Vol. 110, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1983 Pages 313-319

THE RECONSTITUTION REACTION OF NEUROSPORA APOTYROSINASE

Mariano Beltramini & Konrad Lerch

Biochemisches Institut der Universität Zürich,
Zürichbergstrasse 4, CH - 8028 Zürich, Switzerland

Received December 6, 1982

SUMMARY: The reconstitution of Neurospora apotyrosinase was studied in the presence of Cu(I) or Cu(II) ions. The kinetics and the mechanism of reactivation were found to differ markedly for the two metal ions. Thus the reconstitution with Cu(I) was found to be very fast and complete following an all or none process; in contrast the reaction with Cu(II) proved to be rather slow and incomplete with the two Cu(II) ions binding with different rate constants.

Tyrosinase is a copper containing enzyme which catalyzes the o-hydroxylations of monophenols and the oxydation of o-diphenols to o-quinones (1,2). The enzyme contains at the active site a pair of antiferromagnetically coupled Cu(II) ions (2,3). In the reduced state the binuclear center binds reversibly molecular oxygen giving rise to oxytyrosinase (3,4). This binding is responsible for the appearance of two characteristic absorption bands in the visible region (λ = 345 nm, $\varepsilon \sim 17'000$; λ = 590 nm, $\varepsilon \sim 1000$). The copper at the active site of tyrosinase can be removed by cyanide treatment resulting in an apoprotein devoid of enzymatic activity (5,6). In previous studies (5,6), apotyrosinase was shown to be reactivated by Cu(II) ions. In the present paper we report on the influence of the metal valency state on the reconstitution reaction of Neurospora apotyrosinase. The kinetic behaviour of Cu(I) and Cu(II) ions is compared.

MATERIALS AND METHODS

Neurospora tyrosinase was purified as reported previously (7). The enzymatic activity was measured using L-dihydroxy-

0006-291X/83/010313-07$01.50/0

phenylalanine as substrate (8). Protein concentration was measured spectrophotometrically using the coefficient A_{280} (1% 1 cm) = 22.0 (7). Concentration of oxytyrosinase was determined by measuring the ratio A_{345}/A_{280}, the value of 0.17 corresponding to the fully oxygenated protein (7). Apotyrosinase was prepared according to (5). Reconstitution experiments were carried out in 0.15 M potassium phosphate buffer at 0 °C, using $CuSO_4$ (pH = 7.5), $CuSO_4$ in the presence of a 2-fold excess of ascorbate (pH = 5.8) or using the complex $Cu(CH_3CN)_4ClO_4$ in buffer containing 1 - 1.5 M acetonitrile (pH = 5.8). If not indicated otherwise the Cu concentration was in 10-fold excess with respect to the apoenzyme concentration. The $Cu(CH_3CN)_4ClO_4$ complex was prepared according to (9). Copper concentrations were estimated by atomic absorption. All spectroscopic measurements were carried out as reported previously (10).

RESULTS

Neurospora apotyrosinase can be reactivated by incubation with Cu(II) ions. The reconstituted enzyme binds 2 moles of copper per mole of enzyme and its specific activity is about 83% as compared with that of the native enzyme (Table I). Part of the reconstituted tyrosinase is recovered in the oxygenated form as shown by the presence of an absorption band at 345 nm (Fig. 1A, ----). Based on the ratio A_{345}/A_{280} the amount of oxytyrosinase is found to be about 40%. This fraction increases

Table 1: Physico-chemical properties of native, apo and reconstituted Neurospora tyrosinase

Tyrosinase	native	apo	reconstituted Cu^{2+}	reconstituted $Cu^{+}(CH_3CN)_4ClO_4$	reconstituted Cu^{2+}/ascorbate
activity (U mg^{-1})	1200	10-20	1000	1200	1200
A_{345}/A_{280}	0.17[a]	0	0.071 (0.140[a])	0.170	0.170
Cu/protein (g atoms/mol)	2.0	< 0.1	2.0	2.0	2.0
λ emiss.	322	330	332	332	332
Q	0.051[b]	0.129	0.050[b]	0.051[b]	0.050[b]

a) after treatment with H_2O_2 (7)

b) values corrected for quenching of the protein emission excerted by the 345 nm band (10)

λ emiss.: wavelength of the emission maximum, Q: fluorescence quantum yield.

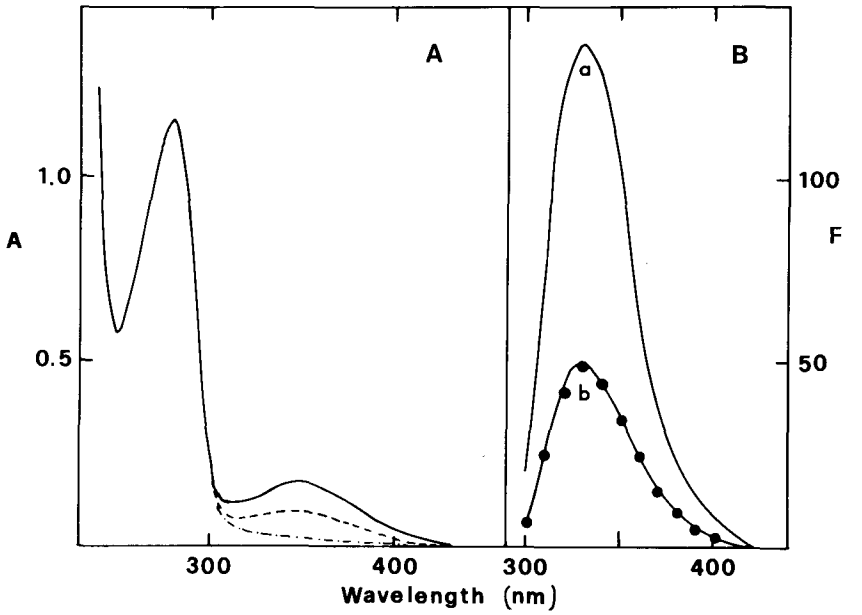

Fig. 1: Absorption (A) and fluorescence (B) spectra of native, apo, and reconstituted Neurospora tyrosinase. A: oxy- (——), apo (-·-), Cu(II) reconstituted tyrosinase (---). The solid line describes also the spectra of the Cu(I) reconstituted tyrosinase. All spectra are normalized to the same protein concentration (0.5 mg/ml). B: emission spectra of apo (a) and native (b) Neurospora tyrosinase. The dots represent the emission spectrum of reconstituted tyrosinase. A, absorbance; F, fluorescence intensity (arbitrary units).

to about 80% on H_2O_2 treatment (Table I). Incubation of apotyrosinase with $Cu(CH_3CN)_4ClO_4$ or with Cu(II) ions under reducing conditions leads to a reconstituted enzyme in the fully oxygenated form, showing the specific activity of native tyrosinase (Table I; Fig. 1A, ——). The binding of copper at the active site of the reconstituted enzyme discloses the same large quenching on the tryptophan fluorescence as documented for the native tyrosinase (10) (Fig. 1B; Table I). In Fig. 2 are shown the kinetics of the reconstitution reaction performed with Cu(II) (A) or Cu(I) (B) ions. In both cases the experimental values can be fitted as first-order processes, however, the apparent rate constant strongly depends on the metal

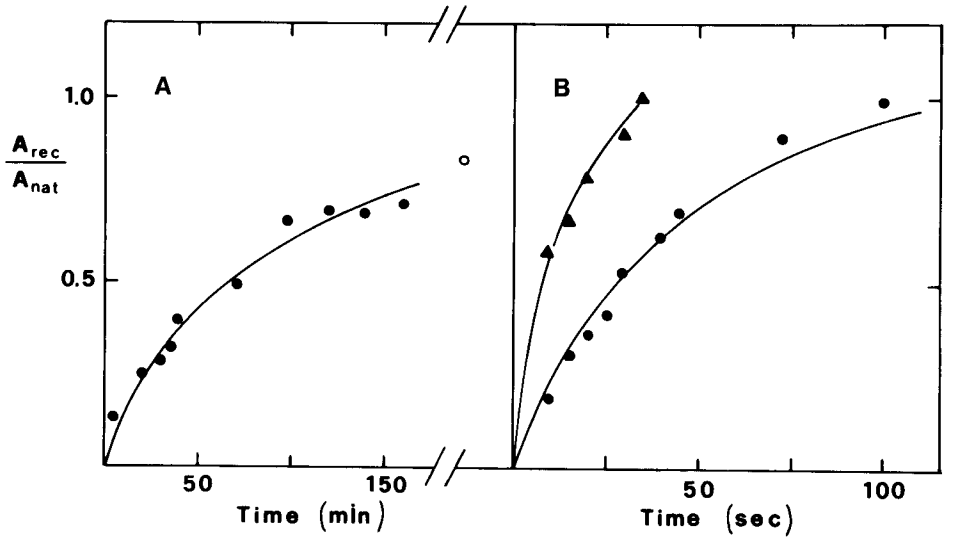

Fig. 2: Kinetics of the reconstitution reaction of Neurospora tyrosinase with Cu(II) (A,●; value after 20 hrs,○), Cu $(CH_3CN)_4ClO_4$ (B, ●) and Cu(II) ascorbate (B,▲). The solid lines refer to first order kinetics with rate constants of 0.123×10^{-3} sec^{-1} (A,●), 25.0×10^{-3} sec^{-1} (B,●) and 80.0×10^{-3} sec^{-1} (B,▲). A_{rec} and A_{nat} indicate the specific activity of the reconstituted and native tyrosinase, respectively.

valency. With Cu(II) the calculated $K_{app} = 0.123 \times 10^{-3}$ sec^{-1} is about two orders of magnitude smaller than the one obtained for $Cu(CH_3CN)_4ClO_4$ ($K_{app} = 25.0 \times 10^{-3}$ sec^{-1}). Cu(II), upon reduction with ascorbate, leads also to very fast reconstitution with a rate constant in the range of 80×10^{-3} sec^{-1}.

Fig. 3 shows the dependency of the specific activity of Neurospora tyrosinase in function of the copper-to-protein ratio (A) or of the fluorescence quenching resulting from copper binding (B). In these experiments, apotyrosinase is incubated with sub-stoichiometric amounts of Cu(I) or, using an excess of Cu(II), protein samples at various stages of the reconstitution reaction were treated with 5 mM EDTA and dialyzed against buffer containing EDTA. The experimental data are compared with two models: an all-or-none model (plots a) or a random binding model (plots b). It is evident that, in contrast to Cu(I), the regain of activity

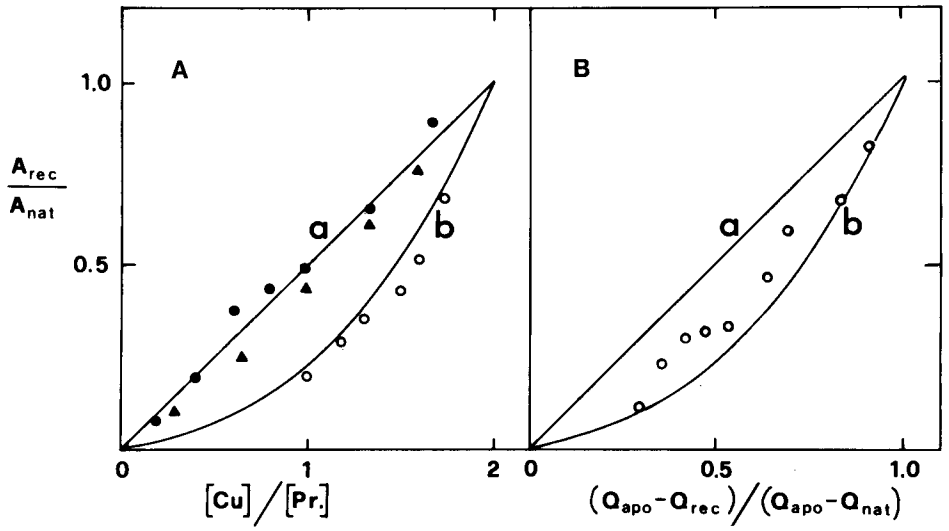

Fig. 3: Dependency of the specific activity of the reconstituted Neurospora tyrosinase in function of the metal-to-protein stoichiometry (A) and of fluorescence quenching (B). The solid lines refer to the plots expected in the case of a all-or-none process (a) or of statistical binding (b). A_{rec} and A_{nat} as in Fig. 2; Q_{apo}, Q_{rec}, Q_{nat} indicate the quantum yields of the apo, reconstituted and native tyrosinase, respectively. The symbols refer to reconstitution experiments carried out with Cu(II) (○); $Cu(CH_3CN)_4ClO_4$ (●); Cu(II)/ascorbate (▲).

with Cu(II) is not a linear function of the copper content or of the observed fluorescence quenching in the reconstituted enzyme.

DISCUSSION

In agreement with previous results (5,6) Cu(II) ions are competent in reconstituting apotyrosinase. As the enzyme can bind molecular oxygen only when the two copper ions are in the reduced state (3,4), the formation of oxytyrosinase during the reconstitution reaction stronly indicates reduction of the Cu(II) ions to Cu(I). This finding is in agreement with the data of Kertesz et al. (6), who explained the disappearance of the EPR signal of Cu(II) in the presence of apotyrosinase as the result of metal reduction, catalyzed by endogenous groups of the protein. The metal reduction, however, is not

strictly required for the reconstitution reaction. As shown in this study, the reconstituted Neurospora tyrosinase has, in fact, a specific activity higher than that indicated by the amount of oxytyrosinase; as a consequence part of the enzyme has to be in the met state [Cu(II)-Cu(II)] (3). The increase of the 345 nm band by addition of H_2O_2 is in agreement with the conversion of met → oxy via copper reduction (4). Hence the Cu(II) reconstituted tyrosinase is similar to the native protein which is mixture of the met and oxy form (4,7). As expected in the case of Cu(I) binding, Neurospora apotyrosinase is reconstituted by $Cu(CH_3CN)_4ClO_4$ or Cu(II)/ascorbate to the oxygenated state. The Cu(II) and Cu(I) ions strongly differ in their reconstitution kinetics, reflecting also the difference in the binding constants for the active site of tyrosinase: 10^{15} M^{-1} and 10^{13} M^{-1} for the cuprous and the cupric ions, respectively, (11). A marked difference between the reconstitution kinetics of Neurospora apotyrosinase and Cu(II) or Cu(I) ions was reported also in a study of the metal transfer from Neurospora copper metallothionein and Neurospora apotyrosinase (12).

From the data presented herein it is evident that the cuprous and cupric ions differ not only kinetically in their reconstitution reactions but also in their reconstitution mechanism. The reconstitution with Cu(I) follows an all-or-none course demonstrating that the population of the partially reconstituted enzyme is composed of fully active and of apo-molecules. Hence the two Cu(I) ions are bound in a pairwise fashion. In contrast, in the case of Cu(II), the nonlinearity between enzymatic activity and copper content indicates that the two copper ions are bound with different rate constants and that half-apo species (3) are present as

intermediates. Furthermore, the binding of the second copper ion at the active site could be the limiting step in the reconstitution reaction. Finally, the non-linear dependency of the recovery of protein fluorescence quenching strongly indicates that the single copper ion present at the active site of the half-apo species contributes to this quenching effect.

ACKNOWLEDGEMENTS

We wish to thank Matthias Birnstiel for his collaboration. This work was supported by the Swiss National Science Foundation (grant 3.709-0.80).

REFERENCES

1. Mason, H.S. (1965) Annu. Rev. Biochem. 34, 595-634.
2. Lerch, K. (1981) in Metal Ions in Biological Systems (Sigel, H., ed.), Vol. 13, pp. 143-186, Marcel Dekker, New York.
3. Himmelwright, R.S., Eickman, N.C., LuBien, C.D., Lerch, K., and Solomon, E.I. (1980) J. Am. Chem. Soc. 102, 7339-7344.
4. Jolley, Jr., R.L., Evans, L.H., Makino, N., and Mason, H. (1974) J. Biol. Chem. 249, 335-345.
5. Rüegg, C., and Lerch, K. (1981) Biochemistry 20, 1256-1262.
6. Kertesz, D., Rotilio, G., Brunori, M., Zito, R., and Antonini, E. (1972) Biochem. Biophys. Res. Comm. 49, 1208-1215.
7. Lerch, K. (1976) FEBS Lett. 69, 157-160.
8. Fling, M., Horowitz, N.H., and Heinemann, S.F. (1963) J. Biol. Chem. 238, 2045-2053.
9. Hemmerich, P., and Siegwart, C. (1963) Experientia 19, 488-489.
10. Beltramini, M., and Lerch, K. (1982) Biochem. J. 205, 173-180.
11. Kidani, Y., Ohkuma, K., Hirose, J., and Noji, M. (1980) Arch. Biochem. Biophys. 200, 452-460.
12. Beltramini, M., and Lerch, K. (1982) FEBS Lett. 142, 219-222.