Replacement of a Conserved Proline and the Alkaline Conformational Change in Iso-2-cytochrome *c*†

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ABSTRACT: Although point mutations usually lead to minor localized changes in protein structure, replacement of conserved Pro-76 with Gly in iso-2-cytochrome *c* induces a major conformational change. The change in structure results from mutation-induced depression of the p*K* for transition to an alkaline conformation with altered heme ligation. To assess the importance of position 76 in stabilizing the native versus the alkaline structure, the equilibrium and kinetic properties of the pH-induced conformational change have been compared for normal and mutant iso-2-cytochrome *c*. The pK_{app} for the conformational change is reduced from 8.45 (normal iso-2) to 6.71 in the mutant protein (Gly-76 iso-2), suggesting that conservation of Pro-76 may be required to stabilize the native conformation at physiological pH. The kinetics of the conformational change for both the normal and mutant proteins are well-described by a single kinetic phase throughout most of the pH-induced transition zone. Over this pH range, a minimal mechanism proposed for horse cytochrome *c* [Davis, L. A., Schejter, A., & Hess, G. P. (1974) *J. Biol. Chem. 249*, 2624–2632] is consistent with the data for normal and mutant yeast iso-2-cytochromes *c*:

$$NH \overset{K_H}{\leftrightarrow} N + H^+ \underset{k_{cb}}{\overset{k_{cf}}{\rightleftharpoons}} A$$

NH and N are native forms of cytochrome *c* with a 695-nm absorbance band, A is an alkaline form that lacks the 695-nm band, K_H is a proton dissociation constant, and k_{cf} and k_{cb} are microscopic rate constants for the conformational change. The Gly-76 mutation increases k_{cf} by almost 70-fold, but k_{cb} and K_H are unchanged. Thus, the mutation does not affect the deprotonation step directly, and the pK_{app} is lowered in the mutant only by coupling to the conformational step. One of the simplest interpretations is that the mutation destabilizes the native protein (NH and N) relative to both the alkaline conformation, A, and the transition state for the N ↔ A reaction.

Although the importance of conserved amino acids for protein structure, function, and folding is widely accepted, the specific roles played by particular residues are often difficult to discern. A residue might be retained for catalytic reasons and contribute little to the tertiary structure. Conversely, some amino acids such as prolines in reverse turns or residue clusters at helix–helix interfaces may be needed to stabilize architectural features of the folded state but have little direct role in catalysis. Learning how mutations alter preferences for different folded states is an important step toward understanding and predicting protein structure and stability.

There are at least three distinguishable conformations for folded cytochrome *c*: acid, native, and alkaline cytochrome *c*. The native form is present between approximately pH 5 and pH 8. pH-induced transitions to the acid or alkaline forms occur with overall p*K*'s of about 3 and 9, respectively, with small p*K* differences between members of the cytochrome *c* family. The native to alkaline transition of cytochrome *c* provides an excellent model system for studies of a conformational equilibrium since changes in heme absorbance are strongly coupled to the conformational change providing a sensitive means of detection. The equilibrium and kinetic properties of the alkaline transition for horse cytochrome *c* are generally consistent with a minimal mechanism involving a rapid deprotonation of the native protein followed by a slow conformational change to the spectroscopically distinct alkaline form (Davis et al., 1974). There is evidence, however, that the transition is more complex at high temperatures where the "alkaline form" may include multiple species (Hong & Dixon, 1989). Changes in populations of native and alkaline species with pH are consistent with a two-state transition, and over a wide range of conditions, the reaction meets the kinetic criteria for a two-state reaction by occurring in a single kinetic phase.

Little is known about the conformation of alkaline cytochrome *c*. The loss of a 695-nm absorbance band, assigned to Met-80 ligation of the heme in the native protein (Schechter & Saludjian, 1967), is strong evidence that Met-80 is no longer a heme ligand in the alkaline protein. The best guess, consistent with the low-spin state of the heme in the alkaline protein, is that a deprotonated Lys ε-amino group displaces Met-80 as a heme ligand (Davis et al., 1974; Gupta & Koenig, 1971; Hettinger & Harbury, 1964; Smith & Millett, 1980; Wilgus & Stellwagen, 1974). An analysis of shifts in p*K*'s

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for various acetimidylated forms of horse cytochrome *c* suggests that the ligand replacing Met-80 in the alkaline form is the ϵ-amino of Lys-72 or Lys-79 (Wallace, 1984). Although the extent of the conformational change that would result from Lys displacement of Met-80 is unknown, a molecular mechanism based on the X-ray structure of tuna cytochrome *c* proposes a drastic rearrangement of the structure on the Met-80 side of the heme (Takano & Dickerson, 1981). Amide proton exchange studies have shown that much of the helical secondary structure is retained in alkaline horse cytochrome *c*, supporting the intriguing proposal that the alkaline conformation is similar to that of a folding intermediate (Dohne et al., 1989; Roder et al., 1988). In addition, kinetic studies of folding of yeast iso-2-cytochrome *c* (iso-2)[1] at high pH indicate that the native to alkaline transition is the final step in folding to the alkaline conformation (Nall, 1986).

Here we report studies of a point mutation which leads to a dramatic shift in the pH-dependent equilibrium between the active nativelike form and the inactive alkaline protein. The mutation involves replacement of highly conserved Pro-76[2] by Gly in a region that forms a type II reverse turn in the native protein. The results strongly suggest that position 76 plays a special role in preferentially stabilizing the nativelike as opposed to the alkaline conformation of iso-2-cytochrome *c*.

Materials and Methods

Growth of yeast and protein purification and characterization have been described previously (Nall & Landers, 1981; Wood et al., 1988a).

While cytochrome *c* is stable for weeks to years at neutral pH, it is necessary to avoid exposure to high pH for long periods of time. pH-induced changes are fully reversible as long as storage of the protein solutions at alkaline pH (pH 9–11) does not exceed 6–8 h.

Equilibrium pH Titrations. Protein samples were prepared by mixing different ratios of two aliquots of protein solutions of equal concentration but extremes in pH such that the final pH falls between the two. A 0.01 mM protein solution in 0.1 M sodium phosphate buffer was prepared, divided into two equal volumes, and adjusted to low and high pH, respectively. For Pro-76 iso-2, the pH extremes were about pH 7 and pH 10.5 and for Gly-76 iso-2, pH 6 and 8.5. Additional samples were prepared at higher and lower pH values to get good base lines for the transition curves. Samples were thermostated at 20 ± 0.1 °C in an HP 8450A UV/visible spectrophotometer and absorbance measurements taken between 200 and 800 nm. The data were analyzed at 696 nm as a function of pH.

Kinetic Measurements. Protein solutions at an initial concentration of 50×10^{-6} M were prepared in 0.1 M sodium phosphate and adjusted to pH 6.0 with phosphoric acid for up-pH jumps. For down-pH jumps, the initial pH was adjusted with NaOH to pH 8.0 for Gly-76 iso-2 or to pH 9.5 for normal iso-2. Protein solutions were mixed in a ratio of 1:5 (protein solution to buffer solution) with 0.1 M sodium phoshate buffer at 20 °C in a Durrum–Gibson D110 stopped-flow apparatus, or by using a manual adder–mixer in a thermostated cuvette of the HP 8450A UV/vis spectrophotometer. Mixing dead times for stopped-flow experiments are 3–5 ms while manual mixing requires 3–5 s. Final conditions for all kinetic experiments are 20 °C, 0.1 M sodium phosphate, and 8.3×10^{-6} M cytochrome *c*. Final pH was varied by adjusting the pH of the mixing buffers over a range and performing trial mixes to determine the actual final pH of the fully mixed solutions. Rates were monitored by changes in absorbance at 418 nm.

Data Analysis. Analysis of both the equilibrium and the kinetic data follows that described by Davis et al. (1974). A linear least-squares analysis was used to fit the pH titration data to the equation:

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{app}} - (1/n) \log [(\epsilon - \epsilon_{\mathrm{H}})/(\epsilon_{\mathrm{L}} - \epsilon)]$$

where ϵ is the molar extinction coefficient of the equilibrium mixture of alkaline and native forms, ϵ_{H} is the molar extinction coefficient for the alkaline form, ϵ_{L} is the molar extinction coefficient for the native protein, and $1/n$ is the slope which is a measure of the number of protons (n) involved in the transition. pH and pK have the usual definitions as the negative logarithms of the hydrogen ion concentration and the proton dissociation constant, respectively.

Apparent rate constants, k_{obs}, are obtained by using an interactive computer program to fit the time-dependent absorbance changes to an equation of the form $\Delta A(t) = \Delta A_0 \exp(-k_{\mathrm{obs}}t)$ where $\Delta A(t)$ is the difference between the absorbance at time t and the final ($t = \infty$) base-line absorbance and ΔA_0 is the total absorbance change associated with kinetic phase k_{obs}. At very high pH, two kinetic phases are observed, and fits are obtained for an equation of the form $\Delta A(t) = \Delta A_0 \exp(-k_{\mathrm{obs}}t) + \Delta A_2 \exp(-k_2 t)$ where ΔA_2 and k_2 are, respectively, the amplitude and apparent rate constant for the second kinetic phase.

In the pH range where the data are described by a single kinetic phase, the nonlinear least-squares program GRIDLS (Bevington, 1969) was used to fit the equation $k_{\mathrm{obs}} = C_1 + [C_2C_3/(C_3 + 10^{-\mathrm{pH}})]$ where C_1, C_2, and C_3 are adjustable parameters. The optimized parameters showed no noticeable dependence on the initial values used for the nonlinear least-squares fits. In the calculation of χ^2 for the fit, instrumental weights of $1/0.1\ k_{\mathrm{obs}}$ were used to account for estimated uncertainties in the apparent rate constants. The procedure is equivalent to that of Davis et al. (1974) with $C_1 = k_{\mathrm{b}}$, $C_2 = k_{\mathrm{f}}$, and $C_3 = K_{\mathrm{H}}$, except that the nonlinear method gives improved error estimates and improved values for the parameters compared to the linear rearrangement of the same equation used by Davis et al. (1974).

Results

Spectral Comparison of Native and Alkaline Forms. In Figure 1 UV/visible spectra of iso-2 are shown at pH 6 in the nativelike conformation and at pH 10 in the alkaline form. Similar spectra have been presented previously for Gly-76 iso-2 [see Figure 2 in Wood et al. (1988a)]. For Pro-76 iso-2 and Gly-76 iso-2, increases in pH lead to loss of the 695-nm absorbance band, indicating that both the normal and mutant

[1] Abbreviations: iso-2, iso-2-cytochrome *c* from *Saccharomyces cerevisiae*; Gly-76, mutant form of iso-2 in which proline-76 is replaced by glycine; iso-1-MS, iso-1-cytochrome *c* treated with methyl methanethiosulfonate; NH and N, protonated and deprotonated forms of iso-2 or Gly-76 iso-2 in the native conformation; A, alkaline conformation of iso-2 or Gly-76 iso-2; k_{obs}, apparent rate constant for the native–alkaline conformational change; k_2, apparent rate constant for a second kinetic phase seen only at very high pH; k_{cf}, microscopic rate constant for the N → A conformational reaction; k_{cb}, microscopic rate constant for the A → N conformational change; K_{H}, dissociation constant for the NH ⇄ N + H^+ deprotonation reaction which precedes and triggers the alkaline conformational change.

[2] The vertebrate cytochrome *c* numbering system is used to denote amino acid positions in order to facilitate comparison between members of the cytochrome *c* family. Iso-1 has five additional amino-terminal residues, and iso-2 has nine additional amino-terminal residues compared to vertebrate cytochromes *c*. Both iso-1 and iso-2 have one residue less on the carboxy terminus. Thus, the vertebrate numbering of iso-1 and iso-2 starts at positions −5 and −9, respectively, and extends to position 103 [see Dickerson (1972) and Hampsey et al. (1986)]. For example, Pro-76 in the vertebrate numbering system corresponds to Pro-85 in the iso-2 numbering system.

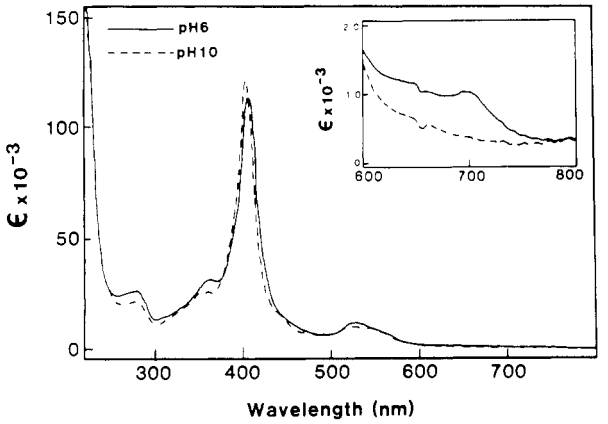

FIGURE 1: Absorbance spectra of the UV/visible region are shown for iso-2-cytochrome *c* at pH 6.0 (—) and pH 10 (---). In the insert, the 600–800-nm region is expanded showing the presence of the 695-nm absorabnce band for nativelike conformations (pH 6) and the absence of the 695-nm band for the alkaline conformations (pH 10). Other conditions are 0.1 M sodium phosphate, 20 °C, and protein concentrations of (10–20) × 10^{-6} mol/L.

proteins pass through similar pH-induced changes in conformation involving the heme–heme ligand "active site" region of cytochrome *c*. At pH 6 (solid lines), both proteins are in a nativelike conformation as indicated by the presence of the 695-nm absorbance band and depressed intensity of the Soret maximum. At pH 10 (dashed lines), both are in the alkaline conformation which shows increased intensity of the Soret maximum and lacks the 695-nm absorbance band. As described previously (Wood et al., 1988a), the pH 6 visible spectrum of Gly-76 iso-2 indicates small amounts of conformational heterogeneity. Weak absorbance at 620 nm for Gly-76 iso-2 suggests the presence of high-spin species. There are some differences in the pH dependence of the Soret maximum for normal and mutant iso-2. Iso-2 (Figure 1) shows an intensity increase together with a shift to lower wavelength (from 408 to 404 nm) while Gly-76 iso-2 shows the increase in Soret intensity but little change in wavelength (the maximum stays at 404 nm).

pH Dependence of Alkaline Conformational Change. Figure 2 shows equilibrium pH titrations for iso-2 (upper curve) and Gly-76 iso-2 (lower curve) in which the 696-nm absorbance is monitored. The decrease in 696-nm absorbance with increasing pH shows a smooth transition from the nativelike to the alkaline conformations with a p*K* of 8.45 for iso-2 and a p*K* of 6.71 for Gly-76 iso-2. Least-squares fits to plots of pH vs $\log[(\epsilon - \epsilon_H)/(\epsilon_L - \epsilon)]$ give slopes indicating a loss of 1.20 and 0.94 protons for iso-2 and Gly-76 iso-2, respectively, on passing from the nativelike to the alkaline conformations. Correlation coefficients for the fits are reasonable, 0.996 for iso-2 and 0.946 for Gly-76 iso-2, considering probable systematic errors in choosing the base-line absorbances for the nativelike and alkaline conformations.

Kinetics of the Nativelike to Alkaline Conformational Change. In Figure 3, the kinetic properties of the conformational change as a function of pH are presented for iso-2. Both amplitudes (Figure 3A) and apparent rate constants (Figure 3B) are presented. Between pH 5 and pH 9, which spans most of the equilibrium transition zone, the kinetics are well described by a single kinetic phase, k_{obs}. In the acid pH region, k_{obs} is independent of pH with a rate of 1.14 × 10^{-2} s^{-1}. As pH is increased, k_{obs} becomes noticeably pH dependent near the equilibrium p*K* and increases rapidly as the pH is raised further into the alkaline region. Near pH 9.5, a second kinetic phase, k_2, is detected. k_2 grows quickly in amplitude

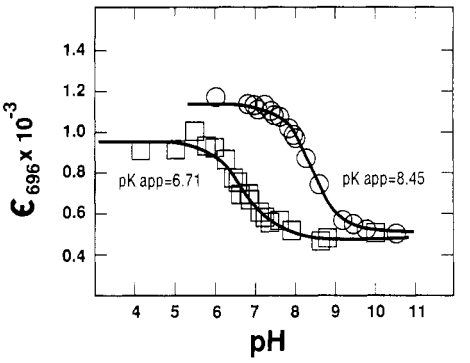

FIGURE 2: pH titrations for iso-2 (O) and Gly-76 iso-2 (□) monitoring the absorbance at 696 nm. The presence of the 695-nm absorbance band (see Figure 1 insert) indicates a nativelike conformation of cytochrome *c* in which the Met-80 sulfur atom is a heme ligand (Schechter & Saludjian, 1967). Loss of the 696-nm absorbance as the pH is raised measures conversion to the alkaline conformation in which Met-80 has been displaced as a heme ligand. The solid line through the circles is the theoretical titration curve for iso-2 in which 1.22 protons are lost with pK_{app} = 8.45. The solid line through the squares is a theoretical pH titration curve for Gly-76 iso-2 in which 0.94 proton is lost with pK_{app} = 6.71. Conditions are 0.1 M sodium phosphate, 20 °C.

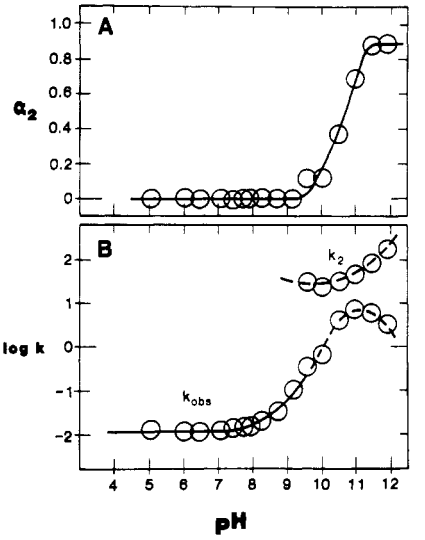

FIGURE 3: Amplitudes (A) and rate constants (B) for the alkaline conformational change of iso-2 measured by pH jumps. The pH jumps end at the indicated pH values and are induced by stopped-flow mixing at 20 °C, 0.1 M sodium phosphate. The initial conditions of the experiments start with the protein in the nativelike conformation (pH 6.0) or the alkaline conformation (pH 9.5). Over most of the pH-induced transition region, a single rate process (k_{obs}) accounts for all of the kinetically detected absorbance change. At high pH, barely above the equilibrium transition region, a second kinetic phase (k_2) is detected with an amplitude (α_2) that increases as pH increases. The solid lines in (B) are theoretical "best fits" to the mechanism of eq 1 using parameters given in Table I. Dashed lines have no theoretical significance.

with increasing pH so that by pH 12 about 90% of the kinetically detected absorbance change occurs in phase k_2.

In Figure 4, the pH dependence of the amplitudes (Figure 4A) and apparent rate constants (Figure 4B) are given for Gly-76 iso-2. At acid pH, k_{obs} for Gly-76 iso-2 is independent of pH. The rate of 1.08 × 10^{-2} s^{-1} is essentially the same as observed for iso-2 in the acid region. The overall behavior of k_{obs} is like that of iso-2 except that the pH dependence of k_{obs} becomes apparent even below neutral pH (i.e., near the equilibrium pK_{app} for Gly-76 iso-2). Again, a second kinetic phase, k_2, is observed at high pH but only when the final pH of the kinetic experiments is on the alkaline side of the

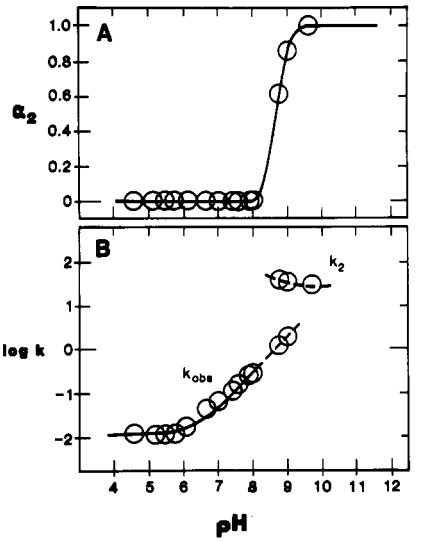

FIGURE 4: Amplitudes (A) and rate constants (B) for the alkaline conformational change of Gly-76 iso-2 measured by pH jumps. The pH jumps end at the indicated pH values and are induced by stopped-flow mixing at 20 °C, 0.1 M sodium phosphate. The initial conditions of the experiments start with the protein in the nativelike conformation (pH 6.0) or the alkaline conformation (pH 8.0). Over most of the pH-induced transition region, a single rate process (k_{obs}) accounts for all of the kinetically detected absorbance change. At high pH, barely above the equilibrium transition region, a second kinetic phase (k_2) is detected with an amplitude (α_2) that increases as pH increases. The solid lines in (B) are theoretical "best fits" to the mechanism of eq 1 using parameters given in Table I. Dashed lines have no theoretical significance.

equilibrium transition region (above pH 8). The amplitude of phase k_2 increases more strongly with pH than for iso-2 until phase k_2 is the only detectable phase for a final pH above pH 9.5.

Discussion

Studies of how point mutations alter tertiary structure suggest that we are a long way from being able to make a priori predictions of how proteins fold from amino acid sequence. Although there are exceptions (Alber et al., 1988; Louie & Brayer, 1989), single amino acid replacements usually have little detectable effect on overall chain folding with mutation-induced changes limited to readjustments of side chains near the site of mutation (Alber et al., 1987; Howell et al., 1986). Surprisingly, these minor structural changes often lead to large changes in thermodynamic stability (Alber et al., 1987) while more extensive structural changes can occur with little effect on stability (Alber et al., 1988). The former suggests that the structural basis of the stability of tertiary structure is either poorly understood or beyond the resolution of X-ray methods; i.e., undetectably small structural differences lead to easily detected stability differences. The latter suggests the existence of structurally distinct conformations with similar free energies. Regardless, we are unable to predict consistently how changes in sequence alter structure and predictions of stability for known variations on the same structure are also problematical.

Understanding how mutations shift conformational equilibria promises to be easier than evaluating effects on overall stability and protein folding. A simple conformational equilibrium involves two energetically important species both of which have stable tertiary structures. Thus, only the relative effects of the mutation on the two separate structures are important. Many tertiary interactions may be common to both conformers so that the focus can be on the interactions that change.

The kinetic mechanisms of conformational changes are much simpler than those for protein folding. Many conformational changes have kinetic properties consistent with a two-state process while kinetic properties of protein folding reactions are only rarely compatible with two-state mechanisms. Kinetic simplicity is vital to the quantitative analysis of the effects of mutations. For simple mechanisms where the number of experimental observables equals or exceeds the number of kinetic parameters, the changes in specific microscopic rate constants or equilibria can be measured, providing a complete quantitative description of mutation-induced changes.

Equilibrium and Kinetic Properties in Accord with a Simple Mechanism. A minimal mechanism proposed by Davis et al. (1974) for the alkaline conformational change in horse cytochrome *c* is consistent with the results for iso-2 and Gly-76 iso-2 over most of the pH range investigated. The mechanism is

$$NH \overset{K_H}{\leftrightarrow} N + H^+ \underset{k_{cb}}{\overset{k_{cf}}{\rightleftharpoons}} A \qquad (1)$$

where NH and N are forms of cytochrome *c* with a 695-nm absorbance band, A is a form that lacks the 695-nm band, K_H is a proton dissociation constant, and k_{cf} and k_{cb} are microscopic rate constants for the conformational change. Starting with eq 1, Davis et al. (1974) derive a relationship between the pH dependence of the absorbance changes and the apparent p*K*:

$$pH = pK_{app} - \log [(\epsilon - \epsilon_H)/(\epsilon_L - \epsilon)] \qquad (2)$$

where ϵ is the molar extinction coefficient of the equilibrium mixture of alkaline and native forms, ϵ_H is the molar extinction coefficient for the alkaline form, and ϵ_L is the molar extinction coefficient for the native protein. For $(k_{cf}/k_{cb}) \gg 1$, the following equation relates pK_{app} to the parameters of eq 1:

$$pK_{app} = p[K_H(k_{cf}/k_{cb})] \qquad (3)$$

To explain the kinetic results, Davis et al. (1974) derive the following equation relating the observed rate constant, k_{obs}, and the parameters of eq 1:

$$k_{obs} = k_{cb} + k_{cf}[K_H/(K_H + [H^+])] \qquad (4)$$

As described under Materials and Methods, nonlinear least-squares methods are used to fit eq 4 to the data, yielding values for k_{cf}, k_{cb}, and K_H (see Table I). The fits are quite good as judged by a comparison of the data to theoretical curves calculated by using the optimized kinetic parameters (Figures 3 and 4). Errors in the kinetic parameters range from 4 to 8% (Table I) and have been estimated as described by Bevington (1969) assuming standard deviations of 10% in the observed rate constants, k_{obs}.

An important check on the validity of the mechanism and methodology is provided by comparing the apparent p*K* calculated from the kinetic parameters, pK^{kin}_{app}, to that obtained from the equilibrium transition, pK^{eq}_{app} (compare columns 6 and 7, Table I). The agreement is good, especially considering that there has been no attempt to account for possible systematic errors in pH, temperature, etc. between the equilibrium and kinetic data sets.

(i) pK for Equilibrium Transition is Depressed in Gly-76 Iso-2. The UV/visible spectra of the low-pH and alkaline forms of iso-2 and Gly-76 iso-2 have much in common, but this alone does not ensure that pH induces the same transition between the same species in the normal and mutant proteins. Visible spectra reflect primarily heme environment and ligation state, which could be similar for somewhat different tertiary

Table I: Equilibrium and Kinetic Parameters for Alkaline Conformational Change[a]

protein	k_{cf} (s^{-1})[b]	$k_{cb} \times 10^2$ (s^{-1})[b]	$K_H \times 10^{12}$ (mol/L)[b]	pK_H[c]	pK^{kin}_{app}[c]	pK^{eq}_{app}[d]
iso-2	52 ± 4	1.14 ± 0.05	1.00 ± 0.08	12.00 ± 0.04	8.34 ± 0.05	8.45 ± 0.02
Gly-76 iso-2	3540 ± 166	1.08 ± 0.05	1.00 ± 0.05	12.00 ± 0.02	6.48 ± 0.04	6.71 ± 0.04

[a] The microscopic rate constants, proton dissociation constants, and derived quantities are given for the mechanism of eq 1. Conditions are 20 °C in the presence of 0.1 M sodium phosphate. [b] Parameters are obtained from the kinetic data (Figures 3 and 4) by a nonlinear least-squares fit to eq 4. Standard deviations in the values of k_{obs} are assumed to be 10% of the magnitude of k_{obs}. Errors are estimated from the fitting program (Bevington, 1969) and are measures of the sensitivity of the quality of the fit to small changes in a given parameter. These errors do not include possible systematic errors in temperature, pH, or other experimental conditions. [c] Quantities derived from k_{cf}, k_{cb}, and K_H. pK_H is the negative of the base 10 logarithm of K_H. $pK^{kin}_{app} = -\log(k_{cf}K_H/k_{cb})$ is the pK_{app} for the transition calculated from parameters obtained from the kinetic data. The errors have been estimated from the errors in k_{cf}, k_{cb}, and K_H by using standard methods for calculating the propagation of errors (Bevington, 1969). [d] pK^{eq}_{app} is the pK_{app} for the transition obtained from a linear least-squares fit of the pH titration data (Figure 2) to eq 2. The equivalence of pK^{eq}_{app} and pK^{kin}_{app} is a test of the mechanism described in eq 1. Errors are estimated from errors in the intercept calculated as described by Bevington (1969). These errors reflect the quality of the fit and do not include possible systematic errors in experimental conditions.

structures. Regardless, the simplest model is that the primary effect of the Gly-76 mutation is to lower the pK for the alkaline conformational change without significant change in mechanism or in the structures of the equilibrating species. The observation that the pH-induced transitions for both proteins indicate loss of one proton is in accord with the simple model.

(ii) Gly-76 Mutation Destabilizes the Native Conformation Relative to the Alkaline Conformation and the Native–Alkaline Transition State. The overall kinetic patterns for normal and mutant iso-2 are the same. For both proteins, rates are pH independent on the low-pH side of the transition, show a slight pH dependence as pH is raised toward the equilibrium pK_{app}, and have a strong pH dependence in the alkaline region. Above the transition region, a new kinetic phase (k_2) is observed for both proteins, and, as pH is raised further, phase k_2 increases in amplitude until it becomes the dominant reaction.

Effects of the Gly-76 mutation on the alkaline conformational change are surprisingly simple. Over the pH range where k_{obs} is the only reaction observed, the quantitative comparison of the kinetic parameters according to mechanism 1 shows that the Gly-76 mutation increases k_{cf} by almost 70-fold in the absence of significant changes in either K_H or k_{cb}. This result together with the theory of absolute reaction rates (Glasstone et al., 1941) suggests two alternative models for the effect of the mutation on the free energies of the species along the reaction coordinate (Figure 5A,B). In Figure 5A, the mutation is assumed to *destabilize* the native conformation (both NH and N) by 2.5 kcal/mol while leaving the transition state (N–A‡) and the alkaline conformation unchanged. The alternative interpretation in Figure 5B shows the mutation as *stabilizing* both the alkaline form (A) and the transition state (N–A‡) by 2.5 kcal/mol while leaving the native conformation (NH and N) unperturbed. The data presented here cannot distinguish between the two possibilities. The fact that the Gly-76 mutation is known to destabilize the nativelike conformation *relative* to the unfolded conformation [but only by 1.2 kcal/mol (Wood et al., 1988b)] appears to support the scheme presented in Figure 5A, but, again, it is not clear whether the primary effect of the mutation is in destabilizing the native conformation or in stabilizing the unfolded conformation.[3]

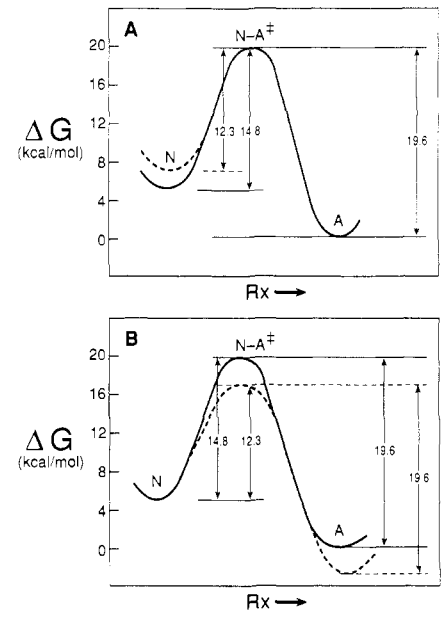

FIGURE 5: Free energy vs reaction coordinate plots showing two alternative interpretations of the effects of the Gly-76 mutation on the equilibrium and kinetic data. In scheme A, the mutation destabilizes the nativelike conformation, N, by 2.5 kcal/mol with no effect on the stability of the transition state, N–A‡, or the alkaline conformation, A. In scheme B, the mutation is assumed to stabilize both N–A‡ and A by 2.5 kcal/mol.

Mutations as Mechanistic Probes of Conformational Change. Studies of additional mutants are needed to obtain a more complete description of the contributions of specific residues to the native to alkaline conformational change. Not only is it important to learn which sites affect which kinetic parameters, but it is also important to test whether different mutations at the same site have analogous effects. For the yeast isozyme, iso-1-MS cytochrome c[4] replacements of Pro-71 lower pK_{app} by 1.5–2.0 pH units depending on the particular mutation. Differences between the values of pK_{app} calculated from the kinetic parameters and those obtained directly by pH titrations indicate that the alkaline transition may be mechanistically more complex for mutants at position 71 than for normal iso-1-MS (Ramdas, 1987; Ramdas & Nall, 1987). A study of the alkaline conformational change for replace-

[3] Effects of the mutation in stabilizing the unfolded form of Gly-76 iso-2 compared to (unfolded) normal iso-2 should be considered too. This is expected to occur through increases in entropy of the unfolded state brought about by replacing the conformationally restrictive proline residue with a conformationally more permissive glycine residue (Alber et al., 1988; Hecht et al., 1985, 1986). The mutation might also alter interaction of the unfolded state with solvent (Shortle & Meeker, 1986, 1989). Changes in solvent interaction with the unfolded form are probably small in the present case since there is little difference in the cooperativity of the guanidine hydrochloride induced transition for iso-2 compared to Gly-76 iso-2 (Wood et al., 1988b).

[4] Iso-1-MS and Thr-102 iso-1 are analogues of normal iso-1-cytochrome c which have been modified to allow functional and physical characterization. Iso-1-cytochrome c as obtained from yeast has a cysteine at position 102. This residue interferes with studies of function and folding through intermolecular disulfide dimer formation and reduction of the heme. For our studies of iso-1-cytochrome c, we have blocked Cys-102 by treatment with methyl methanethiosulfonate which adds an $-SCH_3$ group to Cys-102 via disulfide bond formation (Ramdas et al., 1986). The studies by Pearce et al. (1989) use forms of iso-1-cytochrome c in which Cys-102 is converted to Thr by site-directed mutagenesis (Cutler et al., 1987).

ments of Phe-82 in Thr-102 iso-1-cytochrome *c* shows that mutations at position 82 lower pK_{app} by as much as 1.3 pH units without changes in mechanism. There are at least two kinds of effects of mutations at this site: Ser-82 and Gly-82 variants alter K_H and $K_c = k_{cf}/k_{cb}$, while Ile-82 and Leu-82 variants decrease K_H but have little effect on K_c (Pearce et al., 1989).

So how do we interpret data that show that some substitutions at a single site have more extensive effects than others? If the additional effects arise only when long-range conformational changes are induced by more perturbing substitutions, then mutations that change the least number of kinetic parameters are providing the most "site specific" information. Using this "minimalist's" interpretation, and combining the existing data for the two yeast iso-cytochromes *c*, the primary importance of Pro-76 (and possibly Pro-71) is in stabilizing the nativelike relative to the alkaline conformation. On the other hand, since substitutions at position 82 can depress pK_H with little change in k_{cf} and k_{cb} (Pearce et al., 1989), position 82 is less important for (relative) conformational stability but is likely to be close to the ionizing group that triggers the alkaline conformational change.

References

Alber, T., Dao-pin, S., Wilson, K., Wozniak, J., Cook, S., & Matthews, B. (1987) *Nature 330*, 41–46.

Alber, T., Bell, J. A., Dao-Pin, S., Nicholson, H., Wozniak, J., Cook, S., & Matthews, B. W. (1988) *Science 239*, 631–635.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Cutler, R. J., Pielak, G. J., Mauk, A. G., & Smith, M. (1987) *Protein Eng. 1*, 95–99.

Davis, L. A., Schejter, A., & Hess, G. P. (1974) *J. Biol. Chem. 249*, 2624–2632.

Dickerson, R. E. (1972) *Sci. Am. 226*, 58–72.

Dohne, S. M., Elove, G. A., Roder, H., & Nall, B. T. (1989) *Biophys. J. 55*, 557a.

Glasstone, S., Laidler, K. J., & Eyring, H. (1941) *The Theory of Rate Processes*, McGraw-Hill, New York.

Gupta, R. K., & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1134–1143.

Hampsey, D. M., Das, G., & Sherman, F. (1985) *J. Biol. Chem. 261*, 3259–3271.

Hecht, M. H., Hehir, K. M., Nelson, H. C., Sturtevant, J. M., & Sauer, R. T. (1985) *J. Cell. Biochem. 29*, 217–224.

Hecht, M. H., Sturtevant, J. M., & Sauer, R. T. (1986) *Proteins 1*, 43–46.

Hettinger, T. P., & Harbury, H. A. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1469–1476.

Hong, X., & Dixon, D. W. (1989) *FEBS Lett. 246*, 105–108.

Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science 231*, 1123–1128.

Louie, G. V., & Brayer, G. D. (1989) *J. Mol. Biol.* (submitted for publication).

Nall, B. T. (1986) *Biochemistry 25*, 2974–2978.

Nall, B. T., & Landers, T. A. (1981) *Biochemistry 20*, 5403–5411.

Pearce, L. L., Gartner, A. L., Smith, M., & Mauk, A. G. (1989) *Biochemistry 28*, 3152–3156.

Ramdas, L. (1987) Ph.D. Thesis, University of Texas Health Science Center at Houston, Houston, TX.

Ramdas, L., & Nall, B. T. (1987) *J. Cell. Biochem., Suppl. 11C*, 218.

Ramdas, L., Sherman, F., & Nall, B. T. (1986) *Biochemistry 25*, 6952–6958.

Roder, H., Elove, G. A., & Englander, S. W. (1988) *Nature 335*, 700–704.

Schechter, E., & Saludjian, P. (1967) *Biopolymers 5*, 788–790.

Shortle, D., & Meeker, A. K. (1986) *Proteins 1*, 81–89.

Shortle, D., & Meeker, A. K. (1989) *Biochemistry 28*, 936–944.

Smith, H. T., & Millett, F. (1980) *Biochemistry 19*, 1117–1120.

Takano, T., & Dickerson, R. E. (1981) *J. Mol. Biol. 153*, 95–115.

Wallace, C. J. A. (1984) *Biochem. J. 217*, 601–604.

Wilgus, H., & Stellwagen, E. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2892–2894.

Wood, L. C., Muthurkrishnan, K., White, T. B., Ramdas, L., & Nall, B. T. (1988a) *Biochemistry 27*, 8554–8561.

Wood, L. C., White, T. B., Ramdas, L., & Nall, B. T. (1988b) *Biochemistry 27*, 8562–8568.