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Cloning, expression, crystallization and preliminary X-ray characterization of cytochrome c_{552} from a moderate thermophilic bacterium, *Hydrogenophilus thermoluteolus*

Shin-ichi Ichiki,[a] Shota Nakamura,[b] Tadayasu Ohkubo,[b] Yuji Kobayashi,[b] Jun Hasegawa,[c] Susumu Uchiyama,[d] Hirofumi Nishihara,[e] Keiko Mizuta[a] and Yoshihiro Sambongi[a,f]*

[a]Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan, [b]Graduate School of Pharmaceutical Sciences, Osaka University, Suita 565-0871, Japan, [c]Daiichi Pharmaceutical Co. Ltd, Edogawa-ku, Tokyo 134-8630, Japan, [d]Graduate School of Engineering, Osaka University, Suita 565-0871, Japan, [e]Ibaraki University, Chu-ou 3-21-1, Ami-machi, Inashiki-gun, Ibaraki 300-0393, Japan, and [f]CREST of Japan Science and Technology Corporation, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan

Correspondence e-mail: sambongi@hiroshima-u.ac.jp

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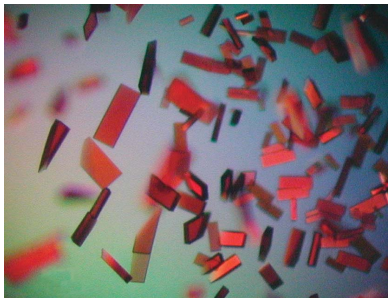

The amino-acid sequence of cytochrome c_{552} (PH c_{552}) from a moderately thermophilic bacterium, *Hydrogenophilus thermoluteolus*, was more than 50% identical to that of cytochrome *c* from an extreme thermophile, *Hydrogenobacter thermophilus* (HT c_{552}), and from a mesophile, *Pseudomonas aeruginosa* (PA c_{551}). The PH c_{552} gene was overexpressed as a correctly processed holoprotein in the *Escherichia coli* periplasm. The overexpressed PH c_{552} has been crystallized by vapour diffusion from polyethylene glycol 4000 pH 6.5. The crystals belong to space group $C222_1$, with unit-cell parameters a = 48.98, b = 57.99, c = 56.20 Å. The crystals diffract X-rays to around 2.1 Å resolution.

1. Introduction

Cytochrome *c* electron-transfer proteins play a central role in energy metabolism in bacteria as well as in mitochondria and chloroplasts and resemble each other. In particular, bacterial soluble mono-haem 'class I' cytochromes *c* are highly homologous and have been isolated from organisms living in a wide temperature range. Bacterial cytochromes *c* are thus appropriate for protein structure and stability studies (Sambongi *et al.*, 2002).

In this context, we have carried out comparative structural and mutagenesis studies on two cytochromes *c*, an 82-residue cytochrome c_{551} (PA c_{551}) from the mesophilic *Pseudomonas aeruginosa* that grows at 310 K and an 80-residue cytochrome c_{552} (HT c_{552}) from the thermophilic *Hydrogenobacter thermophilus* that grows at 345 K (Sanbongi, Ishii *et al.*, 1989). HT c_{552} was more stable than PA c_{551}, although they exhibited 56% sequence identity (Sanbongi, Igarashi *et al.*, 1989). We have also demonstrated that five residues, Ala7, Met13, Tyr34, Tyr43 and Ile78, are responsible for the higher stability of HT c_{552} compared with that of PA c_{551}, in which the corresponding residues are Phe7, Val13, Phe34, Glu43 and Val78 (Hasegawa *et al.*, 1998, 1999, 2000; Uchiyama *et al.*, 2002; Oikawa *et al.*, 2005).

In order to examine protein thermal stability using cytochromes *c*, we searched for a suitable bacterium in addition to *P. aeruginosa* and *H. thermophilus* and found a moderately thermophilic bacterium, *Hydrogenophilus thermoluteolus* (formerly named *Pseudomonas hydrogenothermophila*), that grows optimally at 325 K (Goto *et al.*, 1978). We have already determined the amino-acid sequence of *H. thermoluteolus* cytochrome c_{552} (PH c_{552}) to the 60th residue from the N-terminus (Sambongi *et al.*, 1992); it exhibited high sequence similarity with the sequences of PA c_{551} and HT c_{552}.

In the present study, we cloned the entire gene encoding the PH c_{552} protein and compared the deduced whole amino-acid sequence with those of HT c_{552} and PA c_{551}. We then expressed the PH c_{552} protein in *Escherichia coli*. Crystals of purified PH c_{552} have been grown which, together with the information already obtained for PA c_{551} and HT c_{552}, will facilitate the study of the structural features of protein stabilization.

2. Cloning, expression and purification

2.1. Cloning of the PH c_{552} gene

We used the polymerase chain reaction (PCR) method to clone the PH c_{552} gene and to determine the complete DNA sequence. We first

designed 128 mixed forward primers (PHfw) corresponding to the known sequence Lys-Ala-Lys-Gly-Cys-Met (residues 8–13 according to the PA c_{551} numbering; Fig. 1) and 256 mixed reverse primers (PHrv) corresponding to the Trp-Gly-Pro-Val-Pro-Met sequence (residues 56–61) and used them to amplify *H. thermoluteolus* chromosomal DNA with Ex *Taq* polymerase (Takara Shuzou). PCR with mixed primers PHfw and PHrv using *H. thermoluteolus* chromosomal DNA as a template gave an ∼160 bp DNA fragment, which was then cloned into the pUC19 vector. At least five independent clones were sequenced and the amino-acid sequence (residues 14–50 according to the PA c_{551} numbering) deduced from the DNA exactly coincided with the sequence previously determined by protein chemistry (Sambongi *et al.*, 1992).

We next adopted another PCR-based method (Siebert *et al.*, 1995), which is used for walking uncloned DNA, to obtain the entire PH c_{552} gene. The resulting DNA sequencing revealed that the PH c_{552} gene plus the flanking region had been successfully cloned (DDBJ accession No. AB096874). The mature PH c_{552} consisted of 79 amino acids (Fig. 1) and the N-terminal Asp was preceded by a Sec-dependent periplasmic targeting peptide of 23 amino-acid residues. This indicates that PH c_{552} is synthesized as a precursor and that its signal peptide is cleaved off during translocation to the periplasm of *H. thermoluteolus* cells.

The mature PH c_{552} protein exhibited overall sequence identity of more than 50% to both HT c_{552} and PA c_{551} (Fig. 1).

2.2. Expression and purification

The PH c_{552} gene was inserted into plasmid vector pKK223-3 under the control of the *tac* promoter. The PH c_{552} protein was not expressed as a holoprotein from the gene using its original signal sequence in *E. coli* JCB387 cells. When the original PH c_{552} signal sequence was replaced with that of PA c_{551} by the PCR method described previously (Zhang *et al.*, 1998), however, *E. coli* JCB387 cells co-transformed with pEC86 (Arslan *et al.*, 1998), which carried the *E. coli* cytochrome *c* maturation gene *ccmABCDEFGH* (chloramphenicol resistance), expressed the PH c_{552} holoprotein in the periplasm.

A periplasmic protein fraction of *E. coli* cells was obtained by the cold osmotic shock method (Sambongi *et al.*, 1996) and was then subjected to Hi-Trap Q column chromatography (Amersham Pharmacia Biotech) in 10 m*M* Tris–HCl buffer pH 8.0. The expressed PH c_{552} was washed out with the Hi-Trap Q at pH 8.0 and was further purified by Hi-Trap SP column chromatography (Amersham Pharmacia Biotech), eluting with an NaCl concentration gradient (0–500 m*M*) in 25 m*M* sodium acetate buffer pH 5.0, followed by chromatography on a Superdex 75 column equilibrated and eluted with 25 m*M* sodium acetate buffer pH 5.0. Following these purification procedures, the PH c_{552} protein was purified to homogeneity, as judged by SDS–PAGE, and this protocol consistently yielded 10–15 mg purified protein per litre of cell culture.

The N-terminal amino-acid sequence of the expressed PH c_{552} was determined to be Asp-Glu-Ala-Leu-Ala. This sequence was identical to that of the authentic cytochrome *c* purified from the native organism; thus, the fused signal peptide of PA c_{551} was able to target the PH c_{552} apoprotein to the *E. coli* periplasm and was correctly cleaved off. The visible absorption (400–600 nm) and CD (200–250 nm) spectra of the heterologously expressed PH c_{552} protein were identical to those of the authentic cytochrome *c* (Fig. 2). This indicates that the polypeptide folding and haem insertion were correct in the heterologous host.

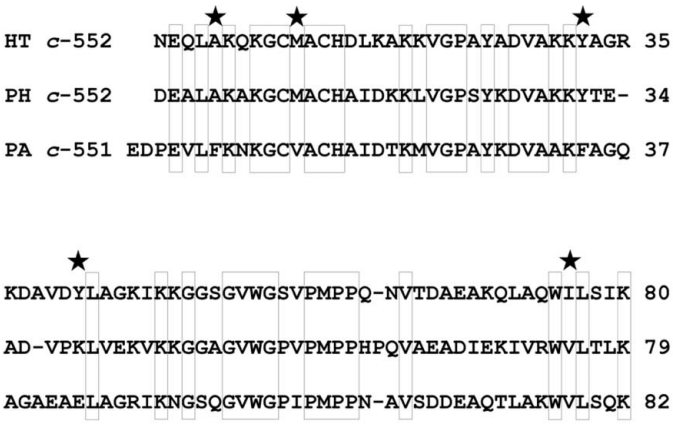

Figure 1
Sequence comparison of PH c_{552}, HT c_{552} and PA c_{551}. Identical amino-acid residues among the three proteins are boxed. The five amino acids marked with asterisks in HT c_{552} (Ala7, Met13, Tyr34, Tyr43 and Ile78) have been predicted to be responsible for the high stability.

3. Crystallization

Initial crystals were obtained using JB Screen (Jena Biosciences) and the vapour-diffusion method at 277 K. Each drop consisted of 1 µl protein solution (40 mg ml^{-1}) and 1 µl reservoir solution. Small plate-

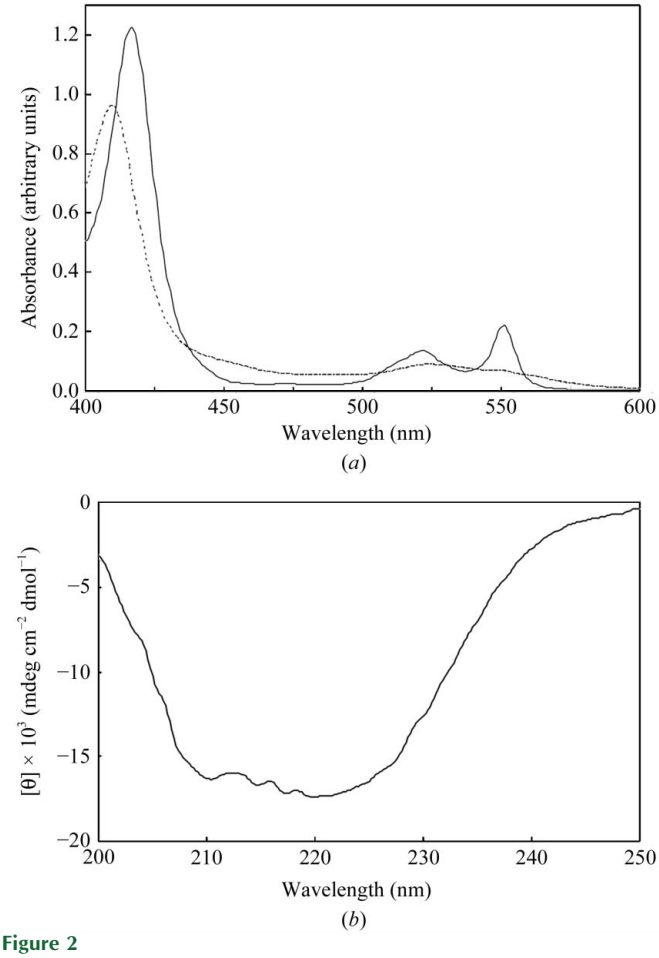

Figure 2
UV–visible and CD spectra. (*a*) UV–visible spectra of dithionite-reduced (solid line) and air-oxidized (dotted line) forms of wild-type recombinant PH c_{552} expressed in *E. coli* were obtained at 298 K and pH 5.0. (*b*) A CD spectrum of the recombinant PH c_{552} expressed in *E. coli* was obtained at 298 K and pH 5.0.

shaped crystals of PH c_{552} grew to typical dimensions of 0.1 × 0.2 × 0.04 mm within a week using 30%(*w*/*v*) polyethylene glycol 4000, 0.1 *M* MES pH 6.5.

The obtained crystals were not single crystals; they became stacked and were not suitable for X-ray diffraction experiments. To improve the quality of the crystals, further screening for crystallization was performed. The screening revealed that the temperature was the most influential factor on the quality of the crystals. Since the reservoir solution was prepared at room temperature and then transferred to a cold room in the initial trials, its temperature was above 277 K. The presence of temperatures higher than 277 K in some steps seemed to make the crystals stacked. Hence, all preparation and crystal observations were performed in the cold room (277 K). As a result, single crystals were obtained using this careful temperature control (Fig. 3).

4. Data collection and processing

Diffraction data were collected on an R-AXIS IV^{++} imaging-plate detector at room temperature (293 K) from a crystal in a glass capillary using Cu $K\alpha$ radiation from a Rigaku MicroMax-007 generator equipped with confocal mirror optics. Data processing was performed using the *CrystalClear* v.1.3.5 software (Rigaku/MSC; Pflugrath, 1997). The crystals, which diffract to a resolution limit of 2.1 Å, belong to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 48.98$, $b = 57.99$, $c = 56.20$ Å. Assuming the presence of one molecule of molecular weight 9.1 kDa in the asymmetric unit ($Z = 8$), the Matthews coefficient is 2.2 Å^3 Da^{-1} and the solvent content is 43% (Matthews, 1968). A molecular-replacement solution for PH c_{552} was found using *MOLREP* (Vagin & Teplyakov, 1997) with the molecular model of PA c_{551} (PDB code 351c). The data statistics are shown in Table 1. Currently, structure determination and model building are in progress.

5. Discussion

After a careful survey of the side-chain interactions between the three-dimensional structures of HT c_{552} and PA c_{551}, we have predicted that for HT c_{552} three spatially separate regions formed by (i) Ala7 and Met13, (ii) Tyr34 and Tyr43 and (iii) Ile78 are responsible for the higher stability with respect to PA c_{551}; this occurs through tighter hydrophobic packing and favorable electrostatic interactions at the atomic level (Hasegawa *et al.*, 1998). This has subsequently been directly proved by a mutagenesis study, in which the corresponding residues in PA c_{551} (Phe7, Val13, Phe34, Glu43 and Val78) were replaced with the residues at the same positions in HT c_{552}. The two double mutations F7A/V13M and F34Y/E43Y and the single mutation V78I in PA c_{551} independently contributed to an overall increase in stability (Hasegawa *et al.*, 1999, 2000; Uchiyama *et al.*, 2002). Furthermore, the reverse mutations in HT c_{552} (A7F/M13V, Y34F/Y43E and I78V) caused destabilization (Oikawa *et al.*, 2005).

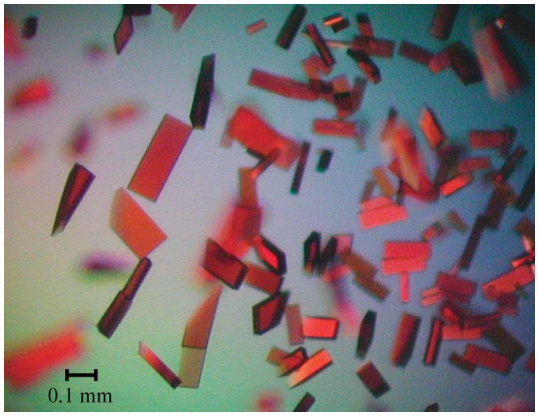

Figure 3
PH c_{552} crystals. Crystals suitable for X-ray analysis were obtained by means of careful temperature control.

Table 1
Data-collection statistics for PH c_{552} crystals.

Values in parentheses are for the highest resolution shell.

Data collection	Home source
Oscillation range (°)	180 × 1
Space group	$C222_1$
Unit-cell parameters (Å)	$a = 48.98$, $b = 57.99$, $c = 56.20$
Resolution limits	31.23–2.10 (2.25–2.10)
No. of observations	18939
No. of unique reflections	4864
Average redundancy	3.89
Completeness (%)	98.5 (95.3)
$\langle I/\sigma(I)\rangle$	16.6 (4.5)
$R_{\rm merge}$ (%)	5.0 (16.4)
MOLREP R factor	0.497
MOLREP correlation coefficient	0.351

It is of note that PH c_{552} has Ala7, Met13 and Tyr34 residues, as found in HT c_{552}, while the position corresponding to HT c_{552} Ile78 and PA c_{551} Val78 was occupied by Val78 in PH c_{552} (Fig. 1). At position 43, the amino-acid residue differed between the three proteins: it is Lys in PH c_{552}, Tyr in HT c_{552} and Glu in PA c_{551}. Thus, PH c_{552} possesses three of the five residues responsible for the enhancement of protein stability revealed by the mutagenesis study on HT c_{552} and PA c_{551}. The results predicted that the stability of PH c_{552} is intermediate between those of HT c_{552} and PA c_{551}.

We have now succeeded in the crystallization of PH c_{552}. We will carry out mutagenesis analysis on PH c_{552} by means of the *E. coli* expression system established in this study, which will be based on the X-ray crystal structure. Together with those on HT c_{552} and PA c_{551}, these studies will shed light on the mechanism of protein stabilization.

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