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Cytochrome $cbb_3$ of *Thioalkalivibrio* is a Na$^+$-pumping cytochrome oxidase

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Cytochrome $c$ oxidases (Coxs) are the basic energy transducers in the respiratory chain of the majority of aerobic organisms. Coxs studied to date are redox-driven proton-pumping enzymes belonging to one of three subfamilies: A-, B-, and C-type oxidases. The C-type oxidases ($cbb_3$ cytochromes), which are widespread among pathogenic bacteria, are the least understood. In particular, the proton-pumping machinery of these Coxs has not yet been elucidated despite the availability of X-ray structure information. Here, we report the discovery of the first (to our knowledge) sodium-pumping Cox (Scox), a $cbb_3$ cytochrome from the extremely alkaliphilic bacterium *Thioalkalivibrio versutus*. This finding offers clues to the previously unknown structure of the ion-pumping channel in the C-type Coxs and provides insight into the functional properties of this enzyme.

Cytochrome $c$ oxidase | sodium pumping | $cbb_3$-type oxidase | alkaliphilic

The known terminal oxidases according to the structure of their active centers and their phylogenetic relations are subdivided into two superfamilies (1). One is composed of numerous representatives containing a heme-copper binuclear active center (BNC). Oxidases belonging to the other superfamily have no copper. This superfamily includes bacterial oxidases of the $bd$ type. The superfamily of representatives with heme-copper BNC is subdivided in turn into two groups, cytochrome $c$ oxidases (Coxs) and quinol oxidases, depending upon the electron donor, which can be either cytochrome $c$ or quinol. Quinol oxidases with a heme-copper BNC are found only in prokaryotes, whereas Coxs are widespread among living organisms of all domains: Eukarya (where they are found in mitochondria and chloroplasts), Bacteria, and Archaea. Although terminal oxidases with heme-copper BNC constitute a diverse group of multisubunit enzymes having from 2 to 13 subunits, conservatism and similar architecture are obviously inherent in their main (catalytic) subunit. The catalytic center of the main subunit always contains two hemes and copper as redox active prosthetic groups and a redox active tyrosine covalently bound to histidine in the polypeptide chain (2–5). Iron of one of the hemes and copper constitute the BNC. Coxs are the best-studied group of terminal oxidases. The basic mechanism of energy transduction by Coxs during respiration consists of the oxidation of cytochrome $c$ by molecular oxygen (O$_2$) coupled to transmembrane pumping of protons (H$^+$). This process results in reduction of O$_2$ to water by the BNC, where O$_2$ is bound. In Coxs, it requires four protons (“chemical” H$^+$ for water production) taken from the inner side of the membrane and can be coupled to the translocation of another four protons (“pumped” H$^+$) from the inner to the outer side of the membrane into the intermembrane or the periplasmic space of mitochondria or prokaryotic cells, respectively, according to the following equation (6–8):

$$4\text{cytc}^{2+} + 4\text{H}^+_{\text{in,chem}} + 4\text{H}^+_{\text{in,pump}} + \text{O}_2 \rightarrow 4\text{cytc}^{3+} + 4\text{H}^+_{\text{out,pump}} + 2\text{H}_2\text{O}.$$  

In A-type Coxs, two H$^+$ pathways in the main subunit were identified, the so-called D channel, conducting all pumped and part of the chemical H$^+$, and the K channel, conducting most of chemical H$^+$ (9). In C-type Coxs, only a K-channel analog was found (10). The described catalytic events are accomplished through generation of a transmembrane difference in H$^+$ potentials (Δ$\mu$H$^+$), which is used as a convertible membrane-linked biological currency. Microorganisms living in an alkaline environment maintain a nearly neutral cytoplasmic pH (11). This presents a problem for alkaliphiles because it gives rise to an inverted pH gradient that decreases the Δ$\mu$H$^+$ (12, 13). Some alkaliphilic microorganisms solve this problem by using an Na$^+$-pumping NADH-CoQ reductase (NOR) (14), and perhaps a Na$^+$-pumping terminal oxidase, as was assumed (15). At present, NOR is the only respiratory chain enzyme for which Na$^+$ pumping has been directly and undoubtedly established (16). However, NOR is absent in the extremely alkaliphilic bacterium *Thioalkalivibrio versutus* AL2, which inhabits an alkaline (~pH 10) Siberian soda lake at saturating salt concentrations.

**Significance**

The majority of aerobic living organisms use oxygen for respiration. The key enzyme, which directly reduces oxygen to water during respiration, is the terminal cytochrome $c$ oxidase. It generates a large portion of the utilisable energy provided by the respiratory chain. Accumulation of biologically available energy by means of cytochrome $c$ oxidases is believed to be due to the proton-motive force across the mitochondrial or bacterial membrane. Details of this energy conversion are still unclear. Here we report the discovery of a sodium-pumping cytochrome $c$ oxidase that converts energy of respiration into sodium-motive force. This finding provides clues to understanding the mechanism of cytochrome $c$ oxidase that is not available when applying knowledge of the proton-pumping versions of the enzyme.


The authors declare no conflict of interest.

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Data deposition: The nucleotide sequences of *Thioalkalivibrio* $cbb_3$ oxidase have been deposited in the EMBL database (accession no. HE575403).

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concentrations (17). T. versutus is a chemolithotroph that oxidizes sulfur compounds and employs Cox as a terminal component of its aerobic electron transport chain. Here we report that T. versutus uses a novel C-type Cox that (i) specifically requires Na⁺ for its activity and (ii) electrogenically exports Na⁺ from cells or right-side-out subcellular membrane vesicles, the process being coupled to oxidation of ascorbate by O₂.

Results and Discussion

Experiments were performed using T. versutus cells (exhausted of endogenous substrates) and the right-side-out subcellular vesicles derived from them. Thus, both cells and vesicles displayed no respiration without substrate addition. To start respiration at the level of Cox in respiratory chain, we used the exogenous substrates Wurster’s blue [N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD)], which is nonspecific for all terminal oxidases, or cytochrome c, which is specific for Cox, both in the presence of a reductant ascorbate. Addition of any of the substrates initiated rapid O₂ consumption (Fig. 1 A and B) and generation of an electric membrane potential (Δψ) (Fig. 1D). Similar to the Na⁺-motived NQR (14), these activities were specifically Na⁺-dependent, showing optimum at alkaline pH (8.5–9.5) (Fig. 1C and D). In contrast, the well-known H⁺-motived Cox from Rhodobacter sphaeroides (7) and Paracoccus denitrificans, used as controls (Fig. S1 A–C), as well as from Pseudomonas stutzeri (18), showed no Na⁺ specificity (SI Text, section S1).

Cytochrome c, unlike TMPD, was inefficient toward T. versutus Cox at high salt concentrations, similar to Cox from other species; therefore, TMPD was used later on. The Cox inhibitor cyanide fully arrested both Cox activities (Fig. 1D and Fig. S1D). Thus, Cox operating in T. versutus membranes is a Na⁺-dependent oxidase (Scox) that can be either a Na⁺-activated H⁺ pump or Na⁺-activated redox loop lacking in H⁺ pump.

To discriminate between these two possibilities, we tested H⁺ pumping by the oxidase. Unlike H⁺-pumping Cox, which acidify the external medium during respiration in the presence of the K⁺ ionophore valinomycin (Fig. S2A) (see also refs. 7 and 19), Scox did not mediate H⁺ extrusion at alkaline pH (Fig. S2A and Fig. S2B), as shown earlier (20). Instead, Scox evoked alkalinization of the medium in response to O₂ pulse when valinomycin was replaced by protonophore, and H⁺ could serve as counter ion instead of K⁺ (Fig. 2B and inset and Fig. S2C). Reversibility of this alkalinization in the time scale (upper green curve, Fig. 2, Inset) points that it cannot be attributed to H⁺ consumption during respiratory chemical reaction of water production because of irreversibility of the latter reaction. These effects could be explained by assuming that the oxidase is Na⁺-motive and electrogenic. In such case, Δψ formed by Na⁺ efflux, in the presence of a protonophore, should be counterbalanced by H⁺ influx, leading to reversible alkalinization of the outer space. Fig. 2D shows these relationships. Consistent with this scheme, a respiration-driven, fully cyanide-sensitive export of 22Na⁺ was found in bacterial cells and vesicles preloaded with this isotope (Figs. 2C and 3 A and B). Export of 22Na⁺ could be stimulated by protonophores [carboxyl] cyanide m-chlorophenylhydrazine (CCCP) and 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), a protonophore operating at alkaline pH (Fig. S3 and SI Text, section S2) as well as valinomycin. Like respiratory activity of T. versutus Scox, export of 22Na⁺ occurred at alkaline pH (8.0–10.0) (Fig. 2C). These data confirm that T. versutus Scox operates as a primary Na⁺ pump rather than an Na⁺/H⁺ antiporter or a K⁺ pump.

To identify the Na⁺-pumping Scox, we considered the three known types of Cox (21, 22): A, B, and C (Fig. 4A), which are represented by cytochromes aa₃/aa₃, ba₃, and cb₃, respectively. Because T. versutus membranes contained hemin B but not heme A (Fig. S4), A- and B-type Cox can be excluded in our case. Instead, we identified an operon of a cb₃ oxidase (C-type Cox)
in the *T. versutus* genome (EMBL accession no. HE575403.1) and detected its expression product (Fig. S5 and SI Text, section S1; for the enzyme pattern, see Fig. 2D), which might operate in this bacterium as a Na\(^{+}\) pump. Consistent with our hypothesis, *P. denitrificans* AO1 vesicles, which lack all Coxs and displayed no active Na\(^{+}\) transport (Figs. 2C and 3C), became capable of

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**Fig. 3.** Evidence of a primary Na\(^{+}\) pump in *T. versutus* (Tv). (A) Substrate-induced Na\(^{+}\) efflux from 22Na-loaded Tv cells, (B) Tv vesicles, (C) *P. denitrificans* AO1 vesicles with Tv cbb\(_3\) (+Scox), and (D) WT *P. denitrificans* vesicles was assessed by radioactivity of 22Na retained in cells or vesicles after substrate addition. (G) Substrate-induced Δψ generation in vesicles from Tv and (H) WT *P. denitrificans* monitored by spectral changes of safranine O (10 μM). A pH 9.2 (A–D and G), 7.5 (E and H), and 9.6 (F and H) reaction medium for “Na\(^{+}\) transport measurements” was used (Materials and Methods). Additions (final concentrations): valinomycin, 0.5 μM (B, D, G, and H) or 2 μM (A and G); protonophores: HQNO, 5 μM (G), 25 μM (B–D and G) or 50 μM (A); KCN, 1 mM (B–D) or 10 mM (A); protein of cells, 2.7 mg/mL (A), vesicles, 0.7 mg/mL (B), 1 mg/mL (C–F), 50 μg/mL (Left) or 76 μg/mL (Right) (G), and 50 μg/mL (H). Arrows at “0” time show substrate addition: TMPD, 40 μM (A, B, G, and H), 50 μg/mL (H) or 3 mM (A and C–F).

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**Fig. 4.** Phylogenetic relationships of representative Coxs based on catalytic subunit protein sequences (Table S1). (A) The brief phylogenetic tree with representative Coxs: A-type (blue), B-type (green), and C-type (pink). *Thioalkalivibrio* clade (yellow, see also B) with R(K)308L(M) substitution is inside C-type Coxs. The sequences given in ref. 10 (gray) cluster separately from C-type Coxs (Unknown). E323 is conserved only in the catalytic subunit of C-type Coxs (see C); residue numbering corresponds to *P. stutzeri* cbb\(_3\) (here and elsewhere). (B) Inset derived from the detailed phylogenetic tree with representative Coxs (Fig. S6); *T. versutus* cbb\(_3\) is orange-contoured. Blue spheres indicate nodes with clade credibility values <90; other nodes have clade credibility values ≥90. (Scale bar: 0.5 substitutions per residue.) (C) Amino acid sequences of helices IX–XI of catalytic subunit of C-type Cox aligned using WebLogo software (23). The upper panels of each pair under an individual α-helix contain representatives of the Na\(^{+}\)-motive-like cbb\(_3\) Cox (yellow-colored in the C-type cluster); the lower panels contain all other representatives of the C-type cluster that are H\(^{+}\)-motive-like (pink-colored in the C-type cluster). Red arrows show R(K)308L(M) substitution and conservative T312, G344, S348, and T389 in the Na\(^{+}\)-motive-like representatives, E323 conserved in both groups, W386 conserved among H\(^{+}\)-motive-like representatives. Red asterisks in the Na\(^{+}\)-motive template indicate residues forming Na\(^{+}\) coordination shell (shown in Fig. 5B).
oxidase-driven primary $^{22}$Na$^+$ pumping (Figs. 2C and 3D) similar to that of $T.$ versutus vesicles (Figs. 2C and 3B) after expression of $T.$ versutus $cbb_3$. We checked whether the observed $^{22}$Na$^+$ export from the energized cells and vesicles could be explained by a $\Delta$ψ-potentiated $^{22}$Na$^+/Na^+$ exchange or specified by a potential-dependent Na$^+$ channel. Because dissipation of $\Delta$ψ by valinomycin, as well as by protonophore (Fig. 3G), increased $^{22}$Na$^+$ export in $T.$ versutus vesicles (Fig. 3B), a passive $\Delta$ψ-potentiated $^{22}$Na$^+/Na^+$ leakage or “Na$^+$ transfer through a $\Delta$ψ-activated Na$^+$ channel can be excluded. Consistent with this conclusion is the absence of $\Delta$ψ-driven $^{22}$Na$^+$ export in $^{22}$Na$^+$-loaded WT $P.$ denitrificans vesicles that lack $T.$ versutus Scox (Fig. 3 E, F, and H). The data also reveal that $P.$ denitrificans $a_{III}$ and $cbb_3$ Scox, expressed in the used growth conditions (Materials and Methods), do not pump Na$^+$. Thus, these experiments directly demonstrated that $T.$ versutus $cbb_3$ is a primary Na$^+$ pump.

The $cbb_3$ oxidases studied previously were shown to operate as H$^+$ pumps (7, 19, 24, 25). However, an individual pathway of pumped H$^+$ analogous to the D channel in A-type Scox has not been identified (10, 24). Only a K-pathway analog, involved in delivering chemical H$^+$ to BNC to produce water in Cox$S$ of all $cbb_3$ members. Additionally, W386 seemed to be fully conserved among the H$^+$-motive-like (all other C-type members). Additionally, W386 seemed to be fully conserved among the H$^+$-motive-like but not in the Na$^+$-motive-like C-type members.

Another indication that the Na$^+$-pumping channel is located within helices IX–XI in the catalytic subunit of $T.$ versutus and of homologs that occupy the same clade: (i) a unique substitution, R(K)308L(M), near the cytoplasmic entrance of the tentative Na$^+$ channel and (ii) conservatism of residues T312, G344, S348, and T389 (Fig. 4C). Accordingly, we propose that within C-type Cox$S$ these five residues serve as a fingerprint that distinguishes two templates of catalytic subunits: Na$^+$-motive-like (Thioalkalivibrio clade) and H$^+$-motive-like (all other C-type members).
Notably, helices IX and X of the catalytic subunit of *P. stutzeri* cbb₃ (H'-motive pattern) as well as of *T. versutus* cbb₃ (Na'-motive pattern) contain features that disrupt regularity of the helices, conferring flexibility that might be necessary for ion transfer (Fig. 5E). Additionally, in *P. stutzeri* cbb₃, the only large cavity seems to be located within helices IX–XI of the main subunit (Fig. 5C) and the recently identified auxiliary subunit U (26) (for details, see SI Text, section S5). When water-filled, this cavity could link the critical residues R(K)308 and E323, providing a water-filled H'-translocation pathway (Fig. 5D). These facts allow us to assume that in the Na'-motive cbb₃ such cavity could serve as the Na'-pumping channel. Finally, we evaluated the Na'-binding capacity of the H'-motive and Na'-motive cbb₃ Coxes using molecular dynamics (MD). MD simulations predicted that the main subunit of *T. versutus*, but not that of *P. stutzeri*, can bind Na⁺ in the E323 site in a redox-linked manner (Fig. 6 A and B and Table S2). According to the prediction, one electron coming into the metal active center switches the enzyme from a Na⁺-nonbinding oxidized (ox) state to a Na⁺-binding reduced (red) state. Under the tentative model, the subsequent entry of H⁺ to the active center via the chemical reaction through the “K channel” results in Na⁺ extrusion (Fig. 6B). The predicted coupling stoichiometry is one electron for each pumped Na⁺, which is consistent with the experimental data (Fig. 2B and Fig. S2 B and C) according to which Scox operation fits in best with the following equation:

\[
4\text{cyt}^{2+} + 4H^+_{\text{chem}} + 4\text{Na}^+_{\text{pump}} + \text{O}_2 \rightarrow 4\text{cyt}^{3+} + 4\text{Na}^+_{\text{out,pump}} + 2\text{H}_2\text{O},
\]

Evidently, the capacity of *T. versutus* Scox to bind Na⁺ is provided by the E323-involving Na⁺-coordination shell (see Fig. 5B), which is absent in *P. stutzeri* Cox due to S348(A) substitution.

The fact that the Na⁺-pumping function was revealed among C-type Coxes may shed light on evolution of Coxes. At present, the phylogenomics-based evolutionary scenario of Coxes remains obscure (1, 22, 34). Therefore, in our reasoning we take into consideration several independent markers of possible Cox evolution and generally accepted facts: (i) geological evidence on oxygen-free atmosphere on the Earth preceded an oxygen atmosphere (35), (ii) close relationship between nitric oxide reductases and C-type Coxes and their far distance from A- and B-type Coxes (1, 36, 37), and (iii) the conclusion that Na⁺ energetics appeared before H⁺ energetics (38). If we believe the mentioned facts and views are true, then C-type Coxes—which include Na⁺-motive and H⁺-motive representatives—might be the ancestors of H⁺-motive A- and B-type Coxes, as assumed earlier (37). C-type Coxes were shown to bind O₂ much more tightly than A-type Coxes (39). Owing to this property C-type Coxes operate under low O₂ conditions and would be the first energy-transducing enzymes capable of oxygen reduction when the early atmosphere of the Earth was formed, being gradually filled with O₂.

**Conclusion**

We provide the first direct demonstration to our knowledge that the *T. versutus* cytochrome cbb₃ (Cox) is a primary Na⁺ pump. This finding is collectively based on the observation of (i) specific Na⁺ dependence of Cox activity, (ii) protonophore- or valinomycin-stimulated Na⁺ pumping in *T. versutus* cells and vesicles, (iii) no H⁺ pumping in them in the presence of valinomycin and reversible alkalinization of the outer space in the presence of protonophore without valinomycin, (iv) expression of the *T. versutus* cbb₃ in *P. denitrificans* cells with the result that membranes of this bacterium are competent in protonophore/valinomycin-activated respiration-supported Na⁺ pumping, and (v) a Na⁺-binding coordination shell in the active center of Cox. These results raise the question of whether Na⁺ pumping is inherent in some other C-type Coxes. In this context, whether the Na⁺ transport in *Vitreoscilla* (40) is evoked by a primary Na⁺-pumping bo-type quinol oxidase or by a secondary pumping Na⁺/H⁺-antipporter is still unclear for the lack of ionophore tests after bo oxidase expression in *Escherichia coli*, which contains self-Na⁺/H⁺-antipporters (41) (for details, see SI Text, section S2). Our findings are consistent with the hypothesis of using Na⁺-motive energy transducers in organisms living under low ΔpH and high salinity (12) and might stimulate further progress in the study of energy-transduction mechanisms of Coxes.

**Materials and Methods**

Cultivation of bacterial strains, phylogenetic analysis, molecular dynamic simulations, and other methods are detailed in SI Materials and Methods.

**Bacterial Strains.** *T. versutus* AL2 and *P. denitrificans* strain A01 [expresses no cytochrome c oxidase activity (42)] and WT strain (PD 1222) were batch-cultured aerobically as previously described (17, 43).

**Membrane Vesicle Isolation.** Right-side-out membrane vesicles were isolated from freshly grown cells in a medium containing 50 mM CAPSO-KOH (pH 9.9), 50 mM K₂SO₄, 0.1 M sucrose, 0.1 mM EGTA, and 0.35 M Na₂SO₄ by disruption in a French press cell according to a standard procedure.

**Expression of Coxes in *P. denitrificans.*** For heterologous expression of *T. versutus* cbb₃ Cox in *P. denitrificans*, the *T. versutus* AL2 ccoNOQP operon was identified (HE575403.1) and amplified by PCR. The PCR fragment was cloned into the XbaI-HindIII-digested derivative of the broad host-range plasmid pBBR1MCS (42, 43) to produce the pBBR1/ccoNOQP plasmid. For protein expression, the recombinant plasmid pBBR1/ccoNOQP was transferred by conjuga
tion into the Paracoccus recipient strain A01. Exogenously expressed *T. versutus* cbb₃ Cox in strain A01 reached a level comparable to that in *T. versutus* AL2 strain, as monitored using Western blotting. Expression of self-cbb₃ Cox in *P. denitrificans* WT strain grown aerobically (this study) reached the same high level as in semiaerobically grown cells (44), as quantified by real-time quantitative PCR. Each self-Cox level, aa₁ and cbb₂, reached 70 pmol Cox/mg of membrane protein as determined by CO-reduced minus reduced spectra using extinction coefficients of 7 mM⁻¹ cm⁻¹.
(aa 595–606 nm) (43) and 7.6 mM 1−CH 3 (cbbs, 558–572 nm) (46). Self-Coxs presence was confirmed by the laser-flash photolysis method (47). Na+ transport measurements were performed with starved bacterial cells exhaustively depleted of Na+ and loaded with 22Na+ (PerkinElmer Life Sciences) on ice as reported (48). Membrane vesicles were loaded with 22Na+ by passive diffusion (incubation medium: 50 mM CAPSO/TRICINE/Mops-KOH, 50 mM K2SO4, 0.1 M sucrose, 0.1 mM EDTA, and 0.6 M NaNO3) at 6 °C overnight. Then 22Na+-loaded cells or vesicles were added to the cold Na+-containing incubation medium and the reaction was started by addition of substrate together with ascorbate (at 22°C). Samples of 25 μL were withdrawn from the incubation mixture at the appropriate time and cells or vesicles were separated from the incubation medium by rapid vacuum filtration (1–3 s) through nitrocellulose filters (Millipore). Radioactivity was counted using a liquid scintillation counter (LKB Wallac 1213 RackBeta). Each curve shown in figures is the average of three to eight independent experiments.

Electrical membrane potential generation in right-side-out membrane vesicles was monitored by the safranine method (49) or by tetraphenylphosphonium-selective electrode (50) at 25 °C.

H+ release in intact cells and membrane vesicles in O2-pulse was assessed by a standard method (51) in 1 mL of anoxic incubation mixture. Respiration of samples was initiated by addition of water (5–20 μL) saturated with air at 25 °C. The evoked changes in pH in the incubation mixture were estimated by titration with argon-saturated 0.5 mM H3SO4.

Respiratory activity was assessed using a Clark-type electrode at 25 °C.

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