Energetics of Heme Binding to Native and Denatured States of Cytochrome b_{562}[†]

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ABSTRACT: Cytochrome b_{562} is a four-helix bundle protein containing a noncovalently bound *b*-type heme prosthetic group. For the first time, energetics of heme binding to an apocytochrome were measured by isothermal titration calorimetry. The heme is tightly bound to native apocytochrome b_{562}, with a dissociation constant (K_d) of approximately 9 nM ($\Delta G° = 11$ kcal mol^{-1}) at 25 °C. Unexpectedly, the thermally denatured apoprotein is also capable of specifically binding heme with modest affinity ($K_d = 3$ μM, $\Delta G° = 7.6$ kcal mol^{-1}). This interaction results in the dependence of holocytochrome b_{562} stability on protein concentration in the submicromolar range.

Proteins containing prosthetic groups, such as cytochromes, globins, peroxidases, flavoproteins, and iron−sulfur proteins perform many crucial cellular functions such as electron transfer, oxygen delivery, and catalysis. Binding of a prosthetic group often confers structural and functional integrity or provides enhanced stability to the holoprotein (Huntley & Strittmatter, 1972; Yip et al., 1972; Leutizinger & Beychok, 1981; Griko et al., 1988; Cocco & Lecomte, 1990; Moore & Lecomte, 1990; Feng & Sligar, 1991; Moore et al., 1991; Feng et al., 1994). Understanding the origins of protein structure and stability and the determinants of specificity in ligand binding reactions are important goals, particularly for applications such as protein engineering or drug discovery. Determining the energetics governing the association between prosthetic groups and apoproteins would be a significant step toward this goal.

Here we study the binding of heme by cytochrome b_{562} (cyt b_{562}) of *Escherichia coli*, a small (12.3 kDa) soluble protein containing a noncovalently bound *b*-type heme group (Itagaki & Hager, 1966). The heme ligands are methionine 7 on helix 1 and histidine 102 on helix 4 (Xavier et al., 1978). X-ray crystallography revealed that ferricytochrome b_{562} exists as a four-helix bundle with pairs of helices nearly antiparallel to each other (Figure 1, left) (Mathews et al., 1979; Lederer et al., 1981). The heme prosthetic group can be readily removed by acidification and extraction (Itagaki et al., 1967; Feng & Sligar, 1991). High-resolution multidimensional NMR showed that the resulting apoprotein retains much of the secondary and tertiary structure of the holoprotein (Figure 1, right) (Feng et al., 1991, 1994). This observation implied that apocytochrome b_{562} could represent an intermediate on the folding/assembly pathway of the holoprotein (Feng et al., 1994). The thermodynamics of denaturation of the apoprotein and oxidized holoprotein have been evaluated using chemical denaturation and van't Hoff analysis (Feng & Sligar, 1991; Fisher, 1991; Robinson & Sligar, 1993), as well as differential scanning calorimetry (DSC) (Robinson et al., 1997a). These data reveal that addition of the heme group under native conditions increases the free energy of denaturation by over 3 kcal mol^{-1}, more than doubling the stability of the protein.

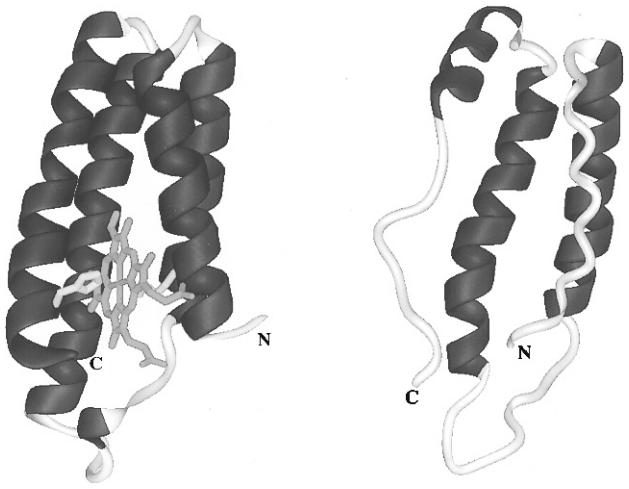

FIGURE 1: Structures of holo- and apocytochrome b_{562} (images contructed using MIDAS Plus). (Left) Ferricytochrome b_{562} [coordinates from Lederer et al. (1981)]. Helices are colored blue, heme is colored red, and side chains of heme ligands methionine 7 and histidine 102 are colored yellow. (Right) Apocytochrome b_{562} [coordinates from Feng et al. (1994)].

To understand the role of heme binding in folding, assembly, and stability, we have employed isothermal titration calorimetry (ITC) to investigate the binding of heme to apocytochrome b_{562}. There are very few data in the literature on the thermodynamics of this type of biologically important prosthetic group−protein interaction. For cyt b_{562}, binding appears to be very tight relative to the stability of the protein, although somewhat weaker than affinities of larger apoglobins for heme (Hargrove et al., 1994, 1996).

By constructing a thermodynamic cycle based upon our measurements, we predict that heme also binds to denatured apocytochrome b_{562} with micromolar affinity. We further

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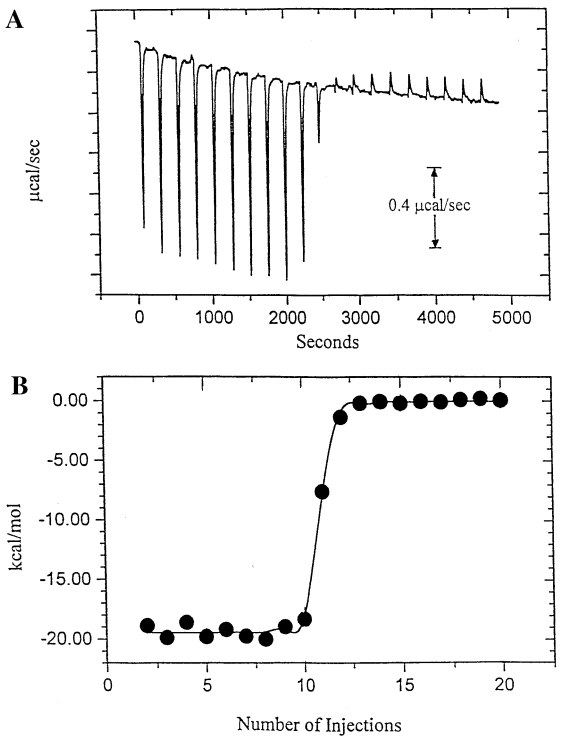

FIGURE 2: Titration of 0.262 mM hematin into 0.010 mM apocytochrome b_{562}. (A) Heat evolved for 20 10-μL injections at 4 min intervals. (B) Heme binding titration curve. The fraction bound was determined by integrating the peaks from the profile shown in panel A (Omega calorimeter software). The results listed in Table 1, 35 °C, first run, were calculated from this curve.

demonstrate this effect by analyzing the dependence of thermal stability upon protein concentration. To our knowledge this study is the first thermodynamic measurement of specific interactions between the denatured state of a protein and its prosthetic group and complements earlier findings with myoglobin (Hargrove & Olson, 1996) and recent laser-induced fast-folding kinetic studies of cytochrome b_{562} (Wittung-Stafshede et al., 1997).

MATERIALS AND METHODS

Titration Calorimetry. All titrations were carried out at pH 8.5 in 20 mM bicine buffer containing 0.1 mM EDTA. Hematin (iron protoporphyrin IX) was purchased from Sigma and used without further purification. The amount of hematin needed for a titration was dissolved in sodium hydroxide and diluted to the desired concentration with buffer, and the pH was adjusted with hydrochloric acid. Exposure to light was minimized. Apocytochrome b_{562} was prepared as described by Feng and Sligar (1991), except that the holoprotein was purified by ion-exchange FPLC just prior to heme extraction.

The titrations were performed with an Omega titration calorimeter (MicroCal, Inc., Northampton, MA). This instrument and its operation have been described in detail by Wiseman et al. (1989). Most titrations involved 20 10-μL injections of 200−400 μM hematin into 10−20 μM apoprotein contained in the cell (volume = 1.3215 mL), with intervals of 4 min between injections. The mean of the last six injections was added to each of the earlier injections as a baseline correction. Curve fitting was performed using the Omega software. The steepness of this curve at the equivalence point (Figure 2B) is caused by the very tight binding of the heme and illustrates the difficulty of obtaining a very accurate binding constant.

The interaction of heme with cytochrome b_{562} turned out to be a more difficult process to subject to isothermal titration calorimetry (ITC) than we had originally expected. In the first place, the binding is very tight, limiting the accuracy of calorimetric measurement of the binding constant to approximately ±25%. It has been shown (Wiseman et al., 1989) that, at least in the Omega calorimeter used in the present measurements, the product of substrate molarity times the binding constant should lie in the 10−1000 range for satisfactory evaluation of the binding constant, whereas in the present experiments this product necessarily exceeded 10^4. However, even an uncertainty in K as large as ±50% would propagate to an error of less than ±0.4 kcal/mol in ΔG.

In the second place, we initially observed values for N, the number of binding sites per molecule in the calorimeter cell, considerably in excess of unity, the expected value. We believe that the higher values of N resulted from aggregation of the heme moiety. We found that if *freshly dissolved* hematin was placed at a suitable concentration (~0.4 mM) in the microsyringe of the calorimeter and apoprotein (~0.02 mM) in the calorimeter cell and the titration was performed as soon as possible, reasonable values for N could be obtained. To combat heme aggregation, fresh solutions were prepared, centrifuged, and filtered just prior to the titrations which were performed under mildly basic conditions (pH 8.5). Three observations indicate that effects of heme aggregation were small in our experiments: (1) Values of N were near unity, indicating stoichiometric binding. This would not be the case if aggregation were occurring. (2) Heats of dilution were small (Figure 2, upper panel). (3) Titrations performed with concentrated apocyt b_{562} in the microsyringe and dilute hematin solution in the calorimeter cell gave values of K and N equivalent to those found when heme was titrated into apoprotein.

Thermal Denaturation. Thermal denaturation experiments were performed essentially as described previously (Bowie & Sauer, 1989; Robinson & Sligar, 1993). Solutions of cytochrome b_{562} were prepared at concentrations between 250 nM and 600 μM, using the same buffer as in the ITC experiments. The protein concentration was measured using a Hewlett-Packard 8452A diode array spectrophotometer, using $\epsilon_{418} = 0.117$ mM^{-1} cm^{-1} (Itagaki & Hager, 1966). Thermal denaturation was monitored using an AVIV CD60S circular dichroism spectrophotometer. The ellipticity at 222 nM was monitored as a function of temperature from 25 to 90 °C. Denaturation curves were fit to yield values of T_m and ΔH, as described previously, using a value of ΔC_p = 1.2 kcal mol^{-1} K^{-1} (Bowie & Sauer, 1989; Robinson & Sligar, 1993). The P_t *vs* T_m profile was fit to a three-state model in which the native holoprotein (NH) is in equilibrium with denatured holoprotein (DH) and denatured apoprotein plus heme (DA + H), using the method described by Robinson et al. (1997b).

For the three-state model

$$\mathrm{NH} \leftrightarrow \mathrm{DH} \leftrightarrow \mathrm{DA} + \mathrm{H} \qquad (1)$$

where

$$K_1 = \frac{[DH]}{[NH]} \qquad K_2 = \frac{[DA][H]}{[DH]} = \frac{[DA]^2}{[DH]} \tag{2}$$

$$P_t = [DA] + [DH] + [NH] = [DA] + \frac{[DA]^2}{K_2} + \frac{[DA]^2}{K_1 K_2} \tag{3}$$

and at $T = T_m$, by definition

$$[NH] = P_t/2 \tag{4}$$

so that eq 3 simplifies to

$$P_t = \frac{K_1 K_2}{(1 - K_1)^2} \tag{5}$$

Values of P_t at different T_m's were calculated from the temperature dependence of K_1 and K_2, and values of ΔH_{1ref}, ΔG_{1ref}, ΔC_{p1}, ΔH_{2ref}, ΔG_{2ref}, and ΔC_{p2} were obtained by fitting the experimental values of P_t and T_m using eqs 1−5 and the nonlinear least-squares algorithm implemented in the program Scientist (MicroMath Scientific Software, Salt Lake City, UT) as described previously (Robinson et al., 1997b).

$\Delta G°$ for heme binding to denatured apoprotein ($\Delta G°_{DH-DA}$) was calculated as $\Delta G°_{NA-NH} - (\Delta G°_{NA-DA} - \Delta G°_{NH-DH})$, where the subscripts NH, NA, DH, and DA refer to native holo, native apo, denatured holo, and denatured apo, respectively. Enthalpy, entropy, and heat capacity changes (ΔH, $\Delta S°$, and ΔC_p) for heme binding to denatured apoprotein were calculated in the same manner.

RESULTS AND DISCUSSION

Titration Calorimetry of Heme Binding to Native Apoprotein. A typical isothermal titration calorimetry (ITC) experiment (the first run at 35 °C listed in Table 1) is illustrated in Figure 2. Panel A shows the heat evolved for 20 10-μL injections of hematin at 4-min intervals, and panel B shows the resulting titration curve. The steeply rising equivalence section of the curve (Figure 2B) limits the accuracy of the binding constant which can be derived from an individual titration to ±25%. The data obtained in 11 titrations in the temperature range 7−45 °C are listed in Table 1. The values of K given in the third column show no definite variation with temperature. They have a mean value of 10.9 ± 1.5 (SE) × 10^7 M^{-1} and a standard free energy at 25 °C of $\Delta G° = 11.0 \pm 0.2$ kcal mol^{-1}, corresponding to a dissociation constant (K_d) of 9 nM. At temperatures below 15 °C values of N (the number of binding sites) lower than 1 were required to fit the titration data. This loss of stoichiometry may be caused by cold denaturation of the apoprotein.

Accurate enthalpies (ΔH) could be obtained from the flat initial and final portions of the titration curves. The values for the enthalpy of binding listed in the fourth column of Table 1 become more negative with increasing temperature. Linear regression of these data gives (in kcal mol^{-1})

$$\Delta H_T = -7.214 - 0.381T \tag{6}$$

with a standard deviation of ±0.887 kcal mol^{-1} and a regression coefficient of 0.974. The slope equals the heat capacity change (ΔC_p) for the binding reaction. Thus the resulting value of $\Delta H°$ at 25 °C is −16.74 kcal mol^{-1}. This value and that for $\Delta G°$ (11 kcal mol^{-1}) at 25 °C lead to a value of $\Delta S° = -19.3$ cal K^{-1} mol^{-1}.

Table 1: ITC Measurements of Binding of Heme to Apocytochrome b_{562} at pH 8.5

T (°C)	N	$K \times 10^7$ (M)	$-\Delta H$ (kcal mol^{-1})
7.0	0.76	15.0	9.76
7.0	0.74	6.4	10.02
7.0	0.78	8.4	9.87
10.0	0.75	10.0	11.04
10.0	0.73	6.7	11.37
15.0	0.93	15.0	13.58
25.0	0.98	7.7	16.43
35.0	1.05	19.0	19.49
35.0[a]	1.06	12.0	19.07
35.0	0.79	14.0	20.34
45.0	1.07	5.7	26.32
mean	0.88	10.9	
SE	±0.05	±1.5	

[a] Data displayed in Figure 2.

The modest entropy decrease and significant change in heat capacity ($\Delta C_p = 0.381$ kcal mol^{-1} K^{-1}) are consistent with a large decrease in solvent-exposed hydrophobic surface area. This decrease arises in part from removing the heme group from solution. Inspection of the structures of ferricytochrome b_{562} and apocytochrome b_{562} indicates that heme binding is also accompanied by substantial conformational changes in the protein which must bury hydrophobic side chains.

Concentration Dependence of Thermal Stability. From previous studies it is unclear whether heme dissociates from cyt b_{562} upon denaturation. Several experiments have produced conflicting evidence. Spectroscopic characterization of the denaturation reaction performed at protein concentrations in the low micromolar range indicates that the heme loses its ligands, suggesting that heme is removed to solution (Feng & Sligar, 1991; Fisher, 1991). In constrast, we have noted recently that no change in T_m occurs with increasing protein concentration in the range between 80 and 500 μM (Robinson et al., 1997a). An increase in stability would be expected from the principle of mass action if heme dissociated upon denaturation. Moreover, no increase in stability is observed when excess heme is present in the solution (Robinson et al., 1997a). To reconcile these findings, we performed thermal denaturation experiments, varying the concentration of cyt b_{562} from 250 nM to 600 μM.

From 250 nM to 4 μM, thermal stability (as monitored by changes in the CD signal at 222 nm) increases (Figure 3A), with T_m rising from 66 to 70 °C (Figure 3B). Above 4 μM, no further increase in thermal stability is observed up to 600 μM and T_m remains constant at about 70 °C (Figure 3). Denaturation appeared to follow a two-state transition in all cases, and values of ΔH, ΔC_p, and $\Delta G°$ obtained from fits of these denaturation experiments are similar to those obtained in other studies of cyt b_{562} (Feng & Sligar, 1991; Robinson & Sligar, 1993; Robinson et al., 1997a). At all concentrations, denaturation was accompanied by loss of about 80% of the ellipticity between 210 and 230 nm (Figure 3A, inset). The reaction was greater than 95% reversible at all concentrations, and denaturation curves monitoring CD overlay with those monitoring changes in visible absorbance

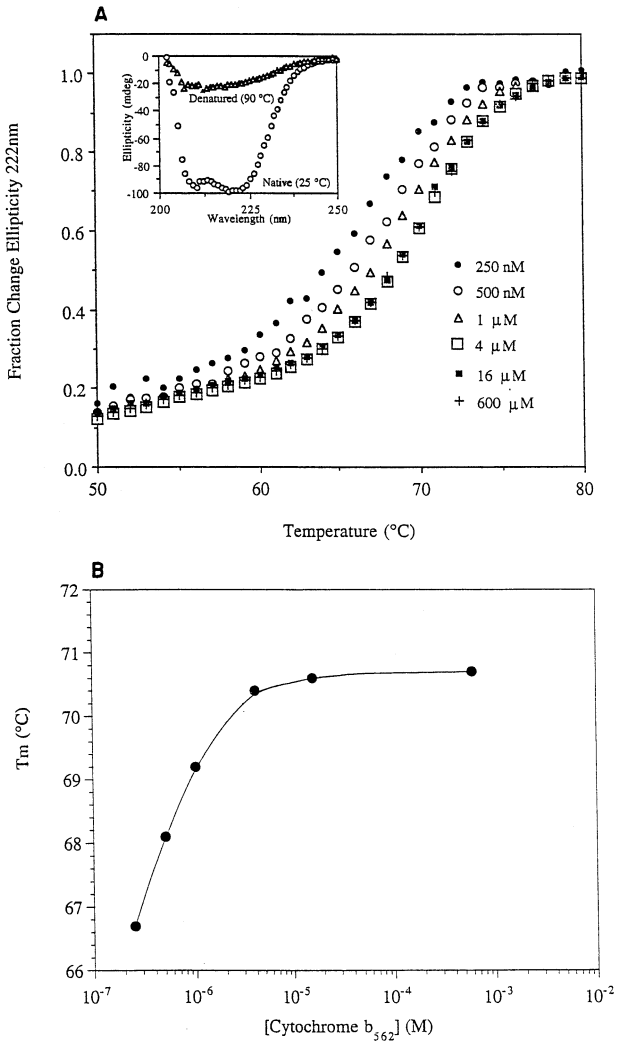

FIGURE 3: (A) Thermal denaturation of holocytochrome b_{562} at increasing protein concentrations. CD ellipticities at 222 nm (ϵ) were converted to fractional ellipticity change which equal ($\epsilon_{25°} - \epsilon_{obs}$)/($\epsilon_{25°} - \epsilon_{90°}$). Inset: CD spectra of native (25 °C) and denatured (90 °C) holocytochrome b_{562}. (B) Variation in T_m with the concentration of holocytochrome b_{562}. T_m values were obtained by fitting thermal denaturation profiles like those shown in panel A. The solid line is a fit of the data to the model shown in Figure 4. Thermodynamic parameters for the fit are listed in Table 2.

spectra (due to loss of heme ligands), indicating a cooperative reaction (Feng & Sligar, 1991; Robinson & Sligar, 1993; Robinson et al., unpublished data). Cyt b_{562} stability was not affected by the presence of non-heme proteins such as BSA (not shown).

Clearly, the denaturation reaction differs in the two concentration regimes. Below 4 μM the increase in stability with increasing protein concentration shows the concentration dependence expected for a reaction in which holoprotein denatures and dissociates to heme plus denatured apoprotein. In principle, such a relationship should continue indefinitely, limited only by solubility. The surprising feature of this profile is that above 4 μM stability becomes concentration independent, indicating that at these concentrations a monomolecular reaction limits stability. No change is observed in reversibility or cooperativity of the reaction. Therefore, we believe the simplest explanation is that below 4 μM heme dissociates from the denatured protein but above this concentration heme binds to the denatured apocytochrome b_{562}.

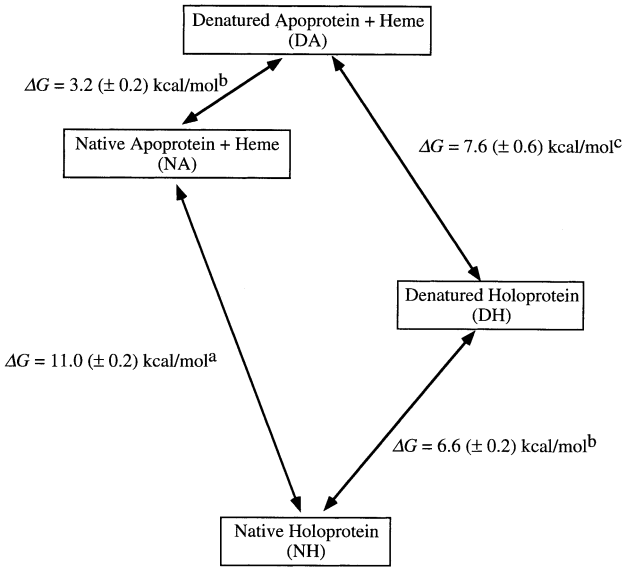

FIGURE 4: Thermodynamic cycle for folding and assembly of cytochrome b_{562}. a, $\Delta G°$ value for heme binding to native apoprotein from ITC measurements; b, $\Delta G°$ values for denaturation of native holo- and apoproteins taken from previous studies (Feng & Sligar, 1991; Fisher, 1991; Robinson & Sligar, 1993; Robinson et al., 1997a); c, $\Delta G°$ value for heme binding to denatured apoprotein calculated from the other three legs of the cycle. Vertical displacements represent approximate differences in free energy.

Heme Binding to Denatured Apoprotein. Binding of heme to denatured apoprotein appears to be a simple bimolecular interaction, suggesting that the binding is site-specific. If more than one heme were bound, cyt b_{562} thermal stability would decrease with increasing protein concentration (Hecht et al., 1984; Robinson et al., 1997b). No such decrease is observed up to 600 μM nor does stability decrease in the presence of excess heme (Robinson et al., 1997a). The presence of BSA had no effect upon stability either. If heme binding to the denatured apocytochrome were nonspecific, BSA should have competed for binding of heme molecules, decreasing holoprotein stability.

Using the thermodynamic parameters determined previously for thermal denaturation of holo- and apocytochromes b_{562} (Feng & Sligar, 1991; Fisher, 1991; Robinson & Sligar, 1993; Robinson et al., 1997a), and those for binding of heme to the native apoprotein determined by ITC, we constructed a thermodynamic cycle for folding and assembly (Figure 4). The cycle is comprised of four states: native holoprotein (NH), native apoprotein (NA), denatured apoprotein (DA), and denatured holoprotein (DH). We use this to calculate that the free energy change ($\Delta G°$) for heme binding to denatured apocytochrome b_{562} is 7.6 (±0.6) kcal mol^{-1}. This corresponds to a dissociation constant (K_d) equal to 3 μM, the approximate concentration at which cyt b_{562} reaches its maximum stability (Figure 3). Other thermodynamic parameters for heme binding to denatured apocyt b_{562} were calculated in the same way and are listed in Table 2. Using this set of parameters, we calculated the predicted dependence of T_m upon P_t (the total protein concentration), using eqs 1−5 for a model in which thermal unfolding proceeds by denaturation of the holoprotein, followed by dissociation of the heme (i.e., NH ↔ DH ↔ DA, right side of Figure 4). The predicted dependence agrees well with the experimental data (Figure 3B).

Table 2: Thermodynamic Parameters for Binding of Heme to Native and Denatured Apocytochrome b_{562} at 25 °C

	K_d (M)	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (kcal mol^{-1} K^{-1})
native apocyt b_{562}[a]	9 (±1) × 10^{-9}	11.0 (±0.2)	17 (±1)	0.019 (±0.01)
denatured apocyt b_{562}[b]	3 (±1) × 10^{-6}	7.6 (±0.6)	2 (±1)	0.018 (±0.07)

[a] Values obtained from ITC meaurements. [b] Values calculated from thermodynamic cycles like that shown in Figure 4.

An alternate model to explain the loss of concentration dependence of protein stability is that hemes aggregate after being released upon denaturation. However, several lines of evidence rule out this possibility: (1) The thermal denaturation reaction remains fully and rapidly reversible even at 600 μM concentration. If hemes were aggregating in this case, refolding of the holoprotein would be inhibited. (2) Cytochrome b_{562} samples incubated at the T_m for 30 min show no change in ellipticity. If heme aggregation was occurring, mass action would cause the holoprotein to gradually and continually denature during such an incubation. (3) No decrease in stability is observed at higher protein concentrations or in the presence of excess heme (Robinson et al., 1997a). All models in which heme aggregates to dimers or any higher oligomeric state predict an inversion of the concentration dependence, i.e., *decreasing* stability at higher protein concentrations, and therefore do not fit the data. The plateau in stability is indicative of a reaction in which the oligomeric state does not change. (4) An independent measure of the affinity of denatured apoprotein for heme obtained from the thermodynamic cycle illustrated in Figure 4 gives essentially the same value as that derived from the concentration dependence of stability.

Circular dichroism (CD) and absorption spectra of denatured cyt b_{562} at 500 nM and 16 μM are indistinguishable, indicating that little change in secondary structure or iron ligation or oxidation state accompanies heme binding to the denatured protein. Presumably enough residual structure is retained in thermally denatured cyt b_{562} to enable binding of the highly nonpolar heme moiety through hydrophobic and van der Waals interactions.

It is interesting to note the partitioning of the free energy for folding and heme binding between the native and denatured states of the cytochrome. Of the ~14 kcal mol^{-1} of free energy stabilizing the native holoprotein with respect to the denatured apoprotein (DA → NH), over 50% (7.6 kcal mol^{-1}) comes from heme binding to denatured state (DA → DH) and less than 25% (3.4 kcal mol^{-1}) [(NA → NH) − (DA → DH)] from interactions between heme and the native apoprotein, including ligation of the iron.

Implications. Binding of heme to the native state of cyt b_{562} imparts a substantial stabilization (3.4 kcal mol^{-1}) to the holoprotein, doubling the free energy required for denaturation. However, the overall affinity of the apocytochrome for heme is 2−5 orders of magnitude weaker than recent estimates for heme-apomyoglobin affinities (Hargrove et al., 1994, 1996).

The novel finding that denatured apo cyt b_{562} binds heme at micromolar concentrations means that folding via the native apoprotein (left side of Figure 4) need not be an obligate intermediate on the folding and assembly pathway of cyt b_{562}. An alternate path for (re)folding is possible, in which denatured apoprotein binds heme to form denatured holoprotein, which then folds to native holoprotein (right side of Figure 4). This alternate scheme may add robustness to the folding pathway, obviating the requirement for a diffusion-limited step and facilitating rapid and efficient folding or refolding. In refolding experiments in the presence of heme, no evidence for a folded apoprotein intermediate is observed, suggesting that the pathway on the right side of Figure 4 dominates.

Previously, heme has been shown to bind nonspecifically to non-heme-containing proteins such as BSA, and at multiple sites, probably by coordination to histine residues (Lecerc et al., 1993, Hargrove et al., 1996, Hargrove & Olson, 1996, and references therein). In contrast, we emphasize here that despite the presence of several alternate histidines in cyt b_{562}, both ITC and thermal denaturation experiments indicate that the stoichiometry of heme binding to native or denatured apoprotein is one to one. Thus the association of heme with denatured apocytochrome b_{562} appears to be a specific interaction and might therefore be an important feature of the folding/assembly reaction.

In vivo, heme binding by denatured apoproteins might allow *E. coli* a more flexible regulation of heme biosynthesis rates (Beck von Bodman et al., 1986; Unger et al., 1986; Springer & Sligar, 1987). Cyt b_{562} is a periplasmic protein and is believed to fold after translocation across the inner membrane (Nikkila et al., 1991). Little is known about the trafficking of prosthetic groups or holoprotein assembly (Mayer et al., 1995). One possibility is that formation of denatured holoproteins allows cotranslation of the protein and prosthetic group, bypassing the need for separate translocation machinery while still allowing posttranslocational folding.

Four-helix bundle proteins have been widely used as templates for protein engineering and design for many years (Ho & DeGrado, 1987; Hahn et al., 1990; Hecht et al., 1990; Bryson et al., 1995). Recently, incorporation of heme into *de novo* designed proteins has been employed both to furnish electrochemical activity (Robertson et al., 1994; Kalsbeck et al., 1996) and to provide an assay for structural specificity (M. Hecht, personal communication). Here we provide a systematic analysis of the affinity of a naturally occurring cytochrome for its prosthetic group. While this interaction is characterized by fairly tight binding, a surprising feature is that specific recognition by the apoprotein in its native state may not necessarily be required to achieve this affinity.

The structure and properties of denatured and partially folded states of proteins are of great interest. Through effects upon protein folding and oligomerization these non-native states can play key roles in protein stability and durability (Shortle, 1996; Smith et al., 1996; Robinson et al., 1997b). We have shown that protein interactions in the denatured state can also be critical for small-molecule recognition, emphasizing the necessity of considering the role of denatured states in assembly and ligand binding reactions.

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