Enhancing the thermal stability of mitochondrial cytochrome b$_5$ by introducing a structural motif characteristic of the less stable microsomal isoform

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Outer mitochondrial membrane cytochrome b$_5$ (OM b$_5$) is the most thermostable cytochrome b$_5$ isoform presently known. Herein, we show that OM b$_5$ thermal stability is substantially enhanced by swapping an apparently invariant motif in its heme-independent folding core with the corresponding motif characteristic of its less stable evolutionary relative, microsomal cytochrome b$_5$ (Mc b$_5$). The motif swap involved replacing two residues, Arg15 with His and Glu20 with Ser, thereby introducing a Glu11-His15-Ser20 H-bonding triad on the protein surface along with a His15/Trp22 π-stacking interaction. The ferric and ferrous forms of the OM b$_5$ R15H/E20S double mutant have thermal denaturation midpoints ($T_m$ values) of ~93°C and ~104°C, respectively. A 15°C increase in apoprotein $T_m$ plays a key role in the holoprotein thermal stability enhancement, and is achieved by one of the most common natural mechanisms for stabilization of thermophilic versus mesophilic proteins: raising the unfolding free energy along the entire stability curve. Keywords: apoproteins/cytochrome b$_5$/m values/residual structure/thermal stability

Introduction

Proteins that are stably folded and functional at high temperatures (thermophilic proteins) continue to attract attention, in part because they challenge our fundamental understanding of protein stability (Sterner and Liebl, 2001; Li et al., 2005; Razvi and Scholz, 2006), but also because of their potential industrial applications (Bruins et al., 2001; Vieille and Zeikus, 2001; de Miguel Bogas et al., 2006). Highly heat stable proteins may be obtained from thermophilic organisms (Vieille and Zeikus, 2001; van den Burg, 2003; Atomi, 2005). Alternatively, they can be generated via engineering approaches commencing with proteins of more modest stability (Renugopalakrishnan et al., 2005; DiTursi et al., 2006; Reetz et al., 2006; Sylvestre et al., 2006), efforts that are sometimes guided by studies comparing orthologous proteins from thermophilic and mesophilic organisms. We have recently initiated a similar rational approach toward thermal stability enhancement (Sun et al., 2005), but based on comparative studies (Silchenko et al., 2000; Altuve et al., 2001; Cowley et al., 2002, 2004, 2005; Altuve et al., 2004) of two paralogous heme proteins that arise via gene duplication in vertebrates (Lederer et al., 1983; Guzov et al., 1996; Wang et al., 2007); the outer mitochondrial membrane and microsomal isoforms of cytochrome b$_5$ (OM b$_5$ and Mc b$_5$, respectively). OM and Mc b$_5$s contain polar heme-binding domains that are separated from membrane anchoring domains via flexible medial regions (Lederer, 1994; Kuroda et al., 1998). Studies with recombinant proteins comprising only the heme-binding domains have revealed that mammalian OM b$_5$s are considerably more thermostable than their Mc counterparts (Silchenko et al., 2000; Altuve et al., 2001, 2004). For example, thermal denaturation midpoints ($T_m$ values) for the tryptic fragments of rat OM cytochrome b$_5$ (rOM b$_5$) and bovine Mc cytochrome b$_5$ (bMc b$_5$) are ~85°C and ~67°C, respectively. Figure 1 compares the amino acid sequences of bMc and rOM b$_5$. An alignment of heme-binding domain sequences for all known mammalian OM and Mc b$_5$ pairs is included in the Supporting Information (Figure S1).

The greater thermal stability exhibited by mammalian OM b$_5$s in comparison with their Mc counterparts probably does not play a direct role in their specialized sub-cellular roles. It is more likely to be a source or consequence of two other notable biophysical differences, one or both of which may be related to their functional divergence. Specifically, OM b$_5$s differ from Mc b$_5$s in having (i) more negative reduction potentials (Altuve et al., 2004), indicating a need for a stronger electron transfer driving force; and (ii) lower polypeptide conformational mobility (Altuve et al., 2001; Lee and Kuczera, 2003; Simeonov et al., 2005), suggesting greater specificity in interactions with redox partners. Our ideas about the possible relationship between OM and Mc b$_5$ stability, redox potential and polypeptide conformational mobility have been detailed in several recent papers (Altuve et al., 2004; Cowley et al., 2004, 2005, 2006; Simeonov et al., 2005).

In light of the divergent stability properties of mammalian OM and Mc b$_5$s, it is noteworthy that the corresponding apoproteins exhibit nearly identical unfolding free energies at pH 7 and 25°C [$\Delta G_N^{\circ}$]$_{\text{rH}}$ ~3 kcal/mol as determined in urea-mediated denaturation studies (Cowley et al., 2004)]. OM and Mc apo-b$_5$s are also similar, in that their empty heme-binding pockets (core 1; see Figs 1 and 2A) are virtually devoid of secondary structure, whereas the remainder of each polypeptide (core 2) adopts a stable holoprotein-like fold (Falzone et al., 1996; Cowley et al., 2004). Studies in our laboratories have indicated that conformationally disordered core 1 is considerably more compact and less dynamic in OM apo-b$_5$ than in Mc apo-b$_5$, however, indicating the population of fewer non-native conformations (Cowley et al., 2004). The stronger heme-binding exhibited by OM apo-b$_5$ relative to Mc apo-b$_5$ may therefore reflect a less unfavorable entropy associated with the accompanying core 1 polypeptide conformational reorganization (Cowley et al., 2004, 2005).

The similar OM and Mc apo-b$_5$ unfolding free energies have arisen by evolution of distinctly different conserved packing motifs, as initially revealed in mutagenesis studies involving residue 71 (Cowley et al., 2002, 2005). Residue 71,
located near the C-terminal end of α5 in core 1 (Figs 1 and 2A), is leucine in all known mammalian OM b5s and serine in their Mc counterparts. Replacing Leu71 in rOM b5 with Ser was found (i) to destabilize the holoprotein, due in large measure to a >2 kcal/mol decrease in apoprotein stability (Cowley et al., 2002); and (ii) to extend conformational disorder in the apoprotein beyond core 1 and into core 2, including disruption of tertiary structure in the vicinity of Trp22 (Cowley et al., 2005). Mc b5s are therefore better able than OM b5s to accommodate Ser at position 71, which we concluded was a manifestation of a more stable core 2. A difference in packing that we predicted might contribute to this greater Mc b5 core 2 stability was identified by comparing β-sheet strands and α-helices, respectively. The residues indicated in bold comprise core 1, the heme-binding pocket. Isoleucine residues are disordered in the rOM b5 X-ray crystal structure. Numbering is based on the scheme introduced by Mathews et al. (1979) for the 93-residue lipase fragment of bovine Mc bs.

Materials and methods

Proteins

The recombinant plasmid pET11a-OM b5, harboring the gene coding for the heme-binding domain of rOM b5, was used as template to construct a gene for the corresponding R15H/E20S double mutant (hereafter rOM R15H/E20S b5). The primers designed for this purpose were 5'-GAAGTTGCGACAATACACCGCGGAATCCACCTGGATG G-3' and 5'-CCATTCAGGTTGATCCGCAGTTCTCGTATTTATTTTGTTGCCTA A CTT-3'. Underlined codons represent mismatches introduced to generate the mutations. The recombinant constructs were transformed into Escherichia coli DH5α competent cells for amplification. Once the mutations had been confirmed by sequencing, the recombinant pET-11a plasmids
carrying the mutant genes were transformed into *E. coli* BL21(DE3) cells for subsequent protein expression. Expression of rOMR<sup>15H/E20S</sup> b<sub>5</sub> and subsequent steps preceding purification were accomplished using the protocol developed for rOM b<sub>5</sub> (Rivera et al., 1992), and purification was achieved using a procedure recently reported for house fly cytochrome b<sub>5</sub> (Wang et al., 2007). Purity was assessed by native and SDS–PAGE. The mutant protein was analyzed by electro-spray ionization mass spectrometry (ESI-MS; KU mass spectrometry laboratory), using conditions that caused essentially complete dissociation of hemin. The experimental mass closely matched the calculated mass of the polypeptide, which includes the initiator methionine and residues (5) to 87: 10 447.7 Da (experimental); 10 447.5 (calculated average MW).

**Holoprotein thermal denaturation**

Thermal denaturation experiments with the holo form of rOMR<sup>15H/E20S</sup> b<sub>5</sub> were performed on a Varian Careee 100 Bio UV/Visible spectrophotometer equipped with a Peltier thermostatted multiple cell holder and a dedicated temperature probe accessory (±0.1°C). Solutions were buffered to pH 7.0 using 50 mM sodium phosphate. Protein concentration ranged from 3–5 μM, and was estimated via the absorbance at the Soret band (λ<sub>max</sub> = 412 nm; ε<sub>max</sub> = 130 000 M<sup>−1</sup> cm<sup>−1</sup>) in the ferric state (Beck von Bodman et al., 1986). Experiments with the ferrous proteins were performed in quartz cuvettes of 1 cm path length and 1 ml sample volume, equipped with tight-fitting PTFE lids. For experiments with the ferrous proteins, we utilized a specially designed side-arm quartz cuvette of 1 cm path length and 3 ml sample volume featuring an opening that could be sealed with a rubber septum. Reduction of the ferric proteins to the ferrous state was accomplished by adding an aliquot of degassed aqueous sodium dithionite, after bubbling of the sample solutions for at least 30 min with N<sub>2</sub> that had been passed through a chromous chloride solution to remove adventitious oxygen. A positive N<sub>2</sub> pressure was maintained throughout the experiment. In all experiments, the temperature was increased in increments of 2°C, and samples were equilibrated for 5 min after reaching each desired temperature. For proteins having thermal unfolding curves that reached a plateau in the denaturing region, thermal denaturation midpoints (<i>T<sub>m</sub></i> values) were obtained by fitting plots of absorbance at the Soret band λ<sub>max</sub> (412 nm for the ferric forms; 423 nm for the ferrous forms) versus temperature to a previously described equation describing a two-state equilibrium (Constans et al., 1998). For proteins having denaturation curves with no plateau in the denaturing region, <i>T<sub>m</sub></i> values were estimated by visual inspection of the data.

**Apoprotein chemical denaturation**

Urea-mediated apoprotein denaturation studies were performed on a PTI QuantaMaster luminescence spectrometer (protein concentrations 0.5–1 μM), monitoring changes in emission (340 nm) of Trp-22 (λ<sub>exc</sub> 295 nm). Samples were buffered to pH 7.0 using 30 mM MOPS, and were incubated at 25°C for 1 h before spectra were recorded at the same temperature. Denaturation data were fit (Kaleidagraph v. 3.5: Synergy Software) to two-state Eq. (1), where [D] is the urea concentration, Δ<i>G</i><sub>N→U</sub> the free energy of unfolding at 25°C when [urea] = 0 and <i>m</i> the sensitivity of the unfolding free energy to urea concentration (Pace, 1986). Urea denaturation midpoints (<i>C<sub>m</sub></i> values) were then determined from the relationship in Eq. (2).

\[
\text{Fraction folded} = \frac{\exp(\Delta G_{N\rightarrow U} - m[D]/RT)}{1 + \exp(\Delta G_{N\rightarrow U} - m[D])/RT}
\]

\[
\Delta G_{N\rightarrow U} = C_n \cdot m
\]

**Differential scanning calorimetry**

Scanning calorimetry experiments with rOMR<sup>15H/E20S</sup> apo-b<sub>5</sub> (0.80 mg/ml) were performed on a VP-DSC microcalorimeter (Microcal Inc.). The sample was extensively diaxylized before use (four changes of buffer every 6 h) against 50 mM potassium phosphate, pH 7.0. Immediately prior to each experiment, insoluble matter was removed by centrifugation at 12 000 g for 5 min and the sample was degassed at 0.5 atm for 15 min. Prior to making measurements, baselines were established via repeated scans in which the sample cell contained buffer solution from the final dialysis step. Scans were performed from low to high temperatures at 1 K/min. Data were analyzed via a statistical mechanics-based deconvolution as implemented in the CSC 5100 software package to obtain the calorimetric enthalpy change (Δ<i>H</i><sub>cal</sub>), and via a non-linear least-squares fit to a two-state model to obtain the van’t Hoff enthalpy change (Δ<i>H</i><sub>VD</sub>).

**Dynamic light scattering**

Dynamic light scattering (DLS) measurements were performed on a BI-200SM research goniometer and laser light scattering system, equipped with a BI-9000AT digital correlator (Brookhaven Instruments Corporation). Incident light of λ = 532 nm (0.3–1.0 W) was used, with scattered light detected at an angle of 90° via a photomultiplier tube. Sample temperature was controlled by means of a thermostatted cell jacket. Samples (100 μM) were passed through 100 nm filters (Whatman, UK) immediately before use. Experiments were performed at 25°C, and samples were buffered to pH 7 with 50 mM potassium phosphate. Each experiment consists of six 30-s runs, and the instrument software reports the results of each experiment as the average of the six runs. Three independent experiments yielded nearly identical results, and therefore we only report the results from one experiment in the appropriate data table. All data could be fitted multimodally, and essentially 100% of the scattering mass was attributed to a single low molecular mass component. The diffusion coefficient (D) and the hydrodynamic radius (<i>R</i><sub>H</sub>) are related by the Stokes–Einstein equation [Eq. (3)].

\[
R_h = kT/6\pi\eta D
\]

where <i>T</i> is the temperature in Kelvin, <i>k</i> the Boltzman constant (1.38 × 10<sup>−16</sup> erg/K) and η the solution viscosity. Values of η for urea solutions were determined by the method of Kawahara and Tanford (1966).

**Results**

**Holoprotein thermal denaturation**

Thermal denaturation of the polypeptide in ferric and ferrous cytochrome b<sub>5</sub> is accompanied by dissociation of the bonds between heme iron and its axial ligands His39 and His63, converting iron from the low-spin to the high-spin state.
reach in our experiments (Figs 3B and 4). We estimate that to C24 the ferric state. Returning the ferrous rOM R15H/E20S b5 is not fully reversible. For this and other reasons (Cowley et al., 2002), the data for those proteins cannot be extrapolated reliably through fits to two-state equations, and Table I therefore only reports their Tm values. Notably, ferric rOMR15H/E20S b5 (Tm = 93°C) exhibits substantially enhanced thermostability relative to ferric rOM b5 (Tm = 85.5°C).

Heme iron reduction enhances cytochrome b5 thermal stability (Hewson et al., 1993), likely because it removes a formal +1 charge on iron and thereby strengthens heme–polypeptide interactions (McLachlan et al., 1986; Banci et al., 1998). Yao et al. (1997) have reported that, in 100 mM sodium phosphate buffer at pH 7, the Tm value of hMc b5 increases from 66°C in the ferric state to 77°C in the ferrous state (ΔTm = 11°C). We obtained a nearly identical result with hMc b5 in 50 mM potassium phosphate buffer at pH 7 (Fig. 3A and B; Table I). Reducing rOM b5 from the ferric to the ferrous state produced a somewhat smaller Tm increase, from ∼86°C to ∼93°C (Fig. 3B; Table I). Reduction was also found to enhance rOMR15H/E20S b5 thermal stability. In fact, ferrous rOMR15H/E20S b5 appears to be ∼80% folded at 99°C, the highest temperature we could reach in our experiments (Figs 3B and 4). We estimate that reduction increases the Tm of the double mutant from ∼92°C to ∼104°C, substantially greater than the effect observed in the ferric state. Returning the ferrous rOMR15H/E20S b5 samples to 25°C at the end of the thermal denaturation experiments reproduced the original spectra, demonstrating (i) absence of heme iron oxidation during the experiments; and (ii) that no solvent was lost to evaporation.

**Apoprotein chemical denaturation**

Urea-mediated denaturation of apo-b5s can be conveniently probed by fluorescence spectroscopy, monitoring changes in fluorescence emission of the lone Trp residue, Trp22. Previously published urea-mediated denaturation data for rOM and bMc apo-b5s at 25°C and pH 7.0 (Cowley et al., 2004) are shown in Fig. 5 and Table II, along with corresponding data for rOMR15H/E20S apo-b5 obtained in the present study. The curves are reported in terms of the fraction of protein that is folded at each urea concentration, and the lines through the curves represent fits to two-state Eq. (1) (see experimental). The results of this study show that rOMR15H/E20S apo-b5 is ∼1.1 kcal/mol more stable than rOM apo-b5 at 25°C. We have reported that Trp22 fluorescence emission intensity decreases substantially upon unfolding of rOM and OM7M apo-b5 (Wang et al., 2006), consistent with the Trp22 side chain moving from a well-ordered region in the folded state to a disordered region in the unfolded state. Trp22 fluorescence emission intensity was observed to be much weaker in the folded forms of bMc and OM7M ap-b5 than in the folded forms of rOM and OM7M apo-b5. Moreover, Trp22 emission intensity was found to increase upon unfolding of OM7M apo-b5, as had previously been demonstrated for bMc apo-b5 (Huntley and Strittmatter, 1972). On the basis of that literature precedent, we concluded that Trp22 fluorescence is quenched by π-stacking with His15 in folded OM7M apo-b5 (Wang et al., 2006), an effect that becomes less efficient as the protein unfolds. The present study showed that Trp22 fluorescence emission intensity is much less intense in rOMR15H/E20S apo-b5 than in rOM apo-b5, and that it increases substantially upon rOMR15H/E20S apo-b5 unfolding (data not shown). We can therefore conclude that Mc b5-like His15/Trp22 interactions are present in rOMR15H/E20S apo-b5.

**Apoprotein thermal denaturation**

Differential scanning calorimetry (DSC) data for rOMR15H/E20S apo-b5 revealed a single transition that is fit well by a

**Table I. Holoprotein thermal denaturation midpoints**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FeIII</td>
</tr>
<tr>
<td>bMc b5</td>
<td>67.6 ± 0.3b</td>
</tr>
<tr>
<td>rOM b5</td>
<td>85.5 ± 0.5b</td>
</tr>
<tr>
<td>rOMR15H/E20S b5</td>
<td>~93c</td>
</tr>
</tbody>
</table>

*Experiments performed at pH 7.0 in 50 mM phosphate buffer. bData from reference (Cowley et al., 2002). cEstimated (see text).
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Table II. Apoprotein urea-mediated denaturation dataa

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cm (M)</th>
<th>m (kcal/mol/M)</th>
<th>ΔG_N−U (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rOM apo-b5</td>
<td>2.52 ± 0.03</td>
<td>1.1 ± 0.1</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>bMc apo-b5</td>
<td>4.09 ± 0.04</td>
<td>0.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>rOMR15H/E20S apo-b5</td>
<td>5.20 ± 0.10</td>
<td>0.7 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>

aData for rOM and bMc apo-b5 are from (Cowley et al., 2004).

bAverage values and errors from two independent runs.

a constant in order to estimate free energies of protein unfolding at temperatures other than the Tm (Freire, 1995).

We calculated values of ΔG from 0°C to 100°C for bMc, rOM and rOMR15H/E20S apo-b5 using Eq. (4), an expanded form of the Gibbs–Helmholtz equation.

\[ \Delta G(T) = \Delta H_m \left[ 1 - \left( \frac{T}{T_m} \right)^m \right] - \Delta C_p \left( T_m - T \right) + T \ln \left( \frac{T_m}{T} \right) \]  (4)

The resultant stability curves (Becktel and Schellman, 1987) are presented in Fig. 7. In light of our assumption of a constant ΔCp, it is worth noting that the relative ΔG_N−U values for bMc, rOM and rOMR15H/E20S apo-b5 determined by extrapolation of DSC data to 25°C (Table IV) are very similar to those obtained in urea denaturation experiments performed at 25°C (Table II). This gives us confidence that relative stabilities of the apoproteins calculated at both higher and lower temperatures are also reliable.

**Dynamic light scattering**

As noted above, urea-mediated denaturation data show that rOMR15H/E20S apo-b5 is ~1.1 kcal/mol more stable than rOM apo-b5 at 25°C. Nonetheless, the data in Fig. 5 and Table II show that the R15H/E20S double mutation (i) greatly increased the concentration of urea required to achieve 50% unfolding of rOM apo-b5 (the Cm value); and (ii) substantially decreased the slope of the unfolding transition as reflected in a diminished m value. The data in Fig. 5 and Table II illustrate our previously reported finding that bMc apo-b5 likewise has a larger Cm value and a smaller m value than does rOM apo-b5 (Cowley et al., 2004). Mutations are known to lower a protein’s denaturant m value if they diminish the change in solvent accessible surface area (ΔASA) that accompanies unfolding (Myers et al., 1995). Previously published DLS data shown in Fig. 8 and Table III have indicated that a smaller ΔASA is a source of the smaller m value of bMc apo-b5 relative to rOM apo-b5 (Cowley et al., 2004). Specifically, bMc apo-b5 unfolding involves a much smaller increase in hydrodynamic radius (Rd value) than does rOM apo-b5 unfolding, largely because urea-denatured bMc apo-b5 is much more compact than urea-denatured rOM apo-b5. Figure 8 and Table III show that the same explanation holds for the smaller m value of rOMR15H/E20S apo-b5 relative to that of rOM apo-b5: urea-denatured rOMr15h/e20s apo-b5 is more compact than urea-denatured rOM apo-b5, and in fact is even more compact than urea-denatured bMc apo-b5. Notably, the double mutation exerted little, if any effect on the Rd value of folded rOM apo-b5, consistent with the fact that both mutated residues are located on the surface of core 2.

We also performed additional DLS studies on OM7M apo-b5 which, as noted in the introduction, unfolds in two
distinct stages. Table III shows that near the end of the first denaturation stage, in 3 M urea, the $R_H$ value of OM$^7M$ apo-b$_5$ is somewhat smaller than those of urea-denatured bMc and rOM$^{R15H}$ apo-b$_5$. On the other hand, the $R_H$ value of OM$^7M$ apo-b$_5$ at the end of its second unfolding stage is similar to that of urea-denatured rOM apo-b$_5$.

Discussion

Effects of the motif swap on holoprotein stability and structure

We have substantially enhanced the thermal stability of rOM b$_5$ in both the ferric and ferrous oxidation states by replacing a conserved mammalian OM b$_5$ core 2 packing motif (Fig. 2C) with the corresponding motif characteristic of the less stable Mc isoform (Fig. 2B). To the best of our knowledge, the resultant mutant rOM$^{R15H/E20S}$ b$_5$ is the most thermostable cytochrome b$_5$ variant reported to date, with $T_m$ values of $93^\circ C$ (Fe$^{III}$) and $104^\circ C$ (Fe$^{II}$) at pH 7.

On the basis of evidence summarized in the introduction, we can assume that the motif swap introduced the following structural features into rOM b$_5$: (i) a Glu11-His15-Ser20 H-bonding triad that appears likely to be quite stabilizing when fully intact, as judged by the 2.8–2.9 Å N(His)–O(Glu) distances in the bMc b$_5$ crystal structure; and (ii) a His15/Trp22 π-stacking interaction in which the His15 imidazole ring is located directly above and parallel to the six-membered ring of the Trp22 indole side chain. This is one of the most commonly observed His/Trp side chain interactions in proteins (Samanta et al., 1999). Figure 9 shows two distinct patterns that are possible for the fully H-bonded Glu11-His15-Ser20 triad. NMR studies have shown that His15 has a pK$_a$ of 8.5 in bMc holo-b$_5$ (Altman et al., 1989), as well as in rat Mc holo- and apo-b$_5$ (Moore et al., 1991). It is therefore safe to assume that His15 in bMc apo-b$_5$ also has a pK$_a$ of ~8.5, and is protonated at the pH of 7.0 utilized in our studies (as in Fig. 9B). The Glu11/His15 H-bond is therefore also a salt bridge, and the His15/Trp22 interaction has cation-π character (Loewenthal et al., 1991; Fernandez-Recio et al., 1997; Berry et al., 2007).

The Glu11-His15-Ser20 triad in rOM$^{R15H/E20S}$ b$_5$ replaced a Glu11-Arg15-Glu20 triad, which appears likely to stabilize OM b$_5$s via ion-pair interactions. Although the relevant N–O distances in the rOM b$_5$ crystal structure are rather long, they can be much shorter (and the electrostatic interactions correspondingly stronger) as demonstrated by the crystal structure of OM$^{SM}$ b$_5$ (Cowley et al., 2002). In all four molecules in the OM$^{SM}$ b$_5$ unit cell, Arg15 NH to Glu11 O$_e$ distances are 2.6–2.7 Å (versus 4.4 Å in rOM b$_5$), and the side chains are much better oriented for H-bond formation. Arg15N$_e$ to Glu20 O$_e$ distances are also shorter in all four OM$^{SM}$ b$_5$ molecules (3.1–3.6 Å) than in the ROM b$_5$ structure (5.5 Å), but are not well oriented for H-bonding. The shorter N–O distances in the OM$^{SM}$ b$_5$ structures than in the rOM b$_5$ structure are primarily a consequence of different Glu11 and Glu20 side chain conformations. In contrast, the Arg15 side chains in rOM b$_5$ and the four OM$^{SM}$ b$_5$ molecules have virtually identical conformations. Analogous to the His15 imidazolyl side chain in the Mc b$_5$ motif described earlier, the Arg15 guanidino moiety in rOM and OM$^{SM}$ b$_5$ engages in parallel π-stacking/cation-π interactions with the six-membered ring of the Trp22 indole group, a particularly common and stabilizing arrangement for Arg and Trp side chains in proteins (Flocco and Mowbray, 1994; Mitchell et al., 1994; Gallivan and Dougherty, 1999; Tatko and Waters, 2003).
Role of the apoprotein in the stability increase

As noted in Results, we are unable to accurately determine thermodynamic parameters for unfolding of the holoproteins in this study. This reflects two factors: (i) most of the denaturation reactions are not fully reversible; and (ii) not all of the unfolding reactions are complete at the highest temperature achievable in our studies (99°C). In principle, however, thermodynamic stability of a given holo-b5 variant can also be estimated from the sum of the free energies of apoprotein folding (ΔG_{U→N} = ΔG_N→U) and of heme binding by the folded apoprotein (ΔG_{bind}). Given that none of the residues in the Mc and OM b5 motifs compared in Fig. 2B and C make van der Waals contact with the heme, we consider it unlikely that the R15H/E20S double mutation enhanced rOM holo-b5 thermostability via strengthened heme–polypeptide interactions. Moreover, the R15H/E20S double mutation did not detectably alter the R_H value of folded rOM apo-b5, suggesting little if any effect on empty core 1. It is therefore reasonable to conclude that the R15H/E20S double mutation had little if any effect on ΔG_{bind}; another parameter we are unable to measure directly. The holoprotein thermal stabilization instead appears to arise primarily from enhancement of apoprotein thermodynamic stability (ΔG_{U→N}), most notably an ~15°C increase in apoprotein T_m.

Three mechanisms have been described which, alone or in combination, can increase T_m of a protein and thereby make it more thermophilic (Nojima et al., 1977; Razvi and Scholtz, 2006): (I) raising ΔG along the entire stability curve; (II) flattening the stability curve by decreasing ΔC_p and (III) shifting the entire stability curve to higher temperatures, for example, by increasing the temperature at which the protein is maximally stable (T_S; where ΔS=0). Figure 7 and Table IV show that (i) rOM and rOM^{R15H/E20S} apo-b5 have nearly identical T_S and ΔC_p values; and (ii) rOM^{R15H/E20S} apo-b5 is more stable than rOM apo-b5 at all temperatures between 0°C and 100°C. The ~15°C increase in rOM apo-b5 T_m resulting from the R15H/E20S double mutation therefore arises almost exclusively via mechanism (I), the most common one utilized by nature for stabilizing proteins from thermophilic organisms relative to their mesophilic counterparts (Razvi and Scholtz, 2006). It is also worth noting that rOM^{R15H/E20S} apo-b5 becomes progressively more stable in comparison with rOM apo-b5 as the temperature increases (ΔΔG ~1.2 kcal/mol at 25°C, ~2.0 kcal/mol at 60°C and ~3.1 kcal/mol at 100°C).

The greater thermal stability exhibited by rOM holo-b5 in comparison with bMc holo-b5 also has a contribution from a higher apoprotein T_m (ΔT_m=5.4°C). rOM and bMc apo-b5 have very similar ΔC_p values and are equal in stability at 34°C, but the T_S value of rOM apo-b5 is ~13°C higher than that of bMc apo-b5. The higher T_m value of rOM apo-b5 relative to bMc apo-b5 therefore occurs mainly via mechanism (II). rOM apo-b5 becomes progressively less stable in comparison with bMc apo-b5 below 34°C (ΔΔG approximately −0.4 kcal/mol at 25°C; approximately −1.6 kcal/mol at 0°C), but progressively more stable relative to bMc apo-b5 above 34°C (ΔΔG ~1.0 kcal/mol at 60°C; ~2.2 kcal/mol at 100°C).

Because the R15H/E20S double mutation appears to have affected ΔG_{U→N} much more than ΔG_{bind}, we consider it

Table III. DLS data

<table>
<thead>
<tr>
<th>Apoprotein</th>
<th>Apoprotein</th>
<th>Apoprotein</th>
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<tbody>
<tr>
<td></td>
<td>0 M urea</td>
<td>3 M urea</td>
</tr>
<tr>
<td></td>
<td>R_H (nm)</td>
<td>P (%)</td>
</tr>
<tr>
<td>rOM</td>
<td>1.83 ± 0.13</td>
<td>5.3</td>
</tr>
<tr>
<td>bMc</td>
<td>2.12 ± 0.13</td>
<td>7.1</td>
</tr>
<tr>
<td>rOM^{R15H/E20S}</td>
<td>1.98 ± 0.09</td>
<td>8.0</td>
</tr>
<tr>
<td>OM</td>
<td>2.67 ± 0.12</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Data recorded at 25°C in 50 mM potassium phosphate buffer, pH 7.0.

Polydispersity, a measure of size distribution.

From (Cowley et al., 2004). Not determined.
likely that relative stabilities of \textit{rOM}^{R15H/E20S} and \textit{rOM} holo-b5 as a function of temperature closely parallel those of \textit{rOM}^{R15H/E20S} and \textit{rOM} apo-b5. In other words, \textit{rOM}^{R15H/E20S} holo-b5 is almost certainly more stable than \textit{rOM} holo-b5 at all temperatures between 0°C and 100°C, and to a greater extent at high temperatures than at more modest temperatures. It can also be argued that \textit{rOM} and \textit{bMc} holo-b5 exhibit greater stability differences at high temperatures than at more modest temperatures, although the relative stabilities of these proteins may actually reverse at a sufficiently low temperature. \textit{rOM} holo-b5 is almost certainly more stable in comparison with \textit{bMc} holo-b5 at any given temperature than the relative apoprotein unfolding free energies at that temperature would indicate, however. Indeed, all available evidence indicates that \textit{rOM} holo-b5 is considerably more stable than \textit{bMc} holo-b5 at 25°C, in spite of our calculations indicating that \textit{rOM} apo-b5 is \textit{~}0.4 kcal/mol less stable than \textit{bMc} apo-b5 at that temperature. As noted in the introduction, this may reflect the fact that conformationally disordered core 1 is more dynamic and expanded in folded \textit{bMc} apo-b5 than in folded \textit{rOM} apo-b5, which increases the entropic penalty associated with heme binding and thereby makes $\Delta G_{\text{bind}}$ less favorable (Cowley et al., 2004).

The motif swap appears to stabilize both the folded and unfolded forms of \textit{rOM} apo-b5

After demonstrating in our previous work that converting \textit{OM}^{SM} b5 to \textit{OM}^{TM} b5 via the H15R/E20S double mutation resulted in substantial increases in both holoprotein and apoprotein stability, we performed the reverse motif swap experiment with \textit{bMc} b5 (Wang \textit{et al.}, 2006). Introducing the H15R/S20E double mutation into \textit{bMc} b5 led to a modest 3.4°C decrease in holoprotein $T_m$, consistent with our expectations. However, \textit{bMc}^{H15R/S20E} apo-b5 was found to be \textit{~}1.8 kcal/mol more stable than \textit{bMc} apo-b5 at 25°C. We suggested that this increase in apoprotein unfolding free energy arose because the H15R/S20E double mutation destabilized unfolded \textit{bMc} apo-b5 to a greater extent than it destabilized the folded apoprotein. Supporting this conclusion was the observation that the H15R/S20E double mutation substantially diminished residual structure present in urea-denatured \textit{bMc} apo-b5 (as evidenced, for example, by an increase in $R_h$), strongly suggesting disruption of energetically unfavorable interactions. This is consistent with results by Pace \textit{et al.} (2000), who showed that stabilizing interactions among ionic residues can lead to unusually compact states for some unfolded proteins, and thereby affect the unfolding free energies of those proteins.

The residues involved in the \textit{bMc} b5 motif highlighted in Fig. 2B clearly favor a compact denatured state, as further demonstrated by the following observations: (i) urea-denatured \textit{bMc} apo-b5 has a considerably smaller $R_h$ value than urea-denatured \textit{rOM} apo-b5 (Cowley \textit{et al.}, 2004); (ii) urea-denatured \textit{rOM} and \textit{OM}^{SM} apo-b5 exhibit nearly identical $R_h$ values (Cowley \textit{et al.}, 2005); (iii) converting \textit{OM}^{SM} b5 to \textit{OM}^{TM} b5 via the R15H/E20S double mutation caused a change in apoprotein unfolding from a cooperative two-state reaction to a process that occurs in two sequential steps (Wang \textit{et al.}, 2006) and (iv) near the end of its first stage of urea-mediated denaturation \textit{OM}^{TM} apo-b5 is more compact than urea-denatured \textit{bMc} and \textit{rOM}^{R15H/E20S} apo-b5, whereas fully urea-denatured \textit{OM}^{TM} apo-b5 has an $R_h$ value nearly identical to that of urea-denatured \textit{rOM} apo-b5 (Table III).

The present study has revealed that urea-denatured \textit{rOM}^{R15H/E20S} apo-b5 is considerably more compact than urea-denatured \textit{rOM} apo-b5, providing strong evidence that the former is more stable. The fact that the R15H/E20S double mutation significantly increased \textit{rOM} apo-b5 unfolding free energy therefore allows us to conclude that it stabilized the folded apoprotein to a greater extent than the unfolded apoprotein. In the absence of this effect on the unfolded apoprotein, the stabilization achieved via the motif swap described herein would almost certainly have been considerably greater than observed.

Possible relevance to stabilization of natural thermophilic proteins

The factors stabilizing natural thermophilic proteins relative to their mesophilic homologues are often subtle, and vary from case to case. Surface electrostatic interactions, most notably salt bridges, have been shown to play a particularly important role, however (Xiao and Honig 1999; Kumar \textit{et al.}, 2000; Szilagyi and Zavodszky 2000). Moreover, recent studies have indicated that the number of salt bridges is generally less important for thermophilicity than their optimization on the protein surface (Greaves and Warwicker, 2007). Indeed, a number of researchers have made mesophilic proteins much more thermophilic by introducing favorable surface ion pairs, often by changing a single residue (Torrez \textit{et al.}, 2003; Gribenko and Makhatadze, 2007).

### Table IV. Apoprotein DSC data

<table>
<thead>
<tr>
<th>Apo-b5</th>
<th>$T_m$ (°C)</th>
<th>$\Delta C_p$ (kcal/mol/K)</th>
<th>$\Delta H_{cal}$ (kcal/mol)</th>
<th>$\Delta H_{cal}/\Delta H_{\text{HI}}$</th>
<th>$T_h$ (°C)</th>
<th>$\Delta G$, 25°C (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{bMc}</td>
<td>44.7 ± 0.5</td>
<td>0.98 ± 0.04</td>
<td>42.9 ± 1.8</td>
<td>41.9 ± 1.7</td>
<td>~5</td>
<td>~2.0</td>
</tr>
<tr>
<td>\textit{rOM}</td>
<td>50.1 ± 0.4</td>
<td>1.07 ± 0.04</td>
<td>35.7 ± 1.5</td>
<td>35.4 ± 1.6</td>
<td>~18.5</td>
<td>~1.7</td>
</tr>
<tr>
<td>\textit{rOM}^{R15H/E20S}</td>
<td>64.9 ± 0.3</td>
<td>0.97 ± 0.05</td>
<td>46.6 ± 1.9</td>
<td>45.3 ± 2.1</td>
<td>~21</td>
<td>~3.1</td>
</tr>
</tbody>
</table>

aData for \textit{rOM} and \textit{bMc} apo-b5 are from (Cowley \textit{et al.}, 2004).

Fig. 9. Two possible sets of interactions for the Glu11-His15-Ser20 H-bonding triad in Mc b5 and in \textit{rOM}^{R15H/E20S} b5.
Recall that the marked thermal stability enhancement obtained in the present work for rOM holo- and apo-b5 resulted from a motif swap in which two spatially close surface residues were mutated. The Arg15 to His mutation almost certainly maintained a positive charge, whereas the Glu11 to Ser mutation resulted in loss of a negative charge. The mutations altered the nature of salt bridging and hydrogen-bonding interactions among surface exposed side chains of residues 11, 15 and 20, and of stacking interactions between the side chains of residues 15 and 22. The R15H/E20S double mutation also entailed loss of four conformationally mobile side chain bonds.

As noted earlier, the substantial thermal stabilization in rOM holo- and apo-b5 resulting from the R15H/E20S double mutation arose by the most common general mechanism employed by nature to achieve high stability in thermophilic versus mesophilic proteins: raising the unfolding free energy across the entire unfolding curve. It is interesting to consider the possibility that it may also have been achieved by one of the most common natural mechanisms at the molecular level, optimization of salt bridges and other surface electrostatic interactions, and that a more favorable entropy change associated with formation of those interactions plays a key role.

Future directions

In future studies, we intend to ascertain the extent to which the various interactions involving His15 (π-stack with Trp22; H-bonds with Glu11 and Ser20) contribute to the greater stability and more compact unfolded states exhibited by rOM versus rOM apo-b5 and OM[R15H/E20S] versus rOM apo-b5. We will also continue our protein engineering studies with rOM b5, with the ultimate goal of rendering both the ferric and ferrous forms of the protein stable in boiling water and under other extreme conditions.

Supplementary Material

Supplementary data are available at PEDS online

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References


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