THIOBACILLUS NOVELLUS CYTOCHROME OXIDASE CAN SEPARATE SOME EUCARYOTIC CYTOCHROMES *c*

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1. Introduction

There is a distinct biological specificity in the reaction of certain redox enzymes with cytochrome *c* and it reflects very well the evolutionary relationship of the organism from which cytochrome *c* is isolated [1–3]. Most eucaryotic cytochromes *c* react very rapidly with cow cytochrome oxidase (EC 1.9.3.1) and yeast cytochrome *c* peroxidase (EC 1.11.1.5) but react very poorly with *Pseudomonas aeruginosa* nitrite reductase (previously called *Pseudomonas* cytochrome oxidase, EC 1.9.3.2), while many procaryotic cytochromes *c* react very poorly with the oxidase and the peroxidase but react rapidly with the reductase. In the previous studies [1], we have predicted that there may occur cytochrome oxidases which are situated between the reductase and the oxidase in terms of the reactivity with cytochromes *c*.

In the present investigation, we purified cytochrome oxidase from *Thiobacillus novellus* and determined its specificity for cytochrome *c* as the electron donor. The bacterial oxidase has haem a as the prosthetic group and its absorption spectrum is very similar to that of mammalian cytochrome oxidase. However, its specificity for cytochrome *c* is considerably different from that of cow cytochrome oxidase.

2. Materials and methods

2.1. *Purification of* T. novellus *cytochrome oxidase and cytochromes*

T. novellus cytochrome oxidase was extracted from the autotrophic cells [4] with 0.2 M phosphate at pH 7.0 containing 0.5% Triton X-100, and the extract obtained fractionated with ammonium sulphate. The precipitate, formed between 35% and 40% saturation of the salt, was dissolved in 0.1 M Tris–HCl buffer, pH 8.5, containing 0.5% Triton X-100. The resulting solution was dialysed against the same buffer as used in the dissolution as described above. The dialysate thus obtained was charged on the DEAE-cellulose column which had been equilibrated with the same buffer as used above. Cytochrome oxidase was adsorbed on the column. After the column was washed with the same buffer as used above, the enzyme adsorbed on the column was eluted with 0.1 M Tris–HCl buffer, pH 8.5, containing 0.5% Triton X-100 and 0.2 M NaCl. The oxidase moved down gradually on the column as a green band and was eluted. The eluate thus obtained was used as the oxidase preparation. The detailed purification procedure of the oxidase will be reported elsewhere.

Highly purified cytochrome *c* (550, *Thiobacillus novellus*) [4], cytochrome *c* (551, *Pseudomonas aeruginosa*) [5], cytochrome *c* (555, *Chlorobium thiosulfatophilum*) [6], cytochrome *c* (552, *Nitrosomonas europaea*) [21] cow cytochrome *c* [7], tuna cytochrome *c* [8] and man cytochrome *c* [9] were obtained by the methods previously established. Cytochrome *c* (*Saccharomyces oviformis*) (*iso*-1-) [10] and cytochrome c_2 (*Rhodospirillum rubrum*) [11] were kindly supplied by Sankyo Co. Ltd (Tokyo, Japan) and Dr T. Horio (Institute for Protein Research, Osaka University, Osaka, Japan), respectively. Horse cytochrome *c* (Type VI) was purchased from Sigma Chemical Co. (USA). Cytochromes *c* were reduced on addition of a small amount of

$Na_2S_2O_4$ and dialysed against 5 mM sodium phosphate buffer, at pH 5.5, for 2 days in a refrigerator.

2.2. *Assay of enzyme activity*

Reactivity of cytochromes *c* with *T. novellus* cytochrome oxidase was determined spectrophotomterically. To a 1.0 ml a solution of each cytochrome *c* was added 0.05 ml 0.45 μM cytochrome oxidase, and the decrease in the absorbance at the α-peak of each cytochrome *c* was followed with time. The concentrations of cytochromes *c* were made substantially constant (12–15 μM). The pH optimum of the reaction was at pH 5.5 regardless of the kind of cytochrome *c*. The spectrophotometric determinations were performed in a Cary spectrophotometer, model 15.

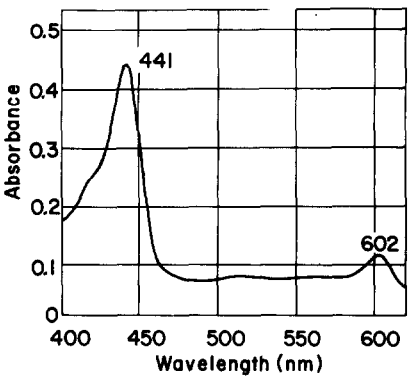

Fig.1. Absorption spectrum of purified *T. novellus* cytochrome oxidase. The oxidase was dissolved in 50 mM Tris–HCl buffer, at pH 8.5, containing 0.5% Triton X-100 and reduced with $Na_2S_2O_4$.

3. Results

As fig.1 shows, *T. novellus* cytochrome oxidase showed an absorption spectrum similar to that of cow cytochrome oxidase [12]; there were peaks at 602 nm and 441 nm in the reduced form. The pyridine ferrohaemochrome of the enzyme showed absorption peaks at 588 nm and 430 nm. This suggests that the enzyme has haem a as the prosthetic group. The molecular features of the enzyme other than the spectral properties as mentioned above are now under investigation and will be published elsewhere.

The enzyme reacted rapidly with cytochrome *c* (550, *T. novellus*), yeast cytochrome *c* and tuna cytochrome *c*, while it reacted very poorly with cytochromes *c* derived from bacteria such as *P. aeruginosa, N. europaea* and *R. rubrum* (table 1).

Table 1
Reactivity of various cytochromes *c* with *Thiobacillus novellus* cytochrome oxidase

Organism	α-Peak (nm)	Relative reactivity		
		P. aeruginosa nitrite reductase[a]	*T. novellus* cytochrome oxidase	Cow cytochrome oxidase[a]
P. aeruginosa	551	100	0.78	0
N. europaea	552	56	0	0
C. thiosulfatophilum	555	55	18.1	19
R. rubrum	550	2.0	1.5	8.5
T. novellus	550	6.0	100	23
S. oviformis	550	4.9	77.1	100
Tuna	550	8.7	93.3	93
Man	550	0.44	13.8	107
Cow	550	0.53	4.7	73
Horse	550	2.5	4.7	124

[a] Cited from ref. [1]

The reactions were performed in 50 mM phosphate buffer at pH 5.5. To 1.0 ml cytochrome *c* solution (12–15 μM) was added 0.03 ml 0.45 μM oxidase, and the decrease in the absorbance at the α-peak of each cytochrome *c* was spectrophotometrically followed with time. The reactivity of cytochrome *c* was expressed as relative value; the molecular activity (mol cytochrome *c* oxidized/mol enzyme) per min was taken as 100% which was observed when the enzyme reacted with *T. novellus* cytochrome *c*.

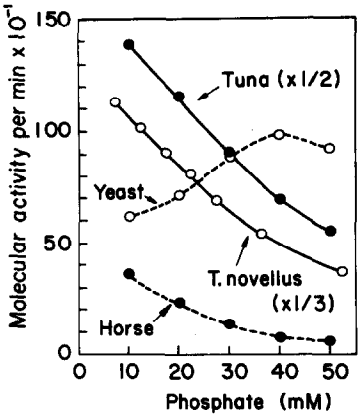

Fig.2. Effect of concentration of phosphate on the reaction rates of cytochromes *c* with *T. novellus* cytochrome oxidase. The reaction conditions were the same as those described in table 1. except that phosphate buffers of various concentrations were used. The values of the molecular activity with *T. novellus* and tuna cytochromes *c* are presented in the reduced scales as indicated in the parentheses.

Surprisingly, it reacted very slowly with cow and horse cytochromes *c* in 50 mM phosphate buffer. Although man cytochrome *c* reacted with the enzyme very slowly its reaction rate was appreciably larger than those of horse and cow cytochromes *c*. The molecular activity (moles of cytochrome *c* reoxidized/mole of haem a in the enzyme) of the oxidase was 2270 per min with *T. novellus* cytochrome *c* in 10 mM phosphate buffer at pH 5.5 and at 18°C.

The reaction rate in the oxidation of cytochrome *c* catalysed by the oxidase was affected greatly by salt concentration. As fig.2 shows, the reaction rates with *T. novellus*, tuna and horse cytochromes *c* decreased greatly as the concentration of phosphate increased. Yeast cytochrome *c* behaved differently; its reaction rate increased with the concentration of phosphate when the salt concentration was below about 40 mM and then decreased with further increase of the salt concentration. Sodium chloride affected also the reaction rate in a similar way as phosphate.

4. Discussion

Haem a containing cytochromes have been obtained from several bacteria; from *Nitrosomonas europaea* [13], *Brevibacterium thiogenitalis* [14], *Mycobacterium phlei* [15] and *Thiobacillus ferrooxidans* [16]. Among them, the oxidases derived from *N. europaea* and *T. ferrooxidans* are known to show the cytochrome oxidase activity although their specificity for cytochrome *c* as the electron donor has not yet been determined. *T. novellus* cytochrome oxidase has haem a as the prosthetic group and reacts rapidly with some of eucaryotic cytochromes *c* as well as with *T. novellus* cytochrome *c*. In these respects, the enzyme is quite similar to the mammalian cytochrome oxidase. However, its specificity for cytochrome *c* as the electron donor differs greatly from that of the mammalian oxidase; the kind of cytochrome *c* which reacts most rapidly with the enzyme appears to shift to the more primitive side in the evolutionary sense as compared to the case of the mammalian oxidase. Namely, the reactivity with the cow oxidase of eucaryotic cytochrome *c* does not substantially vary with the kind of the cytochrome [1,17], while that with the bacterial oxidase reaches a maximum with *T. novellus*, yeast and tuna cytochromes *c*, and then decreases when man, horse and cow cytochromes *c* are used as the electron donors (table 1).

We have determined the reactivities of various kinds of cytochromes *c* with *P. aeruginosa* nitrite reductase and cow cytochrome oxidase [1,2]. As a result, we have reached the conclusion that cytochrome *c* may have coevolved with cytochrome oxidase, and predicted that there might occur various kinds of cytochrome oxidases with different specificities for cytochrome *c* as the electron donor. The present investigation has verified the prediction to some extent.

It seems very interesting that the *T. novellus* oxidase can distinguish some of cytochromes *c* derived from vertebrates from one another. As has been already indicated, cytochromes *c* of bony marine fish differ from the cytochromes of the other vertebrates in that they react with *P. aeruginosa* nitrite reductase at an appreciable rate [1,2]. The reactivity with the bacterial oxidase has shown clearly that tuna cytochrome *c* is distinguishable from cytochromes *c* of the other vertebrates. It appears very interesting that man cytpchrome *c* is distinguished from horse and cow cytochromes *c* in the reactivity with the bacterial oxidase. If we suppose that the more reactive is cytochrome *c* with the enzyme the more

evolutionarily close to the bacterium is the parent organism, man seems closer to the bacterium than horse and cow in terms of the properties of cytochrome *c*.

When we compare primary structures of horse and cow cytochromes *c* with those of tuna and yeast cytochromes *c*, we find that the amino acid residue at 46th is different between the two groups. That is that the 46th residue is phenylalanine in the former group, while it is tyrosine in the latter group. The importance of the 46th tyrosine residue in the reaction of cytochrome *c* with the bacterial oxidase seems to be supported by the fact that man cytochrome *c* which has also the tyrosine residue reacts with the enzyme fairly rapidly. Recently, Kihara et al. [18] have shown that the 46th residue may play an important role on the conformational change of ferricytochrome *c* during the alkaline isomerization.

The reaction of cytochrome *c* with the *T. novellus* oxidase is affected greatly by salt concentration. The similar result is obtained with a cytochrome oxidase preparation from *Rhodopseudomonas palustris* [19]. The reaction with cow cytochrome oxidase is also affected in a similar way by salt concentration when concentration of cytochrome *c* used as the electron donor is very low [20]. In the reaction with the *T. novellus* oxidase of many cytochromes *c*, the reaction rate decreases simply as the salt concentration increases, while the rate yeast cytochrome *c* reaches a maximum and then decreases when the salt concentration is increased. In this respect, yeast cytochrome *c* differs considerably from other eucaryotic cytochromes *c* tested. The cause for this difference should be elucidated in future investigation.

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References

[1] Yamanaka, T. (1972) Advan. Biophys. (Tokyo) 3, 227–276.
[2] Yamanaka, T. (1973) Space Life Sci. 4, 490–504.
[3] Yamanaka, T. (1975) J. Biochem. (Tokyo) 77, 493–499.
[4] Yamanaka, T., Takenami, S., Akiyama, N. and Okunuki, K. (1971) J. Biochem. (Tokyo) 70, 349–358.
[5] Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Nakai, M. and Okunuki, K. (1960) Biochem. J. 77, 194–210.
[6] Yamanaka, T. and Okunuki, K. (1968) J. Biochem. (Tokyo) 63, 341–346.
[7] Yamanaka, T., Mizushima, H., Nozaki, M., Horio, T. and Okunuki, K. (1959) J. Biochem. (Tokyo) 46, 121–132.
[8] Hagihara, B., Tagawa, K., Morikawa, I., Shin, M. and Okunuki, K. (1958) J. Biochem. (Tokyo) 45, 725–735.
[9] Yamanaka, T. and Okunuki, K. (1964) J. Biol. Chem. 239, 1813–1817.
[10] Motonaga, K., Misaka, E., Nakajima, E., Ueda, S. and Nakanishi, K. (1965) J. Biochem. (Tokyo) 57, 22–28.
[11] Horio, T. and Kamen, M. D. (1961) Biochim. Biophys. Acta 48, 266–286.
[12] Okunuki, K. (1966) in: Comprehensive Biochemistry (Stotz, E. E. and Florkin, M. eds) pp. 232–308, Elsevier, Amsterdam.
[13] Erickson, R. H., Hooper, A. B. and Terry, K. (1972) Biochim. Biophys. Acta 283, 155–166.
[14] Sugiyama, Y., Kitano, K. and Kanzaki, T. (1973) Agr. Biol. Chem. 37, 1607–1612.
[15] Revsin, B., Marquez, E. D. and Brodie, A. F. (1970) Arch. Biochem. Biophys. 139, 114–120.
[16] Imai, K., Sugio, T. and Tano, T. (1976) Abstr. 5th Intern. Fermentation Symp. (Berlin) p. 453.
[17] Smith, L., Nava, M. E. and Margoliash, E. (1973) in: Oxidases and Related Redox Systems (King, T. E., Mason, H. S. and Morrison, M. eds) pp. 629–638, Univ. Park Press, Baltimore.
[18] Kihara, H., Saigo, S., Nakatani, H., Hiromi, K., Ikeda-Saito, M. and Iizuka, T. (1975) in: Metalloprotein Studies Utilizing Paramagnetic Effect of the Metal Ions as Probes (Kotani, M. and Tasaki, A. eds) pp. 201–211, Engineering Science, Osaka Univ., Osaka
[19] King, M.-T. and Drews, G. (1976) Eur. J. Biochem. 68, 5–12.
[20] Ferguson-Miller, S., Brautigan, D. L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115.
[21] Yamanaka, T. and Sinra, M. (1974) J. Biochem. (Tokyo) 75, 1265–1273.