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Native or Nativelike Species Are Transient Intermediates in Folding of Alkaline Iso-2 Cytochrome *c*†

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ABSTRACT: Titration to high pH converts yeast iso-2 cytochrome *c* to an inactive but more stable alkaline form lacking a 695-nm absorbance band [Osterhout, J. J., Jr., Muthukrishnan, K., & Nall, B. T. (1985) *Biochemistry 24*, 6680–6684]. The kinetics of absorbance-detected refolding of the alkaline form have been measured by dilution of guanidine hydrochloride in a stopped-flow instrument. Fast-folding species (τ_2) are detected, as in refolding to the native state at neutral pH. An additional kinetic phase (τ_a) is observed with an amplitude opposite in sign to the fast phase. The amplitude of this phase increases and the rate increases with increasing pH. Comparison to pH-jump measurements of the fully folded protein shows that phase τ_a has the same sign, rate, and pH dependence as the alkaline isomerization reaction, suggesting that this new phase involves isomerization of native or nativelike species following fast folding. Absorbance difference spectra are taken at 5-s intervals during refolding at high pH. The spectra verify that nativelike species—with a 695-nm absorbance band—are formed transiently, before conversion of the protein to the alkaline form. Refolding in the presence of ascorbate shows that the transient, nativelike species are reducible, unlike alkaline iso-2. Thus, (1) refolding to the alkaline form of iso-2 cytochrome *c* proceeds through transient native or nativelike species, and (2) a folding pathway leading to native or nativelike forms is maintained at high pH, where native species are no longer the thermodynamically favored product. For the latter stages of folding, the results argue against growth and merge assembly of structure found *only* in the thermodynamically favored product [Harrison, S. C., & Durbin, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4028–4030] and suggest the existence of unique species which direct folding—perhaps along a sequential pathway [Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489].

Harrison and Durbin (1985) have proposed that protein folding is analogous to putting together a jigsaw puzzle: assembly can be initiated from any element of local structure and proceeds by a "growth and merge mechanism" (Karplus & Weaver, 1976) where the participants (elements of local structure, i.e., puzzle pieces) are limited to aspects of local structure found in the thermodynamically determined product of refolding. The stability of the product determines the folding process in that the structure of intermediates is limited exclusively to local structure or substructures found in the thermodynamic result of the refolding process. Thus, folding proceeds directly to the thermodynamically most stable state.

In contrast, Kim and Baldwin (1982) propose that folding is a sequential process involving a unique series of intermediates which direct folding along the most expedient route to the thermodynamically favored product. The structure of sequential intermediates serves to facilitate folding, but this structure may or may not be related to the structure found in the product of folding.

These two proposals are at opposite extremes. Either might be favored by evolution: the puzzle model by providing a highly adaptable folding process as a hedge against point mutations that lead to polypeptides unable to fold (Harrison & Durbin, 1985), sequential folding by charting a pathway through an otherwise uncountable number of possible conformations (Levinthal, 1968). Refolding processes for particular proteins may exhibit properties of both models, perhaps involving a growth and merge mechanism early on, followed by a sequential pathway in the latter, more highly restricted stages of folding.

The jigsaw puzzle model can be tested for proteins that take on different states under different conditions: the "native" form under physiological conditions and a folded but conformationally distinct "isomeric" form under some other conditions. Cytochrome *c* is such a protein: native and alkaline isomeric forms have been described for both horse (Greenwood & Palmer, 1965; Brandt et al., 1966; Davis et al., 1974) and yeast iso-2 cytochrome *c* (Osterhout et al., 1985). The native (neutral pH) form has a 695-nm absorbance band and is reducible by ascorbic acid while the alkaline (pH 9–10) form

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lacks both of these properties. At intermediate pH, the two forms coexist in slow equilibrium with one another. Thus, one can ask whether refolding of iso-2 cytochrome *c* at high pH involves intermediate states with structure found *only* in the alkaline form as demanded by the jigsaw puzzle model or instead passes through intermediate states with native or (nativelike) interactions before conversion to the alkaline form.

Materials and Methods

Growth of yeast (*Saccharomyces cerevisiae*) and purification of iso-2 cytochrome *c* have been described previously as have methods for kinetic measurements and data analysis (Nall & Landers, 1981; Nall, 1983). The final conditions for refolding experiments are 0.1 M sodium phosphate buffer and 0.5 M guanidine hydrochloride (Gdn·HCl).[1] The initial conditions were unbuffered or lightly buffered (0.02 M sodium phosphate) and contained 3.0 or 5.0 M Gdn·HCl. Refolding was induced by Gdn·HCl dilution using manual or stopped-flow mixing with ratios of 1:5 or 1:9 (protein solution to buffer). Buffer pH was adjusted with H_3PO_4 or NaOH. For Gdn·HCl dilution experiments, the final pH was determined for solutions after mixing.

The rate of the alkaline isomerization reaction was determined by pH jumps in the presence of 0.5 M Gdn·HCl and 0.1 M sodium phosphate, the same final conditions as for measurements of refolding rates. To measure rates over a broad (final) pH range, several initial pHs were used. For measurements at low final pH, the initial pH of a lightly buffered protein solution (0.02 M sodium phosphate) was adjusted to pH 9.3 or 7.2. The initial pH was 6.0 or 7.2 for a high final pH. For a final pH of 7.2, the initial pH was 5.0. Rates were independent of initial pH. Protein solutions were mixed in a 1:5 ratio with a mixing buffer containing 0.1 M sodium phosphate. The pH of the mixing buffer was adjusted to give the indicated final pH.

Measurement of ascorbate reducibility was by the absorbance at 550 nm with final conditions of 0.1 M sodium phosphate, pH 8.6, 0.5 M Gdn·HCl, and 5×10^{-4} to 9×10^{-2} M ascorbate. Initial conditions were 0.1 M sodium phosphate, pH 8.6, and 0.5 or 5.0 M Gdn·HCl.

The time constants were determined by comparing digitized kinetic traces to trial functions of the form $\Delta A(t) = A + B \exp[-t/\tau_1] + C \exp[-t/\tau_2] + \ldots$. For the refolding conditions reported here (within the noise and artifact levels inherent in stopped-flow experiments), a two-exponential function provided an excellent fit to the data. For other refolding conditions (pH 7.5–8) where both the alkaline folding reaction (τ_a) and the (neutral pH) slow-folding reaction (τ_1) are detectable, three-exponential terms are needed to account for the fast-phase folding (τ_2) and the two slower phases (B. T. Nall, unpublished results). A single exponential provided an adequate fit for rates of alkaline isomerization of the folded protein. Other procedures relating to instrumentation and data analysis have been described previously (Nall & Landers, 1981; Nall, 1983).

The time dependence of refolding of alkaline iso-2 was monitored by UV–visible difference spectra (Figure 3) taken over a 200–800-nm range with a HP 8450A (diode array) spectrophotometer. On initiation of refolding by manual mixing, absolute spectra were taken at 5-s intervals. A reference spectrum of the same solutions used in obtaining the time-dependent spectra was taken long after kinetic changes had ceased (5 min after initiation of the reaction). The reference spectrum was digitally subtracted from each of the preceding (time-dependent) spectra to generate the time-dependent difference spectra of Figure 3. This procedure is preferable to referencing the time-dependent spectra to a separately prepared reference solution since small systematic errors involving mismatched cuvettes and/or solutions are eliminated.

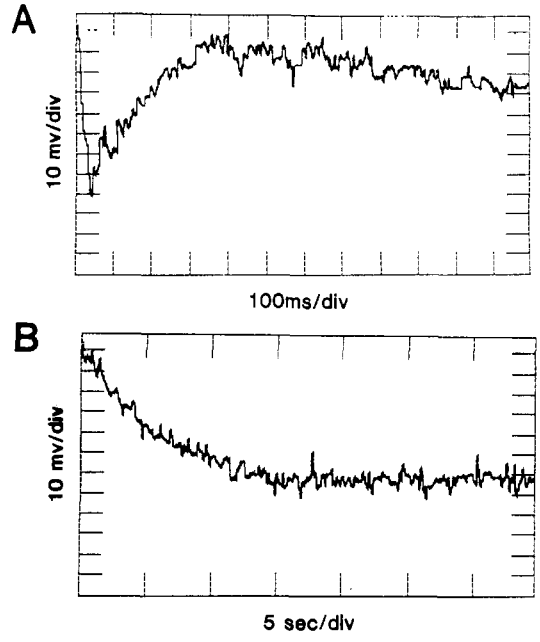

FIGURE 1: Fast (A) and slow (B) kinetic phases detected on refolding iso-2 cytochrome *c* to the alkaline conformation at high pH. Iso-2 is completely unfolded in the initial unfolding conditions: pH 7.2, 0.1 M sodium phosphate, 3.0 M Gdn·HCl. Refolding is induced by stopped-flow dilution using a 1:5 mixing ratio such that complete refolding occurs in 0.5 M Gdn·HCl and 0.1 M sodium phosphate, pH 9.0. Absorbance changes are monitored at 418 nm. Temperature is 20 °C. The final protein concentration is 5×10^{-6} M.

Final protein concentrations were 5×10^{-6} M for fluorescence or absorbance at 418 nm, 30×10^{-6} M for absorbance at 287 nm, 10^{-5} M for ascorbate reducibility, and 44×10^{-6} to 148×10^{-6} M for absorbance in the 695-nm region. Initial protein concentrations were 6–10-fold higher. All measurements were at 20 °C.

Results

Refolding Kinetics of Alkaline Iso-2 Cytochrome c. In Figure 1, the kinetics of absorbance-detected refolding of alkaline iso-2 are shown. In the initial conditions, 3.0 M Gdn·HCl, pH 7.2, the protein is in a random-coil state as judged by ^{1}H NMR spectroscopy. At equilibrium in the final refolding conditions (0.5 M Gdn·HCl, pH 9), the protein is fully folded and about 91% in the inactive alkaline form (Osterhout et al., 1985). The fast kinetic phase, $\tau_2 = 0.137$ s, is the same fast-folding phase observed at neutral pH (Nall & Landers, 1981; Nall, 1983) as judged from the known pH dependence of τ_2 (B. T. Nall, unpublished results). Surprisingly, the fast phase is followed by a slower phase, $\tau_a = 5.7$ s, with an amplitude of opposite sign and comparable magnitude. Slow-folding phases detected at neutral pH (Nall & Landers, 1981; Nall, 1983; Osterhout & Nall, 1985) have smaller amplitudes of the same sign as phase τ_2.

pH Dependence of the Alkaline Isomerization of Iso-2. On titration to high pH, native iso-2 is converted to a folded but inactive alkaline form which lacks the 695-nm absorbance

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; iso-2, iso-2 cytochrome *c* from *Saccharomyces cerevisiae*; τ, time constant of a reaction (reciprocal of the apparent rate constant); NMR, nuclear magnetic resonance.

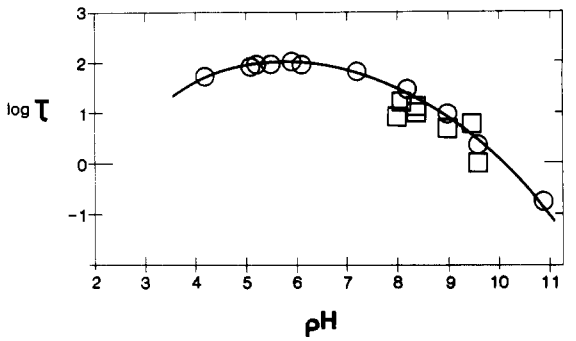

FIGURE 2: pH dependence of the time constants for the alkaline isomerization of the folded protein (○) and for the terminal kinetic phase (τ_a) in refolding to alkaline iso-2 (□). Measurements of the rate of the alkaline isomerization reaction (monitoring absorbance changes at 418 nm) are carried out by pH jumps in the presence of 0.5 M Gdn·HCl. The initial conditions are 0.02 M sodium phosphate, pH 5.0, 6.0, 7.2, or 9.3 depending on the final pH (see Materials and Methods). Final conditions are 0.08 M sodium phosphate and the indicated pH. The initial conditions for refolding experiments are 0.02 M sodium phosphate, pH 7.2, and 3.0 M Gdn·HCl. Refolding is induced by stopped-flow mixing at a 1:5 ratio (protein solution to buffer), giving final conditions with 0.5 M Gdn·HCl, 0.08 M sodium phosphate, and the indicated final pH. Refolding is monitored by absorbance changes at 287 nm. All measurements are made at 20 °C.

band of the native protein (Osterhout et al., 1985). This is the alkaline isomerization reaction which has been thoroughly investigated in homologous cytochrome *c* from horse (Greenwood & Palmer, 1965; Brandt et al., 1966; Gupta & Koenig, 1971; Davis et al., 1974). The kinetics of the alkaline isomerization may be described by a single, pH-dependent time constant. Figure 2 shows the pH dependence of the time constant for the alkaline isomerization reaction of iso-2 and also shows τ_a measured in refolding at alkaline pH. At high pH, the time constants for the alkaline isomerization and for τ_a (measured in refolding) are the same and have the same pH dependence. Furthermore, the amplitudes of the slow phase detected in refolding at alkaline pH (Figure 1B) and the alkaline isomerization reaction have the same sign (not shown). This suggests that these two reactions may involve the same species and the same kinetic steps.

Spectral Changes in Refolding at Alkaline pH. If τ_a measured in refolding (Figure 1B) is identified with the alkaline isomerization reaction, then native rather than alkaline species are a product of fast folding (Figure 1A). As a test, difference spectra from 230 to 800 nm have been obtained at 5-s intervals for refolding to what is predominantly the alkaline form at pH 8.6 (Figure 3A,B). Under these conditions, τ_a is 11 s. The shift in the Soret absorbance band near 410 nm (Figure 3A) and the decreasing intensity of the 695-nm band (Figure 3B) are both consistent with transient formation of species with the absorbance properties of the native protein prior to refolding/isomerization to the alkaline state.

Refolding in the Presence of Ascorbate. Greenwood & Palmer (1965) have shown that the alkaline form of horse cytochrome *c* is not reducible by ascorbate, while the native form of the protein reacts readily. This allows an assay for the fraction of unfolded alkaline iso-2 refolding through transient, ascorbate-reducible species. When alkaline iso-2 refolds in the presence of high concentrations of ascorbate, nativelike species produced in the fast phase (Figure 1A) will be trapped in the (more stable) reduced state, while species folding directly to the alkaline form will be reduced slowly at a rate limited by the alkaline isomerization reaction. At ascorbate concentrations in excess of 5 mM, the slow reduction reaction at alkaline pH is well described by a single-exponential

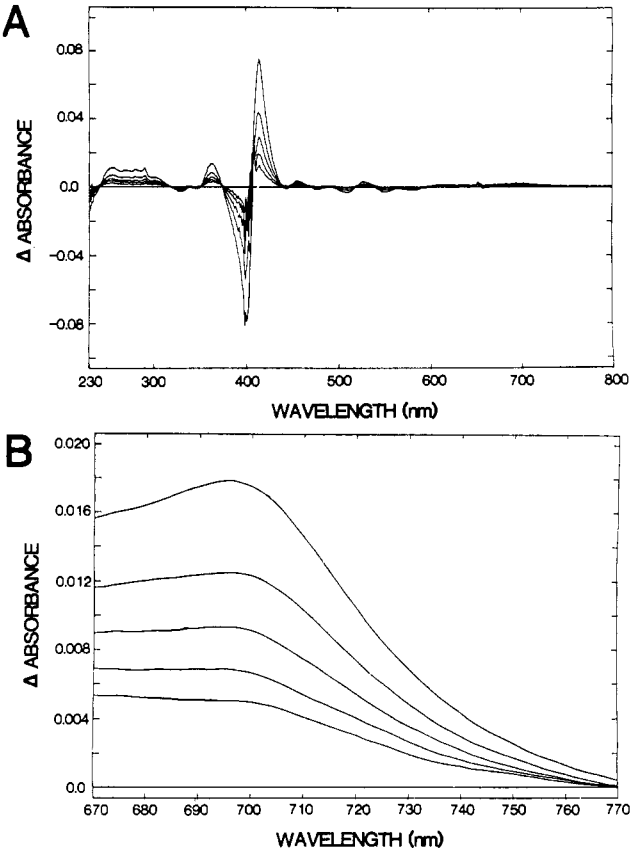

FIGURE 3: UV–visible difference spectra taken during refolding to alkaline iso-2 cytochrome *c*. Spectra are taken at 5-s intervals and are averaged for 5 s each. In order of decreasing spectral differences, the (difference) spectra were taken at 5, 10, 15, 20, and 25 s after initiation of refolding. Differences are with respect to a final spectrum taken after all detectable kinetic changes have ceased—5 min after initiation of refolding. Initial unfolding conditions are 3.0 M Gdn·HCl and 0.02 M sodium phosphate, pH 6.0. Refolding is induced by 1:5 dilution with buffer, giving final conditions of 0.5 M Gdn·HCl and 0.08 M sodium phosphate, pH 8.6. In (A), spectral changes are shown in the UV and Soret regions at a final protein concentration of 17 $\times 10^{-6}$ M. In (B), spectral changes in the vicinity of the 695-nm band are given [absorbance at 695 nm is a measure of Met-80 ligation of the heme (Schechter & Saludjian, 1976)] at a final protein concentration of 148 $\times 10^{-6}$ M. Similar results are obtained for refolding to the same final conditions from initial conditions of 5.0 Gdn·HCl, pH 8.6 (using a 1:9 mixing ratio). All measurements are at 20 °C.

decay and is largely independent of ascorbate concentration (data not shown). The fast, bimolecular reduction reaction is complete within the mixing dead time (about 7.5 s). In Table I, the fraction of total species which pass through an ascorbate-reducible, nativelike state is given for refolding of iso-2 at alkaline pH. A 0.65 fraction of the unfolded iso-2 species refolds through a reducible state and is trapped before conversion to the alkaline form. When fully folded iso-2 is mixed with ascorbate, 0.21 of the species is rapidly reduced compared to an expected 0.20 fraction of native species present at equilibrium at pH 8.6.

Discussion

Folding to Alkaline Iso-2 Proceeds through Native or Nativelike Species. Two kinds of experimental evidence provide a strong case for transient population of native (or nativelike) species in folding to alkaline iso-2. First, refolding of the fully unfolded protein involves formation followed by disappearance of species with the spectral properties and reducibility of the native protein (Figure 3A,B; Table I). In particular, the species present following fast folding have a

Table I: Assay for Reducible Nativelike Species in Refolding to Alkaline Iso-2[a]

initial state[a]	fraction reduced before conversion to alkaline iso-2,[b,c] $1 - \alpha$
folded control,[c] 0.5 M Gdn·HCl, 0.02 M sodium phosphate, pH 8.6	0.21
unfolded,[c] 5.0 M Gdn·HCl, 0.02 M sodium phosphate, pH 8.6	0.65

[a] Two experiments are performed which differ in the initial state of the protein. For the "folded control", folded protein at alkaline pH is mixed with ascorbate as an assay for residual native species. For the "unfolded" initial state, induction of refolding (by Gdn·HCl dilution) and mixing with ascorbate are carried out simultaneously to assay for (reducible) nativelike species formed in the process of refolding. For both initial states, the final conditions are the same: 0.5 M Gdn·HCl, 90 mM ascorbate, and 0.08 M sodium phosphate, pH 8.6. [b] In the presence of high concentrations of ascorbic acid, alkaline species are converted to reduced species in a slow reaction with a rate limited by the alkaline isomerization reaction. The rate of this reaction is largely independent of ascorbate concentration (Greenwood & Palmer, 1965; also see text). Time constants for this reaction are the same (within errors) for the "folded control" and the "unfolded" protein: 43 and 64 s, respectively. The amplitude (α) of the reaction is a measure of the fraction of species in the alkaline form before mixing with ascorbic acid (for protein initially in a folded state) or which fold to the alkaline form before being reduced (for protein initially in an unfolded state). $\alpha = \Delta\epsilon^0(550)/\Delta\epsilon^{eq}(550)$ where $\Delta\epsilon^0(550)$ and $\Delta\epsilon^{eq}(550)$ are kinetic and equilibrium changes in molar extinction at 550 nm, respectively. The kinetic changes are those associated with the terminal, pseudo-first-order phase in reduction. Since all species end up in the reduced form at equilibrium, the quantity reported in Table I (fraction reduced before conversion to alkaline iso-2) is obtained by difference and is equal to $1 - \alpha$. [c] For protein starting in a folded state at high pH, $1 - \alpha$ is a measure of the equilibrium fraction of iso-2 in the native form which is reduced rapidly on mixing with ascorbate. The fraction 0.21 compares favorably to the value 0.20 at pH 8.6 calculated from the apparent pK = 8 for the alkaline isomerization reaction (Osterhout et al., 1985). For protein initially in an unfolded state, $1 - \alpha$ measures the fraction of transient nativelike species reduced by ascorbate after initiation of refolding but before conversion to alkaline iso-2.

695-nm absorbance band (Figure 3B) indicative of methionine ligation of the heme (Schechter & Saludjian, 1976) and the native conformation of cytochrome *c*. Second, these same species disappear slowly at a rate indistinguishable from the rate of the alkaline isomerization reaction of the folded protein, suggesting that the kinetic barrier for the final step in folding to alkaline iso-2 is the same as for the alkaline isomerization of the folded protein. The simplest explanation for the fact that these two reactions have the same rate is that they involve similar reactants, native or nativelike species, being converted to the same product, alkaline iso-2.

Most Unfolded Species Fold through the Nativelike State. The amplitude of the slow phase in refolding may be used to estimate the fraction of total species passing through a native (or nativelike) state in refolding of alkaline iso-2. The first spectrum in Figure 3B, when corrected for a dead time of 7.5 s (5 s mixing plus 2.5 s for the 5 s of time averaging for each spectrum), gives a difference in the molar extinction coefficient indicating that 65% of the species pass through a state with a 695-nm absorbance band. Refolding in the presence of ascorbate (Table I) also shows that 65% of the species pass through a nativelike (reducible) state. The agreement[2] between these two different ways of measuring the fraction of transient nativelike species argues against the alternative interpretation that 100% of the protein folds through a state with partial native properties.

[2] The numerical agreement is probably fortuitous since both methods of measuring the fraction of species passing through the transient nativelike state have estimated errors of ±20%.

Structural Differences of Alkaline and Native Iso-2. The significance of the present results depends on the degree in which native iso-2 and alkaline iso-2 differ in structure. Although the structure of alkaline cytochrome *c* is not known, it is likely that it differs extensively from that of the native protein. The alkaline transition of iso-2 has an apparent pK near 8 (Osterhout et al., 1985) and involves loss of the 695-nm absorbance band believed to be due to methionine ligation of the heme (Schechter & Saludjian, 1976). Consistent with loss of the Met-80 ligand is the disappearance of the paramagnetically shifted methyl signal from Met-80 in the proton nuclear magnetic resonance spectrum of iso-2 on titration to high pH (K. Muthukrishnan and B. T. Nall, unpublished results).[3] Similar properties for the alkaline transition of horse cytochrome *c* have been reported (Greenwood & Palmer, 1965; Redfield & Gupta, 1971) although the pK of the transition for the protein from horse is somewhat higher. Since both optical and NMR properties of alkaline cytochrome *c* show that the heme remains low spin, Met-80 must be replaced by a new strong field ligand, probably Lys-72 or Lys-79 (Hettinger & Harbury, 1964; Gupta & Koenig, 1971; Davis et al., 1974; Wilgus & Stellwagen, 1974; Smith & Millett, 1980). On the basis of the X-ray structure of tuna cytochrome *c*, Takano and Dickerson (1981) have proposed a molecular mechanism for the initial stages of the alkaline isomerization involving the participation of two buried water molecules. One of the buried water molecules weakens the sulfur–Fe(III) bond while (at high pH) the other loses a proton and initiates the alkaline conformational change by displacing the Met-80 ligand. Subsequent replacement of the hydroxyl ion by either Lys-72 or Lys-79 requires a drastic rearrangement of the structure on the left side of the heme (Takano & Dickerson, 1981).

Pathway for Protein Folding? Sequential folding involves a "unique and definite sequence of steps": a folding pathway (Kim & Baldwin, 1982). Sequential folding requires specific, well-defined structures with definite relationships to one another, but with no restrictions on the nature of the structure. The structure of sequential intermediates may be related to that found in the product of folding but can be unique to the process of folding. Complete disruption of the structure of a sequential intermediate (by mutation or by changes in refolding conditions) leads to polypeptides unable to fold or blocked at a specific stage in folding.

The jigsaw model rejects the idea of pathways. Folding is a random process, as is the assembly of puzzles. Pieces may be inserted in varying order, but partially assembled puzzles are required to reflect "aspects" of the completed picture: structural intermediates in folding are composed exclusively from local structure or "substructures" found in the product of the refolding process (Harrison & Durbin, 1985). Puzzle intermediates are not related to one another in a temporal sense but instead share in the structure of the thermodynamically favored product. The *only* substructures (pieces of puzzle) allowed in intermediate states are those found in the resulting folded state. Unique substructures required for the process but not the product of folding are forbidden. The essence of the puzzle model is adaptability (in response to changes in conditions, or mutations), which arises from a largely random assembly process.

[3] In addition to loss of the Met-80 methyl signal, other major differences in the proton NMR spectrum are observed as iso-2 is titrated to high pH. The extent to which the differences in the NMR spectrum (at neutral vs. alkaline pH) reflect changes in structure or changes in paramagnetic properties (e.g., electron *g* tensor) is unknown.

Folding of iso-2 at high pH proceeds by passing through a state that takes on (nativelike) structure absent in alkaline iso-2. Thus, our results violate one of the principal tenets of the puzzle model: that structure found in intermediates be a subset of structure in the thermodynamically favored product of folding. Our results do not prove that the entire folding process follows a sequential pathway but do show that the final stages of folding to either native or alkaline iso-2 are dominated by native or nativelike species.

Conclusions

Folding to the alkaline form of iso-2 cytochrome *c* occurs largely through native or nativelike species under conditions where the nonnative alkaline structure is the thermodynamically favored product. This shows that nativelike species play a major role in directing the final stages of folding to a nonnative state.

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Registry No. Iso-2, 9007-43-6.

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Identification of a Novel Serum Protein Secreted by Lung Carcinoma Cells

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ABSTRACT: The murine anti-human lung tumor monoclonal antibody L3 recognizes antigens found both in the medium of cultured carcinoma cells and in normal human serum. Sequential immunoprecipitation experiments indicate that the L3 antigen is also recognized by a previously described monoclonal antibody directed against a melanoma-associated antigen [Natali, P. G., Wilson, B. S., Imai, K., Bigotti, A., & Ferrone, S. (1982) *Cancer Res. 42*, 583–589]. This antibody precipitated a M_r 76 000 glycoprotein from metabolically labeled extracts of the lung carcinoma cell line Calu-1 and a M_r 94 000 glycoprotein from labeled culture medium. Pulse–chase experiments suggested a precursor–product relationship between these molecules. Analysis of glycosidase sensitivities of the two forms indicated that maturation of carbohydrate side chains correlated with the apparent increase in molecular weights. L3 antigenic activity, measured in a competitive radiometric cell binding assay, was purified more than 90-fold from serum-free medium of Calu-1 cells and more than 3000-fold from normal human serum. The major immunoreactive components purified from culture medium and serum were identical with respect to apparent molecular weight, electrophoretic mobility, p*I*, glycosidase sensitivity, and V8 protease fingerprints. In addition, the sequence of the amino-terminal 16 N-terminal amino acid residues of the major immunoreactive species from both sources was identical. The properties of the L3 antigen did not correspond to those of any known protein, suggesting that this serum protein has not been previously characterized.

The immunological detection of tumor-associated antigens in patient serum can provide clinically useful data for monitoring malignant disease. α-Fetoprotein, an embryonic analogue of serum albumin, is diagnostic for hepatic tumors when found at elevated levels in adult serum, and serum levels of carcinoembryonic antigen (CEA) are widely used to monitor

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