SUBUNIT INTERACTION IN ANTIBODIES AND ANTIBODY FRAGMENTS STUDIED BY CIRCULAR POLARIZATION OF FLUORESCENCE

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1. Introduction

The property of optical activity has been extensively used in the investigation of the conformation of biopolymers [1]. This property is customarily measured by the optical rotatory dispersion or the circular dichroism, CD, of the materials studied. It has been recently shown, however, that the optical activity of asymmetric molecules is also manifested by circular polarization of their luminescence, CPL [2–6]. Since only luminescent 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 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. It may be recalled that in condensed media the fluorescence of a chromophore involves, as a rule, 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 [6,7].

The various chromophores in proteins are not intrinsically asymmetric; they therefore derive their optical activity from asymmetric influence of their environment. This is what makes the optical activity such a useful probe of macromolecular conformation in the vicinity of the chromophores concerned. Thus, CPL was used in the study of the active site of iron-binding proteins [8], of the binding site of antibodies [9] the pyridoxal binding site of phosphorylase [10], and of the adenine-binding site of dehydrogenases [11,12]. Similarly, by analysis of the CPL of antibodies and their fragments it was demonstrated that the Fc fragments undergo a conformational change upon binding of antigen [13]. Fully denatured proteins show no dectectable CPL in their tryptophan emission [6,7], indicating that the asymmetry of the environment of chromophores averages out to zero under these conditions.

We chose to study some characteristics of the interaction between antibody peptide chains by measuring the CPL of protein 315 and its fragments because this protein is a representative homogeneous antibody and its Fab′ and Fv represent progressively smaller fragments of the monomeric protein 315 which still retain complete functional activity but contain different size portions of the heavy and light chains. Marked changes were observed in the CPL of the tryptophan residues upon the interaction between the heavy and light chains to form active antibody. The resistance of the molecules to denaturation by urea was found to increase with increasing length of the interacting chains.

2. Materials and methods

Protein 315, its pepsin produced Fab′ and Fv, V_L and V_H were prepared as described [14]. Heavy and light chains were prepared by reduction of protein 315 with 0.01 M dithiothreitol followed by alkylation with iodoacetamide and separation on Sephadex G-100 in 4 M urea and 1 M propionic acid. The chains were desalted on Sephadex G-25 in 0.1 M NH_4HCO_3.

The instrument for the measurement of CPL was built in our laboratory [15]. The experimental details for the investigation of circularly polarized fluorescence of proteins were given elsewhere [6,7]. The calibration of the instrument was performed by the method of Steinberg and Gafni [15]. The CPL spectrum is expressed by the emission anisotropy factor, g_{em} defined as $g_{em} = \frac{2\Delta f}{f}$, where Δf is the intensity of the circular polarized fluorescence (defined as positive for left-handed circular polarization) and f is the total intensity of the fluorescent light [2,3,5]. The estimated uncertainty in the value of g_{em} is 5×10^{-5}.

3. Results and discussion

The CPL spectra of the 7S monomer of protein 315, of its Fab fragments and of its Fv fragment are shown in fig.1. All of these protein show negative g_{em} values throughout the spectrum; the values differ, however, appreciably from protein to protein. This illustrates the sensitivity of CPL to the environment of the various tryptophan residues. The difference in the CPL spectra of the Fab and Fv fragments is most pronounced in the spectral range 320–350 nm, indicating that at least some of the tryptophan residues present in Fab and absent in Fv emit preferentially in this spectral range and have a relatively highly negative CPL. The difference between the CPL of the intact IgA and its Fab extends through a wider spectral range but is most pronounced at the red part of the spectrum. The value of g_{em} is expected to be constant throughout spectrum for a single tryptophan residue [6,7]. The fact that it is constant for Fv, which contains five Trp residues, indicates that the various emitting residues in Fv have very similar emission spectra. The alternate explanation that they all have g_{em} values of equal magnitude is less likely, since it is known that the various tryptophans have different environments.

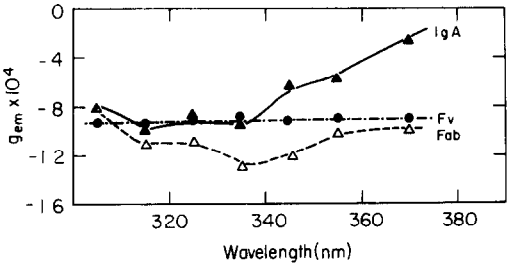

Fig.1. The CPL spectrum of protein 315 and its fragments. The 7S monomer, $4.5 \cdot 10^{-5}$ M (▲); Fab′ fragment $1.7 \cdot 10^{-4}$ M (●) and the Fv fragment $2.6 \cdot 10^{-4}$ M (△). The protein solutions were in 0.15 M NaCl, 0.01 M sodium phosphate pH 7.4.

The CPL spectra of the isolated chains of IgA and Fv are shown in fig.2. The corresponding spectra of IgA and Fv are included in the Figure for comparison. From fig.2 it is obvious that the CPL spectra of L and H, or the sum of the two, are dramatically different from that of intact IgA. Thus, the conformation of the separated chains as viewed via the tryptophan residues, is significantly different from their conformation in the complete IgA molecule. This may be contrasted with the resemblence of distinctive features of the ORD spectra in the peptide absorption range of individual chains and intact immunoglobulins [16]. It may thus be concluded that although there are similarities in conformation between the separated peptide chains and the intact IgA, pronounced differences occur at the environment of the tryptophan residues, and possibly at other locations as well. Some of these differences may of course be at contact regions between the chains, where indeed some of the tryptophan residues are known to be situated [17,18].

The separated chains of Fv also show very different CPL spectra than intact Fv. While g_{em} of Fv is negative, that of V_H is positive and that of V_L is zero within experimental error (fig.2). The conformation of the peptide chains at the vicinity of the tryptophan residues is therefore different when the chains are separated than when they are combined. In the case of V_H and V_L it is not known whether they are in dimer form. The CPL data show, however, that at least V_H has structure and does not resemble in conformation fully denatured proteins, since the latter were invariably found to exhibit

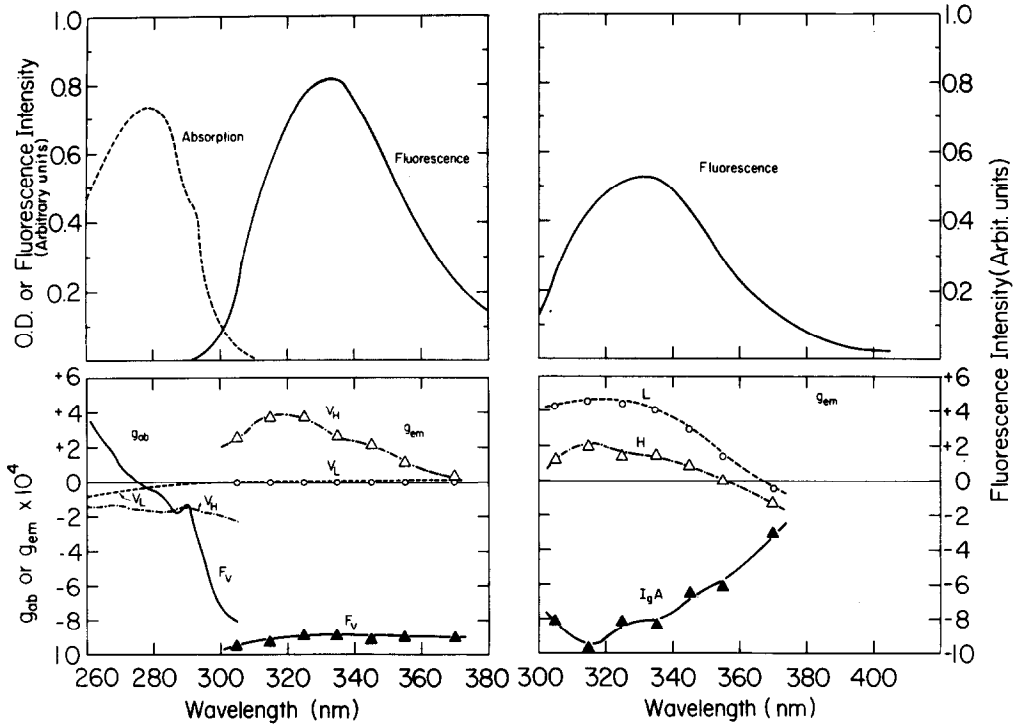

Fig.2. Spectroscopic data for Fv and for protein 315 and their constituent chains. Upper right: absorption ($1.95 \cdot 10^{-5}$ M) and fluorescence ($2.7 \cdot 10^{-5}$ M) spectra of Fv. Upper left: fluorescence spectrum of protein 315 ($5 \cdot 10^{-6}$ M). Lower left: absorption and emission anisotropy factors, g_{ab} and g_{em}, respectively, of Fv ($6 \cdot 10^{-5}$ M for g_{ab} and $2.65 \cdot 10^{-4}$ M for g_{em}) and of V_H ($5.6 \cdot 10^{-5}$ M for g_{ab} and $2.3 \cdot 10^{-4}$ M for g_{em}) and V_L ($1.1 \cdot 10^{-4}$ M for g_{ab} and $4.6 \cdot 10^{-4}$ M for g_{em}). Lower right: g_{em} of protein 315 ($4.5 \cdot 10^{-5}$ M) and of L ($2.8 \cdot 10^{-4}$ M) and H ($8 \cdot 10^{-5}$ M). The protein solutions were in 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4.

negligible values for g_{em}. The change in g_{em} upon recombination of V_H with V_L to form Fv is thus reflected by a pronounced change in conformation as viewed by the tryptophan residues. At least part of this is due to the trytophan residues that are located in between the light and heavy chains of Fv. The trytophan residues which are likely to be located in between the subunits are Trp 48 and Trp 108 of V_H. This follows from homology considerations with Fab' New whose three-dimensional structure was analyzed [17,18]. It is of interest that qualitatively similar conclusions may be drawn from the CD spectra of Fv and its constituent chains (see fig.2). The CD is expressed by the absorption anisotropy factor, $g_{ab} = \Delta\epsilon/\epsilon$, where $\Delta\epsilon$ is the difference between the extinction coefficients for right and left handed circularly polarized light. The differences between g_{ab} and g_{em} of the various materials studied are due to the fact that many more chromophores contribute to the CD than to the CPL measurements, and also possibly to changes in conformation of the tryptophan residues upon electronic excitation [4–6]. It should be noted that under the experimental conditions V_L and V_H as well as L and H may be present as dimers or higher aggregates. Hence the observed changes in CPL should be attributed to the difference in tryptophan environment in the dimers and in the native structures.

The changes induced in the CPL spectrum of protein 315, its Fab' and its Fv fragments in the presence of increasing concentrations of urea are presented in fig.3. Fv is more susceptible to change by urea than Fab', which in turn is more susceptible than IgA. Thus, 1 M urea is sufficient to induce an appreciable change in the CPL spectrum of Fv, but has no effect on Fab or IgA. 2 M urea induces an observable effect in Fab' but not in IgA. On increasing the urea concentration, the changes in

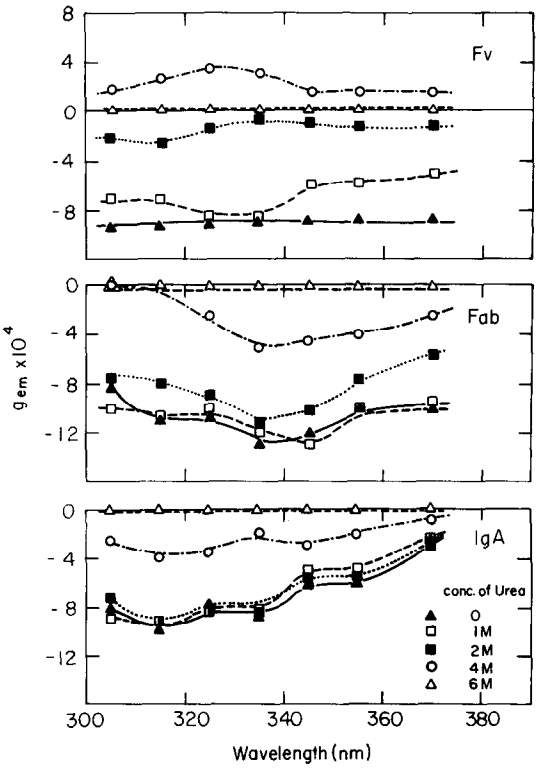

Fig.3. The CPL spectrum of protein 315, Fab' and Fv at various urea concentrations. Protein concentrations: protein 315 $4.5 \cdot 10^{-5}$ M (▲); Fab' $1.7 \cdot 10^{-4}$ M (▲) and $2.65 \cdot 10^{-4}$ M (▲). Urea concentrations in PBS: 1 M (□); 2 M (■); 4 M (○); and 6 M (△). Measurements were carried out between 30 to 60 min after addition of urea.

g_{em} at different regions of the spectrum do not proceed to the same extent. A possible explanation for this behaviour is that different regions of the molecules unfold with unequal ease. Apparently, different tryptophan residues possessing different emission spectra contribute preferentially at different ranges of the spectrum. In all cases, 6 M urea abolishes the CPL spectrum. This is in line with previous findings that fully denatured proteins do not show any detectable CPL in their fluorescence spectra [6] and indicates that IgA, Fab' and Fv all lose organized structure in 6 M urea. It is of interest to note that g_{em} of Fv changes sign from negative to positive upon increasing the urea concentration from 2 to 4 molar. This behaviour cannot be explained by assuming that Fv dissociates in 4 M urea into its constituents chains, since these chains are devoid of any detectable g_{em} in 4 M urea. It may thus be concluded that in 4 M urea Fv exists in a conformation that is different from the native one and that the denaturation is not an all-or-none process.

In conclusion, the separate chains of IgA or Fv have different conformations than the intact parent molecules, at least in the environment of the tryptophan residues. Fv is more susceptible to changes in conformation by urea than is Fab' or IgA. Different regions of these molecules seem to unfold with varying ease. Evidence is presented that at least in the case of Fv the unfolding proceeds through intermediate structures which possess a CPL spectrum that is markedly different from that of the native conformation.

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