Construction and Characterization of Mutant Iso-2-cytochromes *c* with Replacement of Conserved Prolines†

Ladonna C. Wood,‡ Kamalam Muthukrishnan,§ Terry B. White,‖ Latha Ramdas,⊥ and Barry T. Nall*

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284, and Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, Texas 77225

Received April 6, 1988; Revised Manuscript Received July 13, 1988

ABSTRACT: Oligonucleotide-directed mutagenesis has been used to construct two mutant forms of iso-2-cytochrome *c*. In one, Pro-30 is replaced by threonine; in the other, Pro-76 is replaced by glycine. Both prolines are fully conserved among mitochondrial cytochromes *c* and play important structural and functional roles. Yeast with either the Pro-30 or the Gly-76 mutation has appreciable levels of mutant protein in vivo and grows on media containing nonfermentable carbon sources. Thus, neither mutation blocks protein targeting to mitochondria, uptake by mitochondria, covalent attachment of heme, or in vivo function. As judged by ultraviolet–visible spectrophotometry and proton nuclear magnetic resonance spectroscopy, the nativelike conformation of purified Gly-76 iso-2 at pH 6 is almost indistinguishable from that of the normal protein at pH 6. Ultraviolet second-derivative spectrophotometry, however, suggests an increase in the average number of exposed tyrosine side chains, with 2.25 out of 5 residues exposed for the mutant compared to 1.95 for normal iso-2. Above neutral pH, the protein folds to a mutant conformation possibly related to alkaline cytochrome *c*. Nuclear Overhauser difference spectroscopy of the reduced nativelike conformation allows assignment of several proton resonances and comparison of side-chain conformations of the heme ligand Met-80 in the mutant and the normal proteins. The proton chemical shifts for the assigned resonances are the same within errors for Gly-76 iso-2 and normal iso-2 at pD 6, 20 °C. A distorted resonance line shape and small changes in the nuclear Overhauser difference spectrum, however, suggest a minor change in conformation of the mutant protein involving the β'-H of the Met-80 side chain.

Cytochrome *c* is particularly well suited for study of one of the last remaining problems in biochemical information flow, protein folding. Compared to our detailed knowledge of interconversion between the various forms of one-dimensional information, an understanding of the conversion of genetic information into useful three-dimensional structures is very rudimentary. The heart of the protein folding problem is deciphering the tertiary structure code. Although the information contained in an amino acid sequence is believed to be sufficient for specifying tertiary structure, comparisons in one dimension often miss relationships between proteins that are obvious in three dimensions (Doolittle, 1986). Thus, it appears that the tertiary structure code is degenerate, with much of importance depending on context and juxtaposition of a given sequence of amino acids to other sequences (King, 1986). Not only can unrelated sequences encode the same fold but also identical sequences in different surroundings can encode different folds (Kabsch & Sander, 1984). Solving such a code requires a combination of methodologies, all of which are at hand for cytochrome *c*: genetic techniques to manipulate one-dimensional information (Sherman & Stewart, 1978; Montgomery et al., 1980; Hampsey et al., 1986; Pielak et al., 1985), X-ray crystallography for analysis of the extent of mutation-induced changes in tertiary structures (Sherwood & Brayer, 1985; Louie et al., 1988a,b), proton nuclear magnetic resonance (NMR)[1] spectroscopy to monitor changes in dynamics and segmental stability (Moore et al., 1985; Williams et al., 1985; Senn & Wuthrich, 1985; Wand & Englander, 1985; Wand et al., 1986), and fast reaction methods to measure changes in rate-limiting steps in folding (Ikai et al., 1973; Tsong, 1976; Myer et al., 1981; Parr & Taniuchi, 1979; Zuniga & Nall, 1983; Nall & Landers, 1981). In addition, amino acid sequences for close to 100 members of the cytochrome *c* family provide information about the relative importance (if not the structural or functional role) of individual residues.

Our attention is focused on the role of three conserved proline residues. Conservation of these residues among mitochondrial cytochromes *c* indicates that they are important for some aspect of maturation or function. In addition, prolines are believed to play crucial roles in slowing the rates of at least some folding reactions (Brandts et al., 1975). Previous papers

† Supported by grants from the National Institute of General Medical Sciences (GM 32980) and the Robert A. Welch Foundation (AQ-838). L.C.W. is a Robert A. Welch Foundation postdoctoral fellow. T.B.W. acknowledges support from the Robert A. Welch Foundation and from a National Research Service award (GM-09979). This work was done during the tenure of an established investigatorship of the American Heart Association (B.T.N.).

* Address correspondence to this author at the Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760.

‡ Present address: Department of Biological Chemistry, University of California School of Medicine, Center for Health Sciences, 10833 Le Conte Ave., Los Angeles, CA 90024-1737.

§ Present address: Department of Biochemistry, Rice University, Houston, TX 77251.

‖ Present address: Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, TX 77225.

⊥ Present address: Department of Biochemistry, Baylor College of Medicine, Methodist Hospital A601, Houston, TX 77030.

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; iso-2, iso-2-cytochrome *c* from *Saccharomyces cerevisiae*; Thr-30 iso-2, Thr-71 iso-2, and Gly-76 iso-2, mutant forms of iso-2 in which prolines-30, -71, or -76 are replaced by threonine or glycine; iso-1-MS, iso-1-cytochrome *c* from *Saccharomyces cerevisiae* treated with methyl methanethiosulfonate; Val-71 iso-1-MS, Thr-71 iso-1-MS, Ile-71 iso-1-MS, and Ser-71 iso-1-MS, mutant forms of iso-1-MS in which proline-71 is replaced by valine, threonine, isoleucine, or serine, respectively; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; pD, apparent deuteron activity, pH meter reading without correction for isotope effects; [$^2H_4$]TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate.

0006-2960/88/0427-8554$01.50/0

report construction and characterization of mutant proteins with replacements of Pro-71[2] in iso-1 (Ernst et al., 1985; Ramdas et al., 1986; Ramdas & Nall, 1986) and iso-2 (White et al., 1987) cytochromes *c*. Here we describe construction of mutants in which the two remaining conserved prolines at positions 30 and 76 are replaced. Both mutant proteins have been tested for in vivo function and stability. The more stable mutant, Gly-76 iso-2, has been purified and is compared to the normal protein by several spectroscopic methods. The results show that under carefully selected conditions, iso-2 and Gly-76 iso-2 have very similar structures. In the following paper (Wood et al., 1988) an analysis of mutation-induced changes in the folding/unfolding kinetics is presented that shows that folding of Gly-76 iso-2 to its nativelike structure occurs in the absence of the absorbance-detected slow kinetic phase.

Materials and Methods

Strains and Media. *Escherichia coli* strain JM103 [Δ(lac pro A,B), supE, thi, strA, sbcB15, endA, hspR4/F′ traD36, proA,B, lacI^{q}ZΔM15; (Messing, 1983)] was obtained from Bethesda Research Laboratories, Bethesda, MD. Strain RZ1032 [tet^r, supE, dut-1, ung-1, thi-1, rel A, spo T1/F′ lysA] was from George Weinstock. A yeast strain lacking both iso-1 and iso-2 cytochromes *c*, GM-3C-2 [MATα, leu2-3, leu2-112, trp1-1, his4-519, cyc1-1, cyp3-1 (Faye et al., 1981)], and a yeast–*E. coli* shuttle vector, YEpCYC7(Sau3A) (Montgomery et al., 1980), were obtained from Donna Montgomery. The YEpCYC7(Sau3A) plasmid contains the structural gene for iso-2-cytochrome *c* (CYC7) on a Sau3A fragment of the yeast genome inserted into the *Bam*HI site of YEp13 (Broach et al., 1979). Construction of M13mp10 phage (Messing, 1983) containing the nonsense strand of the CYC7 gene (M13-CYC7) has been described previously (White et al., 1987).

Standard media were used to test growth of yeast. These include YPD (1% yeast extract, 2% peptone, and 2% dextrose), YPG (1% yeast extract, 2% peptone, and 2% glycerol), YPGE (1% yeast extract, 2% peptone, 2% glycerol, and 1% ethanol), and YL (0.67% yeast nitrogen base, 0.05% yeast extract, 0.4% casamino acids, and 1% DL-lactate). Except for carbon sources, media components were from Difco.

Site-Directed Mutagenesis. Oligonucleotide site-directed mutagenesis was performed by a modification (Kunkel, 1985) of the procedure of Zoller and Smith (1983). Uracil-containing M13-CYC7 single-stranded template was isolated following two cycles of phage growth on RZ1032. For the Gly-76 mutant, a mutagenic oligonucleotide with a CCT → GGT (Pro → Gly) codon change, 5′-AATATATT(GGT)-GGTACCA-3′, was synthesized on an Applied Biosystems oligonucleotide synthesizer in the oligonucleotide synthesis facility of the Department of Biochemistry and Molecular Biology. For the Thr-30 mutant, an oligonucleotide with a CCT → ACT (Pro → Thr) codon change was prepared: 5′-AAGTTGGA(ACT)AATTTAC-3′. The template and a mutagenic oligonucleotide were used to generate heteroduplexes according to a one-primer mutagenesis protocol (Zoller et al., 1983). These molecules were transfected into competent JM103. Single-stranded phage DNA was isolated from bacteria in the resulting plaques and screened for the presence of the desired mutation by dideoxy DNA sequencing using a sequencing kit from Bethesda Research Laboratories. The replicative form of the mutant phage was isolated as described by Zoller and Smith (1984) and the CYC7-containing *Bam*HI fragment was subsequently cloned into the *Bam*HI site of YEp13. The resulting plasmids, YEp-PG76 and YEp-PT30, were transformed into GM-3C-2 as described by O'Malley and Douglas (1983).

Yeast Fermentation and Protein Purification. Minor changes in previously described procedures for growing yeast (Nall & Landers, 1981; Zuniga & Nall, 1983) improved overall yields of mutant iso-2. Yeast [strain GM-3C-2-(YEp-PG76)] were grown in 180-L batches on YGE media (1% yeast extract, 2% glycerol, and 1% ethanol) with aeration. In some cases, 0.25% peptone was added to increase cell yields. Use of glycerol–ethanol rather than dextrose as the carbon source may decrease cell yields slightly (3–4 kg of cells/fermentation for glycerol–ethanol) but has the advantage that it is a selective medium for yeast containing functional cytochrome *c*. Thus, large-scale fermentations of strain GM-3C-2 (YEp-PG76) could be carried out without significant plasmid loss. Selective media may also improve protein yields due to plasmid amplification: cells with higher than average levels of plasmid will grow more rapidly because of increased in vivo cytochrome *c* activity. Purification of mutant cytochrome *c* was carried out by standard methods used for normal iso-2 (Nall & Landers, 1981; Zuniga & Nall, 1983) except that all steps were carried out at 5 °C. A single fermentation yielded about 100–150 mg of pure Gly-76 iso-2. SDS–polyacrylamide gel analysis (Laemmli, 1970) indicated that the protein was better than 95% pure.

Protein Sequencing. Protein sequencing was performed as described previously (White et al., 1987). The method involves sequencing the mixture of the four cyanogen bromide peptides released from iso-2 or Gly-76 iso-2. The known protein sequence (Montgomery et al., 1980) combined with a comparison of the amino acids released on each sequencing cycle from normal Pro-76 iso-2 and mutant Gly-76 iso-2 allows sequence changes to be verified. In addition, the procedure provides checks on protein purity and shows that the mutant protein has intact amino and carboxy termini.

UV–Visible Spectroscopy. An Aminco spectrophotometer with a low-temperature attachment was used to estimate in vivo levels of mutant cytochromes *c* by low-temperature spectroscopy of yeast cells frozen in liquid nitrogen (Sherman et al., 1968). UV–visible spectrophotometry of purified Gly-76 iso-2 was performed with a Hewlett Packard 8450A UV–vis spectrophotometer as described previously (Ramdas et al., 1986; White et al., 1987). Estimates of the exposure of tyrosine side chains to solvent from second-derivative spectra were by the method of Ragone et al. (1984).

Nuclear Magnetic Resonance Spectroscopy. Samples of iso-2 or Gly-76 iso-2 were prepared in 99.7% deuterium oxide (Cambridge Isotopes) which contained 0.1 M deuteriated sodium phosphate adjusted to an apparent pD 6.0 with deuterium chloride (meter reading without correction for isotope effects). Proton NMR spectroscopy was carried out on a JEOL GX270WB NMR spectrometer by Fourier transformation of averaged free induction decays. Unless indicated otherwise, the temperature was regulated at 20 °C. 32K data points were obtained with quadrature detection and a sampling rate sufficient for a 30-kHz frequency bandwidth. For 1-D

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

spectra, the residual HOD resonance was suppressed with a selective 150-ms presaturating pulse. Truncated-driven nuclear Overhauser (NOE) difference spectra (Dubs et al., 1979; Wagner & Wuthrich, 1979) were obtained by selective preirradiation for 200 ms either on- or off-resonance in alternate sets of 16 scans. Scans obtained with on- and off-resonance preirradiation were averaged in separate areas of computer memory, Fourier transformed, phased with the same phase parameters, and subtracted. For each NOE difference spectrum, 3000–4000 scans, on- and off-resonance, were obtained with recycle times of 1.28 s. In comparing iso-2 to Gly-76 iso-2, we have adhered closely to the assignment strategy used by Senn et al. (1983) to assign the heme and heme ligand resonances of iso-2.

Results

Mutant Construction. When the one-primer method of Zoller et al. (1983) was used, uracil-containing M13-CYC7 phage were mutagenized with 18-base oligonucleotides designed to change the codons Pro-30 and Pro-76 from CCT (Pro) to ACT (Thr) or GGT (Gly), respectively. Transfection of competent JM103 leads to phage plaques enriched for the desired mutations (Kunkel, 1985). Plaques were screened for mutations by DNA sequencing. Although the efficiency of this procedure is such that over half of the resulting plaques can contain the desired mutation (Kunkel, 1985), we found it necessary to screen three isolates to find the Pro-30 → Thr mutant and four isolates for the Pro-76 → Gly mutant.

The Pro-30 → Thr and Pro-76 → Gly mutant genes were cloned into the *Bam*HI site of YEp13 to generate YEp-PT30 and YEp-PG76, respectively. The yeast–*E. coli* shuttle plasmids containing the PT30 and PG76 mutant alleles of the CYC7 gene were subsequently transformed into GM-3C-2, a strain of *Saccharomyces cerevisiae* that lacks both iso-1 and iso-2 cytochromes c.

Characterization of Mutant Cytochromes c in Vivo. In vivo function of the mutant genes was assessed by spot tests for growth on agar plates containing media in which lactate (YL) or glycerol (YPG) was the sole carbon source. GM-3C-2, which lacks functional cytochrome *c*, did not grow on either medium, while GM-3C-2(YEpCYC7), which contains the wild-type allele of the CYC7 gene (iso-2-cytochrome *c*), grew on both media. After 4 days, the strain containing the Pro-30 → Thr mutation, GM-3C-2(YEp-PT30), was negative for growth on lactate but showed some growth on glycerol. The Pro-76 → Gly mutant strain, GM-3C-2(YEp-PG76), showed less growth than wild type on lactate but grew as well as wild type on glycerol. These results were confirmed in liquid media containing glycerol and ethanol (YPGE) where growth rates for wild type and the Gly-76 mutant were similar (about 5-h doubling times) but growth of the Thr-30 mutant was at least 4-fold slower.

In vivo levels of normal and mutant iso-2-cytochromes *c* were estimated from low-temperature visible spectroscopy of whole yeast cells (Figure 1). YPD plates were inoculated with zig-zag streaks of yeast containing the normal or mutant CYC7 (iso-2) alleles and incubated at 30 °C for several days. Samples were prepared from thin pastes of yeast scraped from the plates and frozen in liquid nitrogen (Sherman et al., 1968). While the Gly-76 mutant (Figure 1A) shows a 547-nm band and other features similar to those observed for wild-type strains [for example, see White et al. (1987) Figure 2A], the Thr-30 mutant (Figure 1B) shows depressed levels of mutant iso-2 as well as other heme proteins. These results show that both mutant apoproteins are translocated to mitochondria and can serve as substrates for covalent attachment of heme. The

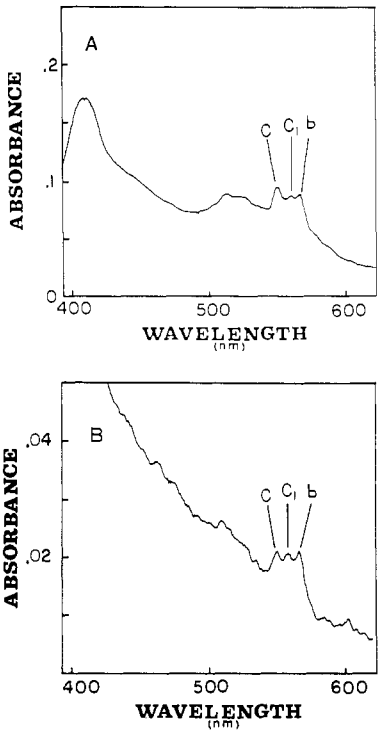

FIGURE 1: Visible absorbance spectra of heme-containing proteins in whole yeast cells frozen in liquid nitrogen (Sherman et al., 1968). Positions of absorbance bands due to particular cytochromes are indicated. The parental yeast strain GM3C-2 (Faye et al., 1981) lacks functional cytochrome *c* and shows little absorbance in this region. Panel A shows GM3C-2 transformed with a plasmid containing the Pro-76 → Gly-76 mutant allele of the CYC7 gene (iso-2-cytochrome *c*): GM3C-2(YEp-PG76). Panel B shows GM3C-2 transformed with a plasmid containing the Pro-30 → Thr-30 mutant allele of the CYC7 gene: GM3C-2(YEp-PT30).

depressed levels of Thr-30 iso-2 suggest either that maturation of the mutant apoprotein is less efficient or that mature Thr-30 iso-2 is less stable than normal iso-2 in vivo.

Purification of Mutant Iso-2. Attempts to purify Thr-30 iso-2 using standard procedures failed to yield sufficient protein for further characterization. Yields of Gly-76 iso-2 were good, although only 20–30% of the yield of normal iso-2. Purified mutant protein was treated with cyanogen bromide, and the resulting four-peptide mixture (see Table I) was subjected to 15 cycles of Edman degradation. Results for Gly-76 iso-2 were similar to those obtained previously in comparing normal iso-2 and Thr-71 iso-2 (White et al., 1987). Except as noted below, the amino acids released on each sequencing cycle were those expected for a protein with the sequence reported for normal iso-2 (Montgomery et al., 1980). Consistent with the DNA sequence of the Gly-76 mutant allele, significant quantities of glycine but not proline were released in cycle 12, confirming the Pro-76 → Gly mutation. As observed before for iso-2 and Thr-71 iso-2 (White et al., 1987), proline was not released efficiently and resulted in significant carry-over to the following sequencing cycle. For Pro-71 in cycle 7, 38% of the proline was carried over and released in cycle 8. For Pro-(−1) in cycle 9, 15% of the proline was released in cycle 10. Incomplete release of proline is well established (Hermodson et al., 1972), and the efficiency of cleavage is known to depend on amino acid sequence (Niall, 1977). Nevertheless, the peptide sequencing experiments confirm the nature of the mutation and show that mature Gly-76 iso-2 has intact amino and carboxy termini.

Characterization of Gly-76 Iso-2 by UV–Visible Spectrophotometry. At alkaline pH, the UV–visible spectrum of

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-Gly-Ser-Ala-Lys-Lys-Gly...
(65)	Ser-Glu-Tyr-Leu-Thr-Asn-Pro-Lys-Lys-Tyr-Ile-Pro-Gly-Thr-Lys...
(81)	Ala-Phe-Ala-Gly-Leu-Lys-Lys-Glu-Lys-Asp-Arg-Asn-Asp-Leu-Ile...
(99)	Thr-Lys-Ala-Ala-Lys-COOH

Cycle	1	2	3	4	5	6	7	8	9	10	11	[12]	13	14	15

[a] The first 15 amino acids are shown for each of the four fragments released on treatment of iso-2 with cyanogen bromide. The position within the iso-2 sequence of the N-terminal residue of each peptide is shown in parentheses. The sequenator cycle in which each set of residues is released is indicated at the bottom of the table. In cycle 12, shown in brackets, the amino acids released from the mutant Gly-76 iso-2 are alanine, glycine, and asparagine instead of alanine, proline, and asparagine.

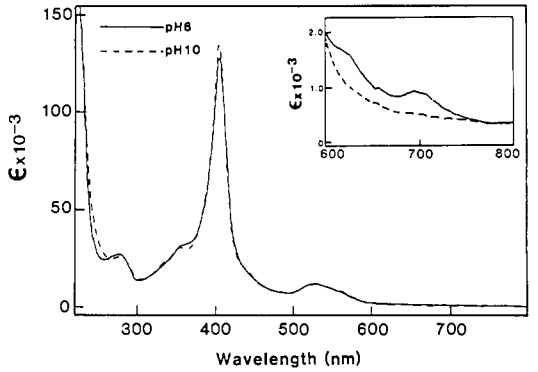

FIGURE 2: UV–visible absorbance spectrum of the oxidized form of purified Gly-76 iso-2 at 20 °C, 0.1 M sodium phosphate. Spectra of the predominantly nativelike conformation (—) at pH 6.0 and of the alkaline conformation (- - -) at pH 10 are shown. The insert is an expansion of the spectra in the neighborhood of the 695-nm absorbance band. The presence of the 695-nm band is an indicator of Met-80 ligation of the heme and the nativelike conformation of cytochrome *c* (Shechter & Saludjian, 1967).

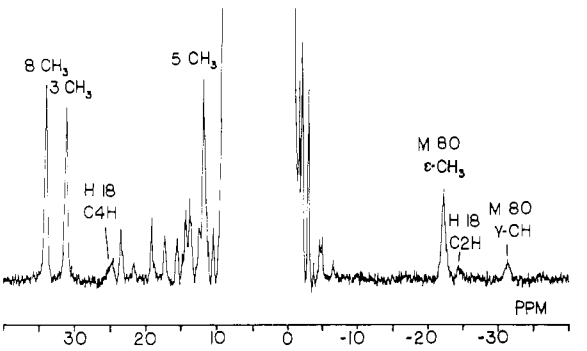

FIGURE 3: ^{1}H NMR spectrum of the paramagnetically shifted resonances of oxidized mutant Gly-76 iso-2 at 20 °C, 0.1 M sodium phosphate, pD 6.0 in D_2O. Positions are indicated for several heme and heme ligand resonances assigned for normal (Pro-76) iso-2 (Senn et al., 1983). Tentative assignment of the resonance near −24.3 ppm to the side-chain C2-H of the heme ligand, His-18, is by comparison with horse cytochrome *c* (Feng and Roder, unpublished results). Between 10 and 25 ppm, there are several resonances from the alkaline conformation, a minor species in equilibrium with the nativelike form.

Gly-76 iso-2 (Figure 2) is much like that of normal iso-2 at high pH. At pH 6, however, there are important differences in the spectra of the two proteins. As with normal iso-2, a prominent 695-nm absorbance band is observed in the 600–800-nm region, indicating that the usual heme ligand, Met-80, is a heme ligand for Gly-76 iso-2. The weak band at 620 nm shows that some high-spin material is present. For normal iso-2 (Osterhout et al., 1985) and iso-1-MS, as well as mutant forms involving substitutions of Pro-71 (Thr-71 iso-2; White et al., 1987; Val-71 iso-1-MS, Ile-71 iso-1-MS, and Thr-71 iso-1-MS; Ramdas, 1987), the high-spin conformation is not evident until below pH 5. At pH 6, the Soret maximum for Gly-76 iso-2 occurs at 406 nm while normal iso-2 has a maximum at 408 nm. For iso-2, spectral changes between pH 6 and 10 involve a blue shift and an increase in the intensity of the Soret maximum, but for Gly-76 iso-2, the blue shift is barely noticeable, although band intensity does increase. These two pH-dependent spectral features, the blue shift and intensity increase, appear to be coupled in iso-2 but are decoupled in the Gly-76 iso-2 spectrum.

Second-derivative spectra in the ultraviolet region may be used to estimate the solvent exposure of tyrosines (Ragone et al., 1984). Second-derivative spectra of iso-2 and Gly-76 iso-2 have been compared at pH 6, 5 °C (data not shown). The ratio of peak to valley heights, $r_n = a/b$, is significantly higher in Gly-76 iso-2 and indicates increased solvent exposure of tyrosines ($r_n = 0.92 \pm 0.02$ and 0.754 ± 0.003 for Gly-76 iso-2 and normal iso-2, respectively).

Comparison of Iso-2 and Gly-76 Iso-2 by ^{1}H NMR Spectroscopy. Figure 3 shows the paramagnetically shifted resonances in the ^{1}H NMR spectrum of oxidized Gly-76 iso-2 at pD 6.0. Positions of assigned heme and heme ligand resonances for ferri-iso-2-cytochrome *c* (Senn et al., 1983) are indicated. In accord with the data of Feng and Roder (unpublished results) for horse ferricytochrome *c*, the resonance near −24.3 ppm is probably the ring C_2H of His-18 instead of Met-80 γ-H. Independent of the specific assignments, strong resonances occur at similar chemical shifts for iso-2 and Gly-76 iso-2, showing that the conformations of the two proteins result in comparable heme magnetic properties. Nevertheless, numerous resonances between 10 and 25 ppm in the spectrum of Gly-76 iso-2 at pD 6 are observed for iso-2 only at higher pD. This suggests that Gly-76 iso-2 at pD 6 is a mixture of nativelike (80–90%) and alkaline (10–20%) forms. Replacement of Pro-71 in iso-2 (White et al., 1987) and iso-1-MS (Ramdas, 1987) also results in small amounts of alkaline-like species under conditions favoring the nativelike conformation.

The ^{1}H NMR spectra of reduced forms of iso-2 and Gly-76 iso-2 are compared in Figure 4. The spectra are strikingly similar, although differences are discernible in both the aromatic and aliphatic regions. Of particular importance are the resolved resonances from the heme meso protons between 9 and 10 ppm and the Met-80 side-chain protons between −1 and −4 ppm, all of which have been assigned in iso-2 by Senn et al. (1983). For short preirradiation times, truncated-driven nuclear Overhauser difference spectroscopy gives intensity changes only for resonances from the nearest neighbors of the preirradiated proton (Wagner & Wuthrich, 1979). The fixed proximity of the heme meso and methyl protons (see Figure

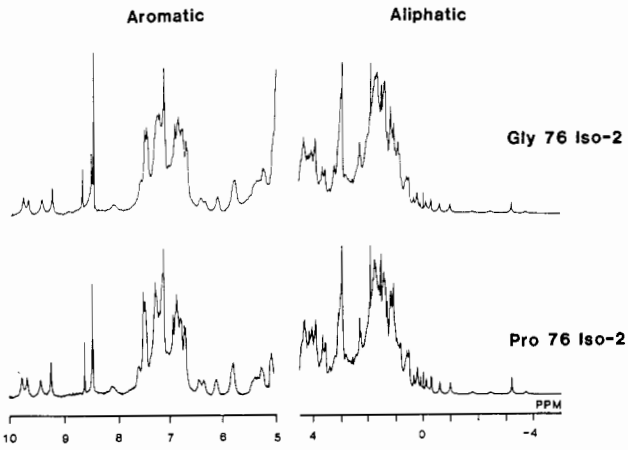

FIGURE 4: Comparison of the ^1H NMR spectra of the reduced forms of mutant Gly-76 iso-2 and normal (Pro-76) iso-2. Relative to the aliphatic regions, the y axes in the aromatic regions have been expanded about 6-fold. Resolved resonances between −1 and −4 ppm are from the side chain of Met-80, a heme ligand.

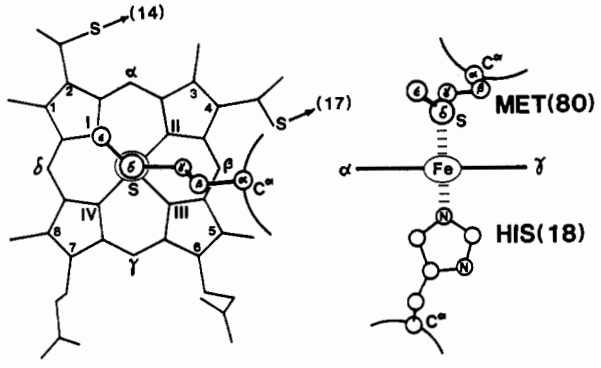

FIGURE 5: Schematic of heme and heme ligands for cytochromes c [after Senn et al. (1983)]. The heme is covalently attached to the polypeptide through thioether bridges to Cys-14 and Cys-17. The Greek letters used in referring to the heme meso protons and to the Met-80 side-chain atoms (Table II and text) are indicated. Heme methyls, thioester bridges, and propionic acid side chains are denoted by clockwise numbering of the β-pyrroles. The strong $(1/r^6)$ distance dependence of difference NOE buildup rates together with the proximity of the heme meso protons to both the heme methyls and components of the Met-80 side chain provides the basis of the NMR spectral assignments (Table II).

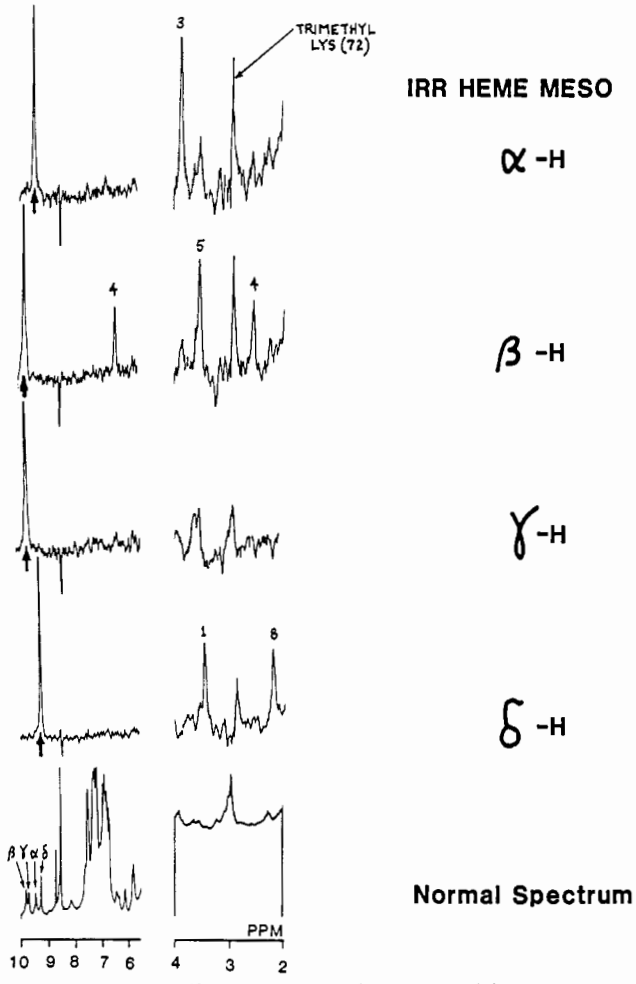

FIGURE 6: NOE difference spectra of the reduced form of mutant Gly-76 iso-2-cytochrome c obtained with 200 ms of selective preirradiation (off-resonance minus on-resonance) of the heme meso protons. Prominent heme proton resonances appearing in the difference spectra have been assigned to heme substituents (Figure 5, Table II) and are numbered by the β-pyrrole atom to which the substituent is bonded. The sharp resonance at 2.95 ppm has been tentatively assigned to N^{ζ}-trimethyllysine-72 and is an inconsistent feature of the difference spectra, probably arising because of an insufficient relaxation delay. Conditions are 0.1 M sodium phosphate in D_2O, pD 6.0, with a protein concentration of about 0.003 M.

5) allows prediction of heme methyl patterns in Overhauser difference spectra obtained with selective preirradiation of particular meso protons. Gly-76 iso-2 (Figure 6) and iso-2 (data not shown; Senn et al, 1983) give the same difference NOE patterns, showing that the same meso proton assignments apply to both proteins. Table II lists chemical shifts for the heme methyl and heme meso protons for both iso-2 and Gly-76 iso-2.

The pattern of difference NOE intensities obtained on selective preirradiation of the resolved resonances between −1 and −4 ppm is essentially the same for iso-2 (Figure 7) and Gly-76 iso-2 (Figure 8). Senn et al. (1983) have previously assigned these resonances in the iso-2 spectrum to specific Met-80 side-chain protons. Our results are in agreement with those of Senn et al. (1983) for iso-2 and, by comparison, provide specific resonance assignments for the Met-80 side-chain protons of Gly-76 iso-2. On preirradiation of the ϵ-methyl of Met-80 (Figures 7 and 8), a triplet appears between 6 and 8 ppm in the NOE difference spectrum for both proteins. On the basis of proximity in the X-ray structure and sequence similarity with other cytochromes c, these resonances are probably ring protons of Phe-82 (Senn et al., 1983). The chemical shifts for the Met-80 and Phe-82 side-chain proton resonances in iso-2 and Gly-76 iso-2 are compared in Table II.

Because of the proximity of Met-80 to the heme (Figure 5), the pattern of NOE intensities in the meso proton region (9–10 ppm region in Figures 7 and 8) provides a sensitive measure of the Met-80 side-chain conformation relative to the heme (Senn & Wuthrich, 1985). The meso proton responses in the NOE difference spectra are similar for iso-2 and Gly-76 iso-2 with one exception. For iso-2, both the β-H and γ-H meso protons show intensity changes on preirradiation of the Met-80 side-chain β'-H proton, but for Gly-76 iso-2, only the β meso proton shows a significant intensity change. In addition, expansion of the spectral region in the neighborhood of Met-80 β'-H (Figure 9) shows that the β'-H resonance line shape is asymmetric for Gly-76 iso-2 but not for iso-2. Taken together, these rather minor spectral differences provide evidence of a small change in the conformation of the Met-80 side chain for reduced Gly-76 iso-2 (see Discussion).

Discussion

Site-directed mutagenesis can be a valuable tool for testing the relationship between primary structure and tertiary

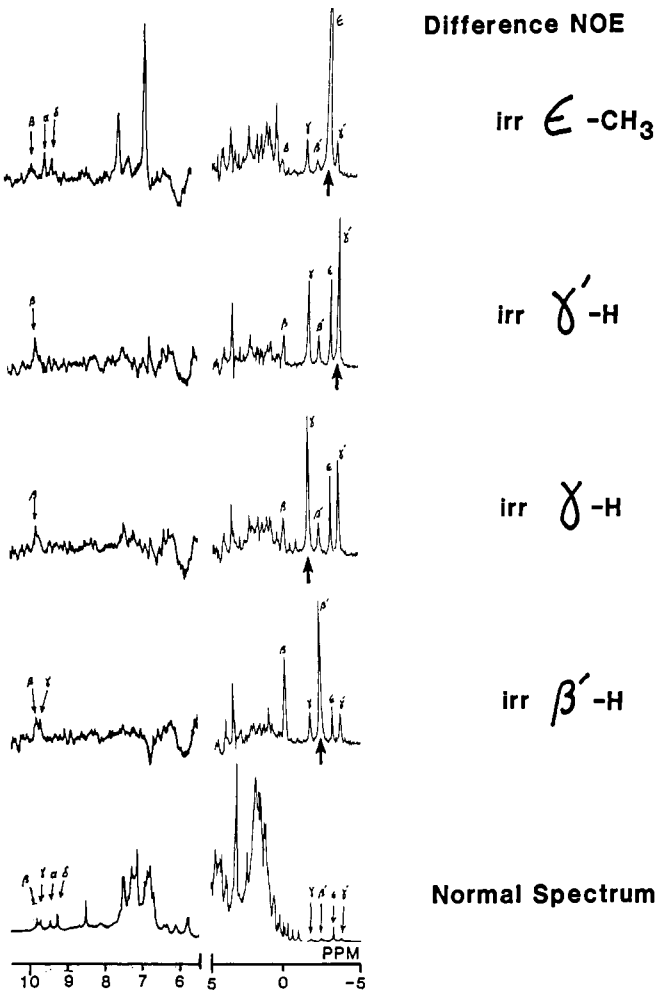

FIGURE 7: NOE difference spectra of the reduced form of wild-type (Pro-76) iso-2-cytochrome *c* obtained with 200-ms preirradiation (off-resonance minus on-resonance) of resolved Met-80 side-chain protons. The observed pattern of NOE difference intensities in the 0 to −4 ppm spectral region supports assignments for these resonances given here (Figure 5, Table II) and elsewhere (Senn et al., 1983). A strong triplet NOE difference is observed between 6 and 8 ppm on preirradiation of the Met-80 ε-methyl. The triplet pattern has been tentatively assigned to the Phe-82 ring protons (Senn et al., 1983). Weaker NOE differences assigned to the heme meso protons are observed between 9 and 10 ppm. The pattern of NOE differences in the 9–10 ppm range provides information on the conformation of the Met-80 side chain relative to the heme (Senn & Wuthrich, 1985). Conditions are the same as for Figure 6.

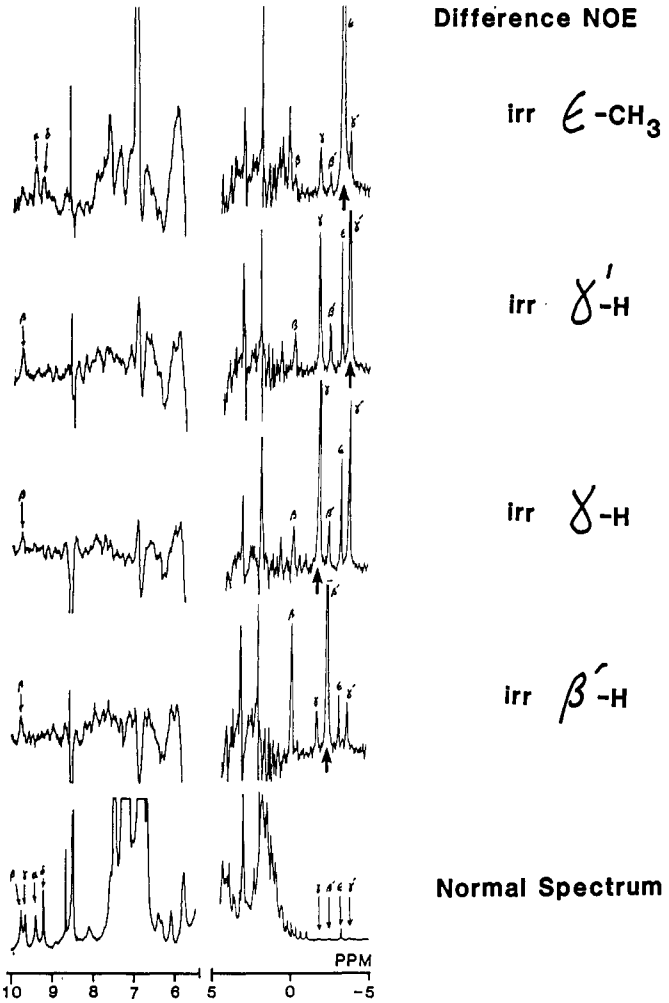

FIGURE 8: NOE difference spectra of the reduced form of mutant Gly-76 iso-2-cytochrome *c* obtained with 200-ms preirradiation (off-resonance minus on-resonance) of resolved Met-80 side-chain protons. The similar patterns of NOE difference intensities (Figure 7 vs Figure 8) and the absence of significant changes in chemical shifts (Table II) suggest that there is little difference between the conformations of the normal and mutant proteins. A possible exception is for preirradiation of the β'-H of Met-80 as indicated by small changes in heme meso proton difference intensities for the normal (Figure 7) and mutant proteins (see text). Conditions are the same as in Figure 6.

structure. In particular, the proline isomerization hypothesis (Brandts et al., 1975) predicts that replacement of a proline should eliminate a slow phase in refolding. However, what if the coupling of folding to imide isomerization depends on protein conformation (Brems & Stellwagen, 1983; White et al., 1987)? Could the loss of a slow phase be due to folding to an alternative (mutant) conformation not requiring the slow step? For example, the presence of a slow phase in folding of normal iso-2 depends on the final conformation: the absorbance-detected slow phase is detected for folding to the native state but not for folding to the alkaline conformation (Nall et al., 1988). Thus, it is important to show that mutant and normal proteins fold to similar conformations when using mutagenesis to test the involvement of specific residues in rate-limiting steps in folding.

Thr-30 and Gly-76 Iso-2-cytochromes c. Altered forms of iso-2 have been constructed in which two highly conserved proline residues have been replaced: Pro-30 and Pro-76. Comparison of protein sequences for a range of cytochromes *c* beyond the mitochondrial forms shows that Pro-30 is more strongly conserved than either Pro-71 or Pro-76 [for example, see Dickerson (1980)]. Instability and poor function in vivo for Thr-30 iso-2 are in accord with the strong preference for proline at position 30 indicated by sequence comparisons. On the other hand, the characteristics observed for the Thr-30 mutant may be due to the choice of the substitute amino acid rather than the loss of the proline residue. Using a fragment complementation system (Taniuchi et al., 1986) in horse cytochrome *c*, Poerio et al. (1986) have found that a Pro-30 → Gly-30 change is less destabilizing than replacement of other less highly conserved residues. Careful analysis of the amino acid preferences of a given location in a protein requires characterization of mutant proteins with as many different replacements as possible (Alber et al., 1987).

The in vivo data do not allow a clear distinction between slow growth as a result of structural instability and slow growth due to decreased specific activity. Fluctuations in gene dosage due to expression of the mutant CYC7 alleles from high-copy yeast episomal plasmids complicate quantitative estimates of in vivo specific activity. Accurate estimates of in vivo function

Table II: Comparison of Reduced Forms of Iso-2 and Gly-76 Iso-2: Assignments and Proton Chemical Shifts for the Heme and for Met-80 and Phe-82 Side Chains[a]

resonance assignment[b]	chemical shift (ppm) Pro-76 iso-2 (normal)	Gly-76 iso-2 (mutant)
heme α *meso*-H	9.40	9.40
heme β *meso*-H	9.76	9.76
heme γ *meso*-H	9.67	9.66
heme δ *meso*-H	9.21	9.21
heme ring methyl 1	3.53	3.55
heme ring methyl 3	3.90	3.91
heme ring methyl 5	3.57	3.57
heme ring methyl 8	2.27	2.27
thioether bridge 2 methyl	1.42	1.44
thioether bridge 2 methine	5.27	5.23
thioether bridge 4 methyl	2.55	2.57
thioether bridge 4 methine	6.40	6.40
Met-80 C_α-H	3.08	3.10
Met-80 C_β-H[b]	−0.21	−0.18
Met-80 $C_{\beta'}$-H[b]	−2.44	−2.42
Met-80 C_γ-H[b]	−1.79	−1.80
Met-80 $C_{\gamma'}$-H[b]	−3.72	−3.69
Met-80 ϵ-methyl	−3.21	−3.18
Phe-82 C2,6-H	6.75	6.76
Phe-82 C3,5-H	7.46	7.46
Phe-82 C4-H	7.18	7.22
Lys-72 ϵ-$N(CH_3)_3$[c]	2.99	2.95

[a] Chemical shifts are in ppm relative to [$^2H_4$]TSP for iso-2 or Gly-76 iso-2 in D_2O at 20 °C, 0.1 M sodium phosphate, pD 6.0. Protein concentrations are about 3 mM. Samples were reduced by bubbling nitrogen through the solutions while in the NMR tube and adding a small amount of sodium dithionite. [b] For the Met-80 C_β and C_γ protons, the higher field resonances have been designated as $C_{\beta'}$-H and $C_{\gamma'}$-H, respectively. [c] Posttranslational modification of a lysine to *N'*-trimethyllysine is assumed to occur at the same location in iso-2 as in yeast iso-1-cytochrome *c* (De Lange et al., 1970).

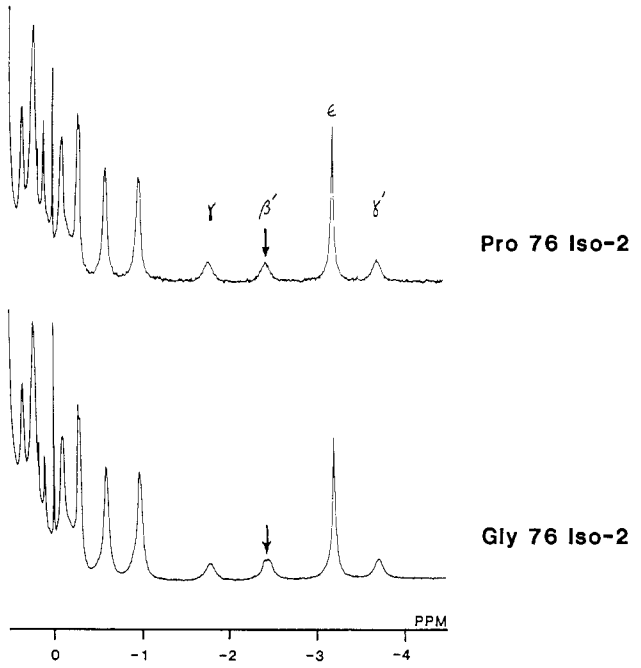

FIGURE 9: Expansion of the spectral regions between 0 and −4 ppm for iso-2 (upper scan) and Gly-76 iso-2 (lower scan) in which resolved resonances from the Met-80 side chain appear. In the upper trace, assignments to specific side-chain protons are indicated (see Figure 5, Table II) for normal (Pro-76) iso-2-cytochrome *c*. In the lower trace, an arrow points out the slightly asymmetric resonance line shape observed for the β'-H of Met-80 for mutant Gly-76 iso-2. The asymmetric liner shape together with changes in the meso proton difference NOE pattern on preirradiation of β'-H (Figure 7 vs Figure 8) suggests a small mutation-induced conformational change in the neighborhood of the β'-H of Met-80 for Gly-76 iso-2 (see text). Experimental conditions are the same as for Figure 6.

require careful comparisons of growth rates of mutant and normal yeast strains with both the genetic background and the gene dosage carefully controlled (Ernst et al., 1985; Schweingruber et al., 1979). Nevertheless, we can conclude that (1) at least partial function is retained by mutant iso-2-cytochromes *c* in which either Pro-30 or Pro-76 is replaced, (2) Gly-76 iso-2 provides higher total activity in vivo than Thr-30 iso-2, (3) Thr-30 iso-2 has a lower steady-state level in vivo than normal iso-2, probably because of decreased stability.

Conformational Heterogeneity of Oxidized Gly-76 Iso-2. Both ^1H NMR spectroscopy and UV–visible spectrophotometry detect small changes in the conformational state of Gly-76 iso-2. For oxidized iso-2 at pH 6, the visible absorbance spectrum has the overall features expected for the nativelike conformation, in particular a 695-nm absorbance band indicating Met-80 ligation of the heme. However, the slight blue shift of the Soret maximum, a small decrease in intensity of the 695-nm band, and a weak 620-nm band indicate that small amounts of alkaline and high-spin forms of the mutant protein are in equilibrium with the nativelike conformation. UV second-derivative spectrophotometry (Ragone et al., 1984) provides a simple but informative measure of changes in solvent exposure of tyrosine side chains in mutant cytochromes *c* (Ramdas et al., 1986; White et al., 1987). For Gly-76 iso-2, the peak to valley ratio, $r_n = 0.92 \pm 0.02$, indicates an average tyrosine exposure of $\alpha = 0.45 \pm 0.01$ or, equivalently, that 2.25 ± 0.04 out of 5 tyosines are exposed to solvent. For normal iso-2 under the same conditions (pH 6, 5 °C), $r_n = 0.754 \pm 0.003$, $\alpha = 0.47 \pm 0.01$, and 1.95 ± 0.01 of 5 side chains are exposed. The increase in the exposure of tyrosine side chains to solvent in Gly-76 iso-2 may be a local effect involving Tyr-74. On the other hand, the increase in tyrosine side-chain exposure, like the weakened 695-nm band and the Soret blue shift, could be an indicator of the presence of small amounts of alkaline species (White et al., 1987).

^1H NMR spectroscopy of oxidized Gly-76 iso-2 is consistent with the results of UV–visible spectrophotometry. The NMR spectrum is dominated by the paramagnetically shifted proton resonances of the heme and heme ligands of the native conformation. Minor resonances show that small amounts of alkaline-like species are in slow exchange with the nativelike conformation. As expected for a native–alkaline conformational equilibrium, on raising the pH these resonances increase in intensity, and the 695-nm absorbance band decreases in intensity. High-spin species are not apparent from the NMR spectrum but would be difficult to detect in small amounts. High-spin and alkaline forms occur for normal iso-2 too, but under more extreme conditions. For normal iso-2, the high-spin transition starts below pH 5, and the alkaline transition begins above pH 7.5.

Changes in Structure of Reduced Gly-76 Iso-2. The ^1H NMR spectrum of reduced Gly-76 iso-2 is very similar to that of normal iso-2. Unlike the spectrum of the oxidized protein, there is little evidence for global conformational heterogeneity. Difference NOE spectra (Figures 7 and 8) show that there is a change in conformation near the β'-H of the Met-80 side chain, but this appears to be a local effect since the chemical shifts of several assigned resonances in the vicinity of the heme are the same for both proteins (Table II). The simplest interpretation of the decrease in the Met-80 β'-H → heme meso γ-H NOE in Gly-76 iso-2 is that the β'-H proton and meso γ-H are further apart in Gly-76 iso-2 than in normal iso-2. However, changes in dynamics or spin diffusion pathways could also give the observed decrease in NOE intensity without

a change in the distance between the two protons [see Wagner and Wuthrich (1979)]. Measurement of the time dependence of the NOE buildup [for example, see Dubs et al. (1979)] should help in distinguishing between the possibilities, but such experiments will require the improved sensitivity of a higher field spectrometer. Difference in the aromatic proton spectra of iso-2 and Gly-76 iso-2 may reflect changes in ring-flip dynamics (or conformation) and warrant a more systematic study of mutation-induced effects on internal dynamics.

ACKNOWLEDGMENTS

This work would not have been possible without yeast and *E. coli* strains provided by other laboratories, and the help and advice of several individuals. In particular, we thank Donna Montgomery, Fred Sherman, George Weinstock, Peter Berget, and Finn Wold. Gary Brayer, Heinrich Roder, and Yiqing Feng provided helpful comments and permission to refer to unpublished results. Efrain H. Zuniga helped with yeast fermentations, protein purifications, and preparation of figures. Christopher C. Q. Chin of the Biochemistry and Molecular Biology Protein Sequencing Laboratory is thanked for the protein sequence analyses.

Registry No. Gly, 56-40-6; L-Pro, 147-85-3; L-Thr, 72-19-5; L-Met, 63-68-3; 5′-AATATATT(GCT)GGTACCA-3′, 116663-11-7; 5′-AAGTTGGA(ACT)AATTTAC, 116668-98-5; cytochrome *c*, 9007-43-6; heme, 14875-96-8.

REFERENCES

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

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 953.

Brems, D. N., & Stellwagen, E. (1983) *J. Biol. Chem. 258*, 3655.

Broach, J. R., Strathern, J. N., & Hicks, J. B. (1979) *Gene 8*, 121.

DeLang, R. J., Glazer, A. N., & Smith, E. L. (1970) *J. Biol. Chem. 245*, 3325.

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

Dickerson, R. E. (1980) *Sci. Am. 242*, 136.

Doolittle, R. F. (1986) *Of URFS and ORFS, A Primer on How to Analyze Derived Amino Acid Sequences*, University Science Books, Mill Valley, CA.

Dubs, A., Wagner, G., & Wuthrich, K. (1979) *Biochim. Biophys. Acta 577*, 177.

Ernst, J. F., Hampsey, D. M., Stewart, J. W., Rackovsky, S., Goldstein, D., & Sherman, F. (1985) *J. Biol. Chem. 260*, 13225.

Faye, G., Leung, D. W., Tatchell, K., Hall, B. D., & Smith, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2258.

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

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., & Walsh, K. A. (1972) *Biochemistry 11*, 4493.

Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73*, 165.

Kabsch, W., & Sander, C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1075.

King, J. (1986) *Bio/Technology 4*, 297.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Louie, G. V., Hutcheon, W. L. B., & Brayer, G. D. (1988a) *J. Mol. Biol. 199*, 295.

Louie, G. V., Pielak, G. J., Smith, M., & Brayer, G. D. (1988b) *Biochemistry 27*, 7870–7876.

Messing, J. (1983) *Methods Enzymol. 101*, 20.

Montgomery, D. L., Leung, D. W., Smith, M., Shalit, P., Faye, G., & Hall, B. D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 541.

Moore, G. R., Robinson, M. N., Williams, G., & Williams, R. J. P. (1985) *J. Mol. Biol. 183*, 429.

Myer, Y. P., Pande, A., & Saturno, A. F. (1981) *J. Biol. Chem. 256*, 1576.

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

Nall, B. T., Osterhout, J. J., Jr., & Ramdas, L. (1988) *Biochemistry 27*, 7310–7314.

Niall, H. (1977) *Proteins (3rd Ed.) 3*, 211.

O'Malley, K., & Douglas, M. G. (1983) *Methods Enzymol. 97*, 344.

Osterhout, J. J., Jr., Muthukrishnan, K., & Nall, B. T. (1985) *Biochemistry 24*, 6680.

Parr, G. R., & Taniuchi, H. (1979) *J. Biol. Chem. 254*, 4836.

Pielak, G. J., Mauk, A. G., & Smith, M. (1985) *Nature (London) 313*, 152.

Poerio, E., Parr, G. R., & Taniuchi, H. (1986) *J. Biol. Chem. 261*, 10976.

Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871.

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

Ramdas, L., & Nall, B. T. (1986) *Biochemistry 25*, 6959.

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

Schweingruber, M. E., Stewart, J. W., & Sherman, F. (1979) *J. Biol. Chem. 254*, 4132.

Senn, H., & Wuthrich, K. (1985) *Q. Rev. Biophys. 18*, 111.

Senn, H., Eugster, A., & Wuthrich, K. (1983) *Biochim. Biophys. Acta 743*, 58.

Sherman, F., Stewart, J. W., Parker, J. H., Inhaber, E., Shipman, N. A., Putterman, G. J., Gardisky, R. L., & Margoliash, E. (1968) *J. Biol. Chem. 243*, 5446.

Sherman, F., & Stewart, J. W. (1978) *Biochemistry and Genetics of Yeast* (Bacila, M., Horecker, B. L., & Stoppani, A. O. M., Eds.) p 273, Academic, New York.

Sherwood, C., & Brayer, G. D. (1985) *J. Mol. Biol. 185*, 209–210.

Taniuchi, H., Parr, G. R., & Juillerat, M. (1986) *Methods Enzymol, 131*, 185.

Tsong, T. Y. (1976) *Biochemistry 15*, 5476.

Wagner, G., & Wuthrich, K. (1979) *J. Magn. Reson. 33*, 675.

Wand, A. J., & Englander, S. W. (1985) *Biochemistry 24*, 5290.

Wand, A. J., Roder, H., & Englander, S. W. (1986) *Biochemistry 25*, 1107.

White, T. B., Berget, P. B., & Nall, B. T. (1987) *Biochemistry 26*, 4358.

Williams, G., Moore, G. R., Porteous, R., Robinson, M. N., Soffe, N., & Williams, R. J. P. (1985) *J. Mol. Biol. 183*, 409.

Wood, L. C., White, T. B., Ramdas, L., & Nall, B. T. (1988) *Biochemistry* (following paper in this issue).

Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468.

Zoller, M. J., & Smith, M. (1984) *DNA 3*, 479.

Zoller, M., Atkinson, T., & Smith, M. (1983) *Manual for Advanced Techniques in Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Zuniga, E. H., & Nall, B. T. (1983) *Biochemistry 22*, 1430.