Registry No. ATPase, 9000-83-3; NH_2-Cys-Tyr-Arg-Gly-Lys-Lys-Arg-Gln, 130469-77-1.

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A Photochemically Induced Dynamic Nuclear Polarization Study of Denatured States of Lysozyme†

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ABSTRACT: Photochemically induced dynamic nuclear polarization (photo-CIDNP) techniques have been used to examine denatured states of lysozyme produced under a variety of conditions. ^{1}H CIDNP difference spectra of lysozyme denatured thermally, by the addition of 10 M urea, or by the complete reduction of its four disulfide bonds were found to differ substantially not only from the spectrum of the native protein but also from that expected for a completely unstructured polypeptide chain. Specifically, denatured lysozyme showed a much reduced enhancement of tryptophan relative to tyrosine than did a mixture of blocked amino acids with the same composition as the intact protein. By contrast, the CIDNP spectrum of lysozyme denatured in dimethyl sulfoxide solution was found to be similar to that expected for a random coil. It is proposed that nonrandom hydrophobic interactions are present within the denatured states of lysozyme in aqueous solution and that these reduce the reactivity of tryptophan residues relative to tyrosine residues. Characterization of such interactions is likely to be of considerable significance for an understanding of the process of protein folding.

Recent advances in the fields of protein engineering, X-ray crystallography, and NMR spectroscopy have increased dramatically our knowledge of the structures of proteins in their native states and of the factors which stabilize them (Eisenberg & Hill, 1989; Shortle, 1989; Wright, 1989). By contrast, relatively little is known about the denatured states of proteins, primarily because of the problems involved in studying them. It is particularly difficult to characterize denatured proteins because the unfolded polypeptide chain interconverts rapidly between many different conformational states of similar energies (Tanford, 1968). As a consequence, crystallization of

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denatured proteins has not been possible, and conventional nuclear magnetic resonance (NMR)[1] methods cannot readily be used. Despite these difficulties, it is of considerable importance that more is learned about the denatured states of proteins, since there is an increasing amount of evidence that they are intimately linked with phenomena such as folding pathways and protein stability (Baldwin, 1986; Shortle & Meeker, 1986).

For the purposes of this paper, the denatured state is defined as the large number of conformations sampled by an unfolded protein. The classic picture of a denatured state is a random coil in which, on average, only random interactions of side chains are present and no single conformational state is significantly favored over any other (Tanford, 1968). Along with immunochemical properties and measurements of side chain reactivities, optical scattering, circular dichroism, and fluorescence techniques enable the deviations of any given denatured state from the idealized random coil to be assessed (Creighton, 1988). It is apparent from such studies that the approximation of a denatured protein to a random coil is an oversimplification (Kuwajima et al., 1985; Shortle & Meeker, 1986; Amir & Haas, 1988; Goto et al., 1990). However, because of the qualitative nature of the methods employed, it has hitherto proved impossible to specify the particular residues involved in nonrandom interactions.

More specific information about the denatured states of proteins should in principle be available from NMR spectroscopy, but poor chemical shift dispersion and the scarcity of interresidue nuclear Overhauser enhancements make conventional assignment techniques difficult. Despite these problems, considerable insight may be gained from comparisons of chemical shifts with those of unstructured model peptides (Dobson et al., 1984; Howarth & Lian, 1984a,b; Roder, 1989; Baum et al., 1989; Evans et al., 1989) and from the rates of exchange of amide protons (Roder, 1989; Baum et al., 1989; Dobson et al., unpublished results).

An NMR technique that has yet to be used extensively in the study of protein folding is photochemically induced dynamic nuclear polarization (photo-CIDNP). The method uses a reversible chemical reaction between the protein and a photoexcited dye to generate nuclear spin polarizations in the side chains of certain amino acid residues (Kaptein, 1982). The polarization is detected as enhancements in the NMR spectrum and is only observed for tryptophan, tyrosine, and histidine residues that are physically accessible to the excited dye (usually a flavin). For the native state of a protein, the method can provide a measure of the exposure of the various residues detected (Kaptein et al., 1978).

The CIDNP spectra of several proteins in unfolded or partially folded states have been studied, usually to shed light on the structure of the native state (Garssen et al., 1978; Canioni et al., 1980; Akasaka et al., 1981; Hincke et al., 1981; Muszkat et al., 1984a,b; Vogel & Sykes, 1984; Weiss et al., 1989). Here we use CIDNP to follow the unfolding transition of a protein and to characterize its denatured state. We describe experiments on hen egg-white lysozyme, an enzyme whose native state has been characterized in great detail: its crystal structure is known at high resolution (Blake et al., 1965; Handoll, 1985), its NMR spectrum has been virtually completely assigned (Redfield & Dobson, 1988), and its CIDNP spectrum has been studied in detail (Hore & Kaptein, 1983; Vogel & Sykes, 1984; Stob et al., 1988). The unfolding transition of lysozyme is a cooperative two-state process with a T_m of approximately 77 °C at pH 3.8 (Pfeil & Privalov, 1976; Dobson & Evans, 1984); under equilibrium conditions, no folding intermediates can be detected (Imoto et al., 1972). Much less information is available about the denatured state. Circular dichroism (Aune et al., 1967) and hydrodynamic volume calculations (Dubin et al., 1973; Nicoli & Benedek, 1976) indicate that the structure of the thermally denatured protein differs significantly from a random coil but the exact nature of the residual interactions has not been determined. Analysis of the NMR spectrum of denatured lysozyme reveals significant residual chemical shift dispersion, particularly in the aromatic region of the spectrum, suggesting the involvement of aromatic residues in nonrandom interactions (Howarth & Lian, 1984b; Evans et al., 1990). In this paper, photo-CIDNP spectroscopy is used to examine lysozyme denatured thermally, chemically and by reduction of its four disulfide bridges.

Materials and Methods

Photo-CIDNP Spectroscopy. ^{1}H CIDNP experiments (Kaptein, 1982) were performed at 400 MHz on a Varian XL-400 NMR spectrometer. A Varian 5-mm ^{1}H probe was modified to permit photolysis of NMR samples from the side by introducing a quartz rod and right-angle prism (McCord & Boxer, 1981). Two light sources were used: a continuous-wave argon ion laser (Spectra-Physics 2016 operating in the multiline mode with principal wavelengths of 488.0 and 514.5 nm) and a pulsed xenon chloride excimer laser (Lumonics TE-861T-4, wavelength 308 nm). Light from the argon ion laser was chopped by means of a mechanical shutter controlled by the spectrometer. Prolonged irradiation of lysozyme samples was found to lower the thermal denaturation midpoint temperature, T_m, presumably as a result of photoinduced damage to the protein. Consequently, a fresh sample was used for each spectrum, and the light intensity was kept as low as possible, consistent with acceptable sensitivity. Thus, the argon ion laser delivered 0.1-s light pulses at 5-W output power while the excimer laser was set up to give five 200-mJ flashes in the space of 0.2 s. Under these conditions, the denaturation temperatures were identical (77 ± 2 °C at pH 3.8) before and after irradiation. "Light" and "dark" free induction decays were recorded alternately and subtracted prior to Fourier transformation to give difference spectra containing only resonances with CIDNP enhancements. For both light sources, 24 light (and 24 dark) scans gave an adequate signal-to-noise ratio. A long relaxation delay between scans was chosen (7 s) to minimize the effects of sample heating by the light pulse. The delay between the light flash and the radio-frequency pulse was set to 50 ms which, together with the short irradiation times, virtually eliminated cross-relaxation effects (Hore et al., 1982). CIDNP was generated by using flavin mononucleotide (FMN) (Sigma), lumiflavin (Sigma), or 2,2′-bipyrazine (Aldrich) as photosensitizers. Unless otherwise stated, samples comprised 1 mM protein and 0.25 mM flavin in 99.8% D_2O at pH 3.8 and were irradiated with the argon ion laser. Chemical shifts were determined relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

Preparation of Samples. Hen egg-white lysozyme, obtained from Sigma, was purified to homogeneity by ion-exchange chromatography before use (Radford et al., 1990). Perdeuterated urea and guanidine deuteriochloride were prepared by repeated lyophilization from 99.8% D_2O. $CM^{6,127}$-Lysozyme was prepared by selective reduction of the 6–127 di-

[1] Abbreviations: DMSO, dimethyl sulfoxide; FMN, flavin mononucleotide; Gdn·DCl, guanidine deuteriochloride; NMR, nuclear magnetic resonance; photo-CIDNP, photochemically induced dynamic nuclear polarization; T_m, midpoint of the thermal denaturation transition; Tris, tris(hydroxymethyl)aminomethane.

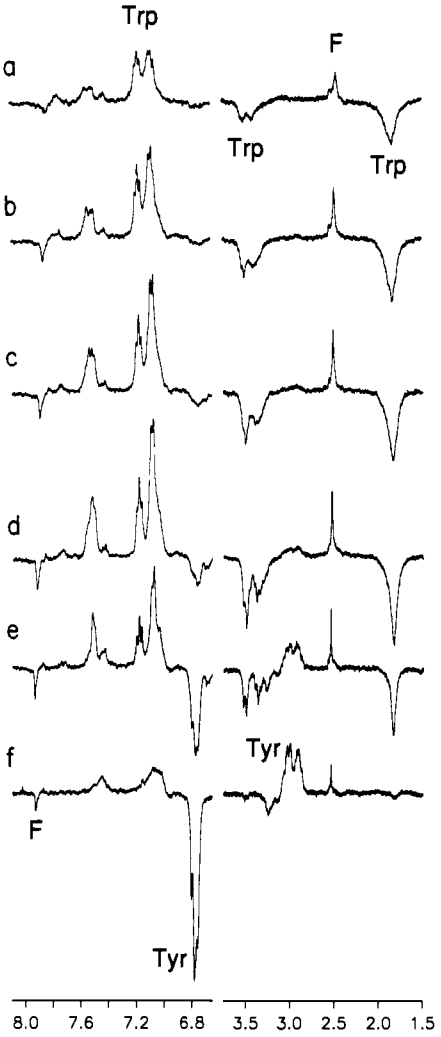

FIGURE 1: Aromatic (left) and aliphatic (right) regions of the photo-CIDNP spectra of 1 mM lysozyme with 0.25 mM FMN at pH 3.8 in D_2O. (a) 30 °C; (b) 40 °C; (c) 50 °C; (d) 60 °C; (e) 70 °C; (f) 80 °C. Resonances labeled F are due to protons of the flavin dye.

sulfide bond in purified lysozyme, followed by carboxymethylation as described by Radford et al. (1990). Fully reduced lysozyme was prepared by a modification of the method of Acharya and Taniuchi (1976). Lysozyme (approximately 20 mg/mL in 0.1 M Tris-HCl, pH 8.5, containing 8 M urea) was incubated for 4 h under anaerobic conditions with β-mercaptoethanol (0.3 M) at 27 °C. The solution was then dialyzed exhaustively against H_2O at pH 2. Fully reduced lysozyme was used immediately or was stored at −20 °C as a lyophilized powder. The fully reduced protein was completely carboxymethylated to make CM-lysozyme by incubating reduced lysozyme (10 mg/mL) at pH 8 in 0.1 M Tris-HCl containing 6 M guanidine hydrochloride with a 10-fold molar excess (over the thiol groups in lysozyme) of iodoacetic acid for 2 h at 37 °C. The modified protein was then purified from the reagents by exhaustive dialysis against 0.1% (v/v) trifluoroacetic acid and lyophilized.

RESULTS

Thermal Denaturation. The effect of temperature on the aromatic region of the photo-CIDNP difference spectrum of lysozyme (1 mM, pH 3.8, with 0.25 mM FMN) is shown in Figure 1. The spectrum at 30 °C is essentially identical with that reported previously (Hore & Kaptein, 1983; Stob et al., 1988). The main features are the absorptive resonances of the directly polarized indole protons (C2H, C4H, and C6H) of Trp-62 between 7.0 and 7.3 ppm and of Trp-123 near 7.5 ppm. Also visible is a weak emissive peak at 6.73 ppm, assigned to the εCH protons of Tyr-23 (Hore & Kaptein, 1983). As the temperature is raised from 30 to 80 °C, the intensity of the tryptophan resonances first increases, reaches a maximum near 60 °C, and then falls dramatically. At the same time, the intensity of the tyrosine emission increases, slowly at first and then more rapidly above 60 °C, until at 80 °C it is the dominant feature of the spectrum, more than twice as intense as the tryptophan resonances. Changes in chemical shifts also occur as the temperature is changed; most notably, there is a reduction in the chemical shift dispersion of both tryptophan and tyrosine resonances between 60 and 80 °C as the protein unfolds. However, even at 80 °C, the chemical shifts of several tryptophan and tyrosine resonances differ significantly from the random-coil values determined from model peptides.

Similar CIDNP effects were found in the aliphatic region of the difference spectra, also shown in Figure 1. At 30 °C, the emissive β-CH_2 resonances of Trp-62 (1.8 ppm) and of Trp-123 (3.4 and 3.5 ppm) are clearly visible. With increasing temperature, the intensities of these peaks grow to a maximum at 60 °C and then decline in parallel with the changes observed for the aromatic protons. By 80 °C, the chemical shift difference between the protons of the two polarized tryptophan β-CH_2 groups has disappeared, leaving an emissive resonance at 3.25 ppm. In accord with observations of the aromatic resonances, an absorptive tyrosine β-CH_2 peak (3.0 ppm) grows strongly between 60 and 80 °C to become the major peak in the spectrum at 80 °C. No enhancements were observed, under any conditions, for protons of the unique histidine (His-15).

The CIDNP spectrum showed a very similar temperature dependence at both pH 2.0 and pH 6.0, except that at the more acidic pH, where the denaturation temperature of lysozyme is reduced from 77 to 69 °C, the effects observed were shifted to lower temperatures by approximately 10 °C. An almost identical temperature dependence was found when FMN was replaced by lumiflavin (also excited with the argon ion laser) or 2,2′-bipyrazine (excited in the ultraviolet) and when the concentration of FMN was varied in the range 0.02–1.00 mM. These experiments show that this behavior is not peculiar to FMN and suggest that it cannot be attributed to changes in the degree of binding, if any, of the dye to the enzyme. Since the same temperature dependence was found with lysozyme concentrations in the range 0.2–2.0 mM at pH 3.8, it is unlikely that protein aggregation contributes to the changes observed.

Amino Acid Mixtures. For the purposes of photo-CIDNP spectroscopy, mixtures of amino acids have been used to model a random-coil protein (Vogel, 1983). Such experiments must be performed above pH 4 to ensure that the rapid degenerate electron-transfer reaction between tryptophan and its radical cation (an intermediate in the chemical reactions that generate CIDNP) does not destroy the tryptophan polarization (Kaptein, 1982; Stob & Kaptein, 1989). This is not a problem in lysozyme itself because of the much smaller rate of electron transfer resulting from the slower translational diffusion of the protein. Since the intensities of peaks in the CIDNP spectra of lysozyme in both the native and denatured states do not change between pH 3.8 and pH 6.0, it is possible to compare directly spectra of amino acid mixtures at pH 6.0 and protein samples at pH 3.8.

Figure 2a shows the aromatic region of the CIDNP spectrum of a mixture of 20 amino acids in the same proportions

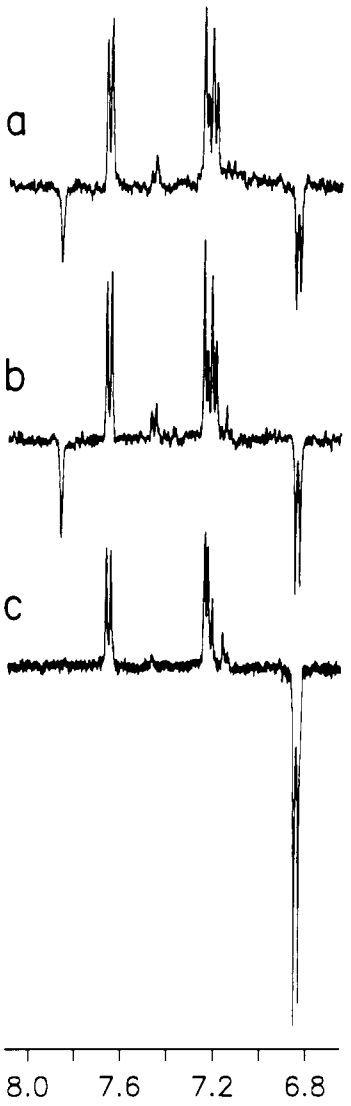

FIGURE 2: Photo-CIDNP spectra (aromatic region) of amino acid mixtures. (a) A mixture of amino acids in the proportions they occur in lysozyme, at concentrations appropriate to 0.5 mM lysozyme with cysteine replaced by cystine, with FMN (0.25 mM) at pH 6.0, 30 °C, in D_2O. (b) *N*-Acetyltryptophan amide (3 mM) and *N*-acetyltyrosine amide (1.5 mM) with FMN (0.25 mM) at pH 6.0, 30 °C, in D_2O. (c) *N*-Acetyltryptophan amide (1 mM) and *N*-acetyltyrosine amide (3 mM), with FMN (0.25 mM) at pH 6.0, 30 °C, in D_2O.

as they occur in 0.5 mM lysozyme, i.e., 3 mM tryptophan, 1.5 mM tyrosine, 0.5 mM histidine, etc., but with cysteine replaced by cystine to prevent irreversible bleaching of the dye (Kaptein, 1982). This spectrum is almost identical with that of a 2:1 mixture of *N*-acetyltryptophan amide and *N*-acetyltyrosine amide (Figure 2b). Absorptive signals from C2H, C4H, and C6H of tryptophan and an emissive doublet from ϵCH of tyrosine are clearly visible together with a weak absorptive enhancement of tyrosine δCH at 7.15 ppm. The only effect of increasing the temperature of the tryptophan/tyrosine mixture to 80 °C was a slight reduction (approximately 10%) in the intensity of all resonances. Compared with the native state of lysozyme at 30 °C, the spectrum of the mixture of amino acids shows a much stronger enhancement for tyrosine relative to that of tryptophan. By contrast, for the thermally denatured state (Figure 1f), the ratio of the intensity of the tryptophan signal to that of tyrosine is much smaller than observed for the amino acid mixture at the same temperature. To reproduce the approximate tryptophan:tyrosine intensity ratio found for thermally denatured lysozyme at 80 °C,

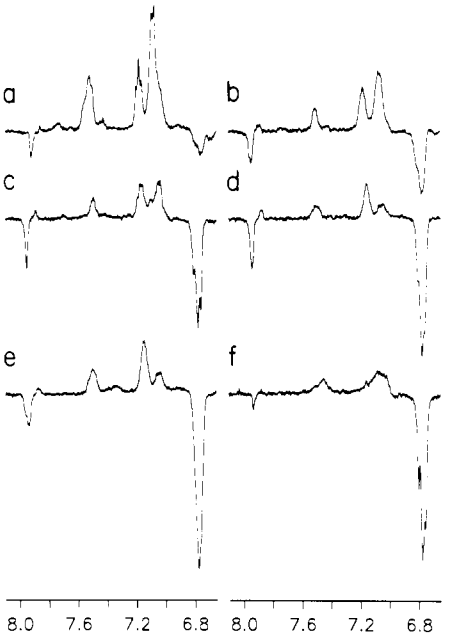

FIGURE 3: Photo-CIDNP spectra (aromatic region) of lysozyme denatured by urea, with lysozyme (1 mM) and FMN (0.25 mM) at pH 3.8 in D_2O. (a) 60 °C, no urea. (b) Urea-d_4 (4 M), 60 °C. (c) Urea-d_4 (6 M), 60 °C. (d) Urea-d_4 (10 M), 60 °C. (e) Urea-d_4 (10 M), 80 °C. (f) 80 °C, no urea.

tryptophan and tyrosine needed to be present in a ratio of about 1:3 (Figure 2c).

Chemical Denaturation. The extent of the residual nonnative interactions in a denatured state has been found to depend on the denaturant conditions (Tanford, 1968; Kuwajima et al., 1985; Dill et al., 1989). The influence of urea on the CIDNP spectrum of lysozyme is shown in Figure 3. The changes in the spectrum are largely similar to those observed during thermal denaturation, although the increase in tryptophan and tyrosine polarizations below the unfolding transition (approximately 6 M urea at 60 °C) is not observed. Increasing concentrations of urea-d_4 at 60 °C cause the tryptophan polarization to fall as urea is added up to 10 M; meanwhile, the tyrosine emission grows steadily, until at 10 M urea the spectrum resembles that of thermally denatured lysozyme at 80 °C in the absence of urea. At 80 °C, when lysozyme is denatured at all urea concentrations, both the tryptophan and tyrosine enhancements remain nearly constant up to about 5 M urea; further addition of urea (up to 10 M) causes the tryptophan CIDNP to increase slightly while the intensity of the tyrosine emission remains the same. Even under these extreme conditions, the spectrum of the denatured protein still differs markedly from that of the amino acid mixture containing 3 mM *N*-acetyltryptophan amide and 1.5 mM *N*-acetyltyrosine amide (Figure 2b).

Changes similar to those seen with urea were found in the presence of guanidine deuteriochloride (data not shown). At 40 °C and 6 M Gdn·DCl, conditions under which the protein is completely denatured, the relative enhancements of the tryptophan and tyrosine resonances are close to those observed at 60 °C in the presence of 10 M urea. The major difference between the CIDNP behavior of lysozyme in the presence of the two denaturants is a general decrease in CIDNP intensity (for both tryptophan and tyrosine) with increasing Gdn·DCl concentration at temperatures above 50 °C, which has been attributed to quenching of the photoexcited flavin (Broadhurst, 1990).

The aromatic region of the CIDNP spectrum of lysozyme denatured in dimethyl sulfoxide (DMSO) at 80 °C, obtained

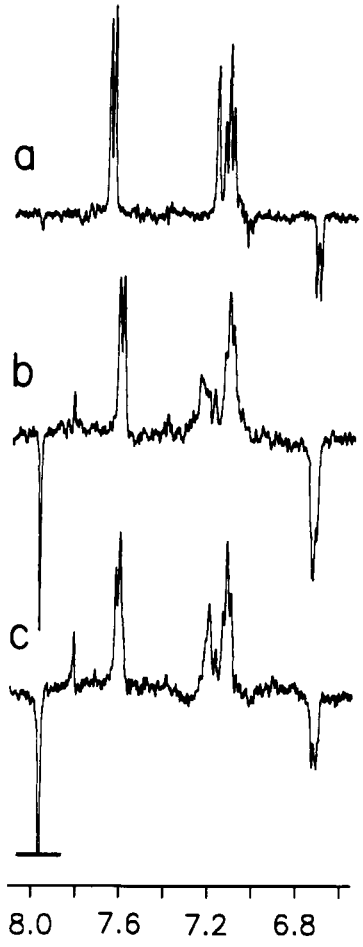

FIGURE 4: Photo-CIDNP spectra (aromatic region) of lysozyme species and amino acid mixtures in DMSO with lumiflavin (0.25 mM). (a) *N*-Acetyltryptophan amide (6 mM) and *N*-acetyltyrosine amide (3 mM), 80 °C. (b) Lysozyme (0.5 mM), 80 °C. (c) CM-Lysozyme (0.5 mM), 80 °C.

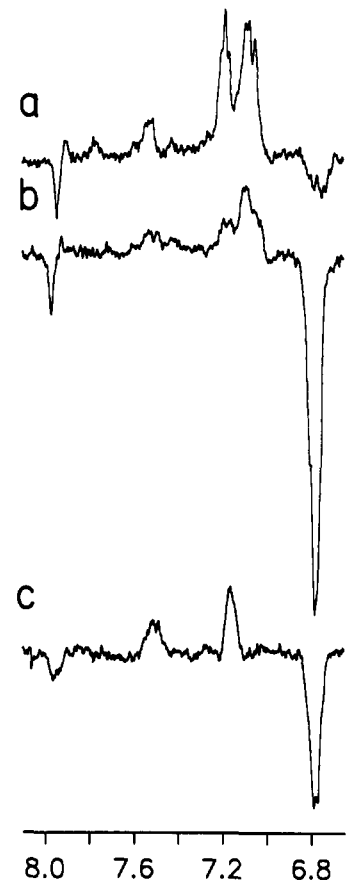

FIGURE 5: Photo-CIDNP spectra (aromatic region) of reduced lysozyme species in D_2O at pH 3.8 with FMN (0.25 mM). (a) $CM^{6,127}$-Lysozyme (0.5 mM), 40 °C. (b) $CM^{6,127}$-Lysozyme (0.5 mM), 60 °C. (c) Fully reduced lysozyme (0.5 mM), 80 °C.

by using lumiflavin rather than FMN (which has low solubility in this solvent), is shown in Figure 4b. Similar spectra were found for temperatures down to 30 °C. Compared with the spectrum of thermally denatured lysozyme in water, there is less chemical shift dispersion, and the shifts of the resonances are closer to random-coil values. Furthermore, the tryptophan peaks are much more intense relative to the emissive tyrosine resonance, and the spectrum resembles that of a 2:1 mixture of *N*-acetyltryptophan amide and *N*-acetyltyrosine amide in DMSO (Figure 4a).

Reduced Lysozyme Species. Lysozyme has four disulfide bonds linking residues 6–127, 30–115, 64–80, and 76–94. In order to examine the contribution made by these cross-links to the CIDNP properties of the denatured states of lysozyme, experiments were performed on partially and fully reduced lysozyme species.

A modified form of lysozyme ($CM^{6,127}$-lysozyme) has been prepared in which the 6–127 disulfide linkage is broken and the thiol groups carboxymethylated (Radford et al., 1990). The denaturation temperature is 53 ± 2 °C at pH 3.8, some 24 °C lower than that of the wild-type protein (Radford et al., 1990). The CIDNP spectra of the native and denatured states of $CM^{6,127}$-lysozyme (at 40 and 60 °C, respectively), shown in Figure 5a,b, have a similar appearance to those of the same states of the wild-type protein (at 60 and 80 °C, respectively) in Figure 1d,f.

When all four disulfide linkages in lysozyme are reduced, the protein is denatured under all conditions studied here. The CIDNP spectrum of fully reduced lysozyme at 80 °C (Figure 5c) is approximately 5 times less intense than that of thermally denatured lysozyme at the same temperature and concentration. However, although there is less chemical shift dispersion than in the spectrum of denatured $CM^{6,127}$-lysozyme (Figure 5b), the relative intensities of the tryptophan and tyrosine polarizations are not greatly changed. A similar reduction in the CIDNP enhancements of samples containing 6 mM *N*-acetyltryptophan amide or 3 mM *N*-acetyltyrosine amide or 1 mM wild-type lysozyme was found on addition of 8 mM cysteine (equivalent to the eight cysteine residues in fully reduced lysozyme). Thus, the loss of intensity in the reduced protein probably reflects quenching of the excited flavin by the cysteine thiol groups (Kaptein, 1982).

In order to assess the effect of the disulfide bonds on the CIDNP spectra of lysozyme in DMSO, the protein was fully reduced and the thiol groups carboxymethylated. The CIDNP difference spectrum of CM-lysozyme in DMSO (Figure 4c) bears a close resemblance to that of the nonreduced protein in the same solvent (Figure 4b). CM-Lysozyme is not soluble in D_2O, so it was not possible to make a direct comparison with the thermally denatured state of wild-type lysozyme.

Discussion

Photo-CIDNP Spectra of Lysozyme. The photo-CIDNP spectrum of hen egg-white lysozyme at 30 °C has been well characterized (Hore & Kaptein, 1983; Stob et al., 1989). Of the 10 potentially polarizable residues (6 tryptophans, 3 tyrosines, and 1 histidine), only 2, Trp-62 and Trp-123, show appreciable polarization. This observation is consistent with estimates of solvent exposure (Glickson, et al., 1971) and dye

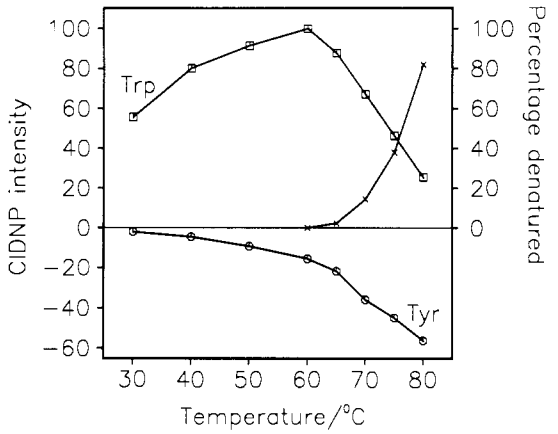

FIGURE 6: Temperature dependence of the integrated CIDNP intensities for tryptophan [(□) 7.80–7.00 ppm] and tyrosine [(○) 6.90–6.50 ppm] signals and the fraction of the thermally denatured state present (×), calculated from the area of the Leu-17 δCH_3 resonance at −0.66 ppm in the corresponding dark spectra.

accessibility (Hore & Kaptein, 1983) based on crystallographic data (Blake et al., 1965), which suggest that approach of the flavin to Trp-62, Trp-63, and Trp-123 should be possible (the absence of CIDNP for Trp-63 is probably the result of its close proximity to the rather more exposed Trp-62). In contrast, little attention has been devoted to the unfolding process and the denatured protein. The experiments described above reveal substantial differences in both chemical shifts and CIDNP intensities between denatured lysozyme in aqueous solution and both the native state and mixtures of amino acids. We divide the following into discussions of the temperature dependence of the CIDNP enhancements above and below the thermal unfolding transition and of the CIDNP behavior observed for the variously denatured states of the protein.

(*a*) *Temperature Dependence.* Figure 6 compares the temperature dependence of the intensities of the tyrosine and tryptophan signals with the fraction of denatured lysozyme present in aqueous solution. Above 65 °C, the tyrosine signal grows, and that of tryptophan falls more or less in parallel with the proportion of unfolded protein. The insensitivity of this behavior to the concentrations of either flavin or protein suggests that neither protein aggregation nor protein–dye binding is responsible. Nor are spin relaxation processes or photoinduced damage to the protein likely to be important under the conditions used (short irradiation periods and delay times). The most plausible explanation for the increase in the tyrosine enhancement above 65 °C is that residues Tyr-20 and Tyr-53, which are buried in the native state, and Tyr-23, which is partially exposed, become more accessible to the dye as the native structure unfolds. In accord with this, the growth of the tyrosine enhancement is shifted to lower temperatures when the T_m of lysozyme is depressed either by reducing the pH to 2, or by addition of urea, or by reduction of the 6–127 disulfide bond.

The decrease in the polarization of tryptophan residues over the same temperature range (65–80 °C) is more surprising. If the denatured state were an unstructured polypeptide chain, one would have expected an increase in the polarization as the four unreactive tryptophan residues became accessible to the dye. That this is not observed is due in part to competition for the photoexcited dye between reactive tryptophan and tyrosine residues in the denatured state. However, as discussed in the next section, this effect is not sufficient to account for the observed behavior, which is principally due to a reduction in the ability of tryptophan residues to react as the protein denatures.

The increase in both tryptophan and tyrosine CIDNP intensities between 30 and 60 °C is less straightforward to interpret. Throughout this temperature range, the equilibrium population of the denatured state is negligible, and there is no evidence for significant perturbations in the native structure (Dobson & Evans, 1984). It therefore seems improbable that this effect is due to modified reactivity of tryptophan and tyrosine side chains brought about by conformational changes in the protein. More plausibly, the temperature dependence reflects some change in the rates of the chemical and physical processes involved in the generation of CIDNP. Similar behavior has been observed for polarized histidine and tyrosine residues in ribonuclease A (Broadhurst, 1990), suggesting that it may be a general feature of the technique rather than something peculiar to lysozyme. Further work is in progress to elucidate these effects.

(*b*) *Comparison with Amino Acid Mixtures.* To a first approximation, the CIDNP behavior of the denatured protein should be mimicked by a mixture of amino acids with the same composition as the intact protein and at the same pH and temperature. Figure 2a suggests strongly that if side chain reactivities are the same in the denatured protein as those in an amino acid mixture of the same composition, then tryptophan and tyrosine residues, but not histidine, would show appreciable CIDNP enhancements. The absence of histidine polarization, previously noted by Vogel (1983), presumably reflects the more rapid reactions of tryptophan and tyrosine side chains with the photoexcited flavin. This competition also affects the observed enhancements of tryptophan and tyrosine. Because these residues compete to react with a small concentration of photoexcited dye molecules, the magnitude of the tyrosine signal in the CIDNP spectrum of a protein reflects not only the number and reactivity of tyrosine side chains but also the number and reactivity of the tryptophans. This complication may be taken into account by comparing the CIDNP spectrum of a denatured state of lysozyme with those of amino acid mixtures. Inspection of Figures 1 and 2 reveals that the amino acid mixtures show much stronger enhancements for tryptophan relative to tyrosine than are observed for the protein in the thermally denatured state. A measure of this effect was obtained by calculating the ratio of the integrated area of the tryptophan signal (7.80–7.00 ppm) to that of tyrosine (6.90–6.50 ppm) (see Figure 7). The intensity ratio of 0.45 for thermally denatured lysozyme at 80 °C compares with a value of 3.8 for the amino acid mixture at the same temperature. The CIDNP spectra of lysozyme denatured in aqueous solution by the addition of urea or guanidine deuteriochloride, or by the reduction of the four disulfide links, all deviate in the same way from the spectrum of the amino acid mixture (tryptophan:tyrosine intensity ratios are 0.54 for lysozyme in 10 M urea at 60 °C, and 0.67 for fully reduced lysozyme at 80 °C). Spectra of *N*-acetyltyrosine amide and *N*-acetyltryptophan amide in different proportions (Figure 2) indicate that the intensity ratio observed for the aqueous denatured states is reproduced approximately by a 1:3 mixture of tryptophan to tyrosine rather than the 2:1 proportion suggested by the six tryptophan and three tyrosine residues in the protein.

Finally, one further detail of the CIDNP method needs discussion. The interactions responsible for CIDNP sort nuclear spins along two pathways such that the polarization of a nucleus in the "recombination product" (formed directly from the radical pair) is equal and opposite to that of the corresponding nucleus in the "escape product" (formed from radicals that have escaped from the radical pair). In the cyclic reac-

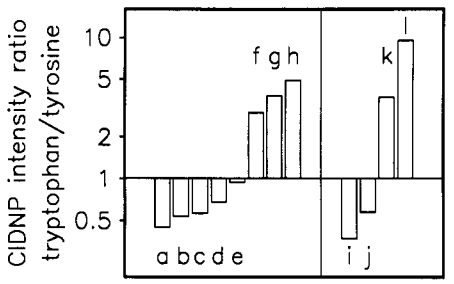

FIGURE 7: Histogram of the tryptophan:tyrosine CIDNP intensity ratio for various denatured states and model systems. (a) Lysozyme in D_2O at pH 3.8, 80 °C. (b) Lysozyme in D_2O with 10 M urea-d_4 at pH 3.8, 60 °C. (c) $CM^{6,127}$-Lysozyme in D_2O at pH 3.8, 60 °C. (d) Reduced lysozyme in D_2O at pH 3.8, 80 °C. (e) Lysozyme in D_2O with 10 M urea at pH 3.8, 80 °C. (f) CM-Lysozyme in DMSO at 80 °C. (g) Lysozyme in DMSO at 80 °C. (h) $CM^{6,127}$-Lysozyme in DMSO at 40 °C. (i) *N*-Acetyltryptophan amide (1 mM) and *N*-acetyltyrosine amide (3 mM) in D_2O at pH 6, 40 °C. (j) *N*-Acetyltryptophan amide (2 mM) and *N*-acetyltyrosine amide (3 mM) in D_2O at pH 6, 40 °C. (k) *N*-Acetyltryptophan amide (6 mM) and *N*-acetyltyrosine amide (3 mM) in D_2O at pH 6, 40 °C. (l) *N*-Acetyltryptophan amide (6 mM) and *N*-acetyltyrosine amide (3 mM) in DMSO at 40 °C. The tryptophan:tyrosine intensity ratio is calculated from the area of tryptophan (7.80–7.00 ppm) and tyrosine (6.90–6.50 ppm) resonances in the CIDNP difference spectrum. Note that the scale is logarithmic.

tions used to generate CIDNP in proteins, these two products are identical (the protein itself) so that there is a tendency for the two types of polarization to cancel, a tendency which is alleviated by nuclear spin–lattice relaxation in the radicals prior to formation of escape products. To be confident in our interpretation of the relative enhancements of tryptophan and tyrosine, we must be certain that this cancellation affects the two types of amino acid to the same extent. To test this, CIDNP spectra both of denatured lysozyme and of amino acid mixtures were recorded with the argon ion laser power reduced by a factor of 10 (Broadhurst and Hore, unpublished results). Attenuation of the light source should reduce the concentration of radicals and hence the degree of cancellation which depends on the rate of the bimolecular reaction of free radicals to form escape products. In neither case was any significant change in the ratio of tryptophan to tyrosine intensities observed.

Structure in the Denatured State. In the experiments reported above, we believe we have eliminated all "trivial" explanations such as spin relaxation, protein–flavin binding, aggregation, competition, cancellation, etc. for the different CIDNP behavior of aqueous denatured lysozyme and amino acid mixtures. This leaves us with the conclusion that, relative to the tyrosine residues, the tryptophan residues in the denatured state are unusually unreactive toward photoexcited dyes. This provides direct evidence that the denatured state of lysozyme in aqueous solution differs significantly from a random-coil model and that CIDNP experiments are highly sensitive to these differences. Thus, the technique is potentially able to probe such states of proteins as well as the more familiar native states.

There is now a wide body of evidence that denatured proteins are not necessarily random coil in nature (Kuwajima et al., 1985; Shortle & Meeker, 1986; Amir & Haas, 1988; Privalov et al., 1989; Goto et al., 1990). The existence of structure in the denatured states of proteins containing disulfide bonds has been rationalized by invoking the conformational constraints imposed by the presence of the cross-links in the denatured protein (Creighton, 1988). In accord with this, complete reduction of all the disulfide bonds in bovine pancreatic trypsin inhibitor leads to the formation of a state that is significantly closer to a random coil than the denatured forms of the nonreduced protein (Roder, 1989). It is interesting in this respect that the tryptophan enhancement observed in the CIDNP spectrum of fully reduced lysozyme in aqueous solution is small relative to that of tyrosine (Figure 5). Thus, by the criteria used here, the residual structure in the aqueous denatured states of lysozyme does not depend critically on the presence of the disulfide bonds. Indeed, only a combination of high temperature and high concentrations of chemical denaturants appears even to begin to make the tryptophan residues in the denatured state approach the reactivity found in amino acid mixtures.

Figure 7 indicates that, of all the denatured states encountered in these experiments, lysozyme in DMSO is the state closest to being an unstructured polypeptide chain, at least as far as the aromatic side chains are concerned. The difference in the tryptophan:tyrosine intensity ratios for the protein in DMSO relative to amino acid mixtures is still more than a factor of 2 (3.9 for the denatured protein and 9.6 for the amino acid mixture) but is much smaller than the 8-fold difference observed for the denatured states and amino acid mixtures in aqueous solution. This suggests that the origin of the reduced reactivity in aqueous solutions could be due to interactions between hydrophobic residues. It is likely that such interactions would be significantly reduced in DMSO relative to aqueous solution. In support of this interpretation, a recent study of the chemical shifts of resonances of specific protons in denatured lysozyme has shown small but significant differences from those expected for an unstructured polypeptide chain (Evans et al., 1990). In only a few cases could the effects be attributed to specific local interresidue interactions (e.g., between Trp-62 and Trp-63). In general, it was concluded that the nonrandom chemical shifts, which are substantially reduced in the presence of DMSO, resulted principally from an averaged effect of the interactions of side chains with aromatic residues (particularly tryptophan) remote in the primary sequence. This supports the conclusions of an earlier ^{13}C NMR study, where perturbations to chemical shift values had also been attributed to hydrophobic interactions between side chains (Howarth & Lian, 1984b).

These results suggest that the dominant interactions which influence the CIDNP intensities are hydrophobic in origin. Although many of the factors that control the enhancements, even in native proteins, are not well understood, there is a strong correlation between CIDNP intensities and the accessibility of the different residues to bulk solvent. The substantially reduced reactivity of the tryptophan residues in the denatured state might well, therefore, be a consequence of the reduced accessibility of these residues because of their involvement, along with other hydrophobic residues, in hydrophobic clusters within a partially collapsed denatured state. Tryptophan is more hydrophobic than tyrosine (Nozaki & Tanford, 1971) and so more likely to be involved in hydrophobic interactions. Assuming that reactivity and accessibility are directly related, a very approximate estimate of the number of accessible tryptophan residues in the denatured state may be obtained by comparison with the spectra of amino acid mixtures. The relative CIDNP intensities for tryptophan and tyrosine indicate that, on average, only about one of the six indole side chains is available for reaction with photoexcited flavin (assuming that all the tyrosine residues are fully exposed to the dye). The CIDNP results are consistent with a range of interpretations, the extremes being one tryptophan residue is accessible all the time or all the tryptophan residues are accessible for one-sixth of the time. If the tyrosine residues are less than fully reactive, then less than one tryptophan

residue on average is available to react with the flavin dye. The extent of the clustering therefore appears to be substantial.

Clustering of hydrophobic residues away from the solvent is an energetically favorable process (Dill, 1985). In the native state, some hydrophobic residues may, by the nature of the restricted conformational properties of the folded state, be exposed to the solvent especially in the region of the active site. Relative to the native state, the denatured state possesses much greater conformational mobility, and it is possible that hydrophobic residues will generate a local environment that is effectively removed from the bulk solvent. The existence of such structures may be of considerable interest from the point of view of protein folding, as they provide an environment suitable for the construction of elements of secondary structure and limit the number of conformations available to the folding polypeptide chain. Recent experiments have suggested that hydrophobic collapse occurs rapidly as a first step during the refolding of cytochrome *c* (Roder et al., 1988) and dihydrofolate reductase (Garvey et al., 1989). The results presented here suggest that at least a degree of hydrophobic collapse characterizes the stable denatured states of lysozyme in aqueous solution. It will be interesting to extend CIDNP experiments on denatured states to a wider range of proteins to discover whether this is a general phenomenon or is confined to a limited range of proteins.

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