Comparative Study of Physicochemical Properties of Two *c*-Type Cytochromes of *Rhodospirillum molischianum**

Torgeir Flatmark, Karl Dus, Henk deKlerk, and Martin D. Kamen

ABSTRACT: The two *c*-type cytochromes of *Rhodospirillum molischianum* (Dus, K., Flatmark, T., deKlerk, H., and Kamen, M. D. (1970), *Biochemistry 9*, 1984) strikingly resemble cytochrome *c* of eukaryotic organisms with regard to p*I*, absorption spectra (at 25° as well as at −190°), and circular dichroism spectra in the Soret and visible wavelength regions. In this respect they differ from previously studied *c*-type cytochromes (cytochromes c_2) of nonsulfur purple photosynthetic bacteria. Although these findings suggest that the chemical nature of the linkages of the hemes to the respective apoproteins may be very similar in the *c*-type cytochromes of *R. molischianum* to that in eukaryotic cytochrome *c*, they should not be taken as evidence for similarity in protein structure surrounding these heme groups. Thus, the oxidation–reduction potentials of the two *R. molischianum* cytochromes are significantly higher than the potential of eukaryotic cytochrome *c*. Midpoint potentials, E_{m7}, of +0.381 and +0.288 V were measured for the small *c*-type cytochrome and cytochrome c_2, respectively. In accordance with these redox equilibria, cytochrome c_2 is slowly oxidized by dissolved molecular oxygen at neutral pH, whereas the small *c*-type cytochrome, which has a substantially higher electron affinity, undergoes autoreduction under the same conditions. Furthermore, in contrast to the two isocytochromes *c* of *Saccharomyces cerevisiae*, the large difference in their oxidation–reduction potentials (about 90 mV) would enable them to function in different electron-transfer sequences within the cell. Neither cytochrome c_2 nor the small *c*-type cytochrome is able to restore any succinate oxidation in rat kidney mitochondria depleted of endogeneous cytochrome *c*.

In the preceding paper (Dus *et al.*, 1970) the isolation, purification and chemical properties of two soluble *c*-type cytochromes from light-grown cells of *Rhodospirillum molischianum* (wild type) were reported. Both heme proteins were obtained in the monomeric form. Despite differences in molecular weight, analyses of their peptide patterns (Dus *et al.*, 1970) strongly suggested structural homology between them, and it therefore became of great interest to compare other physicochemical properties of these cytochromes in order to get a better understanding of the relationship between their structure and function. Furthermore, in view of the large number of well-characterized *c*-type cytochromes, it is important to relate these properties to those established for other cytochromes both of photosynthetic bacteria and of mammalian species.

Materials and Methods

Chemicals. Commercial quinhydrone was recrystallized twice from hot water. Reduced 2,6-dichloroindophenol was obtained by anaerobic reduction with PdH_2. Oxygen was removed from commercial nitrogen by passage through a quartz tube filled with MnO (Hersch, 1960).

Protein Preparations. The *c*-type cytochromes extracted from light-grown cells of *R. molischianum* were partially purified as described (Dus *et al.*, 1970) and obtained in the monomeric form by molecular sieve chromatography on Sephadex G-75 (Flatmark, 1964). From the preparation thus obtained, the two *c*-type cytochromes were isolated by preparative disc electrophoresis on polyacrylamide gel (Flatmark, 1964), and their percentage of the total *c*-type cytochromes was essentially the same as reported previously (Dus *et al.*, 1970). The ferrous and ferric forms of the cytochromes were prepared following the procedure of Yonetani and Ray (1965); Sephadex G-10 was used for removal of the reducing and oxidizing agents by molecular sieve chromatography.

Mammalian cytochrome *c*, extracted from bovine heart muscle by dilute sulfuric acid at pH 4 and 4°, was obtained in the monomeric form by molecular sieve chromatography on Sephadex G-75 (Flatmark, 1964) and subjected to disc electrophoresis on polyacrylamide gel. The preparation separated into the usual four molecular forms (cytochrome I–cytochrome IV) of the protein (Flatmark, 1964).

Measurement of Oxidation–Reduction Potential. Oxidation–reduction titrations were carried out in a suitable thermostated cuvet as described by Paul (1947); the electrodes were Radiometer P 101 and K 4312. Reduced cytochrome and reduced 2,6-dichloroindophenol were mixed in the cuvet to give a final concentration of approximately 20 μM cytochrome and 2 μM indophenol in a total volume of 4.5 ml in 0.1 M phosphate buffer (pH 7.0) at 25°. Anaerobic conditions and stirring were effected during titration by maintaining a slow flow of purified nitrogen through the solution. A stable potential was initially obtained after about 3 hr, and the titration was then started by the addition of increments

* From the Department of Biochemistry, University of Bergen, Bergen, Norway (T. F.), the Biochemistry Division, University of Illinois, Urbana, Illinois 61801 (K. D.), the Laboratoire de Photosynthèse, Gif-sur-Yvette, France (H. dK.), and the Department of Chemistry, University of California, San Diego, La Jolla, California 92038 (M. D. K.). The experimental work described in this paper was supported in part by grants-in-aid from the National Institutes of Health (HD-01262) and the National Science Foundation (GD-7033X) to M. D. K. as well as from Norwegian Research Council for Science and the Humanities to T. F.

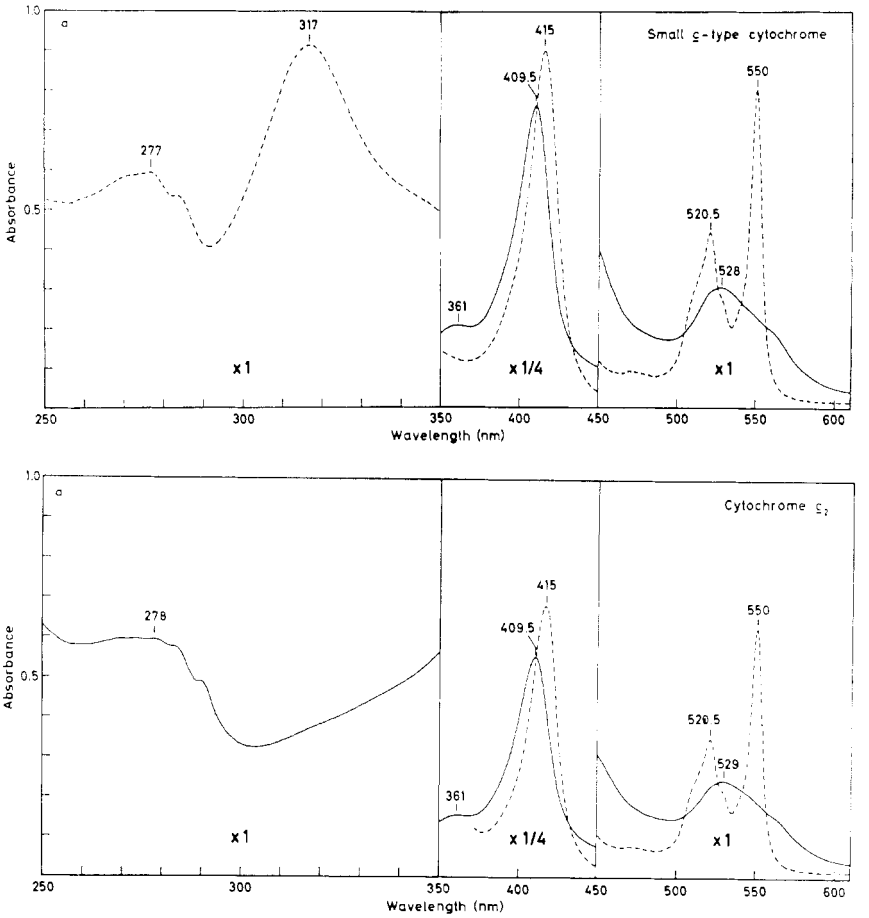

FIGURE 1: Absorption spectra of *R. molischianum* *c*-type cytochromes. The cytochromes were dissolved in 40 mM phosphate buffer, pH 6.9, 25°; (——) ferric forms; (- - - -) ferrous forms. The protein concentrations were 27.6 μM of the small *c*-type cytochrome and 21.1 μM of cytochrome c_2, respectively.

of an oxygen-free solution of potassium ferricyanide to give a stable potential within 15 min. An electron tube potentiometer (Instrumentation Laboratories Deltamatic pH–mV electrometer Model 145) was used for registering the potentials. When equilibrium potentials were reached, the spectrum was recorded. The percentage of reduced *c*-type cytochrome was calculated from the absorption spectrum by using the ratios ϵ_{550} (red):ϵ_{550} (ox) of 3.64 and 3.66 for the small *c*-type cytochrome and cytochrome c_2, respectively. At each potential reading corrections were made for the absorbance contribution due to oxidized dichloroindophenol by assuming a midpoint potential $E_{m7} = +0.217$ V for the mediator The reference electrode (K 4312) was standardized against a saturated solution of quinhydrone.

Spectrophotometry. Absorption spectra were obtained by means of a Shimadzu MPS-50L recording spectrophotometer calibrated with a mercury lamp. Due to the rapid rate of autoreduction of the small *c*-type cytochrome at neutral pH ferricyanide was added (20-fold excess) to the sample as well to the reference cuvet. The concentration of *c*-type cytochromes was determined from the millimolar extinction coefficients ϵ_{550} 29.15 (the small *c*-type cytochrome) and ϵ_{550} 29.76 (cytochrome c_2) (Dus *et al.*, 1970). Absorption spectra and first-derivative absorption spectra at −190° were obtained using a Bausch & Lomb single-beam spectrophotometer. The cytochrome samples were diluted with 0.1 M phosphate buffer (pH 7.0), reduced with a minimum of sodium dithionite, and then mixed with an equal volume of glycerol.

Circular Dichroism. Circular dichroism measurements were performed with a Jasco ORD–UV-5 recording spectropolarimeter with a circular dichroism attachment, in cells of light paths between 1 and 10 mm. The instrument records the difference in absorbance, ΔA, at any wave length for left and right circularly polarized light. The difference in molecular extinction coefficient ($\Delta\epsilon = \epsilon_l - \epsilon_r$) was calculated from the relationship

$$\Delta\epsilon = \Delta A/(cd) \quad (1)$$

where c is the concentration in moles per liter and d is the path length in centimeters. The molecular ellipticity, $[\theta]$ was then obtained from the equation (Moscowitz, 1961)

$$[\theta] = 2.303\frac{4500}{\pi}\Delta\epsilon \quad (2)$$

where the units are deg cm^2 dmole^{-1}. The precision of the measurements is better that ±5% over the entire spectral

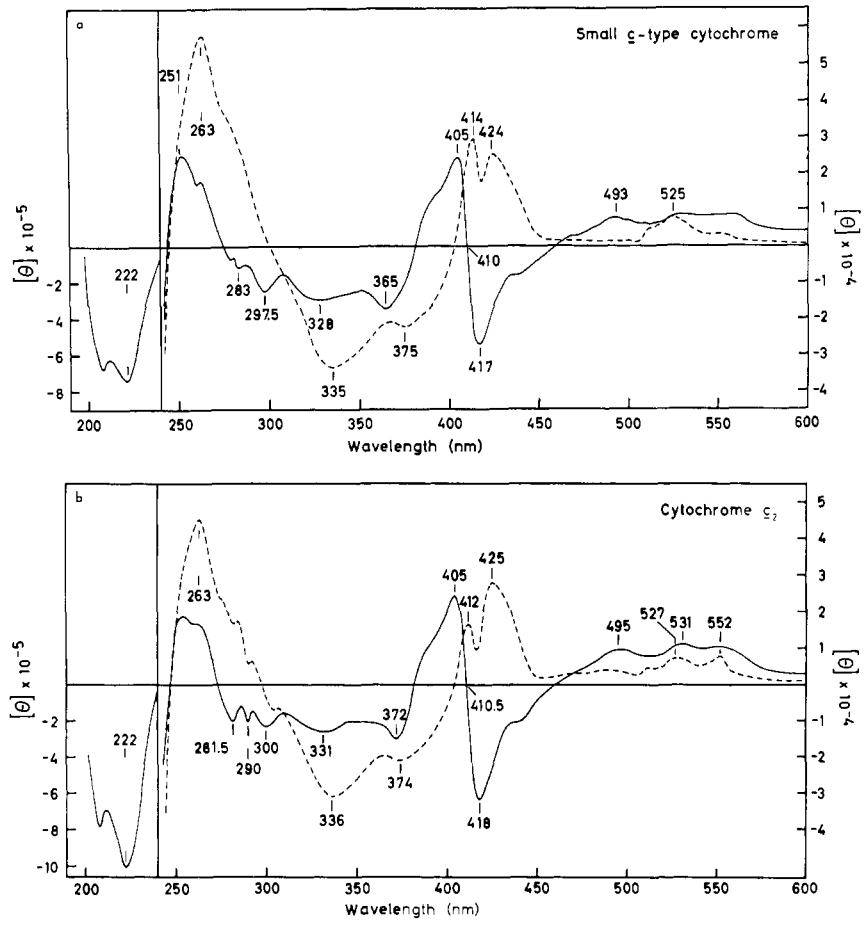

FIGURE 2: Circular dichroic absorption spectra of *R. molischianum* c-type cytochromes. The cytochromes were dissolved in 40 mM phosphate buffer, pH 6.9, 20°; (——) ferric forms; (-----) ferrous forms. The molar ellipticity, $[\theta]$, is given in deg cm^2 dmole^{-1}.

region investigated. For studying the ellipticities at 222 nm as a function of the cytochrome concentration, the instrument was set at the desired wavelength and the amplitude was recorded for 2 min. The observed intensity was taken as the difference between the midpoint of the vertical line drawn by the recorder and the base line given by the solvent. The height of the negative peak in the circular dichroic absorption spectrum around 222 nm was used to estimate the apparent content of α_r helix (Holzwarth and Doty, 1965), by using the value $[\theta]_{222} = -4 \times 10^4$ for helical poly-L-glutamic acid (Cassim and Yang, 1967) as a reference standard.

Isolation of Mitochondria from Rat Kidney. Mitochondrial fractions of rat kidneys were obtained by differential centrifugation essentially as described (Schneider, 1948; Nakano and Danowski, 1966). KCl-washed, cytochrome *c* depleted mitochondria were obtained by the method of Jacobs and Sanadi (1960) and finally suspended in 0.25 M sucrose.

Assay of Succinate Oxidase Activity. The effect of added *c*-type cytochrome on the succinate oxidase activity of cytochrome *c* depleted rat kidney mitochondria was studied as described (Flatmark, 1967). Oxygen consumption was assayed polarographically (Kielly and Bronk, 1958) with a Clark oxygen electrode (Chappell, 1961). The reaction mixture was magnetically stirred in a 3.5-ml vessel supported and covered with a rubber stopper to minimize back-diffusion of air. The electrode was polarized at −0.8 V. Buffer solutions were equilibrated with air, and the solubility of air in the reaction medium was calculated according to Hodgman *et al.* (1962).

Biuret Protein Determination. Mitochondrial protein concentrations were determined by a biuret reaction according to the method of Yonetani (1961).

Results

Absorption Spectra. The absorption spectra of the two *c*-type cytochromes of *R. molischianum* are shown in Figure 1. No difference in the position of the Soret and visible absorption bands was found. Both heme proteins possess a spectrum very similar to that reported for mammalian cytochromes *c* (Margoliash and Schejter, 1966).

Circular Dichroic Absorption Spectra. From Figure 2 it can be seen that both *c*-type cytochromes give complex circular dichroism spectra, both in their oxidized and reduced forms. While a remarkable similarity between the two cytochromes prevails throughout the visible region, differences do exist in the region from 245 to 300 nm due to marked disparity in contents of all aromatic amino acids (Dus *et al.*, 1969). Based on the magnitude of the $\eta - \pi^*$ ellipticity band at 222 nm, an apparent α_r-helix content of ~20%

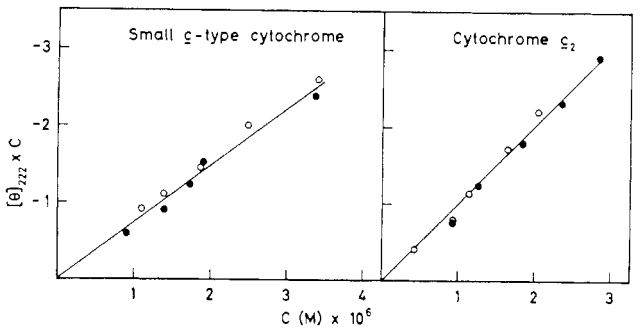

FIGURE 3: Ellipticity at 222 nm as a function of the concentrations of *c*-type cytochromes. (●) Ferric forms; (○) ferrous forms.

was calculated for both heme proteins; no significant changes were found in the magnitude of this band upon oxidation–reduction of either cytochrome (Figure 3).

Light Absorption Spectra at −190°. Upon cooling mammalian ferrocytochrome *c* in a water–glycerol solution to the temperature of liquid nitrogen (−190°) its α and β bands are intensified about six times, and they are shifted by about 2 nm toward the blue (Keilin and Hartree, 1949; Estabrook, 1956; Estabrook, 1961). In addition, the α band is resolved into three satellite bands, termed c_{α_1}, c_{α_2}, and c_{α_3} while the β band is split into as many as nine bands.

A comparison of absorption spectra and first-derivative absorption spectra of the *R. molischianum* *c*-type cytochromes is given in Figure 4. Both cytochromes show splitting of the α band into the same three satellite bands, and there is no significant difference in the location of the maxima of these bands as compared with mammalian cytochrome *c*. The intensity of the c_{α_2} and c_{α_3} bands, however, appears to decrease gslihtly from the small *c*-type cytochrome to cytochrome c_2.

Isoelectric Points. Though the exact p*I* values have not been determined, it can be concluded from the disc electrophoresis experiments that both *c*-type cytochromes of *R. molischianum* are strongly basic proteins. Thus, both cytochromes (in their ferrous forms) concentrated and moved toward the cathode at a pH of 9.4 in the sample gel, and they revealed mobilities close to that of native bovine heart cytochrome *c*.

Oxidation–Reduction Potentials. The results of the potentiometric titrations of the two cytochromes are shown in Figure 5. The shapes of the oxidation–reduction equilibrium curves conform to the symmetry expected for $n = 1$; the midpoint potentials, E_{m7}, are +0.381 and +0.288 V at 25° for the small *c*-type cytochrome and cytochrome c_2, respectively.

Activity of the R. molischianum c-Type Cytochromes in the Mammalian Succinate Oxidase System. Since we observed several physicochemical characteristics common to both *c*-type cytochromes of *R. molischianum* and mammalian cytochrome *c* it was of interest to determine whether these bacterial cytochromes could possibly function as electron carriers in the mitochondrial respiratory chain of eukaryotic organisms.

In the absence of added exogeneous cytochrome *c*, our mitochondrial preparation consumed only negligible amounts of oxygen, *i.e.*, $v = 21.5$ nmoles of O_2 min^{-1} mg of $protein^{-1}$. Addition of native bovine heart cytochrome *c* at a concentration of 3.6 μM had a potent stimulating effect on the specific

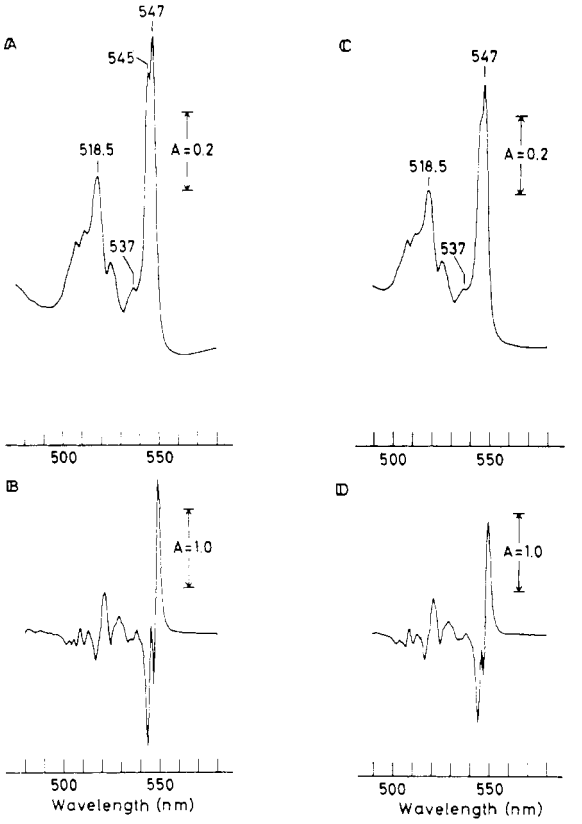

FIGURE 4: Low-temperature spectra of *R. molischianum* *c*-type cytochromes. The spectra of the small *c*-type cytochrome (A and B) and of cytochrome c_2 (C and D) refer to the proteins reduced by sodium dithionite and dissolved in 40 mM phosphate buffer (pH 6.9) and glycerol 50% (v/v), at −190°; optical path of the cuvets was 2 mm. The protein concentrations were 9.3 μM of the small *c*-type cytochrome and 7.1 μM of cytochrome c_2, respectively. The top traces (A and C) are the absorption spectra recorded with a single-beam spectrophotometer; the lower traces (C and D) represent the first-derivative absorption spectra; spectral pass band, 0.5 mm.

respiratory rate ($v = 205$ nmoles of O_2 min^{-1} mg of $protein^{-1}$), while both *c*-type cytochromes of *R. molischianum* were completely inactive, even at ten times higher concentrations (35 μM).

Discussion

The picture which emerges from previous (Dus *et al.*, 1970) and present studies of the two isolated cytochromes of *R. molischianum* is that they are typical *c*-type cytochromes; several of their physicochemical properties closely resemble those of eukaryotic cytochrome *c*. Thus, the close similarity in absorption spectra (throughout the visible wavelength region, at 25° and at −190) and in circular dichroism spectra, of both bacterial cytochromes and bovine heart cytochrome *c* (Flatmark and Robinson, 1968) are compatible with the iron–protein linkages being identical in these three heme proteins. The remarkable correspondence of the spectroscopic properties extends even into the Soret region where the circular dichroism spectra of the bacterial cytochromes and of the protein from bovine heart muscle were found to be almost identical in frequency, sign, and amplitude. Thus,

it would seem permissible to apply the assignments of various transitions in bovine heart cytochrome *c* (Flatmark and Robinson, 1968) also to the two *c*-type cytochromes of *R. molischianum*.

On the basis of all these detailed spectral observations one might be tempted to conclude that similar protein environments surround the heme groups of both these bacterial and the mammalian cytochromes *c*. On the other hand, if we consider that the oxidation–reduction potentials of the two cytochromes of *R. molischianum* differ by as much as 93 mV and that both potentials are significantly higher than that of native bovine heart cytochrome *c* (E_{m7} = +0.25 V) then such an interpretation does not seem to be warranted. At present this discrepancy cannot be completely reconciled but we prefer the view that minor differences in heme environments provided by the three different types of polypeptide chains are more sensitively reflected by the oxidation–reduction potentials characteristic for each of these *c*-type cytochromes. Apparently, both the absorption spectra and the circular dichroism spectra are less sensitive indicators in this respect. Conclusive evidence in this matter, however, can be provided only by a comparison of the three-dimensional structures of all of these proteins, based on their high-resolution electron density maps.

In contrast to the results obtained with the two isocytochromes of the yeast *S. cerevisiae* (Slonimski *et al.*, 1965), the two *c*-type cytochromes of *R. molischianum* differ in several of their physicochemical properties. Aside from the differences in their molecular weights, amino acid compositions and ultraviolet spectra, it is mainly the large difference in their oxidation–reduction potentials (about 90 mV) which leads us to expect distinct differences in their biological functions. The nearly identical visible absorption spectra of the two *c*-type cytochromes, however, preclude a kinetic separation of their light-induced reactions. Unfortunately, no other information on their functional and biological significance is at present available except that they are both completely inactive in the mammalian succinate oxidase system. This is of interest because both *c*-type cytochromes of *R. molischianum* differ from previously characterized *c*-type cytochromes (cytochromes c_2) of nonsulfur purple photosynthetic bacteria in that they resemble mammalian cytochrome *c*, both spectrally and with regard to their strongly basic isoelectric points. These results suggest that, with regard to activity in the succinate oxidase system, the close similarity in p*I* values with that of mammalian cytochrome *c* is not sufficient to reconstitute the activity of cytochrome *c* depleted mitochondria. In their comprehensive studies on relative reactivities of *c*-type cytochromes of many different phyla with bovine and *Pseudomonas* cytochrome *c* oxidases, Yamanaka and Okunuki (1968) found that the *c*-type cytochromes of *R. molischianum* (unresolved) slowly reacts with bovine cytochrome *c* oxidase; they reported 2.6% of the reactivity found for cytochrome *c* of the eukaryotic organism *Saccharomyces oviformis*. In the present study neither the small *c*-type cytochrome nor cytochrome c_2, even at very high concentrations (35 μM), revealed any activity in the succinate oxidase system. It is important to recall that this assay system is more appropriate for testing relative biological activities of *c*-type cytochromes than is the cytochrome *c* oxidase assay, since both the donor and the acceptor specificities of each *c*-type cytochrome is studied at the same time. Indeed, the succinate oxidase assay was found to be a very powerful tool in detecting differences in relative biological activities of chemically modified mammalian cytochromes *c* (Flatmark, 1967).

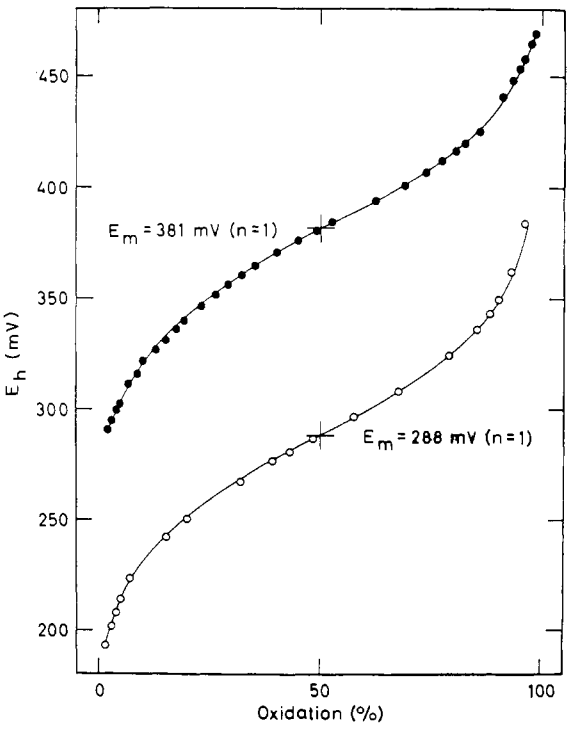

FIGURE 5: Oxidation–reduction titrations of *R. molischianum* *c*-type cytochromes. (●) Small *c*-type cytochrome; (○) cytochrome c_2. The lines represent the theoretical curves for $n = 1$; E_{m7} = +0.381 V for the small *c*-type cytochrome, and E_{m7} = +0.288 V for cytochrome c_2. Oxidative titration was performed with potassium ferricyanide in 0.1 M phosphate buffer (pH 7.0) at 25°; for details, see text.

Acknowledgment

We are indebted to Professor J. S. Singer for the use of his spectropolarimeter. We wish to acknowledge the assistance of Dr. W. A. Cramer in determining the low-temperature spectrum.

References

Cassim, J. Y., and Yang, J. T. (1967), *Biochem. Biophys. Res. Commun. 26*, 58.

Chapell, J. E. (1961), Proceedings of the First IUB–IUBS Symposium Biological Structure Function, Stockholm, 1960, Vol. 2, New York, N. Y., Academic, p 71.

Dus, K., Flatmark, T., deKlerk, H. and Kamen, M. D. (1970), *Biochemistry 9*, 1984.

Estabrook, R. W. (1956), *J. Biol. Chem. 223*, 781.

Estabrook, R. W. (1961), *in* Haematin Enzymes, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., Oxford, Pergamon, p 479.

Flatmark, T. (1964), *Acta Chem. Scand. 18*, 1656.

Flatmark, T. (1967), *J. Biol. Chem. 242*, 2454.

Flatmark, T., and Robinson, A. B. (1968), *in* Structure and

Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo, p 318.
Hersch, P. A. (1960), *Anal. Chem. 32*, 1030.
Hodgman, C. D., Weast, R. C., Shankland, R. S., and Selby, S. M., Ed. (1962), Handbook of Chemistry and Physics, 44 ed., Cleveland, Ohio, The Chemical Rubber Publishing Co., pp 1706, 2142, 3391.
Holzwarth, G., and Doty, P. (1965), *J. Amer. Chem. Soc. 87*, 218.
Jacobs, E. E., and Sanadi, D. R. (1960), *J. Biol. Chem. 235*, 531.
Keilin, D., and Hartree, E. F. (1949), *Nature 164*, 254.
Kielly, W. W., and Bronk, J. R. (1958), *J. Biol. Chem. 230*, 521.
Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.
Moscowitz, A. (1961), *Advan. Chem. Phys. 4*, 67.
Nakano, M., and Danowski, T. S. (1966), *J. Biol. Chem. 241*, 2075.
Paul, K. G. (1947), *Arch. Biochem. 12*, 441.
Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.
Slonimski, P. P., Acher, R., Péré, G., Sels, A., and Somlo, M. (1965), Mechanismes de Régulation des Activités Cellulaires chez les Microorganismes, Marseille, 1963, Paris, Éditions du Centre National de la Recherche Scientifique, p 435.
Yamanaka, T., and Okunuki, K. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo, p 390.
Yonetani, T. (1961), *J. Biol. Chem. 236*, 1680.
Yonetani, T., and Ray, G. S. (1965), *J. Biol. Chem. 240*, 3392.