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INTERACTION BETWEEN ALDOLASE AND CHLORPROMAZINE

FLUOROMETRIC EVIDENCE FOR CONFORMATIONAL CHANGES

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Abstract—Fluorescence studies of aldolase–chlorpromazine mixtures indicate quenching of aldolase emission and modification of the scatter peak. Shifts in the wavelength maxima characteristics of both drug and enzyme, and appearance of a completely new peak characteristic of bound compound further suggest an alteration in the structure of enzyme accompanying the molecular interaction.

Chlorpromazine acts as a fluorescent chromophore in the detection of conformational change in aldolase. Energy transfer from aldolase to chlorpromazine occurs by complex formation. Drug–enzyme complexes are stable at neutral pH and can be distinguished on the basis of its fluorescence spectral properties.

ALTHOUGH it is well known that chlorpromazine (CPZ) has a potent effect on the activity of several enzymes, much remains to be elucidated as to the biochemical mechanism of their effects. In a kinetic study of drug–aldolase interaction Chowdhury *et al.*[1] reported that phenothiazine drugs inhibit rabbit muscle aldolase. Inhibitions were not linearly related to CPZ concentrations and these authors suggest that the unusual kinetics may be due to the occurrence of a conformational change in the structure of the enzyme.

Fluorescence spectroscopy has proved to be a valuable technique in the study of protein interactions.[2,3] Fluorescence procedures for analytical determination of phenothiazine drugs were reported[4–6] and were used on a study of drugs binding to various proteins.[7] The following is an investigation of CPZ–aldolase interaction using this technique to detect alterations in the enzyme structure upon binding to CPZ. In addition, the site of interaction and the relationship of structural change in the CPZ–aldolase complex to inhibition of the enzyme activity have been studied and will be published separately.

MATERIALS AND METHOD

All buffers and reagents were prepared in glass distilled, deionized water. Amongst several media examined, (phosphate, tris, borate, glycylglycine, tes–NaCl, citrate, tricine, bicine), the NaCl, 5 mM tes (*N*-tris-(hydroxymethyl)-methyl-2-amino-ethane sulphonic acid) were selected because all of these exhibited low fluorescence in the regions of CPZ activity and were adequate solvents for the drug and enzyme at pH 7·2 and this range of ionic strength.

Nitrogen gas was bubbled for 10–15 min through a cold solution of tes–NaCl buffer to remove oxygen and this degassed buffer was used to prepare a stock solution of 0·1–2 mg/ml aldolase; and 10^{-6} to 10^{-4} M CPZ, HCl. [Solid CPZ, HCl was a gift from May & Baker (Dagenham) Ltd. and was stored below 0° in the dark.] Aldolase

sample was purchased from Boehringer and Soehne as a crystalline suspension at 10 mg/ml in 2 M ammonium sulphate, pH 6·0. The specific activity of this sample was 10·0–12·0 units at 25°. Gel filtration on Sephadex G200 of this sample showed a homogeneous protein peak, behind the void volume, with parallel distribution of enzyme activity.[8]

Fluorescence emission and excitation measurements were performed with a recording Aminco–Bowman spectrophotofluorimeter (Model No. 4–8202). The grating monochromator for the fluorescent light way calibrated against a mercury arc. Emission spectra reported here are the direct recorder tracings which have not been corrected for the spectral response of the photomultiplier tube (IP 21). The glassware was cleaned with a non-fluorescent detergent (comprox A), concentrated nitric acid and deionized distilled water.

RESULTS

Emission and activation spectra of aldolase

The fluorescence intensity of the aldolase solution excited at different wavelengths is shown (Fig. 1). The principal emission band has a maximum at 345 nm and varies

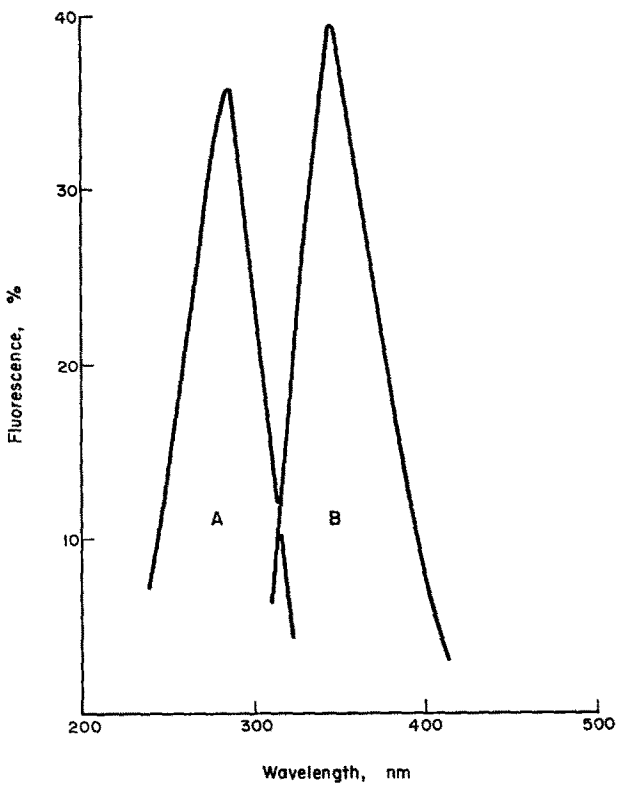

FIG. 1. Change in the activation (A) and emission (B) intensity of a solution of aldolase (100 μg/ml in tes–NaCl buffer at pH 7·2, 4°) at various wavelengths. Activation and emission maxima are 285 nm, 345 nm respectively. The ordinates in this and in subsequent figures are scaled in arbitrary units depending upon the amplification of the signal used in a particular experiment which depends upon concentrations and other experimental conditions.

only in intensity with the activating wavelength. This emission results almost entirely from the presence of the aromatic amino-acids: tryptophan, tyrosine and phenylalanine. The enzyme content of these amino acids was found to be approximately 7, 24, 29 residues/80,000 g[9] and their fluorescence quantum efficiencies are 0·13, 0·14, 0·024[10] respectively. Tryptophan absorbs more strongly than tyrosine, and usually makes the dominant contribution to protein fluorescence. The contribution of a phenylalamine fluorescence is generally small. If there were no residue interaction within the aldolase molecule the tyrosine contribution would appear as a small hump on the lower wavelength side of the tryptophan emission band at pH 7·2. The aldolase emission peak actually appears as a single peak with no distinguishable tyrosine contribution at either of the pH's (3–9) investigated. However at the lower pH the emission maximum is slightly displaced towards shorter wavelengths and its intensity is enhanced.

The wavelengths of light that activate a molecule or complex to fluoresce are the activation spectrum. This also may be measured in intensity and wavelength. The activation maximum of aldolase when the excitation spectrum is scanned at various fixed emission wavelengths over the range (280–420) is at 285 nm (Fig. 1). This corresponds closely to the absorption maximum of the protein (280 nm).

Emission spectra of CPZ. The conditions for maximal enhancement of CPZ fluorescence were found by changing the excitation wavelengths and altering pH(3–9) temperature (4°–25°), solvent and solute composition. In this way it was possible to measure

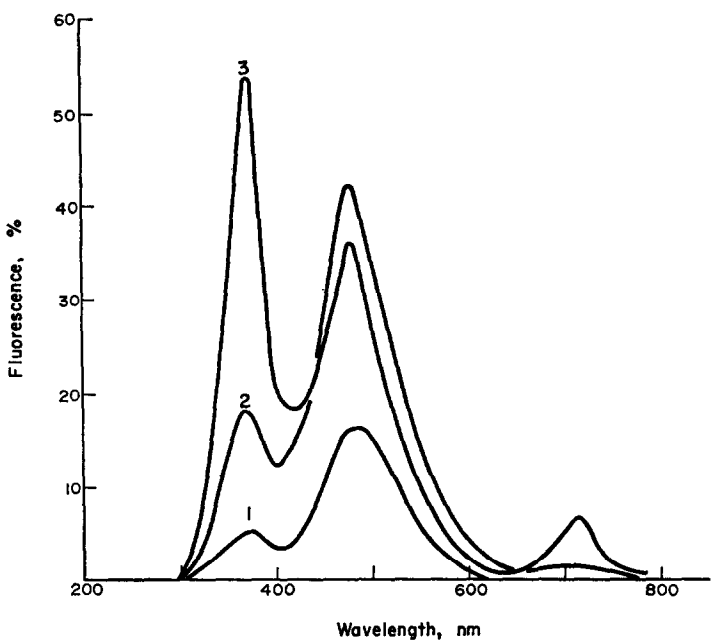

FIG. 2. Emission spectra of 100 μg CPZ at pH 7·2 4° showing the scatter peak (left) principal emission peak (480 nm) and 2nd order scatter peak (right), at excitation wavelengths (1) 285 nm (2) 340 nm, (3) 350 nm. The changes in light scattering intensity are probably due to differential sensitivity of the (IP 28) photomultiplier for these wavelengths. Scatter peak is reduced or eliminated entirely with the use of a single polarizer on the excitation beam, or on double polarizers on the activation and emission sides. (See Fig. 3.)

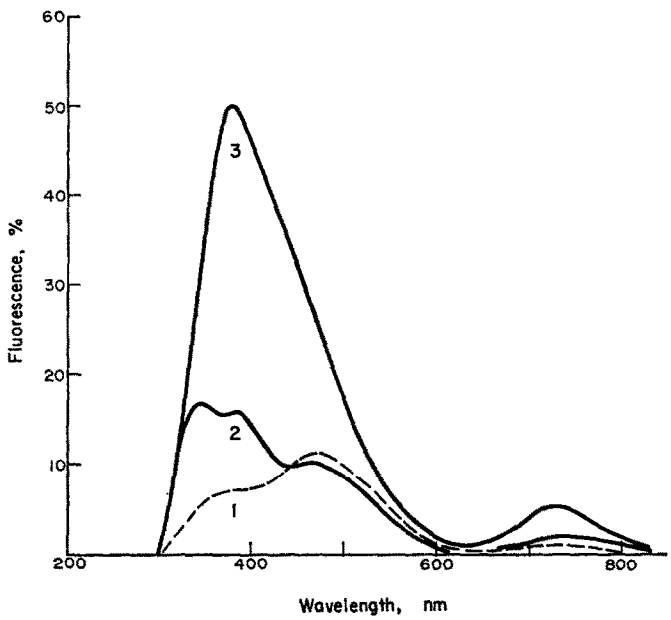

FIG. 3. Emission spectra of aldolase–CPZ mixture (molecular ratio 1:50) which shows 15 per cent quenching of protein peak, excited at 285 nm, analysed with (2) and without (3) polarizers. Spectra (1) shows the emission spectrum of a 100 μg CPZ alone excited at 350 nm under identical conditions to spectra (2). By using double polarizers a better resolution is obtained and the bound drug is more clearly defined.

quantitatively the concentration of CPZ at the levels below $< 10^{-6}$ M (Fig. 2). The lower limit of concentration detectable in the quantitative measurement was limited mainly by fluorescence of impurities in the blank, by scattering of stray light, or by Raman scattering. To overcome this difficulty, particularly when a high concentration of aldolase was present; in some experiments the polarization of scattering was utilised to reduce the intensity of scatter peak (Fig. 3). Decreasing the slit width also reduced the stray light falling on the analysing gratings, but it reduced the output signal proportionately. In contrast, use of polarizers decrease the scatter peak 60–95 per cent, while decreasing sensitivity by only 5–35 per cent.

Emission spectra of aldolase–CPZ mixtures

Under strictly identical conditions 0·1–2·0 mg aliquots of aldolase were exposed to chlorpromazine over a concentration range from 10^{-6} to 10^{-4} M, the concentration at which definite inhibition of aldolase has been reported.[1] To minimize oxidation of CPZ by air, both solutions were nitrogen bubbled and the measurements were performed at 5° or at room temperature with the fast scanning monochromator recording from 200 to 800 nm (30–40 sec). CPZ oxidation is also catalysed by light.[11] Blank titrations in which each component was measured separately under identical conditions of light exposure and temperature for the lengths of time taken by 3–7 scans indicated no change in drug or protein fluorescence spectra.

Addition of increasing amounts of CPZ to a constant concentration of aldolase,

or addition of an increasing amount of the enzyme to a constant concentration of drug, indicates changes in their fluorescence and activation spectra (Fig. 4a, 4b). Each mixture shows a highly reproducible degree of quenching of the activation and emission maxima of aldolase (285 nm, 345 nm). Also a new peak activation maxima (310–330 nm) emission (385 nm) due to the oxidized bound CPZ appears. A further indication of the interaction between CPZ and aldolase is apparent in circumstances

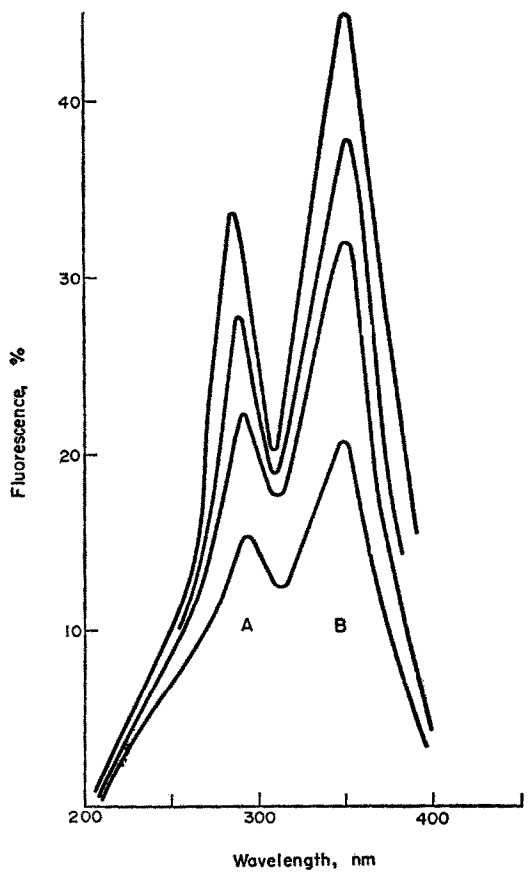

FIG. 4 (a). Activation bound (A) and protein scatter peak (B) of aldolase at 4° activated at 345 nm (the maximum emission wavelength for aldolase). The quenching of this peak for CPZ concentration increasing from $2 \cdot 10^{-6}$ M to $5 \cdot 10^{-5}$ M is shown. For clarity, intermediate spectra are not shown.

where a higher degree of quenching occurs. In these mixtures the appearance of the new peak is accompanied by a blue shift (15 nm) in the activation or emission wavelengths of CPZ (Fig. 5). Also, a smaller change in the fluorescence and activation band of aldolase is observed and the scatter peak of protein and bound compound is greatly altered, suggesting that a dimensional alteration of the macromolecular species has occurred. Bound CPZ is rapidly oxidized on exposure to u.v. irradiation. Dissociation from the protein then occurs, and this is also shown by changes in light scattering observed by continuous scanning.

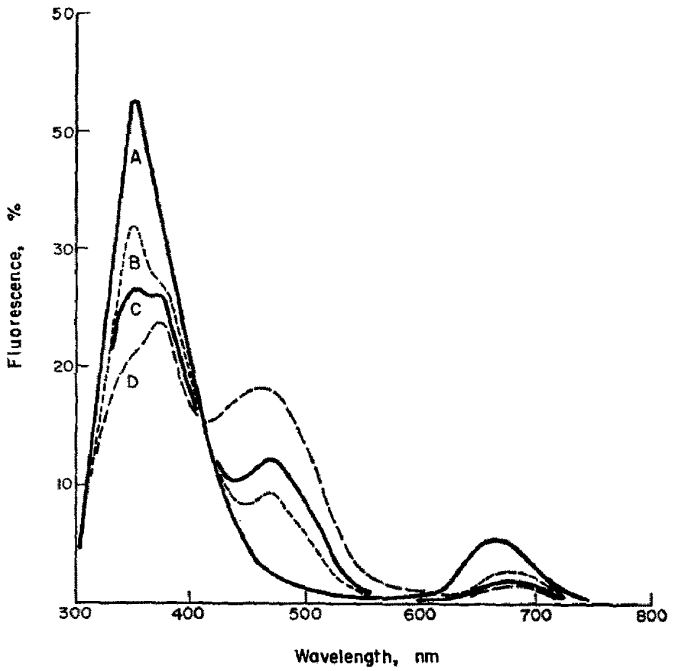

FIG. 4 (b). Emission spectra of aldolase alone (A) and in the presence of increasing amounts of CPZ (B–C–D) excited at 285 nm, showing quenching of aldolase fluorescence peak and the appearance of a new peak at 385 nm due to bound CPZ. Also a shift in the wavelength of CPZ emission is observed.

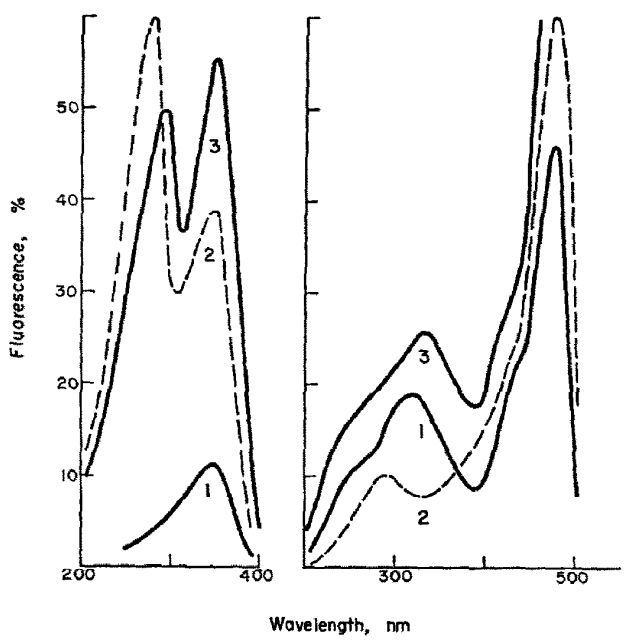

FIG. 5. Activation spectra of 100 μg CPZ alone (1) and in 500 μg/ml aldolase solution (3), together with diagrammatic spectra of protein alone (2) activated at 345 nm (left) and 480 nm (right) showing changes in activation wavelengths. Also the scatter peak of native and bound drug at those wavelengths are demonstrated. The native drug shows much less light scattering than its oxidized or bound drug does. Change in the scatter peak of CPZ upon binding to aldolase is probably due to the dimensional alteration of the macromolecular species.

Fluorescence induction

CPZ has a rather weak native fluorescence upon excitation at 285 nm. When aldolase alone is excited at 285 nm its emission appears at 345 nm, which coincides with the excitation wavelength of CPZ. In the presence of aldolase, CPZ emission is increased following the activation at 285 nm (Fig. 6), while the characteristic tryptophan emission at 345 nm is quenched. These effects suggest that some energy transfer

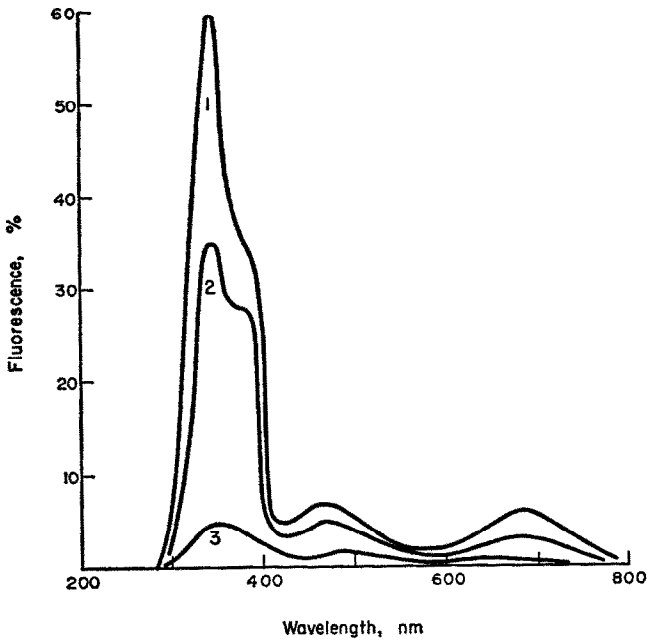

FIG. 6. Emission spectra of 10 μg CPZ alone (3) and in the presence of 500 μg/ml aldolase (2) excited at 285 showing the appearance of the bound compound at 385 and enhancement of the CPZ emission at 480 nm. This pattern is also seen on excitation at 325 nm (the maximum excitation wavelength for the bound drug).

between tryptophan and CPZ occurs by complex formation. This complex is stable at this pH and has been isolated by gel filtration technique.[8]

CONCLUSION

Fluorescence spectroscopy is one of the most sensitive and versatile of the optical techniques that are in use in analysing conformational change which proteins undergo in solution. Since fluorescence of proteins is based on a delicate balance of interacting forces, it is extremely sensitive to changes in the local environment of its aromatic amino acid residues.

Almost all molecular interaction of proteins with small molecules lead to quenching or enhancement of the fluorescence intensity and changes in excitation and emission spectra. The magnitude of these effects are indicative of conformational changes which alter the availability of aromatic amino acid residues to solvent solute, or other functional groups within the molecule.[12]

The spectral changes described above show interaction between aldolase and CPZ. These effects are dependent upon the relative fluorescence and concentration of each

species present in the mixtures. When proteins are bound to small molecules, fluorescence due to tryptophan nitrogen is often quenched,[12] since the fluorescence characteristics of aldolase largely depend on the content and internal relaxation freedom of the indole nitrogen of tryptophan; its quenching by CPZ is by itself adequate evidence for residue interaction. As a result of binding to CPZ, this amino acid residue not only loses its own fluorescence but also can act as an energy sink for transfer of excitation energy from other tryptophan and tyrosine residues in the molecule.[13] The evidence for this interpretation is the strong overlap of the absorption spectrum of the bound CPZ and the emission spectrum of other tryptophan and tyrosine residues which is favourable for resonance transfer energy. The fluorescence of phenothiazine drugs is known to be due entirely to the hetrocyclic nitrogen. The blue shift in the fluorescence spectra when the drug changes its oxidation state upon binding to aldolase appears to be due to the protonation of this nitrogen. Apparently CPZ attachment request protonation of the (fluorescent) nitrogen of the phenothiazine nucleus. If the sulphur is oxidized this protonation is greatly decreased (i.e. fluorescence disappears).

Exposure of the CPZ–aldolase solutions to light also increases the rate of aldolase transformation, which is also demonstrated by a change in light scattering. Relatively a smaller change in the light scattering is observed when either component alone is exposed to light. Since exposure of CPZ solution to light is known to yield an unstable and highly reactive intermediate (semi-quinone free radical)[11] it is possible that this radical favours binding of the drug to the protein.

Evidence for energy transfer from tryptophan to drug molecules is indicated by enhancement of drug emission accompanying the quenching of characteristic tryptophan emission. Such energy transfer only occurs if there is a close association between the fluorescent drug molecule and protein chromophores.[14] In this manner CPZ appears to act as a fluorescent probe[15] in the conformational change of the enzyme. In addition to these observations there is independent evidence that tight binding[16] occurs between CPZ and aldolase, which effects the enzyme activity.[8] Changes in the fluorescence spectra of both drug and enzyme, together with the unusual kinetics of enzyme activity[1] are consistent with a conformational change of enzyme structure. These findings can serve as a basis for a more detailed study of the behaviour of this group of drug–enzyme complex.

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