The Mechanism of Quenching of Liver Alcohol Dehydrogenase Fluorescence Due to Ternary Complex Formation*

William R. Laws and Joseph D. Shore

From the Department of Biochemistry and Molecular Biology, Henry Ford Hospital, Detroit, Michigan 48202

Difference fluorescence emission spectra, reciprocal Stern-Volmer plots, and variable excitation wavelengths have been used to evaluate the selective quenching of the two tryptophan residues/subunit of liver alcohol dehydrogenase. Trp-15, at the surface of the enzyme, is quenched by KI consistent with a collisional mechanism, and has a blue-shifted excitation and red-shifted emission spectrum when compared with the spectral properties of Trp-314, which is in a hydrophobic milieu at the subunit interface of the dimeric enzyme. With excitation at 295 nm, Trp-314 is 80% quenched by formation of a ternary enzyme-NAD⁺-trifluoroethanol complex, and the quenching is essentially additive to that caused by KI. Alkaline pH also results in selective quenching of Trp-314. These results, and considerations of the three-dimensional structure of the enzyme, indicate that the quenching of protein fluorescence of liver alcohol dehydrogenase by either ternary complex formation or alkaline pH is due to resonance energy transfer to tyrosinate. Likely candidates as energy acceptors are the Tyr-286 residues on both subunits. The two Tyr-286 residues are within transfer distance for each Trp-314 residue, as well as being at the surface of the enzyme and 30 Å from the active center zinc atom. Alkaline pH directly ionizes this tyrosine residue, while ternary complex formation causes a conformational change resulting in its ionization.

Oxidized coenzyme binding therefore probably causes a conformational change resulting in protein fluorescence quenching. A stopped flow examination of NAD⁺ binding has indicated that this isomerization is quite rapid (7). Previous studies (5) also demonstrated that the protein fluorescence of liver alcohol dehydrogenase is quenched at alkaline pH with a pKₐ of 9.8 ± 0.2. NAD⁺ was shown to bind to the acid configuration, subsequently causing a further conformational change linked to proton liberation, with formation of a ternary complex with trifluoroethanol resulting in maximal quenching.

Liver alcohol dehydrogenase is a dimeric enzyme consisting of identical subunits, with each monomer having 374 amino acids and a mass of 40,000 daltons. The primary sequence and three-dimensional structure of the apoenzyme and its complexes with coenzyme or inhibitors has been reported (6, 8-11). There are 4 tyrosines and 2 tryptophan residues/submonomer in the horse liver enzyme. The tryptophan residues are in entirely different domains with one exposed to solvent and the other buried in a hydrophobic region at the subunit interface (10). Consequently, with more than one fluorophore and multiple environments, complex emission is to be expected.

Two aspects of the quenching of protein fluorescence of liver alcohol dehydrogenase by the binding of NAD⁺ in the presence of saturating trifluoroethanol have not been resolved. The molecular process that initiates the conformational change on formation of ternary complex is still unknown. It has been proposed that upon binding of NAD⁺, the pKₐ of a titratable group, probably the zinc-bound water, has been altered (10); this concept has been recently modified (12). However, recent studies on pH-dependent changes of the intrinsic fluorescence of chemically modified enzyme (13) indicate that it may not be the water molecule and that lysine 228 is not involved in the alkaline quenching of free enzyme. The other unknown aspect of the quenching process is exactly which residues are being affected and by what process, whether static or dynamic. Therefore, a study was undertaken to identify the quenched fluorophores and to attempt to understand the mechanism by which formation of a ternary complex with NAD and trifluoroethanol, or alkaline pH, causes a decreased emission of protein fluorescence.

MATERIALS AND METHODS

Alcohol dehydrogenase (EC 1.1.1.1) was prepared from horse livers by the method of Theorell et al. (14). Its concentration was determined by absorbance at 280 nm, with verification by activity measurements (15). NAD⁺, grade III, was purchased from Sigma Chemical Corp., purified on Dowex I (15), and then recrystallized (17). Spectrophotometric grade 2,2,2-trifluoroethanol was purchased from Atrich Chemical Corp. All other chemicals were of reagent grade. Sodium phosphate buffer, 0.1 ionic strength, pH 7.2, was used throughout this study. All measurements were taken at room temperature, 23°C.

Fluorescence measurements were performed with an SLM 8000 photon-counting spectrophotometer in the A/B mode. Excitation and
emission bandpasses were 2 and 1 nm, respectively. The uncorrected emission spectra were collected by integrating for 20 s every 0.25 nm step of the monochromator. Quenching studies were done at an emission wavelength of 340 nm, averaging five 50-s integration times for each sample. Salt concentrations were kept constant at 0.5 M by the use of KCl for the studies of the quenching of protein fluorescence by KI. Potassium iodide stock solutions contained 10^{-4} M sodium thiourea to prevent formation of I^- . A triangular cuvette was used with emission through the sample solution to avoid inner filter effects at high KI or NAD^+ concentrations. The reciprocal Stern-Volmer plot was based on the method developed by Lehrer (18), which enables calculation of the quenching constant in proteins containing residues not accessible to solvent, as well as determination of the fraction of the protein fluorescence due to exposed residues.

RESULTS

The quenching of the protein fluorescence of liver alcohol dehydrogenase by KI appears to be quite complex, as indicated by Stern-Volmer plots. Not only is the protein fluorescence quenching nonlinear with increasing [KI], but it also depends on the wavelength of excitation, with excitation at 280 nm showing greater quenching than when the protein is excited at 295 nm. In contrast, the quenching of L-tryptophan by KI is linear and has the same behavior whether excited at 280 or 295 nm. To see if destruction of tertiary structure would allow KI to quench protein fluorescence in a similar manner to tryptophan, the enzyme was incubated (>24 h) in 8 M urea. The Stern-Volmer plot was still nonlinear for the denatured protein, but there was no difference between the two excitation wavelengths used above.

If the results of KI quenching are expressed as a reciprocal Stern-Volmer plot (18), several interesting aspects are revealed (Fig. 1). As expected, the quenching of tryptophan in solution by KI is complete as indicated by the extrapolated intercept of 1, because the fractional amount of quenching can be estimated by the inverse of the intercept. The intercept for the enzyme when excited at 280 nm suggests that KI quenches 53% of the protein fluorescence at 340 nm and even less, 38%, when excited at 295 nm. Although the intercepts and slopes are different, the effective quenching constants (18), \( K_{Q} = \text{intercept/slope} \), are the same within 10% for quenching of enzyme by KI at the two excitation wavelengths. The quenching of enzyme in 8 M urea (Fig. 1) is less than complete and indicates that 80% of the protein fluorescence at 340 nm is quenched on extrapolation to infinite [KI]. Consequently, the enzyme was also placed in 6 M guanidine HCl. While quenching is again more complete than for protein in buffer, 6 M guanidine HCl gives essentially the same results as 8 M urea, with a nonlinear Stern-Volmer curve and an intercept on the reciprocal plot indicating 85% quenching (data not presented).

To characterize further the effect of KI on protein fluorescence, emission spectra were taken with and without 0.25 M KI. The spectra are shown in Fig. 2 and display several interesting features. The spectrum resulting from excitation at 280 nm without KI (Curve A) has a maximum at approximately 335 nm. There are also two shoulders, one at 305 nm due to tyrosine emission, and the other at 320 nm. Upon the addition of KI, as shown in Curve B, there is significant protein fluorescence quenching. While the maximum still occurs at 335 nm, the shoulder at 320 nm is more prominent resulting in a flattening of the peak and a large blue shift in the emission profile.

Excitation at 295 nm with no KI present results in a small blue shift of the emission (Curve C). Curve C has been normalized to Curve A only for visual purposes. Curves B and D correspond to the instrumental settings for Curves A and C, respectively. Although the peak is still approximately at 335 nm, the shoulder at 320 nm is more apparent and there is a decreased amount of intensity on the red edge of the spectrum. As expected from the Stern-Volmer results, there is less relative quenching due to KI with excitation at 295 nm (Curve D). However, the shape of curve D is similar to curve B, with the apparent blue shift in the emission still present.

In order to determine the fluorophores quenched on formation of the ternary complex with NAD^+ and trifluoroethanol, emission spectra were obtained and are shown in Fig. 3. Curves A and C represent the emission profiles excited at 280 and 295 nm, respectively, without any ligands present, and the are the same as those in Fig. 2. Curve B shows the quenched spectrum (excitation 280 nm) due to formation of the ternary complex. The shoulder at 320 nm is less pronounced and there is a red shift in the emission spectrum. While excitation of the ternary complex at 295 nm (Curve D) shows similar behavior as excitation at 280 nm (Curve B), there is more relative quenching due to excitation at 295 nm than at 280 nm; this result is opposite to the results of quenching by KI (Fig. 2).

Fig. 4 displays the normalized difference spectra based on Figs. 2 and 3 and the alkaline difference spectra of the enzyme (pH 7.2 emission minus pH 9.5 emission). It is obvious that KI and ternary complex formation have different effects on
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that caused by KI. This happens despite the even weaker binding of NAD\(^+\) in the presence of iodide.

In order to ensure that the quenching by ternary complex formation is additive to that caused by KI, a reciprocal plot was done as in the KI Stern-Volmer results (18). This is justified by the hyperbolic binding of NAD\(^+\) in the three curves of Fig. 5. As shown by Fig. 6, the intercept is 2 for the data in the absence and presence of KCl reflecting the 50% quenching seen in Fig. 5. The intercept for the KI-ternary complex results is also very close to 2, indicating approximately 48% quenching.

FIG. 5. Protein fluorescence quenching by ternary complex formation; 12 \(\mu\)M enzyme; 10 mM trifluoroethanol; 295 nm excitation. C, no salt; \(\Delta\), 0.25 M KCl; \(\Delta\), 0.25 M KI; \(I_0\), fluorescence intensity at 0 M NAD\(^+\) in either no salt or KCl.

DISCUSSION

The emission spectra shown in Figs. 2 and 3 indicate that the protein fluorescence can be attributed to tryptophan residues present in the liver alcohol dehydrogenase dimer. In each subunit, Trp-15 is buried at the subunit interface, only 4 Å from Trp-314 of the other subunit (10), based on N carbon positions. Consequently, considering the known properties of tryptophan fluorescence (19, 20), the 2 residues are expected to have different emission spectra, with Trp-15 having an emission profile more red-shifted than Trp-314.

The difference spectra (Fig. 4) indicate that KI and ternary complex formation are quenching different tryptophan residues. Since Trp-15 is on the surface of the protein, it will emit at higher wavelengths than tryptophan in a hydrophobic environment and will be more accessible to KI added to the solvent. The spectral and Stern-Volmer results are thus consistent with the protein fluorescence being attributed to tryptophan residues present in the liver alcohol dehydrogenase dimer. In each subunit, Trp-15 resides near the surface in contact with the solvent, while Trp-314 is buried in the protein at the subunit interface, only 4 Å from Trp-314 of the other subunit (10), based on N carbon positions. Consequently, considering the known properties of tryptophan fluorescence (19, 20), the 2 residues are expected to have different emission spectra, with Trp-15 having an emission profile more red-shifted than Trp-314.

The enzyme was titrated with NAD\(^+\) in the presence of saturating trifluoroethanol with excitation at 295 nm and emission at 340 nm. In the case of the enzyme in the absence of salt, the quenching of protein fluorescence rapidly approaches a value of 50% of the initial intensity (5). The sample containing 0.25 M KCl shows the same final level of quenching but indicates slightly weaker binding due to competition of chloride ion for the binding site of the pyrophosphate group of coenzyme (10). In the presence of 0.25 M KI, the initial level of fluorescence is decreased by 22%. However, if the quenching due to KI is added to the curve, as in Fig. 5, the quenching due to ternary complex formation is essentially additive to the protein. The KI difference curve emits to the red of the protein fluorescence with a peak at approximately 340 nm and no apparent shoulders or fine structure. The difference spectrum due to the ternary complex emits at lower wavelengths than the KI curve, and has a relatively flat peak from 320 to 335 nm with a small tyrosine shoulder at 305 nm. Raising the pH of free enzyme from 7.2 to 9.5 results in a difference spectrum similar to that for ternary complex formation, but also causes a decrease in tyrosine emission due to ionization of solvent-exposed tyrosine residues.

FIG. 6. Reciprocal plot of Fig. 5. Conditions as in Fig. 5. Data analyzed after normalizing KI results to start at \(I_0/\Delta I = 1.0\).
sistant with selective quenching of Trp-15 by KI. Conversely, since Trp-314 is in a hydrophobic region, it should behave like tryptophan in a nonpolar solvent and be blue-shifted; this is what is observed when NAD⁺ is added to liver alcohol dehydrogenase in the presence of trifluoroethanol.

Further evidence that the two quenching agents are affecting different residues is given in Figs. 5 and 6 in that the quenching due to ternary complex formation is additive to that already caused by 0.25 m KI. If the same tryptophan residue was quenched by both ternary complex formation and KI, the same final level would have been reached in the NAD⁺ titration with KI as in the absence of collisional quenching agent. A preliminary study (21) reported the same final level and incorrectly interpreted the results as indicating that Trp-15 was quenched by ternary complex formation. However, due to the experimental difficulty of inner filter effect, high enough NAD⁺ concentrations were not used to overcome the inhibition of NAD⁺ binding caused by KI. The triangular cuvette used in our present studies allowed experiments with concentrations as high as 200 μM NAD⁺ to be performed when exciting at 295 nm, elucidating the additive effect.

The difference spectrum in Fig. 4 also shows that alkaline pH quenches the same residue as the ternary complex, Trp-314. Titration of surface groups might be expected to have a greater effect on the environment of Trp-15 and thus result in a difference spectrum similar to that caused by KI. However, it appears that alkaline pH is able to affect the buried tryptophan, while also titrating exposed tyrosine residues to weakly fluorescent tyrosinate ions (20, 22). The fact that pH and ternary complex formation cause similar spectral results implicating a particular residue might have been predicted based on their interrelationship with the conformational states of the enzyme (5).

Half of the emission observed at 340 nm using 280 nm excitation comes from Trp-15, based on the KI results, leaving 50% to be from Trp-314, but in no way reflects the relative quantum yields of the 2 residues. This is exemplified by the amount of quenching due to KI with 295 nm excitation. Assuming Trp-15 is totally quenched at infinite [KI], it contributes only 38% of the fluorescence emission at 340 nm (Fig. 1). Consequently, 62% of the fluorescence comes from Trp-314. This dependence on excitation wavelength and relative intensity of each residue is discussed below.

It is extremely interesting to note that the formation of the ternary complex is able to quench approximately 80% of the fluorescence Trp-314 on excitation at 295 nm. This is based on the intercepts of Fig. 6 which indicate 48 to 50% quenching of the total fluorescence compared to the 62% value indicated for the contribution of Trp-314 with 295 nm excitation. This result is even more intriguing when the fact is considered that the conformational change seen in the enzyme on ternary complex formation with NAD⁺ and trifluoroethanol does not appear to affect the Trp-314 residue itself (11). However, Trp-15 does undergo considerable movement and might be expected to have a change in emission intensity. That the difference spectrum in Fig. 4 does not indicate quenching of Trp-15 upon ternary formation can only suggest that if it is perturbed, the change in intensity is relatively small; selection of appropriate excitation and emission wavelengths to photo-select Trp-15 might be able to resolve the question. Therefore, a quenching process must be present which is very efficient but does not alter the environment of the 2 Trp-314 residues located at the subunit interface.

There are two general methods by which a fluorophore can be quenched. The first involves increasing the rate of nonradiative decay processes. Included in this category are solvent or microenvironment changes, or both, which affect the electronic character of the molecule as well as increased collisions with other molecules in solution, like KI, which deplete the excited state. Since the environment of Trp-314 apparently does not change on ternary complex formation, the nonradiative processes must remain the same and cannot be responsible for the observed quench. However, the quenching of Trp-15 by KI is certainly a dynamic process involving the excited tryptophan residue and iodide ions. Furthermore, as the protein is unfolded in either 8 M urea or 6 M guanidine HCl, Trp-314 also becomes available for collisional quenching with KI, with an extrapolated value of 80% of the total fluorescence quenched at infinite [KI] in urea. The nonlinearity of the Stern-Volmer plot and incomplete quenching of the protein fluorescence in these denaturing solvents might be due to regions of ordered structure remaining after unfolding; residual structure has been discussed by Tanford (23).

Reactions in the excited state can also decrease emission intensities. Although tryptophan is not known to participate in any chemical reactions in the excited state, it can undergo resonance energy transfer (the kinetics of which are equivalent to a two-state irreversible excited state process). However, to be able to have 80% of Trp-314 emission quenched, very efficient energy transfer is required once NAD⁺ is bound in the ternary complex with trifluoroethanol. The three-dimensional structure of the enzyme, and the vicinity of Trp-314 in particular, reveals only three possible acceptors close enough to be very effective. The closest residue is Trp-314 of the other subunit, being only 4 Å away. The reported Förster transfer distance, Rₐ, for Trp → Trp ranges from 5.8 to 16 Å (2). Thus, energy transfer between the 2 Trp-314 residues is probable if the orientation of the dipoles is favorable. Since both tryptophans have the same environment, they should also have the same quantum yield; therefore, a negligible effect is expected due to energy transfer between the 2 Trp-314 residues.

The Trp-286 residues of both subunits are within 17 Å of Trp-314. Energy transfer is therefore possible between tryptophan and tyrosinate ion. The calculated distance, Rₐ, needed for 50% efficient energy transfer between tryptophan and tyrosinate ranges from 8.4 to 13.3 Å (24). These values, however, were obtained using assumed values including tryptophan lifetime, quantum yield, and spectral overlap, and were based on tryptophan and tyrosine free in solution or in small model polypeptides (22, 23, 26). A previous interpretation (2) based on these literature values predicted a very small quenching effect due to the 6 possible tyrosinate residues within 30 Å of either Trp-314. However, the Rₐ for energy transfer from a tryptophan in a hydrophobic environment, such as Trp-314, to tyrosinate is likely to be larger than that obtained in aqueous solution. Being in a hydrophobic environment, Trp-314 will have a longer lifetime and therefore a higher quantum yield, as well as a blue-shifted emission spectrum (Fig. 4). This provides a larger overlap integral with the absorption spectrum of tyrosinate ion, which has a maximum at 295 nm (27). Previous calculations (24) were also based on a random orientation of excitation and emission dipole moments. In a protein, particularly with a buried residue like Trp-314, the orientation between donor and acceptor will be less flexible. Furthermore, examination of the three-dimensional model of the enzyme indicates that the tryptophan and tyrosine residues are positioned such that the predicted oscillators are well aligned. Consequently, quenching of Trp-314 by resonance energy transfer to tyrosinate-286 could be quite efficient. It will be even more efficient when it is considered that, based on probabilities, only 1 Trp-314 residue will be in the excited state in a particular time interval, but 2 tyrosinate-286 residues are available to absorb the energy. A similar energy transfer mechanism has been re-
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The mechanism described above for the quenching of Trp-314 on ternary formation implies that Tyr-286 at neutral pH becomes ionized on NAD⁺ binding in the presence of trifluoroethanol. Thus, the alkaline quench with a pKa of 9.8 (5), the pH dependence of NAD⁺ and alcohol binding (29), and proton release prior to hydride transfer (30) could all be related to the ionization of a tyrosine residue on the surface of the enzyme approximately 30 Å from the active zinc atom.

The difficulty in using protein fluorescence to obtain detailed structural information where multiple fluorophores exist is exemplified by the emission spectrum of the enzyme. As shown by Curves A and C in Figs. 2 and 3, the emission profile changes as a function of excitation wavelength, with a shift in excitation to the red resulting in a blue shift in emission. This behavior has been noted before in liver alcohol dehydrogenase (31) and in Aplysia apomyoglobin (32) which also contains only 2 tryptophans. In the enzyme, it has been observed (31) that in a glass at /K the spectral properties are normal, that is the blue absorbing residue is the blue emitting; the differences in absorption spectra can be attributed to the polarity of the different environments (33). Elevation of the temperature allows the external, blue-excited tryptophan to undergo solvent relaxation (19, 20) shifting its emission to the red of that for Trp-314 which will not be affected by solvent.

The present quenching studies have enhanced the understanding of the protein fluorescence of liver alcohol dehydrogenase. This is particularly evident in the identification of the single tryptophan residue per monomer which is extensively quenched when NAD⁺ binds to the enzyme in the presence of saturating trifluoroethanol, and the verification that it is also the same tryptophan residue quenched by alkaline pH. Various processes have been discussed as to how Trp-314 is effectively quenched, with resonance energy transfer to an ionized tryosine residue being the most probable mechanism. Availability of the three-dimensional structure has been essential to conceiving the tryosinate mechanism by showing that the environment of Trp-314 does not change on ternary complex formation, that Tyr-286 is close enough for energy transfer, and that it is on the surface of the enzyme and can therefore ionize. However, the event at the active site which triggers the proposed conformational change, and how this response is ultimately transferred to Tyr-286, is still unknown and currently is being investigated.

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W R Laws and J D Shore


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