BBA 66525

THE ASCORBATE OXIDASE ACTIVITY OF CAERULOPLASMIN

G. CURZON AND S. N. YOUNG*

Department of Neurochemistry, Institute of Neurology, The National Hospital, Queen Square, London, W.C.1. (Great Britain)

(Received September 15th, 1971)

SUMMARY

1. Evidence that caeruloplasmin exhibits only iron mediated activity towards ascorbate was reinvestigated.

2. The K_m and V of caeruloplasmin towards ascorbate were found to be 4.70 mM and 3.19 electron transfers/Cu atom per min (*e*/Cu per min), respectively, at pH 5.5 and 25° and with EDTA present to chelate contaminant metal.

3. Various possible indirect mechanisms for the oxidation involving the mediation of iron or other contaminating metal ions even in the presence of EDTA were eliminated. Therefore ascorbate appears to be a true caeruloplasmin substrate under the above conditions.

4. However caeruloplasmin oxidised ascorbate in serum at pH 7.35 neither directly nor by an iron-mediated mechanism.

INTRODUCTION

There has been much controversy about the substrate specificity of caeruloplasmin, the blue copper containing oxidase of mammalian plasma, and in particular there have been conflicting reports about its activity towards ascorbate[1–5].

HOLMBERG AND LAURELL[1] reported that ascorbate reversibly decolourised caeruloplasmin in the presence of oxygen and thus considered it a substrate. However, MORELL *et al.*[2] showed that if all solutions used in activity determinations were treated with metal binding resin then caeruloplasmin activity towards ascorbate was very greatly reduced. They suggested that the oxidation of ascorbate was due to the presence of non-caeruloplasmin copper. Nevertheless, OSAKI *et al.*[3,4] showed clear kinetic differences between the catalysis of ascorbate oxidation by caeruloplasmin and by Cu^{2+}.

Previously, CURZON AND O'REILLY[6] found that Fe^{2+} was a caeruloplasmin

* Present address: Department of Psychiatry, McGill University, Montreal, Quebec, Canada.

Abbreviations: *e*/Cu per min, electron transfers/Cu atom per min; DPD, *N*,*N*-dimethyl-*p*-phenylenediamine; [*S*], substrate concentration.

substrate and CURZON[7] showed that Fe^{2+} enhanced caeruloplasmin activity towards *N,N*-dimethyl-*p*-phenylenediamine (DPD). A coupled oxidation mechanism was proposed by which Fe^{2+} was oxidised to Fe^{3+} by caeruloplasmin and the Fe^{3+} oxidised DPD regenerating Fe^{2+}. The increase of activity was prevented by EDTA and apotransferrin. It was suggested that caeruloplasmin might similarly be able to oxidise indirectly any compounds which were oxidised by Fe^{3+}. Finally MCDERMOTT *et al.*[5] showed, using ^{59}Fe, that the concentration of contaminating iron in a caeruloplasmin solution even after treatment with metal binding resin might be sufficient to result in a coupled oxidation and that residual activity towards several compounds was abolished by adding 30 μM EDTA to bind any remaining iron. Reported substrates were classified in 3 groups:

1. Fe^{2+}, which is oxidised directly by caeruloplasmin and is a true substrate.

2. Compounds such as DPD which are true substrates, but the oxidation of which is increased by iron via an iron-caeruloplasmin coupled reaction.

3. Compounds which exhibit only iron-mediated activity and are thus not true substrates. Ascorbate was considered to belong to this class.

Recently it has been shown that caeruloplasmin has a low steric specificity, that V varies only slightly with substrate structure and that K_m values vary with the energy of the highest occupied molecular orbital of the substrate[8,9]. As ascorbate has an energy in its highest occupied orbital which is comparable to that of some substrates[10] this problem has been reinvestigated, and ascorbate has been shown to be a true substrate.

The possibility that caeruloplasmin plays a part in the oxidation of ascorbate in serum under physiological conditions either directly or by an iron-mediated mechanism has also been investigated.

MATERIALS AND METHODS

Caeruloplasmin

All caeruloplasmin used was freshly prepared from plasma. Preparation, treatment and specifications were as described elsewhere[11].

Serum

Pooled residual serum from patients of the National Hospital, London W.C.1. was sterilised before use by filtration through a Millipore GSWP filter with 0.22 μm pore size (Millipore Ltd., Wembley, Middlesex).

Chemicals

All solutions were prepared as described previously[12] and (except for iron solutions) were passed through a 1 cm × 1 cm column of Chelex 100 (100–200 mesh) metal binding resin (Bio-Rad, St. Albans, Herts.) to remove contaminating iron. Ascorbate and ferrous ammonium sulphate solutions were kept under nitrogen.

Oxygen uptake measurements

The activity of purified caeruloplasmin was determined by measuring the rate of oxygen uptake at 25° using a Beckman model 777 oxygen analyser as described elsewhere[11]. Unless otherwise specified, measurements were made on a solution

containing 10 mM acetate (pH 5.5) and 100 μM EDTA. Oxygen uptakes as low as 0.1 μM/min were measurable.

All experiments with serum were performed at 37°. The oxygen concentration of 9 ml of serum in the oxygen electrode chamber was adjusted, by bubbling nitrogen through it, to 60 μM which is between the oxygen concentrations of arterial and venous plasma. The rate of oxygen uptake was measured. Then up to 0.1 ml of ascorbate solution and/or other additives was added to the serum and the rate of oxygen uptake remeasured.

Determination of iron

Concentrations of iron in the reaction medium were determined by an adaption of the method of SMITH *et al.*[13] which utilises the extraction of the chloride salt of the ferrous complex of 4,7-diphenyl-1,10-phenanthroline into amyl alcohol and its subsequent colorimetric determination.

A solution was prepared containing 10 mM acetate pH 5.5, 100 μM EDTA, 30 mM ascorbate and 0.33 μM caeruloplasmin. These concentrations were comparable with those used in activity determinations. To 9 ml of this solution (the volume used in activity determinations) was added 1 ml of 1 M NaCl followed by 0.4 ml of 10 mM 4,7-diphenyl-1,10-phenanthroline. The mixture was shaken for 15 min, 2.5 ml of amyl alcohol added and after a further 15 min shaking the layers were allowed to separate. The $A_{533\ nm}$ of the organic phase was determined spectrophotometrically using 4-cm path length, 2-ml capacity cuvettes. Solutions containing 0–2.5 μM added ferrous ammonium sulphate were carried through the method.

Spectrophotometric measurements

Spectrophotometric measurements were made using Unicam SP 500 and SP 800 spectrophotometers as described previously[12].

RESULTS AND DISCUSSION

The ascorbate oxidase activity of caeruloplasmin

The activity of caeruloplasmin towards ascorbate concentrations up to 10 mM was measured. Above 1 mM ascorbate appreciable activity was detectable. A plot of $1/v$ against $1/[S]$ was obtained (Fig. 1) from which values were derived for the K_m (4.70 mM ascorbate) and V (3.19 e/Cu per min). This is well within the narrow range of V values found for a large range of substrates[9,11], and suggests that ascorbate is a true substrate. The rate of enzymic oxidation is low compared with rates of iron-mediated oxidation[5].

This experiment was performed 3 times using different caeruloplasmin preparations which had been kept from 2 days to 3 weeks and buffer and ascorbate solutions which were freshly prepared each time. Results obtained are shown in Table I. No trend or marked differences between the parameters obtained on different occasions was apparent. Activities obtained were unaffected by raising the EDTA concentration from 100 μM to 1 mM. Six possible explanations for the activity were considered:

1. Molecular oxygen oxidises free Fe^{2+}, present as a caeruloplasmin contaminant, to Fe^{3+}, which then oxidises ascorbate.

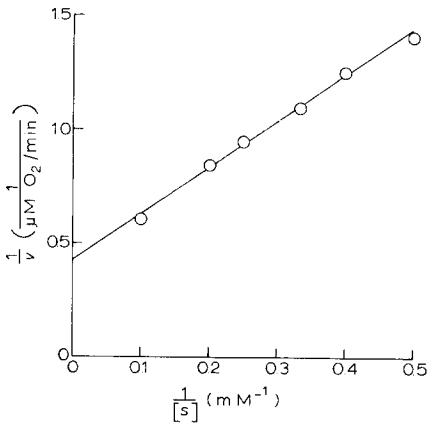

Fig. 1. The oxidation of ascorbate by caeruloplasmin. A plot of $1/v$ against $1/[S]$. The caeruloplasmin concentration was 0.36 μM. Other conditions were as described in MATERIALS AND METHODS. Activity was measured by the rate of oxygen uptake.

TABLE I

K_m AND V VALUES FOR OXIDATION OF ASCORBATE BY CAERULOPLASMIN

Values were determined as shown in Fig. 1.

Age of caeruloplasmin (days)	K_m *(mM)*	V *(e/Cu per min)*
2	5.26	3.67
10	4.70	3.19
20	5.20	4.07

2. Molecular oxygen oxidises Fe^{2+}–EDTA complex to Fe^{3+}–EDTA which then oxidises ascorbate.

3. Caeruloplasmin oxidises Fe^{2+}–EDTA complex to Fe^{3+}–EDTA which then oxidises ascorbate.

4. Caeruloplasmin oxidises free Fe^{2+} in equilibrium with Fe^{2+}–EDTA to Fe^{3+} which then oxidises ascorbate.

5. Oxidation of ascorbate by contaminants other than iron.

6. True enzymic oxidation *i.e.*, through the formation of a caeruloplasmin–ascorbate complex.

While metal ion catalysed oxidations of ascorbate produce hydrogen peroxide, oxidation of substrates by caeruloplasmin does not[14]. Thus Mechanisms 1 or 2 would lead to H_2O_2 formation, detectable on the oxygen electrode by addition of catalase. This was investigated as described below.

Addition of either 12.5 μM Fe^{2+} or 0.5 μM caeruloplasmin to 30 mM ascorbate in 10 mM acetate buffer pH 5.5 with 100 μM EDTA resulted in oxygen uptakes of about 10 μM/min. If 0.01 ml of a saturated solution of catalase (ex beef liver, L. Light and Co., Colnbrook, England) was added the rate of oxygen uptake with iron was inhibited by 39% which is consistent with the decomposition of H_2O_2 produced during the oxidation of ascorbate. However the oxygen uptake with caeruloplasmin was unchanged. Thus the oxidation of ascorbate by caeruloplasmin does not appear

to be due to direct oxidation by contaminating metal ions unless caeruloplasmin has catalase activity. In this case the hydrogen peroxide would be decomposed and catalase might not decrease oxygen uptake. This possibility was eliminated as it was demonstrated that caeruloplasmin did not have catalase activity under the conditions used even towards H_2O_2 concentrations as high as 1 mM.

Mechanism 3 requires caeruloplasmin to oxidise Fe^{2+}–EDTA. However, while caeruloplasmin substrates, *e.g.* Fe^{2+}, cause its absorption peak at 605 nm to disappear reversibly in the presence of oxygen[15] the Fe^{2+}–EDTA complex merely caused a relatively small, rapid and unreversed decrease (Fig. 2). $A_{605\ nm}$ returned to a value also slightly below its original level after addition of Fe^{2+}. Neither EDTA alone nor the Fe^{2+}–EDTA complex affected $A_{605\ nm}$.

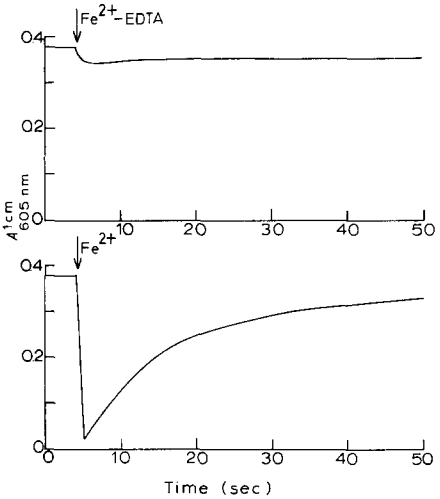

Fig. 2. Effects of Fe^{2+} and Fe^{2+}–EDTA complex on the $A_{605\ nm}$ of caeruloplasmin. At the time indicated by the arrows 0.05 ml of solution containing Fe^{2+} or Fe^{2+} *plus* EDTA was added to 2.45 ml of a 34 μM caeruloplasmin solution in 10 mM acetate buffer (pH 5.5), to give final concentrations of 250 μM iron and 1 mM EDTA.

Mechanism 4 involves mediation by free iron in equilibrium with the iron–EDTA complex. If free Fe^{3+} is responsible for ascorbate oxidation, then adding it to the reaction medium would increase the rate of ascorbate oxidation. However, activity is unaffected by adding Fe^{2+} to the medium until 6 μM Fe^{2+} has been added, when it increases sharply (Fig. 3). This suggests that free Fe^{2+} is not responsible for ascorbate oxidation unless, even when no iron is added, contaminating iron already present saturates the enzyme, the increase in activity above 6 μM added iron presumably being due to a mechanism involving a Fe^{2+}–Fe^{3+} couple.

To determine whether iron could be saturating the enzyme, its concentration in the reaction mixture was determined using the extinction at 533 nm of the iron complex of 4,7-diphenyl-1,10-phenanthroline as described in MATERIALS AND METHODS Fig. 4 shows a plot of $A_{533\ nm}$ against μM iron added to the reaction mixture. The absorbance with no added iron is 0.037 indicating about 0.3 μM iron, which is an upper estimate as free copper (which might be derived from denaturation of caerulo-

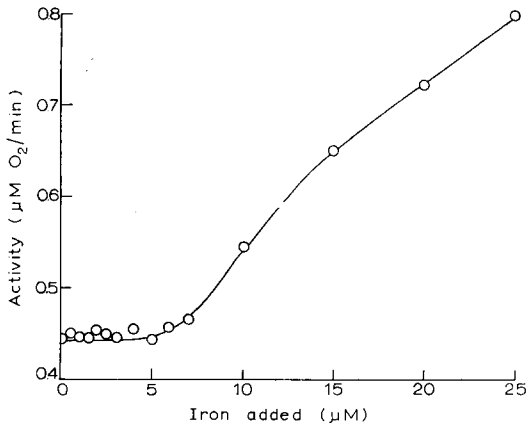

Fig. 3. The effect of iron on the activity of caeruloplasmin towards ascorbate. The caeruloplasmin concentration was 0.3 μM and the ascorbate concentration 25 mM in 9 ml of a medium containing 100 μM EDTA and 10 mM acetate (pH 5.5). The iron concentration of the medium was increased by adding ferrous ammonium sulphate solution in amounts up to 0.1 ml. Activity was measured after each addition of iron solution by the rate of oxygen uptake.

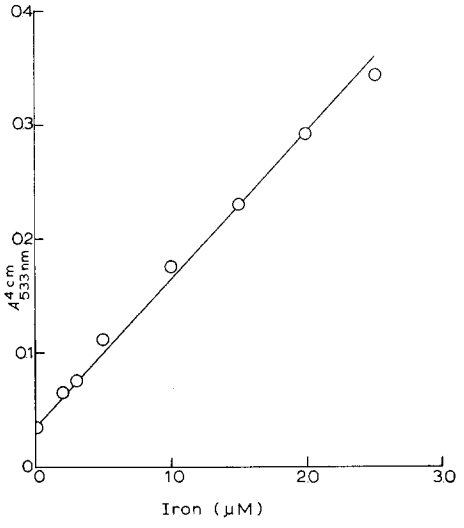

Fig. 4. Absorbance at 533 nm of the 4,7-diphenyl-1,10-phenanthroline complex of iron extracted from the reaction medium plotted against concentration of iron added to the reaction medium. Procedure is described in MATERIALS AND METHODS.

plasmin under the iron assay conditions) also forms a coloured product in the iron assay method[16]. As OSAKI[17] found the K_m of the' low-K_m site' of caeruloplasmin to be 0.6 μM Fe^{2+} the enzyme was presumably not even near saturation. Thus ascorbate oxidation is not likely to have been due to mediation by iron.

According to Mechanism 5 ascorbate is oxidised by contaminants other than iron. The most likely one to be present in any quantity is ionic copper formed by the denaturation of caeruloplasmin. The oxidation of ascorbate by ionic copper as a caeruloplasmin contaminant has been investigated and rejected by others[3,4]. However, in the present study it was also demonstrated, in an experiment similar to that

shown in Fig. 3, that addition of 1 μM Cu^{2+} to the reaction medium did not affect the rate of oxygen uptake.

The evidence against the above five non-enzymic or indirect mechanisms strongly points to ascorbate being a true substrate of caeruloplasmin.

This conclusion is consistent with work on caeruloplasmin substrate specificity. Thus, all the known substrates of caeruloplasmin (except Fe^{2+}) have electrons with a high energy (resonance integral $\beta > 0.8$)[8,11] in their highest occupied orbital. Of more than 30 compounds tested as caeruloplasmin substrates there are no examples of compounds with such high molecular orbital levels which are not substrates[11]. As ascorbate has a molecular orbital energy level comparable with that of known substrates[10] and as caeruloplasmin has a very low steric specificity[9,11], it is hardly surprising that ascorbate is a substrate. The ascorbate oxidase activity of caeruloplasmin was probably not detected in earlier studies[3,4,7] because of the low concentrations of ascorbate used.

The oxidation of ascorbate in serum

In view of the above results and the fact that 20% of the vitamin C in plasma is in the oxidised dehydroascorbate form[18], the possibility that ascorbate is a physiological substrate of caeruloplasmin was investigated.

Serum without any added substrate took up 0.1 μM O_2/min, presumably due to the oxidation of material already present. This oxidation was not due to caeruloplasmin as addition of 1 mM azide, which inhibits caeruloplasmin 98% at the pH of plasma[19], or addition of 2 μM caeruloplasmin, (the concentration already present in serum[20]) had no effect on the oxygen uptake.

Addition of 50 μM ascorbate (a concentration found in serum[21]) to serum increased the oxygen uptake by 0.08 μM/min. However, as addition of neither 1 mM azide nor 2 μM caeruloplasmin affected this uptake, it was not due to caeruloplasmin and may have been non-enzymic. A similar rate of oxygen uptake was obtained when 50 μM ascorbate was added to 50 mM phosphate buffer at pH 7.35.

To investigate whether iron could promote caeruloplasmin oxidation of ascorbate in serum, 20 μM Fe^{2+} was added to serum containing 50 μM ascorbate. There was a rapid (10 sec) drop in oxygen concentration of about 5 μM (equivalent to the oxidation of 20 μM Fe^{2+}) after which the rate of oxygen uptake returned to that occurring before iron was added.

Thus although at pH 5.5 ascorbate is both a true caeruloplasmin substrate and can also be oxidised by an iron-mediated reaction, it is not appreciably oxidised by caeruloplasmin in serum at physiological pH either directly or via an iron mediated reaction.

ACKNOWLEDGEMENTS

We thank the Medical Research Council who supported the work with a research grant.

REFERENCES

1 C. G. HOLMBERG AND C. B. LAURELL, *Acta Chem. Scand.*, 5 (1951) 476.
2 A. G. MORELL, P. AISEN AND I. H. SCHEINBERG, *J. Biol. Chem.*, 237 (1962) 3455.

3 S. OSAKI, C. WALTER AND E. FRIEDEN, *Biochem. Biophys. Res. Commun.*, 12 (1963) 1.
4 S. OSAKI, J. A. MCDERMOTT AND E. FRIEDEN, *J. Biol. Chem.*, 239 (1964) 3570.
5 J. A. MCDERMOTT, C. T. HUBER, S. OSAKI AND E. FRIEDEN, *Biochim. Biophys. Acta*, 151 (1968) 541.
6 G. CURZON AND S. O'REILLY, *Biochem. Biophys. Res. Commun.*, 2 (1960) 284.
7 G. CURZON, *Biochem. J.*, 79 (1961) 656.
8 P. O. GUNNARSSON, G. PETTERSSON AND I. PETTERSSON, *Eur. J. Biochem.*, 17 (1970) 586.
9 G. CURZON AND S. N. YOUNG, *Biochem. J.*, 122 (1971) 32P.
10 A. SZENT-GYORGYI, *Introduction to a Submolecular Biology*, Academic Press, London, 1960, p. 38.
11 S. N. YOUNG AND G. CURZON, *Biochem. J.*, in the press.
12 G. CURZON AND B. E. SPEYER, *Biochem. J.*, 105 (1967) 243.
13 G. F. SMITH, W. H. MCCURDY AND H. DIEHL, *Analyst.*, 77 (1952) 418.
14 E. FRIEDEN, S. OSAKI AND H. KOBAYASHI, *J. Gen. Physiol.*, 49 (1965) 213.
15 W. E. BLUMBERG, J. EISINGER, P. AISEN, A. G. MORELL AND I. H. SCHEINBERG, *J. Biol. Chem.*, 238 (1963) 1675.
16 E. B. SANDELL, *Colorimetric Determinations of Trace Metals*, Interscience, New York, 1959, p. 538.
17 S. OSAKI, *J. Biol. Chem.*, 241 (1966) 5053.
18 H. LINKSWILER, *J. Nutr.*, 64 (1958) 43.
19 R. W. TOPHAM AND E. FRIEDEN, *J. Biol. Chem.*, 245 (1970) 6698.
20 G. E. CARTWRIGHT AND M. M. WINTROBE, *Am. J. Clin. Nutr.*, 14 (1964) 224.
21 Documenta Geigy, *Scientific Tables*, Geigy, Basle, Switzerland, 1970, p. 489.