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Interaction of multitryptophan protein with drug: An insight into the binding mechanism and the binding domain by time resolved emission, anisotropy, phosphorescence and docking

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ABSTRACT

The interaction of antibiotic Tetracycline hydrochloride (TC) with Alkaline Phosphatase (AP) from *Escherichia coli*, an important target enzyme in medicinal chemistry, having tryptophan (Trp) residues at 109, 220 and 268 has been studied using the steady state and time resolved emission of the protein and the enhanced emission of the bound drug. The association constant at 298 K ($\approx 10^6$ $[M]^{-1}$) and the number of binding site (= 1) were estimated using the quenched Trp emission of AP, the enhanced emission and the anisotropy of the bound drug. The values of ΔH^0 and ΔS^0 are indicative of electrostatic and H-bonding interaction. The low temperature phosphorescence of free AP and the protein- drug complex and molecular docking comprehensively prove the specific involvement of partially exposed Trp 220 in the binding process without affecting Trp 109 and Trp 268. The Förster energy transfer (ET) efficiency and the rate constant from the Trp residue to TC = 0.51 and $\approx 10^8$ s^{-1} respectively. Arg 199, Glu 219, Trp 220, Lys 223, Ala 231, Arg 232 and Tyr 234 residues are involved in the binding process. The motional restriction of TC imposed by nearby residues is reflected in the observed life time and the rotational correlation time of bound TC.

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1. Introduction

Escherichia coli Alkaline Phosphatase (AP) is an extensively studied metalloenzyme which rather unspecifically catalyses the hydrolysis and trans phosphorylation of a wide variety of phosphate monoesters. The enzymatic reaction proceeds through a covalent serine phosphate intermediate to produce inorganic phosphate and alcohol [1]. The functional form of AP consists of a homo dimer with an appropriate mass of 94 KDa [2]. Each of the two active sites of the dimeric enzyme contains three metal-binding sites (M1, M2, M3). The M1 and M2 sites are occupied by zinc ions and M3 site is occupied by magnesium ion. The M1–M2 distance is 4 Å, the M2–M3 distance is 5 Å and the M1–M3 distance is 7 Å [3,4]. The three metal ions in three active sites form a catalytic metal triad similar to that of the phospholipase C from *Bacillus cereus* [5] and P1 nuclease from *Penicillium citrinum* [6]. Although M1 is essential for activity, full catalytic efficiency is attained only when all the metal ions are present [7–9]. Several spectroscopic pieces of evidence on the native and metal substituted derivatives indicate that M1 is 5 coordinated, while M2 and M3 are 6 coordinated, probably with water molecules completing the co-ordination sphere [10].

The activity of AP is an indicator for human liver, bile and bone diseases. It is also used in immunoassay such as enzyme-linked immunoabsorbent assays as an antibody conjugate [11]. Alkaline phosphatases play an important role in clinical diagnosis and have become important target enzymes in medicinal chemistry [11,12]. In continuation of our study of time resolved photophysics of sequestered ligand or drug molecules with proteins [13–17], we explore the interaction of AP with an antibiotic Tetracycline Hydrochloride in the present work. The basic molecular structure of tetracycline (TC) is shown in Fig. 1. The ring structure is surrounded by upper and lower peripheral zones where various chemical functional groups can be attached [18–23].

The protein-drug interaction is often characterized by steady-state and time-resolved fluorescence of intrinsic emitting probe tryptophan (Trp) of protein as well as the steady state, time-resolved emission and anisotropy of the bound drug [24–39]. Although the fluorescence spectra of proteins provide significant information regarding binding mechanism and nature of Trp environment, the spectra are usually broad, and the fluorescence

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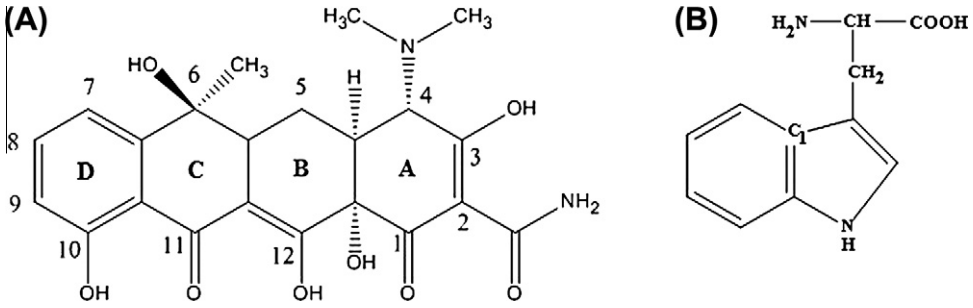

Fig. 1. Structure of (A) tetracycline (B) tryptophan residue.

lifetime even in a protein having single tryptophan exhibits multiexponential decay [25]. However, low temperature (77 K) phosphorescence (LTP) spectra of Trp in proteins in a suitable cryosolvent always give structured spectra with a definite (0, 0) band, characteristic of the tryptophan environment [40–46]. The position, the width of the (0, 0) band, the overall structural features of the spectra and the lifetime provide definitive information regarding the polarity, nature of solvent exposure, the hydrophobicity and the homogeneity of the immediate environment of the Trp. In several cases low temperature (77 K) phosphorescence spectra of multi Trp proteins exhibit more than one (0, 0) bands corresponding to different Trp residues present in the protein [13,41–44,47–54]. This is possible if the Trp residues have different local environment and the intramolecular singlet ↔ singlet non-radiative energy transfer (ET) between the different Trp residues is inherently prevented. Thus LTP along with conventional steady state and time-resolved fluorescence spectra can provide meaningful understanding of protein–protein interaction [13] and protein–ligand interactions [37,55,56].

AP has received much attention because of the fact that it emits room temperature tryptophan phosphorescence having a lifetime of ca. 2 s [57], the longest lifetime thus far reported [58]. Each AP monomer contains three tryptophan (Trp) residues at positions 109, 220 and 268. The long-lived phosphorescence has been assigned to originate from Trp 109 that is buried deep in the hydrophobic core of AP [59–62]. We have previously characterized that Trp residues of AP by LTP (low-temperature phosphorescence) and ODMR (optically detected magnetic resonance) spectroscopy using the wild-type, W220Y mutant (Trp 220 being replaced by tyrosine (Tyr) and Tb-AP (where all the metal ions are substituted by Tb(III) ion) [44]. Trp 109 having almost quasi crystalline environment was found to be involved in the photoinduced energy transfer to bound terbium in Tb-AP [42,44].

In this work we have studied the interaction of the drug TC with AP at pH = 8 at 298 K, 308 K and at 77 K using suitable cryosolvent. The association constant of the complex, the number of binding sites and the thermodynamic parameters of binding have been evaluated using (i) steady state and time resolved fluorescence monitoring the intrinsic tryptophan (Trp) emission of AP, (ii) the enhanced emission of bound TC (iii) the anisotropy of the emission of bound TC at 298 K. In order to find any specific role and perturbation of the Trp/Tyr residues of AP due to binding with the drug, we presented the low temperature phosphorescence spectra of free AP and the complex of AP with TC in 40% ethylene glycol (EG) matrix at 77 K. Also to understand the mechanism of enhanced emission of TC in the AP-TC complex we have investigated the time resolved anisotropy decay of the bound TC; the efficiency and the rate constant of the photoinduced energy transfer (ET) from the excited Trp residues of AP to bound TC by monitoring the Trp emission. The protein-drug docking study is employed to find the probable location of the drug molecule bound to the protein along with ΔG^0 value of binding. Docking study is also exploited to observe any change in the immediate environment of Trps due to binding, and to find the involvement and the perturbation of any other residues near the binding site. The observed rotational correlation time of the bound TC is utilized to reveal the environment of the binding site of TC in the complex.

2. Experimental procedures

2.1. Materials

The Tetracycline Hydrochloride (TC) and Alkaline Phosphatase from *E. coli* (AP) were purchased from Sigma–Aldrich, USA. All the solvents used were of spectral grade and further dried by standard procedures. All solutions were prepared in 50 mM Tris buffer, pH = 8. Temperature during all experiments was maintained at 25 °C ± 0.1 °C, unless otherwise mentioned.

2.2. Instrumentation

2.2.1. Steady state measurements

UV–Vis absorption spectra were recorded on a Hitachi U-4100 UV–Vis-NIR spectrophotometer at 298 K. The steady state emission measurements of 1 μM AP with varying concentration of TC were carried out using a Hitachi Model F-4010 spectrofluorimeter equipped with a 150-W xenon lamp, at 298 K and 308 K using a stopper cell of 0.5 cm path length. The emission measurements of 1 μM AP with varying concentrations of TC were made by exciting the samples at 290 nm and 295 nm (in order to minimize the contribution from tyrosine) using the correct mode of the instrument. Quantum yield of free AP was determined using the procedure described earlier [63]. The steady state emission measurements of 5 μM TC with varying concentrations of AP were carried out by exciting the examples at 370 nm at 298 K. In both the measurement excitation and emission band passes were 10 nm and 5 nm. Fluorescence quantum yield (φ_D) was determined in each case by comparing the corrected emission spectrum of the samples with that of quinine sulfate in 0.1 N H_2SO_4 (φ_D = 0.54) [64]. Inner filter effects have been eliminated in all of the emission spectra.

Emission studies at 77 K were made using a Dewar system having a 5 mm outer diameter quartz tube. The freezing of the samples at 77 K were done at the same rate for all the samples. Phosphorescence was measured in a Hitachi F-7000 spectrofluorimeter equipped with phosphorescence accessories. All the samples were made in 40% ethylene glycol for measurements at 77 K. The samples were excited at 280 nm using a 10 nm band pass, and the emission band pass was 1 nm.

The measurement of steady state anisotropy was performed with manual Glen Thompson polarizer using Hitachi F-7000 fluorimeter. The steady state anisotropy (r) is defined by

$$r = (I_{VV} - G * I_{VH})/(I_{VV} + 2G * I_{VH}) \quad (1)$$

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