Integrity of *Thermus thermophilus* cytochrome *c*₅₅₂ synthesized by *Escherichia coli* cells expressing the host-specific cytochrome *c* maturation genes, *ccmABCDEFGH*: Biochemical, spectral, and structural characterization of the recombinant protein

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Abstract

We describe the design of *Escherichia coli* cells that synthesize a structurally perfect, recombinant cytochrome *c* from the *Thermus thermophilus* cytochrome *c*₅₅₂ gene. Key features are (1) construction of a plasmid-borne, chimeric *cycA* gene encoding an *Escherichia coli*-compatible, N-terminal signal sequence (MetLysIleSerIleTyrAlaThrLeuAlaAlaLeuSerLeuAlaLeuProAlaGlyAla) followed by the amino acid sequence of mature *Thermus* cytochrome *c*₅₅₂; and (2) coexpression of the chimeric *cycA* gene with plasmid-borne, host-specific cytochrome *c* maturation genes (*ccmABCDEFGH*). Approximately 1 mg of purified protein is obtained from 1 L of culture medium. The recombinant protein, cytochrome *rsC*₅₅₂, and native cytochrome *c*₅₅₂ have identical redox potentials and are equally active as electron transfer substrates toward cytochrome *ba*₃, a *Thermus* heme-copper oxidase. Native and recombinant cytochromes *c* were compared and found to be identical using circular dichroism, optical absorption, resonance Raman, and 500 MHz ¹H-NMR spectroscopies. The 1.7 Å resolution X-ray crystallographic structure of the recombinant protein was determined and is indistinguishable from that reported for the native protein (Than, ME, Hof P, Huber R, Bourenkov GP, Bartunik HD, Buse G, Soulimane T, 1997, *J Mol Biol* 271:629–644). This approach may be generally useful for expression of alien cytochrome *c* genes in *E. coli*.

Keywords: cytochrome *c*; *Escherichia coli*; homologous expression; *Thermus thermophilus*

Although studied for many decades as part of the cell's respiratory apparatus (Lemberg & Barrett, 1973; Mathews, 1985; Pettigrew &

Moore, 1987), cytochromes *c* remain of considerable interest as objects in the study of electron transfer reactions (Ferguson-Miller et al., 1979; Pan et al., 1993; Bjerrum et al., 1995; Geren et al., 1995; Winkler et al., 1995) and of protein folding (Sosnick et al., 1994; Bryngelson et al., 1995; Mines et al., 1996; Bai, 1999). In addition, recent evidence indicates that cytochrome *c* released from the mitochondrion is able to initiate apoptosis (Wallace, 1999, and references therein), suggesting that this protein may have functions other than electron transfer. Today, application of modern experimental approaches to cytochrome *c* function can be limited by the general unavailability of suitable expression systems for cloned cytochrome *c* genes. *Escherichia coli* is certainly the organism of choice, but most attempts to express foreign cytochrome *c* genes in this bacterium have been unsuccessful; either the yield is imprac-

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Abbreviations: *rC*₅₅₂, major species of recombinant cytochrome *c* obtained from the expression of the truncated *Thermus cycA* gene in *Escherichia coli*; *rC*₅₅₇, dimeric species of cytochrome *c* obtained from the expression of the truncated *Thermus cycA* gene in *E. coli*; *rsC*₅₅₂, recombinant cytochrome *c* obtained from the expression of the chimeric *Thiobacillus versutus*/*Thermus thermophilus cycA* gene in *E. coli* cells also bearing plasmid pEC86; *ccm*, cytochrome *c* maturation genes; GuHCl, guanidine hydrochloride; IPTG, isopropyl-β-D-thiogalactopyranoside.

tically low or the resulting product does not accurately represent the native cytochrome (Keightley et al., 1998, for review). There are, however, several examples of success in this area: Ubbink et al. (1992), showed that *E. coli* cells, grown under microaerophilic conditions, are able to express the complete *cycA* gene of *Thiobacillus versutus*; the mature protein is obtained in good yield from the periplasm, and proper folding is indicated by the observation that high-resolution NMR spectra of native and recombinant proteins are indistinguishable. Pollock et al. (1998) described high-yield expression of the yeast CYC1 gene in *E. coli* but only when CYC1 was co-expressed with CYC3, the yeast gene encoding for cytoplasmic cytochrome *c* lyase. Finally, Reincke et al. (1999) showed that the membrane bound cytochrome *c*₅₅₂ of *Paracoccus denitrificans* was expressed in *E. coli*, at levels suitable for biophysical study, only when the cells were cotransformed with the *ccm* gene cluster encoding host-specific cytochrome *c* maturation factors (see below). Recent advances in our understanding of cytochrome *c* maturation in prokaryotes (Thöny-Meyer et al., 1994; Page et al., 1997; Thöny-Meyer, 1997; Kranz et al., 1998, for review) suggest it should be possible to develop reliable methods for obtaining recombinant cytochromes *c* and therewith manipulate these proteins using techniques of site-directed mutagenesis.

A number of cytochrome *c* maturation genes (*ccmABCDEFGHI*) are required for the synthesis of cytochrome *c* in *E. coli* (Thöny-Meyer et al., 1995; Grove et al., 1996b). The maturation process consists of the following major steps: a precursor cytochrome is synthesized in the cytoplasm as a pre-apoprotein with an N-terminal signal sequence. The latter is recognized by components of the general secretion (*sec*) pathway that facilitate movement of the protein across the plasma membrane into the periplasm (Batey et al., 2000, and references therein for review). In the case of soluble cytochrome *c*, the signal sequence is removed by leader peptidase (Thöny-Meyer & Künzler, 1997). In the periplasm, the N-terminal cysteine residues of the preapoprotein are "prepared" for reaction with the heme, mainly by being maintained as thiols but possibly also by being held in proximity to heme. CcmG and CcmH are believed to play a role in this step (Grove et al., 1996a; Fabianek et al., 1998, 1999). Heme is also synthesized in the cytoplasm and transported into the periplasm by an as yet, unidentified mechanism. It is then transferred with the help of CcmCD to the heme chaperone CcmE (Schulz et al., 1999), which binds it transiently through a single covalent heme-histidine bond of unknown chemical nature (Schulz et al., 1998). Finally, cytochrome *c* heme lyase, for which the best candidate appears to be CcmF, then catalyzes formation of two heme thioether bonds, and the final folding steps occur to yield mature protein. Toward understanding how cytochrome *c* maturation depends on the function of the *ccmABCDEFGHI* gene products, Arslan et al. (1998) have constructed a plasmid on which this multicistronic operon is expressed under control of the *tet* promoter.

In contrast to the normal path of cytochrome *c* maturation, two examples in which maturation occurs in the cytoplasm of *E. coli* involve the expression of truncated cytochrome *c* genes from the thermophilic organisms, *Hydrogenobacter thermophilus* (Sambongi & Ferguson, 1994) and *Thermus thermophilus* (Keightley et al., 1998). In the case of *H. thermophilus*, expression of the *cycA* gene lacking signal peptide code yields recombinant protein that was first thought to be properly folded (Sambongi & Ferguson, 1994); however, K.L. Bren and K.M. Patel (unpubl. results), using a combination of high resolution NMR and scanning calorimetry, have found that even though both thioether linkages in the isolated

protein are intact, only approximately 10% of the purified protein is folded into a native conformation. In the case of *T. thermophilus*, expression of the *cycA* gene lacking code for the signal peptide also yields a cytochrome *c*-like material. However, careful examination of the protein (Keightley et al., 1998) reveals two major anomalies: the presence of a dimeric cytochrome *c* (*rC*₅₅₇) and a monomeric cytochrome *c* (*rC*₅₅₂) that lacks the split- α peak (*Q*₀₀) in the optical absorption spectrum of reduced heme, the latter being characteristic of native cytochrome *c*₅₅₂ (Hon-nami & Oshima, 1977) (see below).

The central theme of this communication is to demonstrate that these "errors" in the maturation of *Thermus* CycA can be avoided by overexpression of the cytochrome *c* structural gene and cytochrome *c* maturation genes (*ccmABCDEFGHI*) in the same cell. A detailed comparison of the biochemical, spectroscopic, and structural properties of native and recombinant *Thermus* cytochrome *c*₅₅₂ obtained from *E. coli* cells coexpressing the *ccm* genes shows that these two proteins are indistinguishable.

Results and discussion

In the previous communication describing expression of the truncated *Thermus cycA* gene in *E. coli* (Keightley et al., 1998), it was shown that whereas an amount of mature protein suitable for most biophysical studies was easily obtained, both known and possibly undetected types of heterogeneity in the recombinant product seriously limited its usefulness for mechanistic studies of cytochrome *ba*₃ oxidase. In that work, the cytochrome was synthesized in the cytoplasm, and it was conjectured that because of its thermophilic nature, the apoprotein folded properly, bound heme spontaneously and formed the heme thioether bonds without the aid of the periplasmic heme lyase. Clearly, this description of the behavior is incomplete because at least two side reactions were also occurring. First, as will be described in detail elsewhere, the major component *rC*₅₅₂ contains, in a yet to be determined chemical form, the potential to spontaneously convert to a novel cytochrome pigment, p572, characterized by an optical absorption spectrum with peaks at ~430 and 572 nm. Second, the minor, high-molecular weight component *rC*₅₅₇ is a dimer in which the protomers are linked by a Cys11/Cys11 disulfide bond (D.E. McRee, P.A. Williams, V. Sridhar, A. Pastuszyn, K.L. Bren, K.M. Patel, Y. Chen, T.R. Todaro, D. Sanders, E. Luna, & J.A. Fee, in prep.). We demonstrate here that these "errors" in the maturation of *Thermus* CycA are avoided by simultaneous expression of a complete cytochrome *c* structural gene and the cytochrome *c* maturation genes.

Because early studies indicated that the information in the *T. thermophilus* CycA signal peptide was not properly conveyed to the cytochrome *c* maturation proteins of *E. coli* (Keightley, 1993), we reasoned that replacing it with the *T. versutus* CycA signal peptide might result in properly folded, mature cytochrome *c*₅₅₂. Accordingly, a chimeric *cycA* gene beginning with code for the signal sequence of *T. versutus* CycA followed by code for the mature *Thermus* CycA was constructed as described in Materials and methods, and shown in Figure 1. Expression of this gene from plasmid pRSC552 (see Materials and methods) yielded material that did not show the distinct splitting of the *Q*₀₀ band seen in native protein and produced significant quantities of p572 when heated for a short period of time at 70 °C, although there appeared to be very little of the dimeric *rC*₅₅₇ (data not shown). When *E. coli* strain BL21 (DE3) bearing pRSC552 was cotransformed with plasmid pEC86 and both plasmids were maintained by including the

A Sequence of signal peptide from *Thiobacillus versutus* cytochrome *c*₅₅₀.

(Primer 1 see text)

ATG AAG ATC AGC ATC TAT GCC ACT CTC GCC GCC CTG TCC CTC GCC CTG CCC GCC **GTG** GCC
 TAC TTC TAG TCG TAG ATA CGG TGA GAG CGG CGG GAC AGG GAG CGG GAC GGG CGG **CAC** CGG
 Met Lys Ile Ser Ile Tyr Ala Thr Leu Ala Ala Leu Ser Leu Ala Leu Pro Ala **Val** Ala

B Modified sequence of signal peptide from *Thiobacillus versutus* cytochrome *c*₅₅₀.

ATG AAG ATC AGC ATC TAT GCC ACT CTC GCC GCC CTG TCC CTC GCC CTG CCC GCC GGC GGC
 TAC TTC TAG TCG TAG ATA CGG TGA GAG CGG CGG GAC AGG GAG CGG GAC GGG CGG CCG CGG
 Met Lys Ile Ser Ile Tyr Ala Thr Leu Ala Ala Leu Ser Leu Ala Leu Pro Ala **Gly** Ala
 (NarI)

C *Thermus thermophilus* cytochrome *rC*₅₅₂ *cycA* gene open reading frame sequence.

CAA GGC GGC CAG GCG GAC GGG GCC AA (Primer3)
 CAG GCG GAC GGG GCC AAG ATC TAC GCC CAG TGCTAA.
 GAC CGC CTG CCC CGG TTC TAG ATG CGG GTC QCGATT.
 Gln Ala Asp Gly Ala Lys Ile Tyr Ala Gln Cys* . . . (Primer 4 see text)
 (NarI)

D Cytochrome *rsC*₅₅₂ sequence.

ATG AAG ATC AGC ATC TAT GCC ACT CTC GCC GCC CTG TCC CTC GCC CTG CCC GCC GGC GGC CAG GCG GAC GGG
 TAC TTC TAG TCG TAG ATA CGG TGA GAG CGG CGG GAC AGG GAG CGG GAC GGG CGG CCG CGG GAC CGC CTG CCC
 Met Lys Ile Ser Ile Tyr Ala Thr Leu Ala Ala Leu Ser Leu Ala Leu Pro Ala Gly Ala Gln Ala Asp Gly
 GCC AAG ATC TAC GCC CAG TGCTAA
 CGG TTC TAG ATG CGG GTC ACGATT
 Ala Lys Ile Tyr Ala Gln Cys*

Fig. 1. Construction of a hybrid gene containing the cytochrome *c* signal peptide encoding sequence from *T. versutus* placed upstream of and in-frame with the mature *T. thermophilus* cytochrome *c*₅₅₂ structural gene. See text for details.

appropriate antibiotic in the culture medium, a cytochrome *c*₅₅₂ was expressed that showed none of the heterogeneities described above, and, most importantly, it exhibited splitting of the *Q*₀₀ band, as seen with the native cytochrome (see below). We have characterized this protein with a diverse array of relevant chemical and physical techniques. When *E. coli* BL21(DE3) cells are similarly cotransformed with pCYC552, bearing the truncated version of *Thermus cycA* (Keightley et al., 1998), and pEC86, bearing the *ccmABCDEFGH* genes, the resulting cytochrome *c* mixture appears to contain diminished amounts of *rC*₅₅₇ but still forms substantial amounts of p572 on brief heating (data not shown).

Biochemical observations

Approximately 1.0 mg of highly purified recombinant cytochrome *c*₅₅₂ were obtained from 1 L of cell culture. The optical purity ratio *A*₄₀₈/*A*₂₈₀ of our best preparations is 4.7 compared to 4.7 (Hon-nami & Oshima, 1977) and 5.1 (Yoshida et al., 1984) reported previously for the native protein, and gel electrophoresis revealed a single band (data not shown). Figure 2 shows the electrospray *m/z* and mass spectra. The ion spectrum shows three dominant ions at 1,621.8(9⁺), 1,824.6(8⁺), and 2,085.0(7⁺), which indicate a molecular mass of 14,588 ± 2 Da. N-terminal sequence analysis yielded: AspGlyAlaLysIleTyrAlaGln. The predicted mass of the holoprotein having Asp3 as the N-terminus is 14,591

(= 13,974 apoprotein + 617 heme), just outside the expected error limit. These observations show that the periplasmic *E. coli* signal peptidase (Thöny-Meyer & Künzler, 1997) cleaves between Ala2 and Asp3 rather than between Ala(-1) and Gln1 and suggest that the *E. coli* peptidase exhibits a different specificity in the context of the two sequences: (-4)*ProAlaGlyAlaGlnAlaAspGly*—in the chimera and (-4) *ProAlaValAlaGlnGluGlyAsp*—in the *T. versutus* protein. Based on this observation, we assume that the recombinant protein resides in the periplasm and have not sought additional evidence for this. While we are currently mutating the Gly(-3) back to Val(-3) (work in progress), the shorter N-terminus of this recombinant cytochrome *c*₅₅₂ (*rsC*₅₅₂) appears not to cause any of its properties to differ from those of native protein (however, see below).

We used the pyridine hemochrome method (Berry & Trumppower, 1987) to re-determine the reduced-oxidized extinction coefficient of native protein and found Δ*ε*₅₅₂ = 14.39 mM⁻¹ cm⁻¹ while that of *rsC*₅₅₂ yielded a value of 14.26 mM⁻¹ cm⁻¹. These values compare favorably with the previously reported value of 14.3 mM⁻¹ cm⁻¹ (Hon-nami & Oshima, 1980). Using this information, we prepared samples having identical absorbance properties and compared the rate of oxygen uptake in the presence of a fixed concentration of cytochrome *ba*₃ and varying amounts of native or *rsC*₅₅₂; the activity of the recombinant cytochrome was identical to that of native cytochrome at concentrations ranging from ~0.5 to 5.0 μM (Fig. 10 of Keightley et al., 1998).

Fig. 2. Electrospray mass spectrum of recombinant *T. thermophilus* cytochrome *c*₅₅₂.

A further comparison of native and recombinant proteins relevant to their chemical behavior includes the redox potentials. The reduction potential of native cytochrome *c*₅₅₂ was previously determined by titration with ferrous ammonium sulfate (Hon-nami & Oshima, 1977) and reported to be +232 mV. Figure 3 shows cyclic voltammograms for native and recombinant cytochrome *c*₅₅₂. Notably, the two traces overlap and yield potentials of +202 and +200 mV for the native and recombinant proteins, respectively. Because of the way the measurements were made (see Materials and methods), this difference may be real, but additional measurements would be required to establish the error limits. The absolute values have errors of ± 5 –10 mV. The higher value reported earlier (Hon-nami & Oshima, 1977) may have resulted from interaction of the redox buffer, ferrous/ferric ammonium sulfate, with the protein.

Spectral characterization

We now turn to the characterization of the recombinant protein by a series of spectral methods that range in their resonant energies from 40,000 cm⁻¹ to $\sim 10^{-2}$ cm⁻¹ and which probe both the heme and the protein moieties of the cytochrome.

Secondary structural features of the protein component, such as β -sheet and α -helix, are revealed in the circular dichroism (CD) in the 200–250 nm region. It can be seen in Figure 4A that the far-ultraviolet (UV) spectra are indistinguishable, indicating that the secondary structure composition of the two proteins is the same. Similarly, CD in the 300 to 370 nm region provides information about the environment of the tryptophan residues, here the single Trp91, in the native protein. Figure 4B shows that the features arising from tryptophan, even though broad, are essentially

Fig. 3. Cyclic voltammograms of native (smooth line) and recombinant (symbols) *T. thermophilus* cytochrome *c*₅₅₂ in 100 mM potassium phosphate buffer, pH 7. Data were recorded at a pyridine-4-aldehyde-semicarbazone-modified gold electrode with a scan rate of 50 mV/s. The protein concentration was 1 mM.

identical in the two samples. Wittung-Stafshede (1998) reported that a mixture of *rC*₅₅₂ and *rC*₅₅₇ proteins exhibited a profoundly altered CD spectrum in the near-UV spectral region, and we find a similar spectrum from samples of purified *rC*₅₅₂ (not shown). The CD spectra in the Soret region (380–450 nm) have been proposed to arise from both protein-induced deformation of the porphyrin ring (Blauer et al., 1993) and from the interaction of the heme ring with polarizable groups (i.e., aromatic amino acids) in its vicinity (Hsu & Woody, 1971). The Soret CD spectra of the native and recombinant proteins are indistinguishable, with a large positive bands at 405 nm and small negative bands at 417 nm (Fig. 4B). In contrast, the Soret CD spectrum of purified *rC*₅₅₂ has positive (403 nm) and negative (417 nm) bands with similar intensities. The positive Soret CD band of *rC*₅₅₂ also has significantly lower intensity (by a factor of 2) than that for recombinant or native protein (data not shown). These results suggest that the recombinant protein has a native-like heme conformation and heme environment, while the heme conformation and/or environment in *rC*₅₅₂ are perturbed relative to the native protein.

Figure 5 shows the optical absorption spectra arising from the heme in the 380–590 nm region of oxidized and reduced native and recombinant cytochrome *c*₅₅₂ (Fig. 5A,B) and the second derivatives of the spectra of the reduced proteins. Even though the absorption spectra are highly similar, it is important that the second derivatives of the reduced spectra also overlap particularly well across the *Q*₀₀ band. According to the ideas set forth by Vanderkooi and LaBerge (Kohler et al., 1996; Reddy et al., 1996) and Shelnutt et al. (Jentzen et al., 1998), *Q*₀₀ splitting arises from the presence of an asymmetric electrostatic field at the heme and/or a

Fig. 4. (A) Far-UV and (B) near-UV CD spectra of native (smooth line) and recombinant (symbols) *T. thermophilus* cytochrome c_{552} . Each spectrum of the recombinant protein was offset by -2 units of molar ellipticity for this presentation.

combination of *ruffling* and *saddling* of the heme structure. The fact that the splitting is the same in both native and recombinant proteins indicates that the microscopic details of the heme environment in the two proteins must be the same. For comparison to rC_{552} , see Figure 8 of Keightley et al. (1998) where splitting of Q_{00} is notably absent.

The vibrational properties of a heme-protein system can be examined with resonance Raman (rR) spectroscopy (Hu et al., 1993). Kihara et al. (1978) have recorded the rR spectra of *Thermus* cytochrome c_{552} and assigned the principal bands. The latter authors presented spectra obtained from excitation at 514.5 nm while our spectra were obtained with 413.1 nm. rR spectra in the 1,200–1,700 cm^{-1} region of oxidized and reduced forms of the native and recombinant *Thermus* cytochromes c are shown in Figure 6. The spectra presented are highly similar to those previously reported (Kihara et al., 1978). By comparing our spectra, taken in both valence states, it is evident that minute spectral details are conserved in rsC_{552} indicating that those interactions between heme and protein that affect the vibrational properties of the heme are identical in native and the recombinant proteins. Although not shown here, the spectra of rC_{552} deviate significantly from those of the native protein in both the high- and low-frequency regions and in both valence states.

The hyperfine-shifted resonances in high resolution ^1H NMR spectra of the oxidized ($S = 1/2$) cytochromes provide the subtlest structural information about the heme and its environment. Figure 7 shows 500 MHz ^1H NMR spectra of native c_{552} (upper), rsC_{552} (center), and rC_{552} (lower) recorded at pH 7.0. The spectra are similar to 270 MHz spectra recorded by Hon-nami et al. (1980)

under similar conditions and show only ^1H resonances shifted as a result of the hyperfine interaction, primarily heme substituents and heme axial ligands (K.M. Patel & K.L. Bren, unpubl. data). The shifts of these resonances are highly sensitive to the asymmetry of the unpaired electron spin delocalization on the heme, which is determined primarily by the type and orientation of heme axial ligands. The conformation of the heme itself also has been proposed to contribute to these shifts (Shokhirev & Walker, 1998). The one-dimensional (1D) NMR spectra of the native and recombinant proteins in this region are nearly identical, suggesting that the heme conformation and heme-ligand interactions in the recombinant protein are native-like. In contrast, the spectrum of rC_{552} shows some striking differences. Notably, it lacks some peaks found in native, such as those at $+32.9$, $+16.5$, and -11.3 ppm. In addition, it also has peaks that are not present in the 1D spectra of native, such as those at -7.0 ppm, $+12$ ppm, and a number of smaller peaks. The shifts of a number of resonances (for example, the upfield-shifted, intensity-3 resonance attributed to the axial methionine $\epsilon\text{-CH}_3$) also are substantially different in rC_{552} in comparison to native and rsC_{552} (K.M. Patel & K.L. Bren, unpubl. data). Not shown are two-dimensional nuclear Overhauser effect spectroscopy (NOESY) spectra, which provide an assessment of the similarity of overall folding of the proteins. The NOESY spectra for the native and recombinant proteins are nearly identical, indicating a native fold for rsC_{552} in solution. In contrast, the NOESY spectrum of rC_{552} displays extremely weak cross peaks, suggesting that its protein structure is poorly defined. The NOESY of rC_{552} also indicates population of at least two identifiable conformers. The spectra shown here (Fig. 7) were recorded under

Fig. 5. Optical absorption spectra of oxidized and reduced samples and second-derivative spectra of the reduced native and recombinant *T. thermophilus* cytochrome c_{552} . **A:** Oxidized native (smooth line) and recombinant (dotted line) proteins. **B:** Reduced native (smooth line) and recombinant (dotted line) proteins. **C:** Second derivative $[d^2A/d\lambda^2]$ spectra (see Materials and method) of reduced native (smooth line) and recombinant (dotted line) proteins. Note scale difference on left and right panels. The Q_{00} band of cytochromes c generally occurs near 550 nm.

identical experimental conditions. These NMR data provide additional evidence that heme is improperly incorporated in rc_{552} . More detailed evaluation of these spectra is in progress (K.L. Bren & K.M. Patel, unpubl. data).

Crystallographic characterization

The chemical and spectroscopic data presented above suggest that the structure of the recombinant protein should be identical to that of the native protein. However, the former is matured at 37 °C by *E. coli* enzymes while the latter is matured at 70 °C by *Thermus* enzymes, making it possible that details of side-chain and secondary structural element packing, particularly away from the heme cofactor, might not be the same in the two proteins. In 1995, Than et al. (1997) reported a 1.28 Å structure of oxidized, native

T. thermophilus cytochrome c_{552} using single crystals (space group $P4_1$) obtained from ammonium sulfate. In work yet to be published, we have solved the structure of rc_{552} at 2.8 Å using single crystal (space group $P6_5$) obtained from polyethyleneglycol (PEG) solutions. Single crystals of oxidized rc_{552} were also obtained from PEG, X-ray diffraction data were collected to 1.7–1.8 Å, and the structure was solved by molecular replacement using both our earlier data and the coordinates supplied to the Brookhaven Protein Database (1C52) by Than et al. (1997).

Relevant statistical information is given in Table 1, and Figure 8 shows the overall fold of rc_{552} in stereo, colored from red at the N-terminus to blue at the C-terminus, for comparison with Figure 1 of Than et al. (1997). Figure 9A shows a stereo presentation of the electron density map in the region of the heme with emphasis on the two thioether linkages. Figure 9B shows an overlay of the

Fig. 6. High-frequency resonance Raman spectra of oxidized and reduced native (solid lines) and recombinant (dotted lines) *T. thermophilus* cytochrome c_{552} . The excitation wavelength was 413.1 nm. Spectral traces are offset for the purpose of illustration.

native structure of Than et al. (1997) and that of the recombinant structure in the same orientation as in Figure 9A. The two structures are indistinguishable in this region.

Than et al. (1997) analyzed the structure of native protein in great detail and noted particular regions that were unique to *Thermus* cytochrome c_{552} and might be considered, in the current context, indicative of a properly folded protein. Notable among these are: (1) There is a complete absence of internal ordered water molecules, although there is one water molecule near the heme, as shown here in Figure 9 and in Figure 5B of Than et al. (1997). (2) Residue Gln16 is held in an unusual conformation, placing its Φ/Ψ angles outside the normal values. This deviation is due to formation of three H-bonds to the amide group. (3) A salt bridge exists between the carboxylate of the propionyl side chain of heme ring A and Arg125. We have carefully compared the two structures and also find these features in the recombinant protein.

Visually, C_{α} -positions in the recombinant and native structures deviate slightly from each other between residues 24 and 25 and between residues 94 and 103 and 129 to 131. Statistically, the C_{α} positions in these regions have root-mean-square deviation (RMSD) of 0.295 (22–27), 0.716 (90–106), and 0.611 (127–131) Å; excluding these regions (24–25, 94–103, 129–131), the overall RMSD is 0.348 Å; and including these regions, the RMSD of the C_{α} atoms between the two structures is 0.506 (3–131) Å. The origin of the variation in structure in the region of residues 94–103 would appear to be due to differences in intermolecular contacts in the two

crystal forms. On the whole, the differences are minor and occur in nonstructured loops and in the regions of crystal contacts. The X-ray data combined with activity and spectroscopic data indicate that the recombinant and native proteins are indistinguishable.

Finally, a brief comment on the thermal stability of the recombinant protein compared with that of native cytochrome c_{552} . Nojima et al. (1978) reported on the reversible denaturation of this protein in solutions of guanidine HCl. Interpolating from their graphs, the protein is 50% in the unfolded state in ~ 5.8 M GuHCl at 25 °C and is 50% in the unfolded state at ~ 63 °C in 4.2 M GuHCl. We repeated these experiments (K.M. Patel & K.L. Bren, unpubl. data) with the preliminary findings that both native and rsC_{552} are 50% unfolded in ~ 5.3 M GuHCl at 25 °C. Recombinant protein is 50% unfolded in 4.2 M GuHCl at ~ 60 °C, whereas native protein is $\sim 50\%$ unfolded under the same conditions at ~ 70 °C. Thus, at first look, the recombinant protein may be slightly less thermostable than native protein; if real, this may be due to its altered N-terminus.

The system we describe here for expression of *Thermus* cytochrome rsC_{552} will provide abundant amounts of material and thus represents an important objective in our goal to explore the details of *Thermus* cytochrome ba_3 oxidase activity. In a more general sense, when our results are considered with those of Ubbink et al. (1992), Pollock et al. (1998), and Reincke et al. (1999), they suggest that expression of any plasmid-borne *cycA* gene encoding a host-compatible N-terminal signal peptide and a mature CycA protein of foreign origin may, when cotransformed with host-specific cytochrome *c* maturation genes, lead to useful amounts of a properly-folded, alien cytochrome *c*.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strain DH10B from Gibco BRL (Gaithersburg, Maryland) [genotype: $F^- mcrA \phi(mrr^- hsdRMS^- mcrBC) j80dlacZ\phi M15 \phi lacX74 deoR recA1 endA1 araD139 \phi(ara, leu) 7697 galU galK 1^- rpsL nupG$] (Grant et al., 1990)) was used for the ligation and sequencing steps. *E. coli* strain BL21(DE3) from Novagen (Madison, Wisconsin) [genotype: $F^- ompT hsdS_b (r_B^- m_B^-) gal dcm$ (DE3)] (Studier & Moffatt, 1986) was used for the expression of the chimeric cytochrome c_{552} fragment. Plasmid, pET17b, was also obtained from Novagen. The plasmid bearing the complete *T. versutus cycA* gene, pMU18, was described in Ubbink et al. (1992) and was a gift from Dr. Gerard W. Canters. Plasmid pCYC552 carrying the truncated *Thermus cycA* gene was as described previously (Keightley et al., 1998).

Molecular genetics

Procedures were carried out generally as described in Sambrook et al. (1989). Oligonucleotides were purchased from Gibco BRL. The polymerase chain reaction (PCR) was performed using ready-to-go PCR beads (Amersham Pharmacia Biotech, Inc., Piscataway, New Jersey). The PCR products were purified using Qiagen purification kits to remove the mineral oil and excess primer. The QiaExII kit was used for isolating restriction fragments from agarose gels. DNA sequencing was done at the UCSD Molecular Pathology Shared Resource in the UCSD Cancer Center.

Fig. 7. 500-MHz ¹H-NMR spectra of native (A), and recombinant (B) ferricytochromes *c*₅₅₂, and mM *rC*₅₅₂ (C). Samples were in 100 mM potassium phosphate, pH 7.0, with 10% D₂O added.

Construction of a chimeric *cycA* gene

A chimeric *T. versutus*/*T. thermophilus* *cycA* gene was constructed by ligating a fragment of the *T. versutus* gene encoding the N-terminal signal sequence in-frame to a fragment of *T. thermophilus* *cycA* gene encoding the mature cytochrome *c*₅₅₂ sequence. Figure 1 shows the components, and the procedures used were as follows: Figure 1A shows the signal peptide sequence of the cytochrome *c*₅₅₀ gene of *T. versutus*. Figure 1B shows the modified signal peptide sequence. Primer 1 (5'-dTCTCATATGAAG ATCA GCATCTATGCCA-3') and Primer 2 (3'-dGAGCGGGACGGGC GGCCGCGGATC-5') were used to introduce a 5' *Nde*I site, the ATG start codon (underlined in Primer 1) and a 3' *Nar*I site that changes the valine codon to a glycine codon in the signal peptide (underlined in Primer 2). Figure 1C shows partial sequence of the

*rC*₅₅₂ gene, which lacks a signal peptide (Keightley et al., 1998). The reading frame of the cytochrome was amplified by PCR from pCYC552 (cf. Keightley et al., 1995) using Primer 3 (5'-dCAAGGCGCCCGAGGCGGACGGGGCCAA), which includes a 3' *Nar*I site (underlined) and the antisense Primer 4 (3'-dCCCCG AGATTTGCCAGAACT-5') that is located downstream of *cycA* in the pET17B sequence and leaves the *Bam*HI site just downstream of the stop codon of *cycA* intact. Figure 1D shows the final cytochrome *rsC*₅₅₂ sequence. The PCR product from Primers 1 and 2 was restricted with *Nde*I and *Nar*I; this encodes the signal peptide. The PCR product from Primers 3 and 4 was restricted with *Nar*I and *Bam*HI; this encodes for the mature cytochrome *c*. The signal peptide fragment and the mature cytochrome fragment were ligated together and cloned into pET17b, which had been restricted with *Nde*I and *Bam*HI. Primers 1 and 4 were used for PCR amplification of the pre-cytochrome *c* encoding fragment to demonstrate the success of the above manipulations, and DNA sequencing on both strands showed the absence of mutations. We denote this plasmid pRSC552.

Table 1. Data, phasing and refinement statistics for recombinant *Thermus* cytochrome *c*₅₅₂

No. of reflections	92,080
No. of unique reflections	15,100
<i>R</i> -merge (25–1.8 Å)	7.0%
<i>R</i> -merge (1.9–1.8 Å)	30.4%
Completeness (25–1.8 Å)	99.2%
Completeness (1.9–1.8 Å)	98.8%
<i>I</i> / σ (<i>I</i>) (25–1.8 Å)	9.1
<i>I</i> / σ (<i>I</i>) (1.9–1.8 Å)	2.4
<i>R</i> -factor (<i>I</i> > 2 σ)	18.20%
<i>R</i> -factor (<i>I</i> > 0)	20.38%
Free <i>R</i> -factor (<i>I</i> > 2 σ)	23.23%
Free <i>R</i> -factor (<i>I</i> > 0)	26.17%
RMS bonds	0.007 Å
RMS angles	0.905°
Coordinate error (sigma estimation)	0.17 Å

Fig. 8. Overall fold of oxidized recombinant *Thermus* cytochrome *c*₅₅₂ in stereo, colored from red at the N-terminus to blue at the C-terminus.

Fig. 9. **A:** shows in stereo the sigma-weighted $2F_o - F_c$ electron density map in the vicinity of the heme. **B:** Comparison of the structure of the native (taken from Protein Data Base entry 1C52, Than et al., 1997) and the recombinant protein, in stereo.

Cell growth conditions

Escherichia coli strain BL21(DE3) carrying plasmids pEC86 (Cm^r) and/or pSC552 (Amp^r) was grown aerobically at 37 °C in Luria Bertani medium; 1 L of culture medium in 2.8 L, nonfluted Fernbach flasks was subjected to rotary shaking at 110 rpm. Antibiotics were added, where appropriate: chloramphenicol at 30 $\mu\text{g}/\text{mL}$ and ampicillin at 50 $\mu\text{g}/\text{mL}$. IPTG to a concentration of 1 mM was added for induction when cell density reached an OD_{600} of ~ 1.0 . At this point, the medium was supplemented to 0.1% glycerol. The cells were harvested 6–8 h after IPTG induction.

Protein purification

Cytochrome *c* was isolated from the *E. coli* cells essentially as described by Keightley et al. (1998). In brief, this procedure involves two adsorption/desorption steps on cation exchange resins followed by high-resolution gel filtration. Occasionally, a third cation exchange column may be required for final purification. Cytochrome rC_{552} was prepared as described previously (Keightley et al., 1998).

General protein characterization

Optical absorption spectra of cytochrome solutions were recorded using a SLM/AMINCO model DB3500 spectrophotometer. Data were processed using the vector manipulation program, IGOR (WaveMetrics, Lake Oswego, Oregon). Electrospray mass spectrometry was carried out at the Scripps Research Institute of Mass Spectrometry Facility (La Jolla, California) using a Perkin-Elmer SCIEX API III mass analyzer with the orifice potential set at 100 V

(Siuzdak, 1994). Mass spectra were obtained from ion spectra using the Perkin-Elmer program BioMultiView. N-terminal amino acid sequencing was carried out by Dr. Andrzej Pastuszyn in the Protein Chemistry Laboratory, Department of Biochemistry and Molecular Biology at the University of New Mexico as described by Keightley et al. (1995). Measurements of cytochrome ba_3 -cytochrome *c* oxidase activity were carried out as described previously (Keightley et al., 1998), except that solutions of native and recombinant cytochrome c_{552} , at the same concentration as judged by optical absorption spectra, were used in alternating oxygen uptake runs. Individual pO_2 vs. time plots were compared for experimental setups differing only in the origin of the cytochrome c_{552} .

Redox measurements

All electrochemical experiments were carried out in a three-compartment cell using a Bioanalytical Systems (BAS) Model CV 50W electrochemical analyzer. The gold-disk working electrode was polished with 0.05 μM alumina, thoroughly rinsed, then chemically modified by sonication for 30 min in a 0.05 mM aqueous solution of pyridine-4-aldehydesemicarbazone. Potentials were measured vs. SCE. The reference electrode was separated from the working compartment by a Luggin capillary. Both native and recombinant cyt c_{552} gave linear plots of peak current vs. (scan rate) $^{1/2}$, consistent with a diffusion-controlled process.

Circular dichroism spectra

Samples for CD spectroscopy were 10 μM in protein (for far-UV spectra) or 100 μM in protein (for near-UV or Soret spectra) in

100 mM potassium phosphate buffer, pH 7.0. CD spectra were collected on a JASCO J-710 spectropolarimeter at 22 °C on samples in a 0.1 cm pathlength quartz cell.

Resonance Raman (rR) spectra

Spectra were obtained using a custom McPherson 2061/207 spectrograph (set at a focal length of 0.67 m and with a 2400 groove grating) using a Princeton Instruments liquid N₂-cooled (LN-1100PB) CCD detector. Rayleigh scattering was attenuated using a Kaiser Optical holographic super-notch filter. Excitation (413 nm, 5 mW) was provided by a Coherent Innova 302 Kr laser. Spectra were collected in a 90°-scattering geometry on samples at room temperature with a collection time of 5 min. Frequencies were calibrated relative to indene and are accurate to 1 cm⁻¹.

High resolution NMR spectra

Protein samples for ¹H NMR spectroscopy consisted of 1.6–3.6 mM protein in 100 mM potassium phosphate, pH 7.0, in 90% H₂O/10% D₂O. Samples were oxidized with a threefold molar excess of potassium ferricyanide and concentrated using a Centricon-10 device prior to data collection. ¹H NMR spectra were collected on a 500 MHz Varian INOVA instrument. One-dimensional NMR spectra were collected with recycle times of 250 ms or 2 s and presaturation to suppress the solvent resonance. NOESY (Kumar et al., 1980; Macura & Ernst, 1980) spectra were collected with a 80 ppm sweep width with a recycle time of 800 ms and a mixing time of 100 ms. Presaturation during the relaxation delay was used to suppress the solvent signal. All NMR spectra were collected at 25 °C.

X-ray crystallography

Crystals of recombinant *Thermus* cytochrome *c*₅₅₂ were obtained in sitting drops against 36% MPEG 5K (w/w), 0.2 M Imidazole-maleate buffer, pH 6.3, using ~12 mg/mL protein suspended in 25 mM Tris/HCl pH 8.0, 100 mM NaCl. X-ray data to 1.8 Å were collected at the Stanford Synchrotron Radiation Laboratory (SSRL), beamline 7.1. The data were processed using MOSFLM (Leslie, 1992), scaled and further reduced using the CCP4 suite of programs (CCP4, 1994) (see Table 1). Initial electron density maps were generated using the structure of the wild-type protein (Than et al., 1997; 1C52.pdb), and interpreted using Xfit/XtalView (McRee, 1999). The model was refined using SHELX (Sheldrick, 1996) with a test set of 5% of the reflections being used for a *R*_{free} calculation (Brünger, 1992). X-ray amplitudes, phases, and derived atomic coordinates have been deposited with the Protein Data Bank (1DT1).

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