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THE MEMBRANE-BOUND ASCORBATE OXIDASE SYSTEM OF *HALOBACTERIUM HALOBIUM*

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SUMMARY

1. The membrane-bound respiratory components associated with the ascorbate and ascorbate–tetramethyl-*p*-phenylenediamine (TMPD) oxidase systems were investigated by spectrophotometric and polarographic techniques using membranes isolated from whole cells of *Halobacterium halobium*.

2. Membranes were prepared by osmotic lysis with 20 mM $MgCl_2$–50 mM Tris·HCl buffer (pH 8.2) at 0° followed by centrifugation at 30000 × *g*. Electron microscopy on the negatively-stained samples revealed membranes only in the 30000 × *g* residue.

3. The membranes of *H. halobium* contained a predominant complex of *b*-type cytochromes, a low level of a *c*-type cytochrome and two CO-reactive hemoproteins identified as cytochromes *o* and a_1. Two *b*-type cytochromes were tentatively identified as cytochrome b_{561} and cytochrome b_{564}, the former was reduced by either ascorbate or ascorbate–TMPD.

4. Difference spectra (22° and −196°) showed that the membrane-bound *a*- and *b*-type cytochromes were reduced readily by ascorbate or ascorbate–TMPD.

5. Polarographic studies showed that the ascorbate-induced respiration of the membrane-bound cytochromes fraction was inhibited by antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and CO. The antimycin A-insensitive ascorbate–TMPD oxidase activity was inhibited by CN^- and CO.

6. Studies with three strains of halobacteria, *Halobacterium halobium*, *H. salinarium* and *H. cutirubrum* reveal that the ascorbate–TMPD oxidase activity of these halobacteria was about 4 times greater when assayed in the reaction medium containing 3.4 M NaCl than in 70 mM $MgCl_2$. Greater inhibition of the ascorbate–TMPD oxidase activity by CN^- was also observed in the monovalent cation reaction medium.

INTRODUCTION

The structure and chemical composition of the cell envelopes of the Gram-negative extreme halobacteria that grow optimally in a 4–5 M NaCl -containing medium have been studied by various investigators[1]. The evidence suggests that halo-

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

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bacterial membranes are acidic[1,2], deficient in fatty acids[1] and carbohydrate[1]. The main lipid component of one of the halobacteria, *Halobacterium cutirubrum*, was found to be a diether analogue of phosphatidylglycerophosphate[3]. Another unusual property of halobacterial membranes is the phenomenon of electrostatic dissolution of their membranes in solutions of low salt concentrations which could be prevented only by either high concentrations of monovalent cations or by relatively low concentrations of divalent cations[4].

The oxidase systems of the extreme halophiles have only recently been investigated. The respiratory chain system of isolated electron transport particles from *H. cutirubrum*, harvested at early logarithmic growth phase, has cytochromes *o* and a_3 which are reduced readily by ascorbate[5]. This paper describes mainly the membrane-bound ascorbate and ascorbate–tetramethyl-*p*-phenylenediamine (TMPD) oxdiase systems of *H. halobium* and compares its respiratory components with those found in two other extreme halophiles, *H. salinarium* and *H. cutirubrum*.

MATERIALS AND METHODS

Reagents

Antimycin A (Type III), L-ascorbate, crude beef pancreatic deoxyribonuclease (EC 3.1.4.5) and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) were commercially obtained from Sigma Chemical Co., TMPD from the British Drug Houses, Tris from Fisher Scientific Co., and CO from Matheson Corp. of Canada. All other reagents were of analytical grade.

Growth of bacteria and isolation of membrane fraction

H. halobium was grown aerobically in a medium (*cf.* ref. 6) containing 10 g yeast extract (Difco), 7.5 g casamino acids (Difco), 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$, 250 g NaCl, 3 g sodium citrate (dihydrate) and 50 mg $FeSO_4 \cdot 7H_2O$ per l, adjusted to pH 7.8 before sterilization. An erlenmeyer flask (500 ml capacity) containing 150 ml culture medium (pH 7.4) was inoculated with the bacteria, maintained in the same medium solidified with 2 % agar (Difco), and grown at 37° on a rotary shaker (setting at 200 rev./min) for 48 h. Growth was measured by absorbance (*A*) increase (turbidity) with a Coleman Junior spectrophotometer. The freshly grown 48-h cells (150 ml, $A_{660\ nm}$ = 0.04–0.05) were then used to inoculate 2.5 l culture medium (pH 7.4) in a 4-l erlenmeyer flask. The cells were then allowed to grow in the same conditions as described before harvesting at either the mid-logarithmic (70 h) or late-stationary (112 h) growth phase. Foaming was prevented by using Dow Corning antifoam A spray. The cells were harvested by centrifugation (8000 × *g* for 10 min at 0°), washed 3 times with a solution containing 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$ and 2 g KCl per l (*cf.* ref. 7) and then resuspended in 3.4 M NaCl–0.1 M Tris·HCl buffer (pH 7.6) or used for preparing membranes.

The cell membrane preparation was based on the method of BROWN *et al.*[8] using 20 mM $MgCl_2$–50 mM Tris·HCl buffer (pH 8.2) at 0° instead of 20 mM $MgCl_2$ at 4°. The DNA released upon osmotic lysis of intact cells was hydrolized by deoxyribonuclease and the cell membranes were separated by centrifugation (30000 × *g* for 1 h at 0°) of the whole lysate, yielding a red membrane pellet and a light orange supernatant.

Physiochemical measurements

The electron transport components in the membranes were determined without prior extraction of the carotenoid pigments which were reported to be present in this bacterium[9]. All room (22°) and liquid-nitrogen (−196°) temperature difference spectra and the kinetics of cytochrome reduction were recorded with a Phoenix Precision Instrument (PPI) dual/split-beam spectrophotometer. The 22° difference spectra were measured in 10-mm light-path cuvettes; difference spectra at −196° were carried out in 2-mm cells containing reaction medium supplemented to 50% (v/v) in glycerol[10]. Other experimental details are given in the legends to figures. The approximate concentrations of the membrane-bound cytochromes were calculated from difference spectra obtained at 22°. The following wavelength pairs and difference millimolar extinction coefficients were employed: 17.5 (*cf. Escherichia coli* cytochrome b_1 (ref. 11) for the ascorbate or ascorbate–TMPD-reduced cytochrome b_{561} ($A_{561\,nm} - A_{575\,nm}$); 80.0 (*cf.* the Soret cytochrome *o*–CO complex of *Staphyllococcus albus*[12]) for the (reduced + CO *minus* reduced) cytochrome *o*–CO complex ($A_{419\,nm} - A_{434\,nm}$); 24.6 (*cf. E. coli* cytochrome b_{562} (ref. 13)) for the cytochrome b_{564} ($A_{564\,nm} - A_{575\,nm}$) observed in the dithionite *minus* substrate (ascorbate or ascorbate–TMPD) difference spectra and 60.0 (*cf.* the Soret cytochrome a_1–CO complex of *Acetobacter pasteurianum*[14]) for the (ascorbate + CO *minus* ascorbate) cytochrome a_1–CO complex ($A_{427nm} - A_{445\,nm}$).

The reduced pyridine hemochromogens of the membrane-bound cytochromes were formed by treating an equal volume of the membrane suspension with an equal volume of 4.4 M pyridine in 0.2 M NaOH before reduction with 1 mg dithionite. Concentrations of heme *a* ($A_{587\,nm}$—$A_{605\,nm}$) and protoheme ($A_{556\,nm}$—$A_{575\,nm}$) were estimated from the reduced pyridine hemochromogen difference spectra using the millimolar extinction coefficient of 24.0 for heme *a* and 30.0 for protoheme[15]. Heme *c* ($A_{551\,nm} - A_{540\,nm}$), left after extraction of the predominant protoheme and heme *a* in the membrane suspension with acid–acetone and treatment of the acid–acetone residue with an equal volume of an alkaline-pyridine solution, was calculated using the millimolar extinction coefficient of 22.3 (ref. 16).

Protein was determined by the method of LOWRY *et al.*[17] using bovine serum albumin as standard.

RESULTS

Electron micrograph of the negatively-stained samples from H. halobium

Fig. 1 illustrates the membranes in the negatively-stained sample of the membrane fraction used in the investigation of the membrane-bound respiratory components of *H. halobium*. No membranous structures were observed in the supernatant fraction (micrograph not shown.)

Distribution of cytochromes in H. halobium

Fig. 2 illustrates the dithionite-reduced *minus* oxidized difference spectra of the various fractions prepared from whole cells of *H. halobium*. The whole lysate contained cytochrome a_1 (594 nm) (ref. 18) and *b*-type (563 nm) pigment(s), both of which were also present in the membrane fraction. The difference spectrum of the membrane fraction recorded at −196° (–·–) shows the absence of a *c*-type component and the

α-peak of the *b*-type cytochrome appeared at 559 nm instead of 563 nm (22°). No hemoprotein absorption in the α-region was observed in the supernatant fraction but a γ-peak around 428–430 nm (not illustrated) was seen occasionally. This accounted

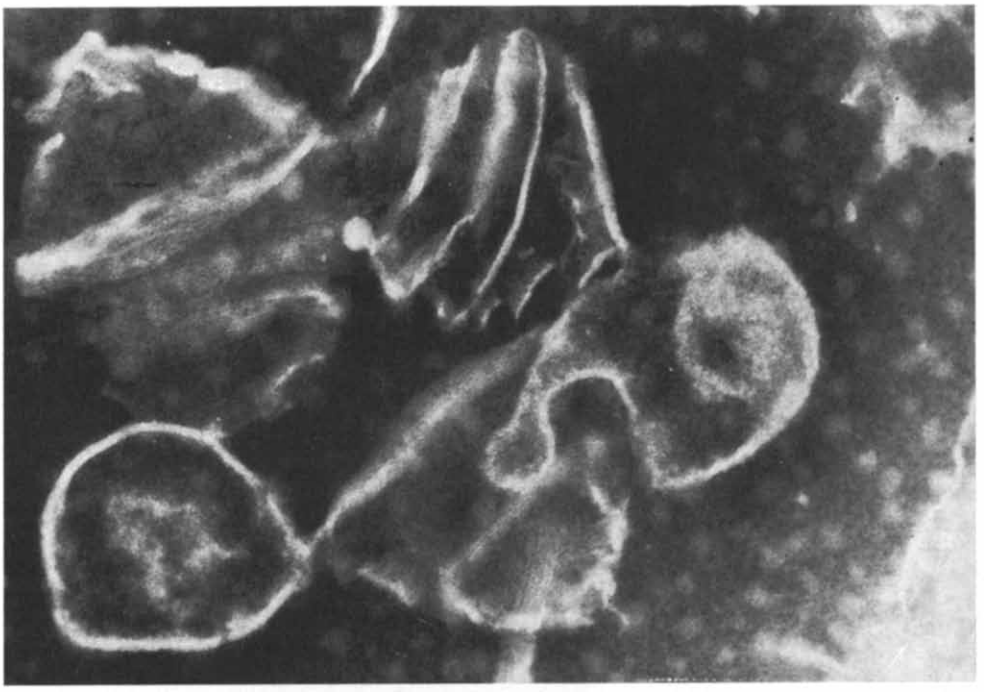


Fig. 1. Electron micrograph of the negatively-stained sample of the membrane fraction from *H. halobium*. Samples previously suspended in 70 mM $MgCl_2$–100 mM Tris·HCl buffer (pH 7.6) were stained for 2 min with 2% phosphotungstic acid (pH 6.8) using Formvar carbon-coated grids before examination with a Philips (Model EM 300) electron microscope as previously described for *H. cutirubrum*[5]. Magnification, 100000 ×.

for about 1–2% of the reduced Soret peak of the *b*-type cytochrome(s) in the membrane fraction. The cytochromes in intact *H. halobium*, which were bound apparently to the membranes, could thus be concentrated in the membrane fraction without any apparent loss, assuming that the components in the whole lysate represent those in the intact cells.

Spectrophotometric studies of the membrane-bound cytochromes in the membrane fraction

Both ascorbate (Fig. 3, ———) and ascorbate–TMPD (Fig. 3, –·—·–) caused the reduction of cytochrome a_1 (peak at about 595 nm, shoulder at about 442 nm) and a *b*-type pigment with maxima at 561 (α), 530 (β) and 430 (γ) nm. The difference spectra obtained with dithionite (Fig. 2, ———), ascorbate (Fig. 3, ———) and ascorbate–TMPD (Fig. 3, –·—·–) suggest the presence of another membrane-bound *b*-type component different from the *b*-type pigment associated with ascorbate or ascorbate–TMPD oxidation. This was clearly illustrated in the difference spectrum obtained with the dithionite–reduced membranes (sample cuvette) recorded against the ascorbate- or ascorbate–TMPD-treated membranes (reference cell). Such a difference spectrum (– – –) has maxima at 564 (α), 535 (β) and 434 (γ) nm. The difference spectrum

(ascorbate–TMPD-reduced *minus* oxidized) of the membranes recorded at −196° (not illustrated) shows only an α-peak at 559 nm, a shift of 2 nm from the 22° difference spectrum (–·—·–), but with no apparent change in the absorption maxima in the Soret.

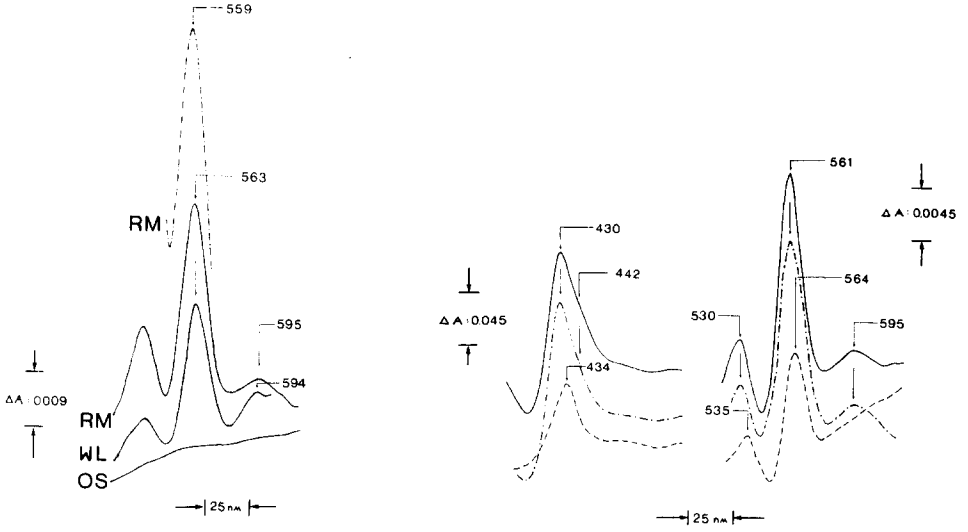


Fig. 2. Difference spectra (dithionite-reduced *minus* oxidized) showing the distribution of cytochromes in *H. halobium*. The 22° difference spectra (———) of the supernatant (OS), whole lysate (WL) and red-membrane (RM) fractions were recorded in 10-mm light-path cuvettes and the −196° difference spectrum of the red-membrane fraction (–·—·–) in 2.0-mm light-path cells. Other experimental details are given in the text. Protein concn. (mg/ml): supernatant fraction, 1.42; whole lysate fraction, 6.88; red-membrane fraction, 1.78 at 22° and 10·24 at −196°.

Fig. 3. Difference spectra of the membrane fraction of *H. halobium*. Both the sample and reference cells (10 mm light-path) contained 2.5 ml membrane suspension (1.78 mg protein/ml) for the difference spectra recorded at 22°. ----, dithionite-reduced *minus* ascorbate-reduced; ———, ascorbate-reduced *minus* oxidized; –·—·–, ascorbate–TMPD-reduced *minus* oxidized. The reference systems were oxidized by air (O_2) bubbling. Final concn.: ascorbate, 2.0 mM; TMPD, 0.16 mM; dithionite, 1.0 mg.

Fig. 4 illustrates that cytochrome a_1 in the membrane fraction of *H. halobium* appeared to be reduced by ascorbate before the *b*-type cytochrome(s). The spectrum recorded at 1.0 min after ascorbate addition to the sample cuvette (A) shows the γ-peak (443 nm) of cytochrome a_1, and that recorded at 2.0 min (B) has both the γ-peaks of cytochrome a_1 (442 nm) and the *b*-type (432 nm) cytochrome(s). On reaching anaerobiosis, the cytochrome a_1 peak appeared as a shoulder at about 442 nm and the *b*-type Soret maximum shifted from 432 to 431 nm (D). The γ-peak of cytochrome a_1 is about 2 nm higher than those reported for other bacteria (refs. 14 and 18) and is not contributed by cytochrome a_3, since no α-peak at about 605 nm was observed in either the ascorbate or ascorbate–TMPD-reduced *minus* oxidized difference spectrum (Fig. 3). The rate of reduction of both types of cytochromes was greatly increased when TMPD was added with ascorbate. Under such conditions it was not possible to observe the individual maximum contributed by the reduced forms of cytochrome a_1 (A) and of cytochrome a_1 and the *b*-type cytochrome (B). In fact, the ascorbate–TMPD-reduced

minus oxidized difference spectrum recorded at 2.0 min resembled that of the ascorbate-reduced *minus* oxidized spectrum observed during anaerobiosis (D).

CN^--reactive pigments in the whole lysate and membrane fractions of H. halobium

The addition of 3 mM CN^- to the whole lysate caused the appearance of peaks at 592 and 440 nm (Fig. 5, –·—·–) which probably represent the CN^- complex of oxidized cytochrome a_1. The 562 and 430 nm peaks could either be contributed by the CN^- complex of a *b*-type cytochrome or reduced cytochrome *b*. Similar addition of CN^- to the membrane fraction (Fig. 5, – – –) only revealed identical absorption peaks of cytochrome a_1. This interesting result suggests that either cytochrome *b* reduction (without CN^- combination) had occurred in the whole lysate but not the membrane fraction or that an essential component involved with CN^- binding of the *b*-type pigment was probably removed from the whole lysate during the preparation of the membranes of *H. halobium*. The ascorbate–TMPD-reduced *minus* CN^--treated difference spectrum (———) shows a marked trough at about 592 nm, a wavelength corresponding to the peak of the CN^- complex of cytochrome a_1 (–·—·–; – – –) and a partial collapse of the Soret maximum (shoulder at about 442 nm) of cytochrome a_1 as compared with that obtained in the ascorbate-reduced *minus* oxidized spectrum (Fig. 3, ———). The Soret peak of the reduced *b*-type pigment was also shifted slightly to a lower wavelength, from 430 (Fig. 3, ———) to 429 nm (Fig. 5, ———). The results tend to suggest that the CN^- complex of oxidized cytochrome a_1 (as present in

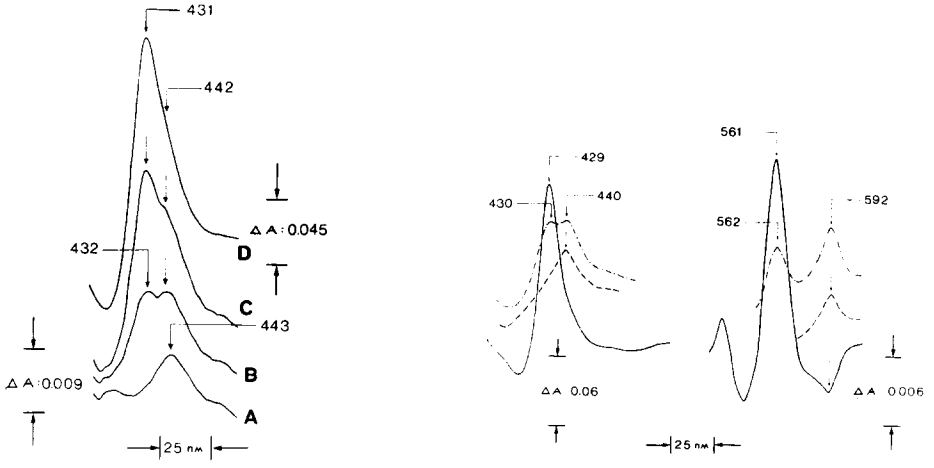


Fig. 4. Time-dependent changes of the ascorbate-reduced *minus* oxidized difference spectrum of the membrane fraction from *H. halobium* (22°). The sample and reference cells (10 mm light-path) contained 2.5 ml membrane suspension (3.3 mg protein/ml) in 3.4 M NaCl–0.1 M Tris·HCl buffer (pH 7.6). 2 mM ascorbate (final concn.) was added to the sample cuvette and the difference spectra recorded at 1 (A), 2 (B), 3 (C) and 22 (D) min after ascorbate addition. The scale of the absorbance changes of Spectra A–C is 5 times less than that of D.

Fig. 5. Effect of CN^- on oxidized whole lysate and membrane fractions of *H. halobium* (22°). –·–, whole lysate + CN^- *minus* oxidized; – – –, membranes + CN^- *minus* oxidized; ———, ascorbate + TMPD *minus* CN^-. The membranes + CN^- *minus* oxidized difference spectrum was carried out with the membranes suspended in 70 mM $MgCl_2$–0.1 M Tris·HCl buffer (pH 7.6) while that reduced with ascorbate–TMPD was in 3.4 M NaCl–0.1 M Tris·HCl buffer (pH 7.6). Protein concn. (mg/ml): whole lysate (–·—·–), 6.88; membranes (– – –), 2.27; membranes (———), 1.78. Final concn.: ascorbate, 2.0 mM; TMPD, 0.16 mM; CN^-, 3.0 mM.

the reference cuvette for Fig. 5, ———), has more absorption at 440 and 592 nm (see Fig. 5, –·—·–) than does the oxidized form of cytochrome a_1 (as present in the reference cuvette for Fig. 3, — — —).

CO-reactive pigments in the membrane fraction of H. halobium

The CO difference spectrum (dithionite + CO *minus* dithionite) of the whole lysate (not shown) showed maxima at 591, 578, 543 and 422 nm and minima at 560 and 443 nm suggesting the presence of cytochromes *o* and a_1. The trough at about 443 nm, a wavelength of about 8–10 nm higher than that normally attributed to an *o*-type pigment alone[5] represents the spectra of the overlapping troughs of the reduced forms of cytochrome a_1 and cytochrome *o* in the reference cuvette. A similar CO difference spectrum was also obtained with the membrane fraction reduced with dithionite. Both of these CO-reactive pigments could be reduced by either ascorbate (not shown) or ascorbate–TMPD as illustrated in Fig. 6.

Previous communication[19] showed that with the halobacteria, CN^- could eliminate the interference caused by the CO complexes of the *a*-type cytochromes in the Soret region, thus revealing the Soret peak of the cytochrome *o*–CO complex. As illustrated in Fig. 7, the addition of CN^- to the membrane suspension pretreated with ascorbate–TMPD *plus* CO resulted in the appearance of the γ-peak (419 nm) of the cytochrome *o*–CO complex. Similar results were obtained when ascorbate was used instead of ascorbate–TMPD. Thus, *H. halobium* cytochrome *o*, unlike the cytochrome *o*

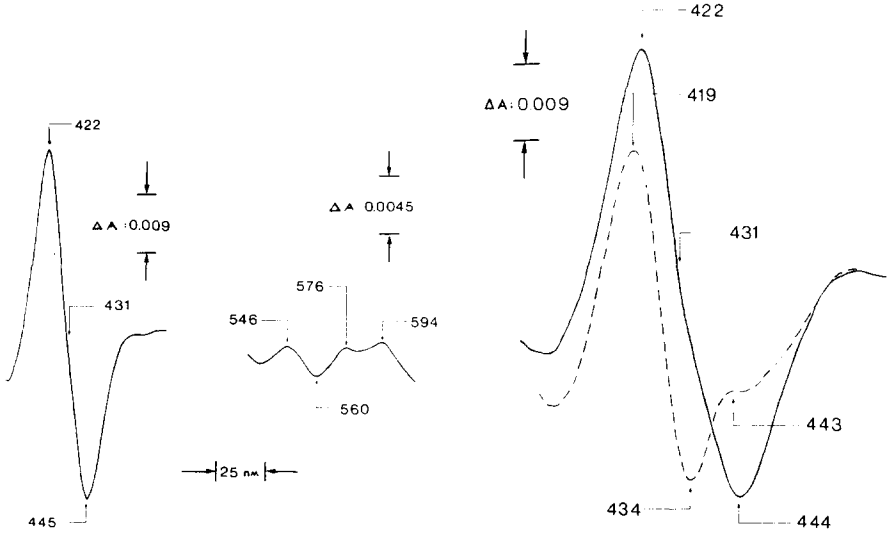


Fig. 6. CO difference spectrum (ascorbate–TMPD + CO *minus* ascorbate–TMPD) of the membrane fraction of *H. halobium* at 22°. The membranes, suspended in 3.4 M NaCl–0.1 M Tris·HCl buffer (pH 7.6), in both the sample and reference cells (10-mm light-path) were reduced with ascorbate–TMPD before CO was bubbled through the sample cuvette for 2.0 min. Final concn.: ascorbate, 2.0 mM; TMPD, 0.16 mM. Protein concn.: 1.78 mg/ml.

Fig. 7. Identification of cytochrome *o*–CO complex in the membrane fraction of *H. halobium* (22°). Experimental conditions were similar to those described in Fig. 6 except that 2 mM CN^- (final concn.) was added to both the sample and reference cuvette after CO treatment. Protein concn.: 2.10 mg/ml.

of the free-living cultured cells of *Rhizobium japonicum* [16] retains its CO complex in the presence of CN^- while cytochrome a_1 cannot.

Formation of pyridine hemochromogens of the membrane fraction of H. halobium

The reduced pyridine hemochromogens formed directly from the membrane fraction (Fig. 8, ———) of the mid-logarithmic growth cells show the presence of heme *a* (around 587 nm) and protoheme (556 nm). No heme *c* was observed, indicating the possible absence of a *c*-type pigment in the membrane fraction. The acid-acetone residue (— — —), however, contained heme *c* (551 nm), which was not observed previously in the reduced pyridine hemochromogen spectrum (———) formed directly from the membrane fraction. Thus, the minor peak of reduced heme *c* was being obscured by the strong superimposed spectrum of the reduced protoheme hemochromogen. With the membrane fraction obtained from cells at the late stationary growth phase, only the α-peak of protoheme was observed. The absence of heme *a* could probably be explained by the low level of cytochrome a_1 at this growth phase. This was supported by the lack of a CN^- complex peak at 592 nm observed with the membrane fraction prepared from cells at the logarithmic growth phase.

Table I illustrates the concentration of the various respiratory components in the membrane fractions prepared from *H. halobium* and *H. salinarium*. With *H. halobium*,

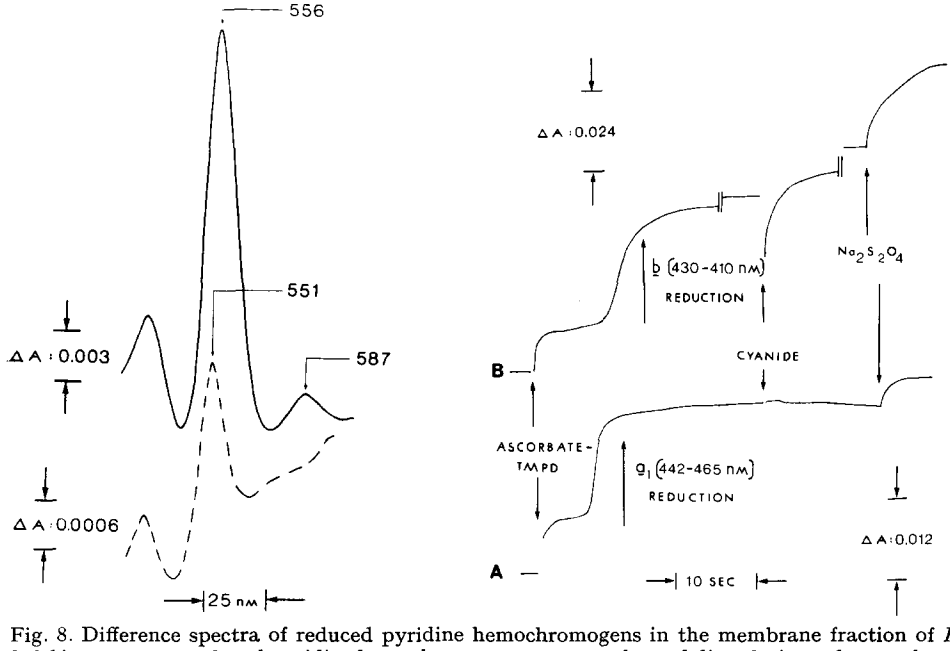


Fig. 8. Difference spectra of reduced pyridine hemochromogens in the membrane fraction of *H. halobium*. ———, reduced pyridine hemochromogen spectrum formed directly from the membrane fraction; — — —, reduced pyridine hemochromogen spectrum of the acid–acetone residue of the membrane fraction. Other experimental details are described in the text. Protein concn. (mg/ml): membranes, 1.22 (———); acid–acetone residue of the membrane fraction, 6.44 (— — —).

Fig. 9. Kinetic reduction of cytochrome a_1 and the *b*-type cytochrome(s) in the membrane fraction of *H. halobium*. The rate of reduction of cytochrome a_1 and the *b*-type cytochrome(s) was estimated using ascorbate–TMPD at 22° in 10-mm light-path cells containing 2.5 ml membranes suspended in 3.4 M NaCl–0.1 M Tris·HCl buffer (pH 7.6). Final concn.: ascorbate, 2.0 mM; TMPD, 0.16 mM; CN^-, 1.0 mM; dithionite, 1.0 mg. Protein concn.: 0.92 mg/ml.

the concentration of protoheme was about 12 times greater than heme *a* and 45 times greater than heme *c* on a protein basis. Thus, the membrane fraction of *H. halobium* has a predominant amount of *b*-type cytochromes. Similarly, there was more protoheme than heme *a* in *H. salinarium*.

TABLE I

CONCENTRATION OF THE MEMBRANE-BOUND RESPIRATORY COMPONENTS IN THE MEMBRANE FRACTIONS OF *H. halobium* AND *H. salinarium*

The concentrations of cytochromes were calculated from 22° difference spectra as described in the text. +, present but not estimated; -, not detected; ?, not observed in the reduced pyridine hemochromogen formed directly from the membrane fraction.

Respiratory components	*Concn. (nmoles/mg protein)*		
	H. halobium		*H. salinarium*
	Mid-logarithmic	*Late-stationary*	*Mid-logarithmic*
Cytochrome a_1	0.21	+	-
Cytochrome b_{561}	0.54***	0.24***	0.45***
Cytochrome b_{561}	0.17	0.08	0.09
Cytochrome *o*	0.27* (0.23§)	0.10*	0.21*
	0.35**	0.17**	
Cytochrome a_3	-	-	0.09
Heme *a*	0.05	-	0.07
Protoheme	0.66	0.38	0.71
Heme *c*	0.014	?	?

*Estimated from (ascorbate–TMPD + CO *minus* ascorbate–TMPD) difference spectra.
**Estimated from (dithionite + CO *minus* dithionite) difference spectra.
***Concentration of cytochrome b_{561} does not exclude that of cytochrome *o*.
§Estimated from CO difference spectrum after elimination of the CO complex of cytochrome a_1 with CN^-.

Kinetic reduction of cytochrome a_1 and the b-type cytochrome(s) in the membrane fraction of H. halobium.

As illustrated in Fig. 9A, ascorbate–TMPD caused the reduction of cytochrome a_1, the aerobic steady state was reached at about 2 sec following ascorbate–TMPD addition, with anaerobiosis occurring at about 10 sec. 1 mM CN^- appeared not to have any effect on the reduced cytochrome a_1 following anaerobiosis, but 1 mg dithionite increased its reduction. Assuming that a 100 % reduction was obtained with dithionite, cytochrome a_1 was reduced by about 30 % in the aerobic steady state by ascorbate–TMPD and 87 % on reaching anaerobiosis.

With the *b*-type cytochrome(s) (Fig. 9B), the aerobic steady state was attained at about 2 sec, the same as that observed with cytochrome a_1, following ascorbate–TMPD addition and anaerobiosis at about 19 sec. The subsequent addition of 1 mM CN^- caused a partial re-oxidation followed by an increase in reduction of the *b*-type component. Dithionite further enhanced the reduction, with complete reduction of the *b*-type cytochrome(s) occurring at about 9 sec after dithionite addition. The *b*-type pigment(s) was thus reduced by about 16 % in the aerobic steady state, 56 % during anaerobiosis and 72 % by CN^- addition on reaching anaerobiosis, dithionite reduction being taken as 100 %.

TABLE II

RESPIRATION OF INTACT *H. halobium* AND THE COMPARATIVE STUDIES OF THE ASCORBATE–TMPD OXIDASE ACTIVITY OF HALOBACTERIAL FRACTIONS

All respiratory activities, expressed as nmoles O_2 per min/mg protein, were measured polarographically with a Clark oxygen electrode in a 3.0-ml reaction vessel at 25°. Ascorbate and TMPD were added to the vessel in 0.01-ml volumes. The sequence of addition in the assay of ascorbate–TMPD oxidase activity was 0.16 mM TMPD, 1.0 μg antimycin A per mg protein, 2.0 mM ascorbate and 0.5 mM CN^-, or ascorbate, antimycin A, TMPD and CN^-. The effect of CO on the respiratory activity was determined using a CO-saturated reaction medium.

Conditions	*Reaction medium (–0.1 M Tris·HCl buffer (pH 7.6))*	*H. halobium*		*H. salinarium (red-membrane fractions)*	*H. cutirubrum (electron transport particles[5])*
		Red-membrane fractions	*Whole cells*		
No substrate	3.4 M NaCl		125.0		
Ascorbate	3.4 M NaCl	31.1			
	3.4 M NaCl (CO-satd.)	20.0			
Ascorbate + antimycin A (3 μg/mg protein)	3.4 M NaCl	25.2			
Ascorbate + HQNO (10 μg/mg protein)	3.4 M NaCl	23.3			
Ascorbate–TMPD	3.4 M NaCl	160.0		177.6	154.0
	0.07 M $MgCl_2$	45.0		47.0	32.0
	3.4 M NaCl (CO-satd.)	128.0			
Ascorbate–TMPD + CN^-	3.4 M NaCl	13.0			
	0.07 M $MgCl_2$	13.4			

Respiratory activities of halobacteria

Table II shows that the ascorbate-induced respiration of the membrane fraction from *H. halobium* was inhibited by about 19 % with 3 μg antimycin A per mg protein, 25 % with 10 μg HQNO per mg protein and 35 % with CO. In the control experiment, similar amount of 95 % ethanol without antimycin A or HQNO had no inhibitory effect on ascorbate oxidation.

The ascorbate–TMPD oxidase activity of the membrane fraction from *H. halobium* was investigated since ascorbate–TMPD like ascorbate was found to donate electrons to cytochrome a_1 and cytochrome *o*, the two likely terminal oxidases of this bacterium. Ascorbate–TMPD could also reduce cytochromes *o* and a_3 in the membrane fraction of *H. salinarium*, the membrane fraction of the colourless mutant *H. salinarium* (Strain 1 M 2)[20], and the electron transport particles of *H. cutirubrum*[5]. The ascorbate–TMPD oxidase activity of the membrane fraction from *H. halobium* was 2.5 times greater in 3.4 M NaCl–0.1 M Tris·HCl buffer (pH 7.6) than in 0.07 M $MgCl_2$–0.1 M Tris·HCl buffer (pH 7.6). Higher ascorbate–TMPD oxidase activity was also found in the NaCl-containing medium with two other extreme halophiles, *H. salinarium* and *H. cutirubrum*. Table II also shows that the ascorbate–TMPD oxidase activity of *H. halobium*, which was slightly higher than the respiration of whole cells without substrate in the NaCl reaction medium, was inhibited by CO and CN^-. CO inhibited 20 % of the ascorbate–TMPD oxidase activity in the NaCl-containing reaction medium while 0.5 mM CN^- blocked 92 % in the NaCl medium but only inhibited 70 % in the $MgCl_2$ reaction medium.

DISCUSSION

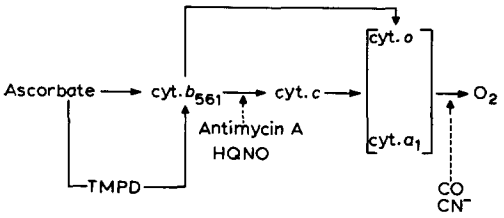
The evidence presented shows that the cytochromes were bound to the membranes of *H. halobium*. Evidence suggests that there are at least three *b*-type cytochromes; a CO-reactive pigment (identified as cytochrome *o*), cytochrome b_{561} (reducible by either ascorbate or ascorbate–TMPD) and cytochrome b_{564} (non-reducible by ascorbate or ascorbate–TMPD). Cytochrome b_{561} appears not to be similar to the cytochrome b_1 reported for other bacteria[21] since the latter was not known to be reduced by either ascorbate or ascorbate–TMPD.

The ascorbate-induced respiration observed with the membrane fraction obtained at the mid-logarithmic growth phase was partially inhibited by antimycin A, HQNO and CO. This was not surprising as ascorbate was demonstrated firstly to donate electrons to the membrane-bound respiratory components at the *b*-type level in the respiratory chain system, which also has a low level of a *c*-type pigment, and secondly to cytochromes *o* and a_1. The ascorbate–TMPD oxidase activity which was insensitive to antimycin A, but sensitive to CO and CN^-, was about 2.5 times greater with membranes in the medium containing 3.4 M NaCl than the respiration observed with membranes in 70 mM $MgCl_2$. Higher ascorbate–TMPD oxidase activity was also found in the monovalent cation medium for *H. salinarium* and *H. cutirubrum*.

Scheme I illustrates the proposed basic ascorbate and ascorbate–TMPD oxidase systems in the membranes of *H. halobium* at the mid-logarithmic growth phase.

The cytochrome b_{561} excludes other *b*-type components which might be associated with the oxidation of other substrates and which are not CO-reactive. The proposed

basic electron transport scheme of *H. halobium* resembles that suggested for the electron transport particles prepared from the early logarithmic growth phase of *H. cutirubrum*[5].



Scheme I.

The major difference between these two halobacterial strains is that *H. cutirubrum*, like *H. salinarium*, has cytochromes *o* and a_3 instead of cytochromes *o* and a_1 as the likely terminal oxidases[19]. Furthermore, the membrane-bound branching respiratory chain system was also demonstrated in the membranes isolated from the colorless mutant, *H. salinarium* (Strain 1 M 2), which has substrate- and dithionite-reducible cytochromes *o* and a_3 and a complex of *b*-type cytochromes[20]. The electron transport systems suggested for the extreme halophiles, harvested from cells at the early and mid-logarithmic growth phases differ from that proposed by LANYI[22,23]. The latter author failed to demonstrate the existence of either substrate or dithionite-reducible cytochrome *o* and/or cytochrome a_3 in *H. cutirubrum*.

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