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Purification and characterization of two soluble cytochromes from the alkalophile *Bacillus firmus* RAB

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A soluble cytochrome *c* and soluble cytochrome *b* were purified from the alkalophilic *Bacillus firmus* RAB. The cytochrome *c*, with an alpha band at 552 nm, had an apparent molecular weight of 16500 and was acidic, with a p*I* of 3.4. At both pH 7.0 and 8.3, the midpoint potential of *c*-552 was +66 mV. Above pH 8.3, the cytochrome exhibited a pH-dependent decrease in midpoint potential. This property, among others, distinguished the cytochrome *c*-552 from other membrane-associated *c*-type cytochromes. The soluble cytochrome *b*, with an alpha band maximum at 558 nm, had a molecular weight of approx. 15500 and was also an acidic protein, with a p*I* of 3.07. It exhibited a pH-independent midpoint potential of +28 mV.

Introduction

Bacilli that grow at extremely alkaline pH face an array of bioenergetic problems that arise from the need to maintain a relatively acidified cytoplasm [1]. Both the Na^+/H^+ antiporter that catalyzes the inward translocation of protons that allows pH homeostasis [2,3] and the F_1F_0-ATPase that accounts for ATP synthesis in these organisms [4,5] must function in a proton poor environment and under conditions in which the electrochemical gradient of protons is quite low [1]. Further challenges to bacilli that grow at pH 10–11 center around the maintenance of a functional membrane under conditions in which the external side of the membrane is in a milieu at pH 10–11, while the cytoplasmic side is at pH 8.5. Issues of functionality also obtain for structures such as flagella and peripheral membrane proteins that are in contact with the highly alkaline medium. The membrane lipids of several alkalophilic *Bacillus* strains have been characterized and found to be quite acidic [6]. The flagellin from alkalophilic *Bacillus firmus* RAB, similarly, was found to have fewer basic amino acids than normally found in bacterial flagellins [7].

Both the bioenergetic problems and the problems of biological function at high pH have focused our interest on components of the respiratory chain. All the alkalophilic bacilli thus far examined possess remarkably high concentrations of membrane-associated respiratory chain components [8,9]. Potentiometric characterizations of the redox species in two obligate alkalophiles suggested complexity, especially among the *b*-type cytochromes [10,11]. Most interestingly, proton

Abbreviations: TEMED, *N,N,N′,N′*-tetramethylethylenediamine; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid.

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pumping by *B. firmus* RAB occurred with apparently high H^+/O stoichiometry at alkaline pH, but with a lower stoichiometry at neutral pH values [12]. Because of our interest in the bioenergetics and membrane biology of the alkalophiles, we have undertaken a systematic characterization of the respiratory chain complexes and components of *B. firmus* RAB. The properties of the cytochrome oxidase from this organism have already been described [13]. In this report, we focus on soluble cytochromes that are released upon rupture of the cells by passage through a French press. The properties of a *c*-type and *b*-type cytochrome, each of which has been purified to homogeneity, are described.

Materials and Methods

Materials. DEAE-cellulose (DE-52) was obtained from Whatman Inc. Sephadex G-50 and CM-Sephadex were obtained from Pharmacia Fine Chemicals. Protein molecular-weight standards, acrylamide and methylene bisacrylamide were obtained from Bio-Rad laboratories.

Organism and growth conditions. Bacillus firmus RAB was grown with shaking at 30 °C on DL-malate in carbonate-buffered medium that was adjusted to pH 10.5 [14]. The cells were grown to the late logarithmic phase of growth, harvested, and washed once with 50 mM Tris-HCl (pH 7.0), 1 mM $MgSo_4$ and 1 mM PMSF.

Preparation of right-side-out and everted vesicles. Right-side-out membrane vesicles were prepared by the lysozyme method of Kaback [15]. Everted vesicles were prepared by passage of washed cells through a pre-cooled French pressure cell at 138 MPa. The supernatant from low-speed centrifugation ($15000 \times g$, 20 min) was subjected to ultracentrifugation at $300000 \times g$ overnight to pellet the membranes.

Assays and chromatography procedures. Absorption spectra were recorded using either an Aminco DW-2a or a Perkin-Elmer 557 dual-beam spectrophotometer (spectral resolution, 1 nm). Protein content of samples was estimated as described by Lowry [16] using lysozyme as the standard. HPLC (high-performance liquid chromatography) was performed on a Shimadzu LC-6A system using a Pharmacia Mono Q HR 5/5 anion exchange and a Pharmacia Superose 12 HR 10/30 gel filtration column. Proteins were desalted and concentrated by use of an Amicon concentrator and Diaflo PM-10 ultrafiltration membranes. Alkaline pyridine hemochrome analysis was performed by the method described by Falk [17].

SDS-polyacrylamide gels. Denaturing SDS-polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli [18] on resolving gels of $12 \times 14 \times 1.5$ cm with a 1-cm stacking gel. Samples were dissolved in 60 mM Tris-Cl (pH 6.8), 60 mM dithiothreitol, 2% SDS, 10% glycerol, 0.02% bromophenol blue and boiled for 2 min before loading on the gel. A set of molecular-weight standards was routinely run that included standards with M_r values of 14200 and 20100. Electrophoresis was carried out overnight at a constant voltage of 50 V. Polyacrylamide gradient gels were poured using a 5–17% sucrose gradient and an inverted TEMED gradient [19]. Gels were stained for at least 2.5 h with shaking in 0.25% Coomassie blue, 50% methanol, 7% acetic acid or silver stained using the silver stain kit from National Diagnostics. The procedure for silver staining followed the National Diagnostics recommended procedure exactly, except that the gels were fixed for 1 h in 10% TCA and then fixed further overnight in destaining solution. Gels were stained for heme using benzidine [20].

Isoelectric focusing. Isoelectric focusing was performed in a Bio-Rad horizontal electrophoresis cell at 25 °C. The composition of the polyacrylamide gel was 4.3% acrylamide, 0.24% bis-acrylamide, 12.5% glycerol and Pharmacia ampholytes. For equilibrium isoelectric focusing, the gel contained 1.9 ml 2.5–5 ampholytes per 30 ml. The gels were polymerized with 0.1% TEMED and 0.03% ammonium persulfate. Samples were applied directly to the gel in their buffer. Equilibrium running conditions were 3.0 h at 1000 V. After focusing, the pH gradient was measured with a surface pH electrode. The gels were fixed in 10% TCA/33% ethanol for 1.5 h, followed by 0.5 h in 5% TCA/33% ethanol and overnight in destaining solution (40% methanol/7% acetic acid).

Oxidation-reduction midpoint-potential titrations. Oxidation-reduction titrations of the soluble cytochrome components were performed electrochemically at 5 °C using an optically transparent gold

electrode in a thin-layer cell as described by Smith et al. [21].

Purification procedures for soluble heme-containing proteins. Three heme-containing proteins were detected in and isolated from extracts of the alkalophilic bacterium *Bacillus firmus* RAB. The supernatant from a double passage through a French press (after low-speed and high-speed centrifugation and dialysis) was chromatographed at 4°C on a DEAE-cellulose column (1.5 × 30 cm) equilibrated with 50 mM Tris/HCl (pH 7.0), 1 mM $MgSO_4$ and 1 mM phenylmethylsulfonyl fluoride (PMSF). After washing with two bed volumes of the application buffer to distribute the proteins through the column, a green protein that possessed catalase activity was eluted with 200 mM NaCl in the same buffer. This catalase-like protein was only present in significant amounts when the cells were broken in the presence of the protease inhibitor PMSF. PMSF has similarly been reported to protect *Neurospora crassa* [22] and spinach catalases [23] against proteolyis. Since many catalase (hydrogen-peroxide : hydrogen peroxide oxidoreductase, EC 1.11.1.6) proteins have been isolated and characterized from plants, bacteria and from mammalian liver and blood, it is only noted that this bacterium possesses a protein with similar properties; its full characterization will be performed at a later time.

A dark red band was left bound to the top of the DE-52 cellulose column. Cytochromes could be eluted with buffer containing 250 mM NaCl. The red band eluted slowly, separating into two distinct bands. The first band was a *c*-type cytochrome and the band that migrated more slowly was a *b*-type cytochrome. The fractions containing a *c*-type cytochrome were concentrated and chromatographed on a Sephadex G-50 column equilibrated with the application buffer plus 500 mM NaCl (pH 7.0). Application in lower ionic strength buffer resulted in extensive non-specific binding to the gel. The purest fractions (A_{280}/A_{410} < 2.0) were pooled, desalted, and concentrated on an Amicon concentrator using a PM-10 Diaflo membrane. The sample was then subjected to a second set of anion exchange and gel filtration chromatography steps before use of HPLC purification on a Pharmacia Mono Q anion exchange column (0.5 × 5 cm). The cytochrome eluted from the column with 150 mM ammonium sulfate in the buffer (pH 7.0). The best fractions were pooled and subjected to HPLC analysis on a Pharmacia Superose 12 gel filtration column equilibrated with 50 mM Tris-HCl (pH 7.0) containing 500 mM ammonium sulfate. Fractions with a purity ratio (A_{280}/A_{410}) less than 0.25 were pooled and used in experiments described below. The *b*-type cytochrome was subjected to the same initial purification scheme as described for the *c*-type cytochrome. The *b*-type cytochrome eluted from the Pharmacia Mono Q anion-exchange column at 200 mM ammonium sulfate. After gel filtration on the Pharmacia Superose 12 column, the fractions with a purity ratio (A_{280}/A_{414}) less than 0.20 were pooled and used in experiments described below.

TABLE I

PURIFICATION OF CYTOCHROMES FROM *BACILLUS FIRMUS* RAB

About 400–500 g of cells *B. firmus* (wet weight) have been used.

Step	Protein (mg)	Purity ratio
Cytochrome C-552		(ratio for A_{280}/A_{410})
I French Press	3021	5.21
II DE-cellulose [a]	1453	2.30
III Sephadex G-50 [a]	333	1.82
IV Mono Q (HPLC) [a]	67	0.52
V Superose 12 (HPLC) [a]	2.3	0.25
Cytochrome *b*-558.5		(ratio for A_{280}/A_{414})
I French Press	1446	3.40
II DE-cellulose [a]	723	2.00
III Sephadex G-50 [a]	166	1.00
IV Mono Q (HPLC) [a]	33	0.40
V Superose 12 (HPLC) [a]	1.1	0.20

[a] Two chromatographic runs.

The most purified fraction of both the *c*- and *b*-type cytochromes moved as single bands on heavily loaded gels and moved as symmetrical peaks on gel filtration columns. The purification procedure for the soluble cytochromes *c* and *b* are summarized in Table I.

Results

Studies of the soluble cytochrome c

Absorption spectra of the purified soluble cyto-

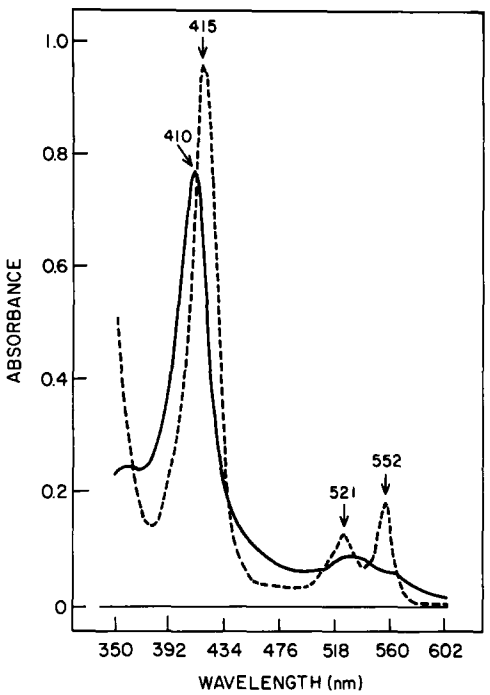

Fig. 1. Absorption spectra of the oxidized and reduced *Bacillus firmus* RAB *c*-type cytochrome. The oxidized cytochrome (———) was reduced (- - - - - -) by the addition of small amounts of solid sodium dithionite. Reaction mixture contained the cytochrome (5 μM heme *c*) in 20 mM Tris-HCl buffer (pH 7.0).

chrome *c* are shown in Fig. 1. The oxidized cytochrome exhibited a Soret band maximum at 410 nm and the reduced cytochrome exhibited maxima at 552 (alpha-band), 521 (beta-band) and 415 nm (Soret band). Isosbestic points (reduced minus oxidized) were located at 410.8, 434, 506, 528, 542 and 559 nm. Alkaline pyridine hemochrome analysis showed a single peak at 549.5 nm, indicating that heme *c* was the only heme prosthetic group present [17]. An absorbance band at 695 nm was observed in the spectrum of the oxidized cytochrome (data not shown). This absorbance band, arising from sulfur : iron charge transfer is indicative of methionine serving as one of the heme axial ligands [24]. Chromatography on a calibrated Sephadex G-50 column, using absorbance at 410 nm to monitor the location of the oxidized cytochrome, yielded a value of $17\,000 \pm 1000$ for the molecular weight of the cytochrome. SDS-polyacrylamide gel electrophoresis gave a value of $16\,000 \pm 500$ for the molecular weight of the purified cytochrome.

Analysis of the amino acid composition (Table II) indicated a notable paucity of lysines and an abundance of aspartic and glutamic residues. Estimates of methionine content were also low. Isoelectric focusing showed that the cytochrome was acidic, yielding a p*I* of 3.42 ± 0.03.

Oxidation-reduction titrations of the cytochrome, which behaved as a one-electron carrier, were performed at four different pH values. As shown in Fig. 2, the cytochrome exhibited a pH-dependent midpoint potential ($E_m = -65$ mV) over the pH range from 7.0 to 8.3 and a pH-dependent midpoint potential (−54 mV/pH) over the pH range from 8.3 to 9.9. At pH 7.0 and 8.3 the cytochrome's midpoint potential was +65 mV ($n = 1.03$), at pH 9.25 $E_m = +8$ mV ($n = 0.93$), and at pH 9.92 $E_m = -20$ mV ($n = 1.07$). All the titrations were fully reversible and the E_m values were independent of mediator concentration. These results indicate that the cytochrome has a p*K* on its oxidized form near 8.3, so that reduction of the cytochrome below pH 8.3 does not involve H^+ uptake, but reduction at pH > 8.3 is accompanied by the uptake of $1H^+$/electron.

In addition to the low-molecular-weight *c*-type cytochrome of the cytochrome oxidase complex [13], membranes of *B. firmus* RAB contained an additional *c*-type cytochrome that could be distinguished from the soluble *c*-552. Right-side-out membrane vesicles were washed with 300 mM ionic strength buffer and sonicated extensively to remove the soluble *c*-552. Heme staining of the gels that were loaded with the sonicated membranes showed one heme-staining band with a molecular weight near 31 kDa and the absence of any band in the 16–18 kDa molecular weight region that would correspond to the soluble heme-containing cytochrome *c* (data not shown). An oxidation-reduction titration of these membranes showed a titratable component with an alpha-band absorption maximum at 551 nm, and a midpoint potential of +67 mV, independent of pH between pH 7.0 and 10.7 (Fig. 3). The differences in molecular weight and in pH-dependence of E_m strongly suggest that the membrane-bound and soluble *c*-type cytochromes are different proteins. Initial electron paramagnetic reso-

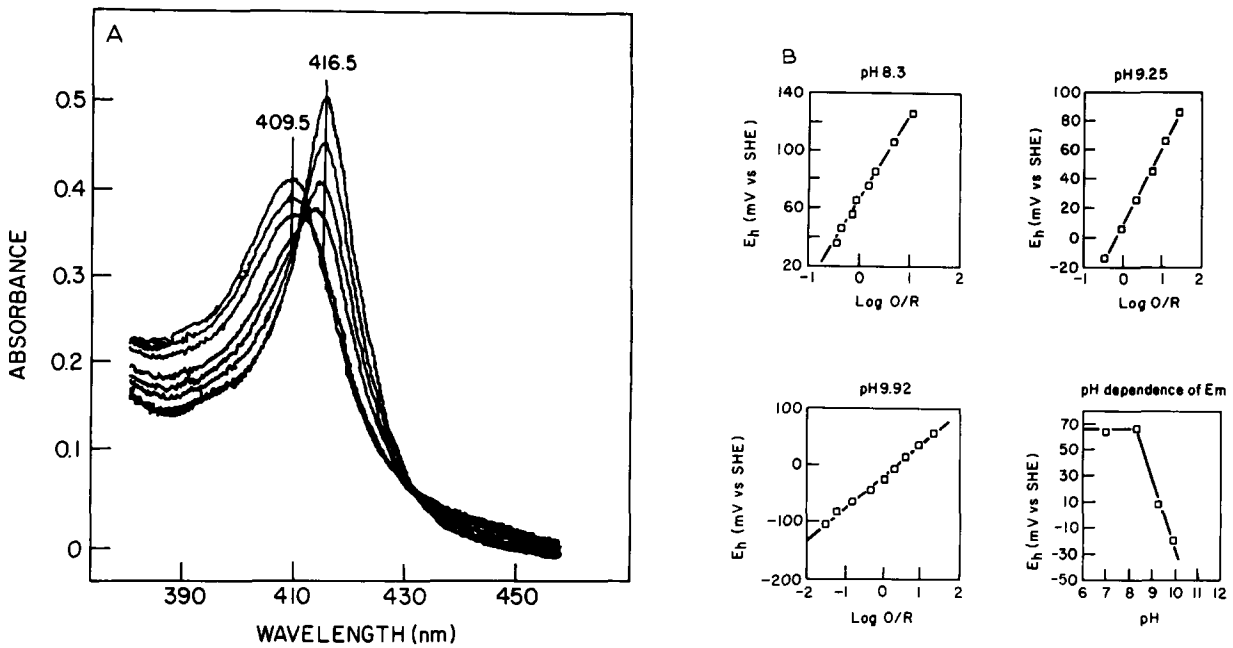

Fig. 2. (A). Oxidation-reduction titration of the *B. firmus* RAB cytochrome *c*-552. The reaction mixture contained the cytochrome (equivalent to 90 μM heme *c*) in 20 mM Tris-HCl (pH 7.0). The following oxidation-reduction mediators were also present: 10 μM duroquinone; 5 μM *N*-ethyl phenazonium ethosulfate; 10 μM 1,2-naphthoquinone and 5 μM *N*-methyl phenazonium methosulfate. The titration was carried out at 5 °C, as described under Materials and Methods, and the oxidation state of the cytochrome was monitored by following the absorbance at 410 nm. (B). Oxidation-reduction titration of the cytochrome *c*-552 at pH 8.3, pH 9.25, pH 9.92 and plot of E_m vs. pH. The titration was carried out as described in (A) above, except that Tricine was substituted for Tris-HCl in the titrations at pH 9.25 and 9.92. Mediators used in the titrations at pH 9.25 and 9.92 were 10 μM DAD, 10 μM 1,2-naphthoquinone, 10 μM 1,4-benzoquinone and 5 μM *N*-methyl phenazonium methosulfate.

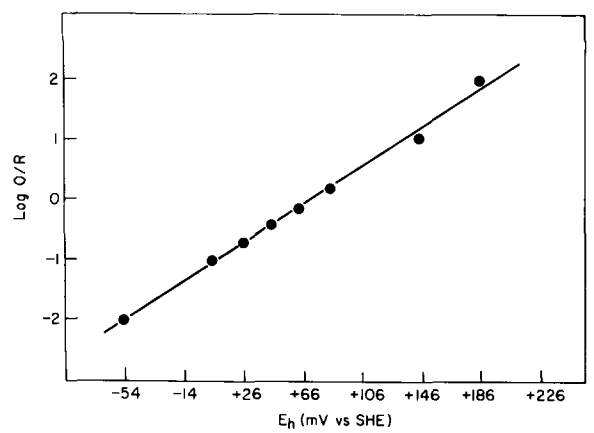

Fig. 3. Oxidation-reduction titration of the *B. firmus* RAB right-side-out membrane vesicles. The reaction mixture contained the membrane vesicles (6.5 mg protein/ml) in 20 mM Tris-HCl (pH 7.0) and 200 mM NaCl. Membrane vesicles had been sonicated and washed extensively with high ionic-strength buffer to remove any remaining soluble proteins. Mediators used were the same as mentioned in Fig. 4A.

nance studies of *B. firmus* RAB membranes indicate the presence of a Rieske iron-sulfur protein and the likely presence of a cytochrome bc_1 complex [25] in this bacterium. Thus the membrane-bound cytochrome *c* could be a cytochrome that is functionally equivalent to cytochrome c_1 [25].

The current results do not indicate whether the *B. firmus* RAB cytochrome *c*-552 should be classified as a cytochrome of the mammalian mitochondrial type, because we lack sufficient sequence or functional data. The cytochrome *c*-552 from *B. firmus* RAB is strongly acidic and has a pH-dependent oxidation-reduction midpoint potential. The protein is also readily auto-oxidizable when exposed to air. Most mammalian mitochondrial *c*-type cytochromes are either basic or very weakly acidic, are not oxidized by O_2 and have oxidation-reduction midpoint potentials that are considerably more positive than that of *B. firmus* RAB cytochrome *c*-552. *Pseudomonas*

aeruginosa cytochrome *c*-551 and *Paracoccus denitrificans* cytochrome *c*-550 are examples of *c*-type cytochromes that are acidic and whose structure and function are analogous to the mammalian and yeast-type cytochromes [26]. Some *c*-type cytochromes from photosynthetic bacteria and algae are acidic but not as acidic as that from *B. firmus* RAB [27–29]. Miki et al. [30,31] reported that two *c*-type cytochromes (*c*-550 and *c*-554) isolated from *B. subtilis* were different from the mammalian mitochondrial type cytochrome *c* with respect to electron-carrying properties. *B. subtilis* cytochrome *c*-550 showed similar properties to those of a *c*-type cytochrome isolated from *B. megaterium* [32,33]. Recently, Wooley [34] has purified three *c*-type cytochromes from *Bacillus licheniformis*. The cytochromes (*c*-551, *c*-552 and *c*-554) have acidic p*I*'s of 4.45, 4.75 and 4.50, respectively, but no clearly established function as yet.

Studies of the soluble cytochrome b

Spectra of the purified cytochrome *b* are shown in Fig. 4. . The oxidized cytochrome exhibited a Soret band maximum at 414 nm and the reduced cytochrome has maxima at 558 (alpha-band), 527 (beta-band), and 425 nm (Soret band). Isosbestic points (reduced minus oxidized) are located at 417, 443, 513, 535, 545 and 568 nm. Alkaline pyridine hemochrome analysis showed a single peak at 556 nm, indicating that protoheme [24] was the only heme prosthetic group present (data not shown). Chromatography on a calibrated Sephadex G-50 column, using absorbance at 414 nm to monitor the location of the oxidized cytochrome, gave a value of 15000 ± 1000 for the molecular weight of the cytochrome. SDS-polyacrylamide gel electrophoresis gave a value of 16000 ± 500 for the molecular weight.

Amino acid composition analysis revealed that this cytochrome lacked any appreciable amount of lysine and had an abundance of aspartic and glutamic residues (Table II). Isoelectric focusing indicated that the *b*-type cytochrome was acidic,

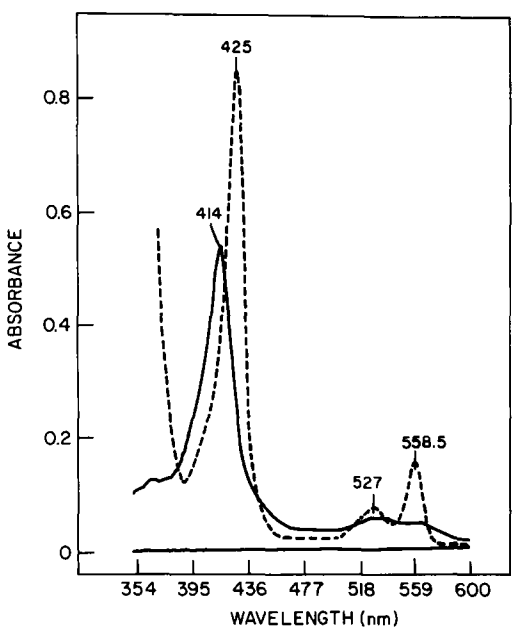

Fig. 4. Absorption spectra of the oxidized and reduced *B. firmus* RAB *b*-type cytochrome. The oxidized cytochrome (———) was reduced (- - - - -) by the addition of small amounts of solid sodium dithionite. Reaction mixture contained the cytochrome (7 μM protoheme) in 20 mM Tris-HCl buffer (pH 7.0).

TABLE II

ESTIMATED MOLAR AMINO ACID COMPOSITIONS OF THE SOLUBLE *c*- AND *b*-CYTOCHROMES FROM *BACILLUS FIRMUS* RAB BASED ON AMINO ACID ANALYSES

n.d., not determined.

Amino acid residue	Estimated residues	
	per mol cytochrome *c*-552	per mol cytochrome *b*-558
Aspartic acid	30	18
Threonine	6	6
Serine	9	12
Proline	12	6
Glutamic acid	36	24
Glycine	21	34
Alanine	36	14
Valine	9	17
Half cystine	0	2
Methionine	1	2
Isoleucine	6	5
Leucine	12	10
Tyrosine	6	6
Phenylalanine	3	4
Lysine	3	1
Histidine	3	4
Arginine	3	1
Tryptophan	n.d.	n.d.

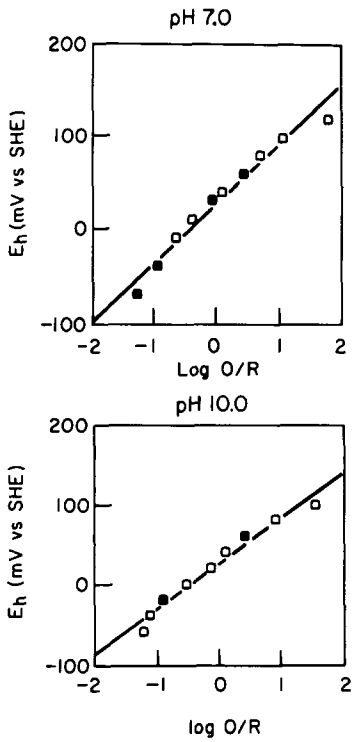

Fig. 5. Oxidation-reduction titration of the *B. firmus* RAB cytochrome *b*-558.5. The reaction mixture contained the cytochrome (equivalent to 15 μM protoheme) in 20 mM Tris-HCl. Titration was carried out as in Fig. 4 at pH 7.0 and 10.0. Open squares are in the reduced direction while closed squares are in the reverse (oxidized) direction.

having a p*I* of 3.07 $\pm$ 0.05. Fig. 5 shows the results of oxidation-reduction titrations of the cytochrome performed at pH 7.0 and 10.0. The midpoint potential for this one electron carrier was +28 mV. All the titrations were fully reversible, and the E_m value was independent of mediator concentration. The function of this soluble cytochrome *b*, as with the acidic, soluble cytochrome *c*, is not yet known, and is the focus of ongoing studies.

Acknowledgments

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