ENZYMIC REDOX REACTIONS OF CYTOCHROMES *c**

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SUMMARY. The two cytochromes *c* for which detailed tertiary structures have been obtained recently--cytochrome *c* from mammalian mitochondria and cytochrome c_2 from *Rhodospirillum rubrum*--have been compared in reactivity in the physiologic redox systems for which the former is the natural substrate. These systems are the mitochondrial DPNH-cytochrome *c* reductase (complex I-III) and cytochrome *c* oxidase (complex IV). In addition, the effects of antimycin A (a specific inhibitor in the reductase system) and L-polylysine (usually employed to inhibit the oxidase system) on the reduction and oxidation of cytochromes *c* and c_2 have been studied. Preliminary data for these two cytochromes, as well as for five others representative of different classes of bacterial and algal cytochrome *c* are presented. Implications of these findings for current attempts to rationalize the structural features of cytochromes *c* are considered.

High resolution x-ray structural studies of mitochondrial cytochrome *c* in both oxidized and reduced forms (1,2), and of an analogous heme protein, *Rhodospirillum rubrum* ferricytochrome c_2 (3), have been successfully completed. Similar results are soon expected for the complete structural analysis of *R. rubrum* ferrocytochrome c_2 (4). Knowledge of the comparative biochemistry of *c*-type cytochromes has been enriched by many exhaustive and systematic researches (5). However, most of the available data have been obtained by testing the reactivity of proteins of widely varying purity in the presence of incompletely characterized mitochondrial preparations. It is requisite to extend these studies with the use of highly purified cytochrome preparations and well-defined enzyme systems, such as the antimycin-sensitive DPNH-cytochrome *c* reductase (complex I-III) (7) and the cytochrome *c* oxidase (complex IV) (8),

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prepared from bovine heart mitochondria. These preparations, which are composed of highly purified and well characterized components, provide an opportunity to extend in depth and precision studies on electron transfer behavior of cytochromes *c*, and offer a reliable basis for inferences from structural features revealed by high resolution x-ray studies. We present in this report preliminary data from such researches initiated recently. In addition to data on the two heme proteins mentioned, we have also included observations on the redox behavior of five other cytochromes *c*, representative of broad classes of the bacterial and algal *c*-type cytochromes. These are: *Euglena gracilis* cytochrome *c*-558 (9) *Chlorobium thiosulfatophilum* *c*-555 (10), *Porphyra tenera* cytochrome *f* (11), *R. rubrum* cytochrome *c'* (12), and *Rhodopseudomonas palustris* cytochrome *c'* (13).

MATERIALS AND METHODS

Cytochromes c. Horse heart cytochrome *c* (Type III, Sigma) as obtained from Sigma Chemical Co. had been purified to about the 99% spectrochemical and chromatographic level by recrystallization. The various bacterial and algal preparations were of the same purity, having been recrystallized numerous times after initial isolation by procedures described elsewhere (9-13). We are indebted to Dr. T.E. Meyer for providing the original samples of bacterial cytochromes *c* and to Dr. R.G. Bartsch for those of bacterial cytochrome *c'* and the algal cytochrome *f*.

Mitochondrial Redox Systems. Preparation and assay of the three systems used--complex I-III (particulate, antimycin- and rotenone-sensitive DPNH-cytochrome *c* reductase), complex IV (cytochrome *c* oxidase), and soluble DPNH dehydrogenase isolated from complex I--were as reported in previous studies (6-8, 14). Protein was determined by the biuret method without correction for the hyperchromicities of complex I-III and complex IV.

Inhibitors. Antimycin A was a gift from Kanegafuchi Chemical Industrial

TABLE I

Reduction of Cytochromes *c* by Complex I-III

Electron Acceptor	Specific Activity*	% Inhibition	
		Antimycin A (1 μM)	Polylysine (5 μM)
cytochrome *c*	24.6	100	> 98
" c_2	13.6	> 98	84
" *c*-558	16.7	100	> 98
" *c*-555	0.45	100	73
" *f*	0.39	None	Activation**
" *c'(R. palustris)*	0.0	----	----
" *c'(R. rubrum)*	0.0	----	----

* μmoles cytochrome reduced x min^{-1} x mg^{-1} complex I-III protein at 38°. The extinction coefficient used for all cytochromes was that of cytochrome *c*, i.e., ΔA (reduced minus oxidized) = 18500 liter x $mole^{-1}$. The wavelengths used for measuring the reduction of the cytochromes, in order of their listing in the Table, were in nm: 550, 550, 558, 555, 554, 550 and 550.

** At this concentration, polylysine activated cytochrome *f* reduction sevenfold. Maximum activation at 10 μM polylysine was 13 fold.

Co., Japan, and L-polylysine hydrochloride (mol. wt. 15,500) was obtained from Schwarz Biochemical.

RESULTS

1. Reduction of cytochromes *c*

Tables I and II summarize results on specific activities for the reduction of various cytochromes *c* by DPNH as catalyzed respectively by the mitochondrial complex I-III and the diaphorase-like, soluble DPNH dehydrogenase isolated from the respiratory chain. The effects of antimycin A and polylysine on the reduction of various cytochromes *c* are also shown in Table I.

2. Oxidation of reduced cytochromes *c*

Table III shows polarographic results on the specific activities for the

TABLE II

Reduction of Cytochromes *c* by Soluble DPNH Dehydrogenase

Electron Acceptor	Specific Activity*
cytochrome *c*	36.3
" c_2	19.5
" *c'(R. palustris)*	0.17**
" *c'(R. rubrum)*	0.48**

* μmoles cytochrome reduced x min^{-1} x mg^{-1} DPNH dehydrogenase protein at 38°. The wavelength used was 550 nm, and extinction coefficient was the same as in Table I.

** Calculated from ΔA_{550} in the initial 30 seconds after addition of enzyme. The rate of reduction declined rapidly and became nil after 1.5 minutes.

TABLE III

Oxidation of Cytochromes *c* (plus Ascorbate-TMPD) by Cytochrome *c* Oxidase

Cytochrome	Specific Activity*	% Inhibition by Polylysine (5 μM)
cytochrome *c* (11 μM)	9.7	100
" c_2 (11 μM)	0.7	72
" c_2 (100 μM)	2.7	90
" *c*-555 (11 μM)	10.7	100
" *c*-558 (11 μM)	19.8	100
" *f* (64 μM)	nil	---

The assay mixture (1.8 ml) consisted of 10 mM potassium phosphate, pH 7.0, 22 μM tetramethyl-*p*-phenylenediamine (TMPD), 1.1 mM sodium ascorbate, and 0.16 mg cytochrome oxidase.

* μmoles cytochrome oxidized x min^{-1} x mg^{-1} cytochrome oxidase protein at 30°.

oxidation of various cytochromes *c*, in the absence and presence of polylysine, by cytochrome oxidase purified from bovine heart mitochondria. In these studies, substrate amounts of ascorbate plus TMPD were used in the assay mixtures for rapid production of reduced cytochromes *c*. In Table IV spectrophoto

metric data are shown for the oxidation of ferrocytochromes *c* by cytochrome oxidase in the absence and presence of polylysine. Cytochromes *c* and *f* were reduced prior to assay by $Na_2S_2O_4$, then filtered through a column of Sephadex

TABLE IV

Oxidation of Ferrocytochromes *c* by Cytochrome Oxidase

Electron Donor	Specific Activity*	% Inhibition by Polylysine (5 μM)
Ferrocytochrome *c*	16.6	100
" c_2	0.88	77
" *f*	0.0	---
" *f* + *c* (ferri)	5.7	100

* μmoles cytochrome oxidized x min^{-1} x mg^{-1} cytochrome oxidase protein at 38°. The extinction coefficient and the wavelengths were the same as in Table I.

G-25 to remove excess dithionite. The reason for the difference in specific activities in Tables III and IV is that the experiments of Table III were conducted at 30°, and those of Table IV at 38°.

CONCLUSIONS

A number of salient observations may be remarked with regard to the results shown in the above Tables.

1. Mitochondrial cytochrome *c* and *R. rubrum* cytochrome c_2 show comparable specific activities in the complex I-III system as well as in the less specific dehydrogenase system (Tables I and II). The K_m values in the former system are 13.3 μM for cytochrome *c* and 23.8 μM for cytochrome c_2, and the characteristic antimycin A inhibition is seen to be complete at 1 μM for both cytochromes. The fact that the antimycin-insensitive, soluble dehydrogenase reacts similarly with both cytochromes, although significantly altered in specificity as witnessed by its reactivity with the high spin, autoxidizable cytochromes *c'* (Table II), is in accord with earlier findings (15,16).

Critical to the observation that cytochrome c_2 reacts so well with the mitochondrial reductase is the fact that it lacks the tyrosine residue no. 74, suggested as involved in electron entry (17); instead it has a phenylalanine residue (no. 77) homologous with the mitochondrial cytochrome *c* tyrosine no. 74. Thus, it appears highly unlikely that this particular mode of reduction occurs, at least in the physiological reduction act.

2. L-Polylysine not only inhibits the oxidase with these two test cytochromes *c*, but also the reductase (Tables I, III and IV). This is inconsistent, again, with the suggestion that reductase and oxidase binding sites are in different places on cytochrome *c* (18).

3. *E. gracilis* cytochrome *c*-558, derived from the algal mitochondrial fraction, reacts well with the mitochondrial redox enzymes (Tables I and III), as expected for a functional mitochondrial protein. However, this cytochrome lacks one of the two characteristic covalent thio-ether links to the heme, since it contains only one cysteine (9). Hence, it appears that sufficient rigidity in heme placement for physiologic function does not require both covalent linkages as in mitochondrial cytochrome *c*.

4. *Chlorobium* cytochrome *c*-555 is a non-respiratory cytochrome. It is functional apparently in the photometabolism of green sulfur bacteria, in analogy with the algal and plant cytochromes *f* (10). Nevertheless, it reacts significantly with the mitochondrial oxidase system (Table III). This finding is in agreement with those of earlier studies, using a less well-defined oxidase preparation (5). On the other hand, a *bona fide* cytochrome *f*, that of the alga *P. tenera*, reacts not at all with the mitochondrial oxidase, but can be coupled through the mammalian cytochrome *c* (Table IV), as originally found for plant cytochrome *f* (20).

5. It is also of considerable interest that cytochrome *f* reacts poorly with the mitochondrial reductase system, but that polylysine *increases* the reducibility of this cytochrome as much as 13 fold (Table I). The reduction of cytochrome *f* by complex I-III is not inhibited upon addition of antimycin A.

Thus, both the stimulating effect of polylysine and the insensitivity to antimycin A suggest that the mode of interaction of cytochrome *f* with the mitochondrial reductase system is different from those of the other cytochromes shown in Table I.

Many other comments could be included concerning the present findings. However, those presented in this report will suffice to indicate the potential significance of the present type of study for eventual clarification of the basis for the structural features revealed in cytochromes by the high resolution x-ray studies. A more detailed report is in preparation (21).

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