vestigated tryptophan in EG:H_2O glass using the method described above. There is only a weak correlation of the ZF parameters with Stokes' shift that is monotonic and continuous.

A similar set of measurements was made on horse liver alcohol dehydrogenase, an enzyme characterized by two structurally distinct tryptophans, one solvent-exposed pair, and one pair inside the contact region of the dimer. The inhomogeneous broadening resulting from statistical heterogeneity is less than the Stokes shift between the structurally distinct sites; the phosphorescence is a partially resolved doublet. Plots of $|D|$ and $|E|$ vs. Stokes shift reveal a clear discontinuity at the wavelength where the O—O bands of the structurally distinct sites merge.

Hen lysozyme, its complex with tri(*N*-acetylglucosamine), human carbonic anhydrase B, and its complex with *m*-acetylbenzenesulfonamide show similar discontinuities in the ZFS parameters plotted against Stokes shift over the O—O bands, even though in lysozyme and its complex no distinct site emissions can be resolved optically.

We conclude that ODMR with narrow band wavelength selection over an inhomogeneously broadened phosphorescence band can reveal, at least in some cases, emission from structurally heterogeneous sites even if these emissions are not optically separable into distinct bands. The success of this method rests upon sufficient differences in the relationship between the ZFS and the Stokes shift between structurally distinct sites.

HETEROGENEITY IN THE CIRCULAR POLARIZATION OF PROTEIN FLUORESCENCE

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Optical activity has been extensively used in the investigation of the conformation of biopolymers. This property is usually measured by the optical rotatory power or the circular dichroism (CD) of the studied biopolymers. We have shown that the optical activity of chiral molecules, including proteins, can be manifested by an additional spectroscopic property, its circular polarization of the luminescence (CPL) (1, 2). CPL is the emission analogue of CD and is related to the molecular conformation of the first electronic excited state in the same way that CD is related to the molecular conformation of the ground state (1, 2). The CPL spectrum is expressed by the emission anisotropy factor defined as $g_{em} = 2\Delta f/f$, where f is the total fluorescence intensity and Δf is the intensity of the circularly polarized component in the emitted light.

If the conformation and the environment of the chromophore are the same in the ground state and excited state, g_{em} should be equal to the absorption anisotropy factor g_{ab} (1, 2) defined as $2(\epsilon_l - \epsilon_r)/(\epsilon_l + \epsilon_r)$ where ϵ_l and ϵ_r are the molar extinction coefficients for left-handed and right-handed circularly polarized light. Since only lumines-

cent chromophores contribute to the CPL spectrum, the information obtained has more specificity than that yielded by CD. In the case of proteins, for example, several chromophores contribute to the observed CD spectrum. These include the peptide bonds, disulfide cross-links, and aromatic side chains, which have strongly overlapping absorption bands. In contrast, CPL is related only to the tryptophan residues, and to some extent to the tyrosine residues, which are fluorescent. Furthermore, the spectral resolution between tyrosine and tryptophan, and among different residues of each kind, are significantly greater in emission than in absorption. The fluorescence of a chromophore usually involves a single electronic transition; the emission spectrum of the chromophore is thus composed of a single electronic band. It is thus clear that the CPL of proteins should be simpler to assign and apply than their CD spectra (2).

The various chromophores in proteins are not intrinsically asymmetric; they derive their optical activity from asymmetric influence of the environment. Therefore detected changes in the CPL spectrum of proteins can reflect conformational alterations in the vicinity of the tryptophan or tyrosine residues that contribute to the protein fluorescence (3–5). According to theory, g_{em} is expected to be constant across the emission band of a single allowed electronic transition (1, 2). We have found that g_{em} is approximately constant across the emission band of single chromophores (2, 3, 6) and across the fluorescence spectrum of proteins that contain a single tryptophan residue per molecule: staphylococcal nuclease (2), azurin (7), and subtilisin Carlsberg (3). Human serum albumin was found to be an exception, probably because of its heterogeneity.

The heterogeneity of protein fluorescence is revealed by the variability of g_{em} across the fluorescence spectrum of the protein. Using this approach, we could resolve the optical activity of tyrosine and tryptophan residues in subtilisin types Novo and Carlsberg and between different tryptophan residues in a single protein: subtilisin Novo, pepsinogen (2), papain, etc. Preferential enhancement of the quantum efficiency of exposed "red" tryptophan (by D_2O) was used to further demonstrate the heterogeneity in the CPL of the tryptophan residues of those proteins (2).

When derivatives of 1-dimethyl-amino-5-naphthalene sulfonyl (DNS) bind to anti-DNS antibodies, no detectable CD is induced in their long wavelength absorption band. The fluorescence of the bound DNS, however, was found to exhibit some circular polarization, reflecting the asymmetric influence of the antibody combining site (8). The value of g_{em} varied across the emission band and depended upon the wavelength of the excitation light. We interpreted these results to indicate the heterogeneity of the combining sites of the population of the antibody molecules (8). The marked difference between g_{ab} and g_{em} demonstrates the change in mode of binding of the hapten in the antibody binding sites that takes place upon electronic excitation of the ligand. In contrast to the axti-DNS-DNS complexes, the emission anisotropy factor of acridine chromophore bound to MOPC-315 antibody via a dinitrophenyl group is approximately constant across the fluorescence spectrum. This and the fact that g_{em} did not depend on the excitation wavelength reflect the homogeneity of the combining site of the myeloma protein (8).

By the analysis of the CPL spectrum of antibodies and their fragments, we could demonstrate the existence of subunit interactions among the light and heavy chains of the antibody molecule (5) and moreover, that the antibody molecule undergoes a conformational change in the Fc portion induced by antigen binding (9). This is of a particular interest since antigen binding to the Fv portion of the Fab fragments is known to trigger specific biological events at the Fc portion (e.g. complement fixation).

To detect a possible conformational change transmitted from the combining site to the Fc portion, we have studied the CPL spectrum of antibodies and their Fab fragments in the presence or absence of their antigens. The antigens chosen were either nonfluorescent (poly(DL-alanyl)-poly(L-lysine) with anti-alanine) or had no tryptophan residues (RNase and anti-RNase). These antigens induced changes in the CPL spectrum of the antibodies and their Fab fragments. The CPL change detected for the Fab fragments upon binding of the corresponding antigens were markedly different than the changes for the whole antibody molecule. A large spectral change at the red part of the CPL spectrum was interpreted to indicate conformational change in the Fc moiety induced by the antigen binding. Reduction of the interchain disulfide bond of the antibody abolished the antigen-induced spectral change due to the Fc portion of the antibody molecule but did not affect the changes observed in the Fab moiety. This suggests that the disulfide bonds at the hinge region of the antibody are required for the transmission of the conformational change from the Fab to the Fc. In fact, reduction of the interchain disulfide bond is known to diminish complement fixation (10).

REFERENCES

1. STEINBERG, I. Z. 1976. *In* Concepts in Biochemical Fluorescence. R. Chen and H. Edelhoch; editors. Marcel Dekker, Inc., New York.
2. STEINBERG, I. Z., J. SCHLESSINGER, and A. GAFNI. 1974. *In* Peptides, Polypeptides and Proteins. E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, editors. John Wiley and Sons, Inc., New York. 351–369.
3. SCHLESSINGER, J., R. S. ROCHE, and I. Z. STEINBERG. 1975. A study of subtilisin types Novo and Carlsberg by circular polarization of fluorescence. *Biochemistry.* **14**:255.
4. SCHLESSINGER, J., and A. LEVITZKI. 1974. Molecular basis of negative co-operativity in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. *J. Mol. Biol.* **82**:547.
5. SCHLESSINGER, J., I. Z. STEINBERG, D. GIVOL, and J. HOCHMAN. 1975. Subunit interaction in antibodies and antibody fragments studies by circular polarization of fluorescence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **52**:231.
6. SCHLESSINGER, J., A. GAFNI, and I. Z. STEINBERG. 1974. Optical rotatory power in the ground state and electronically excited state of diketopiperazines containing aromatic side chains. *J. Am. Chem. Soc.* **96**:7396.
7. GRINVALD, A., J. SCHLESSINGER, I. PECHT, and I. Z. STEINBERG. 1975. Homogeneity and variability in the structure of azurin molecules studied by fluorescence decay and circular polarization. *Biochemistry.* **14**:1921.
8. SCHLESSINGER, J. I. Z. STEINBERG, and I. PECHT. 1974. Antibody-hapten interactions: circular and linear polarization of the fluorescence of dansyl bound to anti-dansyl antibodies. *J. Mol. Biol.* **87**:725.
9. SCHLESSINGER, J., I. Z. STEINBERG, D. Givol, J. HOCHMAN, and I. PECHT. 1975. Antigen-induced conformational changes in antibodies and their Fab fragments studied by circular polarization of fluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2775.
10. SCHUR, P. H., and G. D. CHRISTIAN. 1964. The role of disulfide bonds in the complement-fixing and precipitating properties of 7S rabbit and sheep antibodies. *J. Exp. Med.* **120**:531.