# Optically Detected Magnetic Resonance of Tryptophan Triplet States in Native and Urea-Denatured Proteins and Polypeptides†

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ABSTRACT: Optically detected magnetic resonance (ODMR) and phosphorescence emission spectroscopy of the triplet state of tryptophan were used to examine local order in several native and urea-denatured proteins and polypeptides. We compared the tryptophan environments of the tripeptide model Gly-Trp-Gly, the polypeptide hormones adrenocorticotropin-(1–24) and glucagon, and the following proteins: azurin (*Pseudomonas aeruginosa*), horse liver alcohol dehydrogenase, hen egg white lysozyme and its oxindole-108 and -62 derivatives, and the catalytic subunit of protein kinase (C-subunit) from bovine heart. The results of urea denaturation presented here show that as a protein becomes progressively unfolded, the ODMR lines shift and often broaden, and in the limit of total denaturation the phosphorescence and ODMR parameters approach those of a randomly coiled polypeptide. Our interpretation of the tryptophan triplet state characteristics of the proteins has in large part relied upon what is known from their X-ray crystal structure models. C-subunit is the single protein in this investigation for which no such model is yet available. Based upon what we learned from the other proteins as well as the polypeptides, we find that there are two well resolved classes of tryptophan residues in C-subunit according to ODMR, although this is *not* revealed by the phosphorescence data. In particular, one class is unusual when compared with previous observations: this class has the lowest energy $|E|$ value observed for any tryptophan ($|2E| = 2.319$ GHz), and we propose that the shift is due to a perturbation other than from an amino acid side chain or from solvent, perhaps from the intrinsic molecular bound phosphate.

Elucidation of the unfolded state of a protein is essential to our understanding of the native state, and considerable efforts have been directed toward understanding the process of denaturation and the process by which proteins refold into their native state [cf. reviews by Tanford (1968, 1970), Wetlaufer & Ristow (1973), Baldwin (1975), and Anfinsen & Scheraga (1975)]. In general, the three-dimensional protein structure is thought to be that configuration of the polypeptide chain for which the Gibbs free energy of the entire system, including solvent, is at a minimum.

It has been postulated that nucleation of three-dimensional structure can occur simultaneously at several independent sites in the polypeptide chain (Levinthal, 1968; Wetlaufer, 1973) and that nucleation may be accomplished by formation of a specific pocket in the polypeptide chain stabilized by hydrophobic contacts (Matheson & Scheraga, 1978). Coan et al. (1975) proposed that the aromatic residues of a protein could act as initiators of the folding process, since they have relatively large, rigid contact surfaces for stable, short-range van der Waals interactions and, except for phenylalanine, they have relatively large dipole moments for long-range mutual orientation interactions with other dipoles. Also it has been suggested by Tulinsky et al. (1973) that aggregation of aromatic residues may play an important role in imparting stability to the protease chymotrypsin. Coan et al. (1975) have noted that clustering of aromatic residues occurs in a number of diverse proteins. Comparing trypsin and chymotrypsin, which have generally similar tertiary structures, they noted that about half of the aromatic residues are the same or are replaced by another aromatic residue, that another ~30% are replacements involving a nonpolar or neutral residue, and that the pattern of aromatic clustering is similar in the two proteins. These observations suggest that aromatic character may be a key factor in folding. The highly hydrophobic character of tryptophan, as is evident by its large favorable free energy of transfer from water to organic solvents (Nozaki & Tanford, 1971), further suggests that tryptophan may be very important in the intrinsic stability of a nucleation site. Therefore, it is appropriate to focus on tryptophan as a probe for investigating folding and the process of denaturation.

Recently, we found that a chain-length dependence of folding in the polypeptide hormone glucagon and selected fragments thereof could be detected by the measured shifts in the phosphorescence spectrum and triplet state zero-field splittings (zfs)[1] of the single tryptophan residue (Ross et al., 1976, 1977). It is not clear from these results whether the tryptophan residue itself plays an essential role in the folding process. However, we were able to ascertain that the tryptophan triplet state zfs and phosphorescence are sensitive to changes in the local structure of polypeptides and proteins and that the widths of the optically detected magnetic resonance (ODMR) lines in the tryptophan triplet state depend intimately upon the local environment of the residue (Rousslang et al., 1978; Deranleau et al., 1978). We also examined the effect of guanidine hydrochloride denaturation upon lysozyme, an enzyme with six tryptophan residues (Rousslang et al., 1978). The ODMR lines of the denatured protein were substantially broader than those of the native molecule, and in addition the phosphorescence was blue-shifted with broadening of the vibronic bands. In a subsequent paper, we compared the contributions of Trp-108 and Trp-62 to the ODMR and phosphorescence spectra of lysozyme in its folded state (Rousslang et al., 1979). To briefly recapitulate, the phos-

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[1] Abbreviations used: ODMR, optical detection of magnetic resonance; zfs, zero-field splittings; ADH, horse liver alcohol dehydrogenase; CD, circular dichroism; C-subunit, catalytic subunit of protein kinase; Gdn·HCl, guanidine hydrochloride; EGW, ethylene glycol–water, 1:1 (v/v); ACTH, adrenocorticotropic hormone (1–24); NMR, nuclear magnetic resonance; EDTA, (ethylenedinitrilo)tetraacetic acid.

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phorescence of lysozyme appears to be predominantly due to these two tryptophans. Furthermore, Trp-62, which is in the active-site cleft and adjacent to Trp-63 (Blake et al., 1967), is well exposed to solvent by our criteria (blue-shifted phosphorescence and broad ODMR lines), whereas Trp-108 is largely buried in the tertiary structure of the protein (red-shifted phosphorescence and narrow ODMR lines). According to the comparison between oxindole-62 lysozyme and oxindole-108 lysozyme, Trp-108 is the primary phosphorescence emitter in native lysozyme and Trp-62 is a secondary emitter.

In the present paper, we examine the microenvironments and relative solvent exposure of tryptophan residues in several urea-denatured proteins and polypeptides. These results show that in frozen 50% (v/v) ethylene glycol–water, lysozyme is resistant to complete denaturation, consistent with its behavior in aqueous solution at room temperature [cf. Tanford (1968, 1970)], whereas urea effectively abolishes secondary structure in the hormone, glucagon. Heating the lysozyme solutions to 50 °C prior to freezing facilitates denaturation. Finally, we compare the triplet state of tryptophan in several proteins, for which X-ray crystal structure models exist, in order to make predictions about the tryptophan environments in the catalytic subunit of protein kinase, a protein for which there is no X-ray crystal model at the present time.

## Experimental Section

*Materials.* Crystalline glucagon, purchased from Elanco Products, was purified by ion-exchange chromatography (Ross, 1976). Synthetic adrenocorticotropin-(1–24) (ACTH) was kindly provided by Dr. W. Rittel, CIBA-Geigy, Ltd., Basel, Switzerland. Gly-Trp-Gly was obtained from Vega-Fox. Azurin (*Pseudomonas aeruginosa*) and the catalytic subunit of protein kinase (C-subunit) from bovine heart were respectively the gifts of Professors J. Herriott and E. Fischer, University of Washington, Seattle, WA. Horse liver alcohol dehydrogenase (ADH), made ethanol-free by the procedure of Subramanian & Ross (1978), and chicken egg white lysozyme were obtained from Sigma. Oxindole-62 lysozyme was donated by Professor J. Rupley, University of Arizona, Tucson, AZ, and oxindole-108 lysozyme was prepared according to the procedure of Imoto et al. (1973).

*Phosphorescence and ODMR Measurements.* For spectroscopy and ODMR measurements, sample concentrations were $\sim 10^{-5}$ M in 50% by volume ethylene glycol–water (EGW), buffered in the pH range 6.5–7.4. In denaturation experiments, the urea concentration was 8 M as determined by percent weight. Details of these experiments are given in the text, tables, and figure legends. The optical and microwave configurations used have been discussed in detail elsewhere (Ross et al., 1977; Rousslang et al., 1978).

## Results

*Phosphorescence of Peptides.* Figure 1 shows the typical phosphorescence spectrum of a tryptophan-containing peptide, in this instance the tripeptide Gly-Trp-Gly, excited at 297 nm. The wavelength maxima of the 0–0 vibrational band ($\lambda_{0-0}$) and the highest intensity band of the rest of the phosphorescence emission envelope ($\lambda_m$) are compared in Table I for the polypeptide hormones ACTH and glucagon (each contains only one tryptophan) as well as for the tripeptide, in EGW and 8 M urea–EGW. In the absence of urea, the phosphorescence maxima of ACTH and Gly-Trp-Gly are about the same and 3 to 4 nm to the blue of the glucagon emission bands. In the presence of urea, the maxima of ACTH and Gly-Trp-Gly are negligibly red-shifted (≤0.5 nm), whereas the spectrum of glucagon is significantly blue-shifted (3 to 4 nm). The maxima of glucagon approach but do not quite superimpose upon those of ACTH and Gly-Trp-Gly.

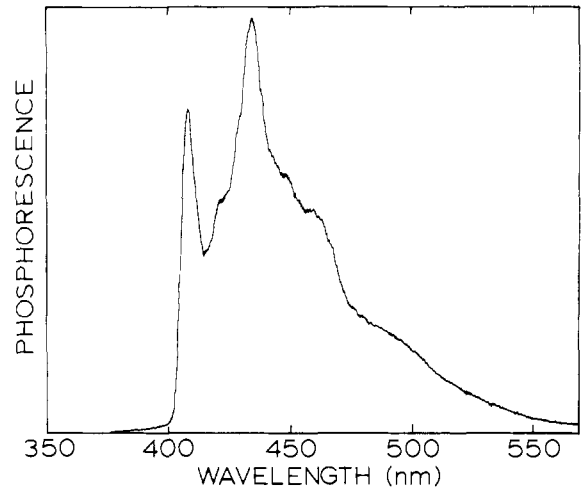


FIGURE 1: Phosphorescence emission spectrum, in arbitrary units, of Gly-Trp-Gly in EGW, buffered at pH 7.4 with 0.1 M $K_xPO_4$. Excitation was at 297 nm with a band-pass of 1.6 nm, and the emission band-pass was 3.2 nm.

*ODMR of Peptides.* The trends in the ODMR data are equivalent to those in the phosphorescence data. As seen in Table I, ACTH and Gly-Trp-Gly have similar zfs and ODMR line widths. For example, in EGW, the triplet state parameters of the tryptophan residue are virtually identical in these two peptides; upon addition of urea, $|D - E|$ *decreases* by nearly 30 MHz in Gly-Trp-Gly and 20 MHz in ACTH, while $|2E|$ *increases* by almost 50 MHz in Gly-Trp-Gly and over 30 MHz in ACTH. By contrast, for glucagon in EGW, the $|D - E|$ transition of the tryptophan is 60 MHz less than the same transition in the other two peptides and $|2E|$ is ~120 MHz larger. Upon addition of urea, the $|D - E|$ transition of glucagon *increases* 30 MHz and the $|2E|$ transition *decreases* over 50 MHz. The zf parameter affected in all cases is $|E|$, with $|D|$ remaining relatively constant. The direction of the shift in $|E|$ of ACTH and Gly-Trp-Gly in urea is precisely opposite the shift that occurs when glucagon is denatured. More importantly, the net effect for glucagon when urea is added to the solvent is that the zfs values become virtually identical with those measured for Gly-Trp-Gly and ACTH under the same conditions. The ODMR line widths of tryptophan in Gly-Trp-Gly are negligibly perturbed by urea; tryptophan lines in glucagon are broadened by 65%. Thus, although there are distinctly different zfs and ODMR line widths associated with the single tryptophan in glucagon and in ACTH, these differences almost vanish in 8 M urea, and in 8 M urea the polypeptide chain of each hormone provides a tryptophan environment similar to that in Gly-Trp-Gly, our model for a fully solvated tryptophan residue [see Deranleau et al. (1978)].

*Phosphorescence of Proteins.* Table II gives the triplet state parameters for four different proteins: azurin ($M_r$ 14000) with one tryptophan (Ambler, 1971); ADH ($M_r$ 84000) with two tryptophans per dimer subunit (Cannon & McKay, 1969); lysozyme ($M_r$ 14000) with six tryptophans (Blake et al., 1967) and its oxindole-62 and oxindole-108 derivatives; C-subunit ($M_r$ 39000) with seven tryptophans (Peters et al., 1977). In native proteins, tryptophan phosphorescence spectra often display narrower lines and more resolved vibrational structure than in smaller polypeptides or in the isolated amino acid tryptophan. As described by Konev (1967), the enhanced resolution disappears in 8 M urea and is usually, although not always, accompanied by a blue shift of a few nanometers. An example of a denatured, blue-shifted, and vibronically

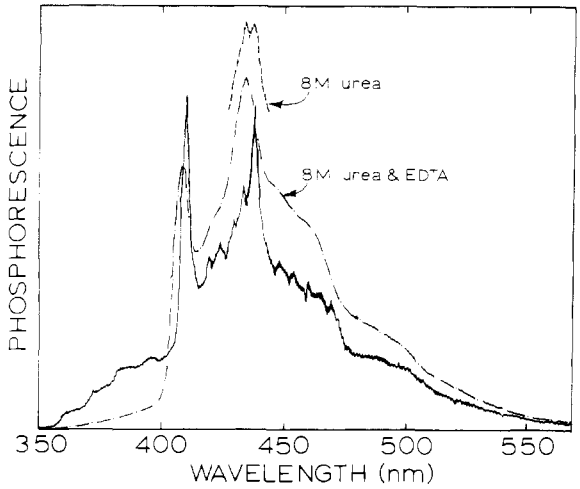


FIGURE 2: Phosphorescence emission spectra, in arbitrary units, of azurin (—) and azurin denatured in 8 M urea in the presence of EDTA (-·-). The inset shows the splitting in the $\lambda_m$ peak of azurin denatured in 8 M urea without EDTA (---). The solvent was EGW buffered at pH 7.4 with 0.1 M $K_xPO_4$. Excitation, with a band-pass of 1.6 nm, was at 290 nm, and the emission band-pass was 1.6 nm.

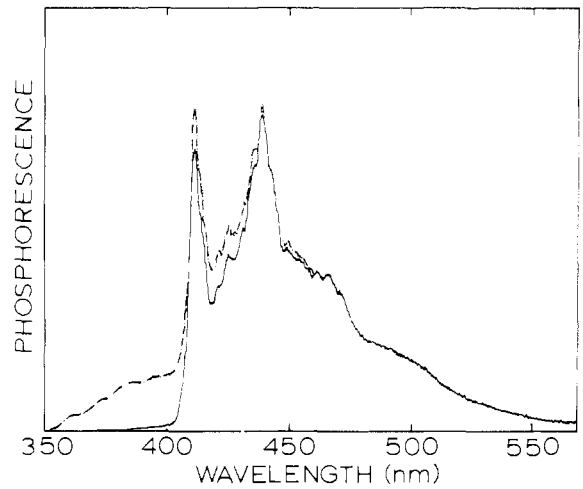


FIGURE 3: Phosphorescence emission spectra, in arbitrary units, of the C-subunit of protein kinase, excited at 297 (—) and 280 nm (-·-), with the emission and excitation band-passes set at 1.6 nm. The solvent was EGW, with 0.5 mM dithiothreitol, 0.05 mM EDTA, and 0.075 M KCl, buffered at pH 6.7 with 0.015 M $K_xPO_4$.

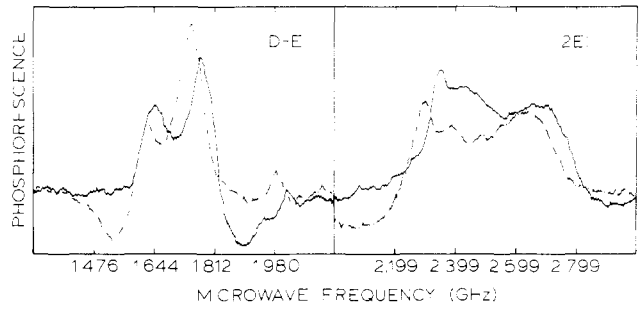


FIGURE 4: Optically detected $|D - E|$ and $|2E|$ zero-field transitions of the C-subunit in the same solvent as described in the legend to Figure 3, excited at 297 nm with a band-pass of 1.6 nm and monitored at 432 nm with a band-pass of 3.2 nm. Signal-averaged scans shown are from passage towards increasing frequency (—) and towards decreasing frequency (-·-), at 8 MHz $s^{-1}$ for $|D - E|$ and at 20 MHz $s^{-1}$ for $|2E|$. The multiple peaks are due to the triplet states of tryptophan residues in different locations in the protein. According to Peters et al. (1977), there are a total of seven possible different tryptophans. One kind of Trp site will typically produce only a single peak for each of the triplet state transitions monitored at 432 nm [see Kwiram et al. (1978)].

broadened phosphorescence spectrum—in this case that of azurin—is shown in Figure 2. The emission spectrum below 400 nm can be attributed to the two tryosine residues (Ambler, 1971), while the lone buried tryptophan [see Adman et al. (1978)] is responsible for the remainder of the envelope. It should be noted (inset in Figure 2) that denatured azurin which has not also been exposed to EDTA, a $Cu^{2+}$ chelating agent, or heated to 50 °C in EGW has a phosphorescence maximum which is a resolved doublet with peaks at 434.0 and 437.8 nm. The blue shifts in the phosphorescence of denatured azurin and C-subunit are qualitatively similar to that of Trp-314 in denatured ADH. Before denaturation this residue has $\lambda_{0-0}$ = 412.1 nm and $\lambda_m$ = 440.2 nm. Trp-314 has been identified by Purkey & Galley (1970) as residing in a buried environment. Unmodified lysozyme and its oxindole-62 derivative show only small blue shifts in 8 M urea (1 to 2 nm). Heating lysozyme in EGW with urea causes no additional shift in the spectrum. However, first heating lysozyme in saturated aqueous urea to 50 °C for an hour and then adding ethylene glycol prior to freezing cause an additional 2–3-nm blue shift.

We observed some red-shifted phosphorescence bands for proteins denatured by urea similar to those reported by Konev (1967). In all cases, the red shifts occur in those species where the native phosphorescence is in part due to what we would call an exposed residue. For example, upon denaturation the exposed tryptophan residue in ADH is red-shifted ~4 nm. Other factors besides the degree of exposure must be important though, because the primary emitting residue(s) in oxindole-108 lysozyme show(s) a 1-nm *red shift* for the $\lambda_{0-0}$ band and a 1-nm *blue shift* for the $\lambda_m$ band.

*ODMR of Proteins.* Comparing the zfs of the native proteins listed in Table II, lysozyme has the lowest energy $|D - E|$ splitting, followed next by oxindole-62 lysozyme. The highest energy $|D - E|$ splittings are associated with the exposed tryptophan in ADH and with one or more of the tryptophans in C-subunit. Monitoring the phosphorescence of ADH at 432 nm, we observed a single broad $|2E|$ transition at 2.544 GHz. The line width, but not the frequency of this resonance, was dependent upon the excitation wavelength. Exciting preferentially Trp-314 ($\lambda_{ex}$ = 297 nm) the line width was 134 MHz, and exciting both Trp-15 and Trp-314 equally ($\lambda_{ex}$ = 280 nm) the line width was 152 MHz. On the other hand, the $|D - E|$ transition is a doublet and clearly corresponds to the well resolved phosphorescence spectrum with distinct $\lambda_{0-0}$ bands for the exposed and buried tryptophan residues. Unlike the phosphorescence spectrum of ADH, the phosphorescence spectrum of C-subunit does not exhibit resolved tryptophan sites (Figure 3). By contrast, the C-subunit ODMR spectrum—both the $|D - E|$ and $|2E|$ transitions—shows well differentiated contributions from different tryptophan sites (Figure 4). In addition, the 2.319-GHz transition of C-subunit is the lowest energy $|2E|$ splitting yet reported for any tryptophan residue. The overall $|2E|$ "line width" of 492 MHz includes both the 2.319- and 2.641-GHz maxima. No attempt was made to resolve the individual contributions, because the ODMR line shape did not appear to be due to just the sum of two overlapping transitions from only two different sites.

The narrowest ODMR lines and the largest $|2E|$ splitting thus far observed for tryptophan are in azurin [see Ugurbil et al. (1977) and Rousslang et al. (1978)]. In comparing any one protein with the others in Table II, one notes a wide range of $|E|$ values from 0.0387 ± 0.0002 $cm^{-1}$ for one emitter (or class of emitters) in C-subunit to 0.0466 ± 0.0003 $cm^{-1}$ in azurin, a range of nearly 0.008 $cm^{-1}$ or 240 MHz. With the

Table I: Comparison of the Triplet State Parameters of Peptides in EGW and EGW + 8 M Urea[a]

| peptide | phosphorescence $\lambda_{0-0}$ (nm) | phosphorescence $\lambda_m$ (nm) | zf transitions $\lvert D-E\rvert$ (GHz) | zf transitions $\lvert 2E\rvert$ (GHz) | zfs parameters $\lvert D\rvert$ (cm$^{-1}$) | zfs parameters $\lvert E\rvert$ (cm$^{-1}$) | ODMR line widths $\gamma\lvert D-E\rvert$ (MHz) | ODMR line widths $\gamma\lvert 2E\rvert$ (MHz) |
|---|---|---|---|---|---|---|---|---|
| Gly-Trp-Gly | | | | | | | | |
| EGW | 407.9 | 434.1 | 1.744 | 2.468 | 0.0993 | 0.0412 | 146 | 258 |
| EGW + urea | 408.4 | 434.4 | 1.717 | 2.516 | 0.0992 | 0.0420 | 146 | 260 |
| ACTH | | | | | | | | |
| EGW | 408.2 | 435.2 | 1.740 | 2.475 | 0.0993 | 0.0413 | 146 | 261 |
| EGW + urea | 408.6 | 434.8 | 1.721 | 2.509 | 0.0992 | 0.0418 | 143 | 266 |
| glucagon | | | | | | | | |
| EGW | 411.6 | 438.9 | 1.682 | 2.592 | 0.0993 | 0.0432 | 94 | 176 |
| EGW + urea | 409.8 | 435.2 | 1.713 | 2.538 | 0.0995 | 0.0423 | 143 | 259 |
| estimated precision of measurement (all samples) | ±0.5 | ±0.5 | ±0.008 | ±0.008 | ±0.0003 | ±0.0002 | ±10 | ±13 |

[a] Explanation of symbols: $\lambda_{0-0}$ and $\lambda_m$ are the wavelengths of the phosphorescence intensity at respectively the maximum of the 0–0 band and the maximum of the highest intensity band of the overlapping lower energy vibrational bands. Excitation in all cases was at 297 nm with a 3-nm band-pass, and emission and ODMR spectra were monitored at 432 nm with a 1.5- or 3.0-nm maximum band-pass. $\gamma$ is the full width at half-height for the ODMR transitions, with scan rates of 8 MHz s$^{-1}$ for $\lvert D-E\rvert$ and 20 MHz s$^{-1}$ for $\lvert 2E\rvert$.

Table II: Comparison of the Triplet State of Tryptophan in Several Native and Urea-Denatured Proteins[a]

| protein and solvent conditions | phosphorescence $\lambda_{0-0}$ (nm) | phosphorescence $\lambda_m$ (nm) | zf transitions $\lvert D-E\rvert$ (GHz) | zf transitions $\lvert 2E\rvert$ (GHz) | zfs parameters $\lvert D\rvert$ (cm$^{-1}$) | zfs parameters $\lvert E\rvert$ (cm$^{-1}$) | ODMR line widths $\lambda\lvert D-E\rvert$ (MHz) | ODMR line widths $\gamma\lvert 2E\rvert$ (MHz) |
|---|---|---|---|---|---|---|---|---|
| azurin[b] | | | | | | | | |
| EGW | 410.0 | 438.1 | 1.624 | 2.792 | 0.1007 | 0.0466 | 42 | 73 |
| EGW + urea | 408.7 | 434.0, 437.8 | 1.630, 1.723 | 2.542, 2.784 | 0.1008, 0.0999 | 0.0424, 0.0464 | 42, 148 | 294 |
| EGW + urea + EDTA | 408.1 | 433.8 | 1.715 | 2.504 | 0.0990 | 0.0418 | 141 | 303 |
| ADH | | | | | | | | |
| FGW | 405.6, 412.1 | 435.0, 440.2 | 1.660, 1.770 | 2.544 | 0.0978, 0.1015 | 0.0424 | 77, 136 | 134 |
| EGW + urea | 410.0 | 436.0 | 1.694 | 2.557 | 0.0991 | 0.0426 | 126 | 276 |
| lysozyme | | | | | | | | |
| EGW | 413.8 | 442.5 | 1.571 | 2.724 | 0.0978 | 0.0454 | 83 | 116 |
| EGW + urea | 413.8 | 441.3 | 1.594, 1.690 | 2.595 | 0.0964, 0.0996 | 0.0433 | 120 | 303 |
| EGW + urea + heat | 413.8 | 441.2 | 1.594, 1.689 | 2.631 | 0.0970, 0.1002 | 0.0439 | 167 | 385 |
| EGW + urea + heat[c] | 411.9 | 438.1 | 1.616, 1.676 | 2.585 | 0.0970, 0.0990 | 0.0431 | 167 | 375 |
| oxindole-62 lysozyme | | | | | | | | |
| EGW | 413.8 | 442.5 | 1.580 | 2.721 | 0.0981 | 0.0454 | 86 | 115 |
| EGW + urea | 413.4 | 440.0 | 1.582, 1.702 | 2.603 | 0.0962, 0.1002 | 0.0434 | 176 | 271 |
| oxindole-108 lysozyme | | | | | | | | |
| EGW | 409.4 | 437.0 | 1.705 | 2.723 | 0.1023 | 0.0454 | 110 | 121 |
| EGW + urea | 410.2 | 436.0 | 1.716 | 2.534 | 0.0995 | 0.0423 | 184 | 329 |
| C-subunit | | | | | | | | |
| EGW | 411.3 | 439.4 | 1.642, 1.769 | 2.319, 2.641 | 0.988, 0.0977 | 0.0387, 0.0440 | 99, 84 | 492 |
| EGW + urea | 410.2 | 436.2 | 1.710 | 2.546 | 0.0995 | 0.0425 | 113 | 300 |

[a] See Table I for estimates of precision, explanation of the symbols, and experimental conditions. [b] The presence of $Cu^{2+}$ in native azurin interfered with excitation at 297 nm, and excitation was at 290 nm. The emission of denaturated azurin, in the presence of EDTA, was red-shifted 1 nm to 435 nm when excited at 297 nm. [c] In this experiment lysozyme was first denaturated in an aqueous saturated urea solution at 50 °C, and the ethylene glycol was added just prior to freezing the sample.

exception of C-subunit, the $|2E|$ lines broaden upon urea denaturation—*the degree of broadening no doubt reflecting the extent of denaturation and the diversity of triplet environments*. The transition frequency of the unusually low-energy (2.319-GHz) transition of C-subunit increases by nearly 230 MHz upon denaturation and combines with the transition(s) from the other residue(s) to give a line at 2.546 GHz approaching that observed for the model compounds representing fully exposed tryptophan. The $|D|$ values of the denatured proteins fall in the range between 0.0990 and 0.1002 $\pm$ 0.0003 $cm^{-1}$, except for lysozyme and its oxindole-62 derivative. Since the $|D|$ value of denatured oxindole-108 lysozyme falls within the range observed for the other proteins and polypeptides in 8 M urea (see below), the low $|D|$ values for lysozyme and oxindole-62 lysozyme must be a consequence of the resistance of lysozyme to urea denaturation *and the persistence of structure in the environment of Trp-108*, the dominant phosphorescent residue (Rousslang et al., 1979).

## Discussion

A number of factors play important roles in the denaturation process, and they have been discussed in detail by Tanford (1968, 1970). The ones we are concerned with here are (1) the interaction between the solvent components, ethylene glycol and urea, and the sample polypeptide or protein, (2) the transition temperature required for conversion to the denatured state, and (3) the effect of intrinsic metal ions, as, for example, the $Cu^{2+}$ in azurin or the $Zn^{2+}$ in ADH.

*Role of the Solvent.* To date, all reported ODMR studies on proteins and polypeptides have utilized ethylene glycol–water mixtures as a cryosolvent. It has been pointed out by Tanford (1968) that proteins are for the most part stable in the presence of ethylene glycol, and the diol concentration must be well over 50% by volume before the first indication of a conformational change can be observed. There has been a considerable precedent for using EGW as a cryosolvent for enzymology studies [see review by Douzou (1977)], and furthermore EGW generally affords reasonably good glasses for ODMR studies. Aqueous snows generally give poor quality ODMR spectra, although we have managed to measure the $|D - E|$ transition of lysozyme in a pH 7.4 phosphate buffer snow and find that the microwave resonance line width and frequency domain are approximately the same as for lysozyme in EGW. We typically use 0.1 M $K_xPO_4$ as the buffer since the pH change with temperature is minimal (Douzou, 1973).

An important consideration with regard to the use of EGW for ODMR and phosphorescence studies of native or denatured polypeptides and proteins is the possibility of ethylene glycol binding and concomitant conformational perturbation. Since in EGW the diol concentration is $\sim$9 M and the water concentration is $\sim$28 M (mole ratio $\sim$1:3) and the sample concentrations are typically in the range $10^{-4}$–$10^{-6}$ M, equilibrium itself will tend to favor an ethylene glycol–protein complex, even if the association constant is only on the order of $RT$ at room temperature ($\sim$0.6 kcal $mol^{-1}$). Lysozyme and ADH, two proteins which have been investigated by ODMR [cf. Zuclich et al. (1973), von Schütz et al. (1974), and Rousslang et al. (1978, 1979)], are known to bind ethylene glycol. ADH can use a variety of alcohols as substrates (Sund & Theorell, 1963; Holzer et al., 1955). In the case of lysozyme, Ikeda & Hamaguchi (1970) have determined that the association constant for the molecule of ethylene glycol which perturbs Trp-62 is 0.4 $M^{-1}$. According to their circular dichroism (CD) measurements, the perturbation is complete at 40% by volume diol. However, there is no effect on the polypeptide backbone below 80% by volume diol. Glucagon, a 29-residue hormone also studied by ODMR (Ross et al., 1976, 1977; Deranleau et al., 1978), shows an increase in helical structure in the presence of ethylene glycol at ambient temperatures (Contaxis & Epand, 1974). The chain length dependent characteristics for acquisition of secondary structure in this hormone at reduced temperatures (near the fusion point of EGW) were determined from the triplet state parameters of the single tryptophan (Trp-25) in glucagon and glucagon fragments of various lengths. It is important to point out that the ODMR results were consistent with the room temperature NMR results of Bundi et al. (1976) which indicated (*in the absence of ethylene glycol*) the persistence of nonrandom structure in the 21–24 (Val-Gln-Trp-Leu) peptide segment of glucagon, and with the CD results of Bromer (1976) which indicated that the last three carboxyl-terminal residues (Met-Asn-Thr) were required for helix formation. In view of these room temperature data, the tendency of ethylene glycol to promote increased helicity in glucagon is probably due to ethylene glycol binding sites elsewhere on the polypeptide chain other than in the immediate region of Trp-25. Nevertheless, some cooperative chain length dependent interaction of glycol with this peptide region, even in the short fragments, cannot entirely be ruled out without additional solvent perturbation studies.

For ODMR denaturation studies in EGW we have used both 6 M guanidine hydrochloride (Gdn·HCl) (Rousslang et al., 1978) and 8 M urea (Ross et al., 1978). The problem with Gdn·HCl at high concentrations is that it tends to precipitate during the time required to freeze the sample (seconds). This is a common problem with cooled salt solutions in a cryosolvent (Douzou, 1977). Urea was chosen for the present investigation because it is not a salt. Although at a concentration of 8 M it tends to crystallize in EGW at room temperature, slightly warmed sample solutions (35 °C), frozen rapidly (seconds), often yield fair glasses, indicating that the solvent environment is probably homogeneous.

*Denaturation in EGW and Temperature Effects.* Urea is a hydrophilic denaturant which destabilizes protein structure primarily by its ability to effectively compete for hydrogen bonds (Schellman, 1955). The extent of urea denaturation—by fully denatured we mean that the native folded protein is converted wholly to a random coil—is both time and temperature dependent; where disulfide bridges exist, the properties of the polypeptide chain should conform to the cross-linked random coil model (Tanford, 1968, 1970).

Not all proteins are readily denatured in 8 M urea. For example, lysozyme at pH 7.0 and 25 °C is resistant to urea denaturation up to a concentration of nearly 8 M and is partly denatured at a concentration of 10 M (Hamaguchi & Kurono, 1963). Raising the temperature increases the extent of denaturation [cf. Tanford & Aune (1970)]. The ODMR results are consistent with these results: lysozyme denatured in EGW–8 M urea has two $|D - E|$ transitions; increasing the temperature of the solution, prior to rapid freezing, results in a decrease in the ODMR line intensity associated with tryptophan in the native protein and an increase in the line intensity in the ODMR spectral region associated with tryptophan in a randomly coiled polypeptide. However, two distinct classes of tryptophan resonances remain, even when lysozyme is first heated in saturated aqueous urea and then ethylene glycol is added just prior to freezing. One possibility is that if denaturation is complete after heating, some degree of the native structure is regained in the few minutes between addition of the diol and freezing of the sample. Another possibility is that some of the tryptophan residues in the denatured (albeit cross-linked) "random coil" are in non fully solvent available

environments as a consequence of the intact disulfide bridges. To distinguish between the contributions of the different tryptophan residues, we denatured oxindole-62 and oxindole-108 lysozymes. The ODMR results for denatured oxindole-62 lysozyme are similar to those for denatured lysozyme. Oxindole-108 lysozyme (in which emission appears to be from exposed residues; Rousslang et al., 1979) has a high value for the zfs parameter, $|D|$, similar to that of the exposed Trp-15 residue of ADH (0.1023 and 0.1015 $cm^{-1}$, respectively). Upon denaturation, $|D|$ decreases in each case to the value observed for tryptophan in the denatured polypeptides (cf. Tables I and II). The value of $|E|$, on the other hand, does not change much for Trp-15 in ADH. $|E|$ does decrease substantially in oxindole-108 lysozyme. When denatured, apparently the most probable environment (in terms of $|D|$ and $|E|$) for either Trp-15 in ADH or the dominant emitters among the remaining five tryptophan residues in oxindole-108 lysozyme is like that of Trp-25 in denatured glucagon. But the range of interactions reflected in the ODMR line width remains somewhat larger in oxindole-108 lysozyme, presumably due to contributions from several tryptophan residues which are still not fully equivalent due to residual structure maintained by the disulfide bridges.

It appears, therefore, that ethylene glycol imparts additional stability to lysozyme. Examination of the X-ray crystal model for triclinic lysozyme from the refinement by Hodsdon and Jensen (unpublished experiments) or the structure solved by Joynson et al. (1970) revealed the intriguing possibility that ethylene glycol can indeed bind in the active site of the enzyme. Depending upon the configuration of ethylene glycol, it could conceivably form a hydrogen-bonded bridge spanning a range from ~4 to 9 Å. Counting the side-chain and backbone interactions in the active site, there are well over a hundred possible atom pairs, within this distance, between a dozen key residues including Trp-62, Trp-63, and Trp-108. Formation of at least some of these links, depending upon steric considerations, may well be the explanation for why ethylene glycol appears to stabilize lysozyme against denaturation by heat and urea.

*Role of Metal Ions.* Azurin is also difficult to fully denature, unless EDTA is available to chelate the protein $Cu^{2+}$. According to the crystallographic model of Adman et al. (1978), the molecule is an eight-stranded $\beta$ barrel, with an external flap containing a short stretch of helix and one disulfide bridge joining the first and second strands of the $\beta$ barrel. Trp-48 is located in the sixth $\beta$ strand, and the indole nitrogen does not appear to be hydrogen bonded (E. T. Adman, personal communication). The $Cu^{2+}$ ligands—Cys-112, His-117, Met-121, His-46, and possibly Gln-18—tie together the eighth, seventh, sixth, and possibly second strands of the $\beta$ barrel. The structural integrity imparted to the protein by this ligation could account for the highly structured environment of Trp-48 as evidenced by the extremely narrow ODMR lines [cf. Ugurbil et al. (1977) and Rousslang et al. (1978)]. Since Trp-48 is in a part of the polypeptide chain binding the $Cu^{2+}$ atom, it is not surprising that the tryptophan ODMR is sensitive to the degree of denaturation. On the other hand, according to the ODMR and phosphorescence results, the stabilizing effect of the $Zn^{2+}$ atoms in ADH (two per subunit) seems to be small for those parts of the enzyme which contain Trp-15 and Trp-314. The two $Zn^{2+}$ atoms do not ligate amino acid residues in the neighborhood of Trp-15 or Trp-314 and are in structurally distinct domains in the native protein [see Brändén et al. (1975)]. In 8 M urea, the tryptophan phosphorescence is essentially the same as that of glucagon in the same solvent. However, the ODMR transition frequencies and line widths indicate that the average environment of the ADH tryptophan residues is still not equivalent to that in a true random coil.

In our discussion above we have considered the role of solvent, temperature, and metal ions in our effort to characterize the ODMR parameters of tryptophan in proteins. We have restricted ourselves to fairly well described systems for which X-ray crystal structure models have been available. In particular, lysozyme has been an important model for a multitryptophan system. We previously determined that Trp-108 is responsible for at least 80% of the phosphorescence of the native molecule (Rousslang et al., 1979). By comparing the phosphorescence and ODMR data of lysozyme, oxindole-62 lysozyme, and oxindole-108 lysozyme, we learned that the remaining 20% of the phosphorescence, although highly structured, is blue-shifted by 4 to 5 nm and has a $|D - E|$ transition frequency and a line width lying between those of glucagon and ACTH or Gly-Trp-Gly. From the X-ray crystal structure model (Blake et al., 1967), the tryptophan residue with the greatest solvent exposure is Trp-62, which shields Trp-63. In native lysozyme, fluorescence studies demonstrate that the Trp-63 first excited singlet state transfers energy efficiently to Trp-62 (Imoto et al., 1972), and, apparently, Trp-108 singlet–singlet transfers to Trp-62 (Formoso & Forster, 1975). Since the remaining 20% blue-shifted phosphorescence in oxindole-108 lysozyme is not observed in native lysozyme, it is conceivable that Trp-62 triplet–triplet transfers to Trp-108. This is consistent with the observation that buried tryptophan residues with blue-shifted fluorescence show red-shifted phosphorescence.

Given the above foundation established with a well-characterized system, we examined the ODMR and phosphorescence of a protein, C-subunit from protein kinase, with seven tryptophan residues. As yet there is no X-ray crystal structure model for this system. The phosphorescence of this molecule is intermediate between that of Trp-48 in azurin and that of the buried residue in ADH, Trp-314. From the phosphorescence spectrum of C-subunit we learn that the primary emitters are not fully exposed to solvent—the phosphorescence envelope is red-shifted and narrow compared to that of Gly-Trp-Gly. Also, *according to the phosphorescence data, there is only a single class of emitters.* By contrast, *according to the ODMR data, there are two main classes of tryptophan residues.* The main two $|2E|$ transitions of C-subunit are the most resolved resonance responses we have observed and clearly correspond to two separate classes of tryptophan residues in a protein. In fact, the 2.319-GHz $|2E|$ line is the lowest $|2E|$ transition energy that we have observed. Indeed, it is even *lower* than our model system Gly-Trp-Gly, which should represent the greatest shift ($|2E|$ = 2.468 GHz) due to maximum solvent exposure. Therefore, one or more of the C-subunit tryptophan residues must be strongly perturbed by a different interaction, perhaps a charged group(s) in the protein. A possible candidate is the protein-bound phosphate [according to Peters et al. (1977), there is 1.7 mol of bound phosphate per mol of enzyme]. On the other hand, the phosphorescence spectrum suggests that the perturbed tryptophan must actually have very little (free) solvent exposure. After denaturation, the phosphorescence and ODMR spectra of the C-subunit are quite similar to those of denatured ADH. From these results we conclude that the unusually perturbed tryptophan environment in native C-subunit is a consequence of tertiary structure. It will be important to see what is borne out by an eventual X-ray crystal structure.

## Conclusion

Proteins with tryptophan residues in distinct environments may display multiple ODMR transitions. Depending upon the relative contributions and separation of these transitions in the spectrum, line narrowing or line broadening can occur upon denaturation. In most cases line broadening is observed; in the case of C-subunit line narrowing is observed. When one tryptophan or one class of tryptophan residues is dominant, line broadening is observed; when there is more than one class strongly contributing, the overall unresolved line may narrow upon denaturation. We can distinguish the extent of denaturation in the vicinity of tryptophan residues from the frequency and width of the ODMR line. In most cases the $|2E|$ line widths were broader than for smaller polypeptides, which reflects an increase in the diversity of tryptophan sites. However, in the limit of total denaturation, in dilute solution, we expect that the phosphorescence and ODMR parameters should be close to those of a randomly coiled peptide.

We have demonstrated in this paper that the tryptophan residue triplet state has excellent resolution as a probe for molecular structure. We have shown that ODMR can be used to detect and resolve specific interactions in the vicinity of tryptophan residues, which arise as a result of tertiary structure, in the absence of an X-ray crystal structure model. ODMR can be used in combination with phosphorescence spectra to study the solution conformational properties of proteins and can provide useful information about interactions which depend upon the three-dimensional structure.

## Added in Proof

After this manuscript was submitted, Shoji et al. (1979) reported the sequence of two phosphopeptide fragments from C-subunit. Considering our proposal that a protein-bound phosphate is responsible for the unusual zfs of at least one Trp residue of C-subunit, it is intriguing to note that one of these peptide fragments has the sequence: Gly-Arg-Thr-Trp-Thr-(P)-Leu-Cys.

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# *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System. Evidence That the Dimer Is the Active Form of Enzyme I†

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ABSTRACT: In vitro kinetic measurements have been performed by using purified HPr, $E_I$, and a membrane fraction of $E_{II}$ from the *Escherichia coli* phosphoenolpyruvate-dependent sugar transport system. These measurements reveal very large lag times in the formation of methyl α-glucoside phosphate which are a function of the $E_I$ and the $E_{II}$ concentrations. The lag times decrease with increasing concentrations of $E_I$ but they increase with increasing concentrations of $E_{II}$. When $E_I$, together with $Mg^{2+}$ and phosphoenolpyruvate, is preincubated at 37 °C before starting the kinetic measurements, the lag time can be decreased or eliminated. We have shown that the process responsible for the lag time is the activation of $E_I$ by dimerization which is influenced by $Mg^{2+}$ and phosphoenolpyruvate.

The *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system is responsible for the concomitant phosphorylation and transport of PTS[1] sugars across the cytoplasmic membrane (Roseman, 1969; Kundig & Roseman, 1971; Postma & Roseman, 1976). The energy source, PEP, is coupled to the phosphorylation and transport processes by a minimum of two enzymatic reactions:

$$\text{PEP} + \text{HPr} \xleftrightarrow[Mg^{2+}]{E_I} \text{P-HPr} + \text{pyruvate}$$

$$\text{hexose}_{out} + \text{P-HPr} \xleftrightarrow[Mg^{2+}]{E_{II}} \text{HPr} + \text{hexose-P}_{in}$$

Procedures have been developed for the complete purification of a number of the proteins in this system (Anderson et al., 1971; Dooijewaard et al., 1979a; Robillard et al., 1979). As a result of these developments, it is now possible to begin studies on the molecular details of the energy coupling and transport mechanism, their regulation, and the kinetics which describe the individual processes. Initial studies on the mechanism of energy coupling have recently been reported (Dooijewaard et al., 1979b). In this present study the kinetics of the phosphorylation of methyl α-glucopyranoside have been monitored under conditions where the rate-limiting component has been HPr and $E_I$ or $E_{II}$. Lag times in α-MeGlc-P formation on the order of several minutes to 1 h were encountered. These could be made a function of the $E_I$ and $E_{II}$ concentrations. These lag times are orders of magnitude longer than the millisecond to second lag times commonly encountered during the establishment of a steady state in an enzymatic reaction. Since pre-steady-state kinetics can help clarify a reaction sequence, subsequent measurements on the origin of these lag times were performed. The results obtained indicate that the observed lag times are due to a slow self-association of $E_I$ to an active dimeric form.

## Materials and Methods

*Bacteria. E. coli* K 235 and *Salmonella typhimurium* SB 2950 were grown and harvested as stated previously (Dooijewaard et al., 1979a).

*HPr* was purified from *E. coli* K 235 according to the procedure of Dooijewaard et al. (1979a).

*$E_I$* was purified from *E. coli* K 235 by the method of Robillard et al. (1979). The ethylene glycol was removed by gel filtration over Sephadex G-75 after which the $E_I$ was stored at −20 °C in the lyophilized form.[2]

*$E_{II}$*. The source of $E_{II}$ was the cytoplasmic membrane fraction of *S. typhimurium* SB 2950. Frozen cells were resuspended in 25 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, and 1 mM $NaN_3$ (1 g wet weight cells/5 mL of buffer) and passed through a French pressure cell at 10 000–15 000 psi. The cell debris was removed by centrifugation for 30 min at 48000*g*. The supernatant was subjected to high-speed centrifugation (200000*g* for 2 h) to collect the membrane pellet. This pellet was washed once after resuspending in the same buffer to the original volume. The final pellet was resuspended in 20% of the original crude cell extract volume by using the same buffer and frozen at −20 °C until use.

*Assay Procedure.* The phosphorylation reaction was carried out at 37 °C in a final volume of 225 μL containing the following components: 5 μmol of potassium phosphate, pH 7.5; 2.5 μmol of KF; 0.25 μmol of DTT; 2 μmol of PEP (cyclohexylammonium salt); 2 μmol of methyl α-glucopyranoside containing an amount of methyl α-D-[U-$^{14}$C]glucopyranoside sufficient to produce 200000 cpm/μmol of α-MeGlc; 0.5 μmol of $MgCl_2$; 0.2 nmol of HPr; the specified amounts of $E_I$ and $E_{II}$. After being incubated at 37 °C, the phosphorylated sugar

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[1] Abbreviations used: PEP, phosphoenolpyruvate; α-MeGlc, methyl α-glucopyranoside; PTS, phosphoenolpyruvate-dependent phosphotransferase system; DTT, dithiothreitol.

[2] $E_I$ purified by this procedure may be associated with ribonucleic acid (unpublished results).

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