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Thermal stability of the polyheme cytochrome c_3 superfamily

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Abstract **The cytochrome c_3 superfamily includes *Desulfovibrio* polyheme cytochromes *c*. We report the characteristic thermal stability parameters of the *Desulfovibrio desulfuricans* Norway (*D.d.*N.) cytochromes c_3 (M_r 13,000 and M_r 26,000) and the *Desulfovibrio vulgaris* Hildenborough (*D.v.*H.) cytochrome c_3 (M_r 13,000) and high molecular mass cytochrome *c* (Hmc), as obtained with the help of electronic spectroscopy, voltammetric techniques and differential scanning calorimetry. The polyheme cytochromes are denatured over a wide range of temperatures: the *D.v.*H. cytochrome c_3 is highly thermostable (T_d = 121°C) contrary to the *D.d.*N. protein (T_d = 73°C). The thermostability of the polyheme cytochromes is redox state dependent. The results are discussed in the light of the structural and functional relationships within the cytochrome c_3 superfamily.**

Key words: Polyheme cytochromes *c*; Thermal stability; Structure/function relationships

1. Introduction

Many polyheme cytochromes – the tetraheme cytochrome c_3 (M_r 13,000) [1,2], the octaheme cytochrome c_3 (M_r 26,000) [3] and a high molecular mass (M_r 65,600) cytochrome *c* (Hmc) with 16 hemes [4–6] – are found in the periplasm of *Desulfovibrio* in which they are involved in the sulfate respiration. These hemoproteins have no structural similarities with the mitochondrial-type cytochrome *c* (class I) and all belong to class III of the *c*-type cytochrome [7]. At least one tetraheme cytochrome c_3 (M_r 13,000) is present in every *Desulfovibrio* species. The resolved 3-dimensional structures [8–11] of various types of cytochrome c_3 (M_r 13,000) show that the arrangement of the 4 hemes in the molecules is highly conserved. Octaheme cytochrome c_3 (M_r 26,000) is a dimer of two identical tetraheme cytochromes and the 3-dimensional structure of the cytochrome from *Desulfovibrio desulfuricans* Norway has been solved [12]: the monomeric subunit has the c_3 folding. Comparison between the arrangement of the heme binding sites (Cys-XX-Cys-His) and the axial histidines in the sequences of cytochromes c_3 and Hmc from *Desulfovibrio vulgaris* Hildenborough has clearly shown the presence of 4 c_3-like domains [5]. The cytochrome c_3 (M_r 13,000) constitutes the structural basic unit of this class of proteins which could be defined as the cytochrome c_3 superfamily.

Thermal denaturation study offers an opportunity to specify the structural relationships within this family, especially to investigate whether the c_3-like domains of the Hmc behave as structurally independent subunits – in that case, several unfolding events should be expected – or if they are tightly interacting. Furthermore, the study of the transition conformational states of a protein may bring some informations about its function.

In this thermostability study, we compare the characteristic heat transition parameters of *Desulfovibrio desulfuricans* Norway (*D.d.*N) cytochromes c_3 (M_r 13,000) and (M_r 26,000) and *Desulfovibrio vulgaris* Hildenborough (*D.v.*H.) cytochrome c_3 (M_r 13,000) and Hmc. We have used (i) electronic spectroscopy which provides information on the heme environment; (ii) voltammetric techniques which give further insight into the heme reactivity; and (iii) differential scanning calorimetry which provides the thermodynamic parameters of protein denaturation. The structural features, which may be relevant for the observed differences in the thermal transition of these polyheme cytochromes, have been analyzed.

2. Materials and methods

2.1. Protein purification

Cytochromes c_3 (M_r 26,000 and M_r 13,000) from *Desulfovibrio desulfuricans* Norway (N.C.I.B. 8310) and cytochrome c_3 (M_r 13,000) and Hmc from *Desulfovibrio vulgaris* Hildenborough (N.C.I.B. 8303) were prepared as described previously in [3,6,13,14], respectively.

2.2. Scanning microcalorimetric measurements

These measurements were carried out on a differential scanning microcalorimeter DASM-4 (NPO Biopribor, Pushchino, Russia) in 0.48 ml cells at a heating rate of 1 K/min. Protein concentrations varied from 0.3 to 1.8 mg/ml. Transition excess heat capacity, associated with structural breakdown, denaturation temperature (T_d), calorimetric denaturation enthalpy (ΔH_{cal}) and effective or Van't-Hoff denaturation enthalpy (ΔH_{eff}) were determined as described in [15]. Technical parameters of our microcalorimeter did not allow to perform heating to temperatures higher than 122°C.

2.3. UV-visible spectroscopy

Thermal denaturation of cytochromes was followed also spectrophotometrically by monitoring the decrease with temperature of the absorbance at 410 and 420 nm of cytochrome solutions using a Beckman DU 7000 spectrophotometer equipped with a water-regulated cell holder. The heating rate was ~1 K/min. The cytochromes c_3 and Hmc were 4 μM and 1 μM, respectively, in 0.1 M Tris-HCl, pH 7.6. The fully reduced state was obtained by adding excess of solid disodium dithionite.

2.4. Electrochemistry

Cyclic voltammetry (CV) and square-wave voltammetry (SWV) were carried out using an EG&G 273 potentiostat modulated by EG&G PARC M270 software. Alternating current voltammetry (ACV) experiments were carried out using the same potentiostat coupled to an EG&G PAR 5208 lock-in analyzer and EG&G PAR Head Start software. A 3-electrode system consisting of a Metrohm Ag/AgCl-saturated NaCl electrode, a platinum wire auxiliary and a pyrolytic graphite working electrode was used throughout. Before each experiment, the working electrode was polished with 0.05 μm aluminum slurry. The solutions were deoxygenated by bubbling with high purity nitrogen. Before each experiment, the cytochrome c_3 and Hmc samples (50 μM and 6 μM, respectively, in 0.1 M Tris-HCl, pH 7.6) were heated in

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sealed tubes using a heating rate of ~0.5 K/min and then maintained for 5 min at the temperature of interest. After about 5 min of cooling, the electrochemical measurements were carried out at room temperature (~ 22°C).

3. Results and discussion

3.1. The polyheme cytochromes are denatured over a wide range of temperatures

Fig. 1 shows the temperature dependence of excess heat capacities for *D.d.*N. cytochrome c_3 (A), *D.v.*H. Hmc (B) and *D.v.*H. cytochrome c_3 (C) at pH 7.6. Thermodynamic transition parameters are given in Table 1. The heat denaturation of *D.d.*N. cytochrome c_3 was irreversible. However, scanning calorimetry shows no sign of detectable aggregation in this case. Comparison of ΔH_{cal} with the value for horse heart cytochrome *c* at the same denaturation temperature shows good coincidence [16]. The ratio $R = \Delta H_{cal}/\Delta H_{eff}$ is a criterion which allows one to estimate how much the average size of a cooperative unit, i.e., the region of a protein molecule which melts more or less independently, differs from the size of the whole protein globule [15,17]. The value of *R* for *D.d.*N. cytochrome c_3 is equal to 1 (Table 1), indicating that the denaturation transition conforms closely to the 2-state mechanism, i.e., one cooperative unit exists within the protein molecule. A similar result was published for cytochrome *c* in [16]. Surprising results were obtained for *D.v.*H. cytochrome c_3 where the heat absorption peak connected with the irreversible thermal denaturation is located in the region of extremely high temperatures (Fig. 1C). The denaturation temperature of this protein differs by 48°C from that of *D.d.*N. cytochrome c_3 (Table 1). It is interesting to note that a similar high temperature of denaturation was obtained for calmodulin complexed with calcium [18]. The heat denaturation of the *D.v.*H. Hmc was also irreversible and scanning calorimetry shows detectable aggregation of denatured molecules after transition peak. In order to check the effect of

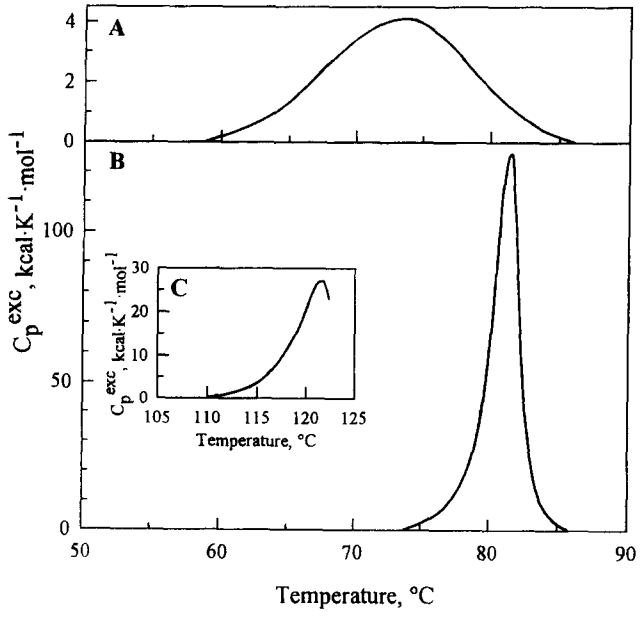

Fig. 1. Transition excess heat capacity versus temperature for *D.d.*N. cytochrome c_3 (M_r 13,000) (A), *D.v.*H. Hmc (B) and *D.v.*H. cytochrome c_3 (M_r 13,000) (C) at pH 7.6 (10 mM Tris-HCl).

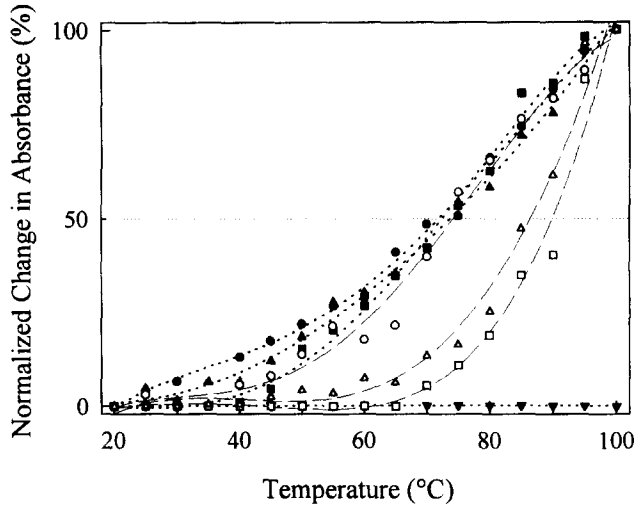

Fig. 2. Temperature dependence of the normalized change in absorbance at 410 nm (dotted line, filled symbols: oxidized cytochrome) and 420 nm (dashed line, open symbols: reduced cytochrome) for *D.d.*N. cytochrome c_3 (M_r 13,000) (●, ○) and (M_r 26,000) (▲, △), *D.v.*H. cytochrome c_3 (M_r 13,000) (▼, ▽) and Hmc (■, □) in 100 mM Tris-HCl, pH 7.6. The absorbance decrease is expressed in percent of the full intensity change

$$\left(\frac{Abs_{t=20°C} - Abs_{t=x}}{Abs_{t=20°C} - Abs_{t=100°C}} \times 100\right)$$

and values are the means of triplicate determinations.

this process on the shape of calorimetric curve (data not shown) and heat denaturation parameters, we changed the protein concentration. On decreasing protein concentration from 1.7 to 0.3 mg/ml the denaturation temperature and calorimetric denaturation enthalpy did not change. Evidently, the aggregation process starts at a temperature higher than the temperature corresponding to the end of the heat denaturation peak.

3.2. The thermostability of polyheme cytochromes is either redox state dependent or not

The optical spectra of *c*-type cytochromes show an intense absorbance signal centered at 410 nm or 420 nm in the oxidized or reduced state, respectively. These features are good markers

Table 1
Parameters of thermal denaturation of cytochromes at pH 7.6 (10 mM Tris-HCl)

Protein	T_d (°C)	ΔH_{cal} (kcal/mol)	ΔH_{eff} (kcal/mol)	R*
*D.d.*N. cytochrome c_3 (M_r 13,000)	73	64	63	1.0
*D.v.*H. cytochrome c_3 (M_r 13,000)	121	~170**		
*D.v.*H. Hmc (M_r 65,000)	81	386	325	1.2
Mitochondrial cytochrome *c*[a]	63.8[b]	38[b]	44[b]	0.86[b]
	80.7	94	88	1.06

ΔH_{cal} and ΔH_{eff} values are accurate within ±7% and T_d values are accurate within ±0.5°C.
*$R = \Delta H_{cal}/DH_{eff}$.
**The approximate ΔH_{cal} value was determined by extrapolation of the left part of the calorimetric peak to higher temperatures (Fig. 1C).
[a] [24].
[b] Low temperature endotherm in the DSC scan of ferricytochrome *c*, *R* values are modified from [24].

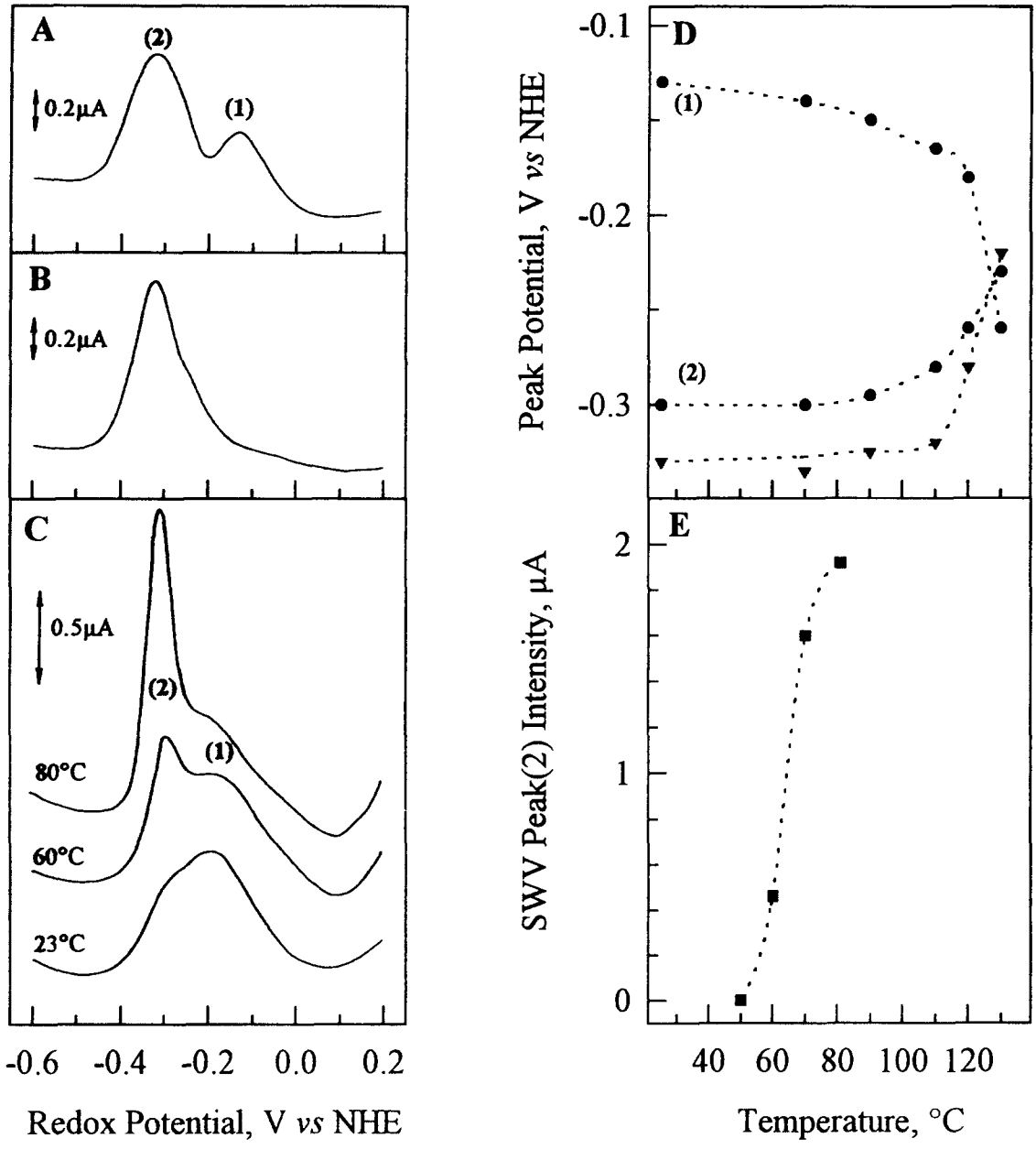

Fig. 3. SW voltammograms of *D.d.*N. (A), *D.v.*H. (B) cytochrome c_3 and *D.v.*H. Hmc (C, at 23, 60 and 80°C). (D) Temperature dependence of the SWV peak (1) and (2) potentials of *D.d.*N. cytochrome c_3 (●) and of the peak potential of *D.v.*H. cytochrome c_3 (▼). (E) Temperature dependence of the intensity of the SWV peak (2) resulting from the degradation of *D.v.*H. Hmc. The redox potentials are expressed versus the normal hydrogen electrode (NHE).

for the integrity of the heme environment. Fig. 2 shows that the *D.v.*H. cytochrome c_3 is very thermostable. No change of the electronic spectrum was observed; even at 100°C the intensity and position of the Soret band remained constant.

Two distinct behaviors could be evidenced with regard to the thermal transition of differently charged cytochromes. On one hand, the denaturation profiles of the cytochrome c_3 (M_r 13,000) were equivalent in both redox states. On the other hand, the reduced *D.d.*N cytochrome c_3 (M_r 26,000) and *D.v.*H. Hmc were much more thermostable than the oxidized form (T_d = 86°C vs. 74°C and 90°C vs. 72°C, respectively). The redox state-dependent stability has been described for mitochondrial cytochrome *c*: the reduced protein is more stable than the ferricytochrome to extreme conditions [19]. Small conformational differences between the two redox states have been evidenced: two possible triggering mechanisms have been proposed [20–22] and in both cases the conformational changes contribute to stabilization of the positive charge which could interact with other charges and dipoles in the protein and solvent [10]. The *Desulfovibrio* cytochromes c_3 (M_r 13,000) are small proteins: the number of hemes/polypeptide chain ratio is 4 times higher than that of monohemic cytochrome *c*. Contrary to cytochrome *c*, the heme of which is buried in the protein, the cytochrome c_3 hemes are highly exposed to the solvent, so the

positive charge which appears in the oxidized state does not need to be stabilized by conformational variations. Conversely, the *D.d.*N. cytochrome c_3 (M_r 26,000) [14] and *D.v.*H. Hmc are multi-domain proteins, the hemes of which are less accessible to solvent. Dynamic fluctuations in the structures could be expected to account for the observed differences in stability in the cytochrome redox states.

Finally, some functional relevance can be proposed about this classification of the cytochrome c_3 in two groups according to their redox state-dependent stability. The tetraheme cytochromes might be present in the periplasm of *Desulfovibrio* in both redox states since they play a key role in the metabolism: they react with hydrogenase in the hydrogen consumption pathway as well as in the hydrogen production. The cytochromes with many hemes, like the Hmc or the octaheme cytochrome c_3, could be viewed as electron storage proteins and the higher stability of their reduced state might be required for this function.

3.3. The temperature denatured cytochromes c_3 retain some redox properties

The thermal stability of *D.d.*N. and *D.v.*H. cytochromes c_3 (M_r 13,000) was studied also with the help of cyclic and square-wave voltammetry. Cyclic voltammetry served to confirm that both cytochromes are fast electrochemical systems as shown in our previous work [23]. Typical square-wave voltammograms (SWV) are given in Fig. 3A and B. No modifications in the shape of CV and SWV curves and peak potentials were observed when the proteins were heated at temperatures lower than 70°C and 110°C, respectively. The temperature dependence of the respective SWV peak potentials is given in Fig. 3D, thus denoting structural transformations of the native species above 70°C and 110°C. The redox potential of the denatured form of the cytochrome c_3 (M_r 13,000) converged towards a value of −220 mV. This shift of potential might be induced by either the lost of the sixth axial iron coordination or a modification of the heme exposition to solvent (some reversible restructurations cannot be excluded also when cooling back the protein samples). No displacement of the axial ligand of the hemes is detectable by the UV-visible analysis: the heme region unfolds with the polypeptide backbone. This behavior contrasts with that of mitochondrial cytochrome *c*, the thermal denaturation of which occurs in two steps (Table 1): the low temperature transition at 60°C mainly represents the destabilization of the heme crevice [24].

In the case of the *D.v.*H. Hmc, no prolonged bubbling for preliminary deoxygenation could be carried out because of the instability of the protein. For this reason, AC voltammetry which is less sensitive to the presence of traces of oxygen was used in place of CV experiments to verify that the cytochrome acts as a fast electrochemical system [6]. Typical SWV curves for three temperatures are given in Fig. 3C. SWV curves remain virtually unmodified up to 50°C. The quasi-reversible response characteristic of intact Hmc molecules begins to disappear at about 60°C, while an additional sharp peak develops denoting the existence of strongly adsorbed species. Such adsorbed species must result from the degradation of the native protein. The temperature dependence of the adsorption peak height is given in Fig. 3E, denoting that the native *D.v.*H. Hmc begins to be drastically transformed when heated at temperatures higher than ~50°C.

3.4. Structural variations induce the thermostability discrepancy within the cytochrome c_3 superfamily

The denaturation temperature values determined by electronic spectroscopy and voltammetric techniques for oxidized forms of cytochromes, as described above, are close to those obtained by scanning microcalorimetry.

The very different thermal stabilities of the two cytochromes c_3 (M_r 13,000) are surprising. In fact their available 3D structures show that the heme cores are well conserved and that the backbones and α-helices are superimposable. Differences have been detected, however, between the polypeptide chains: one major loop of 14 residues is deleted in *D.v.*H. cytochrome c_3. This region in the *D.d.*N. cytochrome c_3 (M_r 13,000) – from residue 20 to 26 – has not a very well defined density in the structure refinement, its temperature factors are high compared to the rest of the molecule [11]. This indicates that this loop shows thermal or structural disorder which can be interpreted by a high flexibility. As reduction in the molecular mobility of a protein enhances stability [25], this surface loop could be responsible for the destabilization of *D.d.*N. cytochrome c_3 (M_r 13,000).

Comparison of denaturation parameters of *D.v.*H. Hmc with those obtained for *D.v.*H. and *D.d.*N. cytochrome c_3 (M_r 13,000) revealed essential differences whereas the ratio *R* equals to 1.2 even for high molecular mass cytochrome consisting of 4 c_3-like domains (Table 1). This fact demonstrates that these domains are tightly interacting at conditions used. Denaturation temperature of Hmc is by 40°C less than that of *D.v.*H. cytochrome c_3. On the other hand, the oxidized forms of the *D.d.*N. cytochromes c_3 (M_r 13,000) and (M_r 26,000) and *D.v.*H. Hmc have similar transition temperatures (about 73°C), as can be seen from the spectrophotometric measurements (Fig. 2). It is generally admitted that intersubunit interactions via ion pairs and hydrophobic contacts provide important contributions to the stability of polymeric proteins [26,27]. However, the dimerization of the *D.d.*N. cytochrome c_3 (M_r 26,000) mainly involves hydrogen bonds and Van der Waals contacts, but no salt bridge [12]. Attempts to solve the structure of the *D.v.*H. Hmc have been reported to be in progress [4]. It has therefore not yet been definitely established whether or not c_3 folding occurs in each Hmc c_3-like domain. Hydrophobic cluster analysis [28] has been carried out [29] to compare the cytochrome c_3 sequences with that of the Hmc domains: the 'N-terminal' parts of domains II, III and IV contain several sets of adjacent hydrophobic residues which could not be identified in the plots of cytochrome c_3 (M_r 13,000). These regions may be hydrophobic contact areas between the various Hmc domains, but these are only structural predictions.

A cytochrome c_3 with 3 hemes – cytochrome c_7 from *Desulfuromonas acetoxidans* – has also been described [30,31] and sequence alignments have evidenced the deletion of the region surrounding the heme 2 binding site. Preliminary X-ray data are consistent with the idea that the heme core of the triheme protein resembles that of hemes 1, 3 and 4 of *D.d.*N. and *D.v.*H. cytochromes c_3 [19]. Increasing the temperature up to 70°C caused the triheme cytochrome c_3 to irreversibly denature [32]. The overall thermostability of these cytochromes primarily depends on the covalently linked hemes which enhance stability by locking the structure like disulfide bridges in other proteins. In a recent work we have evidenced that a single heme axial ligand replacement drastically decreased the thermostability of

D. v. H. cytochrome mutants [33]. The greater effects (stability decrease up to 45°C) were observed in the case of cytochrome mutants with no sixth axial ligand, which indicates that the metal coordination strengthens the protein structure. In the present study it eventually appears that natural structural variations – heme deletion, loop insertion – from the more rigid structure, that of *D. v.* H. cytochrome c_3 (M_r 13,000), destabilize the protein as well as single mutations. Such a study within a well defined structural superfamily of molecules might be a clue to investigate the factors governing the thermostability of proteins.

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