Guanidine Hydrochloride Induced Equilibrium Unfolding of Mutant Forms of Iso-1-cytochrome *c* with Replacement of Proline-71†

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ABSTRACT: Proline-71, an evolutionally conserved residue that separates two short α-helical regions, is replaced by valine, threonine, or isoleucine in at least partially functional forms of iso-1-cytochrome *c* from *Saccharomyces cerevisiae* [Ernst, J. F., Hampsey, D. M., Stewart, J. W., Rackovsky, S., Goldstein, D., & Sherman, F. (1985) *J. Biol. Chem. 260*, 13225–13236]. Treatment of these proteins with a specific sulfhydryl blocking reagent (methyl methanethiosulfonate) to block Cys-102 has allowed investigation of the properties of monomeric forms of the proteins, denoted iso-1-MS. Comparison of the UV–visible absorbance properties (pH 6, 20 °C) shows minor differences between the normal Pro-71 iso-1-MS and two of the three mutant proteins. The Val-71 iso-1-MS protein has absorbance properties indistinguishable from those of the normal Pro-71 iso-1-MS protein, but the Ile-71 iso-1-MS and Thr-71 iso-1-MS proteins show reduced intensity of the 695-nm absorbance band and a small shift in the Soret maximum, from 408 nm for the Pro-71 iso-1-MS and Val-71 iso-1-MS proteins to 406 nm for the Thr-71 iso-1-MS and Ile-71 iso-1-MS proteins. Second derivative spectroscopy is used to assess differences in the polarity of the environment of tyrosine residues. The average degree of exposure of tyrosines to solvent is similar in all four proteins: 0.39 for the normal Pro-71 iso-1-MS and Val-71 iso-1-MS proteins; 0.40 for the Ile-71 iso-1-MS protein; and 0.42 for the Thr-71 iso-1-MS protein. To evaluate the effects of perturbations at position 71 on protein stability, we compared the guanidine hydrochloride induced equilibrium unfolding transitions of the normal and the three mutant forms. At pH 6.0, 20 °C, the midpoints of the guanidine hydrochloride induced transitions (C_m) are 1.07 M for Pro-71 iso-1-MS (wild type), 0.82 M for Val-71 iso-1-MS, 0.72 M for Thr-71 iso-1-MS, and 0.72 M for Ile-71 iso-1-MS. Use of a two-state model allows estimation of the free energy of unfolding under standard conditions: pH 6.0, 20 °C, and no guanidine hydrochloride. The following free energies ($\Delta G°_u$) were determined: normal Pro-71 iso-1-MS, 3.6 kcal/mol; Val-71 iso-1-MS, 2.6 kcal/mol; Thr-71 iso-1-MS, 1.9 kcal/mol; and Ile-71 iso-1-MS, 1.9 kcal/mol; the errors were estimated to be ±0.5 kcal/mol.

The free energy of stabilization of the folded state of proteins is the result of a balance between forces large in magnitude. The major factor contributing to protein stability is hydrophobic interactions, but this is counterbalanced by the conformational entropy of the unfolded protein. Other interactions (e.g., electrostatic interactions, hydrogen bonds) contribute less to net stability (or instability) but are vital in determining the structural details of the product of protein folding. This fine balance of forces suggests that, when necessary to achieve a free energy minimum, a readjustment of molecular structure to a new state (or states) will occur in folding of mutant proteins. Thus measurement of thermodynamic and structural changes in mutant proteins is important for understanding the mechanism of structure–stability compensation and may provide guidelines relating amino acid sequence to three-dimensional structure. Careful studies of the stability of mutant forms of T4 lysozyme (Hawkes et al., 1984) and of tryptophan synthase (Matthews et al., 1980; Matthews & Crisanti, 1981; Yutani et al., 1982a,b; Ogasahara et al., 1984) have been reported. Here we report a similar investigation of yeast iso-1-cytochrome *c*. There are to important differences between our studies and those on T4 lysozyme and tryptophan synthase. First, the presence of a covalently attached heme in cytochrome *c* provides an extremely sensitive (and convenient) probe of functionally important changes in the active site. Second, extensive amino acid sequence homologies of the cytochrome *c* family (Ambler, 1984; Dickerson, 1980) allow focus on conserved sites vital to some (usually unknown) aspect of protein maturation, folding, or function. Juillerat and Taniuchi (1986) have recently reported studies of the effect of substitutions of conserved Leu-32 on cytochrome *c* structure and stability. The experimental approaches differ. They monitor changes in binding affinity of chemically synthesized peptides in a three-fragment complementation system (Juillerat et al., 1980), while our studies are of intact mutant proteins.

Iso-1-cytochrome *c* forms covalent dimers when allowed to stand free in solution. Presumably the dimers are generated by formation of a bimolecular disulfide bond between cysteine-102 residues. Since the folding properties of the dimeric form differ from those of monomeric iso-1[1] (Bryant et al.,

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MS, methyl methanethiosulfonate; iso-1, iso-1-cytochrome *c* from *Saccharomyces cerevisiae*: iso-1-MS, iso-1 treated with methyl methanethiosulfonate; Val-71 iso-1-MS, Thr-71 iso-1-MS, and Ile-71 iso-1-MS, mutant forms of iso-1-MS in which proline-71 is replaced by valine, threonine, and isoleucine, respectively; iso-1-AM, iso-1 with Cys-102 blocked with iodoacetamide; C_m, midpoint of the guanidine hydrochloride induced equilibrium unfolding transition; SDS, sodium dodecyl sulfate.

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1985), it is necessary to eliminate disulfide dimer formation to study the monomeric species. Previously we reported studies of Gdn·HCl[1]-induced folding/unfolding of iso-1 specifically modified with iodoacetamide, iso-1-AM (Zuniga & Nall, 1983). For wild type iso-1-AM, the unfolding transition is reversible and the protein retains functional and optical properties of the unmodified protein. However, treatment of mutant forms with iodoacetamide reduces the intensity of the 695-nm absorbance band, an important indicator of structural integrity (Zuniga and Nall, unpublished data). In the present investigation, methyl methanethiosulfonate (MS) has been used to convert the free S–H group to an S–SCH_3 group (Smith et al., 1975). Both wild type and mutant forms of iso-1-MS retain the UV–visible spectral properties of the unmodified proteins[2] but no longer form disulfide-linked dimers.

Here we report UV–visible absorbance and equilibrium unfolding properties of wild type and three mutant forms of iso-1-MS in which a conserved proline at position 71 is replaced by Val-71, Ile-71, or Thr-71.[3] Pro-71 separates two short α-helical segments in native cytochrome *c* (Dickerson & Timkovich, 1975; Takano & Dickerson, 1981a,b) and thus may be important in local folding by defining the end of one helix and the beginning of another.

Materials and Methods

Growth of yeast (*Saccharomyces cerevisiae*) and protein purification were performed as previously described (Nall & Landers, 1981; Zuniga & Nall, 1983). Wild type iso-1-cytochrome *c* (type VIII) was obtained from Sigma Chemical Co. but was purified further by cation-exchange chromatography. Other chemicals and reagents were from the following sources: dithiothreitol (DTT), Sigma Chemical Co.; 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Aldrich; methyl methanethiosulfonate (MS), Pierce Chemical; guanidine hydrochloride (Gdn·HCl), Heico.

Modification of Wild Type and Mutant Iso-1-cytochrome c. Disulfide dimer formation can be eliminated and spectral properties retained for both mutant and wild type iso-1 by treatment with methyl methanethiosulfonate (Smith et al., 1975) as follows. Twenty milligrams of iso-1 is dissolved in 1 mL of 0.1 M sodium phosphate, pH 7.2, and 10^{-3} M DTT. The solution is allowed to stand at room temperature for 30 min before passage over a Sephadex G-25 column equilibrated at 4 °C with 0.01 M glycine adjusted to pH 7.8 with NaOH. The eluted protein (about 3-mL volume in 0.01 M glycine) is collected in a glass vial. A total of 50 μL of a 0.1 M aqueous solution of methyl methanethiosulfonate is added and the reaction carried out at 4 °C with stirring for 30 min. The reaction mixture is loaded onto a column of Bio-Rex 70 resin equilibrated with 0.05 M sodium phosphate, pH 7.2, and washed with 2–3 volumes of the same buffer containing 0.005 M ferricyanide (to oxidize the protein). The protein is eluted with a 0–0.6 M gradient of NaCl in 0.05 M sodium phosphate, pH 7.2. The earlier eluting monomeric fractions are pooled and concentrated by using Centricon concentrators or by binding to Bio-Rex 70 followed by elution with 0.6 M NaCl/0.1 M sodium phosphate, pH 7.2. The protein is desalted by passing over a Sephadex G-25 column equilibrated with 0.05 M ammonium acetate, pH 6.0, and lyophilized. The resulting protein does not react with DTNB in an assay for free sulfhydryls, can be reversibly unfolded by heat or Gdn·HCl without formation of disulfide-linked dimers (as judged by polyacrylamide–SDS gels run in the absence of sulfhydryl reagents), and is stable as a monomer for months or longer after lyophilization.

UV–Visible Spectral Analysis. Spectra were obtained in a Hewlett-Packard 8450A UV–visible spectrophotometer using quartz cuvettes with a 1-cm optical path. The digitized spectra were stored in memory and transferred to an Apple II+ microcomputer where they were saved as binary (floppy) disk files. Molar extinction coefficients of both mutant and wild type proteins were assumed to be 106.1×10^3 L/(M·cm) at 410 nm and 22.7×10^3 L/(M·cm) at 434 nm (Margoliash & Frohwirt, 1959). Protein concentration was estimated from absorbance at 434 or 410 nm or by averaging measurements at both wavelengths. These wavelengths were chosen since they are isosbestic points for the spectral differences between oxidized and reduced cyotochrome *c* (Margoliash & Frohwirt, 1959). In addition, 410 nm is an approximate isosbestic point for the alkaline isomerization of iso-1-MS and, within errors, is at the visible absorbance maximum for the oxidized protein (408 ± 2 nm). For comparison, all spectra were converted to molar extinction as a function of wavelength. In the ultraviolet region, second derivative spectra were calculated by using the second derivative function of the HP 8450A spectrophotometer. All spectra are taken at 20 °C in 0.1 M sodium phosphate buffer, pH 6.0, at protein concentrations of (5–10) $\times 10^{-6}$ M.

Equilibrium Unfolding. Gdn·HCl-induced equilibrium unfolding transitions are monitored by fluorescence at 340 nm with a Perkin-Elmer (Model MPF-2A) spectrofluorometer using an excitation wavelength of 280 nm. Temperature was controlled at 20 ± 0.2 °C with a circulating water bath. Solutions contain 0.1 M sodium phosphate adjusted to pH 6.0 with H_3PO_4. Reversibility of unfolding was checked by the retention of spectral properties on refolding from concentrated solutions of Gdn·HCl. In most cases the protein samples were preunfolded by brief exposure to high concentrations of Gdn·HCl in order to remove any residual reduced or aggregated species (Nall & Landers, 1981; Nall, 1983).

A two-state treatment of the unfolding transition curves requires estimation of base-line fluorescence for the fully folded and unfolded protein within the transition region. Fluorescence of the folded proteins was assumed to be independent of Gdn·HCl concentration with the following values (relative fluorescence) taken from the transition curves: Pro-71 iso-1-MS, 0.02; Val-71 iso-1-MS, 0; Thr-71 iso-1-MS, 0.04; Ile-71 iso-1-MS, 0.02. Base-lines for the unfolded proteins were determined by a linear least-squares fit to data points at 1.8 M Gdn·HCl and above.

Results

UV–Visible Spectroscopy. Figure 1 shows differences in

[2] A comparison of ferri iso-1-cytochrome *c* and Pro-71 iso-1-MS by ^{1}H NMR suggests that blocked and unblocked forms have similar (and possibly identical) conformations in solution. There are no differences in the fully resolved (paramagnetic) regions of the spectra. In the aromatic and aliphatic regions of the spectra a few resonances do show changes in chemical shifts and line widths. Two alternative explanations for the observed differences are the following: (1) there are small localized differences in the conformation of blocked and unblocked iso-1-cytochromes *c*, or (2) the apparent differences in the ^{1}H NMR spectra are artifacts due to dimerization of the unblocked protein during sample preparation, or during the time required to obtain the NMR spectrum. Experiments in progress should help in resolving this question (Ramdas and Nall, unpublished data).

[3] The vertebrate cytochrome *c* numbering system is used to denote amino acid positions in order to facilitate comparison between members of the cytochrome *c* family. Iso-1 has five additional amino-terminal residues and one residue less on the carboxy terminus compared to vertebrate cytochromes *c*. Thus the numbering of iso-1 starts at position −5 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-76 in the iso-1 numbering system.

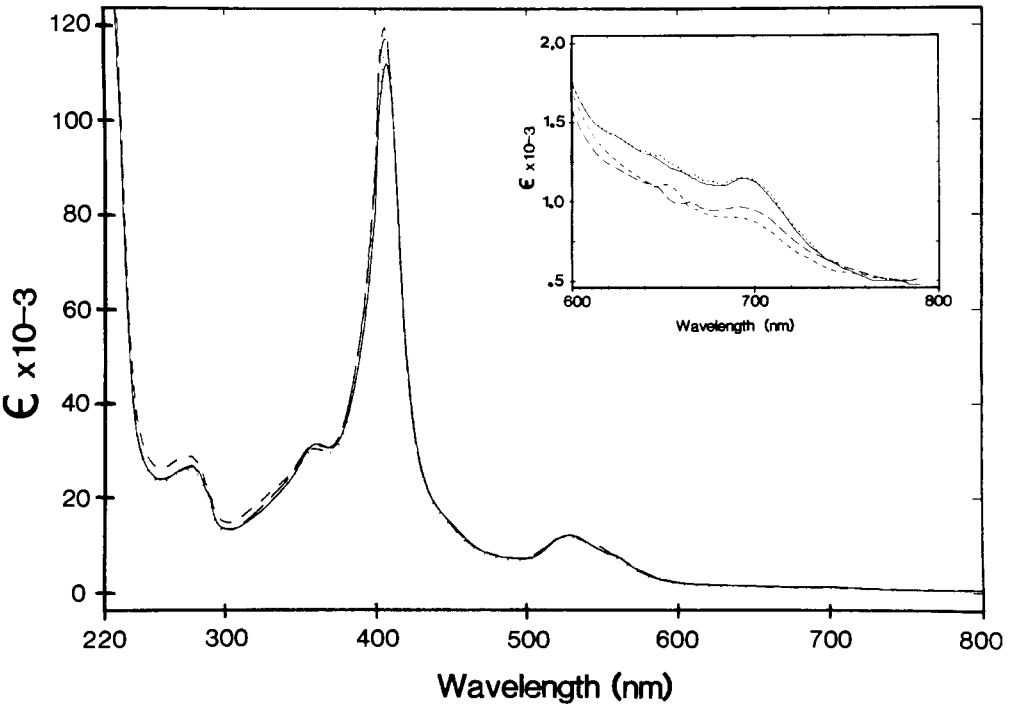

FIGURE 1: Comparison of the UV–visible spectra of oxidized forms of Pro-71 iso-1-MS (—), Val-71 iso-1-MS (···), Thr-71 iso-1-MS (- - -), and Ile-71 iso-1-MS (– –). Molar extinction is plotted vs. wavelength with the 600–800-nm region shown in the insert. The intensity of the 695-nm absorbance band measures Met-80 ligation of the heme (Schechter & Saludjian, 1967). Conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, with protein concentrations in the range of 10^{-5} M.

extinction coefficient as a function of wavelength for oxidized forms of Pro-71 iso-1-MS (wild type), Val-71 iso-1-MS, Thr-71 iso-1-MS, and Ile-71 iso-1-MS. For comparison, the spectra have been normalized to the same value at 410 nm (see Materials and Methods). Between 410 and 600 nm the spectra are the same within errors for all four proteins. In the 600–800-nm region (insert, Figure 1) Pro-71 iso-1-MS (wild type) and Val-71 iso-1-MS are indistinguishable, but important differences are observed for Thr-71 iso-1-MS and Ile-71 iso-1-MS. In particular, the 695-nm absorbance band is reduced in intensity for the mutant forms containing Thr-71 and Ile-71 replacements. This absorbance band is believed to result from Met-80 ligation of the heme iron atom (Schechter & Saludjian, 1967). The 695-nm band is usually correlated with the presence of functional (ascorbate-reducible) conformations of cytochrome *c* (Greenwood & Palmer, 1965) and has been used as a probe of the conformational state of the heme crevice (Kaminsky et al., 1973). Below 410 nm Val-71 iso-1-MS is again indistinguishable from the normal Pro-71 iso-1-MS while Thr-71 iso-1-MS and Ile-71 iso-1-MS exhibit differences. The Soret maximum is shifted slightly from 408 (Pro-71 iso-1-MS and Val-71 iso-1-MS) to 406 nm (Thr-71 iso-1-MS and Ile-71 iso-1-MS), in addition to other spectral differences. This suggests alteration in heme environment or ligation state for the Ile-71 and Thr-71 replacements.

In the ultraviolet region an improved comparison of spectral properties can be made by using second derivative spectroscopy (Ragone et al., 1984). In Figure 2 second derivative spectra are presented. In this spectral region Val-71 iso-1-MS and Ile-71 iso-1-MS are almost identical with wild type while the spectrum for Thr-71 iso-1-MS differs significantly. Nevertheless, analysis of the spectra using methods proposed by Ragone et al. (1984) to estimate the average degree of exposure of tyrosine residues to solvent gives similar fractional exposures for all four proteins (see Discussion; Table I).

Equilibrium Unfolding. In panels A–D of Figure 3 the Gdn·HCl-induced equilibrium unfolding transitions are given for the Pro-71, Val-71, Thr-71, and Ile 71 iso-1-MS proteins, respectively. Changes in both the midpoint and the cooperativity of the transitions are evident. The normal protein, iso-1-MS, has the most cooperative transition and the highest transition midpoint. Val-71 iso-1-MS has the next most cooperative transition and the next highest C_m. Thr-71 iso-1-MS and Ile-71 iso-1-MS do not differ significantly in either cooperativity or transition midpoint (Table II). For an estimated error in C_m of ±0.1 M, the susceptibility of all of the mutant proteins to Gdn·HCl is significantly lower than that for wild type, but differences between the mutant proteins are of marginal significance.

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

protein	r_n[b]	α[c]	av no. of exposed Tyr[c]
Pro-71 iso-1-MS	0.74 ± 0.04	0.39 ± 0.015	1.95 ± 0.08
Val-71 iso-1-MS	0.74 ± 0.01	0.39 ± 0.003	1.95 ± 0.02
Thr-71 iso-1-MS	0.83 ± 0.01	0.42 ± 0.003	2.10 ± 0.02
Ile-71 iso-1-MS	0.79 ± 0.01	0.40 ± 0.003	2.00 ± 0.02

[a] The average exposure of tyrosine side chains to solvent is estimated from second derivative spectra (Figure 2) according to Ragone et al. (1984). Conditions are the same as for Figures 1 and 2. [b] Values for $r_n = a/b$ are ratios of peak to valley heights in the second derivative spectra of the folded proteins (see Figure 2). Ragone et al., (1984) have shown that r_n is a measure of the polarity of the environment of the tyrosine side chains and the tyrosine/tryptophan ratio. Errors are standard deviations for multiple measurements. [c] The average degree of exposure of tyrosines to solvent in the folded states is $\alpha = (r_n - r_a)/(r_u - r_a)$. By use of x = (number of tyrosines)/(number of tryptophans) = 5 for iso-1, $r_u = 2.44$ (the ratio for fully exposed tyrosines) is calculated from eq 1 by using the parameters appropriate for water: $A = 0.21$, $B = 0.66$, and $C = -0.06$. The ratio for completely buried tyrosines, $r_a = -0.325$, is estimated with eq 1 by using parameters appropriate for ethylene glycol: $A = -0.18$, $B = 0.64$, and $C = -0.04$. Since there are five tyrosine residues in iso-1, the average number of exposed tyrosine residues is 5α. Errors are the standard deviations of multiple measurements.

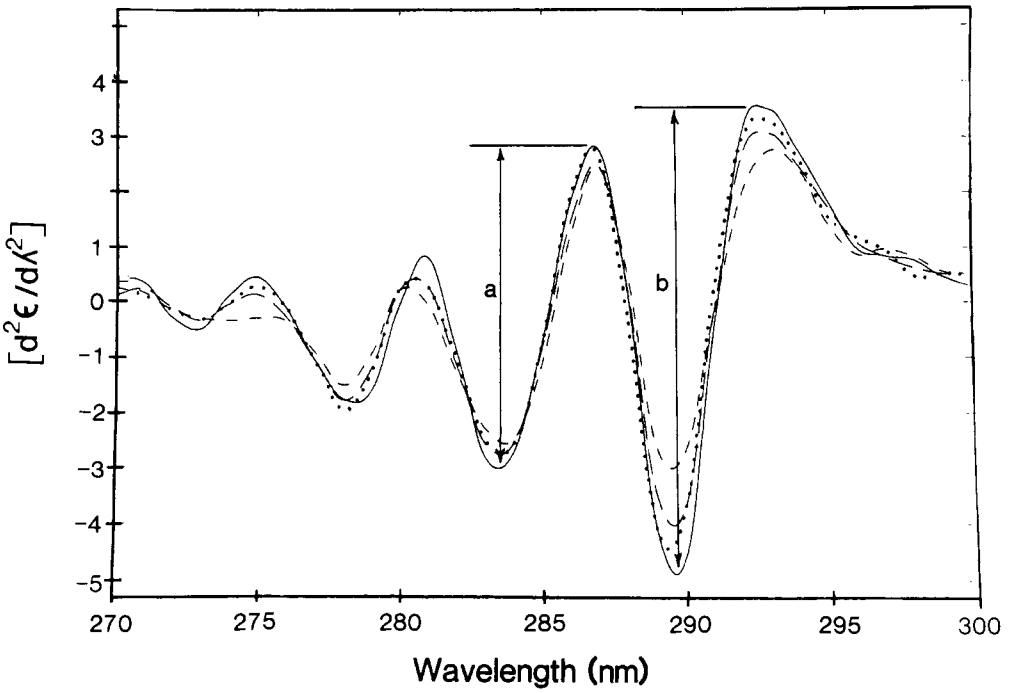

FIGURE 2: Second derivative spectra of Pro-71 iso-1-MS (—), Val-71 iso-1-MS (···), Thr-71 iso-1-MS (- - -), and Ile-71 iso-1-MS (– –). The peak to valley heights *a* and *b* (measures of the polarity of tyrosine side chain environments and the tyrosine/tryptophan ratio, see text) are indicated. Conditions are as in Figure 1.

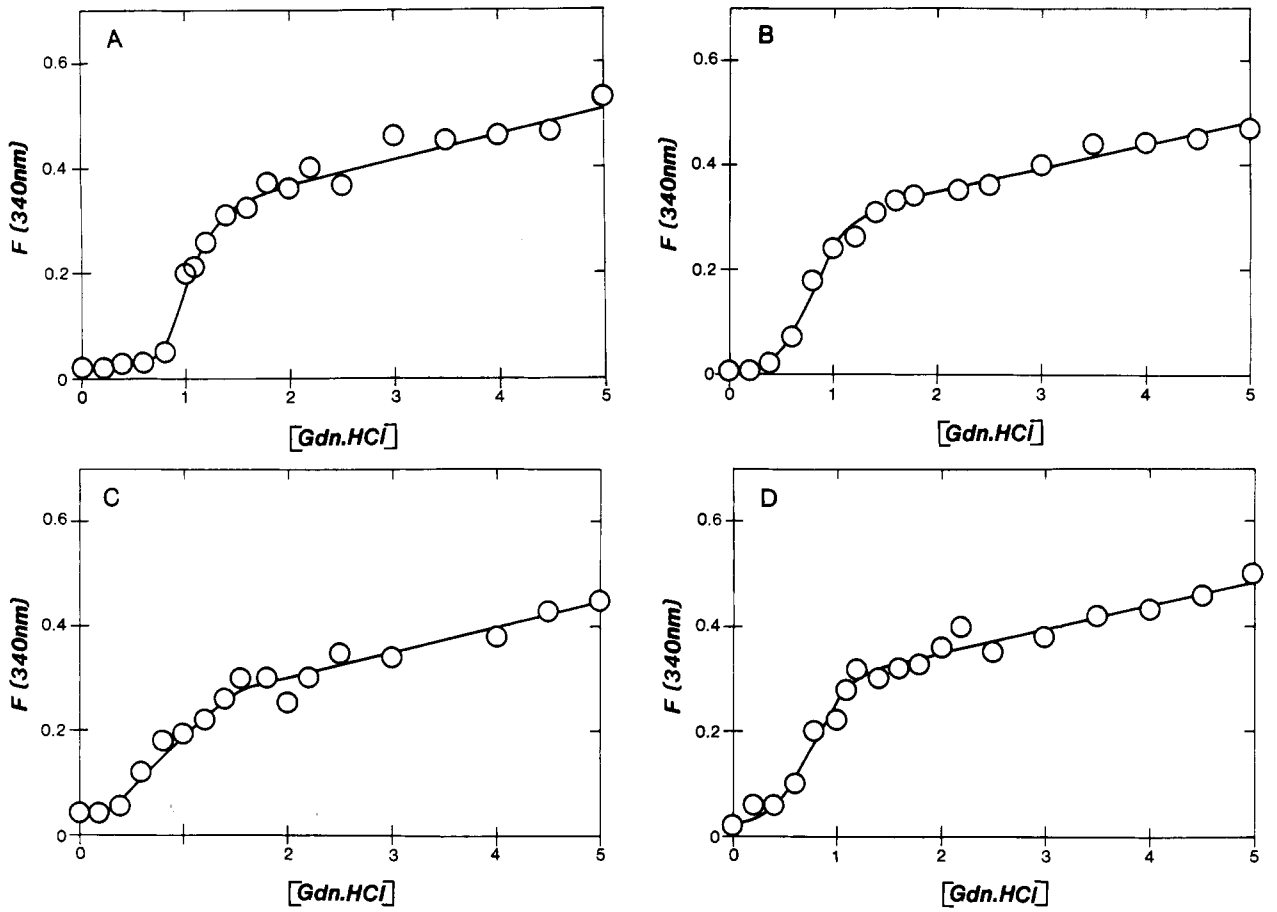

FIGURE 3: Gdn·HCl-induced equilibrium unfolding transitions for (A) Pro-71 iso-1-MS, (B) Val-71 iso-1-MS, (C) Thr-71 iso-1-MS, and (D) Ile-71 iso-1-MS. Fluorescence of the protein solutions (measured at 340 nm with excitation at 280 nm) relative to that of an equal molar concentration of *N*-acetyltryptophanamide is given as a function of Gdn·HCl concentration. Conditions are 0.1 M sodium phosphate, pH 6.0, 20 °C, with protein concentrations of 5×10^{-6} M.

The relative fluorescence of the unfolded protein in the absence of Gdn·HCl can be estimated by extrapolating to 0 M denaturant; the values were as follows: Pro-71 iso-1-MS, 0.28; Val-71 iso-1-MS, 0.26; Ile-71 iso-1-MS, 0.26; Thr-71 iso-1-MS, 0.20; the errors were estimated to be ±0.03. These values for the wild type and mutant iso-1-MS fall in the range

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

protein	C_m[b] (M/L)	$-RT\Delta b_{23}$[b] (kcal·L/M^2)	$\Delta G°_u$[b] (kcal/M)
Pro-71 iso-1-MS	1.07	3.4	3.6
Val-71 iso-1-MS	0.82	3.1	2.6
Thr-71 iso-1-MS	0.72	2.7	1.9
Ile-71 iso-1-MS	0.72	2.6	1.9

[a] The analysis of the Gdn·HCl-induced unfolding transitions (Figure 3) is carried out according to Schellman (1978) with standard conditions of 20 °C, 0.1 M sodium phosphate, and pH 6.0. [b] $\Delta G°_u$ and $-RT\Delta b_{23}$ are obtained from a least-squares analysis of plots of ln K_u vs. C_3 for Gdn·HCl concentrations within the transition region (Figure 3) by using eq 3 and 4. Values for C_m are calculated from $\Delta G°_u$ by using eq 5. Estimated errors are as follows: C_m, ±0.1 M/L; $-RT\Delta b_{23}$, ±0.1 kcal·L/M^2; $\Delta G°_u$, ±0.5 kcal/M.

(0.2–0.3) previously observed for iso-1-AM (Zuniga & Nall, 1983) and iso-2 (Osterhout et al., 1985). The value for Thr-71 iso-1-MS is significantly less than those for the other proteins. This may be due to a systematic error in determining protein concentration by using the same molar extinction coefficient for wild type and mutant proteins.

Discussion

Understanding the sequence–structure relationships of small variations on a known structural theme is a prerequisite to a priori predictions of polypeptide conformation from amino acid sequence. For initial attempts at correlating differences in amino acid sequence with changes in stability, tertiary structure, and function it is important that the folded mutant protein resemble the wild type structure. All three mutant proteins in the present study provide sufficient total activity in vivo to sustain growth of yeast on nonfermentable media (Ernst et al., 1985). Therefore, each of the three proteins containing replacements of Pro-71 fold to native-like proteins, at least to the extent that the structural detail of wild type iso-1 required for in vivo function is retained. The fact that replacement of a conserved residue leads to a functional, native-like structure is of major practical significance: the design and interpretation of experiments can rely heavily on previous results with the wild type protein while the role of an important site in the protein is investigated. The present results comparing different substitutions at position 71 suggest two somewhat contradictory conclusions: (1) replacement of a conserved residue can lead to functional, native-like proteins, and (2) important differences in stability, structure, and function of the mutant proteins are detected easily and depend on the nature of the substituent.

Spectral Properties Consistent with Native-like Structure for Mutant Proteins. The absorbance spectrum of Val-71 iso-1-MS is within error equivalent to that of the normal protein over the entire spectral range (Figure 1). This demonstrates that Pro-71 may be replaced with full retention of the optical properties of normal iso-1-MS. The identity of the spectra of Pro-71 iso-1-MS and Val-71 iso-1-MS in the Soret and 695-nm regions suggests that for all practical purposes (i.e., heme environment and ligation) these two proteins share the same native structure.

Much the same can be said of Thr-71 iso-1-MS and Ile-71 iso-1-MS but with some reservations. For the Thr-71 and Ile-71 substitutions a small shift in the Soret maximum (from 408 to 406 nm) and decreases in intensity of the 695-nm absorbance band (Figure 1) suggest one or all of the following: a folded conformation with less than optimal Met-80 ligation of the heme, an opening of the heme crevice (Kaminsky et al., 1973), or an equilibrium between two or more conformations, some active (i.e., ascorbate reducible) and some inactive (Myer et al., 1980).

An explanation for the small differences in the absorbance spectra of Ile-71 iso-1-MS, Thr-71 iso-1-MS, and Pro-71 iso-1-MS is provided by a well-studied conformational state of the wild type protein: alkaline cytochrome *c* (Greenwood & Palmer, 1965; Brandt et al., 1966; Gupta & Koenig, 1971; Davis et al.,1974). Native cytochrome *c* at neutral pH is converted to a folded but inactive alkaline form by increasing the pH to 9–10. At intermediate pH (pH 7–9) an equilibrium exists between native and alkaline forms. The alkaline form has no 695-nm absorbance band and shows a slight blue shift of the Soret maximum. Thus the shift in the Soret maximum and decreased 695-nm absorbance for Thr-71 iso-1-MS and Ile-71 iso-1-MS can be explained by a decrease in the apparent pK for conversion of these proteins to the alkaline state.[4]

The absorbance properties of intact mutant iso-1-cytochromes *c* are similar to those of the three-fragment complexes of horse cytochrome *c*. Taniuchi and co-workers (Juillerat et al., 1980; Juillerat & Taniuchi, 1986) report shifts in Soret maxima and decreases in 695-nm absorbance in three-fragment complexes of wild type and synthetically prepared mutant forms of horse cytochrome *c*.

Polarity of Tyrosine Environments Similar in Mutant and Wild Type Proteins. Ragone et al. (1984) have provided a simple but sensitive method of estimating the average solvent exposure of tyrosine residues from peak to valley ratios of second derivative spectra. The approach is applicable even in the presence of heme (Fisher & Sligar, 1985) and tryptophan absorbance. As shown in Figure 2, measurement of two peak to valley heights is required: a is measured from a minimum near 283 nm to a maximum near 287 nm, while b is from a minimum near 290 nm to a maximum near 295 nm (293 nm in the present case). A ratio, $r_i = a/b$, is defined for state i. Ragone et al. (1984) have shown that r_i is sensitive to both the tyrosine/tryptophan ratio and to the polarity of the tyrosine environment:

$$r_i = (A_i x + B_i)/(C_i x + 1) \quad (1)$$

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. By use of model compounds, the parameters A_i, B_i, and C_i have been estimated for fully exposed and fully buried residues. Thus, for a protein with a known tyrosine/tryptophan ratio eq 1 can be used to calculate r_i for two limiting states: r_u with all tyrosines fully exposed, and r_a with all tyrosine fully buried. Measurement of r for the native protein, r_n, allows the average degree of exposure of tyrosine residues (α) to be defined (Ragone et al., 1984):

$$\alpha = (r_n - r_a)/(r_u - r_a) \quad (2)$$

In Table I r_n and α values are given for normal and mutant forms of iso-1-MS. The estimates of solvent exposure of tyrosines in the normal and mutant forms of iso-1-MS (α = 0.39–0.42 for five tyrosines/molecule) are in good agreement with that reported for horse cytochrome *c* (α = 0.42 for four tyrosines/molecule) by Ragone et al. (1984). On the basis of the X-ray structure for tuna cytochrome *c*, Dickerson and Timkovich (1975) have classified tyrosine residues at sites homologous to those in iso-1 as buried, half-buried, or exposed. Assuming similar environments for the five tyrosines in tuna cytochrome *c* and iso-1, the average degree of exposure is expected to be about 0.3 compared to our measured values

[4] Measurement of 695-nm absorbance as a function of pH for both mutant and wild type forms of iso-1-MS supports this suggestion. A detailed study of the effect of replacements of Pro-71 on the pH-induced equilibrium between native-like species and alkaline or mutant species is in progress (Ramdas and Nall, unpublished data).

(Table I) of 0.39–0.42. However, a more quantitative estimate of the static accessibility of tyrosine side chains in tuna cytochrome *c* using the method of Lee and Richards (1971) indicates an average degree of exposure of only 0.09 (George D. Rose, personal communication; Rose et al., 1985). This apparent discrepancy may be partially explained by a water molecule buried deep in the protein that forms a hydrogen bond with Tyr-67 (Takano & Dickerson, 1981a). Alternatively, the dynamic accessibility of tyrosines, when averaged over native-like states in solution, may be greater than that estimated from the static, X-ray structure.

Of the mutant proteins shown in Table I, only Thr-71 iso-1-MS has an r_n that differs significantly from that of the wild type protein. In this case the increase in r_n, if attributed to a single tyrosine residue, corresponds to a 15% increase in solvent accessbility of that residue. Since position 71 is bracketed by a fully buried tyrosine at position 67 and a half-buried tyrosine at position 74,[5] it appears that for the present series of substitutions at position 71, the structural integrity of the wild type protein—with regard to tyrosine exposure—is maintained with great tenacity.

Decreases in Stability of Mutant Proteins. A two-state analysis of the unfolding transition curves (Figure 3) allows estimation of the unfolding free energies for wild type and mutant forms of iso-1-MS. Schellman (1978) argues that solvent denaturation can be analyzed by using the following relations (valid in the limit of low protein concentration):

$$\Delta G_u(C_3) = \Delta G^\circ_u + RT\Delta b^\circ_{23}C_3 \quad (3)$$

or

$$\ln K_u = \ln K^\circ_u - \Delta b^\circ_{23}C_3 \quad (4)$$

where ΔG_u is the unfolding free energy, C_3 is the molar concentration of denaturant, $\Delta b^\circ_{23} = -[(\partial \ln K_u)/\partial C_3]_{T,P}$, K_u = (unfolded protein)/(folded protein) is the apparent equilibrium constant, R is the gas constant, and T the temperature in kelvin. Defining the denaturant concentration at the transition midpoint to be C_m, we have

$$\Delta G^\circ_u = -RT\Delta b^\circ_{23}C_m \quad (5)$$

Thus a plot of the natural logarithm of the apparent equilibrium constant vs. the guanidine hydrochloride concentration allows estimation of ΔG°_u and a measure of the cooperativity of the transition, Δb°_{23}. In addition, once ΔG°_u has been determined, C_m can be calculated from eq 5. Data presented in Figure 3, corrected for the Gdn·HCl dependence of the fluorescence of the native and unfolded proteins, have been analyzed by using this model. The results are presented in Table II.

Compared to wild type, the decreases in stability (ΔG°_u) of all of the mutant proteins are comparable. Although the differences between mutant forms are of marginal significance, it is interesting that the order of decreasing stability (Pro-71 iso-1-MS > Val-71 iso-1-MS > Thr-71 iso-1-MS, Ile-71 iso-1-MS) is the same as that for in vivo activity levels estimated from growth rates on lactate media as summarized in Table III (Ernst et al., 1985). The transition midpoints change very little considering the substantial decrease in stability. Reasons for this are that the transitions for the mutant proteins all show significant decreases in cooperativity (compare values for $-RT\Delta b_{23}$, Table II). Such an effect may be attributed to a decrease in the difference between the preferential interaction of the denaturant with the folded state and the preferential interaction of the denaturant with the unfolded state (Schellman, 1978).

Table III: Comparison of in Vivo and Physical Properties[a]

in vivo		in vitro		
protein	approximate sp act. (%)	protein	av no. of exposed Tyr	ΔG°_u (kcal/M)
Pro-71 iso-1	100	Pro-71 iso-1-MS	1.95	3.6
Val-71 iso-1	90	Val-71 iso-1-MS	1.95	2.6
Thr-71 iso-1	60	Thr-71 iso-1-MS	2.10	1.9
Ile-71 iso-1	20	Ile-71 iso-1-MS	2.00	1.9

[a] Physical properties measuring perturbation of the native structure (from Table I) and protein stability (from Table II) are compared to estimates of specific activity in vivo. The comparison is between normal and mutant forms of iso-1-MS and the corresponding forms of iso-1. Estimates of specific activity are obtained by low-temperature spectroscopy of whole yeast cells and growth in lactate medium (Ernst et al., 1985).

Helix Breaker at Position 71? Since position 71 separates two short helical segments in the native protein (assuming structural homology with tuna cytochrome *c*), it is interesting to ask if a residue at this position is required to act as an α-helix breaker. Consistent with this role, P_α values (normalized frequency of occurrence in an α-helix) provided by Levitt (1978) are less than 1 for all of the known substitutions at position 71 that lead to functional proteins (P_α = 1 for random occurrence). Also, the *nonfunctional* mutant iso-1 from which the functional revertants were derived inserts leucine at position 71 (Ernst et al., 1985). Leucine is the *third most likely* residue to occur *within* an α-helix (P_α = 1.30). Parameters from helix–coil transition theory (Zimm & Bragg, 1959) provide measures of the tendency of a given amino acid residue for nucleation (σ) and propagation (s) of an α-helical segment.[6] Scheraga and co-workers have devised methods for measuring these parameters for individual amino acids (Scheraga, 1978). Values at 25 °C have been tabulated [see Creighton (1983)]. Residues found at position 71 in three of the four functional revertants have s values less than 1 and are among the least likely amino acids to initiate helix formation (σ of 10^{-5}–10^{-4}). For comparison, the Pro-71 substitution in the wild type protein would be expected to have both σ and s values near 0 whereas leucine, found at position 71 in a *nonfunctional* mutant, has s = 1.14 and $\sigma = 3.3 \times 10^{-3}$. A possible exception to this trend is provided by the least stable of the functional revertants, Ile-71 iso-1-MS. The helix–coil parameters for isoleucine are $\sigma = 5. \times 10^{-3}$ and s = 1.12. A more detailed analysis from a somewhat different point of view has been presented previously (Ernst et al., 1985) and provides a remarkably successful description of the expected constraints on substitutions at position 71 in terms of short- and long-range perturbations of the native structure.

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[5] The estimates for the degree of exposure of residues 67 and 74 in tuna cytochrome *c* are from Dickerson and Timkovich (1975). George D. Rose (personal communication; Rose et al., 1985) finds the aromatic rings of both residues to be largely buried, with solvent accessibilities of 0.07 and 0.11 for residues 67 and 74, respectively.

[6] The parameter σ is the equilibrium constant for helix nucleation in a region of coil while s is the equilibrium constant for adding one residue of helix onto the end of an existing helical segment.

Registry No. Gdn·HCl, 50-01-1; L-Pro, 147-85-3; L-Val, 72-18-4; L-Ile, 73-32-5; L-Thr, 72-19-5; cytochrome *c*, 9007-43-6.

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