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CHARACTERIZATION OF CYTOCHROME *c* FROM *NITROBACTER AGILIS**

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SUMMARY

Cytochrome *c* from *Nitrobacter agilis* was isolated and purified approx. 60-fold. Absorption spectra of both the oxidized and the reduced Nitrobacter cytochrome *c* and the oxidized *minus* reduced difference spectrum of this cytochrome were essentially identical to the corresponding spectra of horse-heart cytochrome *c*. The redox potential of this cytochrome was determined by spectrophotometric titration with ferrocyanide/ferricyanide and found to be +0.282 V over the pH range 6.0 to 8.7, while a potential of +0.265 V was determined in the same manner for horse-heart cytochrome *c*. The titration also indicated that the Nitrobacter ferrocytochrome is oxidized by a single electron transfer.

INTRODUCTION

ALEEM AND NASON[1] originally demonstrated that nitrite oxidase particles from the chemoautotroph *Nitrobacter agilis* contain cytochrome *c*- and *a*-like components which apparently participate in the oxidation by O_2 of nitrite to nitrate. LEES[2], KIESOW[3] and VAN GOOL AND LOUDELOUT[4] pointed out on the basis of standard redox potentials (E'_0) reported for the nitrite/nitrate[5] and mammalian ferrocytochrome/ferricytochrome *c* (ref. 6) couples that Nitrobacter must work against a theoretical energy gradient in order to oxidize nitrite to nitrate ($E'_0 = +0.43$ V, pH 7.0) *via* cytochrome *c* assuming the latter has the same potential as mammalian cytochrome *c* ($E'_0 = +0.26$ V, pH 7.0). More recently ALEEM[7] and SEWELL AND ALEEM[8] have claimed on the basis of spectrophotometry and the use of inhibitors and uncouplers that nitrite enters the electron transport chain in Nitrobacter at the level of cytochrome a_1 and not at cytochrome *c*; and that reduction of cytochrome *c* involves an energy-dependent reversal of electron transfer from cytochrome a_1.

In the present paper we report on the isolation and characterization of Nitrobacter cytochrome *c* which appears to be spectrally identical to horse-heart cytochrome *c*. The Nitrobacter cytochrome *c* is shown to have an oxidation–reduction potential of +0.282 V and to be involved in single electron transfers.

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MATERIALS AND METHODS

Cytochrome *c* was isolated from frozen *N. agilis* cells (10 g wet wt.) by the butanol method of TISSIERES[9] and purified some 60-fold to an $A_{550\ m\mu}/A_{280\ m\mu}$ ratio between 0.63 and 0.73 by the amberlite and the $(NH_4)_2SO_4$ fractionation procedures of HORIO AND KAMEN[10]. Spectral studies were performed with the Cary Model-14 recording spectrophotometer. The potential of the purified cytochrome was determined by the method of DAVENPORT AND HILL[11], with the exception that the titrations were performed aerobically. The ratio of reduced to oxidized cytochrome was calculated from the absorbance at 550 mμ during the titration with ferrocyanide/ferricyanide and from the absorbance of the fully reduced sample at 550 mμ using the oxidized and reduced extinction coefficients for cytochrome *c* reported by MASSEY[12]. The potential of the ferrocyanide/ferricyanide system was determined in the buffers used for the titration of cytochrome *c* as recommended by CLARK[13], utilizing a Coleman 37A pH meter equipped with a standard calomel and a platinum electrode. The resulting values were employed in the calculation of the potential (E'_0) of the cytochrome preparations. The pH of the solutions was determined at the end of each titration.

RESULTS AND DISCUSSION

The spectra of both the oxidized and the reduced form of the purified Nitrobacter cytochrome *c* are shown in Fig. 1. The spectrum of the oxidized form was characterized by an absorption peak at 411 mμ while that of the reduced form showed absorption maxima at 550, 521 and 417 mμ characteristic of the α, β and γ-peaks, respectively, of reduced cytochrome *c*. Interestingly enough, the spectra of both the oxidized and reduced forms of Nitrobacter cytochrome *c* were superimposable on the corresponding spectra obtained with similar concentrations of oxidized and reduced horse-heart cytochrome *c* (Sigma, Type III) (not shown). The reduced *minus* oxidized difference spectrum of purified cytochrome *c* from Nitrobacter was also identical to that of horse-heart cytochrome *c* (Fig. 1, insert). There was no spectral indication of the presence of either cytochrome *b* or *a* in the purified preparations. The absorption maxima exhibited by Nitrobacter cytochrome *c* in both the reduced and oxidized forms are very close, if not identical to those reported for cytochrome *c* preparations from several other bacteria[9,14] (Table I). However, they vary slightly from those reported for cytochrome *c* preparations from the photosynthetic bacteria[15] (Table I).

It is worth noting that the trough between 455 and 465 mμ observed in difference spectra of nitrite oxidase preparations after reduction is also present in the purified cytochrome *c* preparations of both Nitrobacter and horse-heart cytochrome *c* (Fig. 1, insert); although in the purified preparations, this minimum is blue shifted to 448 mμ. It therefore seems apparent that extreme caution must be exercised in attributing the absorption minimum in the 450–465 mμ region of difference spectra of cytochrome *c*-containing preparations to the bleaching of a flavin component. For example the absorption minimum at 465 mμ observed by VAN GOOL AND LOUDELOUT[4] in difference spectra of whole cells and extracts of *Nitrobacter winogradskyi* obtained after dithionite reduction may well be due to cytochrome *c* and not to a flavin component suggested by these authors. Similarly the decrease in absorbance at 450 mμ

of crude cell-free preparations of *N. agilis* reported by SEWELL AND ALEEM[8] may be related to the concommitant reduction of cytochrome *c* and not to the flavin component which these authors have implicated.

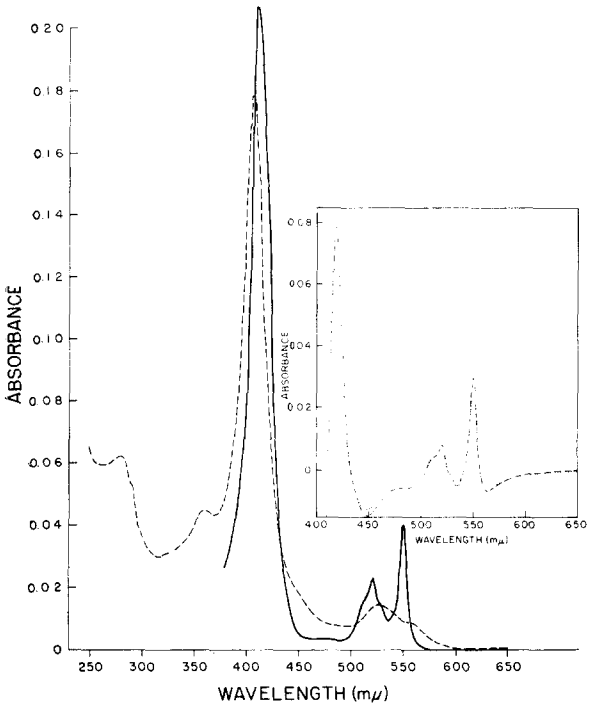

Fig. 1. Absorption spectra of Nitrobacter cytochrome *c*. Absorption spectra were recorded on a Cary Model-14 spectrophotometer using a 0–0.2 *A* slide wire. Cytochrome was dissolved in 1 ml of 0.05 M phosphate buffer (pH 7.0) and scanned in 1-cm light path cuvettes. – – –, oxidized Nitrobacter cytochrome *c*; ———, Nitrobacter cytochrome *c* reduced by several crystals of solid sodium dithionite. Insert: – – –, difference spectra (sodium dithionite-reduced *minus* oxidized) of Nitrobacter cytochrome *c*; ·····, horse-heart cytochrome *c*. The concentration of horse-heart cytochrome *c* is 1.4 μM. The concentration of the Nitrobacter cytochrome *c* was estimated to be 1.4 μM in the oxidized and reduced spectra and 1.7 μM in the difference spectra by using the extinction coefficient for mammalian cytochrome *c* (ref. 12).

Representative results for the titration of Nitrobacter cytochrome *c* and horse-heart cytochrome *c* (in 0.01 M ferrocyanide) with 0.01 M ferricyanide are presented in Fig. 2. Since oxidation of ferrocyanide involves a single electron transfer, the 45° slope of the points from each experiment plotted in this figure with the same units on the ordinate and abscissa indicates a single electron transfer also for the oxidation of the ferrocytochrome. The ratio of ferrocyanide/ferricyanide, corresponding to 50% reduction of the cytochrome, can be determined by extrapolation of the lines in this figure. E_h at this ratio corresponds to E'_0 for the cytochrome at the pH indicated.

The oxidation–reduction potential of Nitrobacter cytochrome *c* was relatively constant at an average value of +0.282 V and a standard deviation of ± 0.0097 V over the pH range investigated (pH 6.0–8.7). Horse-heart cytochrome *c* (Sigma type III) had an average potential, determined in an identical manner, of +0.265 V with a standard deviation of ± 0.0035 V over the pH range 5.95–8.40 (Table II).

TABLE I

COMPARISON BETWEEN THE SPECTRAL PROPERTIES OF *N. agilis* CYTOCHROME *c*, HORSE-HEART CYTOCHROME *c* AND BACTERIAL CYTOCHROMES

Source	*Ref. No.*	*Oxidized peak* λ_{max}	α_{max}	*Relative peak height*	β_{max}	*Relative peak height*	γ_{max}	*Relative peak height*
N. agilis cytochrome *c*	—	411	550	(1.0)	521	(0.575)	417	(5.50)
Horse-heart cytochrome *c*	—	410	550	(1.0)	521	(0.515)	417	(5.10)
Azotobacter vinelandii cytochrome c_4	9	411	551	(1.0)	522	(0.740)	416	(6.60)
Bacillus megaterium cytochrome *c*	14	415	550	(1.0)	520	(0.829)	415	(5.86)
Rhodospirillum rubrum cytochrome c_2	15	410	550	(1.0)	521	(0.605)	415	(5.01)
Rhodopseudomonas palustris cytochrome c_2	15	412	552	(1.0)	522	(0.629)	417.5	(5.10)
Chromatium cytochrome 553	15	410	553	(1.0)	523	(0.684)	416	(8.35)

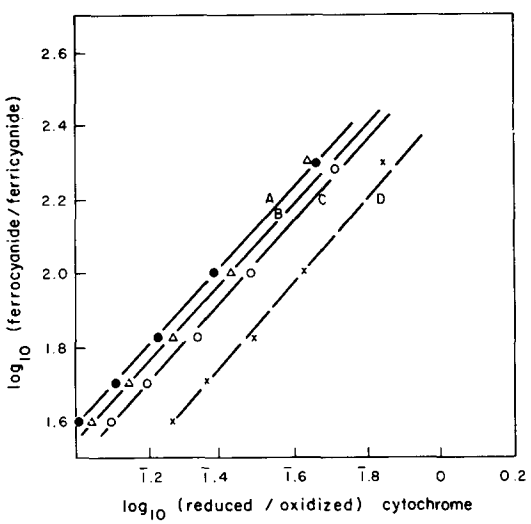

Fig. 2. Oxidation–reduction potentials of Nitrobacter cytochrome *c* and horse-heart cytochrome *c*. A, horse-heart cytochrome *c*, pH 7.0. The remaining points refer to Nitrobacter cytochrome *c*: B, pH 6.5; C, pH 7.4; D, pH 8.5.

At pH 8.90 the potential of horse-heart cytochrome *c* decreased. This decrease and the potential of horse-heart cytochrome *c* are consistent with the results of RODKEY AND BALL[6]. On the basis of the oxidation–reduction potentials of these two cytochromes, it seems possible that they could be interchangeable as electron acceptors in the nitrite oxidase particles.

The difference between the potential of the Nitrobacter ferrocytochrome *c*/ferricytochrome *c* and the nitrite/nitrate couples at pH 7.0 (Δ 0.158 V) represents an

TABLE II

COMPARISON OF THE OXIDATION–REDUCTION POTENTIALS OF NITROBACTER CYTOCHROME *c* AND HORSE-HEART CYTOCHROME *c*

0.05 M phosphate buffer was used over the pH range 6.0–8.0. 0.05 M *N*-tris-(hydroxymethyl)-methylglycine (Tricine, Calbiochem) was used over the pH range 8.4–8.6. The buffer used at pH values above 8.6, was 0.05 M K_2HPO_4 adjusted to the desired pH with 0.05 M NaOH (ref. 6).

Nitrobacter cytochrome c		*Horse-heart cytochrome c*	
pH	E'_o	*pH*	E'_o
6.01	0.291	5.95	0.265
6.05	0.293	6.01	0.265
6.17	0.298		
6.40	0.283	6.50	0.265
6.93	0.275	6.93	0.262
7.10	0.275	6.94	0.264
7.40	0.271	7.40	0.262
7.90	0.281	8.00	0.273
8.50	0.280	8.40	0.265
8.59	0.290		
8.70	0.268	8.90	0.245

energy span of 7.24 kcal calculated from the equation $F' = -nF\Delta E$ when $n = 2$. The recent work of O'KELLEY *et al.*[16] demonstrating the occurrence in nitrite oxidase particles of nitrite–cytochrome *c* reductase activity which proceeds readily in the absence of an added or generated energy source is in conflict with the above thermodynamic consideration. The data presented here demonstrating that the Nitrobacter cytochrome *c* is involved in single electron transfer ($n = 1$) (as are all the other *c* cytochromes which have been studied in this respect) suggest the possibility that there is an intermediate in the biological oxidation of nitrite to nitrate. This intermediate product would have a nitrogen atom with an oxidation number of $+4$ by contrast to that of $+5$ and $+3$ for nitrate and nitrite, respectively, and may eventually provide an explanation to reconcile the above apparent contradiction.

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