Protein Science (1994), *3*:1430–1435. Cambridge University Press. Printed in the USA.

Stabilization of myoglobin by multiple alanine substitutions in helical positions

LAURA LIN,[1] RACHEL J. PINKER,[1] GEORGE N. PHILLIPS,[2] AND NEVILLE R. KALLENBACH[1]

[1] Department of Chemistry, New York University, New York, New York 10003
[2] Department of Biochemistry, Rice University, Houston, Texas 77251

(RECEIVED March 31, 1994; ACCEPTED June 15, 1994)

Abstract

We have carried out a series of multiple Xaa → Ala changes at nonadjacent surface positions in the sequence of sperm whale myoglobin. Although the corresponding single substitutions do not increase the thermal stability of the protein, multiple substitutions enhance the stability of the resulting myoglobins. The effect observed is an increase in the observed T_m (midpoint unfolding temperature) relative to that predicted from assuming additivity of the free energy changes corresponding to single mutations. The stabilization occurs in the presence of urea, as measured by the dependence of the unfolding temperature on urea concentration. The sites that have been altered occur in different helices and are not close in sequence or in the native structure of myoglobin. The observed effect is consistent with a role of multiple alanines in residual interactions in the unfolded state of the mutant proteins.

Keywords: α-helix; protein folding; protein stability; sperm whale myoglobin

Among natural amino acids, Ala has the strongest helix propensity in model helical peptides (Lyu et al., 1990; O'Neil & DeGrado, 1990; Padmanabhan et al., 1990) and substitution of Ala for weaker helix formers has been used extensively to assess the role of α-helix in stabilizing the native state of proteins (Horovitz et al., 1992; Blaber et al., 1993). In the case of the highly α-helical protein, myoglobin, single Ala replacements at surface helical sites tend not to stabilize the native state (Pinker et al., 1993). Replacement of Ala for exposed neutral side chains actually destabilizes the protein. A plausible explanation for this observation is that the propensity differences are smaller than the differences in free energy due to changes in the hydrophobic surface of the protein as bulkier side chains are replaced by alanine. This has been referred to as the lost buried surface effect (Pinker et al., 1993) and can be approximately accounted for by assuming that the native geometry is maintained at the site of mutation. Despite this approximation, the resulting correlation between lost buried surface area and the observed free energies of the corresponding mutant proteins is strong, with a slope equivalent to 20 cal/Å^2 of buried surface, close to the value obtained originally by Chothia (1984).

In carrying out Xaa → Ala substitutions at surface helical sites in myoglobin, we have investigated the additivity of the free energies of substitutions in stabilizing the native protein. The hypothesis that effects of a given Xaa → Ala substitution reflect differences in helix propensity predicts simple additivity of the free energies of mutations occurring at different sites in the same protein. Similarly, differences in lost buried surface area among mutants at remote sites in the protein should be additive. A series of myoglobins containing from 2 to 6 alanine replacements has been constructed and their thermal stability measured in the presence and absence of urea. The data reveal a trend as multiple alanines are introduced into the same protein: the stability of the protein increases, compared to the stability calculated for additive contributions from the single substitutions involved. Thus, mutations that individually exert a destabilizing effect on the stability of the protein collectively stabilize the molecule.

Reprint requests to: Neville R. Kallenbach, Department of Chemistry, New York University, 4 Washington Place, New York, New York 10003; e-mail: kallnbch@acfcluster.nyu.edu.

Results

Thermal stability of proteins

Table 1 identifies the combinations of mutants introduced in the multiply substituted proteins of this study and some basic properties of these. The integrity of the native state in each protein was monitored by measuring the heme absorbance in the Soret band at 420 nm and the CD signal at 222 nm in the UV, reflecting the α-helix content (Woody, 1988). All proteins described have the same Soret band maximum and value of $[\theta]_{222}$ at room temperature in the native state.

Table 1. *T_m's and $\Delta\Delta G^0$'s for myoglobins with multiple alanine substitutions*

Substitutions[a]	T_m	ΔT_m[b]	$\Delta\Delta G^0_{obs}$[c]	$\Delta\Delta G^0_{add}$[d]	$\Delta\Delta G^0_{dif}$[e]	$m' \times 10^{-5}$[f]
$N = 2$						
V66, *G23*	83.0	0.8	−0.45	0.37	−0.82	2.56
V66, V114	81.0	−1.2	0.62	0.70	−0.08	2.86
G129, V66	84.7	2.5	−1.36	−1.8	0.44	2.47
V66, *L149*	80.3	−1.9	0.97	0.85	0.12	3.83
V66, V13	83.8	1.6	−0.86	−0.08	−0.78	2.86
L11, V66	81.1	−1.1	0.57	−0.31	0.88	ND
V66, L137	81.4	−0.8	0.42	1.03	−0.61	3.32
G129, V114	83.9	1.7	−0.92	0.4	−1.32	3.35
V66, L135	71.2	−11.0	4.79			ND
$N = 3$						
V66, V114, V13	80.3	−0.9	0.97	1.37	−0.4	3.08
V114, *L149*, G129	79.4	−2.8	1.4	2.0	−0.6	3.07
V114, *H116*, *S117*	83.5	1.3	−0.7	1.55	−2.25	2.99
V66, G129, *G23*	84.2	2.0	−1.1	−0.68	−0.42	ND
V66, G129, *L149*	82.5	0.3	−0.16	−0.20	0.04	ND
V66, V114, G129	83.4	1.2	0.64	−0.35	0.99	2.20
$N = 4$						
V66, V114, V13, G23	84.9	2.7	−1.48	2.49	−3.97	2.74
V114, G129, V66, *L149*	80.6	−1.4	0.72	1.25	−0.53	ND
V66, G129, *G23*, V13	83.6	1.4	−0.75	−0.01	−0.74	ND
V114, *H116*, *S117*, *G23*	83.1	0.9	−0.48	2.67	−3.15	3.26
$N = 5$						
V114, V66, G129, *L149*, *G23*	79.6	−0.6	0.31	2.37	−2.06	ND
$N = 6$						
V66, V114, Gly 129, *L149*, *G23*, V13	82.9	0.7	−0.37	3.04	−3.41	4.09
V114, *H116*, *S117*, V66, G129, *L149*	83.9	1.7	−0.92	1.35	−2.27	2.92

[a] Italicized residues are not in midhelical positions.

[b] $\Delta T_m = T_{m,mutant} - T_{m,wild\ type}$ (°C). Estimated average error is less than ±0.3 °C.

[c] $\Delta\Delta G^0_{obs} = -\Delta T(\Delta H_{wt} + \Delta C_p \Delta T)/T_m$ (kcal/mol) where T_m is the transition temperature of the wild-type protein (82.2 °C) in the absence of urea (Pinker et al., 1993). Because it refers to conditions without denaturant, this free energy is denoted $\Delta\Delta G_{H_2O}$ by some writers. ΔC_p is 2.8 kcal deg^{-1} mol^{-1} and ΔH_{wt} = 186.5 kcal/mol for myoglobin under the same condition. The buffer used is 20 mM potassium phosphate, 100 mM KCl, 0.5 mM KCN, pH 9.6. All the $\Delta\Delta G^0$ measurements in this paper are done in the same condition and designated as the free energy change in the absence of urea. The error range in observed free energy change is less than ±0.01 kcal/mol, with a ±5% error in ΔH_{wt}.

[d] $\Delta\Delta G^0_{add}$ is the free energy difference between a multiple mutant and the wild-type protein, which would be expected had each mutation contributed independently to the free energy of unfolding of the multiple mutant. $\Delta\Delta G^0_{add}$ is calculated by addition of the corresponding $\Delta\Delta G^0_{obs}$ for the single mutations taken from Pinker et al. (1993).

[e] $\Delta\Delta G^0_{dif} = \Delta\Delta G^0_{obs} - \Delta\Delta G^0_{add}$. For the myoglobins with double alanine substitutions, these values are the free energy of coupling.

[f] Slope of $1/T_m$ versus urea concentrations. The wild-type protein yields a slope of 2.98 × 10^{-5} mol^{-1} K^{-1} under this condition. The variation for different trials with the same protein occurs in the second decimal place (±0.02).

Coupling in multiple substitutions

Interactions between residues in proteins can be analyzed by constructing double mutant cycles (Horovitz & Hersht, 1990; Horovitz et al., 1991). The principle is to compare the free energy change on introducing individual single-site substitutions with the change observed for the double mutant. If a change from the native sequence, denoted as Mb(0, 0), where the 0 represents wild-type side chains at sites *i* and *j* in the protein, to Mb(Ala, 0) has no influence on substitution at the second site, Mb(0, Ala), then the free energy change for the double mutant Mb(Ala, Ala) will correspond to the sum of the 2 single-step free energy changes (Horovitz & Fersht, 1990). The $\Delta\Delta G^0_{dif}$ values in Table 1 for $n = 2$ correspond to the coupling free energies for double mutants of myoglobin, with an average $\Delta\Delta G^0_{dif}$ value of −0.27 kcal/mol. As with other surveys of pairwise site–site interactions in proteins by mutagenesis (Green & Shortle, 1993; Gregoret & Sauer, 1993; Sandberg & Terwilliger, 1993), we find little evidence for nonadditivity or strong coupling in the effects of double surface mutations in myoglobin. Such interactions would naturally be favored if a set of sites that come together in the folded protein is mutated (Horovitz et al., 1992). But as one naively might expect, long range site–site interactions do not seem to play a major role in the native state of myoglobin.

Multiple alanine substitutions

The strategy of comparing a series of single mutations with multiple substitutions via complete mapping of free energy couplings is informative in principle but requires construction of large numbers of mutational intermediates as the number of sites or dimensionality of the cooperative "cycles" (Horovitz & Fersht,

1990) increases. Instead, we have proceeded to introduce additional alanines into nonvicinal sites in the same molecule, in order to see if any systematic trends emerge, without constructing comprehensive networks of intermediates. Table 1 shows the series of proteins containing 2 or more Ala substitutions that have been produced. As in the case of the corresponding single-site substitutions (Pinker et al., 1993), the heme environment and helix content of each multiply substituted protein have been monitored to verify intactness of the native fold. Data on the thermal stability of the proteins are presented in Table 1 as $\Delta\Delta G^0_{obs}$, measured at pH 9.6 where thermal unfolding is reversible (Acampora & Hermans, 1967) in the absence of denaturant. A clearer trend is shown with $n > 2$: multiple substitutions progressively enhance the stability of the protein relative to the ideal $\Delta\Delta G^0_{add}$ values for single substitutions, based on assuming that the effect of these is additive. Figure 1 illustrates the effect of the number of Ala substitutions on $\Delta\Delta G^0_{dif}$, the deviation in free energy from additivity of the multiple mutants calculated as described in the Materials and methods.

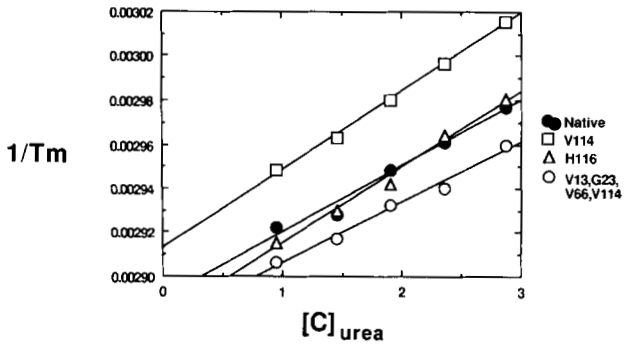

Fig. 2. Melting temperatures of myoglobin and 3 mutant proteins obtained in solutions of 1–3 M urea plotted as a function of urea concentration. The thermal transitions in the presence of urea were measured at pH 11.0, 40 mM glycine-KOH buffer, and the transitions correspond to the change in absorbance at 420 nm, a heme absorbance band. The reciprocal unfolding temperatures in the presence of urea correlate linearly with the molarity of urea. The slopes of these curves yield values of m' for each protein.

Thermal unfolding experiments in the presence of urea

Both thermal and solvent denaturation profiles yield important information about the stabilization of native proteins (Makhatadze & Privalov, 1992; Staniforth et al., 1993). Thermal experiments are conveniently performed with automated instrumentation, whereas isotherms corresponding to urea or guanidine-induced denaturation typically require extended equilibration times and yield values of N-U equilibrium concentrations only by extrapolation to regions well removed from the transition region in the case of a protein as stable as myoglobin (Puett, 1973; Pace & Vanderburg, 1979). Linear extrapolation models do not necessarily provide an adequate description of the variation of free energy with solvent molarity in proteins (Pace, 1986). Part of the problem may be trivial: the solvent conditions in the transition region differ from buffer alone, for example, in ionic strength (Santoro & Bolen, 1992). As a practical alternative, thermal transitions can be monitored for several urea concentrations and the dependence of T_m on activity recorded (Schellman, 1976; Makhatadze & Privalov, 1992). This allows one to evaluate the stability of mutant proteins as a function of both T and urea concentration, avoiding extrapolations of data outside the transition region. Figure 2 shows that myoglobins containing single-site Xaa → Ala substitutions at exposed helical sites exhibit different values of the slope m' of T_m^{-1} versus urea concentration plots relative to the wild-type protein. This is true also of multiple mutants. In a series of mutations at fully buried helical sites in myoglobin (Lin et al., 1993), changes in m' were observed that could be attributed to the interaction of residual structure in the unfolded chain. As discussed below, changes in the slope of the denaturant dependence of free energy of solvent-induced unfolding of staphylococcal nuclease have previously been attributed to effects on the unfolded state of that protein (Green & Shortle, 1993).

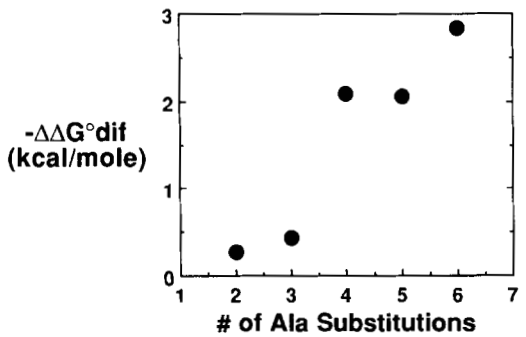

Fig. 1. $\Delta\Delta G^0_{dif}$, the *average* excess free energy of unfolding for myoglobins with multiple alanine substitutions plotted as a function of the number of alanine substitutions. Thermal transitions for each protein were carried out by monitoring the change in the heme absorbance at 420 nm. Free energy of unfolding for each protein was extracted from these profiles. The excess free energy for each multiple mutant ($\Delta\Delta G^0_{dif}$) is the difference between the observed free energy ($\Delta\Delta G^0_{obs}$) and the free energy of unfolding, which is expected from simple addition of the effects of the corresponding single mutations ($\Delta\Delta G^0_{dif}$). The excess free energies are averaged for each set of myoglobins with a given number of alanine substitutions.

Discussion

The main finding of this study is summarized in Figure 1, which shows that introducing multiple Ala side chains at helix positions in myoglobin leads to systematically enhanced protein stability. The stabilization is observed in the presence or absence of urea. In contrast to the corresponding single-site Ala substitutions, most of which are not stabilizing, the effect of multiple substitutions is to stabilize the molecule, with an apparently linear response in the number of alanines over the range of substitutions studied. Pairwise interactions between mutations in proteins have been investigated by several groups and do not reveal systematic trends of nonadditivity (Green & Shortle, 1993).

The stabilization we observe can be interpreted in terms of 2 very different models, which are in fact nonexclusive. In the first, we imagine that progressive Ala substitutions raise the free energy of the unfolded state, possibly by perturbing residual hydrophobic clusters present in U (Shortle et al., 1990; Amir et al., 1992; Neri et al., 1992). In the second, the effect is assumed to arise from interactions between sites in the native state itself. Given the distribution of sites on the surface, the second model requires that interactions propagate through the molecule or

around its surface, via networks of interactions. The latter possibilities for coupling between mutations are certainly imaginable, although they are perhaps less inherently simple to envisage than the former. The unfolding of a protein, either by heat or solvent, does not destroy all residual interactions in the unfolded state (Amir et al., 1992; Shortle, 1993). It is thus reasonable to consider the possibility that Ala substitutions exert a destabilizing effect on the residual structure of the unfolded protein, and hence effectively stabilize N.

One criterion for effects on the unfolded state is based on the dependence of the free energy of the protein on concentration of a denaturing solvent (Shortle & Meeker, 1986, 1989; Green et al., 1992; Shortle, 1993). Analysis of mutant staphylococcal nucleases indicates that the dependence of the free energy of the protein on guanidine hydrochloride concentration (m_{GuHCl}) can be interpreted in terms of differences in solvation by the unfolded form of the protein. Increases in m_{GuHCl} correlate with reduced residual structure in the unfolded protein and vice versa. For experimental convenience, we have measured here the related quantity, m'_{urea}, the slope of plots of T_m^{-1} versus [urea], rather than m_{urea} itself. The 2 quantities are related theoretically via the factor $T_m\Delta H$, where ΔH is the value of the enthalpy of unfolding in the presence of urea at $T = T_m$. This product is not obviously constant, but plots of T_m^{-1} versus [U] are linear with slope m' (Lin et al., 1993). Figure 3A shows that, for single substitutions, there is a correlation between the values of m' and the calculated changes in "lost buried surface area," the differential hydrophobic surface area lost upon mutation. A similar correlation was found for a series of internal substitutions between nonpolar residues in myoglobin (Lin et al., 1993). Figure 3B shows that this correlation is improved as expected if 2 sites that contain polar side chains (Thr 67 and His 116) are excluded. In these cases, the concept of lost buried surface is not applicable (Pinker et al., 1993). The slope m' is a measure of the free energy difference between solvating the folded and unfolded chains, as is m in the isothermal denaturation. This difference is a function of the change in exposed surface area of a protein upon unfolding. As noted above, amino acid substitutions associated with changes in buried surface area between N and U can change the value of m'. For myoglobins with single mutations, solvent-exposed as well as internal substitutions, the changes in m' can be accounted for by calculated changes in surface area of the unfolded protein, estimated from the loss of side-chain area in N as larger side chains are substituted by Ala. In these cases then, there is no reason to suspect that the structures of the unfolded chains are significantly altered.

For myoglobins with multiple alanine mutations, on the other hand, we find no correlation between the calculated changes in surface area and the values of m' (Fig. 3C) and it seems reasonable to infer that the structure of the unfolded chains has been altered. According to this analysis, changes in m' per se do not indicate whether or not changes in the structure of the native or the unfolded state have occurred. Rather, changes in m' that fail to correlate with the anticipated changes in surface area exposed when the protein unfolds indicate either that N is different or that a structural reorganization in the unfolded state is involved.

A second issue concerns the additivity of effects contributing to the value of m' for multiple mutants. We find in all cases that the observed m' values are smaller than those calculated by assuming averaging of the effects of single substitutions. Because the slope m' increases with decreasing hydrophobic surface area in the native structure (as we see in single substitutions), the observed smaller m' in the multiply substituted mutants implies a differential in the exposed surface area. If the multiple mutations affect the surface of the molecule in an additive fashion, we can attribute this effect to residual structural changes in the *unfolded* states of the mutant proteins.

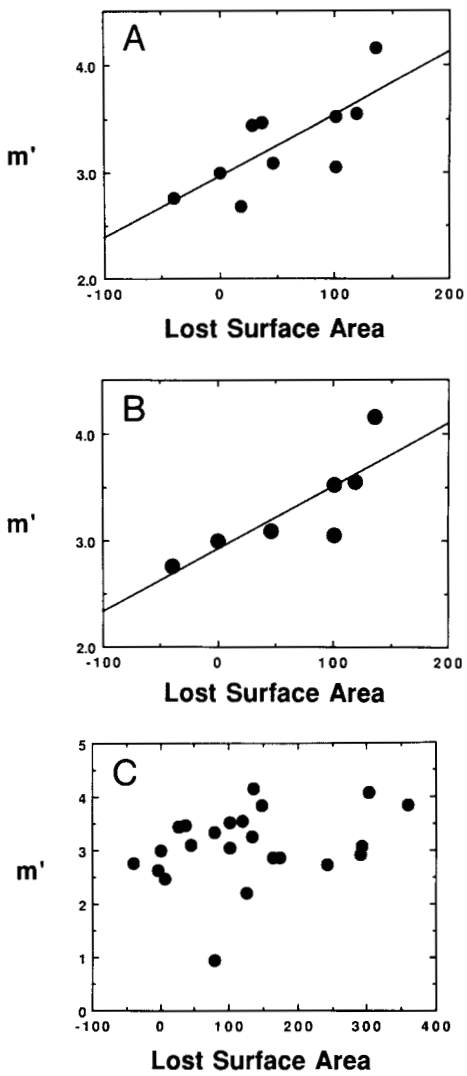

Fig. 3. A: m' values for myoglobins with single substitutions plotted as a function of the lost buried area (in Å^2). Lost buried area is defined as the area that is buried in the native state of the protein and which becomes exposed to solvent upon mutation of the original side chain to alanine. This parameter for each specific substitution is calculated as described earlier (Pinker et al., 1993). The regression line shown corresponds to $m' = 2.96 + 5.75$ Å^2, with $R^2 = 0.55$. **B:** The observed m' values of 7 *nonpolar* single substitutions in myoglobin, plotted as a function of the calculated lost buried area. The regression line drawn corresponds to $m' = 2.92 + 5.87$ Å^2, $R^2 = 0.67$. **C:** The observed m' values for all myoglobins with single and multiple alanine substitutions plotted against the calculated lost buried area.

In summary, alanine substitutions we have studied in myoglobin can affect either or both the folded and unfolded states of the protein, in principle. In the folded state, single mutations

at exposed sites act primarily to destabilize the folded protein, as indicated by the correlation between lost buried surface in N and the m' values (Fig. 3A). On the other hand, multiple mutations act in concert, either to alter N or to destabilize residual structure in the unfolded state, U, of myoglobin, or both. We think the effect is more likely to result from changes in U. As has been pointed out by others (Amir et al., 1992; Neri et al., 1993; Shortle, 1993), the presence of residual structure in unfolded states of a protein has important implications for the search process whereby a molecule acquires its native fold without exploring vast regions of the potential phase space accessible to the chain. Native-like interactions in the unfolded state simplify the search enormously. Even if the residual interactions are not fully native-like, the search for the native state could be enormously restricted relative to a hypothetical one beginning from an extended coil (Amir et al., 1992).

Introduction of multiple alanines at remote sites on the surface of a protein offers one route to stabilizing the folded structure of a protein in general. Because we find no correlation with helix propensity, the effect need not be restricted to helical proteins. We do not know the practical limits over which this stabilization effect persists, for example whether it reaches a plateau or continues as shown in Figure 2. Experiments addressing this question are in progress, as is determination of the structures of multiply substituted proteins by crystallography.

Materials and methods

Mutagenesis

The synthetic sperm whale myoglobin gene from the pMb413 plasmid (Springer & Sligar, 1987) was cloned into the phage vectors M13mp18 and M13mp19. Oligonucleotides (10 ng) bearing the codon for alanine (GCT) were annealed into a uridine-containing M13 template. Phage were transformed into competent JM101 cells. Site-directed mutations were introduced by the method of Kunkel (1985). Mutant sequences were verified by dideoxy sequencing of phage DNA (Sanger et al., 1977). Mutant myoglobins were expressed in a pUC19 vector in *Escherichia coli* TB-1 (Springer & Sligar, 1987).

Protein purification

E. coli TB-1 harboring the pMb413 plasmids were grown at 37 °C. Protein was purified as described by Springer and Sligar (1987). Briefly, cells were harvested, lysed overnight, and sonicated. Cell debris was removed by centrifugation and the supernatant was brought to 60% saturation with ammonium sulfate. The precipitate was collected by centrifugation and ammonium sulfate added to the supernatant to 95% saturation. The precipitate was recentrifuged, resuspended in 20 mM Tris Cl, pH 8.0, 1 mM EDTA, and applied to a Bio-Gel P-100 column. Appropriate fractions were collected, dialyzed to 25 mM phosphate, pH 6.0, and applied to a Whatman CM-52 ion-exchange column. Myoglobin was eluted with a linear gradient from 25 mM phosphate, pH 6.0, to 50 mM phosphate, pH 9.0. Protein purity was verified by SDS/PAGE.

The integrity of the native state in the mutant proteins was determined by the CD spectra of the mutant myoglobins in the ultraviolet region near 200 nm, where the peptide bond absorbs and α-helices have a well-defined spectrum (Woody, 1988). The heme environment was monitored by the absorption spectrum of the heme group in the Soret region (Acampora & Hermans, 1967).

Visible melting curves

The effect of the substitutions on the stability of the protein was monitored by means of thermal transition profiles. Thermal transitions were monitored both by the Soret band heme absorbance at 420 nm and the CD at 222 nm. The resulting profiles are superimposable in the presence of CN as ligand; this ligand stabilizes the wild-type met form of myoglobin by about 3 kcal/mol over the aquomet state. Because the thermal unfolding of myoglobin is not reversible near neutral pH (Acampora & Hermans, 1967), these experiments were carried out at pH 9.6, in 20 mM potassium phosphate with 100 mM potassium chloride in the presence of 0.5 mM KCN as a ligand. Melting profiles were obtained using a thermoelectrically controlled Perkin-Elmer 552 spectrophotometer interfaced to a PC-XT computer for acquisition and analysis of experimental data. The temperature was scanned at a heating rate of 0.5 °C/min.

These melting curves allow us to measure the transition temperatures, T_m, and van't Hoff enthalpies, and to estimate the free energy of the transition, ΔG_{obs}. ΔH was calculated using a 2-state model of protein unfolding, whereas ΔG_{obs} was estimated assuming that ΔH of the transition remains constant with temperature during the transition. Assuming each transition corresponds to a 2-state process, the transition midpoint temperature, T_m, and the apparent van't Hoff enthalpy of unfolding, ΔH_{unf}, can be determined from these profiles (Acampora & Hermans, 1967; Cho et al., 1982). Free energy changes were calculated using the equation:

$$\Delta\Delta G^0_{obs} = -\Delta T[\Delta H_{wt} + \Delta C_p \Delta T]/T_m \quad \text{(kcal/mol)},$$

where T_m is the transition temperature of the wild-type protein, ΔC_p is 2.8 kcal deg^{-1} mol^{-1} for myoglobin (Privalov & Gill, 1988), and ΔH_{wt} = 186.5 kcal/mol.

CD spectra were recorded on a modified Cary 60 spectrometer (Aviv Associates, Lakewood, New Jersey). The temperature was regulated with a Hewlett-Packard 8910000-A temperature controller. Ellipticity at 222 nm versus temperature was monitored in 20 mM potassium phosphate buffer, pH 9.6, in the presence of 0.1 M KCl and 0.5 mM KCN. The temperature was scanned at a heating rate of 0.5 °C/min. T_m from the CD spectra was determined in the same way as from the visible spectra.

Visible melting curves in the presence of urea

For each protein, the transition temperature was measured in solutions with different urea concentrations. Protein from a concentrated stock solution in water was diluted into solutions of 0–3 M urea, 40 mM glycine-KOH buffer, pH 11.0, in the presence of 0.5 mM KCN. Final urea concentration in protein–urea solutions ranged from 0 to 3 M. The solutions were equilibrated overnight at room temperature. Visible and CD melting profiles and the transition temperature for each protein solution were determined as described above.

Differential scanning calorimetry

The total heat of the unfolding transition for native myoglobin and 3 multiple mutants was measured directly with a Microcal MC-2 differential scanning calorimeter (Northhampton, Massachusetts). Excess heat capacity as a function of temperature was measured at 1 °C/min from 25 to 100 °C.

Protein concentrations were about 0.035 mM in 40 mM glycine-KOH and 0.5 mM KCN, pH 11.0. A buffer versus buffer scan was subtracted from the sample scan and normalized for the heating rate. The area of the resulting curve is proportional to the transition heat, when normalized for the number of moles is equal to the transition enthalpy, ΔH_{cal}. The instrument was calibrated with a standard electrical pulse.

Acknowledgments

We thank Drs. G.D. Rose and A. Finkelstein for helpful discussions. This research was supported by grant GM 40746 from the NIH and a Patricia Roberts Harris Fellowship to R.J.P.

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