Evidence for ligand-induced conformational changes in proteins from phosphorescence spectroscopy

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ABSTRACT Phosphorescence spectroscopy on mouse myeloma IgA J539 in rigid solution at 77K revealed the type of anomalous short-lived component in the tryptophan decay originally observed with lysozyme (Churchich, J. E., 1966. *Biochim. Biophys. Acta.* 120:406–412) and seen in a large number of Bence Jones proteins (Longworth, J. W., C. L. McLaughlin, and A. Solomon. 1976. *Biochemistry.* 15: 2953–2958). The decay time of the anomalous component that results from the interaction between tryptophan side chains and disulfide linkages in proteins was observed to significantly lengthen in J539 in response to binding of a galactan antigen. With hen egg-white lysozyme in which the type of fluorescence enhancement on ligand binding seen with J539 has also been observed, phosphorescence measurements revealed a similar lengthening of the decay time of the disulfide-induced anomalous component in the tryptophan decay. These pertrurbations are interpreted as ligand-induced changes to the tryptophan-disulfide proximities that have been shown to exist in these structures. Given the short-range nature of the disulfide perturbation (see following article) the observations suggest, in particular when combined with x-ray crystallographic data, that phosphorescence decay-time measurements of disulfide perturbations can serve as a sensitive spectroscopic indicator of subtle conformational changes in immunoglobulins and other tryptophan-disulfide containing proteins.

INTRODUCTION

Although ligand-induced variations in the intrinsic emission often provide useful indicators of structural changes in protein complexes, the nature of the interactions producing the spectral perturbations is not always apparent. In response to ligand binding, fluorescence quenching in proteins can often be traced to specific effects such as excitation transfer or direct heavy-atom perturbations, but reductions and enhancements in intrinsic protein fluorescence have also been frequently observed with ligands which cannot function in the above roles. Examples of this kind can be found involving the binding of oligosaccharides (Lehrer and Fasman, 1966; Jolley et al., 1973), but with other ligands as well (Pollet and Edelhoch, 1973; Brewer and Weber, 1966). In these cases the changes in intrinsic fluorescence must be occurring as a result of local changes in the environment of emitting aromatic residues or due to structural changes that alter the interaction between the emitting residues and intrinsic quenching groups within the protein.

In addition to the quenching effect of proximal disulfide bridges on the fluorescence of aromatic side chains in proteins (Cowgill, 1967), evidence has been presented for the influence of nonionized carboxyl side chains (Cowgill, 1963), uncharged amino groups (Knibbe et al., 1968), and variation in the polarity and/or polarizability of the local environment (Weinryb and Steiner, 1971). It becomes difficult to interpret such ligand-induced fluorescence changes in terms of specific structural factors.

In sharp contrast to the plethora of interactions which influence excited-state lifetimes in fluid media the tryptophan phosphorescence of globular proteins in rigid solution at 77K is relatively invariant to the local environment of the emitting chromophore. The only intrinsic perturbation known to result in a marked shortening of the phosphorescence lifetimes of both tyrosine and tryptophan at 77K is that with proximal disulfide bridges. A short-lived component in the phosphorescence decay of tryptophan at 77K was observed in lysozyme by Churchich (1966), in γ-globulin fractions (King and Miller, 1975, and references therein), bovine α-lactalbumin (Miller and King, 1975), and in a large number of Bence Jones proteins (Longworth et al., 1976). X-Ray crystallography has confirmed the proximity of disulfide groups to tryptophan side chains in both the lysozyme (Blake et al., 1967) and antibody (Poljak et al., 1973) structures. In addition similar effects of the disulfide linkage on the decay of the indole phosphorescence has been observed

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with simple model systems (Longworth and Hélène, 1975).

Disulfide perturbation on the triplet state of the indole chromophore would appear to involve an endothermic excitation or one-electron transfer with a steep exponential dependence of the quenching constant with separation (Li et al., 1989) arising from electron cloud overlap. Subtle changes in distance between aromatic side chains and perturbing disulfide links can thus be expected to result in significant variations in triplet lifetime.

In the course of our investigation of the emission properties of the Fab' fragment of the anti-galactan antibody J539 in the presence and absence of antigen, the short-lived component in the tryptophan phosphorescence decay previously observed by Longworth et al. (1976) with Bence Jones proteins was apparent. In the present work we describe the changes in the decay time of the short-lived component not only in the immunoglobulin but in hen egg-white lysozyme as well, providing evidence for ligand-induced changes in tryptophan-disulfide interactions within these proteins.

MATERIALS AND METHODS

The three-times-crystallized and dialyzed hen egg-white lysozyme (EC 3.2.1.17) (HEWL)[1] and *N,N',N''*-triacetyl chitotriose (tri-NAG) were purchased from Sigma Chemical Co. (St. Louis, MO) without further purifications. The Fab' of murine immunoglobulin A myeloma protein J539 and the antigen, β-(1 → 6)-D-galactotetraose (gal_4), were obtained from Dr. C. P. J. Glaudemans and were prepared as described by Glaudemans et al. (1984) and references therein. The spectroscopic grade glycerol was obtained from Aldrich Chemical Co. (Milwaukee, WI). The samples were buffered in either 0.01 or 0.07 M phosphate buffer saline (pH 7.4) and mixed with neat glycerol. The glycerol/buffer ratio was rendered 70:30 by weight.

All the phosphorescence experiments were performed at 77 K. The samples contained in 3-mm ID Suprasil quartz tubes were excited with a 100-W high-pressure mercury lamp (Illumination Industry Inc., Sunnyvale, CA). The excitation wavelengths were selected by a 0.25-m grating monochromotor (Bausch & Lomb Inc., Rochester, NY) with a 6-nm bandwidth for obtaining spectra and 12 nm for decay measurements. The excitation and prompt emission beams were separated from the long-lived emission with a mechanical phosphoroscope. The emission wavelengths were selected by a 0.5-m Bausch & Lomb grating monochromotor at a bandwidth of 3 nm. The signals were detected with an EMI 9635QB photomultiplier tube, amplified with a custom-built DC amplifier, captured with a digitizer (Biomation, model 2805 Master, Gould Inc., Oxnard, CA) and displayed on either an oscilloscope (model S54AR, Telequipment, London) or an X-Y recorder (model F-80A, Varian Associates, Inc., Palo Alto, CA). Fluorescence and phosphorescence spectra were not corrected for variations in instrumental sensitivity with emission wavelength.

The decay of the tryptophan phosphorescence was obtained at a number of emission wavelengths by shuttering the excitation with a solenoid shutter and following the time course of the steady-state signal. The decays were slow enough that this could be done with the phosphoroscope in place. Typically 10–12 decay curves were collected and averaged. The averaged decay curve was decomposed into a number of exponentials by first calculating the correlation coefficient from the long-time end of the decay until a maximum value was obtained. The lifetime and relative contribution of the slow, essentially unperturbed component, along with their uncertainties, was obtained from a linear regression analysis of the long-time data. The decay curve resulting from the subtraction of this slow component from the experimental decay was analyzed in the same manner. The decay components in this study were sufficiently well separated in time that a satisfactory decomposition into two exponential contributions was obtained. Emission from the glycerol-buffer solvent made a negligible contribution to the phosphorescence decays with lysozyme where the protein concentration was high. For excitation at wavelengths >300 nm phosphorescence spectra with J539 suggested that there was some solvent contribution at 440 nm. The decays presented were excited at 295 nm where the solvent contribution was <5%, but the pattern of the results was essentially unchanged even for excitation at >300 nm.

The x-ray coordinates of the Fab' fragment of J539 (Suh et al., 1986) were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977), Brookhaven National Laboratories, Upton, NY, and were examined on a Silicon Graphics computer system (model IRIS 3130) using the program Quanta (1987) from Polygen Corp. (Waltham, MA).

[1]Abbreviations used in this paper: gal_4, β-(1 → 6)-D-galatotetraose; HEWL, hen egg-white lysozyme (EC 3.2.1.17); IgA, immunoglobulin A; LADH, alcohol dehydrogenase from equine liver (EC 1.1.1.1); TME, tryptophan methylester; tri-NAG, N,N',N''-triacetyl chitotriose.

RESULTS

Phosphorescence properties of the Fab' fragment of J539

The phosphorescence spectrum of the Fab' in 70:30 wt/wt glycerol buffer at 77 K appears in Fig. 1. Whereas with excitation at 280 nm the spectra shown display a tyrosine contribution, as with most tryptophan-containing proteins

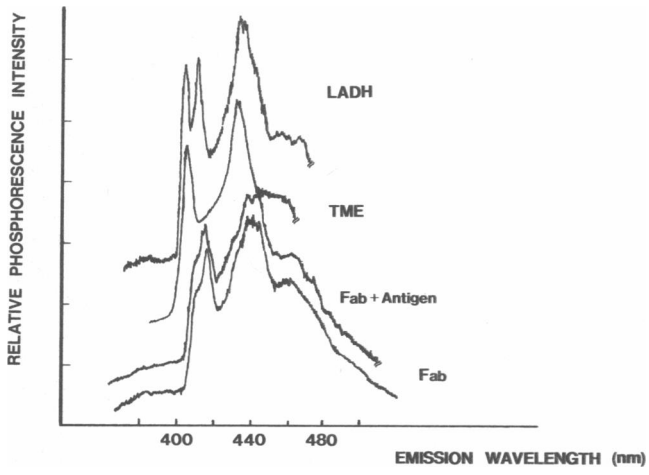

FIGURE 1 The phosphorescence spectra of the Fab' fragment of IgA J539 (1.5×10^{-5} M) in the presence and absence of the gal_4 antigen (1.0×10^{-4} M) using 2:1 glycerol/phosphate buffer (pH 7.4) as the solvent. Tryptophan methyl ester and LADH in the same solvent are shown for comparison. The spectra were all obtained at 77 K, and are displayed with excitation at 280 nm.

the emission is dominated by the structured tryptophan component, and with excitation at 295 nm the spectrum consists entirely of tryptophan. Whereas no attempt was made to calculate the tryptophan phosphorescence quantum yield, comparison of the intensity with tryptophan methylester at the same indole concentration reveals that the efficiency, as with the fluorescence and phosphorescence of a large number of Bence Jones proteins is low (Longworth et al., 1976).

Tryptophan contribution to the spectrum displays spectral heterogeneity

The principal 0-0 transition at 416 nm is unusually red with a blue shoulder visible at 410 nm. The very red peak suggests that the principal emitting groups lie in a rather polarizable local environment. Comparison of the spectrum (Fig. 1) with that of liver alcohol dehydrogenase (LADH) and tryptophan methylester (TME) indicates that there is no clear indication of a contribution from solvent-exposed residues, which tend to display 0-0 transitions below 410 nm.

With excitation at 296 nm, the steady-state phosphorescence at 440 nm from tryptophan in the Fab' showed an unusual nonexponential decay. It could be decomposed into two components: an anomalous short-lived component (0.6 s) accounting for 27% of the initial intensity as well as a more usual long-lived component (4.3 s) making up the remainder (Fig. 2 *a*). By way of comparison the solvent-exposed and buried residues in LADH which give rise to the 405- and 412-nm peaks, respectively (see Fig. 1 and Purkey and Galley, 1970), both decay, as does tryptophan methyl ester, with lifetimes of ~6 s reflecting the relative invariance of the usual decay time to the polarity and/or polarizability of the local environment. The only intrinsic perturbation that has been shown thus far to result in the type of triplet quenching observed here involves the proximity of disulfide bridges to the emitting aromatic residues as is found with lysozyme (Churchich, 1966). In earlier work in which this type of anomalous decay component was observed with a large number of Bence Jones proteins (Longworth et al., 1976), attention was drawn to the tryptophan-disulfide proximity present in immunoglobulin structures, a feature which has continued to reappear in more recent structures (Suh et al., 1986; Satow et al., 1986).

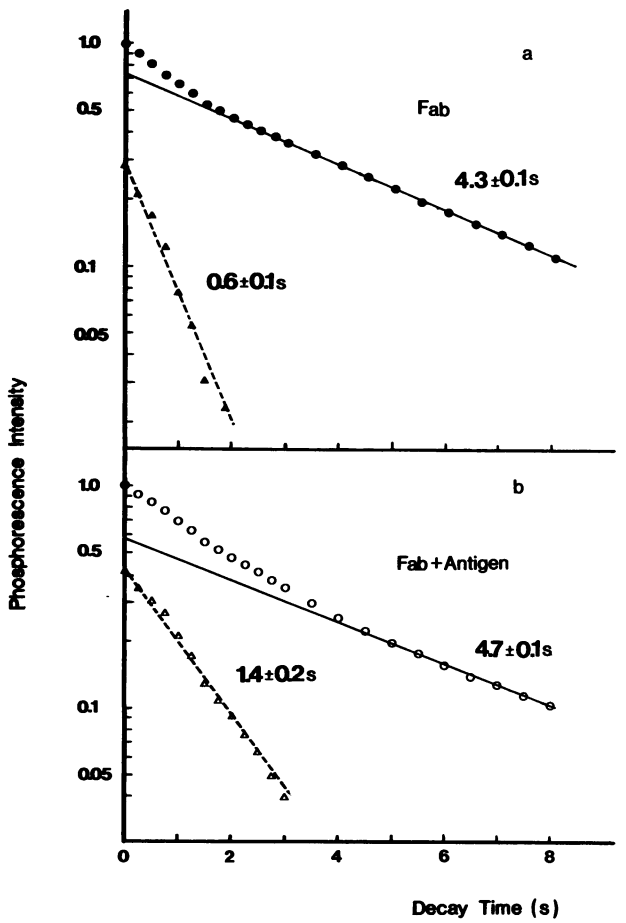

FIGURE 2 The steady-state tryptophan phosphorescence decays of the Fab' fragment of J539 in the (*a*) absence and (*b*) presence of the antigen. The experimental conditions were the same as those indicated in Fig. 1 and the excitation wavelength was 296 nm. The two exponential components arising from the decomposition of the decays are indicated with the dashed lines while the solid line is drawn through the experimental data points.

Evidence for a decreased tryptophan-disulfide interaction in J539 on antigen binding

The phosphorescence spectrum of the antigen-bound Fab displays similar features to that of the free antigen-binding fragment (Fig. 1). On antigen binding the Fab' also showed a two-component steady-state decay for the tryptophan phosphorescence. However, the lifetime of the fast component was consistently found at a number of exciting wavelengths to be increased from 0.7 ± 0.1 to 1.4 ± 0.1 s on binding the galactan. Fig. 2 *b* shows the fast and the slow decay components of J539 excited at 296 nm in the presence of gal_4. It is evident from this increase in lifetime that a reduced tryptophan-disulfide interaction accompanied antigen binding. A reduced tryptophan-disulfide interaction would be expected to manifest itself at the singlet level as well, and likely contributes to the fluorescence enhancements seen in this protein on antigen binding (Jolley et al., 1973; Pollet et al., 1974).

Perturbations to the tryptophan-disulfide interactions in the hen egg-white lysozyme (HEWL) and tri-NAG complex

The similarity of the ligand-induced fluorescence changes in HEWL on binding trisaccharide inhibitors (Lehrer and Fasman, 1966) with those seen in J539, coupled with the known occurrence of phosphorescence decays (Churchich, 1966) characteristic of tryptophan-disulfide proximity, prompted us to examine the phosphorescence properties of lysozyme-inhibitor complexes.

At 77 K, HEWL showed a sharp 0-0 vibronic band at 416 nm, and no tyrosine contribution was observed irrespective of the exciting wavelength. The steady-state phosphorescence decay consists of a 0.95- and a 3.95-s component, with an overall 1/e time of 2.1 s. The relative contribution to the initial intensity from the fast and slow components are 44 and 56%, respectively. These results are consistent with those reported previously (Churchich, 1966; Longworth, 1971).

Upon binding tri-NAG, the 0-0 band was slightly red-shifted, indicating a minor change in tryptophan environment. A 0.5-s increase in the overall steady-state decay time was observed (Fig. 3). After decomposition of the decay, the lifetime for the slow component, comprising 59% of the initial intensity, was increased to 4.21 s, whereas the lifetime of the remaining fast component was increased to 1.22 s, the increase in lifetime of the short-lived component again reflecting a ligand-induced decrease in tryptophan-disulfide proximity, which is probably at the origin of the ligand-induced fluorescence enhancement seen in this protein as well.

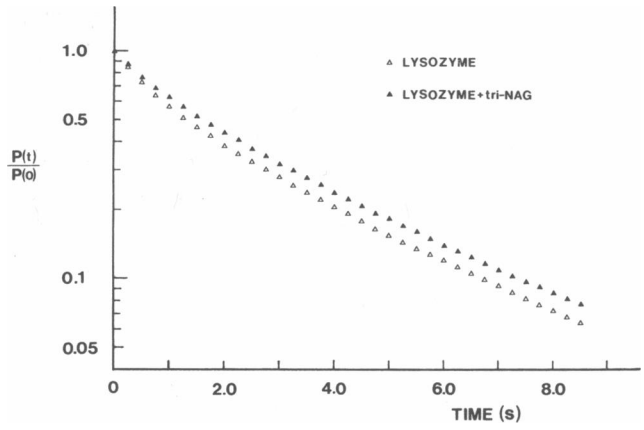

FIGURE 3 Steady-state phosphorescence decays of HEWL (1.0×10^{-3} M) in the absence and presence of tri-NAG (5.0×10^{-3} M) at 77 K in 70:30 glycerol/buffer (ph 7.4). The excitation and emission wavelengths were 290 and 445 nm, respectively.

DISCUSSION

Measurements of the phosphorescence of globular proteins in rigid solution at low temperatures often provides a useful adjunct to emission measurements made under ambient conditions. The facile separation of tyrosine and tryptophan contributions (Longworth, 1971), and the observations of tryptophan environmental heterogeneity in the absence of solvent relaxation, both form straight phosphorescence (Purkey and Galley, 1970) as well as by ODMR (Maki and Zuclich, 1975; Kwiram and Ross, 1982), provide typical examples. On reviewing the factors which are known to, or in principle could, shorten tryptophan triplet lifetimes under these conditions, one finds that compared with the plethora of factors that influence protein fluorescence lifetimes (Cowgill, 1963) under ambient conditions, the short-lived anomalous decay components observed here and in earlier work (Churchich, 1966; King and Miller, 1975 (and references therein); Longworth et al., 1976) are a relatively specific signature of tryptophan-disulfide proximity and provide a sensitive monitor of the conformational state of that part of the protein in which they occur.

The triplet lifetimes of tryptophan in proteins is essentially invariant to the local polarity and/or polarizability of the local environment in rigid media as illustrated with Trp 15 and 314 in LADH. The phosphorescence lifetimes which differ so enormously in solution (Saviotti and Galley, 1974; Strambini, 1987; Vanderkooi et al., 1987) as a result of dynamic factors, have been shown with a large number of examples to lie between 5.5 and 6.5 s in glycol-buffer glasses at 77 K independent of location (Longworth, 1971). Protein phosphorescence lifetimes can be markedly shortened by the presence of certain heavy atoms in the vicinity of the emitting residues (Monsigny et al., 1975; Hershberger and Maki, 1980; Galley et al., 1982, Khamis et al., 1987), by excitation transfer to chromophores which can act as triplet energy acceptors (Galley and Stryer, 1969), or as revealed more recently from stacking with nucleic acid bases (Zang et al., 1987). These perturbing groups, however, are not normally present in proteins and are specifically introduced as extrinsic probes of proximity relationships in protein complexes.

Energy transfer between tryptophans at the triplet level undoubtedly occurs between neighboring tryptophans in proteins, but transfer per se is not a source of quenching. Referring again to the LADH structure the buried tryptophans (314) lie essentially in contact with each other on either side of the twofold axis which relates the two identical subunits so that there is undoubtedly energy

exchange between them. However, the emission from these residues at the 412-nm peak decays with the usual long lifetime. If one tryptophan possesses a triplet energy that is higher than that of a neighboring residue, transfer from the former to the latter could, in principle, be expected to result in a shortened "donor" lifetime. The shortened lifetime would predominate on the blue side of the spectrum where the donor would tend to emit. We know of no demonstration of this effect in proteins to date, and it does not apply in the examples presented here. In both the Fab' of J539 and lysozyme there is relatively more of the short-lived anomalous component to the red (416 nm) than to the blue (410 nm) of the 0-0 transition. These considerations coupled with the observed invariance of tryptophan phosphorescence decay times at 77 K to the polarity and polarizability of the local environment support the contention that the anomalous decay times are characteristic of interactions with neighboring disulfides, and that changes in these decay times reflect alterations in these interactions.

The interaction of disulfide linkages with tryptophan residues both at the triplet and singlet states are short range in nature. The early work of Cowgill (1970) on fluorescence quenching with simple peptides revealed that the perturbation at the singlet level was limited to distances of <7 Å. Similarly at the triplet level there are many proteins such as the serum albumins, which despite the abundance of disulfides in the molecule, display unperturbed tryptophan phosphorescence decays. In addition Longworth and Hélène (1975) observed an unperturbed component in the phosphorescence decay of the cyclic peptide Cys-Trp-Cys, presumably due to conformations in which the indole ring was not sufficiently close to the sulfur atoms. Evidence is provided in an accompanying article (Li et al., 1989) that the interaction at the triplet level appears to involve an endothermic excitation or one-electron transfer to disulfide with a Dexter-type distance dependence characteristic of electron cloud overlap. Employing the parameters obtained from that work reveals that the ligand-induced lifetime changes observed with the systems under investigation here, provide examples of subtle increases in tryptophan-disulfide separations of ~0.3–0.4 Å.

With proteins possessing well-separated tryptophans, the quenched residues, taking into account the prior singlet as well as triplet quenching, can be expected to make only small contributions to the steady-state phosphorescence. These contributions are larger in pulse-induced phosphorescence decay measurements (Li and Galley, unpublished observation) but are still suppressed due to the prior singlet quenching. In such cases it might well be preferable to monitor the disulfide perturbation at the singlet level. Perturbed residues of this type should give rise to significant short-lived contributions in time-dependent fluorescence measurements; the recent observations of Vandeven et al. (1987) on thioredoxin providing an example of this type.

When a number of tryptophans are rather closely associated in a protein so that energy exchange at the singlet and/or triplet levels is possible, the influence of tryptophan-disulfide quenching centers can be expected to be more profound in that they serve as "quenching centers." Lysozyme and the immunoglobulins would appear to fall into this category. The emission quantum yields at both the singlet and triplet levels are very low (Longworth, 1971; Miller and King, 1975) and the overall emission intensity appears to be sensitive to the precise geometry at the quenching centers. The fact that the phosphorescence decays with both lysozyme and J539 contain such a significant fraction of the "quenched" component indicates that excitation tends to be "funnelled in" from other tryptophans in the vicinity of the perturbed residues. As a result decreases such as the ones observed here in the sensitive tryptophan-disulfide interactions can be expected to produce sizeable perturbations to the protein emission at both the singlet and triplet level.

With both J539 and HEW lysozyme the "slow" components in the phosphorescence decays (~4 s) are also somewhat shorter than those found in most globular proteins. We believe this occurs as a result of triplet–triplet energy transfer from other tryptophans to the perturbed residues in proximity to disulfides.

Speculations on antigen binding to J539

J539 has served as a useful model of antigen-antibody interactions in that the x-ray structure of the Fab' fragment has been determined in the absence of antigen to 2.6 Å resolution (Suh et al., 1986). In addition Glaudemans and co-workers have carried out extensive studies on the binding affinities and protein fluorescence changes accompanying the binding of many galactan antigens to J539 and related anti–galactan immunoglobulins (Jolley et al., 1973). These studies have emphasized binding to the antibody structure provided by the x-ray analysis, and a picture involving binding near Trp 33H and Trp 90L,[2] which appear to line the surface of the binding pocket, has emerged (Glaudemans et al., 1984; Glaudemans and Kováč, 1985).

Examination of the x-ray structure of J539 (Suh et al.,

[2]The sequence numbering used here is that found in the x-ray studies on this system (Suh et al; 1986) which differ from the convention proposed by Kabat et al. (1987).

1986) reveals that more deeply within the variable domains invariant Trp 36H and Trp 34L lie in proximity to the intrachain Cys(22H)-Cys(96H) and Cys(23L)-Cys(87L) disulfide bridges, respectively. The indole rings and sulfur atoms are within 1.0 Å of van der Waals contact in both the H and L domains. This interaction, which occurs at the core of the immunoglobulin fold, appears to be an invariant feature of immunoglobulin structures (Poljak et al., 1973; Kabat et al., 1987). We believe these tryptophans to be the major source of the short-lived ligand-perturbed emission seen here. Analogous invariant tryptophans occur in proximity to the intrachain disulfides at the core of the L and H constant domains as well, but very likely do not contribute significantly to the rapid component in the phosphorescence decay in this antibody. The latter domains contain only one other tryptophan side chain and it lies more than 10 Å from either of the "quenching centers" so that the tryptophans at these centers would not receive excitation indirectly by energy transfer as occurs with Trp 36H and 34L in the variable domains where there are five additional tryptophans located within 10 Å. In addition, the invariant tryptophans in the constant domains are located much further from the combining region. Although we cannot rigorously rule out their involvement much larger conformational changes on antigen binding would be required to influence the tryptophan-disulfide interactions in these domains.

As apparent in Fig. 4 the invariant tryptophan residues in the variable domains are buried on either side of the H/L interface but are connected to it. Trp 36H for example is connected through the OH of Ser 35H to a cavity which houses a water molecule in the x-ray structure and which lies below Trp 90L. On the other side Trp 34L is connected to H/L interface through noncovalent interactions involving the side chains of His 33L and Trp 90L. Looking into the mouth of the binding pocket the invariant tryptophans in proximity to the disulfides lie more deeply than the surface tryptophans (33H and 90L) that undoubtedly line the binding pocket. Whereas it is possible that subtle structural changes at the "quenching centers" occur in response to surface binding, we speculate that the perturbations are a reflection of a deeper penetration of the galactan into the binding pocket than envisaged (Glaudemans and Kováč, 1985; Suh et al., 1986), displacing a water molecule observed in the crystal structure (Fig. 4). This would require motions of some side chains such as Trp 90L and Leu 99H lining the pocket, and these motions might be communicated to the quenching centers resulting by way of a "push" and/or "pull" in the increased Trp-disulfide separation that is reflected in the phosphorescence decay. We anticipate that with the use of additional triplet-state approaches it should be possible to more clearly refine the binding geometry and communication pathway to the quenching centers.

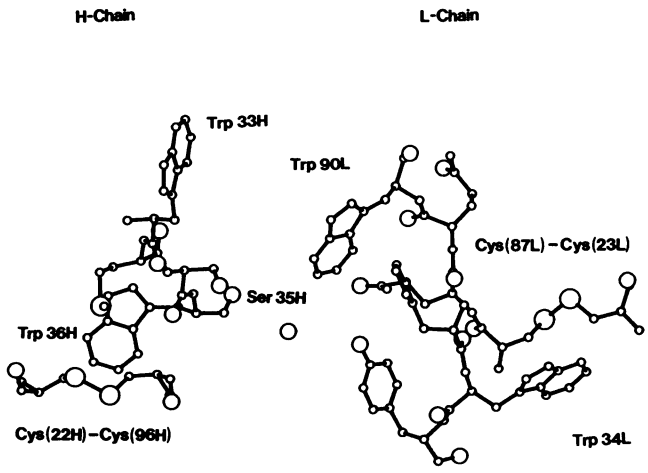

FIGURE 4 An illustration of the tryptophan-disulfide proximities which occur on the VL and VH domains revealed in the x-ray structure of the J539 Fab' fragment. Invariant Trp 36H and 34L located on either side of the H/L interface near the intrachain disulfides are indicated in the figure as are Trp 33H and 90L which appear to line the antigen-binding pocket, entrance to which is from the top of the figure. Connections of the disulfide "quenching centers" to the H/L interface could well occur through some of the side chains illustrated. The oxygen atom in the center of the structure is a water molecule. The figure is based on the x-ray coordinates obtained from the Brookhaven Protein Data Bank.

Perturbations in the lysozyme-triNAG structure

The x-ray picture of the lysozyme structure suggests that in this case the decrease in a tryptophan-disulfide interaction induced by inhibitor binding occurs more directly. The crystal structure reveals that tryptophans 62, 63, and 108 are all located at the binding cleft and are in a position to hydrogen bond with the inhibitor (Blake et al., 1967). Rearrangements of tryptophan side chains lining the bonding cleft in response to inhibitor binding have been suggested from an electron density difference map (Kelly et al., 1979) and NMR data (Perkins et al., 1981). The indole rings of Trp 62 and 63 are shifted towards the cleft. The indole ring of Trp 63, which is in proximity to the Cys(74)-Cys(96) disulfide bridge, shows a displacement away from that disulfide linkage and we believe this to be the perturbation resulting in the phosphorescence decay changes seen in this work as well as the earlier fluorescence enhancements (Lehrer and Fasman, 1966).

While in lysozyme as well as in the Fab more than one tryptophan-disulfide pair exists within the structure, with the data at present we were unable to resolve more than one exponential from the anomalous component of the

steady-state decays. Tryptophan-disulfide pairs throughout these structures are rather similar with respect to separation, but in addition "quenching centers" which do not receive excitation by transfer from elsewhere can be expected to contribute only weakly to the rapid component of the steady-state decay.

We believe the present data provide a demonstration that subtle changes in tryptophan-disulfide proximities can be readily detected from phosphorescence measurements. In addition, given the sensitivity of the tryptophan-disulfide interaction to distance at the singlet as well as the triplet level, the evidence in this work for ligand-induced subtle increases in tryptophan-disulfide perturbations suggests that these are at the origin of the large fluorescence enhancements observed with both these protein systems as well. The invariant nature of the tryptophan-disulfide proximity in immunoglobulin structures suggests that protein emission might well provide a sensitive monitor of subtle ligand-induced conformational changes in a wide variety of antibody structures.

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