presence of the COOH group in DOPA, which is a poor substrate, to a great extent hinders interaction with the enzyme. These findings strongly indicate a bonding of the amine group in the catecholamine side chain to the catalytic centers of ceruloplasmin. It has been suggested[18] that the binding of ceruloplasmin to *p*-phenylenediamines may be not through the amine groups but to the π electrons of the aromatic ring. The binding of catecholamines and *p*-phenylendiamines to ceruloplasmin may therefore be different as also indicated by the results of the kinetic studies reported here.

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