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Tryptophan fluorescence quenching by alkaline pH and ternary complex formation in human $\beta_1\beta_1$ and horse EE alcohol dehydrogenases

Torsten Ehrig[a], Barry B. Muhoberac[b], Thomas D. Hurley[a] and William F. Bosron[a]

[a]*Departments of Biochemistry and Molecular Biology, and Medicine, Indiana University School of Medicine, Indianapolis, IN 46202-5122, USA* and [b]*Department of Chemistry, Purdue University School of Science, Indiana University–Purdue University at Indianapolis, Indianapolis, IN 46205, USA*

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The horse EE and human $\beta_1\beta_1$ alcohol dehydrogenase isoenzymes have almost identical protein backbone folding patterns and contain 2 tryptophans per subunit (Trp-15 and Trp-314). Tyr-286, which had been proposed to quench the fluorescence of Trp-314 by resonance energy transfer at alkaline pH in EE, is substituted by Cys in $\beta_1\beta_1$. The proposed role of Tyr-286 in pH-dependent quenching of EE is confirmed by our observation that tryptophan fluorescence of $\beta_1\beta_1$ is not substantially quenched at alkaline pH. Tyr-286 had also been implicated in the quenching of Trp-314 upon formation of the EE-NAD^+-trifluoroethanol ternary complex. However, $\beta_1\beta_1$ exhibits the same extent of tryptophan fluorescence quenching as EE upon complexation, which strongly suggests that Tyr-286 is not involved in ternary complex quenching.

Alcohol dehydrogenase; Isoenzyme; Fluorescence spectroscopy; Tryptophan quenching

1. INTRODUCTION

Liver alcohol dehydrogenase (ADH) is a dimeric enzyme with a subunit molecular mass of 40,000 [1]. The horse EE isoenzyme has been used as a model system for studies of protein fluorescence, since it contains only two tryptophans per subunit and their positions have been well-defined by X-ray crystallography [2,3]. Two experimental conditions that quench the fluorescence of Trp-314 in the EE isoenzyme have been investigated: (i) titration to alkaline pH (apparent pK_a 9.6–9.8) [4–6] and (ii) binary complex formation with NAD^+ or ternary complex formation with NAD^+ and the substrate analogue trifluoroethanol [4–7]. The mechanisms of both types of quenching have not been unequivocally identified. The quenching at alkaline pH has been interpreted as the result of an ionization of Tyr-286 with subsequent resonance energy transfer from Trp-314 to tyrosinate-286 [5,6]. However, the ligand-induced quenching is more difficult to explain. Since the ligands bind far away from Trp-314 [3,8], it is clear that direct collisional quenching by ligand groups is not involved. It has been suggested that resonance energy transfer from Trp-314 to the ionized Tyr-286 is responsible not only for pH-dependent, but also for ligand-induced quenching [5,9]. Ionization of Tyr-286 upon ligand binding is postulated to result from a ligand-induced conformational change [5,9], since Tyr-286 is not close to the ligand-binding sites [3,8]. On the other hand, it has been questioned whether Tyr-286 participates both in pH-dependent and ligand-induced quenching, since the pH-dependence of ligand-binding suggests that there are two independently operating mechanisms governing these two quenching conditions [6].

We have investigated the protein fluorescence of the $\beta_1\beta_1$ isoenzyme of human ADH and compared it to the corresponding data for the EE enzyme from horse. The replacement of Tyr-286 by Cys in $\beta_1\beta_1$ allows examination of its potential role in the mechanism(s) of pH- and ligand-induced fluorescence quenching.

Abbreviations: ADH, alcohol dehydrogenase; DTT, dithiothreitol; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]; MES, 2[*N*-morpholino]ethanesulfonic acid; ACES, *N*-[2-acetamido]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Correspondence address: W.F. Bosron, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Medical Science Bldg. 405, 635 Barnhill Drive, Indianapolis, IN 46202-5122, USA. Fax: (1) (317) 274-4686

2. MATERIALS AND METHODS

2.1. *Preparation of enzymes*

Horse liver alcohol dehydrogenase was purchased from Boehringer-Mannheim (Indianapolis, IN). The enzyme was dialyzed against 10 mM sodium phosphate buffer, pH 7.0 (5 × 1 l), and cleared by centrifugation at 5,000 × *g* for 5 min.

The human $\beta_1\beta_1$ isoenzyme was expressed in *E. coli* as described previously [10] with the exception that the growth media contained 5 μM $ZnSO_4$. All purification buffers were degassed by flushing with He for 10 min. They contained 1 mM EDTA, 1 mM DTT and 1 mM benzamidine, except in the final purification step. The cells were harvested, lysed and centrifuged at 100,000 × *g* for 35 min [10]. The supernatant was equilibrated with 10 mM HEPES buffer, pH 8.0, using a Minitan concentrator (Millipore, Bedford, MA) equipped with a 30,000 molecular mass cut-off ultrafiltration membrane. The enzyme

solution was then loaded onto a 100 ml S-Sepharose column (Pharmacia, Piscataway, NJ) equilibrated with 10 mM HEPES, pH 8.0, and eluted with 150 mM NaCl in the same buffer. The enzyme solution was dialyzed overnight against 10 mM Tris buffer, pH 8.0, and loaded onto a 50 ml Affi-Gel Blue column (Bio-Rad, Richmond, CA) equilibrated with the same buffer. The enzyme was eluted with 0.4 M NaCl in column buffer and dialyzed against 2 × 2 l of 10 mM Tris buffer, pH 8.6. It was then loaded onto a 100 ml CM-cellulose column and eluted with a 500 ml linear NaCl gradient (20–100 mM) in the same buffer. The enzyme eluted in two peaks, the first of which contained bound NADH as judged from the absorptivity at 320 nm, whereas the second peak was coenzyme-free [11]. Coenzyme-free fractions were pooled and washed with 10 mM phosphate buffer, pH 7.0, using a stirred-cell concentrator with a YM 30 membrane (Amicon, Danvers, MA).

2.2. *Enzyme characterization*

Protein concentration was determined from the 280 nm absorbance of the native enzyme in 10 mM sodium phosphate buffer, pH 7.0. The extinction coefficient used for the horse enzyme was 16.8 $mM^{-1}\cdot cm^{-1}$, and was calculated from the absorbance of 0.42 per mg based on dry weight [12] and a molecular mass of 40,000 [1]. An extinction coefficient of 19.1 $mM^{-1}\cdot cm^{-1}$ was calculated for the human $\beta_1\beta_1$ isoenzyme from the known number of amino acid chromophores (2 tryptophans and 6 tyrosines [13]) [14]. Activity assays [10], active-site titrations [15], and SDS gel electrophoresis [16] were performed as described previously.

2.3. *Fluorescence measurements*

Fluorescence measurements were performed at 25°C with an SLM 8000 spectrofluorometer (SLM, Urbana-Champain, IL) operated in the analogue mode. Excitation and emission bandpasses were 2 nm each, and the excitation wavelength was 295 nm in all experiments in order to selectively excite tryptophans [17]. The emission wavelength was 330 nm. The buffers used for quenching studies with ligands (NAD$^+$ and trifluoroethanol) were recrystallized from water at least twice. The titration solutions contained 100 μM propionaldehyde to prevent unwanted formation of NADH [18].

2.4. *Reagents*

All chemicals and buffers were of reagent grade or higher, and were obtained from the following sources: ethanol, Midwest Grain (Pekin, IL); NAD$^+$, grade I, Boehringer-Mannheim (Indianapolis, IN); isobutyramide, Aldrich (Milwaukee, WI). All other chemicals were from Sigma (St. Louis, MO).

3. RESULTS AND DISCUSSION

Horse EE and human $\beta_1\beta_1$ alcohol dehydrogenase isoenzymes contain the same two tryptophans per subunit, Trp-15 and Trp-314. X-ray diffraction studies show that crystals of the EE–NADH–DMSO ternary complex and the $\beta_1\beta_1$-NAD$^+$ binary complex exhibit almost identical tertiary structures [19]. In both enzymes, Trp-15 is located on the protein surface, whereas Trp-314 is buried at the subunit interface in the hydrophobic interior. There are no substitutions between the two enzymes within 6 Å of the amino acids surrounding Trp-314 or Trp-15, except for Met-303 in EE that is Ile in $\beta_1\beta_1$. Met-303 is about 3 Å from the indole of Trp-314. This substitution does not significantly affect the fluorescence emission intensity of Trp-314, since we found that both enzymes exhibit similar total fluorescence emission intensities, as well as similar relative contributions of Trp-15 and -314 to the total intensity.

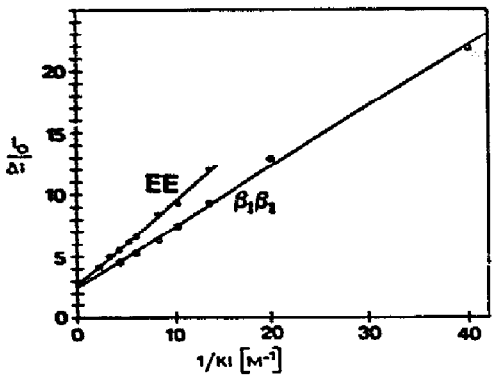

Fig. 1. Modified Stern–Volmer plot of protein fluorescence quenching by KI. The reciprocal of the relative fluorescence quench ($I_o/\Delta I$) for EE horse liver ADH (●) and recombinant human $\beta_1\beta_1$ ADH (○) is shown, and the lines represent linear fits of the data points. Buffer is 50 mM ACES, pH 7.0. Enzyme concentration is 2 μN. Excitation and emission wavelengths are 295 and 330 nm, respectively.

Specifically, the relative contributions of Trp-15 and Trp-314 were determined by quenching the solvent-exposed Trp-15 with the surface quencher, KI. Fig. 1 shows a modified Stern-Volmer plot according to Lehrer [20], in which both enzymes exhibit an almost identical y-axis intercept (2.7 ± 0.15 for EE and 2.5 ± 0.2 for $\beta_1\beta_1$. The reciprocals of the intercepts yield a relative contribution of 37% for Trp-15 in EE and 40% in $\beta_1\beta_1$. A value of 38% had been reported for EE [5].

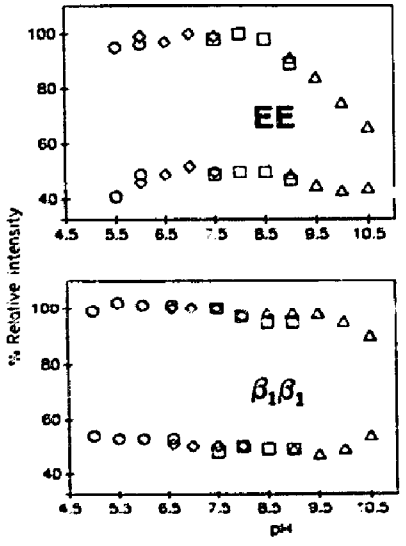

Fig. 2. pH dependence of protein fluorescence of EE and $\beta_1\beta_1$ isoenzymes. The top panel shows the relative fluorescence intensity of EE horse liver ADH and the lower panel that of recombinant human $\beta_1\beta_1$ ADH. Upper profiles, without ligands; lower profiles, in the presence of 10 mM TFE and 10 μM NAD$^+$. Values shown are endpoints of titrations at saturating ligand concentrations. Enzyme concentration is 2 μN. Buffers are MES, ○; ACES, ◇; glycylglycine, □; carbonate, △. All buffers are 50 mM. Emission intensity of unliganded enzyme at pH 7.0 is normalized to 100%. Excitation and emission wavelengths are 295 and 330 nm, respectively.

Titration of EE to pH 10.5 in the absence of ligands causes a 35% decrease in fluorescence ([4,6] and Fig. 2). It was proposed that this quenching is the result of the ionization of Tyr-286 with subsequent resonance energy transfer from Trp-314 to the tyrosinate [5,9]. On this basis one expects little or no quenching at alkaline pH in $\beta_1\beta_1$, since Tyr-286 is substituted by Cys in this isoenzyme. Indeed, Fig. 2 shows that in $\beta_1\beta_1$ the fluorescence intensity at pH 10.5 drops only marginally to about 90% of the value at pH 7.0.

Tryptophan fluorescence in EE is also quenched upon formation of a ternary complex with NAD^+ and trifluoroethanol ([4–6] and Fig. 2). That mainly the 'blue' (buried) Trp-314 is quenched under these conditions was inferred from the red-shift in the emission spectrum profile [5]. X-ray crystallography has shown that binding of coenzyme to EE in a binary complex or of coenzyme and substrate (or substrate analogue) in a ternary complex is accompanied by the 'open' to 'closed' conformational change [3,8]. Fig. 2 shows an almost identical extent (50%) of tryptophan fluorescence quenching upon ternary complex formation in $\beta_1\beta_1$ as is found in EE. Furthermore, it is mainly Trp-314 that is quenched in $\beta_1\beta_1$, since we observed a red-shift of the emission spectrum profile in $\beta_1\beta_1$ (data not shown), which is similar to the one observed with EE. Since Tyr-286 is a Cys in $\beta_1\beta_1$, the explanation that ternary complex formation causes a quenching of Trp-314 by deprotonation of Tyr-286 in the EE isoenzyme seems unlikely. Rather, the data presented here imply two independently operating mechanisms quenching Trp-314: ionization of Tyr-286 in EE is responsible for the quenching at alkaline pH, and another, yet unidentified, mechanism is involved in ternary complex quenching in EE and in $\beta_1\beta_1$.

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