Evidence for cytochrome bc_1 complex involvement in nitrite reduction in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*

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Received 19 March 1984

The cytochrome bc_1 complex which has a quinol : cytochrome c_2 oxidoreductase activity was isolated from a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. When duroquinol (tetramethyl-*p*-benzoquinol) was used as the electron donor, maximum nitrite reduction activity was obtained in an anaerobic mixture containing the isolated cytochrome bc_1 complex, nitrite reductase and cytochrome c_2. Nitrite reduction in chromatophore membranes, as well as in the reconstituted system, was inhibited in the presence of antimycin A. No nitrite reduction occurred in the absence of cytochrome c_2. These results indicated that the cytochrome bc_1 complex involved in photosynthetic electron transfer also donates reducing equivalents to the dissimilatory nitrite reductase via cytochrome c_2 in this photodenitrifier.

Cytochrome bc_1 *complex* *Dissimilatory nitrite reduction* *Denitrification*
Rhodopseudomonas sphaeroides

1. INTRODUCTION

A photosynthetic cyclic electron transfer chain of *Rhodopseudomonas sphaeroides* consists of a ubiquinol : cytochrome c_2 oxidoreductase (a cytochrome bc_1 complex), a photochemical reaction center complex and cytochrome c_2 [1]. Recently, the cytochrome bc_1 complex was isolated from *R. sphaeroides* [2–4]. It contains cytochromes *b* and c_1, iron-sulfur protein and ubiquinone as the essential redox components and is analogous to the mitochondrial cytochrome bc_1 complex in terms of components and function [5].

A photodenitrifier, *R. sphaeroides* forma sp. *denitrificans*, can reduce nitrate to nitrogen as an energy-transforming reaction other than oxygen respiration and photosynthesis [6]. The nitrite reductase involved in denitrification uses reduced cytochrome c_2 as the immediate electron donor [7] and a succinate-reduced minus nitrite-oxidized difference spectrum indicates contribution of both *b*- and *c*-type cytochromes in nitrite reduction [8]. From these findings, we have assumed that denitrifying and photosynthetic electron transfer chains share a common electron transfer chain, the cytochrome bc_1 complex and cytochrome c_2 in this photodenitrifier. Recently, participation of the cytochrome bc_1 complex in anaerobic dissimilatory nitrite reduction in denitrification of *Paracoccus denitrificans* was also suggested from evidence that the nitrite reduction is inhibited by antimycin A [9,10].

Here, we confirmed the above prediction by reconstituting the nitrite-reducing system with the cytochrome bc_1 complex, nitrite reductase and cytochrome c_2.

2. MATERIALS AND METHODS

A green mutant strain of *R. sphaeroides* forma sp. *denitrificans* IL-106 [8] was grown under denitrifying conditions in light as in [7].

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

Chromatophore membranes were prepared by sonication as in [11].

The cytochrome bc_1 complex was isolated essentially as in [3] except that the duration of cholate/deoxycholate treatment was shortened to 2 h to obtain higher activity. The final preparations in 50 mM Tris–HCl (pH 8.0), containing 0.05% sodium cholate and 20% glycerol (v/v) were frozen in liquid nitrogen and stored at −20°C.

Nitrite reductase was purified as in [7] and stored in the presence of 50% glycerol (v/v) at −20°C. Cytochrome c_2 was isolated as in [12].

Nitrite reduction activity was assayed at 25°C under argon by determining nitrite consumed in the reaction medium as in [13]. The reaction was initiated by adding 10 μl of 25 mM duroquinol and terminated by adding 20 μl saturated zinc acetate after 20 min incubation. The activity of cytochrome c_2 reduction was measured spectrophotometrically at 550 nm. Cytochrome content was determined from the reduced minus oxidized difference spectrum with ϵ_{mM} values of 20 and 19.1 at the peaks of the α-band for *b*- and *c*-type cytochromes, respectively [14].

Potentiometric titrations and data analyses were carried out as in [11]. The cytochrome bc_1 complex in 3 ml of 50 mM Tris–HCl (pH 8.0)/0.05% sodium cholate in an anaerobic stirred cuvette corresponded to 0.5 μM cytochrome c_1. In titration of cytochrome c_2, it was 0.5 μM in 50 mM Tris–HCl (pH 8.0).

Duroquinol was prepared from duroquinone (Tokyo Chemical Industry Co.) as in [15]. DDC (*N,N*-diethyldithiocarbamate) and antimycin A were purchased from Wako and Sigma, respectively.

3. RESULTS AND DISCUSSION

The isolated cytochrome bc_1 complex contained both *b*- and *c*-type cytochromes whose α-bands were at 559 and 552 nm, respectively. Potentiometric titrations revealed only 3 cytochrome components (table 1). The *c*-type cytochrome was titrated as a single component with an E_m value of +248 mV which agrees well with that for cytochrome c_1 [3] and is distinct from that for cytochrome c_2 (+309 mV). About equal amounts of two *b*-type cytochromes were found with E_m values of −56 and +86 mV compatible with those reported in [3]. The ratio between *b*- and *c*-type cytochromes was almost 1:1 and compatible with that reported in [4], whereas in [2,3] it was reported that the ratio of *b*- and *c*-type cytochromes was 2. The isolated cytochrome bc_1 complex catalyzed cytochrome c_2 reduction with duroquinol as the electron donor, although duroquinol also reduced non-enzymatically cytochrome c_2. The rate of enzymatic electron transfer through the cytochrome bc_1 complex to cytochrome c_2 was calculated to be 3.71 μmol cytochrome $c_2 \cdot$ nmol cytochrome $c_1^{-1} \cdot h^{-1}$, which is about 60% of that reported in [2]. These results indicated that the isolated cytochrome bc_1 complex is comparable to that of *R. sphaeroides* with respect to cytochrome composition and cytochrome c_2 reduction activity.

Table 1

Potentiometric titrations of cytochromes in the isolated cytochrome bc_1 complex from *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*

Cytochrome	$E_{m8.0}$ (mV)	Relative content (%)
b-type	−56	30
	+86	25
c-type	+248	45
c_2	+309	–

The relative contents of cytochromes in the cytochrome bc_1 complex are given as percent of the total cytochrome content. The redox potential of cytochrome c_2 was obtained as a control experiment by titrating the purified cytochrome c_2 in 50 mM Tris–HCl (pH 8.0)

Table 2 shows the nitrite reduction activities in various reaction media. Maximum activity was obtained in the complete reaction medium containing nitrite reductase, cytochrome c_2 and the cytochrome bc_1 complex. The activity in the reaction medium without the cytochrome bc_1 complex was due to non-enzymatic reaction of duroquinol with cytochrome c_2. Direct electron flow to the nitrite reductase from the cytochrome bc_1 complex did not seem to occur, since the activity without cytochrome c_2 was as low as that without both cytochrome c_2 and the cytochrome bc_1 complex, due to direct reaction of duroquinol with nitrite reductase. These results revealed that the

Table 2

Nitrite reduction activities in the various reconstituted systems

Components of reaction medium	Relative activity (%)
Complete	100
Without the cytochrome bc_1 complex	48
Without cytochrome c_2	20
Without the cytochrome bc_1 complex and cytochrome c_2	13
Without nitrite reductase	0

The complete reaction medium (1 ml) consisted of 50 mM sodium phosphate (pH 7.0), 0.05% sodium cholate, 32 μM cytochrome c_2, the cytochrome bc_1 complex (0.15 μM cytochrome c_1), 2.3 μM nitrite reductase, and 0.13 mM KNO_2. The activities are expressed as percent of NO_2^- consumption in the complete system, the activity of which was 2.48 nmol NO_2^- reduced $\cdot$ min^{-1}

cytochrome bc_1 complex and cytochrome c_2 took part in dissimilatory nitrite reduction as well as photosynthetic cyclic electron transfer in denitrifying *R. sphaeroides*.

Chromatophore membranes prepared from cells grown under photodenitrifying conditions contain nitrite reductase [16]. Nitrite reduction in chromatophore membranes, as well as in the reconstituted system, was inhibited in the presence of antimycin A, which is well-known to interrupt electron flow in the cytochrome bc_1 complex (table 3). This also supported the fact that the cytochrome bc_1 complex actually participates in nitrite reduction of the photodenitrifier. In the presence of DDC, an inhibitor of nitrite reductase [7], nitrite reduction in both the reconstituted system and chromatophore membranes was inhibited.

The photodenitrifier was shown by potentiometric titrations to possess the same composition of *b*-type cytochromes as non-denitrifying *R. sphaeroides* [11] and the cytochrome bc_1 complex from the photodenitrifier was also shown here to

Table 3

Effect of inhibitors on nitrite and cytochrome c_2 reduction in the reconstituted system and in chromatophore membranes from *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*

Inhibitor	Relative activity (%)			
	Reconstituted system		Chromatophore membranes	
	Nitrite reduction	Cytochrome c_2 reduction	Nitrite reduction	Cytochrome c_2 reduction
None	100[a]	100[b]	100[c]	100[d]
Antimycin A (50 μM)	34	30	12	8
DDC (100 μM)	12	–	7	–

[a] 5.86 nmol NO_2^- $\cdot$ nmol cyt.c_1^{-1} $\cdot$ min^{-1}
[b] 3.71 μmol cyt.c_2 $\cdot$ nmol cyt.c_1^{-1} $\cdot$ h^{-1}
[c] 3.73 nmol NO_2^- $\cdot$ mg protein^{-1} $\cdot$ min^{-1}
[d] 0.97 μmol cyt.c_2 $\cdot$ mg protein^{-1} $\cdot$ h^{-1}

Reaction medium for cytochrome c_2 reduction consisted of 50 mM potassium phosphate (pH 7.0), 0.05% sodium cholate, 38 μM cytochrome c_2, 0.15 μM cytochrome bc_1 complex and 100 μM duroquinol. Chromatophore membranes were suspended in 50 mM potassium phosphate (0.29 mg protein/ml) containing 38 μM cytochrome c_2 and 100 μM duroquinol. Nitrite reduction in the reconstituted system was determined as described in table 2. The reaction medium for chromatophore membranes consisted of 50 mM potassium phosphate, 0.25 mM KNO_2, chromatophore membranes (0.29 mg protein/ml) and 200 μM duroquinol. The activity is corrected for that without the cytochrome bc_1 complex and expressed as percent of that without an inhibitor

be identical to that of non-denitrifying *R. sphaeroides* with respect to cytochrome components (table 1). Furthermore, the cytochrome bc_1 complex isolated from cells of denitrifying *R. sphaeroides* grown in light in the absence of nitrate (photosynthetic conditions) gave the same nitrite reduction activity in the reconstituted system as listed in table 2 (not shown). Therefore, as far as the nitrite reduction system is concerned, the difference between the photodenitrifier and the non-denitrifying *R. sphaeroides* seems to be only in the possession of nitrite reductase itself. It is interesting from the viewpoint of evolution of biological energy transformation that the cytochrome bc_1 complex also takes part in anaerobic respiration in addition to photosynthesis and oxygen respiration.

ACKNOWLEDGEMENT

We wish to thank Dr Keizo Shimada for helpful suggestions and discussions.

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