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The thermal stability of the tryptic fragment of bovine microsomal cytochrome b_5 and a variant containing six additional residues

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Thermally induced denaturation has been measured for both oxidised and reduced forms of the tryptic fragment of bovine microsomal cytochrome b_5 using spectrophotometric methods. In the oxidised state, the tryptic fragment of cytochrome b_5 (Ala^7–Lys^{90}) denatures in a single cooperative transition with a midpoint temperature (T_m) of ~ 67°C (pH 7.0). The reduced form of the tryptic fragment of cytochrome b_5 shows a higher transition temperature of ~ 73°C at pH 7.0 and this is reflected in the values of ΔH_m, ΔS_m and $\Delta(\Delta G)$ of ~ 310kJ·mol^{-1}, 900J·mol^{-1}·K^{-1} and 5 kJ·mol^{-1}. Increased thermal stability is demonstrated for a variant protein that contains the first 90 amino acid residues of cytochrome b_5. These novel increases in stability are observed in both redox states and result from the presence of six additional residues at the amino-terminus. The two forms of cytochrome b_5 do not differ significantly in structure with the results suggesting that the reorganisation energy (λ) of the variant protein, as measured indirectly from redox-linked differences in conformational stability, is small. Consequently the reported subtle differences in reactivity between variants of cytochrome b_5 may result from the presence of additional N-terminal residues on the surface of the protein.

Cytochrome b_5; Protein folding; Denaturation; Heme

1. INTRODUCTION

Cytochrome b_5 transfers electrons in the microsomal membranes of cells as an important constituent of the fatty acid desaturase and cytochrome P-450 metabolic pathways [1–3]. The protein is membrane-bound but is released by proteolysis to yield a soluble catalytic domain of 84 amino acids that has been the subject of many biophysical studies [4]. Crystallographic studies reveal this domain as a cylindrical molecule containing both α-helices and β-strands arranged around a non-covalently held haem that is located within a hydrophobic pocket [5,6]. Four α-helices, lying parallel to the cylindrical axis, surround the haem with the remaining two helices together with β-strands maintaining the structure of a second hydrophobic core that forms the base of the haem-binding pocket [7]. NMR studies of the cytochrome b_5 tryptic fragment indicate a highly flexible protein where the aromatic and aliphatic side chains are mobile. It is noteworthy that the conformation of the polypeptide backbone and aromatic side chains of cytochrome b_5 does not differ significantly between the reduced and oxidised states [8,9]. Although high resolution structural studies of cytochrome b_5 in both the solid and solution states have been obtained, the physico-chemical basis for the stability of the protein is not well defined.

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Whole cytochrome b_5 protein and the tryptic fragment have been the subject of studies on protein folding and stability using UV-vis spectroscopy, resonance Raman, circular dichroism, NMR and differential scanning calorimetry [10–14]. Thermal denaturation studies of the tryptic fragment from rabbit microsomal cytochrome b_5 gave a transition temperature 10K higher than that observed for the whole protein whilst resonance Raman and circular dichroism studies show that thermally induced unfolding results in a five coordinate (high spin) ferric haem centre but leaves a polypeptide fold that is of similar conformation to that present at lower temperatures [13]. Additionally 2D ^{1}H-NMR studies of the tryptic fragment of cytochrome b_5 indicate that the removal of the haem leads to a less compact form of the protein but one retaining considerable secondary structure around a hydrophobic core that includes Trp^{26} and Ile^{80} (numbering scheme based on the DNA sequence) [15–17].

Despite these observations, there remains a lack of information on the relative stabilities of the reduced and oxidised states of cytochrome b_5 and the role of the polypeptide fold in conferring protein stability. To investigate these problems, thermal denaturation and refolding have been studied in both the ferric and ferrous states for the tryptic fragment of bovine liver microsomal cytochrome b_5. The reduced form of the protein is characterised for the first time and permits evaluation of the extent of redox linked conformational changes in cytochrome b_5. Concurrently we have produced a variant form of the bovine microsomal protein that contains

six amino acid residues at the N terminus of the protein normally lost during trypsin proteolysis. Both forms of the protein are soluble and in this study we compare the thermal denaturation and stability of the tryptic fragment of cytochrome b_5 (Ala^7–Lys^{90}) with the extended version of the protein (Ala^1–Lys^{90}) in both redox states. An assessment of stability of cytochrome b_5 and the extent of any conformational differences between the reduced and oxidised form is related to the magnitude of the reorganisational energy (λ) for electron transfer. Currently many studies are attempting to evaluate the effect of structural perturbations on rates of long-range electron transfer. In this paper we estimate stability differences between two forms of cytochrome b_5 and suggest that the observed differences may be relevant to biological reactivity.

2. METHODS

2.1. *Protein isolation*

The tryptic fragment of cytochrome b_5 was isolated as described previously from bovine liver microsomes [18]. The cloning, expression and isolation of recombinant protein will be described elsewhere but after column chromatography on DE-52 cellulose and Mono Q FPLC columns, the pure protein exhibited an absorbance ratio (A_{413}/A_{280}) of ~6 and was a single band of ~12 kDa on SDS-PAGE [19].

2.2. *Thermal denaturation*

Thermal denaturation was measured spectrophotometrically between 350 and 600 nm, using an Aminco DW2000 UV-vis spectrophotometer controlled by commercial software. All experiments were carried out at a protein concentration of ~2 μM and in 5 mM MOPS, pH 7.0. The protein concentration was calculated assuming an extinction coefficient of 117 $mM^{-1} \cdot cm^{-1}$ at 412.5 nm for both the tryptic and recombinant ferric forms of the protein. Experiments were carried out over a temperature range of 25 to 95°C and for each experiment a period of ~15 min was left between measurements to ensure the sample had reached equilibrium. The temperature was determined directly at the cuvette holder and was maintained to within ± 0.2°C. At each temperature, three consecutive spectra were recorded to ensure reproducibility and equilibrium. The reversibility of protein refolding was measured by recording spectra at 25°C before raising the sample to the previously estimated T_m. The sample was allowed to equilibrate and a new spectrum recorded before rapidly cooling to 25°C and comparing the final and initial absorbances of the Soret band to estimate the extent of the reversibility of protein refolding. Reduction of cytochrome b_5 was performed most efficiently by adding sodium dithionite to a cuvette maintained in an anaerobic state by purging all reagents with zero grade argon. The efficiency of cytochrome b_5 reduction was checked spectrophotometrically before adding de-gassed mineral oil onto the solution surface to minimise sample evaporation. With this approach, no detectable oxidation of cytochrome b_5 occurred within 8 h.

3. RESULTS

3.1. *Thermal denaturation of the tryptic fragment of cytochrome* b_5

The spectra shown in Fig. 1a reveal that increasing the temperature of a solution containing the oxidised form of the tryptic fragment of cytochrome b_5 leads to a progressive decline in the magnitude of the absorbance between 350 and 600 nm. Most noticeably, the absorbance of the Soret band centred around 413 nm decreases dramatically with increasing temperature. As the temperature increases, the maximum absorbance for the Soret peak shifts from 412.8 nm to 395 nm at 80°C. The absorbance maximum observed at high temperatures is similar to that seen for free haem (λ_{max} = 385 nm) and may indicate that haem dissociation from the protein occurs. Similarly for the reduced protein a representative set of spectra (Fig. 1b) show a corresponding decrease in the intensity of the Soret band (λ_{max} = 423.3 nm at 25°C) with increasing temperature. Again, as the temperature increases a blue shift of the Soret peak occurs of ~6 nm from 423.3 nm to 417.2 nm at 80°C (407.5 nm at 95°C) for the reduced protein.

From the spectra of Fig. 1, thermal denaturation curves can be derived for both the reduced and oxidised forms of the protein. The oxidised protein shows negligible decrease in the absorbance at 413 nm up to a temperature of ~55°C. However, above this temperature the absorbance decreases rapidly, possessing less than 20% of its original magnitude above 80°C. From the plotted spectra, one can estimate the T_m to be approximately 67°C. The profile of this curve indicates that cytochrome b_5 unfolds in a single, cooperative transition between folded (F) and unfolded (U) states. The equilibrium constant is estimated from the following equation:

$$K_{eq} = \{A_F(T) - A(T)\}/\{A(T) - A_U(T)\}$$

where $A_F(T)$ and $A_U(T)$ are the absorbances of the folded and unfolded proteins at temperature T respectively and $A(T)$ is the measured absorbance at this temperature.

The reversibility of protein unfolding of the tryptic fragment of cytochrome b_5 is dependent on the time spent in excess of the midpoint temperature (T_m). For the oxidised form, heating the sample to the T_m followed by rapid cooling to 25°C leads to a recovery of ~85% of the folded state within one hour as indicated by the magnitude of the Soret band absorbance. If the sample is raised to a temperature 10°C above the T_m (results not shown) then, over the same time period, the recovery of the folded state is limited to ~35% of the total protein.

3.2. *Thermal denaturation of the* Ala^1–Lys^{90} *variant*

DNA sequencing of this construct showed that following the ATG codon were a series of bases that translated into the following amino acid sequence: Ala-Glu-Glu-Ser-Ser-Lys-Ala-... The presence of the six additional amino acid residues at the N terminus of the isolated, over-expressed protein and its correct processing by removal of the formyl methionine was verified by Edman sequencing (R. Hewson et al., unpublished results).

Thermal denaturation studies of this variant in either

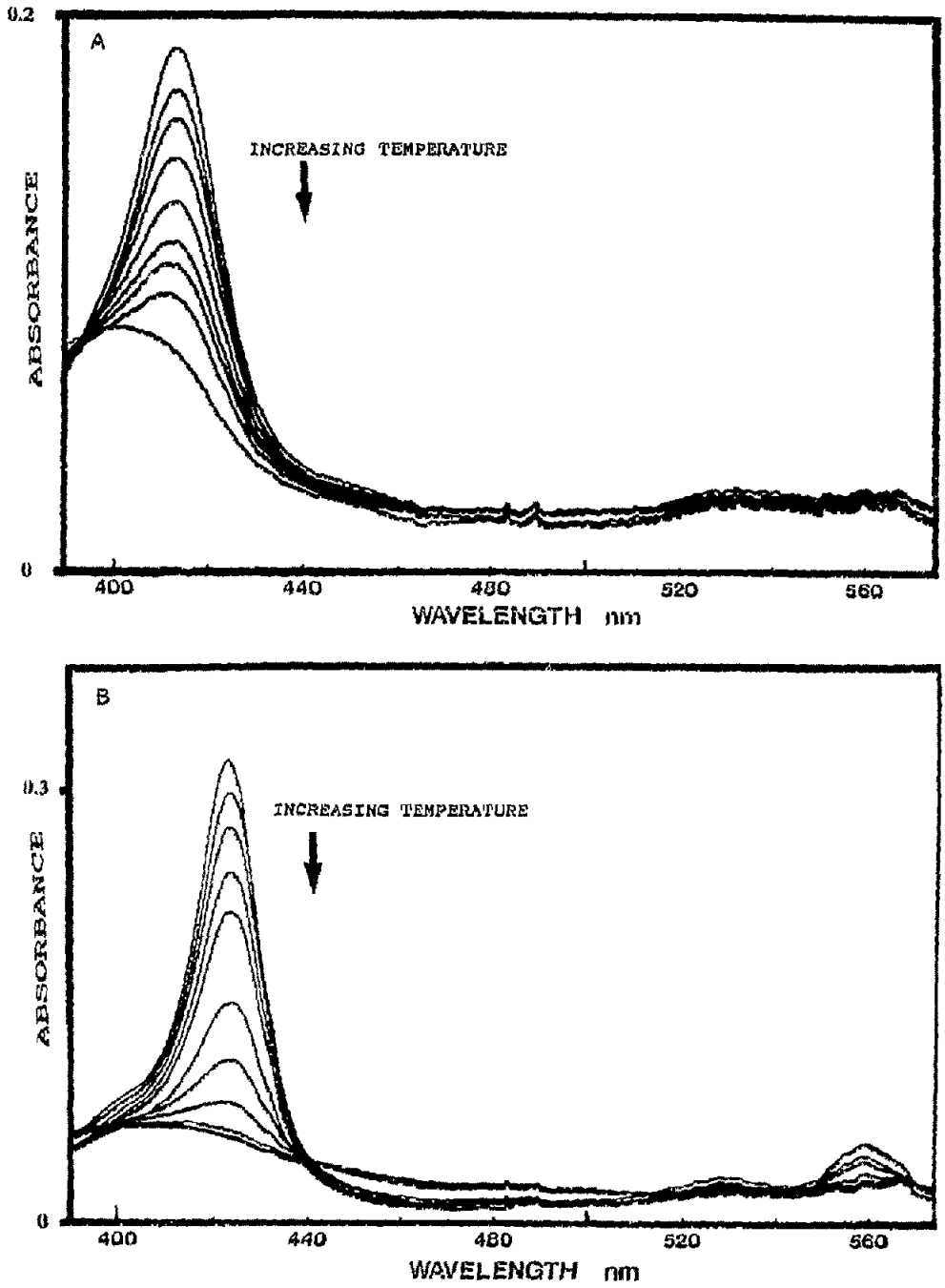

Fig. 1. Absorbance spectra of the tryptic fragment of cytochrome b_5 (Ala^7-Lys^{90}) showing thermal denaturation. (a) ferricytochrome b_5 and (b) ferrocytochrome b_5. The concentration of tryptic ferricytochrome b_5 was ~2 μm in 5 mM MOPS, pH 7.0. Spectra were recorded at temperatures of 25, 55, 57, 62, 64, 66, 68, 70 and 75°C for the oxidised protein and 25, 65, 70, 72, 75, 77, 80, 82, 85, 90 and 95°C for the reduced protein. The absorbance of the oxidised protein at 413 nm and 25°C was initially 0.188 and for the reduced protein, the initial absorbance at 423 nm was 0.325.

the oxidised or reduced states result in a decline in the magnitude of the Soret peak with increasing temperature similar to that seen for the tryptic fragment. The profile of the denaturation curve again describes a single cooperative unfolding process (Fig. 2). However, for both the reduced and oxidised forms of this variant, increased midpoint temperatures are observed when compared with the corresponding form of the tryptic fragment of cytochrome b_5 (Fig. 3). The acquisition of data above 90°C was difficult due to progressive sample evaporation. Consequently it was not possible to obtain data points close to or at 100°C and to thoroughly define the denaturation curve in the unfolded region. The increase in transition temperature would appear to be closely correlated with the presence of the six additional amino acids at the N terminus, since we observe

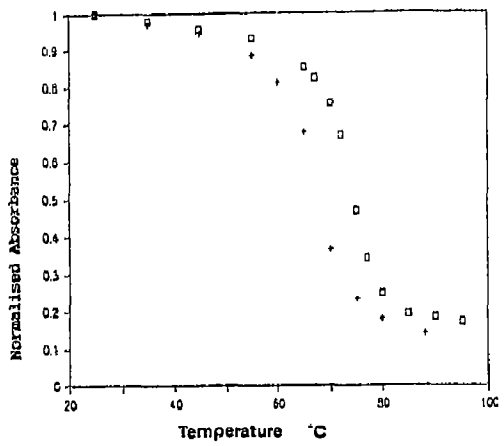

Fig. 2. The change in the absorbance of the tryptic fragment of cytochrome b_5 with increasing temperature. (+) Fractional changes in the absorbance at 412.8 nm for the tryptic fragment of ferricytochrome b_5. (□) Fractional changes in the absorbance at 423.3 nm for the tryptic fragment of ferrocytochrome b_5. The data sets were fit assuming a two state unfolding process and are normalised to aid comparison between proteins.

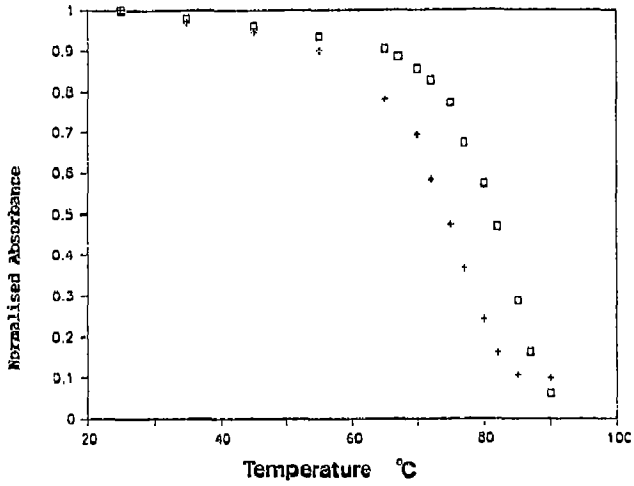

Fig. 3. The change in the absorbance of the Ala^1–Lys^{90} variant of cytochrome b_5 with increasing temperature. (+) Fractional changes in the absorbance at 412.8 nm for the Ala^1–Lys^{90} variant of ferricytochrome b_5. (□) Fractional changes in the absorbance at 423.3 nm for the Ala^1–Lys^{90} variant of ferrocytochrome b_5. Data sets were treated as in Fig. 2.

that a third variant protein (Ala^1–Ser^{104}) exhibits a transition temperature similar to that of the Ala^1–Lys^{90} variant [19]. In comparison with the tryptic fragment both variant proteins refold to a similar extent after heating to their T_m temperatures and cooling.

For the tryptic fragment and variant protein, semilogarithmic plots of the unfolding equilibrium constant ($\ln K_{eq}$) against reciprocal temperature ($1/T$) or plots of free energy (ΔG) against temperature allow estimates of the enthalpy (ΔH_m) and entropy (ΔS_m) from the linear region around the transition temperature (T_m). These values are summarised in Table I for both tryptic and recombinant proteins. The values of $\Delta(\Delta G)$ were estimated from the relationship $\Delta(\Delta G) = [\Delta(T_m)] \times \Delta S_m$ where $\Delta S_m = \Delta H_m/T_m$ and ΔT_m was obtained by comparison with the transition temperature of the oxidised form of the tryptic fragment [20,21].

4. DISCUSSION

The results described above show that both the tryptic fragment (Ala^7–Lys^{90}) and the extended variant of bovine microsomal cytochrome b_5 (Ala^1–Lys^{90}) show a single cooperative transition between the compact folded state and a more disordered conformation. For both proteins, substantial refolding will occur if the fragment is not subjected to extensive incubation times in excess of the transition temperature. Incubation at elevated temperatures inhibits subsequent protein refolding for both fragments of cytochrome b_5 and this may, in view of the shape of the thermal denaturation curves and the absorbance spectra, reflect irreversible protein aggregation that is promoted by haem dissociation. This general pattern of thermal denaturation and

Table I

Thermodynamic data for thermal denaturation of the tryptic fragment of cytochrome b_5 (Ala^7–Lys^{90}) and Ala^1–Lys^{90} variant in the reduced and oxidised states

Cytochrome	T_m (°C)	ΔT_m (K)	ΔH_m (kJ·mol^{-1})	ΔS_m (J·mol^{-1}·K^{-1})	$\Delta(\Delta G)$ (kJ·mol^{-1})
Oxidised tryptic fragment (Ala^7–Lys^{90})	67.4 ± 0.7	–	172 ± 52	505	– (n = 5)
Oxidised variant (Ala^1–Lys^{90})	73.2 ± 0.8	5.8	227 ± 16	655	3.79 (n = 5)
Reduced tryptic fragment (Ala^7–Lys^{90})	73.0 ± 1.1	5.6	311 ± 12	895	5.01 (n = 5)
Reduced variant (Ala^1–Lys^{90})	79.2 ± 1.5	11.8	301 ± 59	854	10.08 (n = 5)

The numbers in brackets represent the number of experiments. Values in the T_m and ΔH_m columns are the mean ($\bar{x}$) and the standard deviation ($\pm \sigma$), respectively for the T_m and ΔH_m values obtained experimentally.

re-folding, recently observed for cytochrome b_{562} [22], has been seen in many other studies and can be explained by a folding process involving native (N), denatured (D) and irreversibly unfolded (I) forms of the protein [23]:

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} I$$

The k_3 step is generally slow and does not significantly perturb the thermal equilibrium (i.e. $k_3 \ll k_2$). Although this scheme may describe the folding/unfolding of the tryptic and variant forms of cytochrome b_5 the present experiments do not permit the solution conformations of these states to be resolved. However, the shifts in haem absorbance maxima for both proteins indicate that the initial stages of the denaturation may involve the loss, or partial dissociation, of the haem from the polypeptide fold. Kitigawa et al. have described spin state changes to the iron upon thermal denaturation, that result in low spin to high-spin iron centre transitions. In the tryptic fragment of rabbit cytochrome b_5 this transition was centred around a midpoint temperature of 66°C, similar to that seen in the present study (67.4°C), and was followed by a slow reversion to a low-spin Fe centre [13,14]. It is tempting to speculate that the first step in this process is associated with haem dissociation whilst the second spin state change is part of a slower irreversible transition occurring in cytochrome b_5 that is also observed here.

Several studies show that the transition temperature of the tryptic fragment of rabbit cytochrome b_5 during thermally induced unfolding is substantially higher (~10K) than that of the detergent solubilised form of the complete protein [10,11,13]. These results are surprising because other studies indicate that the hydrophilic and hydrophobic regions of cytochrome b_5 unfold independently in guanidinium chloride and that a clear structural distinction exists between the two domains [5–7,15]. Here we show that the Ala^1–Lys^{90} variant has an increased transition temperature in both redox states when compared with the tryptic fragment of cytochrome b_5. Furthermore a second variant protein (Ala^1–Ser^{104}) which extends through the assumed linker region connecting the soluble and non-polar domains also shows elevated transition temperatures (T_m = 73.1°C) in the oxidised state, comparable within experimental error to the shorter variant containing only the first 90 amino acids. This result indicates that the higher thermal transition temperature associated with the variant proteins is not attributable merely to the increased size of the domain. Moreover, previous studies indicating that the transition temperature of whole rabbit cytochrome b_5 is lower than that of the tryptic fragment must be incorporated into a model that also allows for the increased stability of domains containing the first 104 amino acids. It is possible that results obtained for the whole protein may be a consequence of detergent solubilisation but it should also be noted that the thirty amino acid residues of the hydrophobic domain, not present in either the tryptic or the variant fragments, could contribute to the observed decrease in thermal stability of the whole protein. With this caveat borne in mind, the data of Table I suggest that the additional six amino acid residues at the N terminus confer additional thermal stability in both redox states to the variant proteins. The thermodynamic data measured here apply to all of the stabilising and destabilising interactions within the protein and it is rarely easy to assess the role of individual residues to conformational stability.

Previously Bendzko and Pfeil observed a $\Delta(\Delta G)$ value of ~7.8kJ·mol^{-1} between whole rabbit cytochrome b_5 and the tryptic fragment [11] and, in view of the similarity of the ΔH values obtained for each protein, these authors suggested that the differences in stability stemmed primarily from entropic contributions. In the present study, the additional amino acid residues at the N terminus of the Ala^1–Lys^{90} variant protein result in changes to both ΔH_m and ΔS_m when compared to the tryptic fragment in the same redox state. The molecular basis for these changes in stability are hard to define due to the large number of potential stabilising interactions resulting from the six additional residues. For the bovine tryptic fragment of cytochrome b_5 it has been shown that the conformation of the main chain and aromatic side chains do not change significantly with redox state. Although this has not been shown for the variants here, preliminary ^{1}H-NMR studies show that the chemical shifts and linewidths of the haem and haem ligand resonances are similar to those of the tryptic fragment and suggest that major conformational differences and protein aggregation do not occur for these variant proteins (D. Whitford, unpublished results). Within the additional six residues at the N terminus are three charged groups (2 Glu and 1 Lys) and an obvious potential for further electrostatic interactions. Ionic interactions have been demonstrated to enhance thermostability when buried within protein interiors but when found on the surface their relative importance is much smaller [24,25]. Moreover, the absence of any large changes in ΔH_m between the variants measured in this study suggests that additional charged interactions do not form in the Ala^1–Lys^{90} variant protein. Other stabilising interactions in proteins can include hydrogen bonding and the hydrophobic effect [26]. The relative importance of H-bonding to increases in thermal stability are presently unclear. Model studies indicate that the magnitude of such changes will be small, whilst mutagenesis studies of T4 lysozyme showed that the formation of additional H-bonds upon the replacement of a Pro residue with Ser or Cys led to no differences in stability betweeen the wild type and mutant proteins [27,28]. Alternatively, *decreases* in thermal stability due to removal of a H-bond have been noted for T4 lysozyme with the mutation Thr^{157}→ Ile [29]. Site-directed

mutagenesis most frequently leads to decreases in the thermal stability of proteins with increased thermal stability being comparatively rare. However this effect can result from the replacement of an internal amino acid with a more hydrophobic residue and one of the best examples is the marked thermostabilisation of yeast iso-cytochrome *c* with the transition $Asn^{52} \rightarrow Ile$ [30]. The present work reveals novel increases in the thermal stability of bovine cytochrome b_5 in the absence of any changes in internal residues. In this instance the enhanced stability of cytochrome b_5 may result from the formation of additional secondary structure at the N terminus which thereby removes non-polar side chains previously accessible to the solvent. The possible significance of such conformational factors to the modulation of the thermal stability of cytochrome b_5 is currently being assessed using NMR spectroscopy. The results raise the interesting possibility that the stability differences noted here for cytochrome b_5 may be reflected in functional differences between the tryptic and other variant proteins. In this respect, Mauk et al. have noted that complex formation between cytochrome *c* and tryptic cytochrome b_5 differs quantitatively from that between cytochrome *c* and the lipase solubilised cytochrome b_5 [31].

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