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Cytochromes *c* of *Nitrobacter winogradskyi* and *Thiobacillus novellus*: structure, function and evolution

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The amino acid sequences of *Thiobacillus novellus* and *Nitrobacter winogradskyi* cytochromes *c* have been compared with those of cytochromes *c* from several other organisms. The two bacterial cytochromes resemble eukaryotic cytochromes *c*; 49 amino-acid residues are identical between *T. novellus* and horse cytochromes *c*, and 50 residues identical between *N. winogradskyi* and horse cytochromes *c*. However, their reactivity with cow cytochrome *c* oxidase is about 80% lower than the reactivity of eukaryotic cytochromes *c* with the cow mitochondrial oxidase, while they react with yeast cytochrome *c* peroxidase as rapidly as eukaryotic cytochromes *c*. The numbers of identical amino-acid residues between *T. novellus* and animal cytochromes *c* are 45–53 and those between *N. winogradskyi* and animal cytochromes *c* 47–53, while those between the two bacterial cytochromes and yeast and protozoan cytochromes *c* are around 40. Thus, *N. winogradskyi* and *T. novellus* cytochromes *c* are more similar to animal cytochromes *c* than to yeast and protozoan cytochromes *c* on the basis of the amino-acid sequence.

Introduction

Previously, we have found that cytochrome *c*-550 purified from *Thiobacillus novellus* reacts fairly rapidly with cow cytochrome *c* oxidase and does so very rapidly with yeast cytochrome *c* peroxidase, while it reacts very poorly with *Pseudomonas aeruginosa* nitrite reductase (cytochrome cd_1) [1]. Therefore, *T. novellus* cytochrome *c* seems more similar to eukaryotic cytochromes *c* than to 'real' bacterial cytochromes *c* such as *Pseudomonas aeruginosa* cytochrome *c*-551 in terms of reactivity with some oxidoreductases. We have also shown that *Nitrobacter winogradskyi* cytochrome *c*-550 is significantly similar to eukaryotic cytochromes *c* in terms of the primary structure [2]. *N. winogradskyi* cytochrome *c* also reacts rapidly with yeast cytochrome *c* peroxidase [3]. Recently, we have almost completed the amino acid sequencing of *T. novellus* cytochrome *c*.

In the present study, we have compared *T. novellus* and *N. winogradskyi* cytochromes *c* with eukaryotic cytochromes *c* in primary structure and reactivity with a few oxidoreductases. Further, we have tried to consider some evolutionary relation of the two chemolithotrophs to other organisms on the basis of the primary structures of their cytochromes.

Materials and Methods

Cytochromes *c* of *T. novellus* and *N. winogradskyi* were purified according to the methods described in Refs. 1 and 3, respectively. The amino acid sequence of *N. winogradskyi* cytochrome *c* was determined by the method as previously described [2]. The N-terminal amino-acid sequences of the peptides derived from *T. novellus* cytochrome *c* were determined by a gas-phase protein sequencer (Applied Biosystems, U.S.A., Model 470A). The details of the sequencing of the cytochrome will be published elsewhere. Cytochrome *c* oxidases of cow, *N. winogradskyi* and *T. novellus* were purified according to Refs. 4, 5 and 6, respectively. Nitrite reductase of *Pseudomonas aeruginosa* was purified by the method as previously described [7], and yeast cytochrome *c* peroxidase [8] was kindly supplied by Dr. T. Yonetani (University of Pennsylvania, U.S.A.).

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Results and Discussion

Structural aspects

From *N. winogradskyi*, two *c*-type cytochromes have been purified; one is soluble cytochrome *c*-550 [2,3] and the other membrane-bound cytochrome *c*-550 (unpublished results). The purification procedure and properties of the latter cytochrome will be published elsewhere. In the present study, the amino-acid sequence of soluble cytochrome *c* [2] has been compared with those of *T. novellus* cytochrome *c*-550 and other cytochromes *c*.

The amino-acid sequence of *N. winogradskyi* cytochrome *c* much resembles *Rhodopseudomonas viridis* cytochrome c_2 [9] and eukaryotic cytochromes *c*, as shown in Fig. 1; 65 and 50 residues are identical between *N. winogradskyi* and *R. viridis* cytochromes *c* [9], and between *N. winogradskyi* and horse cytochromes *c* [10], respectively, if one gap is inserted into 12th position. *N. winogradskyi* cytochrome *c* is the least similar to *Tetrahymena pyriformis* cytochrome *c* [11] in the amino-acid sequence among the cytochromes *c* listed in Fig. 1.

The amino-acid sequence of *T. novellus* cytochrome *c*-550 has been determined except a few residues of the N-terminus, as shown in Fig. 1. *T. novellus* cytochrome *c* is well aligned with *R. viridis*, *N. winogradskyi* and eukaryotic cytochromes *c* if one gap is inserted into 12th position in the numbering on the basis of horse cytochrome *c*. One characteristic for the sequence of *T. novellus* cytochrome *c* is that it has three extra residues between 54th and 55th positions of other cytochromes listed in Fig. 1. This insertion of three residues has been also observed with *Agrobacterium tumefaciens* cytochrome c_2 [12]. The amino-acid sequence of *T. novellus* cytochrome *c* most resembles that of *R. viridis* cytochrome c_2 so far known. It is also similar to those of *N. winogradskyi*, horse and *S. cerevisiae* iso-1-cytochromes *c* [13,14]. On the basis of the sequence, *T. novellus* cytochrome *c* is the least similar to *T. pyriformis* cytochrome *c* among the cytochromes listed in Fig. 1.

Functional aspects

As the primary structures of *N. winogradskyi* and *T. novellus* cytochromes *c* are similar to those of eukaryotic cytochromes *c*, it is expected that the two bacterial cytochromes react rapidly with cow cytochrome *c* oxidase and yeast cytochrome *c* peroxidase. In fact, the reactivity with the peroxidase of the two bacterial cytochromes *c* is as high as that of eukaryotic cytochromes *c* [1,3]. However, the reactivity with cow cytochrome *c* oxidase of the two bacterial cytochromes *c* is considerably lower than that of eukaryotic cytochromes *c*. Many lysine residues on the horse cytochrome *c* molecule have been suggested to be necessary for its reaction with cow cytochrome *c* oxidase [15, 16] and yeast cytochrome *c* peroxidase [17]; lysine residues at positions 7, 8, 13, 25, 27, 79, 86 and 87 are important for horse cytochrome *c* to react with the cow oxidase, and the residues at positions 13, 27, 72, 86 and 87 are important for the cytochrome to react with the yeast peroxidase. As shown in Table I, *N. winogradskyi* cytochrome *c* has all the lysine residues as mentioned above, except Lys-8, while *T. novellus* cytochrome *c* has all the residues except Lys-7, Lys-8 and Lys-86. From these results, Lys-7 or a pair of Lys-7 and Lys-8, appears very important for cytochrome *c* to react with cow cytochrome

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                                                  10        20        30        40
(A) T.novellus c-550  [present study][a]      ??PAKGANVFW-KCMACHAVGEGAKNKVGPELNGIIGRKM
(B) R.viridis c2  [9,25]                      QDAASGEQVFKQ-CLVCHSIGPGAKNKVGPVLNGLFGRHS
(C) Horse c  [10]                             GDVEKGKKIFVQKCAQCHTVEKGGKHKTGPNLHGLFGRKT
(D) N.winogradskyi c-550  [2]                 GDVEAGKAAFN-KCKACHEIGESAKNKVGPELNGLDGRHS
(E) S.cerevisiae iso-1- c  [13,14]      TEFKAGSAKKGATLFKTRCLQCHTVEKGGPHKVGPNLHGIFGRHS
(F) C.krusei c  [20]                  PAPFEQGSAKKGATLFKTRCAECHTIEAGGPHKVGPNLHGIFSRHS
(G) T.pyriformis c-553  [11] GPKEPEVTVPEGDASAGRDIFDSQCSACHAIE--GDSTAAPVLGGVIGRKA
(H) A.tumefaciens c2  [12]                    EGDVAKGEAAFKR-CSACHAIGEGAKNKVGPQLNGIIGRTA

                  50        60        70        80        90        100       107
(A)  GSIEGFNYSDTLKEHNAKGDVWTAEILSQYLANPKGYMPGVKMVFAGLPKEIRADDLEAYLKTFNADGTK
(B)  GTIEGFAYSDANKN---SGITWTEEVFREYIRDPKAKIPGTKMIFAGVKDEQKVSDLIAYIKQFNADGSKK
(C)  GQAPGFTYTDANKN---KGITWKEETLMEYLENPKKYIPGTKMIFAGIKKKTEREDLIAYLKKATNE
(D)  GAVEGYAYSPANKA---SGITWTEAEFKEYIKDPKAKVPGTKMVFAGIKKDSELDNLWAYVSQFDKDGKVKAK
(E)  GQAEGYSYTDANIK---KNVLWDENNMSEYLTNPKKYIPGTKMAFGGLKKEKDRNDLITYLKKACE
(F)  GQAEGYSYTDANIR---AGVEWAEPTMSDYLENPKKYIPGTKMAFGGLKKAKDRNDLVTYMLEASK
(G)  GQEK-FAYSKGMKG---SGITWNEKHLFVFLKNPSKHVPGTKMAFAGLPADKDRADLIAYLKSV
(H)  GGDPDYNYSNAMKKAGGEGLVWTPQELRDFLSAPKKKIPGNKMALAGISKPEELDNLIAYLIFSASSKPAZ
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Fig. 1. Amino-acid sequences of *T. novellus* and *N. winogradskyi* cytochromes *c* aligned with those of cytochromes *c* from several other organisms.
[a] ? means unidentified residue.

TABLE I

Correlation of distribution in the cytochrome c molecules of lysine residues important for their reaction with mitochondrial cytochrome c oxidase and yeast cytochrome c peroxidase

Lys-73 was omitted because its importance in the reaction with mitochondrial oxidase varies between experiments. ○, presence; ×, absence. Reactivities are relative values.

Lysine residue (No.)	Source of cytochrome *c*				
	horse [10]	*N. winogradskyi* [2]	*T. novellus* (present study)	*P. denitrificans* [23]	*R. rubrum* [24]
7	○	○	×	×	×
8	○	×	×	○	○
13	○	○	○	○	○
25	○	○	○	○	×
27	○	○	○	○	○
72	○	○	○	○	○
86	○	○	×	×	×
87	○	○	○	○	○
Reactivity with					
Cow cytochrome *c* oxidase	**100**	16	23	3.7	8.5
Yeast cytochrome *c* peroxidase [a]	**100**	90	108	35	17
T. novellus cytochrome *c* oxidase [b]	31	132	**100**	3.3	3.5
N. winogradskyi cytochrome *c* oxidase [c]	29	**100**	31	n.d. [d]	1.6

Reactions were determined in phosphate buffer of concentration:
[a] 40 mM (pH 6.5).
[b] 20 mM (pH 6.5).
[c] 10 mM (pH 6.0).
[d] Not determined.

c oxidase if the residues other than the lysine residues mentioned above affect only slightly the reactivity of the cytochrome. As the two bacterial cytochromes *c* react with yeast cytochrome *c* peroxidase as rapidly as eukaryotic cytochromes *c*, the suggestion seems really acceptable that the four lysine residues at the positions mentioned above except for Lys-86 are necessary for cytochrome *c* to react with the peroxidase.

T. novellus cytochrome *c* oxidase reacts rapidly with tuna [18], *Saccharomyces oviformis* (= *S. cerevisiae*) [13,14] and *Candida krusei* [19] cytochromes *c* as well as with *T. novellus* cytochrome *c*, while it reacts poorly with cow [20] and horse [10] cytochromes *c* [21]. Further, human cytochrome *c* [22] reacts with the oxidase fairly rapidly [21]. Therefore, we have previously suggested that Tyr-46 in addition to lysine residues mentioned above will be important for cytochrome *c* to react with the *T. novellus* oxidase [21]. However, *T. novellus* cytochrome *c* which reacts rapidly with the oxidase has Phe-46 in place of Tyr-46. This result may be attributable to the extra three residues inserted between positions 54 and 55 of the bacterial cytochrome. It is therefore expected that Tyr-48 in the bacterial cytochrome may be located at the position corresponding to 46th of other cytochromes *c* mentioned above. Although the sequences of *T. novellus* and *N. winogradskyi* cytochromes *c* resemble each other and these cytochromes have the lysine residues important for the reaction with yeast cytochrome *c* peroxidase, the reactivities of the two cytochromes with cytochrome *c* oxidases from the two bacteria are quite different from each other (Table I). The oxidases seem therefore to recognize very minute difference in the conformation of the cytochrome *c* molecule.

Evolutional aspects

As seen from Fig. 1, 50 residues are identical between *N. winogradskyi* and horse cytochromes *c*, and 49 residues identical between *T. novellus* and horse cytochromes *c*. Thus, the two bacterial cytochromes *c* are homologous to eukaryotic cytochrome *c*. However, it seems very interesting that numbers of identical amino-acid residues are, for example, 39 between *S. cerevisiae* and *N. winogradskyi* cytochromes *c* and between *T. pyriformis* and the bacterial cytochromes *c*, respectively (Fig. 1). Thus, *N. winogradskyi* cytochrome *c* is more similar to animal cytochromes *c* than to yeast and protozoan cytochromes *c* (detailed results not shown). These findings seem very curious; *N. winogradskyi* is evolutionarily closer to animals than to yeast and protozoan on the basis of the amino-acid sequences of cytochrome *c*.

The identical amino-acid residues between *T. novellus* cytochrome *c* and animal cytochromes *c* are 45–53 (detailed results not shown), although the identical residues are for example, 41 and 40 between *C. krusei* and the bacterial cytochromes *c* and between *T. pyriformis* and the bacterial cytochromes *c*, respectively. Therefore, it may be said that *T. novellus* is also evolutionarily more similar to animal than to yeast and protozoan on the basis of the amino-acid sequence of cytochrome *c*.

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