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Biochemistry

Cytochrome a_1 of *Acetobacter aceti* is a cytochrome *ba* functioning as ubiquinol oxidase

(bacterial terminal oxidase/purification/proteoliposome/electrochemical proton gradient)

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ABSTRACT **Cytochrome a_1 is a classic cytochrome that in the 1930s had already been detected in *Acetobacter* strains and in the 1950s was identified as a terminal oxidase. However, recent studies did not substantiate the previous observations. We have detected a cytochrome a_1-like chromophore in *Acetobacter aceti*, which was purified and characterized in this study. The cytochrome was solubilized from membranes of the strain with octyl β-D-glucopyranoside and was purified by single column chromatography. The purified cytochrome exhibited a broad α peak around 600–610 nm, which turned to a sharp peak at 589 nm in the presence of cyanide. Carbon monoxide difference spectra of the cytochrome indicated the presence of an *a*-type cytochrome. The cytochrome contained 1 mol each of hemes *b* and *a* and probably one copper ion. These results suggest that the cytochrome purified from *A. aceti* is the so-called cytochrome a_1, and thus the existence of the classic cytochrome has been reconfirmed. The purified enzyme consisted of four polypeptides of 55, 35, 22, and 18 kDa, and it showed a sedimentation coefficient of 6.3 S in the native form. The enzyme had a high ubiquinol oxidase activity (140–160 μmol of ubiquinol-2 oxidized per min per mg of protein). When reconstituted into proteoliposomes, the cytochrome could generate an electrochemical proton gradient during oxidation of ubiquinol. Thus, cytochrome a_1 of *A. aceti* has been shown to be a cytochrome *ba* terminal oxidase capable of generating an electrochemical proton gradient concomitant with ubiquinol oxidation.**

Cytochrome a_1 has a long history, going back to the classic work of Warburg showing that *Acetobacter pasteurianum* (syn. *Acetobacter pasteurianus*) contains a weak band at 589 nm, which is intensified by cyanide and shifts to 592 nm in the presence of carbon monoxide (for reviews, see refs. 1–3). In the 1950s, the earlier finding was confirmed by Chance (4–6), who also showed that cytochrome a_1 works as a terminal oxidase in *A. pasteurianus*. Thereafter, several other bacterial strains were reported to contain cytochrome a_1, judging from the α band, which ranges from 585 to 596 nm (for reviews, see refs. 1 and 2). Recently, however, a_1-like hemoprotein detected in *Escherichia coli* has been shown to be ascribable to heme *b* contained in the cytochrome *d* terminal oxidase complex (7), and that of *Nitrosomonas europaea* has been proved to be a cytochrome aa_3 (8, 9). Furthermore, even in *A. pasteurianus*, a recent study did not prove the presence of cytochrome a_1 but, instead, showed that a_1-like pigment works to donate electrons to cytochrome *d* terminal oxidase (10). Thus, an increasing body of evidence has shown that almost all of these a_1-like hemoproteins are not cytochrome a_1 (for review, see ref. 1), and furthermore there is a report that casts doubt on the presence of cytochrome a_1 itself (9). Until now, cytochrome a_1 has not been biochemically characterized.

Acetic acid bacteria are classified into two genera—*Acetobacter* and *Gluconobacter*; the latter has been shown to contain a ubiquinol-oxidizing cytochrome *o* as the sole terminal oxidase (11, 12). *Acetobacter* has been reported to be subdivided into two classes: one contains cytochrome *d* and the other contains only an a_1-like component (13). Recently, we observed that *Acetobacter aceti* contains an a_1-like cytochrome when the cells are grown with shaking but not when they are grown statically (unpublished data). Thus, in this study, we purify and characterize the a_1-like cytochrome of *A. aceti*. Our results show that the cytochrome is the so-called cytochrome a_1, which is also shown to be a ubiquinol oxidase consisting of hemes *b* and *a*.

MATERIALS AND METHODS

Materials. Ubiquinone 2 (Q_2) was kindly supplied by Eizai (Tokyo) and the reduced form (Q_2H_2) was prepared as described by Rieske (14). Octyl β-D-glucopyranoside (octyl glucoside), valinomycin, and nigericin were purchased from Calbiochem. 3,3′-Diisopropylthiodicarbocyanine [diS-C_3(5)] was from Molecular Probes. Dansylglycine and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were from Sigma, 3-undecyl-2-hydroxy-1,4-naphthoquinone (UHNQ) was from Aldrich, and DEAE-Toyopearl was from Toyo Soda (Tokyo). All other materials were of analytical grade and were obtained from commercial sources.

Bacterial Strain, Growth Conditions, and Preparation of Membranes. *A. aceti* IFO 3284 was grown to the late-logarithmic phase at 30°C in a shaking culture medium consisting of 1% glycerol/0.3% yeast extract/0.2% polypeptone/0.1% KH_2PO_4/0.1% K_2HPO_4. Membranes were prepared by suspending cells in 50 mM potassium phosphate buffer (pH 6.5) containing 5 mM $MgSO_4$ and some DNase, passing them through a French press, and then centrifuging at 80,000 × *g* for 90 min (11).

Purification of Cytochrome a_1. All steps were performed at 0–4°C. Membranes were homogenized with 10 mM potassium phosphate buffer (pH 6.0) at a protein concentration of 10 mg/ml, and Triton X-100 was added to the suspension at a final concentration of 1%. After mixing on ice for 30 min, the suspension was centrifuged at 80,000 × *g* for 60 min. The resultant precipitate was homogenized with 50 mM potassium phosphate buffer (pH 6.5) at a protein concentration of 10 mg/ml, and octyl glucoside was added at a final concentration of 1.25%. The suspension was mixed on ice for 30 min and then centrifuged as described above. The resultant supernatant was directly applied onto a DEAE-Toyopearl

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Abbreviations: Q_n or Q_nH_2, ubiquinone *n* or ubiquinol *n*; octyl glucoside, octyl β-D-glucopyranoside; diS-C_3-(5), 3,3′-diisopropylthiodicarbocyanine; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; UHNQ, 3-undecyl-2-hydroxy-1,4-naphthoquinone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

Table 1. Purification of cytochrome a_1 from the membranes of *A. aceti*

Step	Protein, mg	Q_2H_2 oxidase activity	
		Units	Units/mg
Membrane	118	519	4.4
Washed membrane	89.3	474	5.3
Octyl glucoside extract	18.6	444	23.9
DEAE-Toyopearl	2.8	403	144.0

Enzyme activity is expressed as the activity unit to oxidize 1 μmol of quinol per min at 25°C.

column (≈1-ml bed vol per 2 mg of protein) that had been equilibrated with 50 mM potassium phosphate buffer (pH 6.5). Then, the column was washed with 150 mM potassium phosphate buffer (pH 6.5) containing 1% octyl glucoside at a flow rate of ≈20 ml/hr. The enzyme having Q_2H_2 oxidase activity was eluted at a 3–4 column vol after the elution of nonadsorbed impurities. Fractions having a specific activity >140 units/mg were pooled, concentrated by ultrafiltration (Toyo UK 50 membrane filter), and stored in liquid nitrogen.

Reconstitution of Cytochrome a_1 into Proteoliposomes. Cytochrome a_1 (52 ng) purified as described above was mixed with sonicated *E. coli* phospholipids (5 mg), and octyl glucoside was added to a final concentration of 1.25% in a total vol of 2 ml. The mixture was incubated for 30 min on ice and then dialyzed overnight at 4°C against 500 ml of 50 mM potassium phosphate buffer (pH 6.5). The dialysate was centrifuged at 120,000 × *g* for 3 hr, and the precipitate was suspended with the same buffer in a total vol of 0.4 ml. The suspension was rapidly frozen in liquid nitrogen, thawed, and briefly sonicated immediately before use.

Analytical Procedures. Oxidase activity for Q_2H_2 and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was measured spectrophotometrically at 25°C by following the increase in absorbance at 275 and 520 nm, respectively. The reaction mixture (total, 1 ml) contained 50 mM potassium phosphate buffer (pH 6.5), 30 μM quinol or 3.6 mM TMPD, 0.02% Tween 20, and the enzyme. Activity was calculated by using a millimolar extinction coefficient of 12.25 for quinol or 6.1 for TMPD. Membrane potential (inside negative) and pH gradient (inside alkaline) were measured by following the fluorescence quenching of diS-C_3-(5) and the fluorescence intensity of dansylglycine as described (12). Absorption spectrophotometry was performed with a Hitachi 557 dual-wavelength spectrophotometer. Low-temperature difference spectra were recorded in liquid nitrogen with the same photometer using a cuvette with a 2-mm light path. Heme content was determined by measuring pyridine hemochrome that was prepared by adding pyridine (final concentration, 20%) and then NaOH (final concentration, 0.2 M). Extinction coefficients (reduced − oxidized) used were 20.7 mM^{-1} (555–535 nm) for heme *b* and 21.7 mM^{-1} (586–610 nm) for heme *a* (15). SDS/PAGE was performed in 12.5% acrylamide gels after heating the samples with 2% SDS at 60°C for 30 min. Iron and copper contents were determined by atomic absorption analysis. Protein content was determined by a modified Lowry method (16).

Table 2. Kinetic properties of purified cytochrome a_1

Electron donor	K_m, μM	Turnover no.,* e^- per sec per mol of heme *a*	Inhibitor	K_i, μM
Q_2H_2	19	720–820	KCN	74
TMPD	4500	0.7–0.8	NaN_3	600
			HQNO	4.78
			UHNQ	0.042

*Calculated from the values obtained under standard assay conditions.

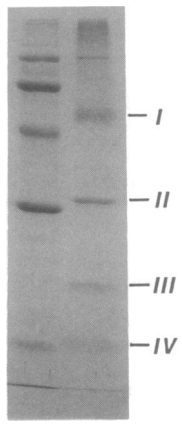

FIG. 1. SDS/PAGE of cytochrome a_1 purified from *A. aceti*. Right lane, purified oxidase (8 μg of protein) was stained with Coomassie blue. Left lane, Coomassie blue-stained marker proteins (2 μg of protein each) of phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme.

RESULTS

Purification and Molecular Properties of Cytochrome a_1. Cytochrome a_1 was solubilized by octyl glucoside from the membranes prewashed with Triton X-100 and then purified by DEAE-Toyopearl column chromatography. This purification procedure is essentially the same as one used for the

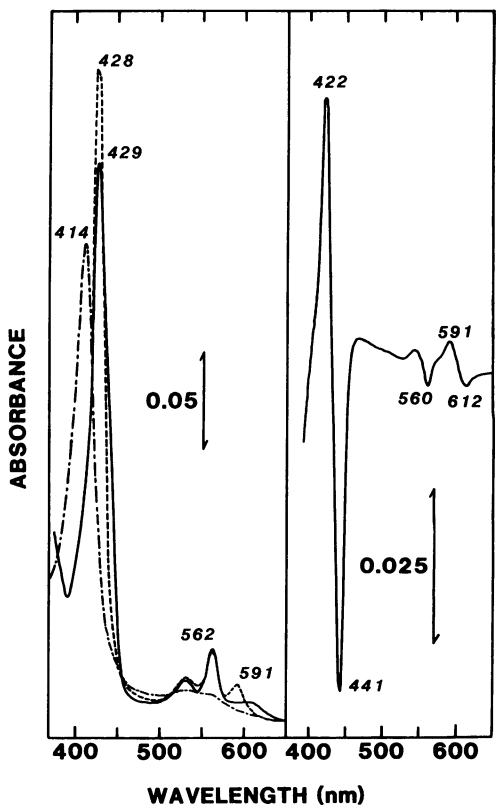

FIG. 2. Absolute absorption spectra and CO difference spectrum of cytochrome a_1 purified from *A. aceti*. Purified oxidase was dissolved in 150 mM potassium phosphate buffer (pH 6.5) containing 1% octyl glucoside at a protein concentration of 0.268 mg/ml. (*Left*) Absorption spectra of the oxidized (-----), reduced (——), and reduced + CO (---) forms are shown. (*Right*) Reduced + CO minus reduced difference spectrum is shown.

purification of cytochrome *o* from *Gluconobacter suboxydans* (12). The principle of the procedure is to use an affinity interaction of such a hydrophobic protein with the column resin (12). Thus, ubiquinol oxidase activity was purified >30-fold from the membranes with high recovery (Table 1). The oxidase thus purified catalyzes the oxidation of ubiquinol, Q_2H_2, of which turnover number is comparable to that of *E. coli* or *G. suboxydans* cytochrome *o* (12, 17, 18) (Table 2), but it exhibits almost negligible activity for TMPD (Table 2) and no activity for reduced horse cytochrome *c* (data not shown). The purified oxidase was intensively inhibited by typical inhibitors for terminal oxidase—cyanide and azide (Table 2). Furthermore, the quinone analogue HQNO or UHNQ was also a potent inhibitor for the oxidase, as in the case of other ubiquinol-oxidizing terminal oxidases (12, 17). As shown in Fig. 1, the purified oxidase exhibits four polypeptides in SDS/PAGE and the apparent molecular masses of these polypeptides were estimated to be 55, 35, 22, and 18 kDa. The molecular size of the first subunit may be underestimated, since the band was diffused and thus suggested to be very hydrophobic by analogy with cytochrome *o* or *d* (12, 17, 19). The molecular size of the native enzyme in the presence of 1% octyl glucoside was examined by sedimentation velocity analysis, in which the oxidase showed a sedimentation coefficient of 6.3 S. Thus, the molecular mass of the enzyme is estimated to be from 130 to 150 kDa. The purified cytochrome contained 6.5 nmol each of hemes *b* and *a* per mg of protein (see below), and 10.7 ± 0.8 nmol of iron and 3.5 ± 0.2 nmol of copper per mg of protein. Judging from the molecular mass, the cytochrome thus appears to consist of 1 mol each of hemes *b* and *a*. Data also suggest that the cytochrome probably contains one copper ion, although the copper content in it is lower than the value expected for one atom and thus needs to be examined more thoroughly.

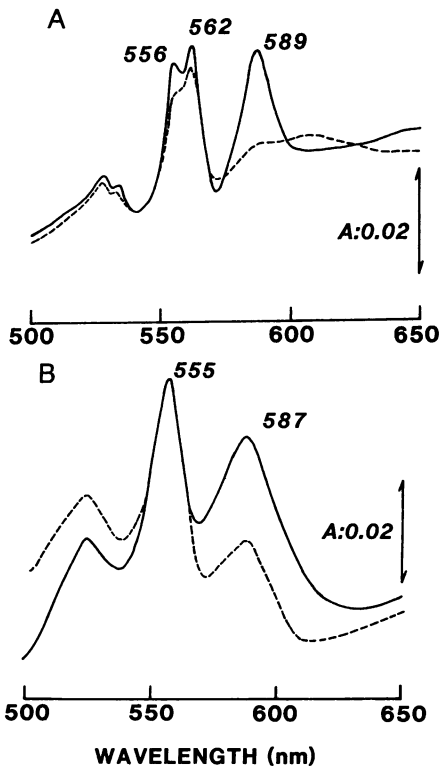

FIG. 3. Low-temperature reduced-minus-oxidized difference spectra (*A*) and pyridine hemochromogen spectra (*B*) of cytochrome a_1 purified from *A. aceti*. (*A*) Purified oxidase (0.76 mg per ml of protein) was reduced with sodium dithionite in the presence (——) or absence (---) of 1 mM cyanide. Thereafter, reduced minus oxidized difference spectra were taken at liquid nitrogen temperature. (*B*) Pyridine hemochromogen was prepared as described with purified oxidase at a protein concentration of 0.268 mg/ml. Difference spectra of the hemochromogen were taken: dithionite reduced minus non-treated (---) and reduced minus ferricyanide oxidized (——).

Spectral Properties of Cytochrome a_1. The absorption spectrum of the purified enzyme exhibits a sharp α band at 562 nm and a small and broad α band around 600–610 nm in the reduced state (Fig. 2). Exposure of the reduced enzyme to CO causes the latter α band to shift to 591 nm and the γ band, which also shifts from 429 to 428 nm, to increase. The CO difference spectrum exhibits a peak at 422 nm and a trough at 441 nm (Fig. 2). The reduced minus oxidized difference spectrum of the enzyme measured at liquid nitrogen temperature exhibits a split α band at 556 and 562 nm and also a small and broad α band from 590 to 610 nm (Fig. 3*A*). Addition of cyanide to the reduced cytochrome induces a dramatic change of the latter band, which turns to a sharp band at 589 nm. Pyridine hemochromogen of the cytochrome exhibits two distinct α bands at 555 and 587 nm, which correspond to hemes *b* and *a*, respectively (Fig. 3*B*).

Reconstitution of Cytochrome a_1 into Proteoliposomes. The purified oxidase was reconstituted into proteoliposomes with phospholipids prepared from *E. coli* by the octyl glucoside dialysis method. Proteoliposomes thus reconstituted exhibited a respiratory control; Q_2H_2 oxidase activity was 88.5 units/mg and 131.2 units/mg in the absence and presence of uncoupler, valinomycin plus nigericin, respectively. The proteoliposomes could generate a membrane potential (inside negative) during oxidation of ubiquinol, Q_1H_2, which can be monitored by fluorescence quenching of the carbocyanine dye diS-C_3-(5). Although data are not shown, the quenching was enhanced with nigericin and diminished with valinomycin; cyanide disturbed the generation of fluorescence quenching. As shown in Fig. 4, the membrane potential generation by the proteoliposomes is dependent on the concentration of quinol, and the maximal value, −147 mV, is obtained at 20 μM. Furthermore, a pH gradient generation was also observed by measuring the fluorescence increase of dansylgly-

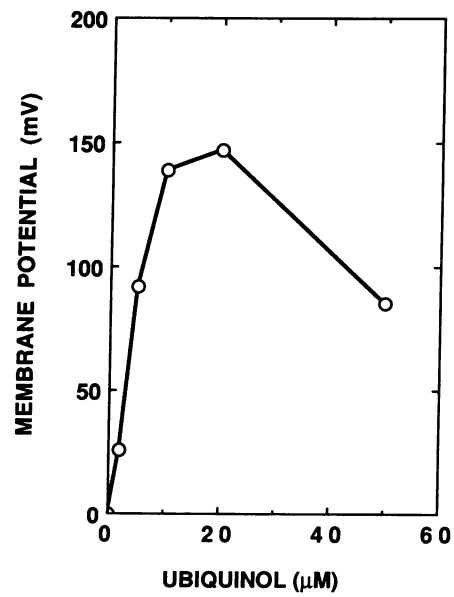

FIG. 4. Ubiquinol-dependent membrane potential generation with proteoliposomes containing cytochrome a_1. The fluorescence quenching of diS-C_3-(5) was measured with proteoliposomes containing 0.37 μg of protein. The reaction was initiated by the addition of given concentrations of Q_1 in the presence of 2 mM dithiothreitol. Membrane potential (mV) was calculated from a calibration curve that was prepared with valinomycin-dependent potassium diffusion potentials (17).

Table 3. Some properties of bacterial *a*-type cytochrome terminal oxidases

Cytochrome	Bacterial strain	Heme present	Absorption maxima			Electron donor	Ref(s).
			Reduced	+ CO (α)	+ CN (α)		
a_1	*A. aceti*	*b* and *a*	429, 562, 590–610	591	589	Ubiquinol	Present work
ba_3	*T. thermophilus*	*b* and *a*	427, (442), 560, 611	593	ND	Not detected	23
aa_3 (a_1)*	*N. europaea*	*a*	443, 597	595	597	Cytochrome c	8, 9
aa_3	*P. denitrificans*	*a*	445, 605	605	ND	Cytochrome c	24
caa_3	Thermophilic PS3	*c* and *a*	416, 444, 550, 604	593	ND	Cytochrome c	25

ND, not determined.
*Originally called a_1, now called aa_3.

cine (data not shown). The proteoliposomes generated the pH gradient of 1.98 (inside alkaline) during oxidation of 20 μM Q_1H_2, which is induced only in the presence of valinomycin.

DISCUSSION

Recent evidence has enabled us to classify the bacterial respiratory chain into two categories; one has cytochrome *c* oxidase at the terminal end and the other has ubiquinol oxidase. The former system is found in the respiratory chain of the so-called "oxidase positive" bacteria such as *Pseudomonas, Azotobacter, Paracoccus*, and so on, where cytochrome aa_3 or *co* functions as the terminal oxidase (for review, see ref. 1). On the other hand, the latter system occurs in "oxidase negative" *E. coli* (for review, see ref. 1) or *G. suboxydans* (12), the respiratory chain of which contains cytochrome *o* or *d*. Recently, *A. aceti* has been shown to contain ubiquinol oxidase, but not cytochrome *c* oxidase, in the terminal end of the respiratory chain, which changes between *o*-type and a_1-like cytochromes depending on the conditions—i.e., shaking or static (unpublished data). The organism in the shaking culture produces a_1-like ubiquinol oxidase, which was characterized in this study.

The data obtained here indicate that the terminal oxidase of *A. aceti* purified in this study is a ubiquinol oxidase that is similar to other ubiquinol oxidases such as cytochrome *o* or *d* in terms of kinetics for ubiquinol or inhibitors (12, 17–20). The ubiquinol oxidase has also been proved to directly react even with native ubiquinol of *A. aceti*, Q_9H_2, which has been shown by reconstitution of ethanol oxidase respiratory chain from quinoprotein alcohol dehydrogenase, Q_9, and the purified oxidase (unpublished data). Furthermore, the oxidase has been shown in this study to be capable of generating an electrochemical proton gradient, the function of which has also been found in other ubiquinol oxidases (12, 17, 21, 22). Thus, it is concluded that the terminal oxidase of *A. aceti* grown in shaking conditions is an energy-generating ubiquinol oxidase.

The results presented here also indicate that the ubiquinol oxidase purified from *A. aceti* is a so-called cytochrome a_1, judging from the following criteria. (*i*) The oxidase contains heme *a* in addition to heme *b*. (*ii*) The enzyme exhibits the weak and broad α band, which is largely intensified and changes to the sharp band at 589 nm after addition of cyanide. As mentioned by Keilin (3), the cyanide-induced intensification of the α band, which may be due to the formation of a compound between ferrous heme and cyanide, is characteristic of cytochrome a_1 as well as the weak α band. (*iii*) The cytochrome *a* component of the enzyme is able to react with CO, which is clearly observed in its CO difference spectrum. Thus, the present work reconfirmed the original evidence that cytochrome a_1 is a terminal oxidase functioning in *Acetobacter* species, although evidence against its occurrence had accumulated (for review, see ref. 1; ref. 9).

Thus, cytochrome a_1 of *A. aceti*, cytochrome *ba* oxidase catalyzing ubiquinol oxidation, is closely related to cytochrome *o* ubiquinol oxidases, where both have four similar polypeptides and the only variation is that the CO-binding component is heme *a* instead of heme *b*. On the other hand, as summarized in Table 3, cytochrome a_1 of *A. aceti* is distinctive from other *a*-type cytochrome terminal oxidases in terms of spectroscopic characters, heme composition, and the electron donor. Cytochrome aa_3-type cytochrome *c* oxidases—e.g., from *Paracoccus denitrificans* or thermophilic bacterium PS3—exhibits a sharp α peak around 605 nm in the reduced state, mainly due to heme *a* but not to heme a_3, in the absence of CO or CN, which is a feature clearly different from cytochrome a_1. Cytochrome aa_3 of *N. europaea*, originally called cytochrome a_1, exhibits an atypical α peak at 597 nm in the reduced state, the wavelength of which is close to that of cytochrome a_1, but the peak of which is sharp without CO or CN and is not affected by their addition. Thus, cytochrome a_1 seems to lack the heme *a* moiety, reacting with reduced cytochrome *c*, present in cytochrome aa_3; instead it has the heme *b* moiety reacting with ubiquinol. Recently, similar species of cytochrome have been purified from *Thermus thermophilus* and termed cytochrome ba_3, which is shown to consist of a single polypeptide of 35 kDa containing one heme *a* molecule, one heme *b* molecule, and two copper ions (23). Since the catalytic function of cytochrome ba_3 has not been identified, however, the relation between both cytochromes purified from *A. aceti* and *T. thermophilus* is obscure at this moment. Anyway, we would like to use the term cytochrome a_1 (ba_1) for the oxidase of *A. aceti* for the historical reason described in the Introduction.

In this study, therefore, the classic cytochrome a_1 originally found by Warburg has been reconfirmed and demonstrated to occur in the respiratory chain of an *Acetobacter* species, *A. aceti*, and furthermore the cytochrome a_1 has been shown to be a ubiquinol oxidase containing both heme *a* and heme *b*. Such a cytochrome a_1 may be widely present in other "oxidase negative" bacteria exhibiting a_1-like spectra or having heme *a*.

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