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Overexpression, purification, and enthalpy of unfolding of ferricytochrome c_{552} from a psychrophilic microorganism

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Abstract

The psychrophilic, hydrocarbonoclastic microorganism *Colwellia psychrerythraea* is important in global nutrient cycling and bioremediation. In order to investigate how this organism can live so efficiently at low temperatures (~4 °C), thermal denaturation studies of a small electron transfer protein from *Colwellia* were performed. *Colwellia* cytochrome c_{552} was overexpressed in *E. coli*, isolated, purified, and characterized by UV-visible absorption spectroscopy. The melting temperature (T_m) and the van't Hoff enthalpy (ΔH_{vH}) were determined. These values suggest an unexpectedly high stability for this psychrophilic cytochrome.

Keywords

Psychrophile; cytochrome c_{552} ; van't Hoff enthalpy; thermal denaturation

Here, we report the first studies of cytochrome c_{552} from the psychrophilic microorganism *Colwellia psychrerythraea*. We describe its overexpression, purification, UV-vis absorption spectrum, and enthalpy of unfolding determined by thermal denaturation.

In contrast with the extensive literature on proteins from thermophilic organisms, only a few proteins from psychrophilic organisms have been characterized to date, with few consistent broad conclusions [1–8]. Elucidating the molecular basis of psychrophilicity is important for expanding fundamental understanding of protein folding dynamics and biological electron transfer processes. Psychrophilic organisms such as *Colwellia psychrerythraea* play essential roles in global nutrient cycling and bioremediation; *Colwellia* species were a major component of the microbial community response in the deep, cold waters of the Gulf of Mexico following the 2010 *Deepwater Horizon* oil spill [9].

The published genome of the hydrocarbonoclastic (hydrocarbon-degrading), obligately psychrophilic (cold-loving) bacterium *Colwellia psychrerythraea* 34H reveals the presence of a gene (gene ID 637282778) coding for a homologue of the electron-transfer protein cytochrome c_{552} (*Cpcyt c₅₅₂*) [10]. The amino acid sequence is provided in the Supplementary Information (Figure S1). The structure is predicted to be that of a globular

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cytochrome; its closest match in a BLAST search of the PDB is cyt *c*₅₅₂ from the mesophilic marine bacterium *Marinobacter hydrocarbonoclasticus* (formerly known as *Pseudomonas nautica*) [11–13]. The structure, folding, and dynamics of globular cytochromes *c* from a wide variety of mesophilic [14–17] and thermophilic [18–22] organisms have been well studied, making this protein an excellent model system for an examination of psychrophilic adaptations. The bright red color and unmistakable iron porphyrin absorption spectrum aid in isolation, purification, and characterization of cytochrome *c*; and since the heme group is covalently bound to the protein through a conserved Cys-X-X-Cys-His motif, the heme remains attached to the amino acid chain even under denaturing conditions.

A synthetic gene for *Cpcyt c*₅₅₂ was co-transformed into *E. coli* with the pEC86 heme cassette [23] to overexpress a properly folded cytochrome with a covalently bound heme. Experimental details describing cloning, protein overexpression, and protein purification are provided in the Supplementary Information. Spectroscopic experiments are performed in 100 mM sodium phosphate buffer, pH 7.0, in septum-topped quartz cuvettes using a Varian Cary 5000 UV-vis-NIR spectrophotometer with a Peltier temperature-controlled sample compartment. The sample compartment and optics are purged with a flow of dry dinitrogen gas to prevent water condensation on the cuvette at low temperatures.

The mass of purified *Cpcyt c*₅₅₂ has been confirmed by MALDI-TOF mass spectrometry (Scripps Center for Mass Spectrometry, La Jolla, CA); calculated mass 8832 Da, measured *m/z* 8834. The N-terminal signal peptide is correctly cleaved in *E. coli* and does not appear in the purified protein. The UV-visible absorption spectrum of Fe^{III}*Cpcyt c*₅₅₂ is shown in Figure 1. The primary features are the Soret band ($\epsilon \sim 104,000 \text{ M}^{-1}\text{cm}^{-1}$) at 411 nm, and the Q-bands between 500–600 nm, with a maximum at 525 nm ($\epsilon \sim 9500 \text{ M}^{-1}\text{cm}^{-1}$). Also visible in the spectrum of Fe^{III}*Cpcyt c*₅₅₂ is the band at 698 nm that is attributed to Fe-S(methionine) coordination (inset, Figure 1) [24, 25].

The shape, intensity, and position of these bands are commonly used as a probe of protein folding. Since we are most interested in thermal effects on protein folding for this psychrophilic protein, we have used heat to denature *Cpcyt c*₅₅₂, monitoring the unfolding by UV-vis absorption spectroscopy (Figure 2). Spectra were collected every 2 K from 275–359 K (2–86 °C), with a 10-minute temperature equilibration period between each measurement. The thermal ramp and spectral data collection are computer controlled using software provided by Agilent. Upon heating and protein denaturation, the Soret band shifts to higher energy and the Q-bands broaden and shift. The Fe^{III}-S(Met) band at 698 nm in the oxidized protein disappears upon heating, indicating the loss of heme Fe–methionine coordination when the protein unfolds. The thermal denaturation of *Cpcyt c*₅₅₂ is irreversible. For the current analyses, we assume that the Lumry-Eyring model holds, *i.e.*, that reversible protein unfolding is followed by irreversible changes in the unfolded protein at high temperatures [26].

A thermal denaturation curve can be produced by plotting the absorbance at 411 nm (λ_{max} of the Soret band for the folded protein) vs. temperature. Using the method of John and Weeks [27], we have fit the first derivative of each thermal denaturation curve to a modified form of the van't Hoff equation. Fitting the first derivative rather than the raw data minimizes the effect of the baseline on the fitting results [27, 28]. From these fits, we have determined the van't Hoff enthalpy (ΔH_{vH}) and the melting temperature (T_{m}) for *Cpcyt c*₅₅₂ (Table 1). Since at T_{m} , $\Delta G = 0$ and $\Delta S = \Delta H_{\text{vH}}/T_{\text{m}}$, we can also calculate the unfolding entropy ΔS for this protein (Table 1). Limited enthalpy data are available in the literature for thermal denaturation of cyt *c* at neutral pH [29, 30]; here we will compare to ferricytochromes *c* from the mesophilic eukaryotes yeast and horse and the thermophilic bacterium *Thermus thermophilus*.¹

For yeast iso-1-cytochrome *c*, a plot of ΔH_{vH} vs. T_m is linear in the range of pH 3–5 [31, 32]. Extrapolation to pH 7 yields $\Delta H_{vH} = 398$ kJ/mol and $T_m = 333$ K (Table 1). It is of note that *Cpcyt c₅₅₂* has a T_m that is identical (within error, 334 ± 2 K) to that of yeast iso-1-cytochrome *c* at pH 7, while ΔH_{vH} for *Cpcyt c₅₅₂* is lower by ~ 90 kJ/mol than that of yeast iso-1-cytochrome *c* ($\Delta H_{vH} = 309 \pm 33$ kJ/mol). In the case of horse heart cytochrome *c*, both T_m and ΔH_{vH} are high [33]. By contrast, a thermophilic *cyt c* from *T. thermophilus* has both a higher T_m and a significantly lower ΔH_{vH} (Table 1) [34].

One might expect that if a thermophilic *cyt c* is characterized by lower ΔH and ΔS and higher T_m values when compared to a mesophilic homologue, then psychrophilic proteins would be characterized by lower T_m , and increased values of ΔH and ΔS . The results reported here, however, indicate that this is not the case. Our findings reveal that psychrophilic *Cpcyt c₅₅₂* has a lower ΔH_{vH} and unchanged T_m compared to similar mesophilic proteins and suggest that there exist mechanisms of protein stabilization in the psychrophile that are unexpected based on sequence alone. It will be important to examine the three-dimensional structure of the protein for insights into the molecular basis of this stabilization. In particular, it has been suggested that psychrophilic proteins have decreased intramolecular interactions (*e.g.*, hydrogen bonds) in order to increase flexibility, with an expected concomitant decrease in stability [6, 35, 36]. The large number of alanines in the amino acid sequence of *Cpcyt c₅₅₂* (16 residues, 20.3 %) is consistent with the prediction of greater protein flexibility. The relatively short, hydrophobic alanine side chain should decrease steric conflicts and increase overall protein chain flexibility, as demonstrated by the intrachain diffusion studies of Kiefhaber and coworkers [37, 38]. Our work suggests that the predicted flexibility of psychrophilic *Cpcyt c₅₅₂* does not correlate with overall protein stability toward unfolding. Protein dynamics (flexibility) and protein stability are not simply coupled in this protein. Further calorimetric, spectroscopic, and structural studies to investigate these issues are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

$\lambda_{\max}$	wavelength of maximum absorbance
<i>Cpcyt c₅₅₂</i>	<i>Colwellia psychrerythraea</i> cytochrome <i>c₅₅₂</i>

¹ Table S1 presents a multiple sequence alignment for *Cpcyt c₅₅₂* with *cyt c* from these organisms. Although the sequence identities with *Cpcyt c₅₅₂* are low (15.7–20.6 %), the structures of these cytochromes are all very similar to that of the predicted structure of *Cpcyt c₅₅₂*. Also included in the alignment is the sequence of *cyt c₅₅₂* from the mesophile *M. hydrocarbonoclasticus*, the closest match to *Cpcyt c₅₅₂* in the PDB.

ΔH_{vH}	van't Hoff enthalpy
IP	isopropyl β -D-1-thiogalactopyranoside
T_m	melting temperature

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Synopsis

Thermal denaturation of ferricytochrome c_{552} from *Colwellia psychrerythraea*; monitored by UV-vis absorption spectroscopy; calculations of T_m and van't Hoff enthalpy

Highlights

- The first monoheme cytochrome from a psychrophile was overexpressed and purified
- The T_m and van't Hoff enthalpy of unfolding are reported from thermal denaturation
- This cyt c_{552} has a lower ΔH_{vH} and unchanged T_m compared to mesophilic cytochromes
- Predicted protein flexibility does not correlate with overall protein stability
- Protein stability and dynamics are not coupled simply, in contrast with predictions

Figure 1. UV-visible absorption spectrum of ferricytochrome *c*₅₅₂ from *Colwellia psychrerythraea* in sodium phosphate buffer, pH 7.0. The inset highlights the band at 698 nm attributed to Fe–methionine(S) coordination.

Figure 2. Thermal denaturation of ferricytochrome c_{552} from *Colwellia psychrerythraea* (sodium phosphate buffer, pH 7.0), monitored by UV-vis absorption spectroscopy.

Table 1Thermodynamic parameters for ferricytochrome *c* unfolding.

Protein	ΔH_{vH} (kJ/mol)	T_m (K)	ΔS (kJ/mol K)	Reference
<i>C. psychrerythraea</i> cyt c_{552} ¹	309±33	334±2	0.925	This study
Yeast iso-1-cyt c ²	398	333	1.20	32, 33
Horse heart cyt c ³	410	357	1.15	34
<i>T. thermophilus</i> cyt c_{552} ⁴	130	389	0.334	35

¹ Determined spectroscopically; Soret band. Data reported ± 95% confidence level.² Determined spectroscopically; Soret band. Extrapolated from low pH data.³ Determined by differential scanning calorimetry.⁴ Determined spectroscopically; methionine ligation.