Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 66-71

FERROXIDASE II. THE ESSENTIAL ROLE OF COPPER IN ENZYMATIC ACTIVITY

A. GARNIER[*+], L. TOSI[*] and M. STEINBUCH [Δ]

[*] *DEPARTEMENT DE RECHERCHES PHYSIQUES, Université Pierre et Marie Curie, Laboratoire associé au C.N.R.S. n° 71, 4, Place Jussieu, 75230 Paris Cédex 05*

[+] *U.E.R. DE MEDECINE ET BIOLOGIE HUMAINE, Université Paris XIII, Bobigny*

[Δ] *DEPARTEMENT DE FRACTIONNEMENT, Centre National de Transfusion Sanguine, Orsay*

Received November 12,1980

SUMMARY : One of the biological roles of ceruloplasmin is the oxidation of Fe(II) to Fe(III). It has therefore been called Ferroxidase I whereas a cupri-lipoprotein has been called Ferroxidase II. It is shown that this protein can bind several Cu(II) but only one being essential for its enzymatic activity. The hypothesis that ferroxidase activity arises from lipo-hydroperoxides has been ruled out.

INTRODUCTION : One of the important steps of iron metabolism is the ferroxidase reaction catalyzing the oxidation of Fe(II) to Fe(III) which can then become chelated by transferrin, the specific iron transport agent of plasma.

In 1966, Osaki et al. (1) observed that in plasma this ferrous oxidizing activity is achieved by ceruloplasmin while Rabinovitz et al. (2) demonstrated by immunoelectrophoresis of normal sera that this activity is in fact controlled by two proteins : ceruloplasmin and a low-density lipoprotein. In 1970, a non ceruloplasmin ferroxidase (Ferroxidase II) with azide-resistant activity has been identified and isolated by Topham and Frieden (3). For several years, Topham et al. worked to characterize this enzyme and their results are reported in a serie of papers (4-7). Their results indicate that the enzyme contains protein-bound lipids and copper, both of them being essential for its catalytic activity. Concurrently Frieden et al. (8,9) considered the possibility of Ferroxidase II being a peroxidized lipoprotein. Its oxidase activity towards Fe(II) arising then from the reduction of hydroperoxydes by Fe(II) in the classical reaction between the metal iron and free radicals reduction of lipohydroperoxides.

In this paper we report the isolation and purification of a copper containing lipoprotein exhibiting ferroxidase activity. It contains one cupric

Abbreviations used: CD, circular dichroism ; RR, resonance Raman ; DTC, diethyldithiocarbamate ; PL, peroxidized lecithin.

0006-291X/81/010066-06$01.00/0

ion which is essential for its catalytic activity and we characterize spectroscopically the metallic site. Furthermore, using peroxidized lecithin it is shown that the oxidase activity exhibited by this compound towards Fe(II) cannot account for the ferroxidase activity of the isolated enzyme. Presuming the identity of the protein isolated by us and Topham 's Ferroxidase II we shall call it hereafter Fox II.

MATERIALS AND METHODS : Apotransferrin was prepared from freshly isolated human transferrin, some experiments were done with a commercial product obtained from Behring (Hoechst-Germany) as were anti-NHS and anti-β-lipoprotein. L-α-phosphatidylcholine from egg yolk type V-E (lecithin) was a product of Sigma (St-Louis, U.S.A.) and PEG 6 000 was obtained from BDH (Poole, England). Iron (II) ammonium sulfate, manganese and magnesium chloride were from Merck (Darmstadt, Germany) whereas heparin (Na-salt) was a product of OSI (Vitry, France). All others products were of reagent grade.

Enzyme preparation. Topham et al. (3) used fraction IV-1 obtained in Cohn's 6 + 9 fractionation scheme as starting material. We localized Fox II in our fraction III obtained by a slightly modified Nitchmann's procedure (10). Topham's purification method proved to be unsuitable with this starting material. A new technique was developed associating 2 PEG precipitation steps with an isolation procedure for lipoproteins as described by Burstein et al. (11). Heparin is used as a precipitating agent once in the presence of manganese chloride, reprecipitation being done in the presence of magnesium chloride. The product is then submitted to ion exchange chromatography on DEAE-Sephadex at pH 7.7. Table I shows the flow-sheet for the purification of Fox II. The enzyme enrichment was monitored by activity measurements as described below. Its homogeneity was assessed in cellulose acetate and PAGE electrophoresis indicating a single major band corresponding to a molecular weight of approximately 550 000 daltons. Protein concentration was determined by the method of Lowry et al. (12) using bovine serum albumin as standard. In immunoelectrophoresis a single precipitation line is seen with anti-NHS, the enzyme being also precipitated by anti-β-lipoprotein serum.

Peroxidized lecithin. Lecithin in buffer (Tris-HCl, pH 7.5, 5×10^{-2} M) solution was sonicated in the presence of air for approximately 15 min. in an MSE 150 W Mk 2 sonicator operating at power 2.5 ; the temperature of the sonication bath was maintained at 0°C. Peroxidation was attested by the appearance of an absorption band at 227 nm (ε = 25 000) due to diene conjugation (13). Taking into account that, on the average, one molecule of lecithin out of three contains a pair of non conjugated bonds (14), the absorbancy measurements at 227 nm indicates that after 15 min. of sonication all the non conjugated double bonds are peroxidized.

Activity determination. The ferroxidizing activity of Fox II (and also of peroxidized lecithin) was checked spectrophotometrically. The formation of the Fe(III)-transferrin complex was followed up by the absorbance change at 460 nm. Ceruloplasmin being present in the starting material 1 mM azide was added to the 0.2 M acetate solution (pH 6) to inhibit its activity. This substance having no effect on Fox II all measurements were done in its presence. The O_2 concentration measurements were performed under the same conditions using a YSI 5331 oxygen probe.

Spectrophotometric measurements. Absorption spectra were recorded on a Cary 219 spectrophotometer ; CD spectra on a Jobin Yvon Dichrograph model Mark III

Table I

Flow-sheet for the isolation of Fox II as by-product of routine fractionation

Fraction III
Fresh paste suspended in NaCl 0.9 % (1:5 w:v), pH 6

Supernatant
+ PEG 6000 (6 % final concentration)

Precipitate discarded

Precipitate
dissolved in 0.1 M sodium acetate, pH 6
+ PEG 6000 (4 % final concentration)

Precipitate
dissolved in sodium acetate, pH 7.4
+ heparin (0.3 % final concentration) and $MnCl_2$ (1 M), 0.075 v:v

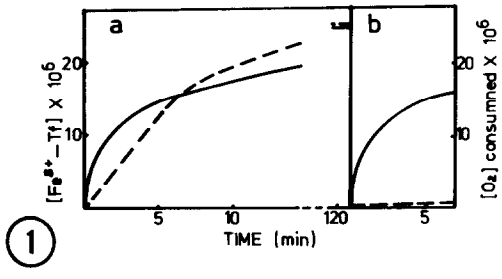
Precipitate
dissolved in 0.02 M Tris-HCl buffer, pH 7.7
+ $MgCl_2$ (2.5 M ; 0.05 M final concentration)

Precipitate
dissolved in NaCl 1 %, 0.02 M Tris-HCl buffer, pH 7.7
Ion exchange chromatography (DEAE-sephadex) ; the peak presenting the highest activity being selected.

interfaced with a Nicolet 1171 signal averager. RR spectra were obtained by means of a Coderg PHO spectrometer equipped with holographic gratings, using the exciting lines of an Ar^+ laser.

Copper content was determined by two different methods : by atomic absorption spectroscopy and by spectrophotometry of the DTC complex of Cu(II) extracted from the protein ($Cu(DTC)_2$ absorption spectrum exhibits a band at 430 nm with $\varepsilon = 8\,000$).

RESULTS AND DISCUSSION : In order to test Sexton's hypothesis, i.e. that ferroxidase activity arises from lipoperoxide decomposition, we compared the time courses of Fe (III) - transferrin formation and O_2 consumption in presence of Fox II and/or PL. The latter was used because lipids present in Fox II are essentially phospholipids. The results recorded in Figure 1 show that, under the experimental conditions, the reactions are nearly complete within 15 min. The striking point is that, in presence of Fox II, one mole of molecular oxygen is necessary for the oxidation of one Fe (II), whereas in presence of PL no detectable oxygen consumption is seen during Fe(III)-transferrin formation. The molar activity defined as the number of moles of Fe(III)-transferrin formed during the first minute of the reaction is 280 per mole of enzyme and 0.55 per mole of peroxide.



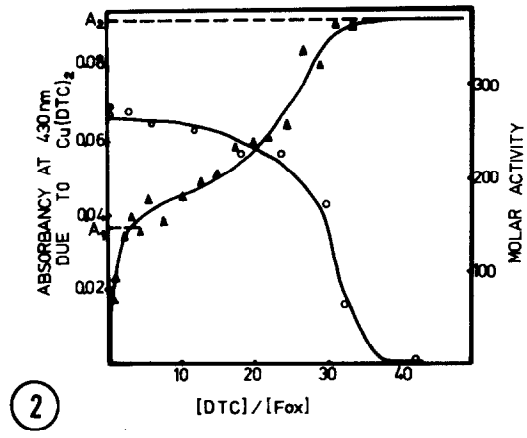


Figure 1. Time course of Fe(III)-transferrin formation (curve a) and oxygen consumption (curve b).
Reaction conditions : 25 µM Fe(II), 50 µM transferrin, 236 µM O_2 in 0.2 M acetate buffer pH 6 at 30°C, —— plus 0.08 µM Fox II, - - plus 21 µM peroxidized lecithin.

Figure 2. Absorbancy at 430 nm (Δ Δ)and molar activity (o o) as a function of molar ratio [DTC]/[Fox II].
Reaction conditions : (Δ Δ) 7.8 µM Fox II in tris buffer pH 7.7 ; (o o) 35 µM Fe(II), 75 µM transferrin, 236 µM O_2, 0.08 µM Fox II in 0.2 M acetate buffer pH 6 at 30°C.

We believe that these experiments completely rule out Sexton's hypothesis for two reasons : i) the difference in oxygen uptake ; ii) the quantity of lipoperoxides we can estimate to be present in Fox II in the most favourable case can account for only one fourth of Fox II molar activity.

To determine the K_M value for Fe(II) and for Fox II the initial rate of Fe(III)-transferrin formation was measured at constant O_2 concentration (236 µM) and various Fe(II) and transferrin concentrations : Fe(II) concentration values varied between 3.6 and 36 µM and the molar ratio [transferrin]/[Fe (II)] was maintained at 2. Using a Eadie, Hofstee plot we obtained K_M = 16 ± 3 µM. This value as well as those of molar activity are in good agreement with those published by Topham et al. (5).

The second aim of this work was to determine if copper was necessary for enzymatic activity. The number of cupric ions bound per enzyme, determined by the two methods described, depends largely on the enzyme sample under consideration. So we used the following procedure : increasing concentrations of DTC were added to a Fox II solution varying the molar ratio [DTC]/[Fox II] from 0 to 100. For each molar ratio the difference of absorbance at 430 nm between the DTC treated and DTC untreated Fox II solution was measured. The results are reported in Figure 2 where the absorbancy at 430 nm versus [DTC]/[Fox II] values has been plotted. A two steps curve is obtained :

calling A_1 the absorbancy corresponding to the first step and A_2 that corresponding to the second one, it appears that the value A_1/[Fox II] depends on the enzyme sample while that of A_2-A_1/[Fox II] is constant whatever the sample. Furthermore when the enzymatic tests are performed in presence of DTC,ferroxidase activity vanishes only when the second step is reached (figure 2). These results may be interpreted as arising from the presence of two kinds of cupric ions in the enzyme : the first species,easily removed at low DTC concentrations is not essential for ferroxidase activity, so they may be called "parasitic" cupric ions. In contrast, the second species or "prosthetic" cupric ions, is essential for ferroxidase activity. Its removal needs much higher values of DTC concentration. Since for the second step A_2-A_1/[Fox II] equals 8 000 one can conclude that there is only one prosthetic copper per protein molecule. These results corroborate those of Topham reporting that Cu(II) is necessary for enzymatic activity. They show, in addition, that although Fox II can bind several Cu(II), only one is in fact a prosthetic copper.

The absorption spectra of Fox II exhibit a strong band in the 460 nm region with several shoulders at 486, 457, 431, 406 and 325 nm which have been assigned by Topham et al. (3) to Cu(II) transitions.This spectral patterns, however strongly remind that of carotenoids. The presence of the latter in Fox II is confirmed by RR measurements :two strong peaks at 1526 and 1158 cm^{-1} and a weaker one at 1070 cm^{-1} characteristic of carotenoid RR spectra (6) are resonance enhanced upon excitation by means of the laser lines of an Ar^+ laser.

The CD spectrum of Fox II in the visible region exhibits a large negative band at 530 nm ($\Delta\varepsilon$ = - 0.7) with a shoulder at 620 nm ($\Delta\varepsilon$ = - 0.3). This signal is not modified by the addition of DTC at [DTC]/[Fox II] < 10, i.e. at DTC concentrations that release "parasitic" copper only, but the signal disappears at DTC concentrations high enough to remove the "prosthetic" copper,i.e. at [DTC]/[Fox II] > 50. These results strongly support the contention that the visible dichroic signal is due to the "prosthetic" cupric ion. CD data are compatible with metal coordination through nitrogen containing ligands(17).

ACKNOWLEDGMENTS. This work was supported by an ATP CNRS grant (ATP Coordination Chemistry n° 3311).

References

1. Osaki, S., Johnson, D.A. and Frieden, E. (1966) J. Biol. Chem. 241, 2476-2482
2. Rabinovitz, M., Chayen, R., Schen, R.J. and Goldschmidt, L. (1966) Clin. Chim Acta 14, 270-275

3. Topham, R.W. and Frieden, E. (1970) J. Biol. Chem. 245, 6698-6705
4. Sung, C.S.M. and Topham, R.W. (1973) Biochem. Biophys. Res. Comm. 53, 824-829
5. Topham, R.W. and Johnson, D.A. (1974) Arch. Biochem. Biophys. 10, 647-654
6. Topham, R.W., Sung, C.S.M., Morgan, F.G., Prince, W.D. and Jones, S.H. (1975) Arch. Biochem. Biophys. 167, 129-137
7. Lykins, L.F., Akey, C.W., Christian, E.G., Duval, G.E. and Topham, R.W. (1977) Biochemistry 16, 693-698
8. Sexton, R.C., Osaki, S. and Frieden, E. (1974) Fed. Proceed. 33, 1569
9. Frieden, E. and Hsieh, H.S. (1976) Adavances in Enzymology and Related Areas of Molecular Biology, Meister, A., Ed., John Wiley, New-York, V. 44, 187-236
10. Nitschmann, H., Kister, P. and Lergier, W. (1954) Helv. Chim. Acta 37, 867-871
11. Burstein, M., Scholnick, H.R. and Morfin, R. (1970) J. Lipid Res. 11, 583-595
12. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
13. Schreiber, J. (1979) Pharmazie 34, H1, 37-40
14. Lundberg, B. (1973) Acta Chem. Scand. 27, 3545-3549
15. Johnson, D.A., Osaki, S. and Frieden, E. (1967) Clin. Chem. 13, 142-150
16. Inagaki, F., Tasumi, M. and Miyazawa, T. (1975) J. Raman Spectrosc. 3, 335-343
17. Garnier, A. and Tosi, L. (1975) Biopolymers 14, 2247-2262