Changes in Conformation and Slow Refolding Kinetics in Mutant Iso-2-cytochrome *c* with Replacement of a Conserved Proline Residue†

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ABSTRACT: Using oligonucleotide-directed mutagenesis, we have produced a mutant form of iso-2-cytochrome *c* of yeast in which threonine (Thr-71) replaces a conserved proline residue (Pro-71) located between two short α-helical segments in the native protein. Optical spectroscopy indicates that, at pH 7.2, Thr-71 iso-2-cytochrome *c* folds to a nonnative conformation possibly related to the alkaline form of the native protein. On titration to pH 5.2, Thr-71 iso-2-cytochrome *c* regains many of the optical properties of the normal protein. We have shown that the proline residue at position 71 has no effect on the kinetics of fluorescence-detected slow refolding. However, between pH 5 and pH 7.2 the amplitude for absorbance-detected slow folding is strongly pH dependent in the mutant protein but is largely independent of pH in the normal protein. We believe this to be due to the folding of Thr-71 iso-2-cytochrome *c* to a nonnative conformation at pH 7.2 that does not require the slow, absorbance-detected conformational changes observed in folding to the more native-like state at pH 5–6.

The amino acid sequence of a protein determines its three-dimensional structure in a manner that is currently unknown. In order to understand the contribution of specific amino acids to the process of structure formation, it is necessary to identify the effects of single amino acid replacements on specific kinetic phases in protein folding. Similarly, the contribution of specific amino acids to the stability of the native protein can be determined by comparing equilibrium unfolding transitions for point mutant and wild-type forms of a protein. In this report, we present both types of data in a partial analysis of the involvement of proline-71[1] in the tertiary structure formation of iso-2-cytochrome *c* (iso-2)[2] from the yeast *Saccharomyces cerevisiae*.

Proline residues are believed to play an important role in tertiary structure formation. Imide bond isomerization, for example, has been proposed to cause a kinetic block in protein folding (Brandts et al., 1975). Experiments with ribonuclease A have shown that equilibration between the fast and slow folding portions of an unfolded population can be catalyzed by strong acid, is independent of urea or guanidine hydrochloride concentrations, and has an activation enthalpy of 21 kcal/mol (Schmid & Baldwin, 1978; Schmid et al., 1984). These properties are those to be expected if the equilibration were governed by imide isomerization. In addition, one might expect a relationship between kinetic phases and proline content, and indeed, the refolding kinetics of 11 nonhomologous proteins were shown to be qualtitatively related to their proline content (Stellwagen, 1979). Other experiments have concentrated on comparing homologous proteins that differ in proline content as well as other amino acids. Carp parvalbumin contains one proline residue and has been observed to undergo a slow kinetic phase during refolding which is absent in the refolding of homologous parvalbumins devoid of proline (Lin & Brandts, 1978). However, kinetic phases and proline content are not always directly related: the number of kinetic phases observed during refolding of homologous cytochromes *c* or ribonucleases A is not proportional to proline content (Babul et al., 1978; Krebs et al., 1983).

As the results described above suggest, it is difficult to draw conclusions regarding the involvement of specific proline residues in rate-limiting folding steps. Cis–trans isomer-specific proteolysis can be used to assign specific prolines to kinetic phases, but only in a limited number of special cases (Lin & Brandts, 1983). Studies using nonhomologous proteins suffer from difficulties in interpretation due to differences in tertiary structures which could be the source of changes in folding kinetics. A similar problem arises for homologous proteins. Homologues sufficiently divergent to differ in proline content generally exhibit numerous other sequence differences. Furthermore, differences in proline content in homologous proteins occur, by definition, at variable positions in the pri-

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[1] 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.

[2] Abbreviations: Gdn·HCl, guanidine hydrochloride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; iso-2, iso-2-cytochrome *c* from *Saccharomyces cerevisiae*; Thr-71 iso-2, a mutant form of iso-2 in which proline-71 is replaced by threonine; iso-1-MS, iso-1-cytochrome *c* from *S. cerevisiae* treated with methyl methanethiosulfonate; Val-71 iso-1-MS, Ile-71 iso-1-MS, and Thr-71 iso-1-MS, mutant forms of iso-1-MS in which proline-71 is replaced by valine, isoleucine, and threonine, respectively; C_m, midpoint of the guanidine hydrochloride induced equilibrium unfolding transition; τ_i, time constant of kinetic phase *i* (reciprocal of the apparent rate constant); α_i, amplitude of a reaction expressed as the fraction of the total observable kinetic change associated with τ_i; HPLC, high-performance liquid chromatography.

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mary structure. Residues occurring at variable sites are expected to play a less crucial role in folding than residues occurring at conserved locations. Ideally, the involvement of proline residues in protein folding should be examined in proteins differing only in the conserved proline residue(s) of interest. With the advent of site-specific mutagenesis, such experiments are feasible.

This report describes the construction and the initial thermodynamic and kinetic characterization of a mutant iso-2-cytochrome *c* of yeast designed to study the effects of the replacement of a conserved proline residue on the stability and rate of formation of tertiary structure. We designed this mutant to contain a threonine in place of an evolutionarily conserved proline residue at position 71. The effects of this change on the slow protein folding reactions and on the structure of the mutant protein are discussed.

Materials and Methods

Strains and Media. Escherichia coli strain JM103 [Δ(*lac pro*A,B), *sup*E, *thi*, *str*A, *sbc*B15, *end*A, *hsp*R4/F′, *tra*D36, *pro*A,B, *lac*I^{q}ZΔM15; Messing, 1983] was obtained from Bethesda Research Laboratories, Bethesda, MD. A yeast strain that contains neither functional iso-1 nor iso-2-cytochrome *c*, GM-3C-2 (α, *leu*2-3, *leu*2-112, *trp*1-1, *his*4-519, *cyc*1-1, *cyp*3-1; Faye et al., 1981), and a yeast–*E. coli* shuttle vector, YEpCYC7(*Sau*3A) (Montgomery et al., 1980), were provided by Donna Montgomery. YEpCYC7(*Sau*3A) contains a *Sau*3A fragment of the yeast genome containing the structural gene for iso-2-cytochrome *c* (CYC7) inserted into the unique *Bam*HI site of YEp13 (Broach et al., 1979).

Single-Stranded Vector Construction. The *Sau*3A fragment containing the coding region for iso-2-cytochrome *c* was isolated from the plasmid YEpCYC7 described above and treated with the Klenow fragment of DNA polymerase I in the presence of a mixture of the four deoxynucleotide triphosphates to produce blunt ends. DNA linkers containing a restriction site for *Bam*HI were then ligated to these blunt ends, and the resulting fragment was treated with *Bam*HI restriction endonuclease and ligated into the unique *Bam*HI site in M13mp10 (Messing, 1983). The CYC7 insert in this phage, M13-CYC, was sequenced by the method of Messing et al. (1983) to determine the orientation of the inserted fragment and to verify its integrity. M13-CYC phage particles contain the nonsense strand of the CYC7 gene.

Oligonucleotide-Directed Mutagenesis. An 18-base oligonucleotide was designed that changed the codon for Pro-71 (CCA) of the wild-type sequence to ACA. The oligonucleotide, 5′-G ACG AAC (A)CA AAG AAA TA-3′, which was obtained in purified form from OligoChemCorp, Denton, TX, was annealed to the M13-CYC phage DNA and utilized in double-primer in vitro synthesis as described by Zoller et al. (1983). The molecules were transfected into *E. coli* strain JM103 which was made competent for uptake of DNA by the method of Mandel and Higa (1970). Single colonies of infected cells were picked and screened for presence of the desired mutation by the dot–blot hybridization technique detailed by Zoller et al. (1983). The 18-base mutagenic oligonucleotide was treated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and hybridized to phage DNA from putative mutants bound to a nitrocellulose filter. The filter was washed at successively higher temperatures and subjected to autoradiography between washes. It was assumed that mutant phage DNA would bind the ^{32}P-labeled mutagenic oligonucleotide at higher temperatures than would the wild-type DNA sequences. DNA from candidate mutants was sequenced to confirm the mutation. The *Bam*HI fragment from the desired mutant phage was subsequently cloned into the unique *Bam*HI restriction site of YEp13. The resultant plasmid, YEp-PT71, was transformed into *S. cerevisiae* strain GM-3C-2, under selection for leucine prototrophy, by a modification of the method of Hicks et al. as described by O'Malley and Douglas (1983).

Protein Purification. Procedures for growing yeast and purification of cytochrome *c* have been described previously (Nall & Landers, 1981). In the present case the final purification step made use of cation-exchange HPLC. Partly purified iso-2-cytochrome *c* obtained by batch elution of the protein from coarse Bio-Rex 70 resin was passed over a Sephadex G-25 column equilibrated with 0.1 M sodium phosphate, pH 6.0, and 1 mM DTT and loaded onto a preparative cation-exchange HPLC column (PolyCat-A from Custom LC, Houston) that had been equilibrated with the same buffer. The protein was eluted with a 0.2–0.6 M linear gradient of NaCl over 50 min at a rate of 1 mL/min. The major peak of iso-2-cytochrome *c* was collected, and the protein was oxidized with a slight excess of ferricyanide and desalted by passing over a Sephadex column. The protein was reloaded onto the preparative HPLC column and eluted as before except that DTT was left out of the buffer so that the protein eluted in the oxidized form. SDS–polyacrylamide gel (Laemmli, 1970) analysis indicated that the protein was more than 98% pure.

Protein Sequencing. A Beckman Model 890D automatic sequencer with the 0.1 M Quadrol program was used to compare the amino acid sequence of the mutant and wild-type forms of iso-2. The method employed (Chin and Wold, unpublished results) involves sequencing the mixture of four cyanogen bromide peptides (Erhard, 1967) released from iso-2-cytochrome *c*. From the known sequence of this protein (Montgomery et al., 1980) the amino acids removed on each sequencing cycle can be predicted, allowing verifiction of sequence changes. In addition, this procedure confirms that the mutant protein has an intact amino terminus and carboxy terminus.

UV–Visible Spectral Analysis. Optical spectroscopy was performed as previously described (Ramdas et al., 1986). In the 600–800-nm region large systematic errors in base lines occurred at the low concentrations of Thr-71 iso-2 available. (Protein absorbance in this region was of the order of 3×10^{-3} to 5×10^{-2} for Thr-71 iso-2, with base-line errors as large as 1.5×10^{-2}.) Assuming that changes in absorbance at 800 nm are negligible compared to the base-line errors, the spectra (inserts, Figures 3 and 4) have been corrected as follows. With a concentrated protein solution the molar extinction at pH 7.2 for Pro-71 iso-2 was determined to be $\epsilon_{800} = 0.3 \times 10^3$ L/(M·cm). Spectra of Thr-71 iso-2 were offset to $\epsilon_{800} = 0.3 \times 10^3$ L/(M·cm) by adding or subtracting a constant (wavelength-independent) factor from the spectra. Adjustments of $\pm 1.5 \times 10^3$ L/(M·cm) or less were required. A pH-induced conformational change in the folded protein was monitored at both 418 and 696 nm (panels A and B, respectively, of Figure 5). For the limited amounts of protein available, the data obtained at 418 nm showed less scatter and did not require base-line corrections.

Second derivative spectra were analyzed according to Ragone et al. (1984). For protein in folded state n, values for the parameter $r_n = a/b$ are obtained from ratios of peak to valley heights in the second derivative spectra (Figure 6). Ragone et al. (1984) have shown that r_i is a measure of the polarity of the environment of tyrosine side chains and the tyrosine/tryptophan ratio for a protein in state i. Generally,

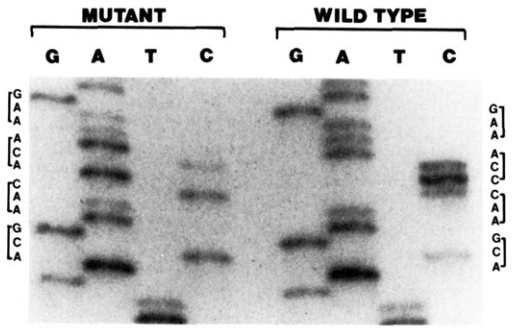

FIGURE 1: M13 DNA sequencing of genes encoding wild-type (Pro-71) and mutant (Thr-71) iso-2-cytochrome *c*. Cytochrome *c* containing M13 phage which bound ^{32}P-labeled oligonucleotide at high temperatures (55 °C) during dot–blot hybridization were sequenced by the method of Messing (1983). The sequence of the mutant gene is ACGAAC*ACA*AAG and the sequence of the wild-type gene is ACGAAC*CCA*AAG.

$r_i = (A_i x + B_i)/(C_i x + 1)$, where x is the molar ratio between tyrosine and tryptophan and A_i, B_i, and C_i are parameters dependent on the polarity of the tyrosine environment. For a protein with a known tyrosine/tryptophan ratio, r_i can be calculated for two limiting cases: r_u with fully exposed tyrosines and r_a with fully buried tyrosines. For Pro-71 iso-2 or Thr-71 iso-2 x = (number of tyrosines)/(number of tryptophans) = 5. For unfolded protein $r_u = 2.44$ is calculated by using parameters appropriate to water: $A = 0.21$, $B = 0.66$, and $C = -0.06$. For completely buried tyrosines $r_a = -0.325$ is obtained by using parameters appropriate to ethylene glycol: $A = -0.18$, $B = 0.64$, and $C = -0.04$. The average degree of exposure of tyrosines to solvent, α, is defined to be $\alpha = (r_n - r_a)/(r_u - r_a)$. Since there are five tyrosines in iso-2, the average number of exposed tyrosine residues is 5α.

Equilibrium and Kinetic Measurements of Folding/Unfolding. Methods for monitoring the equilibrium unfolding transition by changes in relative fluorescence have been described (Zuniga & Nall, 1983; Osterhout et al., 1985; Ramdas et al., 1986). The base line (Figure 7) for the unfolded protein was determined by a least-squares fit to data obtained for Pro-71 iso-2 between 2.5 and 5.0 M Gdn·HCl. The base line for the folded protein was assumed to be independent of Gdn·HCl concentration with a relative fluorescence of 1.8%. Procedures described by Schellman (1978) have been used to estimate the stabilities of the folded proteins under standard conditions in the absence of denaturants.

Procedures for monitoring slow kinetic phases in refolding by fluorescence and absorbance have been described (Osterhout & Nall, 1985). For kinetic measurements by absorbance, a wavelength of 418 nm was chosen since, for limited protein, this wavelength is one of the most sensitive to changes in the folded state. Comparable sensitivity in the ultraviolet spectral region requires almost twice the final protein concentration. For Pro-71 iso-2, we have shown previously that the same absorbance-detected slow folding phase is observed at 287, 418, and 695 nm (Nall, 1983; Osterhout & Nall, 1985).

RESULTS

Construction of Thr-71 Mutant Iso-2-cytochrome c. The cloned CYC7 gene in M13-CYC was mutagenized with an 18-base oligonucleotide designed to change the codon for Pro-71 from CCA (Pro) to ACA (Thr) according to the two-primer method of Zoller et al. (1983). Mutant candidates were identified as phage which hybridized preferentially to labeled mutagenic oligonucleotide. An autoradiogram demonstrating the DNA sequence of the region flanking the codon for Pro-71 in both the wild-type gene and the desired mutant is shown in Figure 1. The entire CYC7 gene of this mutant (M13-PT71) was resequenced, and no other changes were identified (data not shown).

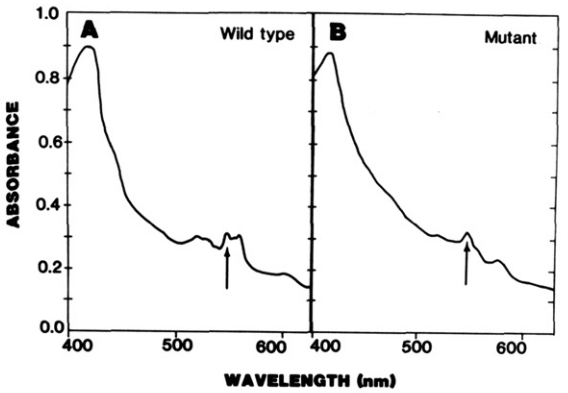

FIGURE 2: Low-temperature spectroscopy of *S. cerevisiae* strain GM-3C-2 containing wild-type or mutant iso-2-cytochrome *c*. The absorbance spectrum at low temperature of yeast cells containing YEp13 with the wild-type (A) or mutant (B) gene was measured from 400 to 700 nm. Yeast containing the mutant gene display an absorbance maximum at 547 nm, typical for cytochrome *c* (indicated by arrows). Yeast containing the vector YEp13 without the cytochrome *c* gene do not have an absorbance maximum at 547 nm (results not shown).

The altered gene was cloned into the *Bam*HI site of YEp13 to generate YEp-PT71 and transformed into GM-3C-2, a leucine auxotroph of *S. cerevisiae* that makes no detectable cytochromes *c*, under selection for leucine prototrophy as described under Materials and Methods. The presence of cytochrome *c* in the transformed strain was examined by low-temperature absorbance spectroscopy of intact cells (Figure 2). Although the absorbance was reduced in the mutant-carrying strain when compared to wild type, the 547-nm absorbance maximum characteristic for cytochrome *c* in vivo (Sherman et al., 1968) was observed. Thus, YEp-PT71 codes for an apocytochrome *c* polypeptide which is translocated into the mitochondria and which attaches heme. Absorbance maxima characteristic for cytochromes aa_3 and *b* (603 and 559 nm, respectively; Sherman et al., 1968) were not detected in the strain carrying the mutant cytochrome *c*.

Mutant cytochrome *c* was isolated and purified from transformed yeast as described under Materials and Methods. The purified mutant protein was cleaved with cyanogen bromide, and the resulting peptides were subjected as a group to automated sequential Edman degradation. Wild-type protein was isolated and subjected to the same treatments for comparison. The same amino acid residues for the first six degradative cycles were observed for the wild-type and mutant proteins (Table I). The absence of proline in cycle 7 in the mutant peptide mixture confirms the inference from the DNA sequence that Pro-71 is replaced with another amino acid. The amino acid detection method employed allows efficient detection of all amino acids other than threonine or serine, so the low yield of threonine, along with the absence of anomalous amino acids in the mutant protein, is consistent with the DNA sequencing result indicating a threonine substitution at position 71. The amino acids detected in the other sequencing cycles were the same for both proteins with the exception of cycle 8, in which some proline was detected in the wild-type peptide mixture but not in the mutant peptide mixture. This is probably due to incomplete release of proline on cycle 7 (Hermodson et al., 1972). The efficiency of cleavage is known to depend on amino acid sequence and is particularly low when proline is followed by a hydrophobic amino acid (Niall, 1977).

Table I: Cyanogen Bromide Fragments of Wild-Type Iso-2-cytochrome c[a]

(-9) NH_2-Ala-Lys-Glu-Ser-Thr-Gly-Phe-Lys-Pro•••
(65) Ser-Glu-Tyr-Leu-Thr-Asn-Pro-Lys-Lys•••
(81) Ala-Phe-Ala-Gly-Leu-Lys-Lys-Glu-Lys•••
(99) Thr-Lys-Ala-Ala-Lys-COOH

cycle	wild type (nm)[b]		mutant (nm)[b]		ratio (mutant/wild type)
1	Ala	1435	Ala	1485	1.03
	Ser	nd	Ser	74	
	Thr	nd	Thr	31	
2	Lys	814	Lys	789	0.97
	Glu	167	Glu	161	0.96
	Phe	730	Phe	627	0.86
3	Glu	291	Glu	284	0.98
	Tyr	247	Tyr	253	1.02
	Ala	837	Ala	868	1.04
4	Ser	125	Ser	198	1.58
	Leu	568	Leu	611	1.08
	Gly	244	Gly	290	1.19
	Ala	129	Ala	239	1.85
5	Thr	227	Thr	280	1.23
	Leu	1110	Leu	1102	0.99
	Lys	144	Lys	173	1.20
6	Gly	226	Gly	213	0.94
	Asn	148	Asn	146	0.99
	Lys	759	Lys	920	1.21
7	Phe	550	Phe	598	1.09
	Pro	121	Pro	nd	0.0
	Lys	709	Lys	792	1.12
	Thr	nd	Thr	117	
8	Lys	780	Lys	767	0.98
	Glu	253	Glu	267	1.06
	Pro	133[c]	Pro	nd	0.0
9	Pro	510	Pro	508	1.00
	Lys	904	Lys	1108	1.23

[a] nd indicates not detected. [b] Yields are estimated by integration of HPLC peaks. [c] Proline in cycle 8 from the wild-type protein is probably due to incomplete cleavage of the proline in cycle 7 (see text).

Such a sequence-dependent effect on cleavage efficiency (and, possibly, the overall yield of proline) is apparent in comparing cycles 7 and 8 with cycle 9.[3] Proline released in cycle 7 is followed by a trimethyllysine (DeLang et al., 1970) while the proline released in cycle 9 is followed by a glycine. In all other respects the residues released were consistent with the known sequence of iso-2-cytochrome c. The results of cycle 9 show that proline was detectable in both the wild-type and mutant peptide mixtures.

Spectral Properties of Purified Proteins. The absorbance spectra of wild-type and mutant proteins were compared. Figure 3 depicts the spectra of the oxidized forms of the proteins at pH 7.2. The spectral properties were similar. Absorbance at 695 nm of the oxidized mutant protein, however, was somewhat decreased compared to absorbance of the wild-type protein. Absorbance at this wavelength is indicative of the degree of ligation of Met-80 to the heme iron (Shechter & Saludjian, 1967); we interpret the decrease in the absorbance spectrum of the mutant protein as a decrease in the extent of ligation of this residue to the iron atom.

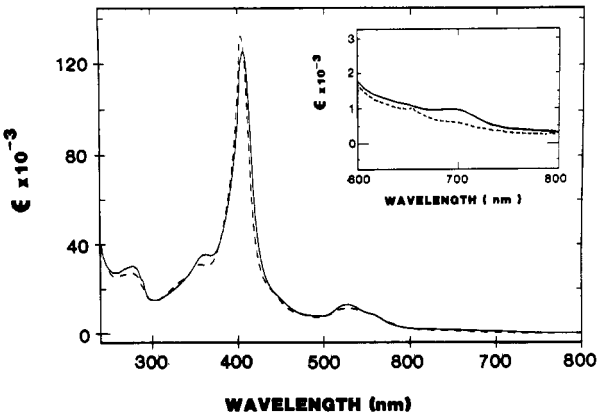

FIGURE 3: Absorbance spectrum of wild-type (—) and mutant (---) cytochrome c. Molar extinction of oxidized mutant or wild-type iso-2-cytochrome c is plotted vs. wavelength. The 600–800-nm region is shown in the insert. The mutant protein possesses a greater absorbance in the Soret region and a decreased absorbance at 695 nm, compared to wild-type protein. The conditions were 0.1 M sodium phosphate, pH 7.2, and 20 °C, with protein concentrations in the range of 10^{-5} M.

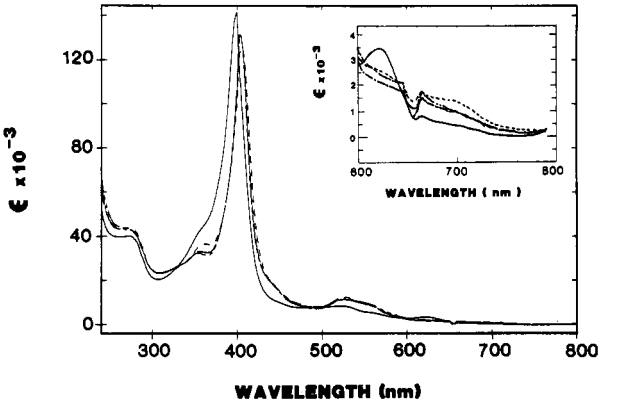

FIGURE 4: Absorbance spectrum of Thr-71 iso-2-cytochrome c in acidic, neutral, and alkaline conditions. Molar extinction is plotted vs. wavelength for mutant cytochrome c at pH 3.1 (—), 5.5 (---), 7.2 (-·-), and 8.8 (-··-) in 0.1 M sodium phosphate at 20 °C. The 600–800-nm region is included in the insert. The discontinuity near 650 nm is an instrumental artifact.

Because the absorbance spectrum of the mutant protein at pH 7.2 appeared similar to that of the wild-type protein in alkaline conditions (Osterhout et al., 1985), we examined the absorbance of the mutant protein in increasingly acidic conditions (Figure 4). The pH dependence of the molar extinction coefficient at 696 and 418 nm is given in Figure 5. The extent of heme ligation by Met-80 as revealed by absorbance at 696 nm increased as the pH decreased, reaching a maximum between pH 5 and pH 6. At pH 4.2 and below, changes in ϵ_{418} and ϵ_{696} (panels A and B of Figure 5) along with the appearance of a 620-nm absorbance band (Figure 4) indicate partial conversion of the protein to a high-spin state.

An additional measure of structure can be found in the second derivative of the absorbance spectra. Ragone et al. (1984) have shown that examination of second derivative spectra provides an estimate of the average solvent exposure of tyrosine residues. The second derivatives of the absorbance spectra of wild-type and mutant proteins were examined under various pH conditions. Figure 6A depicts the second derivative spectra of wild-type and mutant cytochrome c at pH 7.2. Figure 6B is a similar comparison at pH 5.2. Comparison of the peak to valley heights can be used to calculate the average

[3] For iso-2 incomplete release of proline in cycle 9 was not determined directly: sequencing was carried out to only nine residues. However, sequencing of a mutant iso-2 in which proline-76 is replaced by glycine (Gly-76 iso-2) was extended well past cycle 9 (L. C. Wood, unpublished results). Consistent with the present results (Table I), high carry-over was observed between cycles 7 and 8 (37%). Significant, but lower, carry-over was observed between cycles 9 and 10 (15%). We have no explanation for the fact that the total amount of proline released in cycles 7 and 8 is about half of that observed in cycle 9 (for both iso-2 and Gly-76 iso-2). One possibility is a sequence-specific side reaction leading to preferential loss of proline when it is followed by trimethyllysine at position 72.

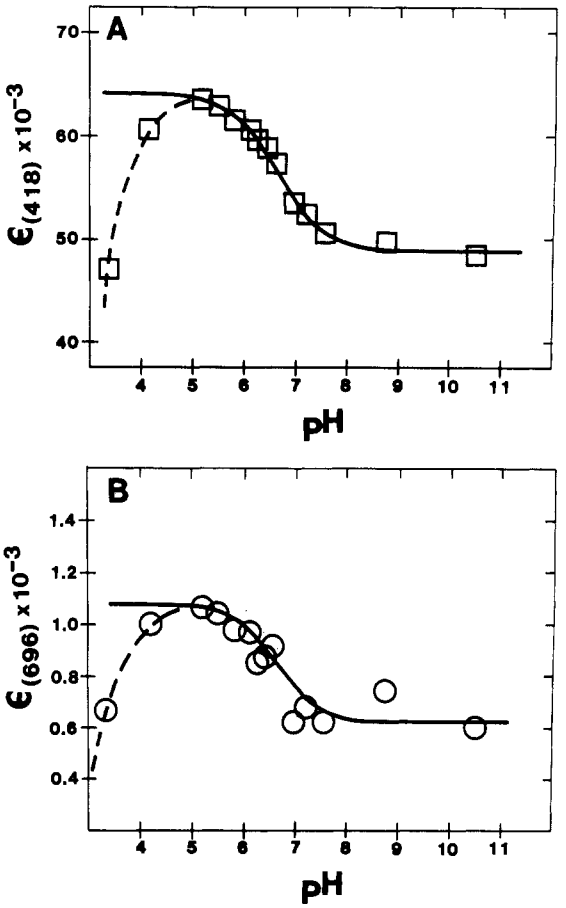

FIGURE 5: Effect of pH on the absorbance properties of Thr-71 iso-2-cytochrome *c*. Absorbance changes are given at (A) 418 (□) and (B) 696 nm (○). The solid line (—) represents a titration curve with pK = 6.63. Conditions are 20 °C and 0.1 M sodium phosphate, with a protein concentration of 8 × 10^{-6} M.

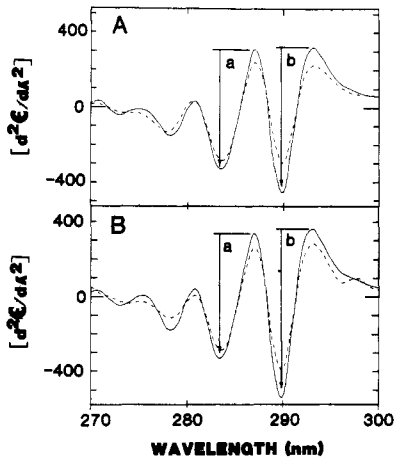

FIGURE 6: Second derivative spectra of Pro-71 (wild-type; solid line) and Thr-71 (mutant; dashed line) iso-2-cytochrome *c* at (A) pH 7.2 and (B) 5.2. Second derivative spectra were calculated by using the second derivative function of the HP 8450A spectrophotometer. The peak to valley heights *a* and *b* (measures of the polarity of tyrosine side-chain environments and the tyrosine/tryptophan ratio) are indicated. Conditions were 0.1 M sodium phosphate and 20 °C.

number of exposed tyrosine residues (see Materials and Methods). The spectra in Figure 6 suggest that while there are obvious structural differences, under acidic conditions the mutant protein more closely approaches the conformation of the wild-type protein.

Equilibrium Unfolding. The stability of the Thr-71 mutant iso-2-cytochrome *c* at pH 7.2 was measured by a fluorescence-detected guanidine hydrochloride induced unfolding transition (Figure 7, Table II). The transition for the wild-type protein is included for comparison (Osterhout et al., 1985). Changes in fluorescence occurring during the unfolding transition have been attributed to a decrease in the quenching of the unique tryptophan residue by the heme, presumably occurring as the protein unfolds and the average distance between the tryptophan and the heme increases (Tsong, 1974). Mutant cytochrome *c* was shown to be slightly less stable than wild type. Both proteins appear to be completely unfolded in 3.0 M guanidine hydrochloride and fully folded in 0.3 M guanidine hydrochloride; these conditions were used in subsequent refolding kinetic experiments as the initial and final states.

Table II: Two-State Analysis of Protein Stability[a]

protein	C_m[d] (M/L)	$-RT\Delta b_{23}$[d] (kcal·L/M²)	$\Delta G°_u$[d] (kcal/M)
Pro-71 iso-2[b,c]	1.49	2.6	3.8
Thr-71 iso-2[c]	1.19	2.5	3.0

[a] Analysis of the Gdn·HCl-induced unfolding transitions (Figure 7) is carried out according to Schellman (1978) with standard conditions of 20 °C, 0.1 M sodium phosphate, and pH 7.2. [b] Pro-71 iso-2 unfolding data is from Osterhout et al. (1985). [c] For the standard conditions, the folded state of Pro-71 iso-2 exhibits a 695-nm absorbance band, but the folded state of Thr-71 iso-2 lacks a 695-nm absorbance band (Figure 3). [d] Estimated errors are as follows: C_m, ±0.1 M/L; $-RT\Delta b_{23}$, ±0.1 kcal·L/M²; ΔG_u, ±0.5 kcal/M.

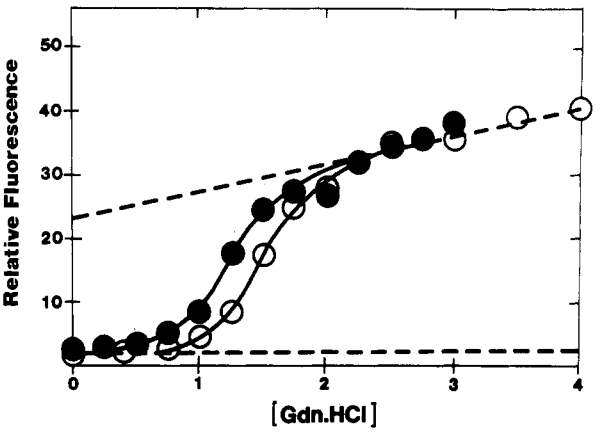

FIGURE 7: Gdn·HCl-induced equilibium unfolding transition of Thr-71 iso-2-cytochrome *c* (●). Fluorescence of the protein solutions (measured at 340 nm with excitation at 280 nm) relative to that of an equimolar concentration of *N*-acetyltryptophanamide is plotted relative to Gdn·HCl concentration. For comparison, similar data from Osterhout et al. (1985) are included for Pro-71 iso-2 (○; excitation at 287 nm, emission at 350 nm). The proteins were in 0.1 M sodium phosphate, pH 7.2, 20 °C.

Absorbance-Detected Slow Folding Kinetics. The isomerization of proline residues to the conformation required in the native state putatively allows a closer association of the polypeptide chain to the heme, causing changes in the heme environment that are detected by absorbance changes at 418 nm. Because the mutant cytochrome *c* is missing one of the three highly conserved proline residues, we wished to know whether the absorbance-detected slow folding phase seen for wild-type iso-2-cytochrome *c* (Osterhout & Nall, 1985) was present in our mutant protein. The results of these slow folding experiments are shown in Figure 8 and Table III. A slow phase was observed for wild-type iso-2-cytochrome *c* at both pH 5.5 and pH 7.2. For the mutant protein the slow phase, if present, was below the level of detection for refolding at pH 7.2. At pH 5.5 a slow phase was detected for the mutant protein, although the rate was slower than for the normal protein (Table III).

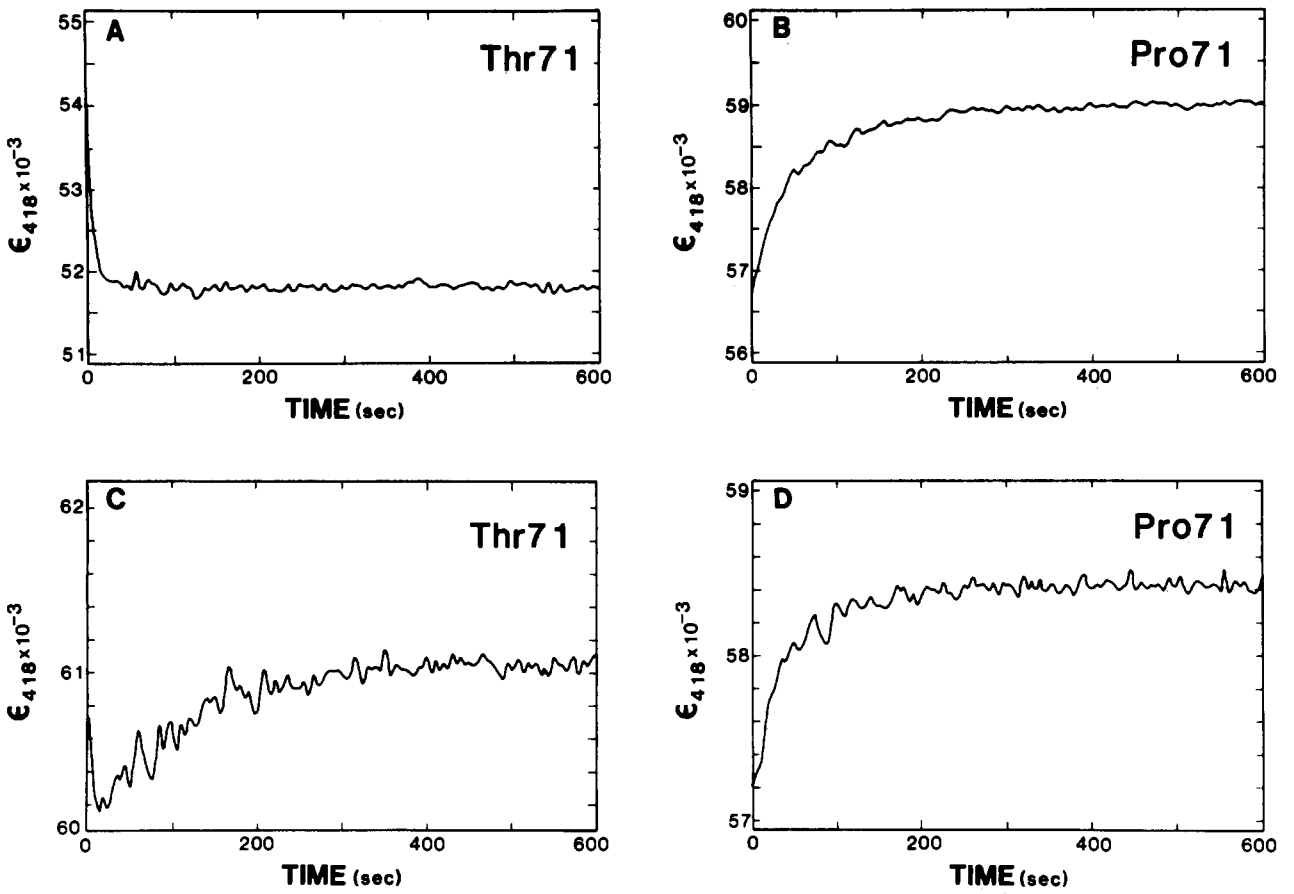

FIGURE 8: Slow refolding kinetics of Pro-71 (wild-type) and Thr-71 (mutant) iso-2 as measured by absorbance at 418 nm. In (A) the absorbance changes are shown for refolding of mutant protein following a 3.0 to 0.3 M Gdn·HCl concentration jump at pH 7.2 and 20 °C in the presence of 0.1 M sodium phosphate. The absorbance changes for the wild-type protein under the same conditions are shown in (B). Absorbance changes during the refolding of mutant and wild-type protein from 3.0 M Gdn·HCl at pH 7.2 to 0.3 M Gdn·HCl at pH 5.5, 20 °C, in the presence of 0.1 M sodium phosphate are shown in (C) and (D), respectively.

Table III: Amplitudes and Time Constants for Slow Refolding[a]

protein/property	final pH	amplitudes $\Delta\epsilon_{418}$[b]	amplitudes ΔF_{340}[b,c]	τ (s)
Pro-71 iso-2				
absorbance, λ = 418	7.2	3.3×10^3		99
nm	5.5	2.2×10^3		50
fluorescence, λ = 340	7.2		13	13
nm[c]	5.5		16	13
Thr-71 iso-2				
absorbance, λ = 418	7.2	nd ($<2 \times 10^2$)		nd
nm	5.5	2.2×10^3		119
fluorescence, λ = 340	7.2		21	11
nm[c]	5.5		23	11

[a] Data are from Figures 8 and 9. Conditions for refolding are T = 20 °C, 0.3 M Gdn·HCl, 0.1 M sodium phosphate, and the indicated pH. nd indicates that a kinetic phase was not detected. [b] Total amplitudes are given as changes in molar extinction at 418 nm for absorbance-detected slow folding and in arbitrary units of fluorescence at 340 nm for fluorescence-detected refolding. Since the fast-phase folding was not monitored, the relative amplitudes for slow refolding of Thr-71 iso-2 are unknown. For Pro-71 α_{1a} = 0.26 for absorbance-detected slow folding and α_{1b} = 0.11 for fluorescence-detected slow folding at 20 °C, pH 7.2, and 0.4 M Gdn-HCl (Osterhout & Nall, 1985). [c] The apparent difference in total amplitude between Pro-71 iso-2 and Thr-71 iso-2 for fluorescence-detected slow folding may not be significant. These amplitudes are subject to large systematic errors in determining the mixing dead time (about 5 s) and in estimating the protein concentrations for mutant and normal proteins with different absorbance spectra.

Fluorescence-Detected Slow Folding Kinetics. When fluorescence (280-nm excitation, 340-nm detection) is used as a probe, a slow phase in a 5–20-fold faster time range is observed for wild-type iso-2-cytochrome *c* (Osterhout & Nall, 1985). Figure 9 depicts the fluorescence changes occurring in Pro-71 (wild-type) and Thr-71 iso-2-cytochrome *c* during the slow phase of refolding from 3.0 to 0.3 M guanidine hydrochloride at both pH 7.2 and pH 5.5. The time constants and amplitudes for fluorescence-detected slow refolding (Table III) are similar for the mutant and the normal protein and are independent of the final pH.

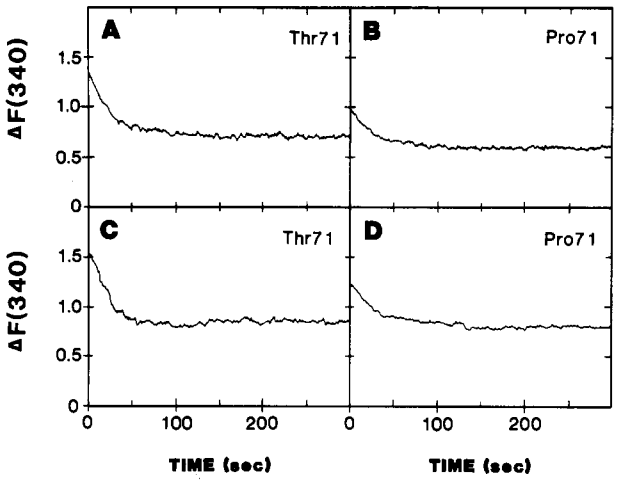

FIGURE 9: Slow refolding kinetics of Pro-71 and Thr-71 iso-2-cytochrome *c* as measured by fluorescence. Fluorescence changes at 340 nm (excitation at 280 nm) following a 3.0 to 0.3 M Gdn·HCl concentration jump at pH 7.2 and 20 °C in the presence of 0.1 M sodium phosphate are plotted for mutant (A) and wild-type (B) protein. Fluorescence changes during refolding of mutant (C) and wild-type (D) protein from 3.0 M Gdn·HCl at pH 7.2 to 0.3 M Gdn·HCl at pH 5.5 in 0.1 M sodium phosphate are also shown.

Table IV: Exposure of Tyrosine Residues to Solvent[a]

protein	pH	r_n[b,d]	α[b,d]	av no. of exposed Tyr[c,d]
Pro-71 iso-2	5.2	0.74	0.39	1.93
Thr-71 iso-2	5.2	0.78	0.40	2.00
Pro-71 iso-2	7.2	0.82	0.41	2.07
Thr-71 iso-2	7.2	1.00	0.48	2.40
Pro-71 iso-2	10.0	1.07	0.51	2.52
Thr-71 iso-2	10.3	1.03	0.49	2.45

[a] The average exposure of tyrosines to solvent is estimated from second derivative spectra (see Figure 6) according to Ragone et al. (1984). Conditions are the same as for Figure 6 at the indicated pH. [b] r_n is a measure of the polarity of the environment of tyrosine side chains and the tyrosine/tryptophan ratio. α is an estimate of the degree of exposure of tyrosines to solvent (see Materials and Methods). [c] Since there are five tyrosines in iso-2, the average number of exposed tyrosine residues is 5α. [d] Approximate errors are as follows: r_n, ± 0.03; α, $\pm$ 0.01; average number of exposed tyrosines, ± 0.06.

Discussion

Creation of Thr-71 Iso-2-cytochrome c. We have constructed a mutant of yeast iso-2-cytochrome *c* with a threonine residue replacing the evolutionarily conserved Pro-71. Amino acid analysis of total protein hydrolysates showed that mutant protein was detectably higher in threonine content and lower in proline content than the wild-type protein (results not shown). The results from DNA and protein sequence analyses demonstrate conclusively that threonine replaces proline at position 71 in the mutant protein and that the primary structures of the two proteins were otherwise identical.

Structure of Thr-71 Iso-2. Compared to that of wild-type protein (Figure 3), the absorbance spectrum of Thr-71 iso-2 at pH 7.2 shows a blue shift in the Soret maximum (408 nm for wild type to 404 nm for mutant) and decreased absorbance at 695 nm, suggesting the loss of Met-80 as a heme ligand. The absorbance spectrum of the mutant protein at pH 7.2 is similar to that of the wild-type protein at alkaline pH (pH 9 or 10; Osterhout et al., 1985). The differences in the spectra of wild-type and mutant proteins at pH 7.2 may be explained by a decrease in the apparent p*K* for conversion to the alkaline state from pK_{app} = 8.45 for Pro-71 iso-2 (L. Ramdas, unpublished results) to pK_{app} = 6.63 for Thr-71 iso-2 (Figure 5). This hypothesis is supported by estimates of the average solvent exposure of tyrosine residues in the mutant protein in neutral and acidic conditions as revealed by second derivative spectra (Figure 6, Table IV). Pro-71 iso-2 (wild type) at pH 7.2 is estimated to contain an average of 2.07 tyrosine residues exposed to the solvent. Thr-71 iso-2 at pH 7.2, however, contains 2.40 exposed tyrosine residues. This degree of exposure is comparable to that of wild-type protein in the alkaline state, which contains 2.52 exposed tyrosine residues at pH 10.0 (Table IV). Only under relatively acidic conditions (pH 5.2) does the number of exposed tyrosine residues in Thr-71 iso-2 approach that of Pro-71 iso-2 (2.00 vs. 1.93, respectively).

Stability of Thr-71 Iso-2. The stability of the mutant protein in the presence of guanidine hydrochloride is slightly less than that of wild-type protein. A decrease in stability is to be expected from the substitution of a threonine residue for Pro-71. The proline residue at this position in the wild-type protein is partially buried and, as a hydrophobic residue, could contribute up to 2500 cal/mol to the stability of the protein (Brandts, 1964). Threonine, however, is not as hydrophobic and thus is expected to contribute less to the mutant protein's stability, which agrees with our results (Figure 7). There is no significant change in the cooperativity of Gdn·HCl-induced unfolding as judged by values of $-RT\Delta b_{23}$ (Table II).

For fluorescence, the estimate of the free energy of unfolding of Pro-71 iso-2 (3.8 kcal/mol; Table II) is somewhat larger than the value of 3.1 kcal/mol reported for absorbance-detected unfolding (Nall & Landers, 1981). The free energy difference for the two methods is similar in magnitude to expected errors of ± 0.5 kcal/mol, where the major source of (systematic) error is in determining base lines of the fully folded and unfolded states of the protein. The values in Table II are probably more reliable because the base lines for the folded and unfolded proteins are better behaved for fluorescence than for absorbance. Alternatively, the difference could be because fluorescence and absorbance detect different distributions of equilibrium folding intermediates. If so, the two-state estimates given in Table II are not a proper measure of protein stability.

The two-state estimates of stability (Table II) may be compared to those obtained for replacements in the homologous iso-1-MS cytochrome *c* at pH 6.0 (Ramdas et al., 1986): Pro-71 iso-1-MS, 3.6 kcal/mol, and Thr-71 iso-1-MS, 1.9 kcal/mol. The data suggest that replacement of Pro-71 by threonine is more costly (in terms of unfolding free energy) in iso-1-MS than in iso-2. This difference may be an artifact of the choice of standard states: pH 6.0 for iso-1-MS and pH 7.2 for iso-2. The choice is important since the folded states for both mutant proteins are native-like at pH 6.0 (intact 695-nm absorbance band) but alkaline-like at pH 7.2 (no 695-nm absorbance band). Thus the free energy estimate for unfolding of Thr-71 iso-2 is for unfolding of an alkaline-like mutant protein, while the estimate for Thr-71 iso-1-MS is for unfolding from the native-like conformation. The difference in standard-state pH is unlikely to be important for the normal proteins since Pro-71 iso-1-MS ($\Delta G°_u$ = 3.6 kcal/mol, at pH 6.0) and Pro-71 iso-2 ($\Delta G°_u$ = 3.8 kcal/mol, at pH 7.2; Table II) have native-like conformations at both pH 6.0 and pH 7.2.

Refolding Kinetics of Thr-71 Iso-2. The kinetics of refolding of mutant protein during dilution from 3.0 to 0.3 M guanidine hydrochloride were examined. Refolding was performed at pH 7.2 to enable comparison with the well-documented refolding kinetics of wild-type iso-2-cytochrome *c* (Nall, 1983; Nall & Landers, 1981; Osterhout & Nall, 1985). An absorbance-detected slow phase was not detected for refolding of Thr-71 iso-2 at pH 7.2. When the mutant protein was refolded to pH 5.5, however, an absorbance-detected slow phase was observed with an amplitude near that of the normal protein (Table III). The slow folding phase for Thr-71 iso-2 detected by fluorescence is unchanged in both amplitude and rate, showing that this slow phase is not due to isomerization of Pro-71. There are two possible explanations for the absence (or decreased amplitude) of the absorbance-detected slow phase. First, this slow phase could reflect removal of a kinetic barrier to slow folding due to cis-trans isomerization of Pro-71. Proline residues with an isomeric state in the unfolded protein other than that found in the native protein may be required to isomerize before folding can proceed to completion, resulting in a slow reformation of the fully native protein. Loss of a slow phase is expected for replacement of such a residue with an amino acid (Thr-71) that does not isomerize in the unfolded protein. On the other hand, the replacement of Pro-71 could favor (pH-dependent) changes in the final conformation of the protein such that the adjustments reflected by the slow phase no longer need occur. This hypothesis is supported by correlation of the absorbance spectrum of the mutant protein with absorbance-detected slow folding. At pH 7.2, where the spectrum is similar to that of the alkaline form of wild-type iso-2, slow folding is not de-

tected. Reappearance of the slow phase during refolding of the mutant protein at pH 5.5 occurs in conjunction with return of the spectral properties of functional cytochrome *c* (presence of a 695-nm absorbance band). Furthermore, we have observed that when wild-type iso-2 is refolded in alkaline conditions, the amplitude of the absorbance-detected slow phase decreased to zero with increasing pH (unpublished results). Thus, the inability to detect a slow phase by absorbance during refolding of the mutant protein at pH 7.2 is probably due to the assumption of a nonnative conformation, such as an alkaline form, in the final state. At a lower pH, the mutant protein assumes more of a native conformation, and the absorbance-detected slow phase reappears.

It is still entirely possible that one or more of the remaining prolines in iso-2 cytochrome *c* is responsible for the absorbance-detected and/or fluorescence-detected slow folding phases. For this reason, we have altered the remaining two evolutionarily conserved prolines and are conducting studies to determine whether either of these prolines is directly involved in generation of slow folding phases.

Alkaline Isomerization in Thr-71 Iso-2. The pH-dependent spectral changes of mutant Thr-71 iso-2 (Figures 4 and 5) are similar to those observed for normal Pro-71 iso-2 but occur at lower pH [pK_{app} = 6.63 for Thr-71 iso-2, Figure 5; pK_{app} = 8.45 for Pro-71 iso-2, Osterhout et al. (1985) and Ramdas (unpublished results)]. This conformational change between two folded states of cytochrome *c* usually occurs between pH 8 and pH 10 and has been termed the alkaline isomerization (Greenwood & Palmer, 1965). The reaction probably involves replacement of Met-80 with another low-spin heme ligand, but little else is known about the conformation of alkaline cytochrome *c*. Assuming that analogous optical changes indicate a similar (pH-induced) conformational change in Thr-71 iso-2, then an important effect of replacement of Pro-71 is a decrease in the pK_{app} for this reaction. On the basis of a detailed kinetic analysis of the alkaline isomerization in horse cytochrome *c*, Davis et al. (1974) have presented a minimal mechanism for the reaction. Their scheme for formation of alkaline cytochrome *c* from the native form involves two coupled, sequential reactions with loss of a proton (K_H) followed by a conformational reaction (K_c). If the two-state approximation to the overall reaction is valid, the observed titration curve is described by $pK_{app} = -\log (K_H K_c)$. Thus the lower apparent p*K* for Thr-71 iso-2 may be attributed to shifts in protonation equilibria or conformational equilibria (or both) favoring formation of alkaline cytochrome *c*. Analysis of the pH dependence of the kinetics of the alkaline isomerization [e.g., Davis et al. (1974)] is required to distinguish between these possibilities. For replacements of Pro-71 in iso-1-MS cytochrome *c*, such an analysis is underway (Ramdas, unpublished experiments).

Comparison with Folding/Unfolding for Mutants of Iso-1-MS Cytochrome c. A detailed investigation of the kinetics of fluorescence-detected refolding has been reported for replacements of Pro-71 by threonine, valine, and isoleucine in iso-1-MS cytochrome *c* (Ramdas & Nall, 1986). For the iso-1-MS mutants stopped-flow mixing techniques have been used to monitor both fast and slow kinetic phases. Consistent with the present results, replacements of Pro-71 in iso-1-MS do not alter the rates of fluorescence-detected slow folding. Instead, the major change is in a fast kinetic phase in folding/unfolding, which is increased in rate by 5–20-fold, depending on the residue replacing Pro-71. It is not known whether similar changes in fast folding/unfolding occur for Thr-71 iso-2. Conversely, the results are not yet complete for absorbance-detected slow folding of Pro-71 replacements of iso-1-MS. Nevertheless, available data are consistent with a previous suggestion that the qualitative features of protein folding reactions may be conserved among homologous proteins (Nall & Landers, 1981). Perhaps this principle can be extended to effects of amino acid replacements at the same position in homologous mutant proteins.

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Registry No. Pro, 147-85-3; iso-2, 9007-43-6; Thr, 72-19-5.

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Differential Detergent Solubility Investigation of Thermally Induced Transitions in Cytochrome *c* Oxidase†

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ABSTRACT: The thermal denaturation of membrane-reconstituted cytochrome *c* oxidase (EC 1.9.3.1) occurs at ~63 °C as determined by high-sensitivity differential scanning calorimetry. The heat capacity profile associated with this process is characterized by the presence of two well-defined peaks, indicating that all the enzyme subunits do not have the same thermal stability. This thermal denaturation of the enzyme complex is coupled to a change in its solubility properties. This change in solubility allows separation of the native and denatured protein fractions by detergent solubilization followed by centrifugation under conditions in which only the native fraction is solubilized. Using this principle, it has been possible to study the denaturation of membrane-reconstituted cytochrome *c* oxidase and quantitatively identify the protein subunits undergoing thermal denaturation using computer-assisted gel electrophoresis analysis. This technique allows calculation of single-subunit thermal denaturation profiles within the intact enzyme complex, and as such, it can be used to obtain transition temperatures, molecular populations, and van't Hoff enthalpy changes for individual protein subunits, thus complementing results obtained by high-sensitivity differential scanning calorimetry.

A wide variety of physical techniques, including differential scanning calorimetry, NMR, and optical methods, have been used to study the thermal unfolding of proteins. Most of these studies have examined the temperature-dependent denaturation of small globular hydrophilic proteins and to a lesser extent complex multidomain hydrophilic proteins (Privalov, 1979, 1982). Until now, however, very little information is available on the thermal denaturation of heterologous multisubunit proteins or integral membrane proteins. In a previous paper, we have studied by high-sensitivity differential scanning calorimetry the melting process of the mitochondrial enzyme cytochrome *c* oxidase reconstituted into phospholipid bilayer vesicles (Rigell et al., 1985). It was shown that the denaturation process was accompanied by changes in the solubility of individual subunits of the enzyme complex and that the native subunits could be separated from denatured subunits by differential detergent solubilization following thermal denaturation. In this paper, we describe a further development in the application of differential solubility thermal gel analysis to the study of protein melting in membranes. In this paper, it is shown that differential solubility thermal gel analysis can be used in a quantitative fashion to measure the population of individual protein conformational states as a function of temperature. These individual subunit melting profiles can be used to calculate apparent thermodynamic parameters for individual subunits in complex systems. This method offers a natural complement to differential scanning calorimetry studies and particularly to the resolution of complex peaks into individual components.

Cytochrome *c* oxidase is the terminal member of the electron-transfer chain of the inner mitochondrial membrane, and it catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen while simultaneously contributing to the proton gradient across the inner membrane. Bovine cytochrome *c* oxidase is a multisubunit integral membrane protein containing 13 subunits (Kadenbach et al., 1983), the 3 largest of which are encoded on the mitochondrial genome whereas the remaining subunits are nuclear gene products (Schwal et al., 1972; Sebald et al., 1972).

In a previous report, we presented a calorimetric characterization of the thermal unfolding of cytochrome *c* oxidase. Those experiments were complemented by gel electrophoresis of the protein in which the thermal denaturation of the subunits of the enzyme was followed by examining thermally induced solubility changes of the enzyme complex in a detergent-containing medium (Rigell et al., 1985). In this paper, we present the technique of differential solubility thermal gel analysis as a means of measuring the denaturation of individual subunits of the multisubunit enzyme cytochrome *c* oxidase. This technique when applied to the membrane-reconstituted enzyme allows a positive identification of the thermal denaturation peaks observed by differential scanning calorimetry as well as quantitative determination of transition tempera-

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