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Purification of cytochrome a_1c_1 from *Nitrobacter agilis* and characterization of nitrite oxidation system of the bacterium

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Abstract. Cytochrome a_1c_1 was highly purified from *Nitrobacter agilis*. The cytochrome contained heme *a* and heme *c* of equimolar amount, and its reduced form showed absorption peaks at 587, 550, 521, 434 and 416 nm. Molecular weight per heme *a* of the cytochrome was estimated to be approx. 100,000 – 130,000 from the amino acid composition. A similar value was obtained by determining the protein content per heme *a*. The cytochrome molecule was composed of three subunits with molecular weights of 55,000, 29,000 and 19,000, respectively. The 29 kd subunit had heme *c*.

Hemes *a* and *c* of cytochrome a_1c_1 were reduced on addition of nitrite, and the reduced cytochrome was hardly autoxidizable. Exogenously added horse heart cytochrome *c* was reduced by nitrite in the presence of cytochrome a_1c_1; K_m values of cytochrome a_1c_1 for nitrite and *N. agilis* cytochrome *c* were 0.5 mM and and 6 μM, respectively. V_{max} was 1.7 mol ferricytochrome *c* reduced/min · mol of cytochrome a_1c_1. The pH optimum of the reaction was about 8. The nitrite-cytochrome *c* reduction catalyzed by cytochrome a_1c_1 was 61% and 88% inhibited by 44 μM azide and cyanide, respectively. In the presence of 4.4 mM nitrate, the reaction was 89% inhibited. The nitrite-cytochrome *c* reduction catalysed by cytochrome a_1c_1 was 2.5-fold stimulated by 4.5 mM manganous chloride. An activating factor which was present in the crude enzyme preparation stimulated the reaction by 2.8-fold, and presence of both the factor and manganous ion activated the reaction by 7-fold.

Cytochrome a_1c_1 showed also cytochrome *c*-nitrate reductase activity. The pH optimum of the reaction was about 6. The nitrate reductase activity was also stimulated by manganous ions and the activating factor.

Key words: *Nitrobacter agilis* – Cytochrome a_1c_1 – Nitrite dehydrogenase – Nitrate reductase

Nitrobacter agilis acquires energy by oxidizing nitrite to nitrate (Buchanan and Gibbons 1974). Aleem and Nason (1959) proposed an electron transfer system for the oxidation of nitrite in the bacterium; it was composed of cytochromes a_1, aa_3 and *c*. Aleem (1970) and Aleem and Sewell (1981) suggested that cytochrome a_1 might be located at the electron entrance of the system. Cytochrome a_1 is a heme *a*-containing cytochrome which has an α peak around 590 nm (Keilin 1966) and is generally assumed to be a cytochrome oxidase (Morton 1958). However, as the cytochrome has not been purified, nothing has been known about its molecular properties[1]. It has been an important problem to be solved whether cytochrome a_1 of *N. agilis* is a cytochrome oxidase or a nitrite dehydrogenase.

Aleem (1970, 1977) indicated that reduction of cytochrome *c* (horse heart) by nitrite is very difficult since the midpoint redox potential of NO_3^-/NO_2^- system (+0.421 volt) is considerably higher than that of cytochrome *c* (+0.26 volt). They have insisted that reduction of cytochrome *c* by nitrite is an energy-dependent reversed electron transfer mediated by cytochrome a_1, while O'Kelley et al. (1970) have reported that the reduction is energy-independent.

Cytochrome aa_3 (Yamanaka et al. 1979, 1981; Chaudhry et al. 1980) and cytochrome *c* (cytochrome *c*-550) (Yamanaka et al. 1982; Tanaka et al. 1982), which are also the components of the electron transfer system for nitrite oxidation, have been purified and characterized physicochemically and enzymatically. In order to elucidate the electron transfer mechanisms coupled with nitrite oxidation, characterization of another component, cytochrome a_1, seemed to be essential.

In the present studies, cytochrome a_1 was highly purified, and its molecular and enzymatic properties were determined. Although the cytochrome a_1 preparation was electrophoretically almost homogeneous, it was always accompanied by a *c*-type cytochrome which was different from cytochrome *c*-550. The *c*-type cytochrome moiety was not separable from cytochrome a_1 by gel electrophoresis, and it appeared to be cytochrome c_1 on the basis of a similarity to cow cytochrome c_1 in some properties. Therefore, the cytochrome a_1 preparation obtained in the present studies should be called cytochrome a_1c_1.

Materials and methods

Growth and harvest of bacterial cells

Nitrobacter agilis (ATCC 14123) was grown in the medium described by Aleem and Alexander (1958) with slight modifications (Yamanaka et al. 1981). Large-scale cultivation of the organism was performed as previously described in 800 l of the above medium in a stainless steel fermenter of 1000 l capacity (Yamanaka et al. 1981). After 6-day cultivation at 28 – 30° C, the cells were harvested by means of a Sharples continuous centrifuge. Usually, a yield of centrifugally packed cells of about 80 g (23 g dry weight) was obtained from the 800-l cultivation.

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1 Although Erickson et al. (1972) have obtained "cytochrome a_1" from *Nitrosomonas europaea*, it seems to be a modified cytochrome aa_3, because the cytochrome shows its α peak at 600 nm in the cells.

Physical and chemical measurements

Spectrophotometric measurements were performed with a Cary spectrophotometer, model 16 or a Hitachi spectrophotometer, model 124, using 1 cm-light path cuvettes. Polyacrylamide gel electrophoresis in the absence of SDS (sodium dodecyl sulfate) was performed by the methods of Dingjan et al. (1973). In order to recover cytochrome a_1c_1 from the gel after electrophoresis, the green band was cut out, put into dialysis tubing and dialyzed against 10 mM Tris-HCl buffer, pH 8.0 containing 1% Triton X-100 and 1 mM $NaNO_2$. The gel electrophoresis in the presence of SDS was carried out according to Laemmli (1970). The amino acid composition was analyzed with an automated amino acid analyzer (Irica Instruments, Inc., model A-3300, Kyoto, Japan).

Enzyme assays

Nitrite-cytochrome c reductase. Nitrite-cytochrome *c* reductase activity was determined by method of O'Kelley et al. (1970). The assay was performed anaerobically using Thunberg-type cuvettes. The standard reaction mixture consisted of 0.1 ml of 0.8 mM horse ferricytochrome *c*, 2.0 ml of 0.1 M Tris-HCl buffer, pH 8.0, 0.1 ml of 1 M $NaNO_2$ and 0.1 ml of enzyme solution. A total volume of the reaction mixture was 2.25 ml. Reaction was started by addition of $NaNO_2$ or cytochrome *c* or both, and the increase in the absorbance at 550 nm was followed with time. The absorbance change during first 2 min was taken to calculate the enzymatic activity.

Cytochrome c-nitrate reductase. The assay was performed according to Straat and Nason (1965). The reaction mixture contained 0.8 ml of 0.1 M appropriate buffer, 50 µl of 1 M $NaNO_3$, 50 µl of 0.8 mM ferrocytochrome *c* and appropriate amount of enzyme in a total volume of 1.0 ml. The reaction was started by the addition of $NaNO_3$, and the activity was measured by the decrease in the absorbance at 550 nm. Usually, cytochrome *c* oxidase activity of the cytochrome a_1c_1 preparation was negligible as compared with the nitrate reductase activity.

To determine stoichiometric relation between cytochrome *c* oxidized and nitrite formed, higher concentrations of cytochrome *c* were used and a total volume of the reaction mixture was adjusted to 1.0 ml. The reaction was terminated by addition of 0.1 ml of 1% sulfanilic acid dissolved in 3*N* HCl. Then, 0.1 ml of 0.02% naphthylethylenediamine was added, and after 10 min the developed color was measured by the absorbance at 540 nm.

Special reagents

N. agilis cytochromes aa_3 and *c* were prepared as previously described (Yamanaka et al. 1981; Tanaka et al. 1982). Cow cytochrome c_1 was kindly supplied by Mr. Takeda and Dr. H. Matsubara (Osaka University, Osaka, Japan). Horse cytochrome *c* (type VI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Purification of cytochrome a_1c_1

Frozen cells (about 25 g, centrifugally packed state) of *Nitrobacter agilis* were suspended in 25 ml of deionized water, treated with a sonic oscillator (20 kHz, 250 W) for 20 min, and then treated twice with a French pressure cell at 400 kg/cm^2. The suspension obtained was first centrifuged at 3,000 × *g* for 10 min and the resulting supernatant was further centrifuged at 100,000 × *g* for 60 min. The particles obtained between at 3,000 × *g* and 100,000 × *g* were suspended in 80 ml of 5 mM Tris-HCl buffer, pH 8.5. Triton X-100, phenylmethylsulfonylfluoride, $NaNO_2$ and $(NH_4)_2SO_4$ were added to the suspension to the final concentrations of 1%, 1 mM, 1 mM and 0.5 M, respectively. The pH of the suspension was adjusted to 8.5 with 2*N* NaOH. After gentle stirring overnight at 4° C, the suspension was centrifuged at 100,000 × *g* for 30 min. The supernatant thus obtained, which contained cytochromes a_1c_1, aa_3, *c*-550 and *c*-551, was dialyzed against 10 mM Tris-HCl buffer, pH 8.0 containing 1 mM $NaNO_2$ and 0.5% Triton X-100. Presence of nitrite during the preparation was essential to obtain cytochrome a_1c_1. The dialyzed extract was subjected to chromatography on a DE-32 (DEAE-cellulose DE-32, Whatman) column (6 cm × 25 cm) which had been equilibrated with the same solution as used for dialysis. Cytochromes *c*-550 and *c*-551 were passed through the column. The column was washed extensively with the same solution as used for above dialysis containing 20 mM NaCl, and cytochrome aa_3 was eluted slowly by the washing. When the concentration of NaCl in the washing solution was raised to 75 mM, cytochrome a_1c_1 was eluted. Cytochrome a_1c_1 was separated from cytochrome aa_3 by this chromatography with DE-32 column. To the eluate containing cytochrome a_1c_1 were added 1 M Tris-HCl buffer, pH 8.5 and 10% deoxycholate to the final concentrations of 50 mM and 0.25%, respectively. Then, 16 g per 100 ml of solid $(NH_4)_2SO_4$ was added (final, 27% saturation) slowly to the resulting solution. After centrifuged, the pellet was discarded which contained a small amount of cytochrome aa_3. To the resulting supernatant was added $(NH_4)_2SO_4$ to 50% saturation. Cytochrome a_1c_1 was precipitated as oily pellet by centrifugation. The pellet thus obtained was dissolved in a minimal volume of 10 mM Tris-HCl buffer, pH 8.0 containing 1 mM $NaNO_2$. The concentrated cytochrome a_1c_1 preparation was subjected to gel filtration with Sephacryl S-200 column (2.2 cm × 100 cm) which had been equilibrated with 50 mM Tris-HCl buffer, pH 8.5 containing 1 mM $NaNO_2$, 0.5% Triton X-100 and 20% saturated $(NH_4)_2SO_4$. A green pigment which contaminated the preparation was separated in this process. Cytochrome a_1c_1 was eluted at the void volume of the column. The elution profiles from the DE-32 column and the Sephacryl S-200 column are shown in Fig. 1. Cytochrome a_1c_1 fraction eluted from Sephacryl S-200 column was 50% saturated with $(NH_4)_2SO_4$ and the precipitate appeared collected by centrifugation. The pellet obtained was dissolved in a small volume of 10 mM Tris-HCl buffer, pH 8.0 and the resulting solution was desalted on a Sephadex G-25 column which had been equilibrated with 10 mM Tris-HCl buffer, pH 8.0 containing 0.1% Triton X-100. The preparation thus obtained was used as the purified cytochrome a_1c_1.

Results

Purification

Cytochrome a_1c_1 was purified to an electrophoretically almost homogeneous state. Its purification is summarized in Table 1. In the course of the purification, recovery of the

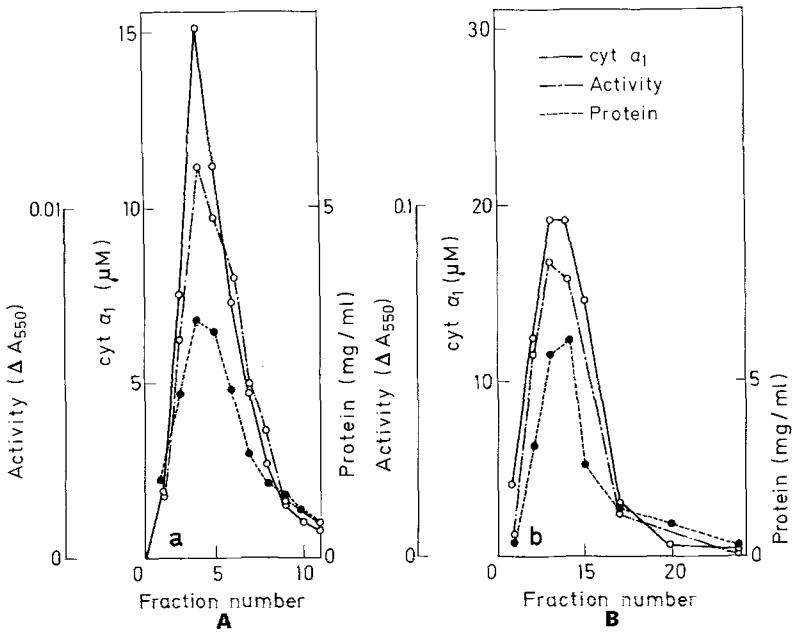

Fig. 1 A, B
Elution profiles of *Nitrobacter agilis* cytochrome a_1c_1 from DE-32 column **A** and Sephacryl S-200 column **B**. Cytochrome a_1 represents cytochrome a_1c_1

Table 1. Summary of purification of *Nitrobacter agilis* cytochrome a_1c_1

Step	Total protein (mg)	Cyt a_1 (nmol) [recovery (%)]	Activitiy[a] [recovery (%)]	Activation by 4.5 mM $MnCl_2$ (%)
Cell free extract	899	1420 [100]	8.19 [100]	70
Membrane fraction	609	1240 [87]	5.07 [62]	67
Solubilized fraction	236	446 [31]	6.80 [83]	58
DE-32	75.1	245 [17]	0.293 [3.6]	270
$(NH_4)_2SO_4$ (27–50% saturation)	43.6	176 [12]	0.330 [4.0]	130
Sephacryl S-200	27.4	160 [11]	–	–
Final preparation	–	141 [9.9]	0.102 [1.2]	270

[a] μmol of Cyt *c* (horse) reduced per min

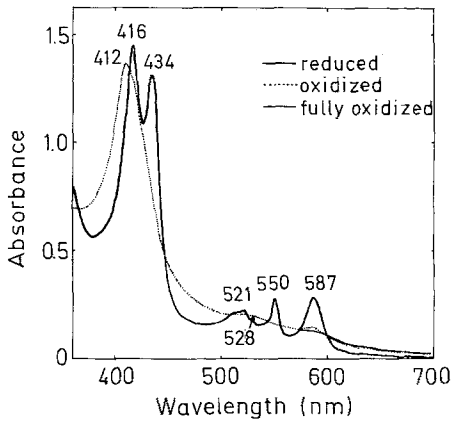

Fig. 2. Absorption spectrum of *Nitrobacter agilis* cytochrome a_1c_1. Oxidized cytochrome was prepared by desalting the ferricyanide-oxidized cytochrome on a Sephadex G-25 column equilibrated with 10 mM Tris-HCl, pH 8.0 containing 0.1% Triton X-100. The reduced cytochrome was prepared by addition of a small amount of dithionite. Fully oxidized means the spectrum determined in the presence of excessive amount of ferricyanide

activity was smaller than that of cytochrome a_1 at the step of chromatography with DE-32 column. The unadsorbed fraction in the DE-32 column chromatography contained a factor which activated cytochrome a_1c_1. Purified cytochrome a_1c_1 was activated by $MnCl_2$, while it was rather inhibited by the salt at earlier steps of the purification (Table 1). In elution profiles from the DE-32 column and Sephacryl S-200 column, cytochrome a_1 concentration, enzymatic activity and protein concentration changed almost parallelly (Fig. 1). Cytochrome a_1c_1 recovered from the gel after polyacrylamide gel electrophoresis in the absence of SDS also had the nitrite-cytochrome *c* activity.

Spectral properties

The cytochrome a_1c_1 preparation contained heme *a* and heme *c* of nearly equal amount based on the absorption spectrum of its pyridine ferrohemochrome. Oxidized cytochrome a_1c_1 showed absorption peaks at 528 and 412 nm. On addition of $Na_2S_2O_4$ to the preparation, peaks appeared at 587, 550, 521, 434 and 416 nm. The absorption spectrum of the cytochrome a_1c_1 are shown in Fig. 2. Addition of CO or KCN to the reduced preparation did not cause any clear spectral changes.

The pyridine ferrohemochrome of cytochrome a_1c_1 showed absorption peaks at 588 and 550 nm in the α peak region. The heme responsible for the absorption peaks at 587 and 434 nm in the reduced cytochrome was extractable with acid acetone (HCl:acetone = 0.1:100), while the heme responsible for the peaks at 550, 521 and 416 nm was not. The results obtained above suggested that cytochrome a_1c_1 had heme *a* and heme *c*. The millimolar extinction coefficient (ε_{mM}) of the α peak at 587 nm of the reduced cytochrome a_1c_1 was determined to be 30 on the basis of ε_{mM} at the α peak of pyridine ferrohemochrome *a* (26, Morrison et al. 1960) and ε_{mM} at 550 nm to be about 24 on the basis of ε_{mM} at the α peak of pyridine ferrohemochrome *c* (29.1, Drabkin 1942).

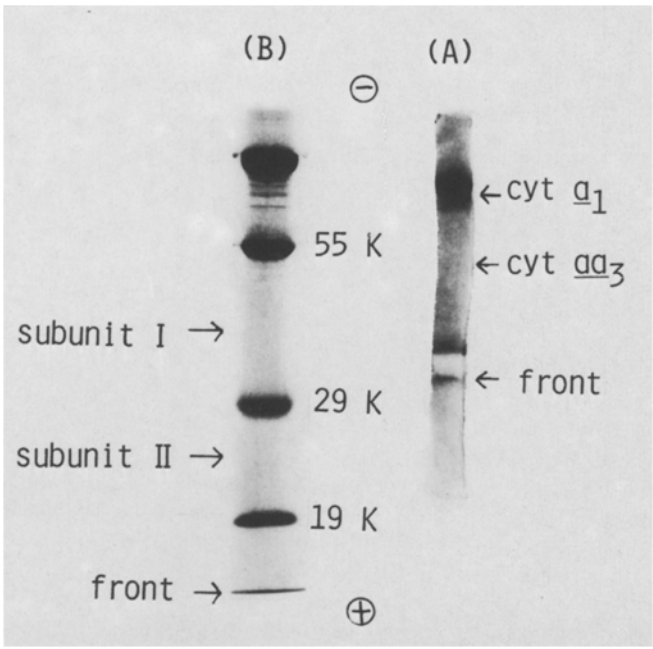

Fig. 3. Polyacrylamide gel electrophoresis of *Nitrobacter agilis* cytochrome a_1c_1 in the absence *A* and presence of SDS *B*. Both gels were stained with Coomassie brilliant blue. In order to show that cytochrome a_1c_1 is distinct from cytochrome aa_3, mobilities corresponding to cytochrome aa_3 and its subunits I and II are shown by arrows. Cytochrome a_1 represents cytochrome a_1c_1

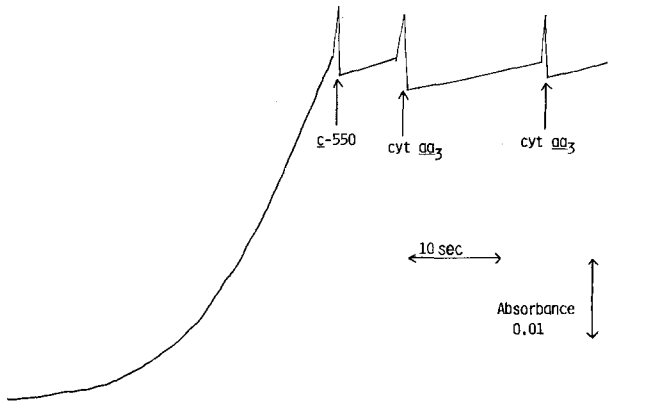

Fig. 4. Oxidation of cytochrome c_1 moiety in *Nitrobacter agilis* cytochrome a_1c_1. Absorbance change at 550 nm was followed with time. Cytochrome a_1c_1 was reduced by additional of a small amount of dithionite. After 3-fold dilution with 10 mM phospate buffer, pH 6.5, *N. agilis* cytochrome aa_3 (2 times) and *N. agilis* cytochrome *c*-550 were added to the final concentrations of 40 nM (each time) and 180 nM, respectively

Heme *c* in the reduced cytochrome a_1c_1 was easily oxidized with potassium ferricyanide, while complete oxidation of heme *a* occurred hardly. On addition of $Na_2S_2O_4$ to cytochrome a_1c_1 which had been oxidized by potassium ferricyanide, heme *c* was fully reduced rapidly while heme *a* was not reduced completely. Addition of a small amount of solid $Na_2S_2O_4$ to cytochrome a_1c_1 which was not treated with ferricyanide, the heme *a* was reduced more easily than heme *c*.

Molecular properties

Cytochrome a_1c_1 was nearly homogeneous in polyacrylamide gel electrophoresis in the absence of SDS (Fig. 3). Its mobility was less than that of cytochrome aa_3. The cytochrome a_1c_1 band in the gel was scarcely stained with Nadi stainining (Keilin 1927), while the cytochrome aa_3 band was well stained. Recovered cytochrome a_1c_1 from the gel still retained heme *a* and heme *c*. When the cytochrome a_1c_1 preparation was subjected to SDS-polyacrylamide gel electrophoresis, 4 main bands appeared in the gel when the gel was stained with Coomassie brilliant blue (Fig. 3). They corresponded to proteins of 100,000, 55,000, 29,000 and 19,000 daltons. The 100 kd band was hardly observed with cruder preparations and became more intensely stained as the purification preceeded, while the intensity in the staining of other three bands was almost constant through the purification steps. Therefore, the 100 kd band was attributed to an aggregate of other protein species. Namely, the cytochrome a_1c_1 molecule seemed to be composed of three kinds of subunits of 55 kd, 29 kd and 19 kd. The 29 kd band sometimes colored in red and was stained with the heme staining (Connelly et al. 1958). Cytochrome a_1c_1 recovered from the gel as mentioned above also showed 4 bands in SDS-polyacrylamide gel electrophoresis.

Molecular weight per heme *a* of cytochrome a_1c_1 was calculated to be about 130,000 from the amino acid composition. The value was roughly consistent with that determined on the basis of the protein and heme contents. Cytochrome c_1 moiety free from cytochrome a_1 was obtained by gel filtration with Sephacryl S-200 of cytochrome a_1c_1 in the presence of mercaptoethanol, while the cytochrome a_1 moiety was not obtained usually.

Enzymatic properties

Although the cytochrome a_1c_1 preparation obtained in the present studies showed a slight cytochrome *c* oxidase activity, the oxidase activity per heme *a* decreased rapidly as the purification proceeded. Therefore, cytochrome a_1 itself in the cytochrome a_1c_1 preparation seemed not to be autoxidizable, and very slow oxidation in air of reduced heme *c* in the preparation was attributed to the presence of cytochrome *c*-550 and cytochrome aa_3 as contaminants. Indeed, heme *c* in cytochrome a_1c_1 was readily oxidized aerobically on addition of a small amount of both cytochrome *c*-550 and cytochrome aa_3 (Fig. 4). The cytochrome c_1 moiety accelerates the nitrite-cytochrome *c* reduction catalyzed by cytochrome a_1c_1. Cow cytochrome c_1 also accelerated the reaction when it was used in place of the cytochrome c_1 moiety.

Heme *a* in the oxidized cytochrome a_1c_1 was reduced slowly on addition of $NaNO_2$ but the reduction did not proceed completely. NaN_3, KCN and $NaNO_3$ inhibited the reduction. Heme *c* in the oxidized cytochrome a_1c_1 was also reduced by $NaNO_2$ but to less extent than the reduction of heme *a*. The reduced heme *a* and heme *c* of cytochrome a_1c_1 were easily oxidized on addition of $NaNO_3$.

Table 2 shows nitrite-cytochrome *c* reductase activity of the cytochrome a_1c_1 preparation. The reduction of cytochrome *c* catalyzed by the preparation proceeded even aerobically. Although the reaction of *Nitrobacter agilis* cytochrome aa_3 with cytochrome *c* has been known to be very dependent on the ionic strength of the reaction mixture

Table 2. Nitrite-cytochrome *c* reduction catalyzed by cytochrome a_1c_1

Reaction mixture	A_{550nm}/15 min		
	No addition	+4.5 mM $MnCl_2$	+4.5 mM $MnCl_2$ + Activating factor[a]
1. Complete (anaerobic)[b]	0.079	0.198	0.553
2. − NO_2^-	0	ND[c]	ND
3. − Cytochrome a_1c_1	0	0	0
4. Aerobic	0.062	ND	ND

[a] Activating factor (see text) of 600 μg was added
[b] Complete system composed of 36 μM horse ferricytochrome *c*, 0.09 M Tris-HCl buffer, pH 8.0, 44 mM $NaNO_2$ and 22 nM cytochrome a_1c_1
[c] ND: not determined

Table 3. Effect of inhibitors on the activity of cytochrome a_1c_1

Inhibitor	Final concentration	Relative activity (%)	Inhibitor	Final concentration	Relative activity (%)
NaN_3	44 μM	39	Iodoacetic acid amide	4.4 mM	82
	8.8 μM	58			
	4.4 μM	72	$NaNO_3$	44 mM	0
KCN	44 μM	12		4.4 mM	11
	8.8 μM	59		0.44 mM	50
	4.4 μM	75		44 μM	89
o-Phenanthroline	0.89 mM	78	Ammonium sulfate	7.3 mM	60
	8.9 mM	72		15 mM	52
NaSCN	0.89 mM	65		36 mM	42
	4.4 mM	70		73 mM	29
CO	bubbling for 2 min	90		150 mM	22

Table 4. Effect of metal salts on the activity of cytochrome a_1c_1

Compound	Final concentration (mM)	Relative activity (%)	Compound	Final concentration (mM)	Relative activity (%)
$MgCl_2$	0.89	96	$NiSO_4$	4.4	71
$MgCl_2$	0.89	93	$ZnSO_4$	4.4	100
+ATP	+0.89		EDTA	4.4	82
$MgCl_2$	4.4	100	$MnCl_2$	4.4	
$MnCl_2$	4.4	340	+ EDTA	+4.4	100
$CaCl_2$	4.4	158	$CaCl_2$	4.4	
$CuSO_4$	4.4	0	+ EDTA	+4.4	125
$CoCl_2$	4.4	59			

(Yamanaka et al. 1981), the nitrite-cytochrome *c* reductase activity of cytochrome a_1c_1 was less dependent on the ionic strength. The pH optimum in the nitrite-cytochrome *c* reduction was around 8.0. The reaction rate in Tris-HCl buffer was faster than in sodium-phosphate buffer at the same concentration and at the same pH.

The purified enzyme was not stable when it was kept in an ice bath; half of its activity was lost in one day, and the absorption attributable to heme *a* decreased slowly. The enzymatic activity of the membrane fraction was stable. The enzyme preparation seemed stable when it was kept in liquid nitrogen.

The activity of cytochrome a_1c_1 was inhibited by low concentrations of KCN and NaN_3 (Table 3). $NaNO_3$ also inhibited the activity and the inhibition was competitive with $NaNO_2$ as shown by Lineweaver-Burk plots (results not shown). O'Kelley et al. (1970) have observed that these reagents inhibit the enzyme activity of a crude extract of *N. agilis*.

Effects on the nitrite-cytochrome *c* reduction by cytochrome a_1c_1 of some metal ions and ATP are summarized in Table 4. ATP did not activate the reaction at all. It did not activate the enzyme activity even in cruder extracts of the bacterium (results not shown). $MnCl_2$ accelerated greatly the reaction and $CaCl_2$ also activated it.

K_m value for nitrite of cytochrome a_1c_1 was calculated to be 0.54 − 2.3 mM and V_{max} was 0.56 − 1.1 mol of ferricytochrome *c* (*N. agilis*) reduced/min · cytochrome a_1. When 4.6 mM $MnCl_2$ was added to the cytochrome a_1c_1 preparation whose K_m and V_{max} values were 0.54 mM and 1.1 mol of ferricytochrome *c* reduced/min · cytochrome a_1, respectively, the values were changed to 0.63 mM and 2.1 mol of ferricytochrome *c* reduced/min · cytochrome a_1, respectively. Specificity for cytochrome *c* of cytochrome a_1c_1 was determined using *N. agilis*, tuna and horse cytochromes *c*; although V_{max} values scarcely varied with these cytochromes *c*, K_m value for *N. agilis* cytochrome *c*-550 was 6.1 μM and much smaller than the values for other two cytochromes *c* (ca. 35 μM).

Reduction of cytochrome *c* by nitrite is expressed as follows.

$$2 \text{ cytochrome } c^{3+} + NO_2^- + H_2O \rightarrow 2 \text{ cytochrome } c^{2+} + NO_3^- + 2H^+$$

Degree of cytochrome *c* reduction was 56% and 36% with 44 mM and 4.4 mM $NaNO_2$, respectively, when the reaction reached the equilibrium. From the results,

$$\frac{[H^+]^2[\text{cytochrome } c^{2+}]^2[NO_3^-]}{[\text{cytochrome } c^{3+}]^2[NO_2^-]}$$

was calculated to be 3.9×10^{-20} M^2 when 10 μM ferricytochrome *c* was used and the reaction was performed at pH 8.0. This value agreed roughly with the value, 1.8×10^{-20} M^2, which was calculated on the basis of thermodynamic data, assuming that $E_{m,7}$ of cytochrome *c* to be +0.25 V and that of NO_3^-/NO_2^- to be +0.42 V.

Cytochrome a_1c_1 also showed a nitrate reductase activity. Ferrocytochrome *c* was oxidized by the cytochrome preparation in the presence of nitrate. The pH optimum of the reaction was around 6. When 1 mol of nitrite was formed from nitrate, 2 mol of ferrocytochrome *c* were oxidized. Oxidation of ferrocytochrome *c* by this enzyme was inhibited by KCN, NaN_3 and higher concentrations of $NaNO_2$. The enzymatic activity was also activated by $MnCl_2$ and the activating factor mentioned in the preceding section.

Characterization of activating factor

A factor which activated nitrite-cytochrome *c* reductase and nitrate reductase activities of cytochrome a_1c_1 was included in the passed solution at the chromatography with DE-32 column as mentioned in the preceding section. When the eluate from the DE-32 column was adjusted to pH 6.5 with dilute acid and charged on a CM-32 column, cytochromes *c*-550 and *c*-551 which contaminated the eluate was adsorbed on the column but the factor was not. The factor was resistant to boiling and overnight trypsin digestion at 40° C, and remained in the inner solution after overnight dialysis against 10 mM Tris-HCl, pH 8.0 using a cellulose dialysis tubing through which substances less than 50 kd come out. It was eluted at the void volume in the Sephacryl S-200 column chromatography.

Discussion

A cytochrome a_1c_1 complex has been purified from *Nitrobacter agilis*. In the complex, the two cytochromes are bound so tightly to each other that they are not separable by polyacrylamide gel electrophoresis. The cytochrome a_1 moiety in the complex shows absorption peaks characteristic of cytochrome a_1; its reduced form has α and γ peaks at 587 and 434 nm, respectively. The cytochrome a_1 in the complex differs greatly from cytochrome aa_3 in various properties although it does have heme *a* as the prosthetic group. Cytochrome a_1c_1 is not autoxidizable, migrates in the gel electrophoresis at different rate from cytochrome aa_3, and its subunit constitution differs from that of cytochrome aa_3. These findings show that cytochrome a_1 in the cytochrome a_1c_1 complex is not derived from cytochrome aa_3 by modification but distinct from the latter.

As judged from inhibition by CN^- of the nitrite-cytochrome *c* reductase activity of cytochrome a_1c_1, it seems probable that the reagent combines with the cytochrome a_1. However, the spectrum of the cytochrome a_1 does not change when CN^- or CO is introduced to the cytochrome a_1c_1 preparation. Cytochrome a_1 of other bacteria, e.g. *Escherichia coli* is probably a terminal oxidase, and different from the cytochrome a_1 of *N. agilis*. Poole et al. (1981) have reported that *E. coli* cytochrome a_1 combines with CO.

The present studies have verified the findings made spectrally with cells or particles that *N. agilis* cytochrome a_1 does not function as a cytochrome oxidase but its acts as a "nitrite dehydrogenase" (Aleem 1970, 1977). Cytochrome a_1c_1 reduces exogenously added cytochrome *c* in the presence of nitrite. Although its molecular activity is very low, this is attributable to the difference in the redox potential between nitrate/nitrite and cytochrome *c*. In vivo, the cytochromes of *N. agilis* will be kept almost completely oxidized by a very active cytochrome aa_3 (Yamanaka et al. 1981). Therefore, it is expected the turnover of cytochrome a_1 is much accelerated in vivo unlike the in vitro experiment with a limited amount of ferricytochrome *c*. Further, the content of cytochrome a_1 in *N. agilis* is very high: ca. 10% of the total cell protein is cytochrome a_1. This will make it possible to transfer much electrons from nitrite to the cytochrome system even though the turnover of cytochrome a_1 is low. As addition of ATP does not affect the enzymatic activity of cytochrome a_1c_1 even with the cruder preparations, the nitrite-cytochrome *c* reduction seems not to require energy.

Cytochrome a_1c_1 shwos also a cytochrome *c* – nitrate reductase activity. As the activity is also observed with the cytochrome a_1c_1 preparation which is recovered after gel electrophoresis, it is obvious that cytochrome a_1c_1 itself, but not a contaminant, has the activity. Thus, the nitrate reductase activity of cytochrome a_1c_1 is inhibited by cyanide and azide, and activated by manganous ion and the activating factor as the case of the nitrite-cytochrome *c* reduction. Further, the nitrate reductase preparation obtained by Straat and Nason (1965) contains cytochrome a_1 although they have not checked if it shows a nitrite-cytochrome *c* reductase activity. The pH optimum differs between the two reactions catalyzed by cytochrome a_1c_1; the nitrite-cytochrome *c* reduction has the pH optimum at pH 8 while the cytochrome *c*-nitrate reduction has it at pH 6. Therefore, for active nitrification by *N. agilis*, the environmental pH should be kept around 8.

Based on the results obtained in the present studies together with findings previously reported (Yamanaka et al. 1981, 1982), the electron transfer system which participates in the nitrite oxidation by *N. agilis* will be as follows:

$$\begin{array}{l} NO_2^- + H_2O \\ \qquad\downarrow \\ NO_3^- \end{array} \longrightarrow \text{cyt } a_1 \rightarrow \text{cyt } c_1 \rightarrow \text{cyt } c \rightarrow \text{cyt } aa_3 \longrightarrow \begin{array}{l} 2H^+ + {}^1/_2O_2 \\ \qquad\downarrow \\ H_2O \end{array}$$

This scheme will be supported by the results obtained by Sundermeyer and Bock (1981) that autotrophic cells of *Nitrobacter winogradskyi* are able to generate ATP by oxidation of nitrite in the presence of rotenone.

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References

Aleem MIH (1970) Oxidation of inorganic nitrogen compounds. Annu Rev Plant Physiol 21:67–90

Aleem MIH (1977) Coupling of energy with electron transfer reactions in chemolithotrophic bacteria. In: Haddock BA, Hamilton WA (eds) Microbial energetics. Cambridge University Press, London, pp 351–381

Aleem MIH, Alexander M (1958) Cell-free nitrification by *Nitrobacter*. J Bacteriol 76:510–514

Aleem MIH, Nason A (1959) Nitrite oxidase, a particulate cytochrome electron transport system from *Nitrobacter*. Biochem Biophys Res Commun 1:323–327

Aleem MIH, Sewell DL (1981) Mechanism of nitrite oxidation and oxidoreductase systems in *Nitrobacter agilis*. Curr Microbiol 5: 267–272

Buchanan RE, Gibbons NE (1974) Bergey's manual of determinative bacteriology, 8th ed. Williams and Wilkins Co, Baltimore

Chaudhry GR, Suzuki I (1980) Cytochrome oxidase of *Nitrobacter agilis*: Isolation by hydrophobic interaction chromatography. Can J Microbiol 26:1270–1274

Connelly JL, Morrison M, Stotz E (1958) Hemins of beef heart muscle. J Biol Chem 233:743–747

Dingjan PG, Postoma T, Stores JAP (1973) Quantitative differentiation of human serum alkaline phosphatase isoenzymes with polyacrylamide disc gel electrophoresis. Z Klin Chem Klin Biochem 11: 167–171

Drabkin DL (1942) Spectrophotometric studies. X. Structural interpretation of the spectra of cyanide, pyridine, and carbon monoxide derivatives of cytochrome *c* and hemoglobin. J Biol Chem 146:605–617

Erickson RH, Hooper AB, Terry K (1972) Solubilization and purification of cytochrome a_1 from *Nitrosomonas*. Biochim Biophys Acta 283:155–166

Keilin D (1927) Influence of carbon monoxide and light on indophenol oxidase of yeast cells. Nature 119:670–671

Keilin D (1966) The history of cell respiration and cytochrome. Cambridge University Press, London

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Morrison M, Connelly J, Petix J, Stotz E (1960) Properties and structural consideration of hemin *a*. J Biol Chem 235:1202–1205

Morton RK (1958) The cytochromes. Rev Pure Appl Chem 8:161–220

O'Kelley JC, Becker GE, Nason A (1970) Characterization of particulate nitrite oxidase and its component activities from the chemoautotroph *Nitrobacter agilis*. Biochim Biophys Acta 205:409–425

Poole RK, Scott RI, Chance B (1981) The light-reversible binding of carbon monoxide to cytochrome a_1 in *Escherichia coli* K12. J Gen Microbiol 125:431–438

Straat PA, Nason A (1965) Characterization of nitrate reductase from chemoautotroph *Nitrobacter agilis*. J Biol Chem 240:1412–1426

Sundermeyer H, Bock E (1981) Energy metabolism of autotrophically and heterotrophically grown cells of *Nitrobacter winogradskyi*. Arch Microbiol 130:250–254

Tanaka Y, Fukumori Y, Yamanaka T (1982) The complete amino acid sequence of *Nitrobacter agilis* cytochrome *c*-550. Biochim Biophys Acta 707:14–20

Yamanaka T, Fujii K, Kamita Y (1979) Subunits of cytochrome *a*-type terminal oxidases derived from *Thiobacillus novellus* and *Nitrobacter agilis*. J Biochem 86:821–824

Yamanaka T, Kamita Y, Fukumori Y (1982) Molecular and enzymatic properties of "cytochrome aa_3"-type terminal oxidase derived from *Nitrobacter agilis*. J Biochem 89:265–273

Yamanaka T, Tanaka Y, Fukumori Y (1982) *Nitrobacter agilis* cytochrome *c*-550: Isolation, physicochemical and enzymatic properties, and primary structure. Plant Cell Physiol 23:441–449

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