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pH Dependence of Folding of Iso-2-cytochrome *c*†

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ABSTRACT: Starting from a standard unfolded state (3.0 M guanidine hydrochloride, pH 7.2), the kinetics of refolding of iso-2-cytochrome *c* have been investigated as a function of final pH between pH 3 and pH 10. Absorbance in the ultraviolet and visible spectral regions and tryptophan fluorescence are used to monitor folding. Over most of the pH range, fast and slow folding phases are detected by both fluorescence and absorbance probes. Near neutral pH, the rate of fast folding appears to be the same when monitored by absorbance and fluorescence probes. At higher and lower pH, there are two fast folding reactions, with absorbance-detected fast folding occurring in a slightly faster time range than fluorescence-detected fast folding. The rates of both fast folding reactions pass through broad minima near neutral pH, indicating involvement of ionizable groups in rate-limiting steps. The rates of slow folding also depend on the final pH. At acid pH, there appears to be a single slow folding phase for both fluorescence and absorbance probes. At neutral pH, the absorbance-detected and fluorescence-detected slow folding phases separate into distinct kinetic processes which differ in rate and relative amplitude. At high pH, absorbance-detected slow folding is no longer observed, while fluorescence-detected slow folding is decreased in amplitude. In contrast, the equilibrium and kinetic properties of proline imide bond isomerization, believed to be involved in the slow folding reactions, are largely independent of pH. The results suggest that the pH dependence of slow folding involves coupling of pH-sensitive structure to proline imide bond isomerization. The dependence of kinetic properties on pH suggests that one factor governing the rates of both fast and slow folding reactions involves ionizable groups which alter the stability of folding intermediates. Differences in the pH sensitivity of key intermediates may influence relative rates and thus the selection of pathways in folding, leading to appearance and disappearance of kinetic phases.

Protein folding is often viewed as a relatively simple reaction. Folding starts in an unfolded state composed of astronomical numbers of unstructured polypeptide chains with similar free energies and proceeds directly to a unique (but dynamic) folded state at the global free energy minimum. This two-state view has been surprisingly successful in understanding equilibrium folding/unfolding transitions. Nevertheless, the molecular gymnastics required to fold a protein suggest considerable mechanistic complexity. Evidence of this complexity is provided by experimental observations of a multitude of kinetic phases in folding. Kinetic experiments prove that several (kinetically distinct) species are present but the importance of these species in directing the process of folding remains controversial.

Criteria for judging the importance of kinetically detected species are provided by folding to different final conditions. If the species are important in guiding folding, then changes in the final conditions should affect the relative stability of the intermediates and thus the rates and amplitudes for folding reactions. The experimental question reduces to whether or not there are qualitative differences in the kinetic patterns for folding under different final conditions.

For horse cytochrome *c*, it is well-known that the number and amplitudes of kinetic phases depend strongly on experimental conditions such as the presence or absence of external heme ligands, the type of denaturant, and pH (Brems &

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Stellwagen, 1983; Myer, 1984; Tsong, 1973; Dyson & Beattie, 1982). At low pH, the kinetic changes appear to be linked to the presence of additional folded species or local conformational changes in the unfolded protein (Dyson & Beattie, 1982; Robinson et al., 1983; Tsong, 1977). Little is known about refolding of cytochrome *c* from horse in the alkaline pH range.

We present experiments with iso-2-cytochrome *c* from *Saccharomyces cerevisiae* to test for changes in the kinetics of folding over a wide range of final pH. There have been previous hints of a dependence on final conformation and conditions: at alkaline pH, refolding involves a new kinetic process (Nall, 1986) related to a well-known pH-induced (alkaline) isomerization between folded cytochrome *c* conformers (Greenwood & Palmer, 1965; Brandt et al., 1966; Davis et al., 1974; Osterhout et al., 1985). In addition, folding of a proline to threonine mutant form of iso-2 *to a nonnative conformation* at neutral pH occurs without the usual absorbance-detected slow phase, but the slow phase returns when folding proceeds to a more nativelike form at slightly lower pH (White et al., 1987).

Other experiments focus on the unfolded protein. Both the amplitudes and time constants for folding at neutral pH are independent of *initial* pH between pH 3 and pH 9 and *initial* guanidine hydrochloride (Gdn·HCl)[1] concentration between 2 and 5 M in the unfolded protein[2] (Osterhout & Nall, 1985). Thus, the kinetic features appear to be largely independent of the *initial* unfolded state of the protein. The present experimental design allows changes in the kinetic pattern of folding to be attributed solely to differences in the population of species in the *final* conditions for folding. The initial conditions for unfolding the protein are held constant (above the Gdn·HCl-induced unfolding transition near neutral pH) while the *final* conditions of refolding are varied over the pH range from pH 3 to pH 10.

Materials and Methods

Yeast Fermentation and Protein Purification. Iso-2 was purified from strain B4926 (mat α cycl-1 CYC7-H1 trp1-1) of *Saccharomyces cerevisiae* obtained as a gift from Fred Sherman (University of Rochester School of Medicine and Dentistry). Yeast were grown on YD media (1% yeast extract/1% dextrose) in 180-L fermentations at 30 °C with vigorous aeration. The cells were harvested 8–16 h after growth had leveled off in the stationary phase. Prior to being harvested, low-temperature visible spectra (Sherman et al., 1968) were taken of thick suspensions of cells to ensure full induction of mitochondrial proteins. Cell yields were in the range of 1.7–3.5 kg of cells (wet weight) from which 200–400 mg of purified iso-2 was obtained. Additional details of the growth of yeast and the purification of iso-2-cytochrome *c* are provided elsewhere (Nall & Landers, 1981; Zuniga & Nall, 1983).

Kinetic Measurements. Stopped-flow mixing experiments were performed by using a modified Durrum D-100 stopped-flow apparatus with a 1:5 mixing ratio. In the initial conditions, the protein solutions contained 3.0 M Gdn·HCl and 0.04 M sodium phosphate, pH 7.2. The mixing buffer was prepared such that (following mixing of 1 part protein solution with 5 parts buffer solution) the final conditions contained 0.5 M Gdn·HCl and 0.091 M sodium phosphate. The pH of the mixing buffer was varied to obtain a final pH in the range 3–10. Folding was monitored by fluorescence changes at 350 nm (excitation at 287 nm) or by absorbance changes at 418 and 287 nm. The initial unfolded protein concentration was about 6×10^{-5} M, and the final concentration following mixing was about 10^{-5} M.

For times longer than 100 s, the base-line stability for stopped-flow mixing experiments is poor. To improve the accuracy of the absorbance-detected slow folding data, manual mixing experiments were performed in a Hewlett-Packard 8450A UV/Vis spectrophotometer. In a few instances, fluorescence-detected slow folding data were obtained by manual mixing. Dead times for manual mixing are estimated to be about 5 s. The same protein and buffer solutions were used for both stopped-flow and manual mixing kinetic experiments. All experiments were carried out at 20 ± 0.2 °C.

Data Analysis. Methods for analyzing kinetic data by peeling off exponential phases have been described previously (Nall & Landers, 1981; Nall, 1986; Ramdas & Nall, 1986). At high pH, folding to the nativelike form is followed by a slow conformational rearrangement to the nonnative alkaline form. This conformational change is strongly pH dependent and may be studied separate from folding by pH jumps in the absence of denaturants. The reaction is usually called the "alkaline isomerization", but we will refer to it as the alkaline conformational change in order to distinguish it from proline imide bond isomerization. The alkaline conformational change is characterized by large changes in heme absorbance with an amplitude opposite in sign to that of remaining (folding) kinetic phases (Nall, 1986). To focus on folding processes leading to the native (or nativelike) protein, absorbance changes due to the alkaline conformational change were subtracted from the data prior to analysis. The pH dependence of the rate of the alkaline conformational change and its relation to folding at high pH have been described elsewhere (Nall, 1986).

The data points in Figures 2 and 3 are averages of at least three measurements. The size of the symbols in the figures is of the order of the standard deviations. Errors, however, are largely systematic with the scatter of points from one set of conditions to another being slightly greater than the deviations for a fixed set of conditions. Significant differences in the rates of absorbance-detected and fluorescence-detected fast folding occur below pH 5–5.5 and above pH 8–8.5. Continuity of the data suggests small differences in rates of absorbance-detected and fluorescence-detected fast folding between pH 5.5 and pH 8, but such differences are too small to be verified by experiment.

Results

Slow Folding at Neutral and Alkaline pH. In Figure 1, the slow folding kinetics of Gdn·HCl-unfolded iso-2 are compared at pH 7.2 (Figure 1A,C) and pH 9.5 (Figure 1B,D). In the initial conditions, the protein is fully unfolded in the presence of 3.0 M Gdn·HCl. The final refolding conditions have been chosen so that refolding is complete (0.5 M Gdn·HCl, pH 7.2 or pH 9.5). Two optical probes of folding are monitored: heme absorbance changes at 418 nm (Figure 1A,B) and tryptophan fluorescence at 350 nm (Figure 1C,D). At neutral pH, slow folding occurs with the expected pattern: different kinetic phases are observed with the different probes,

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; iso-2, iso-2-cytochrome *c* from *Saccharomyces cerevisiae*; Thr-71 iso-2, mutant form of iso-2 in which proline-71 is replaced by threonine; τ, time constant of a reaction (reciprocal of the apparent rate constant); α, amplitude of a reaction expressed as the fraction of the total observable kinetic change associated with a particular time constant.

[2] Above pH 8, the Gdn·HCl concentration must be above 4 M before the (apparent) relative amplitude is independent of the initial pH. Below 4 M Gdn·HCl, large absorbance changes from isomerization of residual alkaline iso-2 interfere with measurement of the amplitude for slow folding (Osterhout et al., 1985).

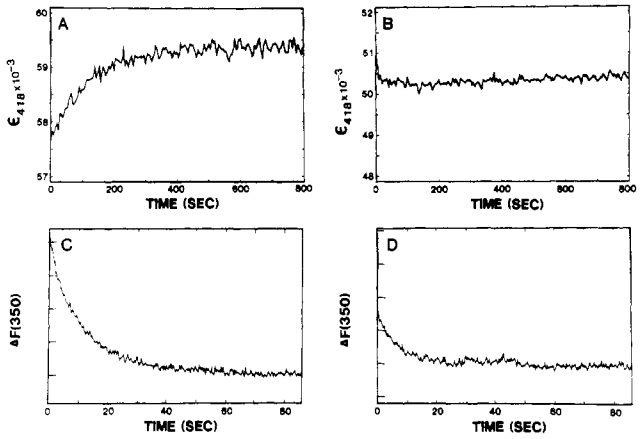

FIGURE 1: Absorbance- and fluorescence-detected slow folding phases at neutral and alkaline pH. The absorbance-detected slow phase in folding at pH 7.2 (A) is no longer detected for folding at pH 9.5 (B). The fluorescence-detected slow phase in folding at pH 7.2 (C) occurs with the same rate but with decreased amplitude at pH 9.5 (D). In the initial conditions, the protein is fully unfolded in 3.0 M Gdn·HCl, 0.1 M sodium phosphate, pH 7.2. Folding is initiated by 1:5 dilution with sodium phosphate buffer adjusted to give a final pH of 7.2 (A, C) or 9.5 (B, D) after mixing with the unfolded protein solution. Folding is monitored by absorbance (A, B) at 418 nm or by fluorescence (C, D) at 350 nm (excitation at 287 nm) at a final Gdn·HCl concentration of 0.5 M. Other conditions are temperature of 20 °C and final iso-2 concentration of 10^{-5} M.

absorbance-detected slow folding occurring on a 10–20-fold slower time scale than fluorescence-detected slow folding (Nall, 1983). Under these conditions (neutral pH), both slow reactions have many properties expected for folding involving proline imide isomerization (Osterhout & Nall, 1985). At alkaline pH, absorbance-detected slow folding is no longer observed (Figure 1B), and fluorescence-detected slow folding occurs with a much smaller amplitude (Figure 1D). Except for a proline flanked by amino acids with titrating side chains, the equilibrium and kinetic properties for imide bond isomerization are expected to be independent of pH (Grathwohl & Wuthrich, 1981).

Fluorescence-Detected Folding: pH Dependence of Rates and Amplitudes. In Figure 2, the relative amplitude (Figure 2A) and time constants (Figure 2B) for the fluorescence-detected fast and slow phases are plotted vs the final pH. In the initial conditions, the protein was fully unfolded (3.0 M Gdn·HCl, pH 7.2). With the exception of data near pH 3, refolding is complete in the final conditions (0.5 M Gdn·HCl, indicated pH). At pH 3 and 0.5 M Gdn·HCl, iso-2 has entered the Gdn·HCl-induced unfolding transition zone [See Osterhout et al. (1985)].

Over the pH range investigated, only two kinetic phases are detected by fluorescence. The slow phase (τ_{1b}) increases slightly in rate with increasing pH below pH 7 but is largely independent of pH between pH 7 and pH 10. The log of the fast folding time constant (τ_{2b}) shows a bell-shaped pH dependence, decreasing in rate with increasing pH between pH 4 and pH 7 and then increasing in rate above pH 7. The amplitude (α_{2b}) for the fast folding reaction increases from about 0.8 near pH 4 to nearly 0.95 by pH 10.

Absorbance-Detected Folding: pH Dependence of Rates and Amplitudes. Both fast and slow absorbance-detected folding phases exhibit a pH dependence that differs from that monitored by fluorescence. In Figure 3, the rate of absorbance-detected slow folding (τ_{1a}) is seen to be independent of pH between pH 3 and pH 5 but decreases in rate between pH 5 and pH 7. τ_{1a} appears to be independent of pH above pH 7. Accurate measurement of τ_{1a} is difficult above pH 7 since

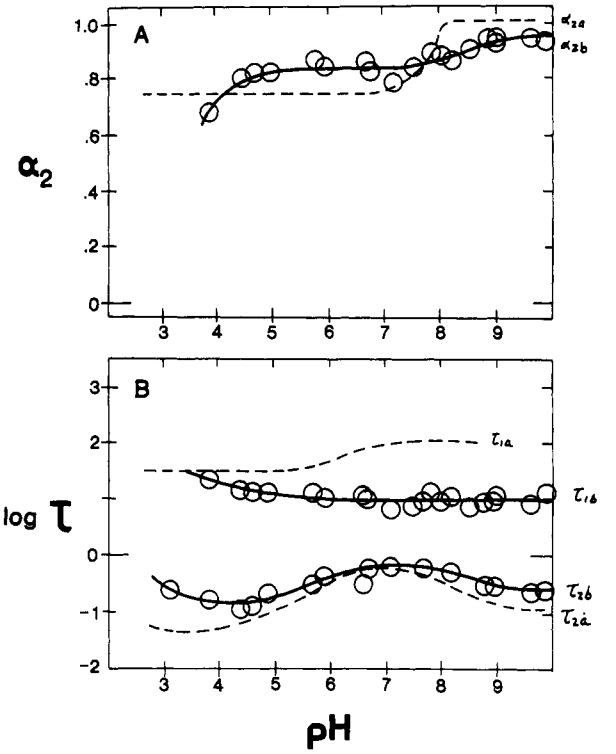

FIGURE 2: pH dependence of fluorescence-detected folding. The open circles (O) show the final pH dependence of (A) the relative amplitude (α_{2b}) for the fast folding reaction and (B) the time constants for the fast (τ_{2b}) and slow (τ_{1b}) kinetic phases in fluorescence-detected folding. The solid line (—) through the data points has no theoretical significance but is reproduced in Figure 3 for comparison. The dashed line (- - -) shows the relative amplitude and time constant behavior for absorbance-detected folding (Figure 3). In the initial conditions, iso-2 was fully unfolded in 3.0 M Gdn·HCl, 0.04 M sodium phosphate, pH 7.2, 20 °C. Refolding was initiated in a stopped-flow apparatus by mixing the unfolded protein solution at a 1:5 ratio with phosphate buffer so that the final refolding conditions were 0.5 M Gdn·HCl, 20 °C, and 0.091 M sodium phosphate. The pH of the dilution buffer was adjusted to give the indicated final pH after mixing with the unfolded protein solution. Fluorescence changes were monitored at 350 nm (excitation at 287 nm). The final protein concentration was about 10^{-5} M with an initial unfolded protein concentration 6-fold higher.

the amplitude associated with this reaction is decreasing rapidly. The log of the time constant for absorbance-detected fast folding (τ_{2a}) shows a bell-shaped pH dependence with a minimum in rate near pH 7. Although absorbance-detected fast folding (τ_{2a}) and fluorescence-detected fast folding (τ_{2b}) have the same rate near neutral pH, the rates differ in both the alkaline and acid pH regions, with absorbance-detected fast folding occurring on a more rapid time scale. (Compare solid and dashed lines in Figures 1 and 2.) The amplitude for absorbance-detected fast folding (α_{2a}) is largely independent of pH below pH 7. Above pH 8, absorbance-detected slow folding is no longer observed, and only fast folding phases are detected in the stopped-flow time range.

Discussion

Two strategies have been used to define the number and characteristics of kinetically distinct species in protein folding (Utiyama & Baldwin, 1986): (1) different probes have been used to monitor folding to distinguish between species differing in physical properties; (2) the initial (or final) conditions of the kinetic experiments have been held constant so that changes in the kinetic pattern can be attributed unambiguously to changes in species in the final (or initial) conditions (Labhardt & Baldwin, 1979). Previous work with iso-2-cytochrome *c*

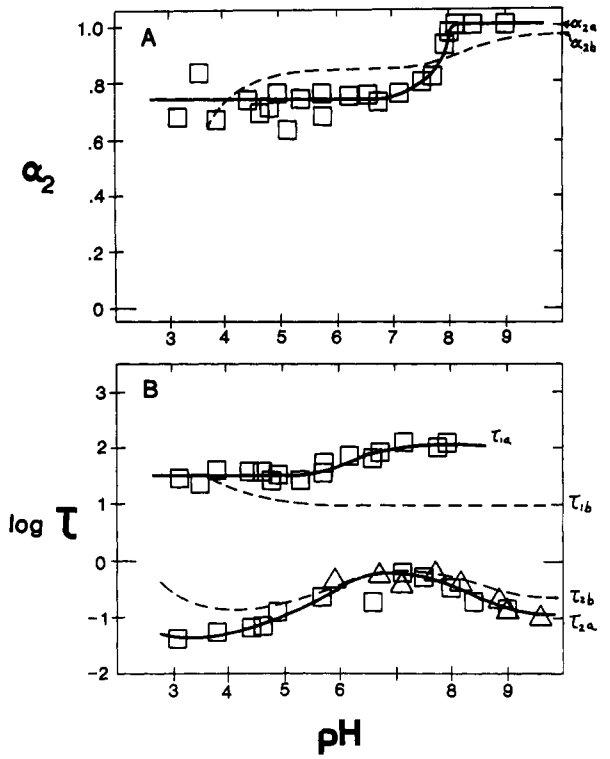

FIGURE 3: pH dependence of absorbance-detected folding. The final pH dependence is given for (A) the relative amplitude (α_{2a}) of the fast phase and (B) the time constants for the fast (τ_{2a}) and slow (τ_{1a}) phases in refolding. Folding is monitored by the absorbance at 418 (△) and 287 nm (□). The solid line (—) through the data points has no theoretical significance but is reproduced in Figure 2 for comparison. The dashed line (- - -) gives the final pH dependence of the relative amplitude and time constants for fluorescence-detected refolding (Figure 2). Other conditions are given in Figure 2.

showed that, at sufficiently high Gdn·HCl concentration, the kinetic properties of folding to a fixed final state are independent of the initial pH of the unfolded protein (Osterhout & Nall, 1985). Thus, the distribution between kinetically distinct species present in the unfolded protein is independent of pH. The present experiments in which the initial unfolding conditions are held constant and the final conditions varied test for pH-dependent changes in the distribution of species formed in the final refolding conditions.

New Species Detected in Fast Folding at Acid and Alkaline pH. At neutral pH, numerous probes of protein conformation detect the same fast folding rate process throughout the folding/unfolding transition zone (Nall & Landers, 1981; Nall, 1983). Probes used include ultraviolet absorbance, visible absorbance, 350-nm fluorescence, 695-nm absorbance, and reaction with ascorbate. Since different probes measured the same fast folding rate at neutral pH and since this phase produced the native conformation (i.e., a molecule reducible by ascorbate), it was generally held that cytochrome *c* attained its native conformation in a fast phase in a single highly cooperative process.[3] The current experiments (Figures 2 and 3) show that in the acid and alkaline pH ranges the absorbance-detected and fluorescence-detected fast phases are no longer coincident, indicating the involvement of additional kinetic species. Fits of the data to single-exponential functions are good. The fast phases, however, may be composed of unresolved, kinetically distinct species. If so, the kinetic measurements give average rates for folding of fluorescence-detected or absorbance-detected subsets of species.

pH Dependence of Folding. The dependence of the rates for both fluorescence- and absorbance-detected fast folding is similar with minima near pH 7. Increases in rate away from the minima suggest involvement of histidine or carboxylic acid groups at low pH and lysine or tyrosine at high pH. These ionizable groups may increase folding rates by stabilizing structural intermediates which facilitate folding. Heme ligand exchange might also be involved. Since the low-spin heme of the unfolded protein has intrinsic protein ligands, probably neutral histidines, at the fifth and sixth positions (Babul & Stellwagen, 1971), at some stage in the folding process Met-80 will be required to displace a heme ligand. Protonation of the imidazole nitrogen precludes involvement in an iron–nitrogen ligand bond. Thus, on the acid side of neutrality, protonation could facilitate histidine displacement by Met-80 and catalyze folding.

The rate of fluorescence-detected slow folding shows a 2.5-fold increase in rate as the pH is increased from pH 4 to pH 6 and is constant above pH 6. Rate changes in the pH 4–6 region might indicate involvement of side chain carboxyl groups of the propionic acid constituents of the heme. Such behavior is to be expected for kinetic processes dominated by intrinsically slow events like proline imide bond isomerization with weak coupling to nearby ionizing side chains or pH-dependent secondary structure. At high pH (pH 6–10), there is a significant decrease in the relative amplitude for fluorescence-detected slow folding, suggesting either a decrease in the population of these species or a smaller (relative to fast folding) change in fluorescence, possibly due to quenching.

pH-dependent changes in the rate of absorbance-detected slow folding are modest: about 4-fold over the entire pH range, with the faster rates in the acid region. The log of the time constant titrates in a sigmoidal manner with bends near pH 5.5 and pH 6.5 suggesting involvement of one or more histidine side chains, possibly with coupling to some intrinsically slow process such as imide isomerization. Between pH 7 and 8, the amplitude for absorbance-detected slow folding decreases to zero. Thus, at alkaline pH, either the absorbance changes are no longer coupled to slow folding or a competing faster pathway has emerged.

Does Slow Folding Involve Protein Conformational Changes, Proline Isomerization, or Both? Previous experiments demonstrated that both slow refolding phases have properties indicating the involvement of proline imide bond isomerization (Osterhout & Nall, 1985). Here we show that at alkaline pH the fluorescence-detected slow refolding phase is diminished and the absorbance-detected slow phase is lost completely (Figures 2 and 3). If prolines generate the slow phases, these results require either that at alkaline pH the optical response is uncoupled from proline isomerization (proline isomerization is still required to attain the final conformation but is not observed spectrally) or that the structure of alkaline cytochrome *c* is indifferent to the isomerization state of the proline.[4] The same reasoning can be

[3] Temperature jump experiments at pH 7.2 have detected an additional fast phase with a time constant of about 1 ms (Nall & Landers, 1981). This reaction can be observed by Soret changes but either is absent or is below the level of detection for temperature jumps monitoring ultraviolet absorbance or tryptophan fluorescence (Nall, 1983). The insensitivity of the rate to Gdn·HCl concentration suggests that this reaction is likely to involve perturbation of a heme spin state equilibrium in either the folded (Dyson & Beattie, 1982; Robinson et al., 1983) or the unfolded (Tsong, 1975, 1977) protein.

[4] Preliminary results support the latter possibility. Double-jump experiments suggest that folded, alkaline iso-2 is composed of at least two sets of species which, when rapidly unfolded, have distinct, absorbance-detected refolding rates at neutral pH (L. Ramdas, unpublished experiments).

used to explain the absence of absorbance-detected slow folding in a Pro-71 to Thr-71[5] mutant form of iso-2 on folding to a nonnative (alkalinelike) form at neutral pH. As with the normal protein, the slow phase returns on folding to a more nativelike conformation at slightly lower pH (White et al., 1987). Changes in the state of heme ligation may be linked to proline isomerization and changes in kinetic properties. Brems and Stellwagen (1983) observe the loss of certain slow kinetic phases in folding of horse and tuna cytochromes *c* under conditions (pH or presence of extrinsic heme ligands) affecting heme ligation.

Summary. Proline imide isomerization is probably involved in both absorbance- and fluorescence-detected slow folding at neutral pH. However, the amplitudes and rates for folding of iso-2-cytochrome *c* depend on the pH at which refolding occurs, indicating involvement of titratable groups in rate-limiting steps and/or stabilization of folding intermediates. At alkaline pH, absorbance-detected folding occurs entirely on a fast time scale, suggesting that structures formed during folding are insensitive to slow proline imide isomerization. Fluorescence-detected folding at alkaline pH still involves a slow process in a time range appropriate to imide isomerization, but a decrease in amplitude indicates either that the slow fluorescence changes are selectively quenched or that a reduced fraction of species fold via the slow fluorescence-detected pathway.

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[5] 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-2 has nine additional amino-terminal residues and one residue less on the carboxy terminus compared to vertebrate cytochromes *c*. Thus, the numbering of iso-2 starts at position −9 and extends to position 103 [see Dickerson (1972) and Hampsey et al. (1986)]. For example, Pro-71 in the vertebrate numbering system corresponds to Pro-80 in the iso-2 numbering system.