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SHORT COMMUNICATION

Fluorescence Quenching and Time-resolved Fluorescence studies of α-Mannosidase from *Aspergillus fischeri* (NCIM 508)

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Abstract Apart from the vital role in glycoprotein biosynthesis and degradation, α-mannosidase is currently an important therapeutic target for the development of anticancer agents. Fluorescence quenching and time-resolved fluorescence of α-mannosidase, a multitryptophan protein from *Aspergillus fischeri* were carried out to investigate the tryptophan environment. The tryptophans were found to be differentially exposed to the solvent and were not fully accessible to the neutral quencher indicating heterogeneity in the environment. Quenching of the fluorescence by acrylamide was collisional. Surface tryptophans were found to have predominantly positively charged amino acids around them and differentially accessible to the ionic quenchers. Denaturation led to more exposure of tryptophans to the solvent and consequently in the significant increase in quenching with all the quenchers. The native enzyme showed two different lifetimes, τ_1 (1.51 ns) and τ_2 (5.99 ns). The average lifetime of the native protein (τ) (3.187 ns) was not affected much after denaturation (τ) (3.219 ns), while average lifetime of the quenched protein samples was drastically reduced (1.995 ns for acrylamide and 1.537 ns for iodide). This is an attempt towards the conformational studies of α-mannosidase.

Keywords α-Mannosidase · Fluorescence · Solute quenching · Denaturation · Lifetime

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Introduction

Mannosidases are involved in both glycoprotein biosynthesis (Golgi mannosidases) and degradation (lysosomal mannosidases) from yeast to human beings. [1, 2]. The absence of this enzyme causes the genetic lysosomal storage disease α-mannosidosis in humans and cattle [3]. The enzymes are categorized as class I and class II mannosidases and based on the sequence alignments; they belong to family 47 and family 38 glycosidase, respectively in Henrissat's glycosidase classification [1, 4]. A number of these enzymes have been cloned and sequenced; also some success has been achieved in their high level expression. There has been widespread interest in α-mannosidase in recent years, in particular, mammalian Golgi mannosidase-II involved in glycoprotein biosynthesis and is currently an important therapeutic target for the development of anticancer agents [5]. Microbial α-mannosidases have been studied widely [6] and categorized into two groups. In group A, the enzyme hydrolyzes non-reducing terminal α-mannosidic linkages regardless of the aglycon moiety. It includes those enzymes which act on para-nitro phenyl α-D-mannopyranoside (pNPM) and do not have strict linkage specificity i.e. they act on α(1–2), α(1–3), α(1–4) and α(1–6) linkages although with different rates. In group B, the enzymes hydrolyze specifically linked α-D-mannosidic bonds. These enzymes have strict stereo-chemical requirements. α-Mannosidases have also been classified as class 1 and class 2 enzymes, class 1 having similarity with group B enzymes and class 2 with group A [4]. During last 10 years several reports on fungal α-mannosidases have been published [7–12]. However there are hardly any reports on conformational studies of the enzyme.

Studies of indole fluorescence quenching by added solutes have provided valuable information regarding the

structure and dynamics of proteins in solution [13–15]. The α-mannosidase from *A. fischeri* is a homo-hexamer, with an apparent molecular mass 412 kDa. The enzyme has been purified; characterized and preliminary studies on chemical modification have been carried out earlier [16, 17]. Apart from pNPM, the enzyme also hydrolyzed mannobioses with the rate in the order of Man-α-(1–3) Man>Man-α-(1–2) Man>Man-α-(1–6) Man. In the present paper, we report, on the basis of steady state fluorescence, solute quenching technique and time-resolved fluorescence, the exposure and environment of the tryptophan residues in the enzyme.

Materials and methods

Production and purification of α-mannosidase from *A. fischeri*

The fermentation of *A. fischeri* was carried out as described by Keskar et al. [18] using 2% yeast extract as a carbon source as well as an inducer. The purification of the enzyme was carried out as described by Gaikwad et al. [16] with minor modifications.

Enzyme assay

Activity of α-mannosidase was estimated by incubating suitably diluted enzyme with 500 μM pNPM in a total volume of 1.0 ml at 50°C for 15 min in 50 mM phosphate buffer, pH 6.5. The reaction was terminated by adding 2 ml of Na_2CO_3 and the *p*-nitro phenol released was determined from its absorbance at 405 nm. A unit of activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitro phenol per min under the assay conditions.

Modification of Trp residue with NBS

Enzyme solution (1 ml, 5.8 μM) was titrated with freshly prepared N-bromosuccinimide (NBS) (2 mM) with different installments (5 μl each) till the protein to NBS ratio reached 1:10. Modification of protein with NBS was accompanied by a decrease in the absorbance of modified protein at 280 nm. The NBS modification was also carried out under denaturing conditions (6 M guanidium hydrochloride (Gdn-HCl)) and number of Trp residues modified were determined spectrophotometrically, assuming the molar absorption coefficient of 5,500 $M^{-1}cm^{-1}$ for the modified Trp at 280 nm [19].

Solute quenching studies by steady state fluorescence

Fluorescence measurements were performed for native and denatured protein with different quenchers like acrylamide (5 M), succinimide (2.5 M) (neutral quenchers), iodide (5 M) and cesium ions (5 M) (charged quenchers), on Perkin-Elmer LS 50B spectrofluorimeter at 30°C. α-Mannosidase samples of <0.1 OD were excited at 295 nm and emission spectra were recorded in range of 300–400 nm. Slit widths of 7.0 nm each were set for excitation and emission monochromators. Small aliquots of quencher stocks were added to protein samples and fluorescence spectra were recorded after each addition. Iodide stock solution contained 0.2 M sodium thiosulfate to prevent formation of tri-iodide (I^-_3). For quenching studies with denatured protein, the protein was incubated with 6 M Gdn-HCl overnight at room temperature. Fluorescence intensities were corrected for volume changes before further analysis of quenching data.

The steady-state fluorescence quenching data obtained with different quenchers were analyzed by Stern–Volmer (Eq. 1) and modified Stern–Volmer (Eq. 2) equations in order to obtain quantitative quenching parameters [13].

$$F_o/F_c = 1 + K_{sv}[Q] \tag{1}$$

$$F_o/\Delta F = f_a^{-1} + 1/[K_a f_a(Q)] \tag{2}$$

Where F_o and F_c are the relative fluorescence intensities in the absence and presence of the quencher, respectively, (Q) is the quencher concentration, K_{sv} is Stern–Volmer quenching constant, $\Delta F = F_o - F_c$ is the change in fluorescence intensity at any point in the quenching titration, K_a is the quenching constant and f_a is the fraction of the total fluorophores accessible to the quencher. Equation (2) shows that the slope of a plot of $F_o/\Delta F$ versus $(Q)^{-1}$(modified Stern–Volmer plot) gives the value of $(K_a f_a)^{-1}$ and its *Y*-intercept gives the value of $f^{-1}{}_a$.

Lifetime measurement of fluorescence decay

Lifetime measurements were carried out on Edinburgh Instruments' FLS-920 single photon counting spectrofluorimeter. An H_2 flash lamp of pulse width 1.0 ns was used as excitation source and a Synchronization photomultiplier was used to detect the fluorescence. The diluted Ludox solution was used for measuring Instrument response function (IRF). The samples (1 mg/ml) were excited at 295 nm and emission was recorded at 336 nm. Slit widths of 15 nm each were used on the excitation and emission monochromators. The resultant decay curves were analyzed by a Reconvolution fitting program supplied by Edinburgh Instruments.

The bimolecular quenching constant K_q was calculated as $K_q = K_{sv}/\tau$ where τ is the average life time of the protein in the absence of a quencher. The average life time was calculated using the formula,

$$(\tau) = \sum\nolimits_i \alpha_i \tau_i^2 \Big/ \sum\nolimits_i \alpha_i \tau_i \quad \text{where } i = 1, 2 \ldots\ldots \tag{3}$$

Results and discussion

As described in our earlier report [17], one tryptophan residue is essential for the activity of α-mannosidase from *A. fischeri*. In the present studies, kinetics of the NBS modified enzyme with 45% residual activity was studied. There was no change in the K_m values (100 μM for native and 97.5 μM for modified enzyme) coupled with a decrease in the V_{max} values (0.286 μmol/min/mg for native and 0.122 μmol/min/mg for the modified enzyme) indicating that the tryptophan residue can be essential for the catalytic conformation. Tryptophan is also shown to be essential for the activity of α-mannosidase from *Phaseolus vulgaris* [20].

The number of tryptophans detected by NBS modification in the native enzyme and under denaturing conditions was found to be two and seven, respectively. Thus, five tryptophans seem to be present in the hydrophobic interior of the protein and two are exposed to solvent. The studies of tryptophan environment were taken up further.

Steady-state fluorescence of α-mannosidase and quenching studies

The spectrum of the native enzyme (Fig. 1a, spectrum 1) shows maximum fluorescence intensity between 333 nm to 348 nm indicating several populations of Trp, few in the hydrophobic environment and others, differentially exposed to the polar environment. The denatured protein shows λ_{max} red shifted to 355 nm upon denaturation indicating polar environment due to exposure of the tryptophans (Fig. 1b, spectrum 1).

Tryptophan residues appear to be uniquely sensitive to quenching by a variety of solutes as a result of a propensity of the excited indole nucleus to donate electrons while in the excited state. Fluorescence spectra of the native and denatured α-mannosidase recorded in the absence and in the presence of increasing concentrations of acrylamide displayed higher extent of quenching in the presence of 6 M Gdn-HCl, clearly revealing that unfolding results in a significant increase in the accessibility of the tryptophan residues to the quencher. Also, denaturation led to a significant increase in the extent of quenching with the other quenchers used in this study, namely succinimide, iodide and cesium (Table 1). The percentage quenching was calculated on the basis of raw data.

Of the four quenchers used, acrylamide was the most effective, quenching 67.0% of the total intrinsic fluorescence of the protein whereas bulkier succinimide quenched only 35.0% of the total available fluorescence. The ionic quenchers, iodide and cesium ion, which can not penetrate into the protein matrix and can access only surface exposed tryptophans were found to quench only 31.0% and 15.0%, respectively, of the total fluorescence intensity of *A. fisheri* α-mannosidase.

Significantly lower quenching observed with the neutral quencher succinimide, must be due to the bulkier nature of the molecule. The lower quenching with charged quenchers (I^- and Cs^+) with native α-mannosidase indicated most of the fluorescent tryptophan residues in the protein to be buried in the hydrophobic core of the protein. Additionally, lowest quenching observed with Cs^+ appears to be due to the inability of this quencher to access the fluorophores. This may be due to the presence of positively charged residues in the vicinity of some of the exposed (or partially exposed) tryptophan residues, which repel the positively charged cesium ion, but allow the neutral acrylamide and succinimide and the negatively charged iodide ion to approach the indole moieties of the tryptophan residues in their neighborhood.

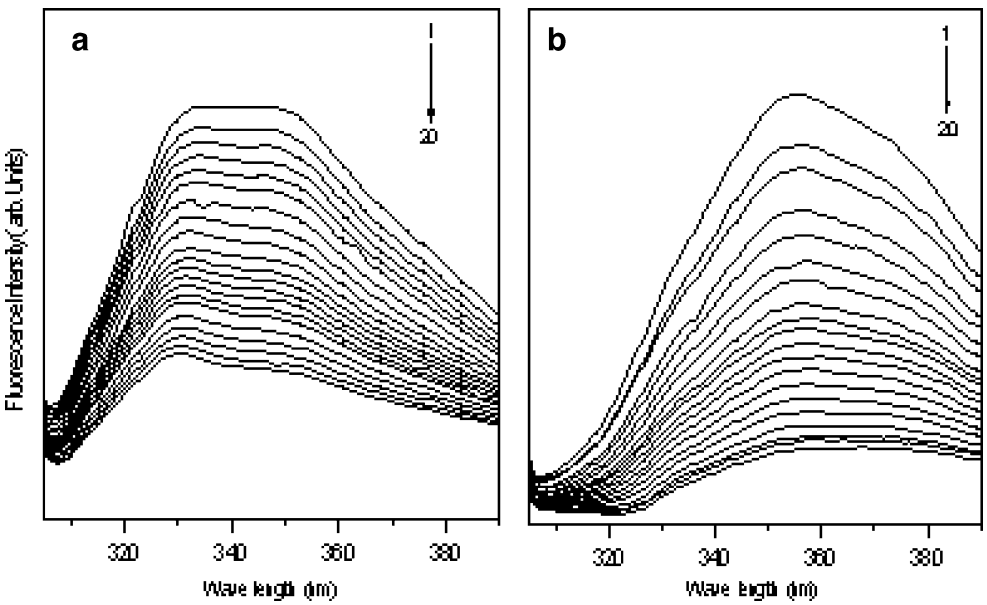

Fig. 1 Fluorescence spectra of α-mannosidase in the absence and presence of acrylamide. **a** Under native conditions, **b** under denaturing conditions (6 M Gdn-HCl). Spectrum 1 corresponds to protein alone and spectra 2–20 correspond to the protein in presence of increasing concentrations of acrylamide. The final concentration of the quencher in both **a** and **b** is 0.46 M

Table 1 Extent of fluorescence quenching of *A. fisheri* α-D-mannosidase with different quenchers

Quencher	Quenching (%)[a]	
	Native	In 6 M Gdn-HCl
Acrylamide (0.46 M)	67.0	85.0
Succinimide (0.30 M)	35.0	57.0
KI (0.30 M)	31.0	57.0
CsCl (0.30 M)	15.0	30.0

[a] Quenching % was calculated from raw data.

The iodide ions could get concentrated in a positively charged environment in the vicinity of tryptophans which increases the probability of iodide ions colliding with them and quenching the fluorescence. Besides, the inherently low quenching efficiency of Cs^+ may also be partly responsible for the lower quenching observed with it [21].

Denaturation resulted in a significant increase in the quenching by all the four quenchers, with the extent of quenching observed being 85.0%, 57.0%, 57.0% and 30.0% with acrylamide, succinimide, iodide and cesium ion, respectively (Table 1). Even after denaturation, some residual conformation was present in the enzyme which prevented the access of the neutral quenchers to the tryptophan therein.

Analysis of fluorescence quenching Data

The Stern–Volmer plots for the quenching of the protein with different quenchers are shown in Fig. 2. The quenching profiles obtained for native and denatured protein with acrylamide follow linear dependence on the quencher concentration (Fig. 2a) indicating collisional type of quenching. The profiles obtained with succinimide, iodide and cesium ion for native protein exhibited negative curvature (Fig. 2b–d) showing that certain tryptophans are selectively quenched before others in a protein. At low concentration of quencher, the slope of the Stern–Volmer plots reflects largely the quenching of the more accessible residues. At higher concentrations, the easily quenched fluorescence has been depleted, and those tryptophans having lower quenching constants become dominant. Similar quenching patterns have been observed for several multi-tryptophan proteins [13, 22, 23].

For denatured protein, all the four quenchers showed linear Stern–Volmer plots making it clear that upon denaturation, even buried tryptophans become exposed to solvent and are more accessible for quenching. These results indicate the presence of tryptophans in *A. fischeri* α-mannosidase in different environments where some tryptophans are partially or fully exposed to solvent while others are buried inside hydrophobic environment. These observations are in good agreement with the fluorescence spectrum of the native enzyme and also with the results of chemical modification experiments with NBS, where only two tryptophan residues in α-mannosidase could be modified under native condition and seven tryptophan residues could be modified upon denaturation of protein. This heterogeneity has also been shown by fluorescence life time measurements which are discussed later.

From the slopes of the two linear components of the Stern–Volmer plots, collisional quenching constants, K_{sv1} and K_{sv2} were obtained for succinimide, I^- and Cs^+ and are listed in Table 2. K_{sv1} is considerably greater than the K_{sv2} in case of succinimide, iodide and cesium giving substantial evidence for selective tryptophan quenching by these quenchers. Upon denaturation, the K_{sv1} increased nearly twice in acrylamide and iodide ion, and slight increase in the value was observed for succinimide and cesium ion (Table 2). The K_{sv} value for Cs^+ is lower than that for I^-. The higher K_{sv} value of I^- as compared to Cs^+ indicates higher efficiency of quenching by

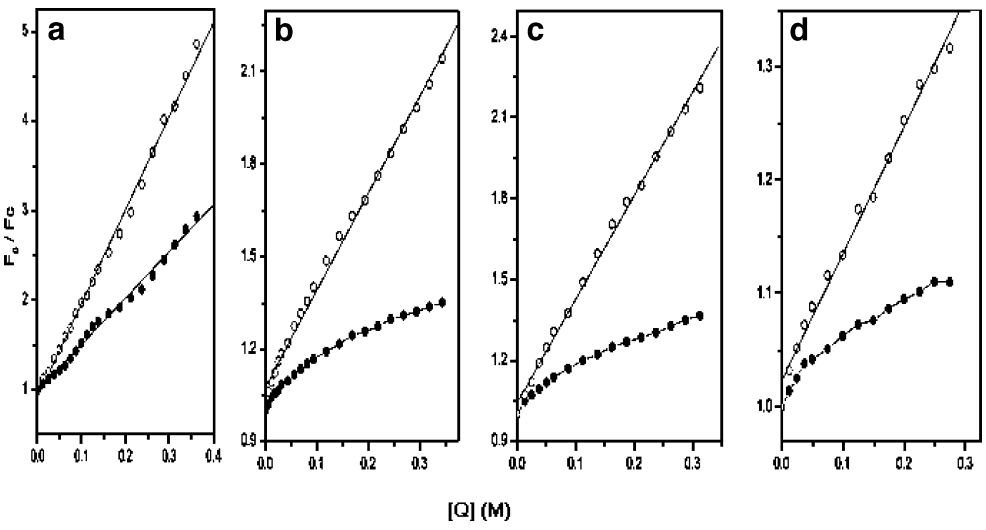

Fig. 2 Stern–Volmer plots for the quenching of the intrinsic fluorescence of α-Mannosidase with different quenchers **a** acrylamide, **b** succinimide, **c** cesium ion, and **d** iodide ion of the native enzyme (*closed circles*) and in the presence of 6 M Gdn-HCl (*open circles*). Titrations were carried out as described in the "Materials and methods" section

Table 2 Summary of parameters obtained from the intrinsic fluorescence quenching of *A. fischeri* α-D-mannosidase with different quenchers

Quencher and condition	K_{sv1} (M^{-1})	$K_{q1}\times10^{-9}$ (M^{-1} S^{-1})	K_{sv2} (M^{-1})	$K_{q2}\times10^{-9}$ M^{-1} S^{-1})	f_a	K_a
Acrylamide						
Native	3.41	1.07			0.73	6.72
Native+Gdn-HCl 6 M	10.63	3.30			1.00	8.57
Succinimide						
Native	3.12	0.98	0.55	0.17	0.29	11.55
Native+Gdn-HCl 6 M	3.15	0.98			0.57	12.12
KI						
Native	1.82	0.57	0.84	0.26	0.33	9.65
Native+Gdn-HCl 6 M	3.67	1.14			0.71	7.90
CsCl						
Native	1.02	0.32	0.31	0.1	0.12	10.32
Native+Gdn-HCl 6 M	1.05	0.33			0.38	5.84

$K_{sv1}$and K_{sv2} are Stern–Volmer quenching constants, K_{q1} and K_{q2} are bimolecular quenching constants, f_a is the fraction of accessible residues and K_a is the quenching constant obtained from modified Stern–Volmer analysis.

I^- due to the presence of an electropositive environment around Trp residues in the enzyme.

Modified Stern–Volmer plots obtained with all four quenchers are shown in Fig. 3 from which f_a or fractional accessibility and K_a, quenching constant were obtained according to Eq. 2 and listed in Table 2. Based on the f_a values of the four quenchers used, 73.0% and 29.0% of the total fluorescence was found to be accessible to acrylamide and succinimide respectively, and 33.0% and 12.4% was accessible to I^- and Cs^+ , respectively.

Denaturation of protein with 6 M Gdn-HCl led to 100% accessibility with acrylamide. For succinimide, I^- and Cs^+, the fraction accessible increased to 57.0%, 71.0% and 38.0%, respectively. The complete accessibility of tryptophan residues to acrylamide and increased accessibility to other quenchers (succinimide, iodide and cesium) clearly indicated that the protein unfolds upon treatment with 6 M Gdn-HCl. The extent of the quenching achieved with Cs^+ is significantly lower than that observed with I^-, as explained above; it is quite likely that a larger fraction of the tryptophan residues have positively charged residues in their close proximity.

The bimolecular quenching constants K_{q1} and K_{q2} are also presented in Table 2. The K_q values for acrylamide and iodide quenching of the native enzyme do increase after denaturation while they are not affected in case of succinimide and Cs^+ quenching. Due to some residual conformation present even after denaturation, succinimide, the bigger molecule will not have an easy access to deeply buried tryptophans and quenching by Cs^+ ions will still be restricted due to positive charge around trypotophans. Low K_q values reflect low collisional frequency.

Fluorescence lifetime measurement

The dynamics of the steady state fluorescence of α-mannosidase from *A. fischeri* was resolved to correlate the photo physical parameters of the protein to the structural properties. The intrinsic emission decay of the enzyme was

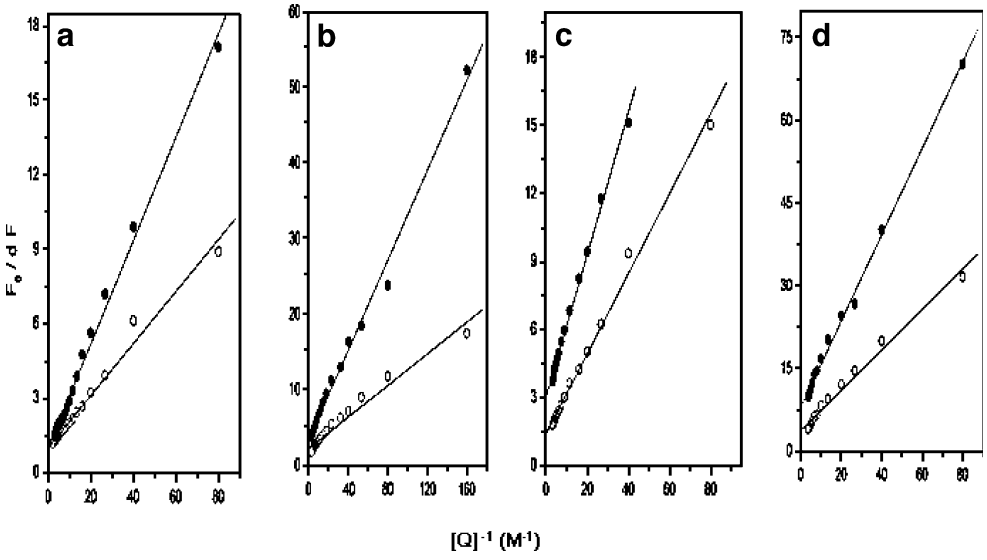

Fig. 3 Modified Stern–Volmer plots for the quenching of the intrinsic fluorescence of α-mannosidase with different quenchers **a** acrylamide, **b** succinimide, **c** cesium ion, and **d** iodide ion of the native enzyme (*closed circles*) and in the presence of 6 M Gdn-HCl (*open circles*). Titrations were carried out as described in the "Materials and methods" section

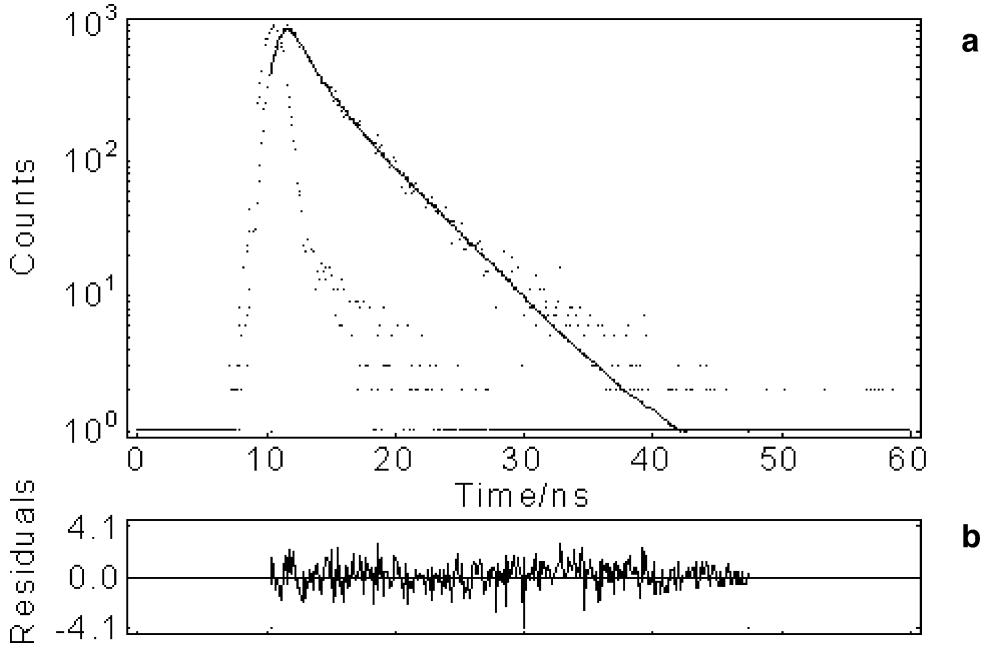

Fig 4 **a** Time-resolved fluorescence intensity decay of α-mannosidase under native conditions. Typical fluorescence decay data obtained at 25°C using an excitation wave length of 295 nm (slit width 15 nm) and observing the fluorescence emission at 333 nm (slit width 15 nm). The protein concentration was 1.0 mg/ml in 50 mM potassium phosphate buffer of pH 6.5. The calibration time for each channel was 1.0 ns. On the *Y*-axis, the photon counts are presented in a logarithmic scale. The *fast decaying, noisy solid line* represents the IRF (instrument response function) used as the excitation source. The *slower decaying line* represents the experimental fluorescence decay curve. **b** Plot of the auto correction function of the weighted residual used to judge the goodness of fitting

studied in nanosecond domain (Fig. 4) and could be described by two decay components τ_1 and τ_2. The corresponding relative amplitudes α_1 and α_2 were obtained from reconvolution fit. The decay curves obtained from the lifetime measurement of intrinsic fluorescence of α-mannosidase could be fitted well into a bi-exponential curve (χ^2<1.01), since mono-exponential curves gave large errors (χ^2>1.2). From these fits, two decay times τ_1 (1.51 ns) with 37.19% contribution and τ_2 (5.992 ns) with 62.81% contribution for the Trp fluorescence of the native enzyme were obtained (Table 3), indicating the presence of more than one fluorophores differentially emitting the energy. In the present enzyme, the components having longer lifetime have major contribution to the total quantum yield.

Table 3 Lifetimes of fluorescence decay of *A. fischeri* α-D-mannosidase under different conditions

Sample description	α_1	τ_1	α_2	τ_2	(τ)	χ^2
Native	0.073	1.51	0.011	5.992	3.187	1.083
NBS modified	0.070	1.28	0.030	5.880	4.320	1.001
Denatured (6 M Gdn-HCl)	0.080	1.16	0.028	4.656	3.219	1.006
Native+0.5 M acrylamide	0.115	1.04	0.006	5.452	1.995	1.036
Native+0.5 M KI	0.178	0.446	0.018	3.082	1.537	1.007
Native+0.5 M CsCl	0.092	1.144	0.013	4.112	2.153	0.942

For the denatured enzyme, again two decay times τ_1 (1.16 ns) and τ_2 (4.656 ns) with 39.28% and 60.72% contribution to the total fluorescence, respectively were observed. This can be due the change in the conformation of the protein. The fact that this cannot be correlated with the steady state fluorescence spectrum of the denatured enzyme reflects the heterogeneity of the tryptophan environment of the multitryptophan enzyme.

The enzyme with one Trp modified (with NBS) and 40% residual activity was analyzed for the lifetime measurement of fluorescence decay. The shorter lifetime was reduced to 1.28 ns from 1.51 ns while longer lifetime was reduced to 5.88 ns from 5.99 ns. But, the contribution of shorter fluorescence lifetime component was decreased nearly by 4%, from 39.48% of native to 35.62% of the NBS modified, whereas for longer fluorescence lifetime component, same amount of contribution, about 4.0%, was increased from 60.72% of the native to 64.38% for NBS modified one. Thus, active site Trp does contribute to the fluorescence to marginal extent. Steady state fluorescence titration of the native enzyme with NBS (1:8 enzyme: NBS ratio) had shown 4% decrease in the total fluorescence (data not shown) confirming the marginal contribution of the active site Trp to the total fluorescence of the protein.

The average lifetime (τ) of the fluorescence decay of the native enzyme (3.187 ns) does not change much after denaturation (3.219) although both shorter and longer life times show decrease in the values. The average lifetime drastically decreases for all the samples of enzyme treated with the quenchers (1.995 ns for acrylamide, 1.537 ns for iodide quenched samples). The contribution of shorter lifetime component increases from 37.19% to 79.63% for acrylamide, to 58.64% for I^- and to 66.82% for Cs^+ quenched samples. The contribution of the longer life time decreases from 62.81% to 20.37% for acrylamide, to 41.36% for I^- and to 35.18% for Cs^+ treated samples. The fluorescence seen after maximum quenching could be due to the inaccessible tryptophans. This could be due to the differentially exposed tryptophans in the protein.

In summary, α-mannosidase, a multi-tryptophan protein from *A. fischeri* shows maximum fluorescence intensity between 333–348 nm indicating several populations of tryptophans, few of which are in the hydrophobic environment and others differentially exposed to the polar environment. These tryptophans are inaccessible to the neutral quencher under native conditions. The quenching by neutral quencher was found to be collisional. Tryptophans on the surface of a protein have predominantly positively charged environment and differentially accessible to I^-. The heterogeneity of the tryptophan environment is reflected into the lifetime of the fluorescence of the native as well as after treatment of the enzyme with denaturant or quenchers.

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