pH-Dependent Quenching of the Fluorescence of Tryptophan Residues in Class A β-Lactamase from *E. coli* (TEM-1)

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Z. Naturforsch. **59 c,** 824–827 (2004); received May 11/June 16, 2004

We performed an investigation of the pH-dependent quenching of the fluorescence of tryptophan residues of TEM-1 β-lactamase from *E. coli* by uncharged and charged quenchers. pH-dependent Stern-Volmer constants (K_{SV}/pH) of tryptophan residues allowed us to determine subtle but discrete structurally and functionally important processes.

Key words: β-Lactamase TEM-1, Fluorescence Quenching, Conformational Changes

Introduction

β-Lactamase (EC 3.5.2.6) from *E. coli (*strain TEM-1) catalyzes the hydrolysis of the β-lactam ring in penicillin and cephalosporin antibiotics and belongs to class A β-lactamases (Majiduddin *et al.,* 2002). The three-dimensional structure of the enzyme shows that its 263 amino acid residues are organized in two spatial domains. The first one ($\alpha\beta$-domain) is formed from a large five-stranded β-sheet and three attached α-helices (H1, H10 and H11). This domain contains two tryptophan residues, Trp 229 and Trp 290. The indol moieties of these residues are very close and the interaction among them and the histidine residues nearby quenches a big part of the tryptophan fluorescence emission. The second domain (called α-domain) is built of eight α-helices (H2–H9) and several loops, one of which (Ω-loop) is important functionally (Jelsch *et al.,* 1992, 1993; Matagne and Frere, 1995). The number of tryptophan residues is also two (Trp 165 and Trp 210). Trp 210 is situated on the H9 helix in the neighbourhood of the single disulfide Cys77–Cys123 bridge – a very strong internal quencher of the fluorescence. Trp165 is part of the Ω-loop structure and is in a direct connection with Glu166, a residue in the active site of the enzyme (Matagne and Frere, 1995). The macromolecule has two clefts on its surface formed between the domains (Jelsch *et al.*, 1992; Matagne and Frere, 1995). One of the clefts is used for substrate binding and is formed by residues of both domains and the Ω-loop. Trp 165, present in this part of the molecule, can be used as a "natural fluorescent label" allowing the investigation of local structural changes, which are functionally important.

Quenching of fluorescence is a process of decreasing of the fluorescence intensity of the fluorophores. The quenching of the fluorescence of tryptophan residues in proteins is very intensively studied by both theoretical and experimental methods (Eftink and Ghiron, 1981; Lakowicz, 1983; Callis and Vivian, 2003; Liu and Callis, 2004). It is a very useful method to detect conformational changes of proteins in different conditions and protein-ligand interactions (Lakowicz, 1992; Szegedi *et al.,* 2000; Sivendran and Gierasch, 2001; Flowers *et al.,* 2003; Iwamoto *et al.,* 2003). The collisional interaction (appropriate electronic orbital overlapping) between a fluorophore and the quencher molecules is a basic requirement for effective quenching. It can be realized in two ways: within a *static* fluorophore-quencher "dark" complex or within a *dynamic* collisional short-living "light" complex with dissolved quencher (Lakowicz, 1983). The last bimolecular reaction between the excited counterpart and the quencher usually follows quasi-first order: a linear dependence of quenching (F_0/F) at low quencher concentrations ([Q]). The Stern-Volmer constant of proportionality (K_{SV}) is an important variable in the interpretation of collisional quenching processes, but its value in protein studies is measured at fixed pH

0939–5075/2004/1100–0824 $ 06.00

values usually without an effective analysis of electrostatic interactions in proteins (Eftink and Ghiron, 1981). Two essential factors determinate the *dynamic* K_{SV}: the steric accessibility of a fluorophore to the quencher and the electrostatic accessibility of the quencher. To separate these two effects we use uncharged and charged quenchers, respectively.

It is shown in this study that a combined investigation by uncharged and charged quenchers of pH-dependent Stern-Volmer constants (K_{SV}/pH) of *E. coli* TEM-1 β-lactamase tryptophan residues allows us to determine that changes in the microenvironment around tryptophan residues exist, which are pH-dependent and could be important to understand better the electrostatic interactions in this protein. These results could be used in both experimental and theoretical studies to improve our knowledge about molecular mechanism of this enzyme.

Materials and Methods

The enzyme β-lactamase from *E. coli* (TEM-1) was kindly provided by M. Makinen and A. Sosa (Sosa-Peinado *et al.,* 2000). Acrylamide (AcAm), $EuCl_3$, KI, and Tris [tris-(hydroxymethyl)-aminoethane] were obtained from Fluka. Mes (morpholinoethane sulfonic acid) and Gly (glycine) from Serva, NATA (*N*-acetyltryptophan amide) from MERCK were used.

The protein concentration was measured on a UV-VIS SPECORD (Carl Zeiss, Germany) spectrophotometer using $\varepsilon_{mM} = 2.93\ mM^{-1}\ cm^{-1}$ at 280 nm. Fluorescence intensity measurements at ambient temperature were made on a Perkin-Elmer LS-5 spectrofluorimeter equipped with a Data Station Model 3600 in the range, where emission was linear correlated to protein concentration. The absorbance of all solutions was < 0.1 at the excitation wavelength (295 nm). Fluorescence intensities were corrected for the dilution and the absorbance of AcAm and $EuCl_3$ at 295 nm. The three component buffer was used in all experiments which covered a pH interval from 1.5 to 12.5 and contained 10 mM of each Mes, Tris, Gly and 50 mM KCl. The pH values were measured on a Radiometer, model PHM83 Autocal pH meter, as they were corrected with aqueous solutions of NaOH or HCl.

The classic Stern-Volmer equation relates the drop in fluorescence (F_0 in absence, F in presence of the quencher) to the concentration of collision quencher (Q) as:

$$F_0/F = 1 + K_{SV}\,[Q] = \tau_0/\tau = 1 + k_q\,\tau_0\,[Q],$$

where τ_0 and τ are the fluorescence lifetimes in the absence and the presence of Q, K_{SV} is the Stern-Volmer constant for the collision quenching process and k_q is the bimolecular rate constant for the quenching process (Eftink and Ghiron, 1981). We used this equation to calculate the K_{SV} from the initial slopes of the titration curves obtained in a wide range of pH values. The uncharged quencher AcAm was used to study possible conformational alterations, leading to changes of accessibility of the indole ring. The charge environment around fluorophore(s) with alteration of pH values was analyzed by quenching with Eu^{3+} and I^- ions. Because of their nature it is known that the AcAm molecules penetrate through the proteins matrix, while the charged quenchers interact with tryptophan fluorophores on/or near the protein surface.

Results and Discussion

In Fig. 1 fluorescence spectra of the intact enzyme (curve 1) and of the model compound NATA (curve 4) are presented. The protein spectra have a maximum at 348 nm and a bandwidth of 53.5 nm, unlike the model compound, whose fluorescence maximum is at 360 nm. The protein fluorescence spectrum is quenched fully by I^- ions (curve 2) and the fluorescence difference

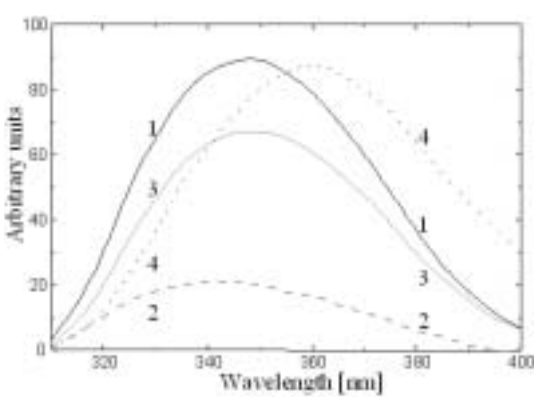

Fig. 1: Fluorescence emission spectra of TEM-1 β-lactamase: native spectrum (curve 1), quenched spectrum with iodide anions (curve 2), difference spectrum 1–2 (curve 3), fluorescence spectrum of NATA (*N*-acetyltryptophan amide) (curve 4). All solution were in 30 mM Mes–Tris–Gly buffer and 50 mM KCl. $\lambda_{exitation}$ = 295 nm. Protein concentration 2 μM.

spectrum between the intact and the quenched spectrum (curve 3) shows that the emission maximum of the former is blue shifted (342 nm), while that of the latter is slightly red shifted with about 3 nm. About 76% of native β-lactamase fluorescence is quenched by I^- ions. These data suggest that a considerable part of fluorescence is due to the tryptophan residue(s) exposed to the solvent and only a small fluorescence emission remains unquenched and is the result of buried in the protein structure fluorophores.

Fig. 2 shows the K_{SV} *vs* pH plot for quenching with AcAm. In the absence of pH-dependent protein structural changes a lack of dependence of the Stern-Volmer constant on pH could be expected, like the AcAm quenching of NATA. Nevertheless, the values of K_{SV} are pH-dependent in the region from pH 4 to pH 9. The amplitude of this change is not large (K_{SV} increases from 6.75 to 9.25 M^{-1}), but the extent of the pH interval of change supposes that ionization of more than one group should be responsible for the effect. Thus, the curve fits two Henderson-Hasselbalch curves for the ionization of a single residue with $pK_{eff,\,1} \approx 5.7$ and $pK_{eff,\,2} \approx 8.2$, respectively (Fig. 2). It can be assumed that during the two pH-induced processes Trp 165 undergoes structural changes that increase the accessibility of this residue to the quencher AcAm, as the K_{SV} values remain less than the K_{SV} of the model compound ($K_{SV,\,NATA} = 18 \pm 2\ M^{-1}$). A comparison of these results with the pH dependence of the enzyme specificity constant (k_{kat}/K_m) of the TEM-1 β-lactamase (not shown) demonstrate that the obtained pK_{eff} coincides very well with the pK obtained from the alkaline slope of (k_{kat}/K_m)/pH dependence (Makinen *et al.,* private comunication). Furthermore, the observed ratio (2:1) of the changes of K_{SV} for the two processes in Fig. 2 allows us to conclude that the change in accessibility is higher in the pH step of acylation.

The experimental results for accessibility of the tryptophan residues in native structure, discussed above, determine a ratio between $K_{SV,\,pH\,6} = 7.3\ M^{-1}$ and this one of the model compound ($K_{SV,\,NATA} = 18 \pm 2\ M^{-1}$) equal to <SA> = 0.41 compared with theoretically calculated average static accessibility of $<SA_{Trp165}> = 0.58$. The 29% increase of the latter corresponds to the ratio of molecular radii of AcAm and water molecules. The interval of change in accessibility of Trp165 from low pH (0.38) to high pH (0.52) is only Δ<SA> = 0.14, which is a subtle alteration in accessibility of the Trp165 residue.

The pH effects of tryptophan fluorescence quenching for charged I^- and Eu^{3+} ions are shown in Fig. 3, curves 2 and 3. According to the charge sign the curves have opposite courses, but same $pK_{eff} \approx 4.4$. For both quenchers the model compound $K_{SV,\,NATA}$/pH line (Fig. 3, line 1) does not cross the protein curves at a pH value near to the iso-electric point (pI 4.9) of the enzyme. It could be assumed that the charge potential in the proximity of Trp 165 in this pH region is not zero and with an increase of the number of charges at the

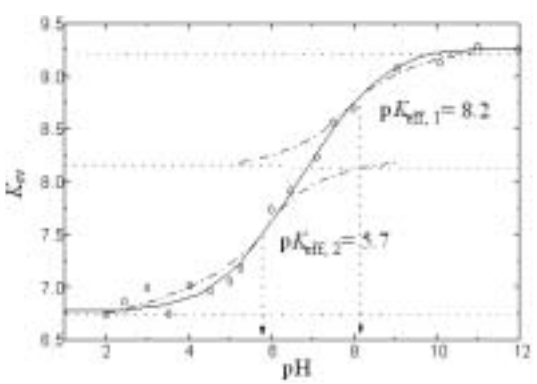

Fig. 2: pH-dependence of Stern-Volmer constants for acrylamide quenching of TEM-1 β-lactamase. Protein samples were prepared as reported in Fig. 1. K_{SV} was calculated from the initial slope of F_0/F *vs* [AcAm] plots for the corresponding pH value.

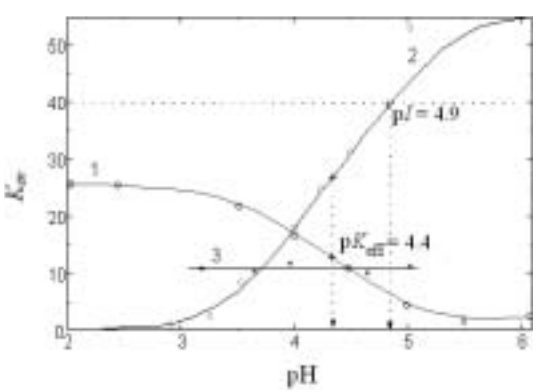

Fig. 3: K_{SV} *vs* pH of TEM-1 β-lactamase. Quenching by iodide anions ($[I^-]$) (curve 2) and europium cations (Eu^{3+}) (curve 3), the same for model compound NATA (*N*-acetyltryptophan amide) quenched by I^- (curve 1). Protein samples were prepared as indicated in Fig. 1. K_{SV} was calculated from the initial slope of F_0/F *vs* $[I^-]$ or $[Eu^{3+}]$ plots for the corresponding pH value.

ions (I^- or Eu^{3+}) the difference in "electrostatic accessibility" increases too.

The data obtained allows an estimation of the Coulomb interaction by dynamic contact between the charged quencher and the excited tryptophan residue. For the relative changes of the "charge accessibility" for single charged negative ions we obtained $\Delta K_{SV} = 5\ M^{-1}$. For quenching with Eu^{3+} ions a six-fold increase of ΔK_{SV}, which means $\Delta K_{SV} = 30\ M^{-1}$, could be expected. The measured pH dependence of the Stern-Volmer constants for both quenchers corresponds to simple Henderson-Hasselbalch curves with the same $pK_{eff} \approx 4.4$ (Fig. 3, curves 2 and 3). It can be concluded that the interaction of the quenchers are changes of local charge potential, like the changes in the formation of the enzyme-substrate complex.

Acknowledgement

This work was supported by grants: MU-X-1001 and MU-X-12002 from the National Council "Scientific Researches", Sofia, Bulgaria.

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