Characterization of the Guanidine Hydrochloride-Denatured State of Iso-1-cytochrome *c* by Infrared Spectroscopy†

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ABSTRACT: Infrared spectroscopy has been used to monitor residual ordered structure in the denatured state of wild-type and two mutants of iso-1-cytochrome *c*. The technique used involves a careful digital subtraction procedure that removes spectral contributions from buffer, water vapor, and the denaturant guanidine hydrochloride. Reliable and reproducible spectra can be produced using these methods. The data for iso-1-cytochrome *c* show upon denaturation a shift of the structure-sensitive amide I infrared band away from the spectral region associated with random structure. Second-derivative resolution enhancement of the amide I absorption band uncovers several bands which can be associated with various residual ordered structures in the denatured state. Gradual changes in the amide I band after denaturation are also observed as the guanidine hydrochloride concentration is increased. Two single-site mutants of iso-1-cytochrome *c*, which have been shown to have more compact denatured states than the wild-type protein, exhibited denatured-state infrared spectra with significant differences from the wild-type protein spectra. These observations provide new insight into the characteristics of protein denatured states.

A major concern of research in our laboratory is investigation of the role of the denatured state of a protein in modulating its native/denatured-state equilibrium (Bowler et al., 1993). A significant body of research during the last decade has indicated that the denatured state should not be ignored in this equilibrium (Dill & Shortle, 1991; Shortle, 1993). Many elegant crystallographic studies have provided a strong basis (Matthews, 1993) for interpreting stability changes which occur upon mutation of a protein in terms of structural changes to the native state of that protein. However, to date, the main evidence for denatured-state effects on protein stability comes from thermodynamic parameters such as the rate of change of the free energy of unfolding as a function of denaturant concentration, dG/dC, for chemical denaturation (Dill & Shortle, 1991) and the change in heat capacity upon denaturation, ΔC_p, from thermal denaturation studies (Shortle et al., 1988). Relatively few studies exist which attempt to directly correlate thermodynamic evidence of denatured-state contributions to protein stability with structural effects in the denatured state [see, for example, Shortle and Meeker (1989) and Betz and Pielak (1992)].

The difficulty is that obtaining structural data on the denatured state of a protein, which in general contains very little temporally-persistent structure, is not straightforward. The number of studies which have obtained such data is few. Small-angle X-ray scattering data have demonstrated that the radius of gyration of denatured proteins is not that of a random coil (Flanagan et al., 1992; Sosnick & Trewhella, 1992). Similarly, distance measurements by fluorescence spectroscopy imply a compact denatured state for staphylococcal nuclease (James et al., 1992). Infrared (IR)[1] data on heat-denatured RNase A also indicate residual secondary structure (Sosnick & Trewhella, 1992), and new NMR methods have allowed characterization of a persistent hydrophobic cluster for the 434 repressor protein dissolved in 7 M urea (Neri et al., 1992).

In an attempt to address the lack of structural methods available to probe the denatured state of a protein, we have developed methods to observe the IR spectrum of proteins in the presence of denaturing concentrations of guanidine hydrochloride. A major advantage of IR spectroscopy for monitoring the denatured state of a protein is the fast time scale of the process (10^{-13} s) which should minimize conformational averaging effects and hence allow it to be more sensitive to small changes in the ensemble of configurations of the denatured state of a protein caused by single-site mutation. This advantage has been demonstrated clearly by the ability of IR spectroscopy to detect significant structural differences between oxidized and reduced cytochrome *c* (Dong et al., 1992; Bowler et al., 1993; Schlereth & Mantele, 1993) while X-ray crystallography (Berghuis & Brayer, 1992) and NMR spectroscopy (Gao et al., 1991) did not detect significant structural changes. The main disadvantage of IR spectroscopy is the relatively low structural resolution of the technique at its current level of development. Data on the bulk secondary structure of a protein are available; however, the precise location of a particular secondary structure within the primary sequence of the protein cannot currently be defined by this method. In this sense IR spectroscopy provides an important complement to NMR methods under development (Neri et al., 1992) to study the denatured state. NMR techniques will provide high structural resolution, but can be expected to have significant difficulties with structural averaging due to the comparatively slow time scale of NMR spectroscopy.

In this paper we demonstrate the feasibility of observation of the structure-sensitive amide I infrared absorption band of a protein, in the presence of denaturing concentrations of

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[1] Abbreviations: GdnHCl, guanidine hydrochloride; IR, infrared; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

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GdnHCl. Although studies of denatured proteins by infrared spectroscopy are not unusual, the majority of these have been of thermally denatured proteins (Bandekar, 1992; Sosnick & Trewhella, 1992; Surewicz et al., 1987, 1990; Muga et al., 1991) or proteins denatured by organic solvents (Bandekar, 1992; Purcell & Susi, 1984). In these cases, the denaturing agent does not interfere with the peptide bond vibrational frequencies of interest. GdnHCl does overlap with the amide I infrared band. However, recent advances in infrared spectroscopy (Susi & Byler, 1986) have permitted us to develop reliable methods for accurate subtraction of the GdnHCl absorbance from the amide I band. Given the wide use of GdnHCl as an agent for reversible protein denaturation, the availability of a rapid and sensitive method to monitor the denatured state of a protein in its presence is of great importance.

Due to the interest in the possible effects of mutation on the energetics of the denatured state of a protein, we have applied our IR method to wild-type iso-1-cytochrome *c* and two mutants of iso-1-cytochrome *c* which from thermodynamic analysis appear to have more compact denatured states than the wild-type protein (Bowler et al., 1993; Szep and Bowler, unpublished data). The significance of the differences observed in the denatured-state amide I IR spectra of these proteins will be discussed.

EXPERIMENTAL PROCEDURES

Materials. GdnHCl was ultrapure from US Biochemical and was used without further purification. Deionized water was further purified by glass distillation through a Corning MP-6A glass distillation apparatus. All buffer salts were reagent grade. Wild-type and mutant iso-1-cytochromes *c* were prepared and isolated as previously described (Bowler et al., 1993) from the *Saccharomyces cerevisiae* cell line GM-3C-2 (cytochrome *c* deficient; Faye et al., 1981), bearing the iso-1-cytochrome *c* gene cloned into the pRS425 yeast shuttle vector (Christianson et al., 1992). Final purification was by HPLC cation-exchange chromatography (Waters SP 8HR column). Buffers used in HPLC chromatography were (A) 50 mM sodium phosphate, pH 7.2, and (B) buffer A + 1.0 M NaCl. The gradient was as follows: 10 min at 0% B; 0–30% B in 40 min; and 20 min at 30% B; flow rate 2.0 mL/min. The wild-type and mutant proteins all contain the replacement Cys102Ser so that intermolecular disulfide dimerization of iso-1-cytochrome *c* is not a complication in interpretation of the physical properties of these proteins.

Preparation of Samples for IR Spectroscopy. Iso-1-cytochrome *c* mutants were oxidized with a large excess of $K_3Fe(CN)_6$ (5 mg/mg of protein) for a minimum of 2 h. The protein was then isolated from the iron hexacyanide by G-25 chromatography using 20 mM Tris-HCl, pH 7.5, and 40 mM NaCl as the running buffer. Protein concentration and degree of oxidation were evaluated spectrophotometrically (Margoliash & Frohwirt, 1959) using the wavelengths 550 nm (9.0×10^3 M^{-1} cm^{-1}, oxidized), 541.8 nm (9.0×10^3 M^{-1} cm^{-1}, isosbestic point), 526.5 nm (11.0×10^3 M^{-1} cm^{-1}, isosbestic point), 360 nm (28.5×10^3 M^{-1} cm^{-1}, oxidized), and 339 nm (20.9×10^3 M^{-1} cm^{-1}, isosbestic point). The degree of oxidation in separate experiments ranged from 93% to 99% by this measure. The eluant was then concentrated in a Centricon-3 ultrafiltration device (Amicon), rediluted with 2 mL of the buffer, and reconcentrated to approximately 100 μL. The final concentration of the protein stock was 35–38 mg/mL. Samples for IR spectroscopy were prepared by mixing together a 6.0 M stock of GdnHCl (concentration evaluated by refractive index measurements; Nozaki, 1972), the protein stock, distilled water, and a 10× buffer stock to produce a protein solution with the desired concentration of GdnHCl in 20 mM Tris, pH 7.5, and 40 mM NaCl. The final protein concentration in the samples ranged from 15 to 18 mg/mL in a total sample volume of 40 μL.

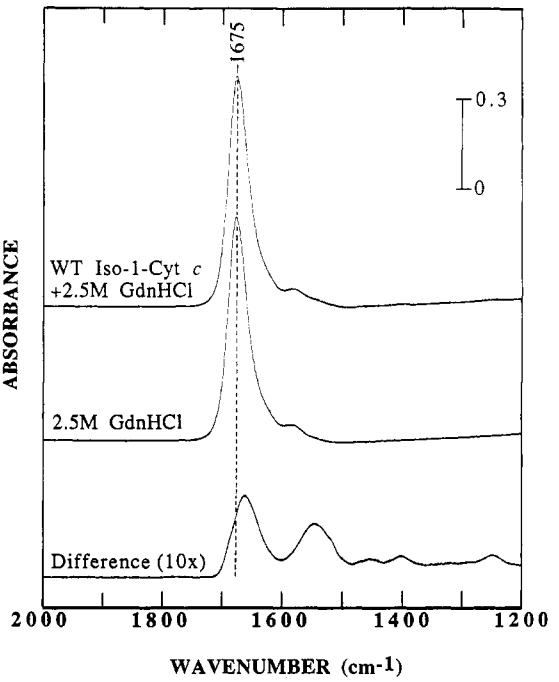

FIGURE 1: IR spectra of wild-type iso-1-cytochrome *c* in 2.5 M GdnHCl before (top) and after (bottom) subtraction of GdnHCl. The middle spectrum is that of 2.5 M GdnHCl used for subtraction.

Acquisition and Analysis of IR Spectra. Samples were placed in a Beckman FH-01 cell with CaF_2 windows and a 6-μm path length for infrared measurement. Spectra were recorded at 20 °C with a Perkin-Elmer Model 1800 Fourier transform IR spectrophotometer equipped with a Hg/Cd/Te detector and interfaced with a Perkin-Elmer 7700 computer. For each sample a 1000-scan interferogram was accumulated in a single-beam mode at a resolution of 2 cm^{-1}. The raw spectrum was then subjected to a careful digital subtraction procedure. First buffer and water vapor backgrounds were subtracted according to previously published methods (Bowler et al., 1993; Dong et al., 1992; Dong & Caughey, 1994). Finally, a background spectrum of aqueous GdnHCl of the same molarity as the sample was subtracted. Subtraction of GdnHCl was done so as to produce a smooth amide I band shape with no discontinuities and to maintain the amide I to amide II peak intensity ratio close to that observed for the native protein. Improper subtraction is evidenced by a doubled peak in the amide I region or an amide I peak with a concave discontinuity. The final difference spectrum was smoothed with a nine-point Savitsky–Golay function (Savitsky & Golay, 1964) to remove possible white noise. Spectra were resolution-enhanced by second-derivative methods using Savitsky–Golay derivative software for a five data point window (Dong et al., 1992; Dong & Caughey, 1994; Susi & Byler, 1986).

RESULTS

In Figure 1, we show the spectrum of wild-type iso-1-cytochrome *c* in 2.5 M GdnHCl solution (top), the spectrum of 2.5 M GdnHCl (middle), and the spectrum of denatured iso-1-cytochrome *c* after subtraction of the GdnHCl (bottom). The peak of the GdnHCl infrared spectrum is separated by

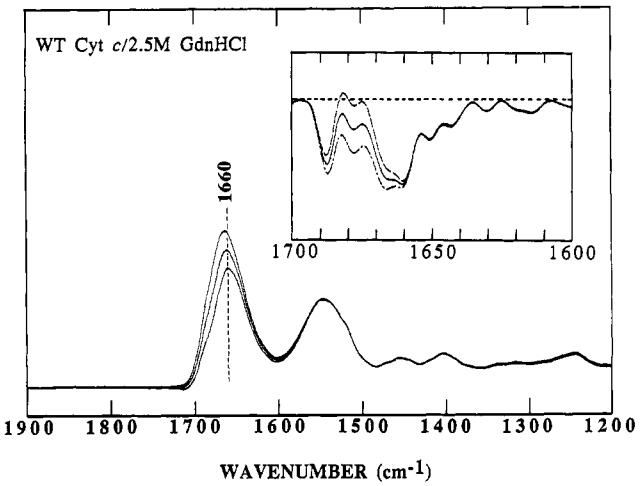

FIGURE 2: Effects of significant undersubtraction (upper spectrum) and oversubtraction (lower spectrum) of the GdnHCl spectrum from the amide I band of wild-type iso-1-cytochrome *c* measured in the presence of 2.5 M GdnHCl. The middle spectrum represents standard GdnHCl subtraction conditions. The inset shows the second derivatives of the spectra in the main panel.

15–18 cm^{-1} from the peak of the amide I spectrum of iso-1-cytochrome *c*, which makes the subtraction relatively straightforward despite the fact that the amide I band constitutes only about 4% of the intensity of the raw spectrum. When the amount of amide I and GdnHCl intensity becomes comparable during the subtraction, the spectrum shows two distinct peaks, allowing the GdnHCl intensity to be removed with a good degree of certainty.

We have intentionally oversubtracted and undersubtracted the denaturant spectrum to investigate the effect of such errors on the line shape and intensity of the amide I spectrum (Figure 2). In the case of both undersubtraction (upper curve, Figure 2) and oversubtraction (lower curve, Figure 2) the relative intensities of the amide I and amide II bands deviate significantly from those observed for the native protein. For the case of oversubtraction a concave feature begins to appear in the amide I contour due to excess subtraction of the GdnHCl spectrum. The upper and lower curves therefore represent cases that we would consider to be outside the range of error in the accuracy of denaturant subtraction. The effects of over- and undersubtraction are best evaluated after resolution enhancement by second-derivative methods (inset, Figure 2). It is apparent that the major effects of inaccurate subtraction are limited to the wavelength region between 1690 and 1665 cm^{-1}. In fact, at frequencies below 1660 cm^{-1} the line shapes and intensities of the components of the amide I band are completely unaffected by incorrect subtraction. Even within the region 1690–1660 cm^{-1}, it is clear that oversubtraction and undersubtraction affect the intensity but not the identity of the components of the amide I band in this region. Thus, a qualitative description of the amide I band is possible even in this region of the amide I spectrum.

Although we cannot be certain of the absolute accuracy of the denaturant subtraction, by using consistent methods, we can achieve a high degree of precision in our subtraction that should allow comparison of differences in the denatured-state spectra of closely related proteins even in the region between 1690 and 1665 cm^{-1}. In Figure 3 the amide I spectra from two separate measurements at 2.5 M GdnHCl after second-derivative resolution enhancement are overlaid for wild-type iso-1-cytochrome *c* and for a Lys73Trp mutant protein. The second-derivative curves from separate experiments at 2.5 M GdnHCl match quite well in terms of both curve shape and

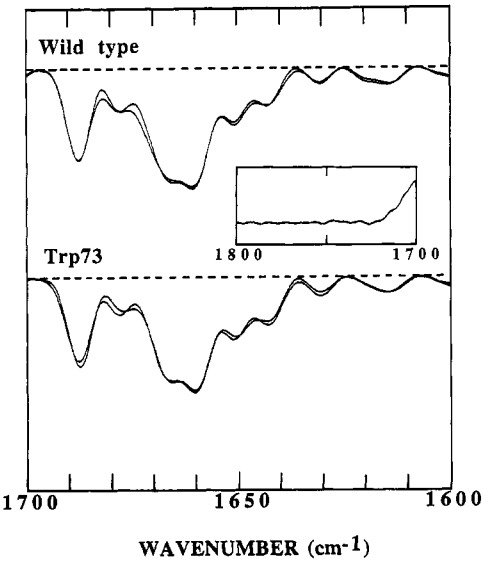

FIGURE 3: Typical reproducibility of the IR second-derivative spectra of iso-1-cytochrome *c* after GdnHCl subtraction. Overlay of two separate trials at 2.5 M GdnHCl is shown for wild-type (upper) and Trp 73 (lower) iso-1-cytochromes *c*. An inset of the IR second-derivative spectrum in the region 1800–1700 cm^{-1} is shown for the wild-type protein (upper) to demonstrate signal-to-noise and the efficacy of water vapor subtraction. The *x*-axis of the inset is compressed relative to the main figure, but the scale of the *y*-axis is unchanged.

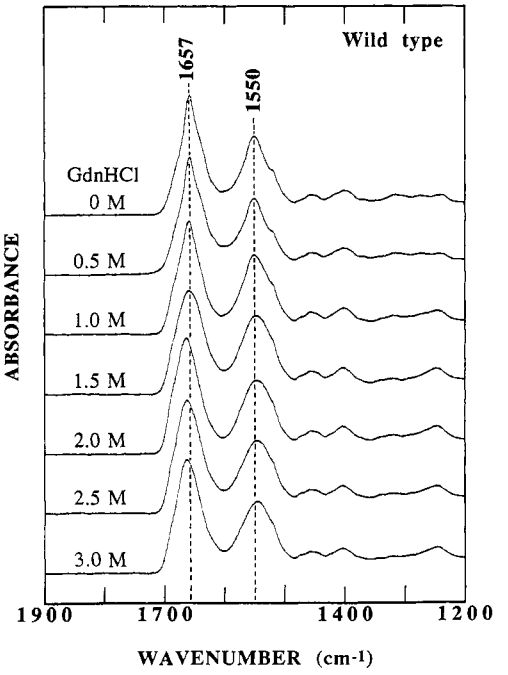

FIGURE 4: IR spectra in the amide I, II, and III regions of wild-type iso-1-cytochrome *c* as a function of the concentration of GdnHCl.

intensity. The largest deviations occur near the position of the GdnHCl band at 1675 cm^{-1} as one might expect if the subtraction is not absolutely precise. What is clear from this figure is that slight imprecisions in the subtraction of GdnHCl have little impact beyond ±5 cm^{-1} from the position of the GdnHCl band.

IR spectra were acquired at a series of concentrations from 0 to 3.0 M GdnHCl. GdnHCl concentrations below, in the midst of, and above those required to unfold iso-1-cytochrome *c* were included in this series. Figure 4 shows the IR absorbance spectrum of iso-1-cytochrome *c* in the region between 1900 and 1200 cm^{-1} as a function of GdnHCl

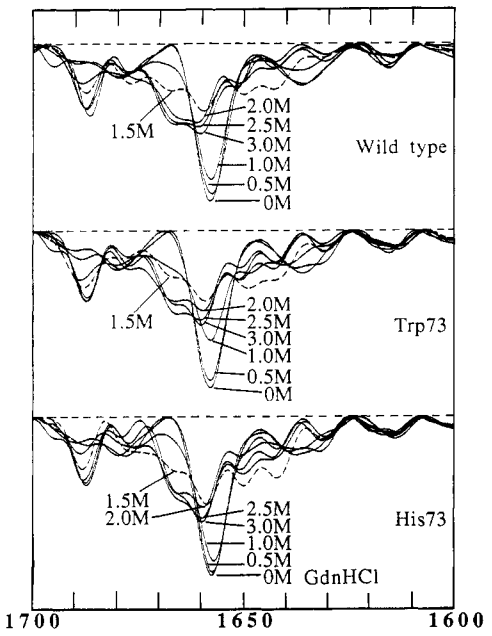

FIGURE 5: IR amide I second-derivative spectra of wild-type (top), Trp 73 (middle), and His 73 (bottom) iso-1-cytochromes *c* at different concentrations of GdnHCl.

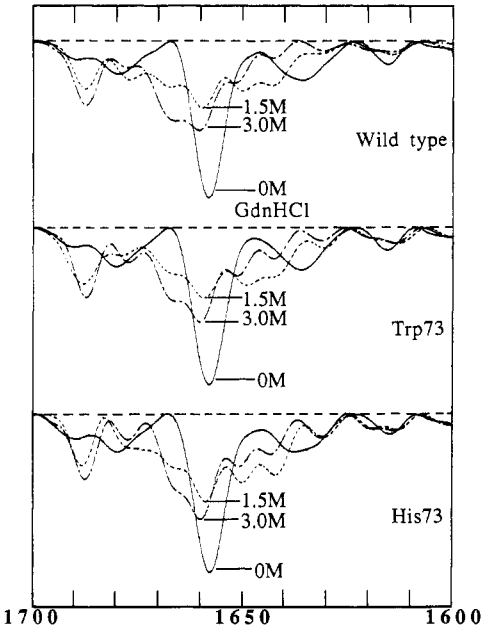

FIGURE 6: IR amide I second-derivative spectra from Figure 5 for only the 0, 1.5, and 3.0 M GdnHCl concentrations.

concentration. For wild type iso-1-cytochrome *c* the midpoint of the unfolding transition as monitored by CD spectroscopy is 1.16 M GdnHCl and the transition zone ranges from approximately 0.8 to 1.5 M GdnHCl (Bowler et al., 1993). In Figure 4, it can be seen that the peak shape and position of the amide I band centered at 1657 cm^{-1} are similar for 0 and 0.5 M GdnHCl. At 1.0 M GdnHCl the peak has broadened and shifted slightly. At 1.5 M GdnHCl the amide I band has broadened significantly and shifted to slightly higher wavenumber, and for 2.0–3.0 M GdnHCl the peak position shifts more. Previous data using CD spectroscopy to monitor unfolding show that at 1.5 M GdnHCl approximately two-thirds of the folded-state ellipticity at 220 nm had been lost. By 3.0 M GdnHCl more than 80% of the folded-state ellipticity at 220 nm had been lost (Bowler et al., 1993). The changes we observe in the IR spectrum after the end of the unfolding transition are thus consistent with previous observations by CD spectroscopy. It is interesting to note at this point that experimental data from several sources suggest that random structure will have a peak at 1645 ± 5 cm^{-1} in the amide I infrared region (Susi & Byler, 1986; Dong et al., 1992; Dong & Caughey, 1994). In fact, the peak of the amide I band of iso-1-cytochrome *c* shifts away from this region upon GdnHCl denaturation. Although inaccurate subtraction of the denaturant spectrum can cause slight shifts in the amide I peak position, even the extreme oversubtraction shown in Figure 2 does not reverse this trend.

In addition to the wild-type protein, we have studied two mutants of iso-1-cytochrome *c* which replace the highly solvent-exposed lysine 73. The Trp 73 and the His 73 mutants decrease the slope of free energy of unfolding versus [GdnHCl] plots by 15% (Bowler et al., 1993) and 26% (Bowler and Szep, unpublished data), respectively, suggestive of more compact denatured states than found for the wild-type protein (Dill & Shortle, 1991). Figure 5 presents second-derivative amide I IR spectra of all three proteins as a function of GdnHCl concentration. Generation of the second derivative allows resolution of the features underlying the absorption bands shown in Figure 4 (Susi & Byler, 1986; Surewicz & Mantsch, 1988). In general, the gross features of the denaturation of all three proteins as monitored by second-derivative amide I IR spectroscopy are similar as might be expected for three proteins that differ only by a single amino acid. The major band at 1657 cm^{-1} due to α-helix is lost gradually and replaced by three main features at 1687, 1666, and 1660 cm^{-1} in the spectra of the denatured proteins. The loss in intensity of the α-helix band at 1657 cm^{-1} occurs at lower concentrations of GdnHCl for the Trp 73 protein compared to the other two proteins, consistent with the GdnHCl denaturation midpoint of 1.02 M for this protein (Bowler et al., 1993). The GdnHCl denaturation midpoint of the His 73 protein is indistinguishable from that of the wild-type protein (Bowler and Szep, unpublished data). It is evident that the second-derivative spectra continue to change after the unfolding transition for all three proteins, suggesting that the structure of the unfolded state continues to change as the GdnHCl concentration increases. One noteworthy feature related to the changing denatured state is a small peak at 1695 cm^{-1} that is observed only at 2.0 M GdnHCl for all three proteins. The 1695-cm^{-1} peak is fully reproducible in a total of seven different samples of iso-1-cytochrome *c* and its mutants measured at this concentration. It therefore must be a structural feature of iso-1-cytochrome *c* characteristic of this concentration of GdnHCl.

Closer scrutiny of the second-derivative spectra of these three proteins in the denatured state ([GdnHCl] ≥ 1.5 M) shows some significant differences in detail. These differences are seen more clearly in Figure 6, where only the 0, 1.5, and 3.0 M GdnHCl spectra are shown. First, note the high degree of similarity of the native-state spectra of all three proteins, indicative of nearly identical native-state structures. Much more significant differences can be noted in the denatured-state spectra. Comparison of the two bands near 1650 and 1640 cm^{-1} in the denatured-state spectra shows that the persistence of these two bands, particularly of the 1640-cm^{-1} band, as a function of the concentration of GdnHCl follows the order wild type < Trp 73 < His 73. A difference of lesser magnitude can also be seen for a band near 1630 cm^{-1}, a region normally associated with β-sheet. This band is most

persistent for the His 73 protein and is slightly more persistent for the Trp 73 protein than for the wild-type protein. With some degree of caution, we note that comparison of the relative intensities of the bands at 1660 and 1666 cm^{-1} shows that the relative intensity of the band at 1660 cm^{-1} increases in the order wild type < Trp 73 < His 73, the same trend noted above.

DISCUSSION

The first point to consider regarding the spectra of denatured proteins in the presence of GdnHCl is the reliability of the subtraction procedure. Several points make us quite confident of our ability to accurately subtract the contribution of GdnHCl to the spectrum. Spectra at 0.5 M GdnHCl, before the unfolding transition, are essentially identical to the native spectra at 0 M GdnHCl. Tests of intentional oversubtraction and undersubtraction indicate that the region between 1690 and 1665 cm^{-1} is most affected, whereas outside this region effects are negligible. Quantitative interpretation of peaks in the second-derivative spectrum between these two frequencies should therefore be approached cautiously. Although absolute accuracy of the subtraction cannot be assured, the use of consistent methods gives good reproducibility of spectra at a given concentration, which should permit qualitative comparison of the denatured-state spectra of closely related proteins even between 1690 and 1665 cm^{-1}. One might also worry that guanidine molecules bound to the protein could have a different vibrational spectrum than unbound molecules, leading to artifacts in amide I spectra obtained in the presence of GdnHCl. However, the 0.5 M GdnHCl sample shows no evidence of any additional peaks due to protein-bound guanidine, suggesting that such effects are minimal. Similarly, protein-bound water molecules do not seem to have an altered vibrational spectrum from unbound water molecules (Venyaminov & Kalnin, 1990). Given the above considerations, this technique should be limited currently to qualitative comparison of denatured-state spectra. Rigorous quantitative analysis of these denatured-state spectra at the present level of development of this technique could lead to unwarranted conclusions. Another limitation of the method is that the Hg/Te/Cd detector saturates near 4.0 M GdnHCl at the cell path length used, which presently will prevent application of this method to proteins that are fairly resistant to GdnHCl denaturation.

The qualitative features of the denatured-state IR spectra of iso-1-cytochrome *c* have several interesting characteristics. In Figure 5 it is evident that the amide I IR spectra of each of these proteins continue to change after the unfolding transition is complete ([GdnHCl] ≥1.5 M, unfolding at 1.5 M GdnHCl is in the range of 90–95% complete for all three proteins; Bowler et al., 1993; Bowler and Szep, unpublished results). Such a result is inconsistent with the denatured state of this protein being a random coil. If the initial unfolded state of the protein were random, then further changes to the infrared spectrum would not occur as the GdnHCl concentration is increased. These IR data are consistent with Dill's Heteropolymer theory, which predicts that the denatured state of a protein will continue to expand as the denaturant solution becomes a better solvent for the unfolded protein (Alonso & Dill, 1991; Dill & Shortle, 1991). These data are also consistent with the progressive increase in fluorescence intensity observed after completion of the GdnHCl-induced unfolding transition of cytochrome *c*. These fluorescence data indicate that the Trp 59 to heme distance in denatured cytochrome *c* continues to increase as the concentration of GdnHCl is increased (Tsong, 1974, 1975). Similarly, a study of the rate of deuterium exchange of the backbone amide protons of oxidized iso-1-cytochrome *c* by NMR spectroscopy demonstrates that the free energy needed for local unfolding to occur, so as to permit H/D exchange, is higher than the global denaturation free energy for some main-chain amides (Marmorino et al., 1993). This result is also consistent with the presence of residual ordered structure after the unfolding transition of oxidized iso-1-cytochrome *c*. The IR data presented here also demonstrate that the expansion of compact denatured states is coupled to redistributions of residual ordered structure in the denatured state of a protein. The changing nature of the structure of the denatured state is most evident in the 1695-cm^{-1} band that appears only at 2.0 M GdnHCl, the band at 1660 cm^{-1} that gradually grows in intensity as the GdnHCl concentration increases above 1.5 M, and in the bands near 1650 and 1640 cm^{-1} which are initially rather prominent in the denatured state and then decrease in intensity at higher GdnHCl concentrations. Other bands such as the prominent band at 1687 cm^{-1} and the small band near 1630 cm^{-1} seem to be more stable as a function of GdnHCl concentration after denaturation.

Published IR spectra of denatured proteins are relatively common (Bandekar, 1992). In comparing our GdnHCl-denatured protein to spectra of proteins denatured by other means, we will limit our comparisons to solution-phase examples. In several studies of thermally-denatured proteins a commonly observed feature is a strong band near 1615 cm^{-1} (Byler & Purcell, 1989; Muga et al.,1991; Surewicz et al., 1987, 1990). This band has been assigned by these authors to extended β-structure resulting from protein aggregation. Interestingly, IR spectra of thermally-denatured RNase A acquired under conditions where the denaturation is completely reversible show no band at 1615 cm^{-1} (Sosnick & Trewhella, 1992). The second-derivative IR spectra of GdnHCl-denatured iso-1-cytochrome *c* (Figures 5 and 6) do not show a significant component near 1615 cm^{-1}, suggesting that aggregation is not occurring in the presence of GdnHCl. Denaturations in the presence of methanol show strong bands near 1685 and 1615 cm^{-1} for several proteins (Purcell & Susi, 1984). Its seems that aggregation may be an important process in these cases as with thermal denaturation. We do observe a strong band near 1685 cm^{-1}. This band frequency is commonly assigned to β-turn structures (Krimm & Bandekar, 1986; Bandekar, 1992; Dong et al., 1992; Susi & Byler, 1986), suggesting that turn structures may be a common feature of denatured states. Recent studies of protein denaturation in DMSO (Jackson & Mantsch, 1991) and halogenated alcohols (Jackson & Mantsch, 1992) have shown that at moderate mole fractions of organic solvent in D_2O the IR spectra resemble those observed for methanol denaturation, with a strong band near 1620 cm^{-1} attributed to protein aggregated into intermolecular β-sheets. At high mole fractions the main feature of the denatured-state IR spectra is a band near 1663 cm^{-1}. It is possible that, at high mole fractions, these organic solvents induce structures that resemble to some extent the types of denatured states produced by GdnHCl.

The IR spectra of thermally-denatured horse heart cytochrome *c* have been published previously (Muga et al., 1991). Comparison of these data with IR data for GdnHCl-denatured iso-1-cytochrome *c* shows substantial spectral differences between the two denatured states. Upon thermal denaturation the amide I band of horse heart cytochrome *c* shifts toward 1640 cm^{-1}, opposite in direction to the shift of the amide I peak for GdnHCl denaturation of iso-1-cytochrome *c*. An

absorbance near 1615 cm^{-1} is a prominent feature, and a shoulder near 1685 cm^{-1} is observed. Obviously, substantial differences exist between these two types of denatured states. The only common feature is the contribution near 1685 cm^{-1} for both, suggesting that both types of denatured states have significant amounts of turn structure. Although the native folds of iso-1-cytochrome *c* and horse heart cytochrome *c* are substantially the same (Bushnell et al., 1990), it should be noted that there are significant primary sequence differences (about 60% homology) between these two proteins which could affect the characteristics of the denatured state. Some of the observed differences between the GdnHCl-denatured and thermally-denatured IR spectra may be due to the primary sequence differences and not the mode of denaturation.

In comparing the wild-type protein to the two mutant proteins, differences in several regions of the spectrum were noted: the overall intensities of the bands near 1650, 1640, and 1630 cm^{-1} and the relative intensities of the 1666- and 1660-cm^{-1} bands. In each of these cases the prominence of the effect followed the order His 73 > Trp 73 > wild type. This progression is also the order from most compact to least compact denatured state as judged from the slope of a free energy of unfolding versus [GdnHCl] plot (see above). This qualitative correlation suggests that the differences in the structures of the denatured states of these proteins may indeed derive from the relative compactness of their denatured states.

Precise assignment of the structures associated with each of the bands in Figures 5 and 6 is not a simple issue. While several empirical methods have been quite successful in evaluating the secondary structure of proteins (Dong et al., 1990, 1992; Dong & Caughey, 1994; Susi & Byler, 1986; Kalnin et al., 1990), there has been some concern recently about the difficulties inherent in these methods and the possibility of exceptions to the empirical assignments (Surewicz et al., 1993). Calculations of amide I bands of proteins based on floating oscillator (Torii & Tasumi, 1992a) and doorway state (Torii & Tasumi, 1992b) models question the absoluteness of some of the empirical assignments of amide I band components to particular secondary structure types. In general, bands in the region of 1658–1650 cm^{-1} have been assigned to α-helix, bands in the regions 1640–1620 cm^{-1} and 1695–1690 cm^{-1} to β-sheet, bands between 1690 and 1660 cm^{-1} to turn structures, and bands near 1663 cm^{-1} to 3_{10} helices (Dong et al., 1992; Dong & Caughey, 1994; Susi & Byler, 1986; Kalnin et al., 1990, Krimm & Bandekar, 1986; Bandekar, 1992; Venyaminov & Kalnin, 1990). Some studies suggest a band near 1675 cm^{-1} may represent β-sheet structure (Susi & Byler, 1986), and a couple of studies have suggested that 3_{10} helices may produce bands at 1639 cm^{-1} for spectra obtained in D_2O (Holloway & Mantsch, 1989; Prestrelski et al., 1991a). The assignment of random structure is more variable. Recent IR work on superoxide dismutase in H_2O, which contains about 44% unordered loop structure in crystals (Trainer et al., 1982), suggests that bands near 1647 and 1658 cm^{-1} both represent unordered structure (Dong et al., manuscript in preparation). Similarly, work in D_2O on the platelet-derived growth factor (Prestrelski et al., 1991b) has also suggested that loop structures may produce bands in the 1655–1650 cm^{-1} region [it is typical to observe shifts of about 5 cm^{-1} to lower wavenumber for spectra in D_2O relative to H_2O; see, for example, Holloway and Mantsch (1989)]. Previous work had indicated that random coil is normally associated only with bands near 1648 cm^{-1} (Dong et al., 1992; Dong & Caughey, 1994; Susi & Byler, 1986). Another possible ambiguity in accurate assignment of band frequencies to secondary structure types arises from the possibility that interactions of GdnHCl with the protein may shift the characteristic secondary structure frequencies of the amide I band somewhat. Given this context, assignment of the major band at 1687 cm^{-1} of denatured iso-1-cytochrome *c* to β-turn structure is fairly safe. The assignment of the other bands is more ambiguous. The band near 1666 cm^{-1} could represent turn, or 3_{10} helix structure, the bands at 1660 and 1650 cm^{-1} random structure or α-helix, and the bands at 1640 and 1630 cm^{-1} β-sheet or contributions from the E_1 component of vibration due to α-helix structure (Torii & Tasumi, 1992a,b). Although it is difficult to make precise assignments for the amide I vibrational spectra of the GdnHCl-denatured state of iso-1-cytochrome *c*, it is clear from the above discussion of possible assignments that GdnHCl-denatured iso-1-cytochrome *c* is not predominantly random coil and that significant ordered structure persists after unfolding. We note that much of this ordered structure is likely transient in nature, considering the time scale of infrared spectroscopy.

The recent doorway state calculations of Torii and Tasumi (1992b) provide an intriguing interpretation of our unfolded amide I spectra. Native iso-1-cytochrome *c* is predominantly an α-helical protein. Their data suggest that the band at 1657 cm^{-1} be assigned to the A_1 vibrations of α-helices and that some of the intensity below 1640 cm^{-1} be assigned to the E_1 vibrations of α-helices. Calculation of the position of the A_1 and E_1 band frequencies as a function of helix length indicates that both will shift to higher frequencies as the helices become shorter (to maximum values of 1663 and 1648 cm^{-1}, respectively). Either or both of the bands at 1666 and 1660 cm^{-1} could represent shortened α-helices produced upon denaturation, and either or both of the bands at 1650 and 1640 cm^{-1} might represent their related E_1 components. In this context the more compact denatured states of the Trp 73 and His 73 proteins might be affecting the amount of denaturation-resistant short α-helix structures. This interpretation is not unreasonable given the high helical content of folded iso-1-cytochrome *c* and the apparent correlation between the loss in intensity of the α-helix band at 1657 cm^{-1} with appearance in intensity in the 1666- and 1660-cm^{-1} bands.

CONCLUSIONS

The experiments presented here have demonstrated that reliable and reproducible IR spectra of GdnHCl-denatured proteins in the structure-sensitive amide I region can be produced. The data support the conclusion that the denatured state of iso-1-cytochrome *c* is not a random coil, an observation that has been made for thermally-denatured RNase A as well (Sosnick & Trewhella, 1992). We also have observed some significant changes relative to the wild-type protein in the amide I IR spectra of two mutants of iso-1-cytochrome *c* which have more compact denatured states according to thermodynamic analysis (Bowler et al., 1993). The magnitude of the differences observed appears to correlate with the relative compactness of the denatured state, suggesting that changes in the compactness of a denatured state have specific structural consequences.

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