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Fluorescence quenching of human orosomucoid

Accessibility to drugs and small quenching agents

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The fluorescence behaviour of human orosomucoid was investigated. The intrinsic fluorescence was more accessible to acrylamide than to the slightly larger succinimide, indicating limited accessibility to part of the tryptophan population. Although I^- showed almost no quenching, that of Cs^+ was enhanced, and suggested a region of negative charge proximal to an emitting tryptophan residue. Removal of more than 90% of sialic acid from the glycan chains led to no change in the Cs^+, I^-, succinimide or acrylamide quenching, indicating that the negatively charged region originates with the protein core. Quenching as a function of pH and temperature supported this view. The binding of chlorpromazine monitored by fluorescence quenching, in the presence and in the absence of the small quenching probes (above), led to a model of its binding domain on orosomucoid that includes two tryptophan residues relatively shielded from the bulk solvent, with the third tryptophan residue being on the periphery of the domain, or affected allotopically and near the negatively charged field.

INTRODUCTION

Orosomucoid (α_1-acid glycoprotein; OMD) is a small acute-phase glycoprotein (M_r 41000) that is very negatively charged at physiological pH, contains 40% carbohydrate and has up to 16 sialic acid residues (10–14%, by weight) (Jeanloz, 1972; Schmid, 1975). OMD has demonstrable activity in a number of important physiological systems, and interacts with a variety of ligands. Thus it may inhibit or promote platelet aggregation (Andersen & Eika, 1980), it has been reported to be immunosuppressive (Bennett & Schmid, 1980), and it sequesters both Δ^4-oxosteroids (Westphal, 1971) and basic drugs (Piafsky *et al.*, 1978; Glasson *et al.*, 1980). With some 60 drugs identified as interacting with OMD, it has become recognized as probably the only high-affinity carrier for most of these in serum, and therefore knowledge of the mechanism of binding becomes important, not only in their clinical uses, but also in the analogous descriptions of their physiological receptors.

OMD contains three tryptophan residues, which mainly account for the fluorescence properties of the protein (Kute & Westphal, 1976). That same study showed that one of the tryptophan residues is in or very near the steroid-binding site and that the native tryptophan fluorescence is quenched by progesterone. Work in our laboratory has indicated that tryptophan is involved in drug binding (Friedman *et al.*, 1985) and that the steroid-binding and basic-drug-binding sites overlap significantly (Kirley *et al.*, 1982). Fluorescence spectroscopy with the use of low-M_r quenchers has become a valuable tool in examining the environments of tryptophan residues in proteins (Eftink & Ghiron, 1981; Omar & Schleich, 1981; Lakowicz, 1983), and the research described in the present paper examines the quenching effects of Cs^+, I^-, acrylamide and succinimide on OMD intrinsic fluorescence. The dependence of the intrinsic fluorescence on pH and temperature as well as drug binding is also examined.

MATERIALS AND METHODS

Chemicals and buffers

Chlorpromazine hydrochloride and dipyridamole were purchased from Sigma Chemical Co. Electrophoresis-grade acrylamide was obtained from Bio-Rad Laboratories, optical-grade CsCl from Calbiochem, certified A.C.S. KI from Fisher Scientific Co. and succinimide from Eastman Kodak. All buffer salts were reagent grade or better. Phosphate-buffered saline solution used in all the studies was 0.14 M-NaCl/0.01 M-sodium phosphate/1 mM-EDTA, pH 7.4.

Protein preparation

OMD was prepared from the urine of individuals with nephrotic syndrome as described previously (Halsall *et al.*, 1982). The purified protein was fully characterized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, immunoelectrophoresis, analytical gel-filtration chromatography, ultracentrifugation, a spin-labelled-propranolol-binding assay and the sialic acid content determined by the Warren (1959) assay as modified by O'Kennedy (1980). Concentrations were determined spectrophotometrically, by using $\epsilon_{278} = 38750\ M^{-1} \cdot cm^{-1}$ (Kute & Westphal, 1976) and an M_r of 41000.

Abbreviation used: OMD, orosomucoid.

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Fluorescence spectra and data analysis

The fluorescence spectra were recorded on a Perkin–Elmer 650-10S spectrofluorimeter with the temperature being controlled by a circulating-water bath. For the temperature-dependence work, the temperature in the cell was monitored with a Doric Trendicator 400 type T/°C digital thermometer. All other work was at 20–23 °C. Control experiments established that 7 min of continuous irradiation resulted in the loss of 3–7% of the protein intrinsic fluorescence. Therefore, after determination of spectral shifts, the emission spectrum was monitored only between 20 nm each side of the $\lambda_{max.}$, to minimize photodecomposition. Excitation was at 285 nm and the spectral bandwidths of both the excitation and emission monochromator did not exceed 10 nm. Peak heights were measured at $\lambda_{max.}$. All transfers were made with calibrated Pipetman microlitre pipettes (Rainin Instruments).

Although protein concentrations were kept low to minimize inner-filter effects, intensities were corrected by (Lakowicz, 1983):

$$F_c = (F_{obs.}) \cdot \text{antilog}[(A_{exc.} + A_{em.})/2] \quad (1)$$

where $F_{obs.}$ and F_c are the observed and corrected fluorescence intensities, and $A_{exc.}$ and $A_{em.}$ are the absorbances at the excitation and emission wavelengths respectively.

The Stern–Volmer equation (Stern & Volmer, 1919; Eftink & Ghiron, 1981; Lakowicz, 1983):

$$F_0/\{F_c \cdot \exp(V[Q])\} = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F_c are the corrected fluorescence intensities in the absence and in the presence of quencher, Q, and V and K_{sv} are the static quenching constant and Stern–Volmer constant respectively, and the modified Stern–Volmer equation (Lehrer, 1971; Eftink & Ghiron, 1981; Lakowicz, 1983):

$$F_0/\Delta F = (1/fa) + 1(faK_Q[Q]) \quad (3)$$

where $\Delta F = F_0 - F_c$, fa is the maximum fraction of quenchable fluorescence and K_Q is the effective quenching constant, were used to analyse the quenching titration curves. Quenching parameters were obtained from linear-regression analysis of plots according to eqn. (3).

U.v.-absorption spectroscopy

A Gilford model 240 spectrophotometer equipped with a thermal programmer was used to monitor the denaturation of OMD to determine T_d and $\Delta H_{(vH)}$ where, formally, T_d is the temperature at which one-half of the protein molecules are denatured and $\Delta H_{(vH)}$ is the van't Hoff enthalpy. The absorbance at 285 nm was monitored as a function of temperature. The heating gradient was 1 °C/min.

Binding measurements

Chlorpromazine-binding data were obtained by the method of Hummel & Dreyer (1962). The binding isotherms were taken from a column (16 mm × 310 mm) (Whatman) packed with Sephadex G-25–150 (Pharmacia, supplied via Sigma Chemical Co.), which was run in the dark to prevent photodegradation of the drug. The applied ligand concentration was varied in the phosphate-buffered saline running buffer, whereas the protein concentration was kept constant. The number of mol of ligand bound per mol of protein was determined for each experiment by comparison of the experimental trough area with a calibration plot of trough area versus mol of ligand obtained by blank injections to the column in the presence of ligand. Corrections for the contribution of protein to the total volume (Fairclough & Fruton, 1966) were insignificant, and not made.

RESULTS

Quenching of OMD intrinsic fluorescence by low-M_r quenchers

The fluorescence-emission spectrum of orosomucoid in phosphate-buffered saline is due primarily to tryptophan even with excitation at 280 nm (Kute & Westphal, 1976). The absence of a shoulder at 308–310 nm further shows minimal involvement of tyrosine in the observed fluorescence spectra (Omar & Schleich, 1981). Quenching studies with acrylamide (an efficient neutral quencher) showed that all the intrinsic fluorescence of OMD is accessible to quenching, as expected (Fig. 1). However,

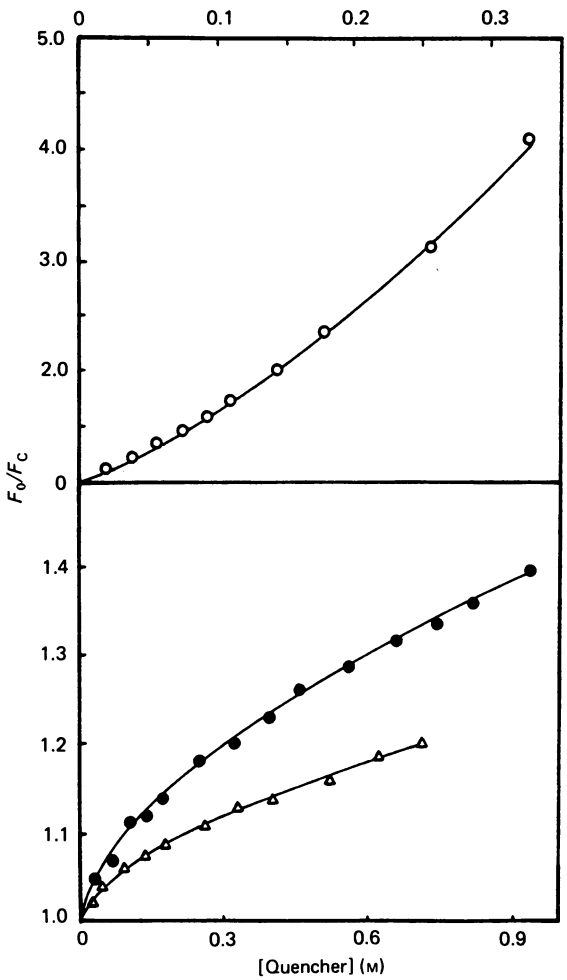

Fig. 1. Stern–Volmer plots of the quenching of OMD fluorescence by low-M_r quenchers

The intrinsic fluorescence of 1 μM-OMD was monitored with addition of acrylamide (○), Cs^+ (●) and succinimide (△). Eqn. (2) was used in plotting the data. The titrations were carried out as described in the text. Excitation was at 285 nm, and emission intensity was measured at 340 nm. The lines are visual aids only.

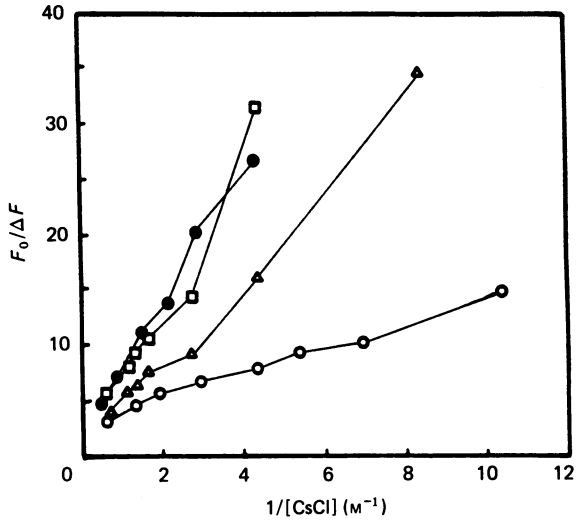

Fig. 2. Modified Stern–Volmer plot of Cs^+ quenching of OMD fluorescence

Cs^+ quenching of 1 μM-OMD was monitored in the absence (○) and presence of chlorpromazine (●) and dipyridamole (△). Cs^+ quenching of 1 μM-asialo-OMD (> 90% desialylated) in the presence of chlorpromazine is also shown (□). The concentration of each drug was 1 μM, and at these concentrations more than 70% of the binding sites are occupied. Fluorescence data were obtained as described in Fig. 1 legend and the text. The data were plotted according to eqn. (3). The lines are visual aids only.

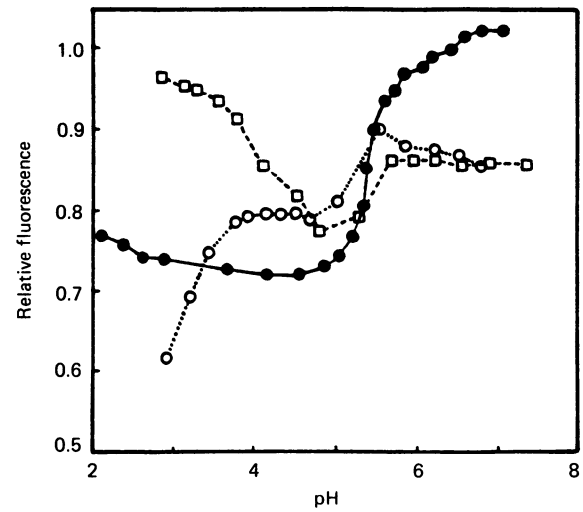

Fig. 3. pH-dependence of OMD fluorescence in the absence and in the presence of quenchers

The fluorescence of 1 μM-OMD was monitored (as described in the text) as the pH was lowered by addition of 0.1 M-HCl. The fluorescence intensities in the absence of quenchers (●) denote the ratio of fluorescence at pH 7.4 to the fluorescence at that pH. The fluorescence intensities in the presence of succinimide (□) and Cs^+ (○) (both kept constant at 0.45 M) denote the ratio of quenched to unquenched fluorescence at that pH. Identical titrations were performed while the pH was monitored. A red-shift of less than 6 nm was observed on lowering the pH to 4.0. The lines are visual aids only.

succinimide (a neutral quencher approx. 20–30% larger than acrylamide) quenched approx. 20% of 1 μM-OMD intrinsic fluorescence when at 1 M. The Stern–Volmer plot for succinimide quenching is curved downward, which is indicative of selective quenching (Fig. 1) (Eftink & Ghiron, 1981; Lakowicz, 1983).

Charged quenchers (I^- and Cs^+) were used to determine the accessibility of the tryptophan residues in OMD to solvent. I^- was found to quench less than 5% of the intrinsic fluorescence of 1 μM-OMD when at 1 M. Cs^+ proved to be a much better quencher, and the Stern–Volmer plot showed a downward curvature (Fig. 1). When the Cs^+ quenching data are plotted according to eqn. (3), the intercept and slope yielded $K_Q = 3.05$ and $fa = 0.322$ at both 25 °C and 37 °C (Fig. 2). Thus approx. one-third of the intrinsic fluorescence is accessible to Cs^+. Quenching studies on asialo-OMD (with an average of 1 sialic acid residue per protein molecule) showed no change in acrylamide, succinimide, I^- and Cs^+ quenching abilities (Halsall *et al.*, 1985).

Acidic pH-dependence of OMD intrinsic fluorescence

OMD intrinsic fluorescence was monitored as the pH was lowered from pH 7.4 (Fig. 3). Between pH 6.0 and pH 4.0, the intrinsic fluorescence was enhanced by 30–40%. A red-shift in emission maximum wavelength of less than 6 nm was observed between pH 6.0 and pH 4.0; however, below pH 4.0 there was a decrease in the fluorescence intensity with a larger red-shift in emission maximum. The Stern–Volmer plot of acrylamide quenching showed increasing upward curvature with decreasing pH (results not shown). Both succinimide and Cs^+ quenched more of the protein fluorescence in the region of enhancement (Fig. 3). Below pH 4.5, Cs^+ quenching declined rapidly, whereas succinimide quenching remained constant until below pH 4, where unfolding of the protein allowed succinimide to quench more. The Stern–Volmer plots for succinimide and Cs^+ become more linear below pH 4.0 (results not shown).

Temperature-dependence of OMD intrinsic fluorescence

OMD intrinsic fluorescence monitored as a function of temperature showed a decrease (Fig. 4) with a deviation from linearity at approx. 53 °C and a return to linearity above 70 °C. In the presence of Cs^+ (279 mM) and acrylamide (18.7 mM), relative fluorescence profiles of the same general form were obtained (Fig. 4). However, although the efficiency of acrylamide quenching increases somewhat as the OMD unfolds, that for Cs^+ decreases slightly through the unfolding transition. $\Delta H_{(vH)}$ and T_d for OMD in the presence of the above concentrations of quenchers were unchanged (results not shown). A small amount of polymerization occurs upon heating the protein (Halsall & Kirley, 1981, 1982); however, neither quencher altered this level.

Quenching of OMD intrinsic fluorescence by drugs

Titration of OMD with chlorpromazine or dipyridamole yields quenching curves indicative of either multiple binding sites or the involvement of more than one class of fluorophore (results not shown). Independent determination of the stoichiometry of chlorpromazine binding by using the Hummel–Dreyer method gave only a single high-affinity site for chlorpromazine. It has been reported that dipyridamole has a single high-affinity

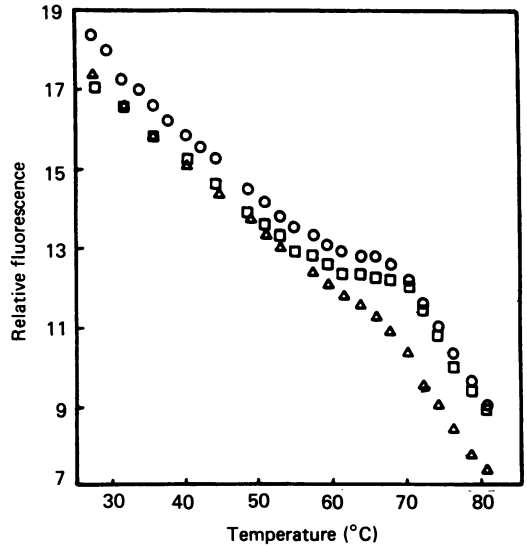

Fig. 4. Temperature-dependence of OMD fluorescence

The fluorescence of 1 μM-OMD was monitored in the absence (○) and in the presence of acrylamide (△) and Cs^+ (□) with increasing temperature. The concentrations of acrylamide and Cs^+ were 18.7 mM and 279 mM respectively. The fluorescence was monitored as described in the text.

binding site ($K_{ass.} = 10^7\ M^{-1}$) and a lower-affinity site (El-Gamel *et al.*, 1982). Modified Stern–Volmer plots for the titration of the OMD intrinsic fluorescence by both drugs showed $fa = 2/3$. Quenching of OMD intrinsic fluorescence at a chlorpromazine/OMD molar ratio of 1:1 (> 70% of the drug-binding sites occupied at the concentrations used) by acrylamide showed 100% of the fluorescence still accessible, although protection occurs at low acrylamide concentrations (results not shown). The efficiency of Cs^+ quenching of dipyridamole/OMD and chlorpromazine/OMD each at a molar ratio of 1:1 (> 70% of the drug-binding sites occupied at the concentrations used for both drugs) was decreased substantially ($K_Q = 0.71$ and $K_Q = 0.33$ respectively) compared with the efficiency of Cs^+ quenching of protein with no drug present (Fig. 2). The fraction of accessible fluorescence to Cs^+ quenching is unchanged in the presence of either ligand.

DISCUSSION

A number of groups in the past 10 years have attempted to establish the solvent accessibility of the three tryptophan residues in OMD (Kute & Westphal, 1976; Schmid *et al.*, 1976; Kalal & Kalous, 1984). As Eftink & Ghiron (1981) have shown, neutral and charged quenchers of intrinsic fluorescence can give environmental information for the different classes of tryptophan residues in proteins containing more than one of this fluor. The three tryptophan residues of OMD are quenched by acrylamide, and appear to be in similar environments with respect to acrylamide, as shown by an upward-curving Stern–Volmer plot (Fig. 1). As the protein unfolds (by thermal or acidic denaturation), the efficiency of acrylamide quenching increases (Fig. 4). This is expected, since the tryptophan residues become more exposed upon unfolding, and thus more accessible to quenching. Succinimide is not as efficient as acrylamide in quenching tryptophan fluorescence, reflecting steric limitations to diffusion, and/or the polarity of the local microenvironment around the tryptophan residues (Eftink & Ghiron, 1984), and is therefore a more discriminating probe. The Stern–Volmer plots for succinimide quenching of OMD intrinsic fluorescence suggest that at pH 7.0 the tryptophan residues of OMD are not equally accessible to succinimide (Fig. 1).

pH studies using difference spectroscopy (Karpenko *et al.*, 1968) and circular dichroism (Schmid *et al.*, 1976) showed that the major conformational changes in OMD structure occur with unfolding below pH 4.0. Consistent with this are the changes seen with succinimide and Cs^+ quenching below pH 4 (Fig. 3). However, the changes in the relative fluorescence (Fig. 3) and Cs^+ and succinimide quenching between pH 7.4 and pH 4 demonstrate that there are microenvironmental changes occurring about the tryptophan residues.

The reason for the enhancement of fluorescence intensity with decreasing pH (Fig. 3) is unclear at this time. The effect of changing pH on fluorescence intensity in proteins varies widely, with both enhancement and depression being observed (Longworth, 1971). Assorted mechanisms have been suggested to account for the results observed, but it is apparent that the complicated and interacting effects of conformational change, pH-dependent quenching, and tyrosine-residue emission prevent any but conjectural explanations for the behaviour of multifluor-containing proteins such as OMD. However, Fig. 3 may be interpreted with some confidence to mean that the fluorescence enhancement is Cs^+-quenchable. Thus the increased succinimide and Cs^+ quenching in the area of enhancement would again indicate microenvironmental changes occurring in the region of the solvent-accessible tryptophan.

At pH 7.4, Cs^+ is capable of quenching only one-third of the intrinsic fluorescence of OMD, which it does with a higher efficiency than found for the majority of proteins (Altekar, 1977). It is likely that this Cs^+-quenchable fluorescence is from a single tryptophan residue, and that the high K_Q results from a favourable electrostatic interaction with one or more negatively charged groups in the vicinity of it. The loss of Cs^+ quenching below pH 4.5 (Fig. 3) suggests that the group(s) responsible for the interaction is an aspartic acid or glutamic acid side-chain carboxy group. Such an interaction would account for the decrease in Cs^+ quenching efficiency seen upon thermal unfolding (Fig. 4), the charge moving away from the tryptophan residue as unfolding occurs.

The quantum yields of the individual tryptophan residues are unknown, and hence interpretive models based on these data must be considered speculative. As a starting point, then, we offer the following. We suggest that two of the three tryptophan residues in OMD are involved in the hydrophobic binding domain of the basic drugs, since both chlorpromazine and dipyridamole quench approx. 60% of the intrinsic fluorescence of OMD. A significant fraction of the remaining fluorescence can be quenched by Cs^+, but with a greatly reduced efficiency (Fig. 2), suggesting that the favourable electrostatic interaction has been modified. A simple screening of the region of negative charge by the ligand seems less likely than modification by allotopic interaction (Halsall, 1980), since it has been reported that the association of

dipyridamole and of chlorpromazine with OMD results in conformational change in the protein (El-Gamel *et al.*, 1982, 1983). Although the present data cannot distinguish whether this Cs^+-quenchable tryptophan residue is proximal or distal to the drug-binding domain, it is apparently close to a region of negative charge originating with the protein core.

This model would also account for some of the differences between steroid binding and basic-drug binding. Kute & Westphal (1976) suggest that there are tryptophan residues near the steroid-binding site, since progesterone quenches approx. 17% of OMD intrinsic fluorescence. Kirley *et al.* (1982) have demonstrated that although the steroid-binding and basic-drug-binding domains overlap significantly, basic-drug binding is far less temperature-dependent, suggesting the contribution of an electrostatic interaction.

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