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FLUORESCENCE QUENCHING OF Trp-314 OF LIVER ALCOHOL DEHYDROGENASE BY OXYGEN

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The quenching of the fluorescence of liver alcohol dehydrogenase (LADH) by molecular oxygen has been studied by both fluorescence lifetime and intensity measurements. This was done in the presence of 1 M acrylamide which selectively quenches the fluorescence of the surface tryptophan residue, Trp-15, thus allowing us to focus on the quenching of the deeply buried tryptophan, Trp-314, by molecular oxygen. Such studies yielded a Stern-Volmer plot of F_0/F with a greater slope than the corresponding τ_0/τ plot. This inidicates that both dynamic and static quenching of Trp-314 occurs. The temperature dependence of the dynamic quenching of LADH by oxygen was also studied at three temperatures, from which we determined the activation enthalpy for the quenching of Trp-314 to be about 10 kcal/mol. The oxygen quenching of a ternary complex of LADH, NAD^+ and trifluoroethanol was also studied. The rate constant for dynamic quenching of Trp-314 by oxygen was found to be approximately the same in the ternary complex as that in the unliganded enzyme.

1. Introduction

Horse liver alcohol dehydrogenase (LADH) is a dimeric protein of M_r 80 000 which possesses only two types of tryptophan residues. These residues differ markedly in their microenvironment. Trp-15 is located on the surface of the protein [3] and is accessible to solute fluorescence quenchers such as iodide and acrylamide [1,4,9,17,19]. Trp-314, on the other hand, is buried deeply within the protein at the intersubunit interface and is virtually inaccessible to polar quenchers.

Molecular oxygen (O_2) is a much smaller and less polar quenching probe which is thought to be able to penetrate more rapidly into protein structures than the above-mentioned polar quenchers [16]. We have previously studied the O_2 quenching of the fluorescence of LADH and have found downward-curving Stern-Volmer quenching profiles due to the different degree of accessibility of the two residues to O_2 [8]. The fluorescence of the surface residue, Trp-15, was found to be quenched by O_2 with a rate constant of 3.5×10^9 M^{-1} s^{-1}. Trp-314 was found to be less accessible to O_2, but nevertheless, O_2 was found to penetrate into the protein to quench Trp-314 with an apparent rate constant (from fluorescence intensity measurements, see below) of approx. 1×10^9 M^{-1} s^{-1}. Diffusion of O_2 into LADH certainly occurs on the nanosecond time scale, but this rate constant is the lowest published value for the O_2 quenching of tryptophan fluorescence in a protein and thus characterizes Trp-314 of LADH as being one of the most deeply buried tryptophan residues in a studied globular protein.

This buried tryptophan residue, and its ability to be quenched by O_2, thus provide a means of probing the dynamic properties of the protein matrix surrounding this residue (see also recent studies of the fluorescence lifetime and anisotropy of Trp-314 for structural/dynamic information on LADH [6,14,19]). Below we will use the O_2

quenching of Trp-314 to gain further insight into the structural/dynamic properties of LADH by studying the temperature dependence of the reaction (to obtain an activation enthalpy describing the fluctuations of the protein matrix) and by studying the effect of binding specific ligands to the protein's active site. Also, we will investigate the existence of static quenching of Trp-314 by O_2 by measuring the drop of fluorescence lifetime and intensity as O_2 is added. In our earlier studies [8] we obtained some preliminary data that suggested that static quenching does occur. Here we report more thorough investigations of this matter.

The fluorescence of LADH is, of course, the sum of contributions from both Trp-15 and -314. In order to focus on the fluorescence of the latter residue we will use the strategy of adding 1 M acrylamide to the solution. Acrylamide selectively quenches the fluorescence of the surface residue, Trp-15, and at this concentration approx. 90% of the fluorescence of this residue is quenched. At most, only about 5% of the fluorescence of Trp-314 is quenched by this amount of acrylamide [9].

2. Experimental

2.1. Materials

Crystallized liver alcohol dehydrogenase was obtained from Boehringer-Mannheim, F.R.G., and from Calbiochem-Behring, San Diego, CA as a 10% ethanolic suspension. Before use, LADH was dialyzed for approx. 36 h against three changes of 0.03 M sodium phosphate buffer (pH 7.2).

NAD^+ was obtained from Boehringer-Mannheim as the lithium salt and was used without further purification. Gold-labeled trifluoroethanol (TFE) from Aldrich Chemical Co. (Milwaukee, WI) was used. Acrylamide was recrystallized from ethyl acetate. All solutions were prepared with distilled, deionized water.

2.2. Fluorescence spectroscopy

Fluorescence lifetimes were measured using an SLM cross-correlation, phase-modulation fluorometer as described by Spencer and Weber [20]. All studies were done using a Corning 7-60 emission cutoff filter. An excitation wavelength of 295 nm with a slit width of 0.5 nm was used in all cases to ensure photoselection of only tryptophan residues. Excitation frequencies of 18 or 30 MHz were used for all measurements. Steady-state fluorescence intensities were always recorded as the ratio of the fluorescence intensity of the sample to the intensity of the reflected excitation light.

Oxygen quenching studies were performed using the phase-modulation fluorometer described above and a stainless-steel high-pressure cell [15] with oxygen pressures up to 1500 lb/in^2. A 2×2 quartz cuvette was used in all experiments and the solutions were allowed to equilibrate for 45–60 min with mild stirring at each oxygen pressure. Only three or four data points (i.e., different oxygen pressures) were taken with a protein sample. Fresh samples were used to ensure the integrity of the protein samples. This also allows us to cut down on the long-term drift of the instrument, especially the modulation signal instability, and to avoid complication associated with the slow aggregation of the protein.

2.3. Data analysis

For a homogeneously emitting system, steady-state fluorescence intensity (F) data can be analyzed according to the following modified Stern-Volmer equation

$$\frac{F_0}{F} = (1 + K[Q])e^{V[Q]} \tag{1}$$

where F_0 and F are the intensities in the absence and presence of quencher (Q), and K and V dynamic and static quenching constants [7], respectively. Fluorescence lifetime values in the absence (τ_o) and presence (τ) of quencher reflect only the dynamic quenching process as given by the following relationship [16].

$$\frac{\tau_0}{\tau} = 1 + K[Q] \tag{2}$$

From the dynamic quenching constant, K, the bimolecular rate constant, k_q, for the quenching reaction can be obtained as $K = k_q\tau_o$. If there is no static quenching (i.e., $V = 0$) then F_0/F and τ_o/τ

plots should be identical. If static quenching occurs F_0/F will be greater than τ_o/τ at all [Q] (note that this is strictly true only for a homogeneous system).

For a heterogeneously emitting system the quenching process is more complicated and intensity data can be described by the following equation, where f_i, K_i and V_i, respectively, are the fractional fluorescence intensity(in the absence of quencher), dynamic and static quenching constants for residue i.

$$\frac{F}{F_0} = \sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q])e^{V_i[Q]}} \qquad (3)$$

Fluorescence lifetime quenching data for a heterogeneously emitting system are even more complicated, as we have discussed elsewhere with respect to lifetimes measured by phase-modulation fluorometry [8].

2.4. Oxygen concentration determination

Oxygen concentrations were calculated by plotting known concentrations of oxygen at 1 atm pressure at 5 °C, 0.001933 M; 15 °C, 0.001541 M; 25 °C, 0.001275 M; and 35 °C, 0.00103 M [15,23] in 0.01 M salt solution. These points lie on a straight line; by interpolation the molar concentrations needed at our experimental temperatures were determined. By using the Winkler method [23] for measuring oxygen concentration we have found that the presence of 1 M acrylamide in our phosphate buffer solution decreases the oxygen concentration by only approx. 3%. This represents a relatively small amount so no corrections were made for the reported oxygen molarities in the presence of 1 M acrylamide.

3. Results and discussion

In fig. 1 are shown data for our reinvestigation of the O_2 quenching of the fluorescence, both intensity and lifetime, of LADH in the absence (A) and presence (B) of acrylamide as well as a study of the O_2 quenching of an LADH ternary complex (C). The curved Stern-Volmer plot for the

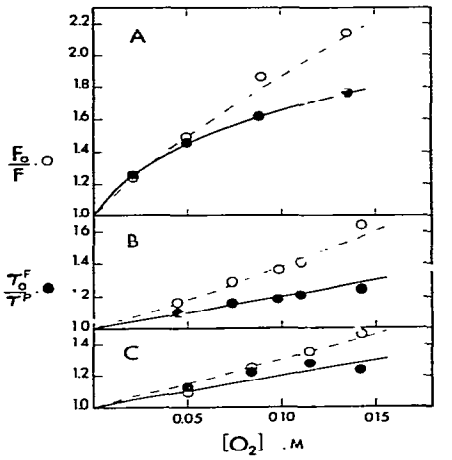

Fig. 1. Fluorescence intensity (●) and lifetime (○) Stern-Volmer plots for O_2 quenching of LADH at 20 °C, 0.03 M phosphate buffer (pH 7.2). (A) LADH alone. The dashed line is a fit of eq. 3 to the data using $K_{314} = 2.0$ M^{-1}, $V_{314} = 1.4$ M^{-1}, $K_{15} = 30$ M^{-1}, $V_{15} = 0$, $f_{314} = 0.4$. The solid line through the lifetime data is a fit of eq. 1 of ref. 8 with the above parameters and $\tau_{o,314} = 4.0$ ns (corresponding to a phase angle, θ_{314}, of 24.34° at 18 MHz), and $\tau_{o,15} = 7.0$ ns ($\theta_{15} = 38.37°$). (B) LADH in the presence of 1 M acrylamide. The dashed and solid lines are fits with $K_{314} = 2.0$ M^{-1}, $V_{314} = 1.4$ M^{-1}, $\tau_{o,314} = 4$ ns, and $f_{314} = 1.0$. (C) LADH in the presence of 2.8×10^{-4} M NAD^+, 1×10^{-2} M trifluoroethanol and 1 M acrylamide. The dashed and solid lines are fits with $K_{314} = 2.0$ M^{-1}, $V_{314} = 0.8$ M^{-1}, $\tau_{o,314} = 1.5$ ns and $f_{314} = 1.0$. All lifetimes are phase lifetimes corresponding to a modulation frequency of 18 MHz. Intensity and lifetime data were obtained with an excitation wavelength of 295 nm with emission being observed through a Corning 7-60 filter.

quenching of LADH is in agreement with our previously reported data [8] and those of Calhoun et al. [4] and indicates that the two classes of tryptophan residues in this protein have significantly different quenching constants. The solid line in fig. 1A is a fit of a two-component model to the intensity and lifetime data as described below.

Also, as we previously observed [8], when 1 M acrylamide, which selectively quenches approx. 90% of the fluorescence of Trp-15, is added, the resulting O_2 quenching Stern-Volmer plot reflects predominantly the O_2 quenching of Trp-314. The τ_o/τ O_2 quenching plot in the presence of acrylamide is found to have a significantly lower slope

than the F_0/F plot (see fig. 1B), confirming our earlier observation [8]. In cases where the fluorescence is homogeneous, such a deviation is indicative of the presence of a static quenching process [15].

The addition of 1 M acrylamide to LADH renders the fluorescence of this protein more homogeneous by abolishing most of the emission from Trp-15. Nevertheless, the fluorescence of the protein in the presence of 1 M acrylamide is not completely homogeneous due to the small (about 10–15% of the total intensity) remaining contribution from Trp-15. Before interpreting the above-mentioned difference between the F_0/F and τ_o/τ plots, the effect of this remaining Trp-15 contribution on the shape of the quenching profiles will be considered. The lifetime of the remaining Trp-15 fluorescence should be about 1 ns (decreased from its value of ~ 7 ns due to dynamic quenching by acrylamide). If the rate constant for O_2 quenching of this residue is about 4×10^9 M^{-1} s^{-1}, a typical value for a surface tryptophan residue, then the quenching constant for Trp-15 (in the presence of acrylamide) will be 4 M^{-1}. The apparent quenching constant for the F_0/F data in fig. 1B (in the presence of acrylamide) happens to be approximately this value. Intuitively, therefore, one can see that the O_2 quenching of the residue fluorescence of Trp-15 cannot be a cause of the discrepancy between the F_0/F and τ_o/τ data.

Eliminating the above technical consideration we are left with the conclusion that the discrepancy between the F_0/F and τ_o/τ plots for LADH in the presence of acrylamide is due to the existence of a static quenching process by O_2. Reports of static quenching by O_2 of protein fluorescence are few [5,12,18]. Most of the proteins studied by Lakowicz and Weber [15,16], in their pioneering O_2 quenching studies, were multi-tryptophan proteins, where the inherent heterogeneity of the emission leads to complex quenching patterns. Lakowicz and Weber did show an apparently large static component for the O_2 quenching of the single-tryptophan protein, human serum albumin. Those data, the work of Coppey et al. [5] and Jameson et al. [12] with Hb^{desFe} and Mb^{desFe}, the work of Mantulin and Pownall [18] with apolipoprotein AI-phospholipid complexes, and the present study suggest that static quenching by O_2 may be a common process. More work with single fluorophore proteins is certainly needed. The fact that the deeply buried Trp-314 of LADH is statically quenched by O_2 leads one to consider O_2 molecules as being partitioned into the relatively oily interior of this protein. Using eqs. 1 and 2, the intensity and lifetime quenching data for Trp-314 can be described by $K = 2.0$ M^{-1} and $V = 1.4$ M^{-1} at 18 MHz. (With a modulation frequency of 30 MHz similar values of $K = 1.8$ M^{-1} and $V = 1.25$ M^{-1} were found.) From this K value a k_q of 0.5×10^9 M^{-1} s^{-1} is calculated (since τ_o for Trp-314 is approx. 3.5–4 ns) [19]. Thus, the rate constant for O_2 quenching of Trp-314 is even lower than the value previously calculated from intensity quenching data [8]. We note that the value of $k_q = 0.5 \times 10^9$ M^{-1} s^{-1} for Trp-314 is remarkably close to the value of 0.6×10^9 M^{-1} s^{-1} determined by Calhoun et al. [4] for the rate constant for O_2 quenching of the phosphorescence of LADH at room temperature.

Using the above K and V values for O_2 quenching of Trp-314 obtained in the presence of acrylamide, the data in fig. 1A for the O_2 quenching of LADH in the absence of acrylamide, where there are contributions to the fluorescence from both Trp-15 and -314, can be fitted to a two-component model. The dashed line through the intensity data in fig. 1A is a fit of eq. 3 with $K_{314} = 2.0$ M^{-1}, $V_{314} = 1.4$ M^{-1}, $K_{15} = 30$ M^{-1}, $V_{15} = 0$, $f_{314} = 0.6$ and $f_{15} = 0.4$. Similarly, the solid line through the lifetime data is a fit using eq. 1 of ref. 8 with $\tau_{o,314} = 4.0$ ns (phase angle of 24.34° at 18 MHz) and $\tau_{o,15} = 7.0$ ns (phase angle of 38.37° at 18 MHz), in addition to the above parameters. The f_{314} and f_{15} values used in this fit were determined independently by an acrylamide quenching study using an excitation wavelength of 295 nm and a Corning 7-60 filter as described elsewhere [8,9].

The static quenching constant of $V = 1.4$ M^{-1} found for Trp-314 is not large and it may be more correct, at least in this case, to consider O_2 not to be excluded from the protein matrix, rather than being strongly partitioned into the protein. This does, however, raise a question as to the mechanism of the dynamic quenching process. That is, it is not clear whether dynamic quenching occurs by

diffusion of O_2 molecules from the solvent (where the concentration is known) into the protein, or if quenching occurs by diffusion of O_2 molecules sequestered in some distal region of the protein (where the local O_2 concentrations would be undetermined) through the protein to the tryptophan site. The latter mechanism probably operates in certain systems, such as the lipoprotein aggregates studied by Mantulin and Pownall [18], and the present results suggest that this mechanism must at least be considered for simple proteins such as LADH. Gratton et al. [11] have recently presented a model for the quenching of the fluorescence of fluorophores located within globular proteins. Their model includes the partitioning of quencher into and diffusion of quencher through a protein.

Fig. 1C shows the quenching profile for the ternary complex; LADH-NAD^+-TFE. For this ternary complex the K_{314} for O_2 quenching is found to be about 2.0 M^{-1} at 18 MHz; at 30 MHz K_{314} is found to be 1.3 M^{-1} for this complex. The binding of these ligands, NAD^+ and TFE, quenches the fluorescence of LADH primarily by quenching Trp-314 [17]. The mechanism for the quenching of Trp-314 by NAD^+ and TFE is uncertain. Wolfe et al. [24] proposed that the quenching may be due to increased solvent exposure of Trp-314. X-ray crystallographic structure studies show no significant change in the microenvironment of Trp-314 upon coenzyme binding [2]. Our acrylamide quenching studies have also revealed no increase in the accessibility of Trp-314 upon ligand binding [9].

The value of K for oxygen quenching Trp-314 in the ternary complex also seems to support the conclusion that Trp-314 remains in essentially the same environment upon ligand binding. However, a comparison of the k_q values for the oxygen quenching of Trp-314 for the ternary complex and free protein is difficult due to the uncertainty in the τ_o value for Trp-314 in the complex. Our measured τ_o (phase lag), in 1 M acrylamide plus NAD^+-TFE, is about 1.1 ns, from which one would calculate k_q to be approx. 1.8×10^9 M^{-1} s^{-1}, a value that is small for an oxygen quenching rate constant, but still somewhat larger than the k_q for oxygen quenching of Trp-314 in the free protein.

The above-mentioned value of 1.1 ns for τ_o is expected to be lower than the actual τ_o value for Trp-314, however. This is due to the fact that this measured τ_o value contains some contribution from Trp-15, with its low τ value in the presence of 1 M acrylamide. This contribution of Trp-15 to the measured lifetime will be larger in the ternary complex than in the free protein (i.e., contributions of $\approx 20\%$ to the ternary complex as compared to $\approx 10\%$ in the free protein, since the binding of NAD^+-TFE quenches Trp-314). Also inherent in phase-modulation lifetime calculations is the fact that the phase lifetime is always less than the weighted average lifetime [20]. Ross et al. [19] determined the τ_o of Trp-314 to be about 2.4 ns in the NAD^+-pyrazole ternary complex. The fluorescence yield of the TFE ternary complex is lower that that of the pyrazole complex so that the expected τ_o for Trp-314 in the former should be shorter than 2.4 ns. Our unpublished analysis of phase and modulation data for LADH at multiple frequencies, using the alogarithm of Weber [22], is consistent with a τ_o of approx. 1.5 ns for Trp-314 in the TFE ternary complex. Using this value of 1.5 ns for τ_o in the TFE ternary complex, the k_q for oxygen quenching of Trp 314 becomes approx. 1.3×10^9 M^{-1} s^{-1}, (for 30 MHz data, k_q is approx. 0.7×10^9 M^{-1} s^{-1}). In view of the uncertainty concerning the lifetime of Trp-314 in the TFE ternary complex and the noise in the data in fig. 1C, we conclude that the exposure of Trp-314 to O_2 is essentially unchanged upon ligand binding.

From a study of the temperature dependence of the quenching of Trp-314 of LADH by oxygen (in the presence of 1 M acrylamide), we find an activation enthalpy, $\Delta H^{\ddagger}$, for the oxygen quenching rate constant of 10 ± 1 kcal/mol (from lifetime measurements; a value of 12 ± 3 kcal/mol is obtained from intensity measurements), as illustrated by the Arrhenius plot in fig. 2. Previous works of Lakowicz and Weber [13,15] have shown the $\Delta H^{\ddagger}$ for the oxygen quenching of tryptophan in water and certain proteins to be of the order of 3–4 kcal/mol. Our value of 10 kcal/mol is thus significantly larger than other reported $\Delta H^{\ddagger}$ values. However, in a recent work, Lakowicz and co-workers [14] reported data for the temperature

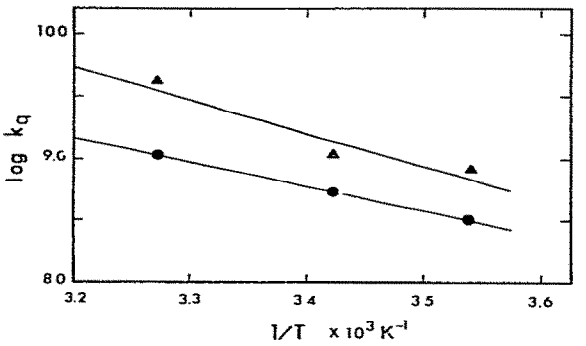

Fig. 2. An Arrhenius plot for the apparent rate constant for the O_2 quenching of LADH fluorescence in the presence of 1 M acrylamide. Rate constants were obtained from phase lifetime (●) and intensity (▲) Stern-Volmer plots. In the latter case, the static quenching component was neglected in determining the rate constant, and thus these values are larger than those from the lifetime data.

dependence of the oxygen quenching of LADH (i.e., the quenching of the total fluorescence from LADH, not just Trp-314) from which one can calculate a $\Delta H^{\ddagger}$ of 8 kcal/mol. We believe the large $\Delta H^{\ddagger}$ value we find for the quenching of Trp-314 to be due to the fact that this residue is very deeply buried in this relatively large protein. By comparison, most single-tryptophan-containing proteins are quite small and thus may physically be able to provide less shielding for their tryptophan residues. For example, in our unpublished work with cod parvalbumin, a single tryptophan protein of molecular weight 12000, we find a $\Delta H^{\ddagger}$ of 6–7 kcal/mol for oxygen quenching. This value is much closer to other published $\Delta H^{\ddagger}$ values, even though the tryptophan residue in this protein appears to be in the interior of this protein. For oxygen to quench the fluorescence of a molecule it must come into contact with the molecule. For O_2 to diffuse into the matrix of globular proteins, rapid structural functions must occur to facilitate the formation of pores or channels leading to the internal tryptophan residue. This must involve the breaking or stretching of bonds, including hydrogen bonds, which is an energetic process. Since Trp-314 is very deeply buried at the intersubunit interface of LADH, one would expect that more bonds (or stronger bonds) must be stretched or broken for oxygen to penetrate the three-dimensional structure of this protein, resulting in a relatively large $\Delta H^{\ddagger}$ value for the quenching process.

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