Properties of copper in purified cytochrome *a*

EICHEL *et al.*[1] and GREEN AND CRANE[2] have reported that their cytochrome oxidase preparations contain a high concentration of copper as well as heme iron. On the other hand, many investigators[3–5] have observed from dietary experiments that copper-deficient tissues had a low cytochrome oxidase activity and a decreased content of heme *a*. Based on the above results, it has been considered that the copper in the cytochrome oxidase of typical cytochrome systems plays an important role in the mechanism of the cytochrome oxidase reaction. However, there is no direct evidence to support the above role of copper. The present communication deals with some properties of copper in the preparation of cytochrome *a* purified by our method[6].

In a previous report[6], we have also reported that our cytochrome *a* preparation contains copper in an amount such that the molar ratio of copper to heme iron is 4.5. Later, we repeated analyses of the copper content of various preparations and found that about 80 % of the total copper can be removed from the protein moiety by dialysis against cyanide, but the cyanide-treated preparation shows the same oxidase activity as that of an untreated preparation after the complete removal of cyanide by re-dialysis of the preparation against 0.1 *M* phosphate buffer containing 0.5 % Emasol 4130 at pH 7.4. From these results it seems likely that a large part of the total copper is not required for oxidase activity, and it may be adsorbed on the protein during the purification. In order to determine whether or not this is true, cytochrome *a* was prepared according to our method by using water from which copper was removed, and the copper content of the preparation thus obtained was determined. As illustrated in Table I, five samples of Fraction S_4 of cytochrome *a* contain equimolar amounts of copper and cytochrome *a* or iron. The relation between cytochrome *a* and copper contents during the purification process are also illustrated in Table II. All the results indicate that the molar ratio of copper to cytochrome *a* is always 1. About 50 % of the cytochrome oxidase activity is inhibited by copper-chelating agents such as ferrocyanide, salicylaldoxime, or ethylxanthate as illustrated in Table III. However, the copper in cytochrome *a* is not released by dialysis against cyanide or salicyladoxime and the activity is not altered by the treatment as described the above. The activity after the above treatment is also inhibited by the above copper-chelating agents at

TABLE I

COPPER AND IRON CONTENTS IN FRACTION S_4 OF CYTOCHROME *a*

Preparation No.	*Contents (10^{-2} μatoms or μmoles/mg protein N)*			$\frac{Cu}{Fe}$	$\frac{Cu}{Cyt.\ a}$
	*Cu**	*Fe***	*Cyt. a****		
1	6.35	6.07	5.65	1.05	1.12
2	5.68	5.65	4.91	1.01	1.16
3	5.91	5.65	5.36	1.04	1.10
4	5.58	4.89	4.23	1.10	1.32
5	6.27	5.95	5.09	1.05	1.23
Average	5.96	5.64	5.05	1.05	1.18

* The copper content was determined by the method of MCFARLANE[8].

** The iron content was determined by the method of SANDELL[9].

*** The concentration of cytochrome *a* was determined spectrophotometrically assuming[10] that $\varepsilon^{(reduced)}_{605-630\ m\mu} = 16.5 \cdot 10^3\ M/cm$.

TABLE II

RELATION BETWEEN COPPER AND CYTOCHROME *a* CONTENTS DURING THE PURIDICATION PROCESS

*Stage of purification**	S_1	S_2	S_3	S_4
Turnover number** (μmoles O_2/μmole cyt. *a*/min)	114.2	150.0	42.2	36.4
Cytochrome a (10^{-2} μatom/mg N)	1.05	1.07	3.75	5.09
Copper (10^{-2} μatom/mg N)	1.10	1.43	4.76	6.09
Copper/cytochrome *a*	1.04	1.33	1.27	1.19

* The nomenclature of the fractions is the same in a previous report[6].
** The activity was measured manometrically in 1 % Emasol 4130[10].

TABLE III

EFFECT OF COPPER-CHELATING AGENTS ON THE ACTIVITY OF CYTOCHROME *a*

Reagent	*Concentration (M)*	*Inhibition** (%)*
KCN	10^{-5}	60
	10^{-4}	100
NaN_3	10^{-3}	72
$K_4Fe(CN)_6$	10^{-2}	50
Salicylaldoxime	10^{-2}	50
Ethylxanthate	10^{-2}	58

* The activity was measured manometrically at 30° by using hydroquinone as substrate. Fraction S_4 was used as purified cytochrome *a*.

the same ratio. Acid treatment of cytochrome *a* can easily release the copper from the protein moiety, while heat treatment cannot even at 100° for 30 min.

To check the valency state of copper in cytochrome *a*, color development by the addition of 2,2′-diquinolyl or 2,9-dimethyl-1,10-phenanthroline was also examined. By the addition of either of the above reagents, cytochrome *a* in Emasol 4130 strongly exhibits the specific color due to Cu^+. However, as in the case of iron in succinic dehydrogenase[7], the color is not developed in the presence of *p*-chloromercuribenzoate. Based on these results, it appears that the copper in cytochrome *a* exists as in the cupric state. Cytochrome *a* in its oxidized form shows a spectrum having absorption maxima at 280, 340, 424, and 600 mμ. Absorbancy at the 340 mμ peak is decreased by dialysis against cyanide and recovered by the addition of Cu^{++} but not by the addition of Cu^+. However, there is no direct evidence that the 340 mμ peak is due to the combination of cytochrome *a* with copper. We are now studying the valency change of copper in cytochrome *a* in the presence of reduced or oxidized cytochrome *c* to investigate the possibility that the copper can act as an electron carrier. The results of these studies will be presented elsewhere.

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Studies on the electron transport system
XIV. The isolation and properties of soluble cytochrome c_1. Addendum

Since the publication of the paper on the isolation and properties of cytochrome c_1[1] it has been pointed out to the authors[2] that the calculations of the oxidation–reduction potential of cytochrome c_1 were not based on the most reliable E_0 value for the benzoquinone–hydroquinone system (see p. 44 and Table VI in GREEN *et al.*[1]). When the more correct value of + 0.69607 V[3] is used for the E_0 of the reference quinone and 0.060148 for the factor 2.30258 RT/F, the mid-point potentials for cytochromes c_1 and c shown in the accompanying table are obtained. This table is a reproduction of our original table, but with the revised values substituted for the earlier ones. The mean E_0' for the cytochrome c_1 system is + 0.220 V, and for the cytochrome c system + 0.248 V. This latter value has to be compared with E_0' values obtained by others, which range from + 0.254 V to + 0.262 V.

TABLE I

DETERMINATION OF THE OXIDATION-REDUCTION POTENTIAL OF CYTOCHROMES c_1 AND c

The measurements were made in 0.1 *M* phosphate at 30°. The *p*-benzoquinone and hydroquinone solutions were prepared in ethanol from recrystallized samples.

Concentration of quinone-hydroxyquinone mixture (μmoles/ml)	*Cytochrome*	*pH*	*E_0' of quinone system (V)*	*Total concentration of hemoprotein (μmoles/ml)*	*Final concentration of hemoprotein*		*$0.060148 \times \log \frac{ox}{red}$*	*E_0' (V)*
					oxidized (μmoles/ml)	*reduced (μmoles/ml)*		
1	c_1	6.73	+ 0.2913	0.034	0.0314	0.0026	0.0651	+ 0.226
1	c_1	7.55	+ 0.2419	0.034	0.026	0.008	0.0308	+ 0.211
0.5	c_1	7.55	+ 0.2419	0.034	0.023	0.011	0.0193	+ 0.223
6	c	6.73	+ 0.2913	0.105	0.081	0.024	0.0321	+ 0.259
6	c	7.55	+ 0.2419	0.105	0.058	0.047	0.0055	+ 0.236

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