Fluorescence Quenching as an Indicator for Structural Fluctuations in Liver Alcohol Dehydrogenase†

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ABSTRACT: *N*-Acetyltryptophanamide (NATA), when illuminated anywhere within the 280-nm absorption band, has an emission lifetime of 3.1 ns. The tryptophan residues in liver alcohol dehydrogenase (LADH), however, when excited at 280 nm exhibit two lifetimes of $\tau_1 = 2.2$ and of $\tau_2 = 5.7$ ns. Excitation at 300 nm yields a single decay of 5.0 ns. It is shown that at the latter wavelength, only the two (equivalent) tryptophan residues buried within the LADH structure are excited. The reaction rate of the NATA fluorescence quenching by ionic and nonionic quenchers is practically independent of the temperature (between 5 and 41 °C). The same substances were used to quench the tryptophan fluorescence in LADH. Here (in the same temperature range), the quenching rate decreases drastically with a decrease in temperature. These findings are discussed in terms of conformational fluctuations in LADH, whereby the temporal movement of the polypeptide chains opens channels through which the above quencher molecules can diffuse and reach the tryptophan residues located within the enzyme structure.

X-ray diffraction studies describe the three-dimensional structure of proteins in the crystalline state. For such a structure, the location of the amino acid residues and, hence, the interactions between various groups within the protein are well defined. Recently, however, it has been suggested that proteins in solution undergo rapid conformational changes. Such structural fluctuations have been invoked to account for hydrogen-exchange data (Lumry and Rosenberg, 1976; Wickett et al., 1974), for the results of NMR studies on the rotational movement of aromatic amino acid residues (Wagner et al., 1976), and for the quenching of fluorescence (Lakowicz and Weber, 1973) in proteins. In the quenching studies, it has been demonstrated that the fluorescence of tryptophan residues buried within the protein is easily quenched by oxygen, and it was concluded that the diffusion of O_2 through the protein network is facilitated by the above rapid structural fluctuations. It can be assumed that the movements necessary to create diffusional channels within a closely packed protein coil alter appreciably the interactions between some of its constituent reactive groups.

In the present study, the fluorescence quenching of tryptophan residues and its temperature dependence in the enzyme alcohol dehydrogenase (obtained from horse liver), LADH,[1] are described. The results are correlated with the accessibility of the protein interior to quenching substances.

The structure and function of liver alcohol dehydrogenase have been reviewed in detail by Branden et al. (1974), and reference will be made to their article wherever structural aspects of the enzyme are discussed. LADH is composed of two subunits, each of which contains one tryptophan residue (Trp-314) which is buried within the hydrophobic area of interaction between the subunits. A second tryptophan residue in each subunit (Trp-15) lies in the periphery of the protein and is probably exposed to the solvent. It is possible to distinguish between the optical properties of the two kinds of tryptophan (Purkey and Galley, 1970), and in the present study the fluorescence and quenching of the tryptophan buried within the enzyme were selectively followed.

Materials and Methods

N-Acetyltryptophanamide (NATA) was obtained from Miles Laboratories, Elkhart, Ind. The liver alcohol dehydrogenase (LADH) was a Boehringer, Mannheim, Germany, product and was supplied as a suspension of the enzyme in 0.02 M phosphate buffer. All other chemicals were of analytical grade. Triply distilled water was used throughout. LADH solutions (OD $\simeq$ 0.05 at 300 nm) were prepared by dissolving some of the enzyme suspension in 5×10^{-3} M (pH 7.2) buffer (about 0.25 mL of the suspension into 10 mL of buffer). The solution was filtered to remove any undissolved residues. Solutions were refrigerated and could be used for 3 days without deterioration. LADH activity was determined from the rate at which it catalyzes the NAD (nicotinamide adenine dinucleotide) reduction by ethanol (procedure supplied by the Boehringer Laboratory). The activity was about 2.5 units/mg. It was redetermined from time to time and found to be constant over a period of at least 3 months.

The stability of LADH in solution toward heat denaturation was checked by heating the system for 15 min to 41 °C and again measuring its activity at room temperature. The activity decreased from the initial 2.5 units/mg to a value of 2.1 units/mg, and it was concluded that the enzyme did not deteriorate appreciably by the above treatment. It must, however, be mentioned that prolonged heating did affect the enzyme activity to a greater extent.

KI solutions, when freshly prepared, could be stored in the refrigerator for 2 days. They contained about 10^{-4} M thiosulfate to prevent I_3^- formation (Lehrer, 1971).

Fluorescence intensities were measured by following the emission with a Hamamatsu R-462 photomultiplier and a Brookdeal-Ortec photon counting system. The solutions were excited at 300 nm (see below). Fluorescence lifetimes were measured by illuminating the solutions with a TRW nanosecond light source. The lamp response and the fluorescence were monitored by a DUVP Amperex photomultiplier coupled to a sampling oscilloscope and, hence, to a computer of average transients (CAT). In order to minimize errors due to electronic

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[1] Abbreviations used are: LADH, liver alcohol dehydrogenase; NATA, *N*-acetyltryptophanamide; NAD, nicotinamide adenine dinucleotide.

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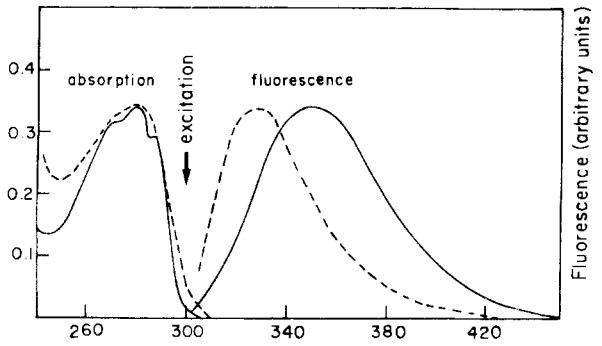

FIGURE 1: Absorption and normalized emission spectra of NATA and of LADH in aqueous pH 7.1 solution when excited at 300 nm: (—) NATA; (- - -) LADH.

drift, the signals from the sample and from the scattering solutions were alternately measured for 10-s intervals and the data stored in separate compartments of the CAT. The lifetime was obtained by convoluting the lamp response curve with a mono- or double-exponential decay function and fitting it to the experimental decay curve (Barboy and Feitelson, 1977).

Results

The fluorescence of tryptophan residues within the LADH structure, their quenching by foreign substances, and the temperature dependence of these processes were studied. The data were compared with those for *N*-acetyltryptophanamide (NATA) in aqueous solution, which served as a model for tryptophan residues in a polypeptide chain.

The normalized absorption and fluorescence spectra of *N*-acetyltryptophanamide (NATA) and of the dehydrogenase (LADH) are shown in Figure 1. It is seen that the absorbance of the enzyme lacks the residual structure found in NATA. Also, the absorption peak extends to longer wavelengths than that of NATA in aqueous solution.

NATA. The fluorescence peak of NATA in aqueous, pH 7.1 solution lies at 350 nm. Neither the fluorescence yield nor lifetime depends on the excitation wavelength within the long-wavelength absorption band centered at 280 nm. The data between 5 and 41 °C are presented in Table I. The quantum yields are based on a value of $\phi = 0.13$ at 22 °C obtained by comparison with the fluorescence of tryptophan (Tatischeff and Klein, 1975). The decrease in quantum yield with temperature corresponds to that in the fluorescence lifetime. A similar, though somewhat larger, effect on the quantum yield has previously been found for other indole derivatives (Feitelson, 1970).

The quenching rate of the NATA fluorescence by ionic and by nonionized compounds is shown in Table II. Quenching was studied at comparatively low concentrations of the quenching substances. The rate constants k_q were obtained from linear Stern–Volmer plots and from lifetimes of NATA at the corresponding temperatures. It is seen that, except for CsCl, all the rate constants approach the diffusion-controlled limit. The rate constants at 22 °C were also determined from lifetime measurements, and it was found that the decrease in quantum yield approximately equals the decrease in lifetime. Rate constants obtained from lifetime measurements at 22 °C are presented in parentheses in the appropriate column of Table II. It is further seen from the data in Table II that, within the experimental error range of ±10%, the quenching rate constants for NATA do not depend on the temperature between 5 and 41 °C.

LADH. The fluorescence peak of LADH in an aqueous solution of pH 7.1 lies at 330 nm. The width at half-height of the emission band is 0.8 times smaller than that of NATA. At 280 nm, in addition to the tryptophan residues, the tyrosines present in LADH also absorb light. However, no contribution of the tyrosine fluorescence (λ_{em} (max) 305 nm) to the tryptophan emission peak at 330 nm was observed. This indicates that the tyrosine excitation energy is dissipated in a nonradiative manner, which might include energy transfer to a tryptophan residue.

Contrary to NATA, the lifetime of the tryptophan residues in LADH when illuminated in the long-wavelength absorption band depends on the excitation wavelength. When exciting at the 280-nm absorption peak, two lifetimes of $\tau_1 = 2.2$ and $\tau_2 = 5.7 \pm 0.2$ ns were obtained. Excitation in the tail of the absorption band at 300 nm produced one lifetime of $\tau = 5.0 \pm 0.2$ ns only. Purkey and Galley have shown that above 300 nm only the tryptophan residue buried in the interior of the LADH molecule is excited (Purkey and Galley 1971). This was verified by quenching the tryptophan fluorescence with iodide (Lehrer, 1971). In 0.1 M KI at 22 °C, both excitations at 280 and at 300 nm yield only one lifetime of $\tau = 4.3$ ns. This shows that the tryptophan of shorter lifetime is preferentially quenched. The value of $\tau = 4.3$ ns corresponds to the longer ($\tau = 5.0$ ns) lifetime shortened by KI quenching with a Stern–Volmer constant of $K_{sv} = 1.8\ M^{-1}$ (see Table III). It is therefore assumed that the $\tau = 2.2$ ns lifetime belongs to the easily accessible tryptophan residues located on the periphery of the enzyme, while the $\tau = 5.0$ ns lifetime represents the inner, buried tryptophan residues. Since we are interested in the accessibility of the latter "inner" tryptophan residues only, these residues were excited selectively by using 300-nm illumination.

The fluorescence yields and lifetimes of LADH are presented in Table I. It is seen that, contrary to NATA, the

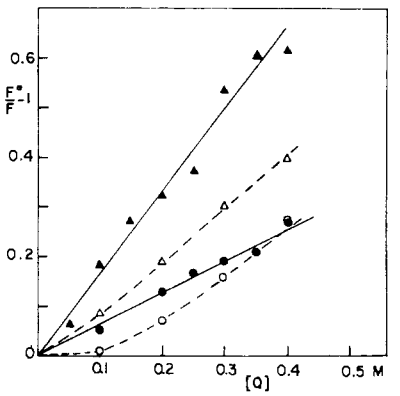

FIGURE 2: Stern–Volmer quenching plots of LADH fluorescence by (a) (△) KI and (○) CsCl at increasing ionic strength and (b) (▲) KI and (●) CsCl at constant ionic strength (by adding KCl up to a total salt concentration of 0.4 M). Temperature = 22 °C.

TABLE I: Quantum Yields (ϕ) and Lifetimes (τ) of NATA and of TRP Residues in LADH in Aqueous pH 7.1 Phosphate Buffer (5×10^{-3} M).[a]

		5 °C	22 °C	34 °C	41 °C
NATA	ϕ	0.145	0.13	0.105	0.90
	τ (ns)	3.7	3.1		2.2
LADH	ϕ	0.25	0.25	0.24	0.23
	τ (ns)	5.0	5.0		3.9

[a] Excitation at 300 nm.

TABLE II: Rate Constants for the Quenching of NATA Fluorescence in Aqueous pH 7.1 Phosphate Buffer (5×10^{-3} M).[a]

	$k_q = K_{sv}/\tau$					
	5 °C		22 °C[b]		41 °C	
quench. subst	K_{sv}	$k_q \times 10^{-9}$ (M^{-1} s^{-1})	K_{sv}	$k_q \times 10^{-9}$ (M^{-1} s^{-1})	K_{sv}	$k_q \times 10^{-9}$ (M^{-1} s^{-1})
KI	9.9	2.9	10.0	3.3 (3.1)	7.0	3.2
CsCl	1.6	0.45	1.8	0.6	1.1	0.5
thiourea	8.4	2.45	7.0	2.3 (2.1)	5.1	2.3
acetone	9.7	2.85	10.0	3.3 (3.1)	8.0	3.6

[a] Estimated error is ±10%. [b] Quantities in parentheses (at 22 °C) are rate constants obtained from lifetime measurements at a quencher concentration of 0.2 M.

TABLE III: Rate Constants for the Quenching of Tryptophan Fluorescence in LADH in Aqueous pH 7.1 Phosphate Buffer (5×10^{-3} M).

	$k_q = K_{sv}/\tau$					
	5 °C		22 °C[b]		41 °C	
quench. subst	K_{sv}	$k_q \times 10^{-9}$ (M^{-1} s^{-1})	K_{sv}	$k_q \times 10^{-9}$ (M^{-1} s^{-1})	K_{sv}	$k_q \times 10^{-9}$ (M^{-1} s^{-1})
KI[a]	0.3	0.06	1.7	0.35 (0.3)	3.4	0.9
CsCl[a]	≤0.1	~0.018	0.6	0.12 (0.16)	1.1	0.29
thiourea	≤1.0	0.2	7.8	1.5 (1.1)	12.0	3.15
acetone	≤0.5	0.12	5.5	1.1	11.4	3.0

[a] KI and CsCl solutions of constant ionic strength, obtained by adding KCl up to a total salt concentration of 0.4 M. [b] Quantities in parentheses (at 22 °C) are the rate constants obtained from lifetime measurements at a quencher concentration of 0.2 M.

tryptophan quantum yield in LADH is very little affected by the ambient temperature. A small decrease in lifetime is observed at 41 °C but this is presumably due to partial heat denaturation of the enzyme during the lengthy (up to 1 h) lifetime measurements.

The quenching of the inner tryptophan fluorescence by ionic and by neutral substances is shown in Table III. The fluorescence quenching did not obey the linear Stern–Volmer relation when the tryptophan residues of LADH were excited at 280 nm. For excitation at 300 nm, linear plots were obtained at comparatively low quenching substance concentrations. These did not exceed 0.4 M for KI and for CsCl and 0.1 M for thiourea and for acetone. The ionic quenchers KI and CsCl yield a concave quenching plot even at low concentrations, unless the ionic strength is kept constant (Figure 2). The latter was therefore adjusted by adding KCl so that the total KI or CsCl plus KCl concentration was 0.4 M over the whole quenching range. KCl (0.4 M) itself did not affect the fluorescence of LADH.

From Table III it is seen that the quenching rate constants of tryptophan residues in the interior of LADH differ strikingly from those of NATA in solution. While the latter are hardly, if at all, affected by the ambient temperature, the quenching within the LADH molecule increases drastically with temperature. The fluorescence at 5 °C is only quenched to a minute degree, whereas at 41 °C an almost diffusion-controlled quenching rate is attained.

Figure 3 shows that only one tryptophan residue is quenched when illuminating the solution at 300 nm. It has been shown by Lehrer that in a protein containing more than one tryptophan residue the number of such residues accessible to quencher molecules can be determined from a transformed Stern–Volmer equation

$$F^0/(F^0 - F) = 1/f_a K_{sv}[Q] + 1/f_a \quad (1)$$

Here F^0 and F are the fluorescence intensities in the absence and in the presence of quencher, K_{sv} is the quenching constant, and f_a is the number of the above accessible tryptophan residues (Lehrer, 1971). The intercept in Figure 3 shows that this number is unity when LADH is excited at 300 nm.

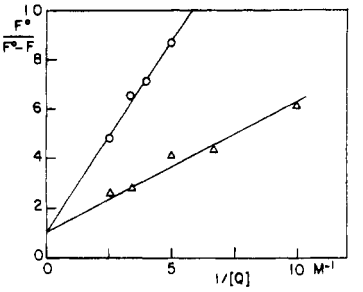

FIGURE 3: Modified Stern–Volmer plots for LADH fluorescence quenching by (○) CsCl and by (△) KI (see eq 1). Temperature = 22 °C.

Discussion

Under Results it was shown that excitation at 300 nm affects only the tryptophan residues located in the interior of the LADH molecule. The following similiarities and differences between the fluorescence properties of tryptophan residues in solution (NATA) and the above "inner" tryptophan residues were observed.

(1) The fluorescence peak of NATA in aqueous solution lies at 350 nm, while the tryptophan residues in LADH emit at 330 nm. From model studies it has been shown that the 330-nm fluorescence might be due to exciplex formation between the tryptophan indole rings and the polar peptide bonds within the protein (Lasser et al., 1977).

(2) Both for NATA and for LADH similar quenching constants are obtained both from fluorescence intensity and from decay measurements. It can therefore be concluded that the quenching is of a dynamic nature. This means that, although it is possible that a given quencher substance is unevenly distributed between the protein interior and the ambient solution phases, the quenching process is not brought about by

the formation of a complex between quencher and fluorophore but necessitates the diffusional movement of small molecules through the protein network.

(3) A fairly strong decrease in fluorescence yield with an increase in temperature is found for NATA in solution. Similar effects in indole derivatives have at least in part been attributed to electron ejection from the indole ring in aqueous solution (Grossweiner and Joschek, 1965; Feitelson, 1970). No measurable temperature effect was observed in the fluorescence of tryptophan residues in LADH. This is taken to indicate that in the nonaqueous protein interior of LADH no solvated electrons are formed or, if electrons are temporarily detached from the tryptophan residue, they immediately recombine with the *excited* indole ring.

(4) The most important observation in this study is the striking temperature dependence of fluorescence quenching by ionic and by nonionic substances in LADH; no such dependence was found in solutions of NATA. As shown above, the quenching process requires a diffusional movement of quencher molecules into and through the protein structure. At 5 °C this movement is evidently much impeded but, as seen from Table III, its rate increases greatly already at room temperature. If, as is generally assumed, the structure, and hence most of the interactions between various parts of the polypeptide chains, is preserved at room temperature, the above diffusional process can only take place if the protein structure undergoes local dynamic changes. The movement of polypeptide chains enables small molecules to pass easily through temporarily formed openings in the network but leaves the overall protein structure intact. Our results indicate that such structural fluctuations do take place on the nanosecond time scale and that they are strongly temperature dependent. It is difficult to determine the activation energy for the quenching process in LADH, since at 41 °C the diffusion controlled quenching rate is already approached. A rough estimate of E_a can, however, be obtained from the measurements at 5 and at 22 °C only. The Arrehenius equation yields an activation energy of 13.5 ± 1.3 kcal/mol for quenching by different substances. The fact that a similar value is obtained both for ionic and for nonionized species, although the actual rate constants differ widely, indicates that the temperature-dependent process can indeed be related to the protein structure rather than to a specific quencher molecule. The activation energy of 13.5 kcal/mol can be compared to the values of 17–37 kcal/mol for the activation enthalpy obtained for the rotational movement of aromatic amino acid residues in the basic pancreatic trypsin inhibitor protein from high-resolution NMR measurements (Wagner et al., 1976). There too is the activation energy attributed to the necessity for breathing motions of sizable amplitude, which enable the above rotational movement to take place. The somewhat higher value of E_a might well be due to the exceptionally heat-resistant structure of the trypsin inhibitor in which structural distortions would be less likely to take place.

Lakowicz and Weber in their study on the tryptophan fluorescence quenching in proteins used oxygen as an expressly indifferent quencher which does not interact with the protein network (Lakowicz and Weber, 1973). In the present case, substances were used which can interact with the protein environment and thus, it is hoped, might shed light on the interactions experienced by these molecules in their diffusional pathway. No great importance should be attached to the differences in absolute values between the rate constants in Table III. The distribution of small molecules and in particular of ionic species between the solution and the protein phases might somewhat differ from one case to another. Therefore, for a given quencher concentration in solution its concentration within the protein and thus the quenching rates could differ for different substances. However, the effects of ionic strength are important. The three-dimensional structure of LADH, as seen in stereo photographs (Branden et al., 1970), shows a likely diffusional channel which, beginning from the enzyme periphery at His-105, passes the ionized residues Glu-107, Lys-185, and Arg-312 and finally leads to Trp-314, the residue located near the interface between two LADH subunits. Figure 2 shows clearly that at low ionic strength the diffusion of Cs^+ ions is impeded (probably by positively charged residues). The quenching and hence the diffusion rate do increase with increase in CsCl concentration, i.e., with an increase in ionic strength. The effect disappears when a constant ionic strength is maintained (Figure 2), and the fixed ionic groups are electrically screened so as to enable the Cs^+ ions to pass unhindered.

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