Fluorescence Quenching of Liver Alcohol Dehydrogenase by Acrylamide†

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ABSTRACT: The quenching of the fluorescence of liver alcohol dehydrogenase by acrylamide has been studied as a function of excitation and emission wavelength. Downward curving Stern–Volmer plots are found, providing further support for the notion that Trp-15 of the protein is surface exposed and that Trp-314 is extensively buried within the protein. The acrylamide quenching of the binary complex formed between the protein and NAD^+ was also studied. The quenching pattern in this case is found to be complicated due to the interaction of acrylamide with the binary complex. Independent evidence for the fact that acrylamide binds to the binary complex is obtained from enzyme inhibition studies and from NAD^+ binding studies showing acrylamide to bind with positive cooperativity with respect to the coenzyme. When the interaction of acrylamide with the binary complex is taken into consideration, however, the quenching data can be interpreted as indicating that the binding of NAD^+ to the protein does not induce a conformational change that leads to the exposure of Trp-314.

Liver alcohol dehydrogenase (LADH)[1] has been an attractive protein for luminescence studies. It is composed of two identical subunits and possesses only two types of tryptophanyl residues, Trp-15, which X-ray crystallographic studies show lies near the surface, and Trp-314, which is extensively buried near the subunit interface region (Bränden et al., 1975). Experimental evidence confirming the fact that the two types of tryptophanyl residues reside in entirely different domains comes from fluorescence (solute) quenching studies using KI (Laws & Shore, 1978; Abdallah et al., 1978; Ross et al., 1981), acrylamide (Eftink & Selvidge, 1980), and other quenchers (Hershberger, 1980). In most all cases downward curving Stern–Volmer plots are observed, consistent with the selective quenching of Trp-15. Studies by Saviotti & Galley (1974) of the quenching of the room temperature phosphorescence of LADH by O_2 have also been interpreted as indicating that Trp-314 is extensively buried, being quenched only as a result of the partial unfolding of the protein.

The fluorescence of LADH is also sensitive to the presence of specific ligands. Bound NADH quenches ~70% of the fluorescence of LADH at pH 7.0 via an energy-transfer quenching mechanism (Theorell & Tatemoto, 1971; Geraci & Gibson, 1967). The binding of NAD^+ to LADH also results in a quenching of the protein's fluorescence, although to a lesser degree than that of the reduced coenzyme. The binding of NAD^+ quenches primarily the fluorescence of Trp-314 (Laws & Shore, 1978; Abdallah et al., 1978; Ross et al., 1981), but the mechanism of the quenching process is uncertain. Among the proposed mechanisms are that (a) an induced change in the conformation of the protein occurs upon NAD^+ binding, increasing the exposure of Trp-314 (Wolfe et al., 1977), (b) an induced deprotonation of a tyrosine residue takes place upon NAD^+ binding, thus leading to the quenching of tryptophanyl fluorescence by energy transfer to tyrosinate (Shore et al., 1975), (c) the binding process induces a slight conformational change, bringing the indole ring of Trp-314 in at least transient contact with a quenching group such as Met-803 (with its potential quenching heavy sulfur atom; Ross et al., 1981) or a carbonyl group (Abdallah et al., 1978), or (d) energy transfer occurs between Trp-314 and the bound NAD^+, despite the relatively small overlap integral (Abdallah et al., 1978; Ross et al., 1981).

In the present paper, we will report detailed studies of the quenching of the fluorescence of LADH and its complexes by the polar quencher acrylamide. These studies were initiated in hopes of gaining insight concerning the possible mechanisms of the quenching of LADH fluorescence upon NAD^+ binding mentioned above. In particular, by measuring the accessibility of the protein fluorescence to acrylamide in the absence and presence of NAD^+, one should be able to determine if the binding of the coenzyme induces a conformation change that leads to the increased exposure of Trp-314. In addition, the present studies were attempted in order to further characterize the relative degree of exposure of Trp-15 and -314 to a small quencher such as acrylamide. As will be seen, in the course of our studies with this system, we found that acrylamide actually binds to the protein, forming a LADH–coenzyme–quencher ternary complex, thus complicating the quenching process.

Materials and Methods

Acrylamide was recrystallized from ethyl acetate. NADH (grade III), NAD^+ (grade III), adenosine 5′-diphosphoribose (ADPR), and nicotinamide mononucleotide (NMN) were obtained from Sigma Chemical Co. NAD^+ was usually used without purification. On ocassion, the NAD^+ was purified according to the procedure of Stinson & Holbrook (1973). There was no significant difference in the results (acrylamide quenching pattern or NAD^+ association constant) obtained by using purified and nonpurified NAD^+. Trifluoroethanol (gold label) was obtained from Aldrich Chemical Co. Isobutyramide was recrystallized from water. All other chemicals were reagent grade. Solutions were prepared with distilled,

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[1] Abbreviations: ADPR, adenosine 5′-diphosphoribose; LADH, liver alcohol dehydrogenase; NAD^+ and NADH, the oxidized and reduced forms of the coenzyme nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide; TFE, 2,2,2-trifluoroethanol; Tris, tris(hydroxymethyl)aminomethane.

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deionized water. NaS_2O_3 was added to KI solutions to prevent I_3^- formation. pH measurements were made with a London PHM4 pH meter.

Lyophilized horse LADH was obtained from Sigma Chemical Co. Solutions of this commercial protein were found to become cloudy with aging. This property of lyophilized LADH has recently been the subject of a study by Ross et al. (1979). We have found that heating a solution of LADH (~3 mg/mL) at a pH between 7 and 9 at 50 °C for 15 min results in the formation of a white, flocculent precipitate. Presumably this heat treatment hastens the aggregation of a certain population of LADH molecules [the aggregation process being endothermic (Ross etal., 1979)] without denaturing the protein [the T_m of LADH being ~82 °C (Koch-Schmidt & Mosbach, 1977)]. After the precipitated material (about 25–40% of the original protein) was removed by filtration or centrifugation, the remaining protein solution was found to have a specific activity that was typically 25–30% higher than that of the original solution. Thus some of the proteinaceous material removed by this treatment appears to be inactive (or less active) LADH. The activity of LADH was measured by using the spectrophotometric procedure of Dalziel (1957). By use of the formula given by Dalziel, the LADH prepared by this heat treatment was found to be in the range of 92–97% pure. This protein was also found to be >95% active by using the NADH–isobutyramide titration method of Theorell & McKinney-McKee (1961a). Heat-treated LADH solutions were found to remain clear for at least 2 weeks at 4 °C. Crystallized LADH, obtained from Boehringer Inc., was used in a few studies and gave a nearly identical fluorescence emission spectrum, acrylamide quenching pattern, and NAD^+ titration pattern to that of the above described heat-treated, lyophilized enzyme.

Fluorescence quenching and NAD^+ binding studies were performed by using a Perkin-Elmer MPF 44 spectrophotofluorometer equipped with a thermostated cell holder. All studies were performed at 25 °C by using 5-nm excitation and emission bandwidths. Emission spectra were not wavelength corrected. In the acrylamide quenching studies, aliquots of an 8 M acrylamide solution were added directly to the LADH sample in the fluorescence cuvette by using a Gilmont micrometer syringe. Fluorescence values were corrected for any dilution effects and for absorptive screening by acrylamide or NAD^+, as described elsewhere (Parker, 1968; Eftink & Ghiron, 1976). The acrylamide/I^- double quenching experiment was performed by using a 4-mm path-length fluorescence cuvette with an excitation wavelength of 300 nm. Both measures were taken to minimize the rather large amount of absorptive screening that apparently results from the formation of a charge-transfer complex between I^- and acrylamide. The acrylamide quenching of the LADH–NMN binary complex was also performed by using a 4-mm cuvette due to the high absorbance of the concentrated NMN solution employed. Quenching data were analyzed according to the Stern–Volmer relationship by plotting F_0/F vs. [Q], where F_0 and F are the unquenched and quenched fluorescence intensities and [Q] is the concentration of quencher.

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

For a homogeneously emitting system, the quenching process can be described by the dynamic and static quenching constants, K and V. Both of these constants can provide information concerning the solvent accessibility of a tryptophanyl residue in a protein (Eftink & Ghiron, 1976, 1981). For a protein containing more than one type of fluorescent tryptophanyl residue, the Stern–Volmer equation (eq 1) must be rewritten as

$$\frac{F}{F_0} = \sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q])e^{V_i[Q]}} \qquad (2)$$

where f_i, K_i, and V_i are the fractional protein fluorescence and quenching constants for fluorescent residue i. The shape of plots of F_0/F vs. [Q] is characteristic of the relative degree of exposure of residues in a multitryptophan containing protein (Eftink & Ghiron, 1976, 1981; Lehrer & Leavis, 1978). In the particular case in which a protein possesses one class of accessible and one class of nonaccessible residues, a downward curving plot of F_0/F vs. [Q] will be observed.

A pH of 9 was selected for the quenching studies due to the need to work at a pH at which the protein can easily be saturated with NAD^+ and due to the desire to avoid titration of the pK_a = 9.8 group on the protein that quenches the fluorescence of Trp-314 (Laws & Shore, 1978). Acrylamide quenching studies have also been performed at pH 7, yielding Stern–Volmer plots very similar to those at pH 9. An emission wavelength of 323 nm was used in many of the studies rather than the λ_{max} 327 nm in order to avoid the Raman scattering peak when exciting at 295 nm.

Studies of the binding of NAD^+ and NADH to LADH were performed by using a direct fluorescence titration method (Theorell & Tatemoto, 1971; Stinson & Holbrook, 1973). Both of these ligands quench the fluorescence of the protein. The degree of quenching [given by $(F_0 - F)/(F_0 - F_\infty)$, where F_0 and F_∞ are the relative fluorescence at zero and saturating concentrations of the ligand], however, is not linearly related to the fractional degree of saturation, ν, of the binding sites (Theorell & Tatemoto, 1971; Stinson & Holbrook, 1973). The binding of coenzyme to the first binding site quenches more of the protein's fluorescence than does binding of the second ligand; the parameter δ describes the ratio of the quenching coefficient for the first ligand divided by that for the binding of both ligands [see Theorell & Tatemoto (1971)]. For determination of ν from fluorescence quenching studies, the following relationship, obtained by combining eq 9 and 13 in the article by Theorell & Tatemoto (1971), was used.

$$\frac{F_0 - F}{F_0 - F_\infty} = \nu + \left[\frac{(1 - \delta)}{3}\right](-1 + \sqrt{1 + (1 - \nu)12\nu}) \qquad (3)$$

The value for the parameter δ for NADH binding was obtained by comparing the protein fluorescence quenching and NADH fluorescence enhancement titration curves in the presence of isobutyramide (Theorell & Tatemoto, 1971). A value of δ = 0.70 was obtained for the binding/quenching of LADH fluorescence by NADH, using excitation and emission wavelengths of 295 and 323 nm. In studies of the binding of NAD^+, a δ value of 0.7 was also assumed. On the addition of NAD^+ to a LADH solution, we invariably noticed a slow (time scale of minutes) drop in the protein's fluorescence following the initial rapid fluorescence drop. The slow fluorescence drop was accompanied by an appearance of fluorescence at 430 nm (and was not observed if TFE was added first to the protein solution), suggesting that a small amount of the NAD^+ is being converted to NADH. An oxidizable substrate must be present in our solution; however, we were not able to remove this coreactant by chromatographic purification of NAD^+ by the method of Stinson & Holbrook (1973), recrystallization of the buffer components, glass distillation of the water used, dialysis of protein solution, or pretreatment of the protein with NAD^+, followed by dialysis. The slow fluorescence drop was observed for both lyophilized

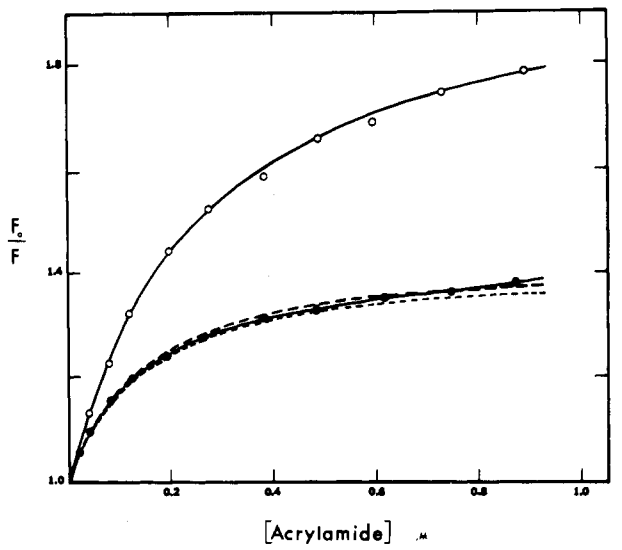

FIGURE 1: Stern–Volmer plots for the acrylamide quenching of LADH at emission wavelengths of 323 (●), and 350 nm (○). Other conditions: pH 9, 0.05 M bicine buffer, 25 °C, and excitation wavelength of 295 nm. For the 323-nm emission data, three fits to the data are shown, as follows: (---) $f_1 = 0.295$, $K_1 = 10.9\ M^{-1}$, $V_1 = V_2 = K_2 = 0$; (--) $f_1 = 0.28$, $K_1 = 9.0\ M^{-1}$, $V_1 = 0.9\ M^{-1}$, $V_2 = K_2 = 0$; (—) $f_1 = 0.26$, $K_1 = 11.0\ M^{-1}$, $V_1 = 1.1\ M^{-1}$, $K_2 = 0.04\ M^{-1}$, $V_2 = 0.004\ M^{-1}$. Note that the latter fit is not necessarily unique.

and crystallized protein samples. We judge this small degree of NAD^+ to NADH conversion to not qualitatively affect our acrylamide quenching studies, although the determination of the binding constant for NAD^+ and the degree of quenching caused by NAD^+ binding may be affected somewhat (since NADH quenches the protein's fluorescence to a greater extent than does NAD^+).

LADH concentration (needed for ν determinations) was determined by using a molar extinction coefficient of $3.74 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm (on the heat-treated protein) or by the isobutyramide–NADH titration method of Theorell & McKinney-McKee (1961a).

Enzyme kinetics studies were performed at 25 °C by observing the increase or decrease in the OD at 340 nm with a Perkin-Elmer 200 spectrophotometer.

Results

In Figure 1 are shown the Stern–Volmer plots for the quenching of LADH fluorescence by acrylamide with excitation at 295 nm and emission at 323 and 350 nm. As can be seen, there is a dramatic turnover in such plots, indicating, as expected, that there are two types of tryptophanyl residues in LADH, one readily accessible to quencher and one not. On the basis of other studies, these residues can be assigned to Trp-15 and -314, respectively (Laws & Shore, 1978; Saviotti & Galley, 1974). There is a greater relative amount of quenching seen at 350 nm than at 323 nm, indicating that the fluorescence of Trp-15 is red shifted with respect to that of Trp-314 (i.e., the difference spectrum for quenching by acrylamide shows a maximum at 335 nm, as compared to a maximum at 327 nm in the absence of quencher). Quenching studies in the presence of 50% glycerol show the initial slope of the Stern–Volmer plot to be decreased by a factor of ~3, indicating that the quenching of Trp-15 occurs primarily by a dynamic process. In addition, fluorescence lifetime studies (M. R. Eftink and D. M. Jameson, unpublished results) further support the contention that the quenching is primarily dynamic.

The simplest analysis of the data in Figure 1 involves the assumption that Trp-314 is completely buried (dynamic quenching constant for Trp-314, K_{314}, equals zero) and that there is no static quenching for either residue. With these assumptions, a fit of eq 2[2] to the data (323 nm emission λ) yields $f_{15} = 0.295$, $f_{314} = 0.705$, and $K_{15} = 10.9\ M^{-1}$. One of the dashed curves in Figure 1 illustrates this fit, which is satisfactory at low [Q] but not at high [Q]. A somewhat better fit to the data is obtained by assuming that the Trp-15 is also quenched statically (and again $K_{314} = V_{314} = 0$). As discussed elsewhere (Eftink & Ghiron, 1981), one can expect a static quenching constant to be approximately one-tenth of the value of the dynamic quenching constant. When this assumption is made, a fit to the data is obtained with $f_{15} = 0.28$, $f_{314} = 0.72$, $K_{15} = 9.0$, and $V_{15} = 0.9$. Since static quenching by acrylamide has been found for almost every single tryptophan containing protein studied, the second fit is a much more reasonable one.

The best fit, however, is obtained when it is assumed that the quenching constants for Trp-314, K_{314} and V_{314}, are nonzero. Values of $f_{15} = 0.26$, $f_{314} = 0.74$, $K_{15} = 11.0\ M^{-1}$, $V_{15} = 1.1\ M^{-1}$, $K_{314} = 0.04\ M^{-1}$, and $V_{314} = 0.004\ M^{-1}$ yield the fit given by the solid line in Figure 1. The buried Trp-314 may thus be quenched by acrylamide with a very low but nonzero quenching constant.

When the excitation wavelength is increased from 290 to 303 nm, the Stern–Volmer plots show a relative decrease in the fraction of quenchable fluorescence. On analysis of the data for each excitation wavelength (assuming for sake of simplicity that only Trp-15 is quenched), the parameters given in Table I are obtained. The K_{15} values are found to be about the same for each wavelength. The f_{15} values are found to vary

Table I: Parameters Describing the Fluorescence Quenching of LADH by Acrylamide[a]

condition/excitation λ	emission λ = 323 nm f_{15}	emission λ = 323 nm K_{15} (M^{-1})	emission λ = 350 nm f_{15}	emission λ = 350 nm K_{15} (M^{-1})
290 nm	0.50	9.5	0.62	8.9
295 nm	0.28	9.0	0.48	8.7
300 nm	0.21	8.7	0.38	9.1
303 nm	0.22	9.8	0.37	11.2
50% glycerol	0.29	3.5		
crystallized LADH	0.29	8.5		
lyophylized LADH[b]	0.36	8.2		
pH 7.0[c]	0.28	10.3		
NAD^+ binary complex	(see text)			
NAD^+–TFE ternary complex	0.51	8.3		
ADPR binary complex[d]	0.27	9.8		
NMN binary complex[e]	0.27	10.4		
I^- as quencher, 300 nm	0.19	12.5	0.37	11.8

[a] All studies performed at 25 °C, pH 9.0, and 0.05 M bicine buffer with an excitation wavelength of 295 nm, unless stated otherwise. Similar results were obtained in those cases in which the experiments were performed in 0.1 M Tris-HCl, 0.05 M glycine, or 0.1 M borate buffers. The borate buffer was found to inhibit the binding of NAD^+, however. [b] Refers to the non-heat-treated lyophilized commercial protein from Sigma Chemical Co. Although the f_1 and K_1 values reported for this sample do not appear to differ significantly from the heat-treated sample, at 1 M acrylamide, a F_0/F value of 1.65 is approached for the former compared to a value of 1.4 for the latter. [c] Phosphate buffer (0.033 M), pH 7.0. [d] ADPR (6.4×10^{-4} M), pH 7.0, and phosphate buffer. [e] NMN (9×10^{-3} M), pH 7.0, and phosphate buffer.

[2] This type of analysis, with the assumptions mentioned, corresponds to the analysis described by Lehrer (1971) in an earlier study with lysozyme.

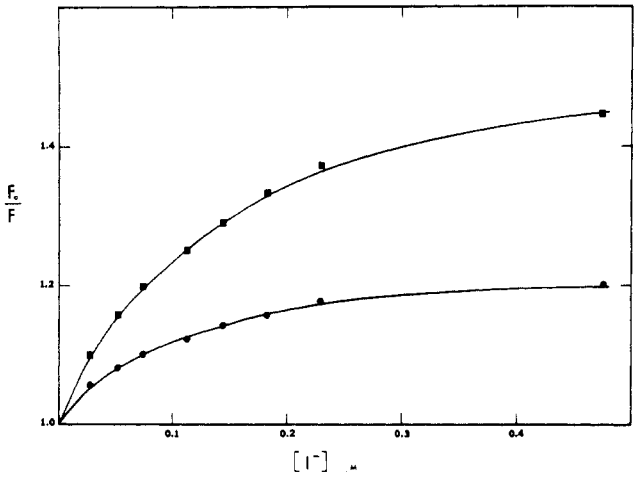

FIGURE 2: Stern-Volmer plots for the I^- quenching of LADH fluorescence, using an excitation wavelength of 300 nm and emission wavelengths of 323 (●) and 350 nm (■). The salt concentration was maintained at 0.5 M with KCl. Studies performed at 25 °C, pH 9.0, and 0.1 M Tris-HCl buffer (total ionic strength 0.6 M).

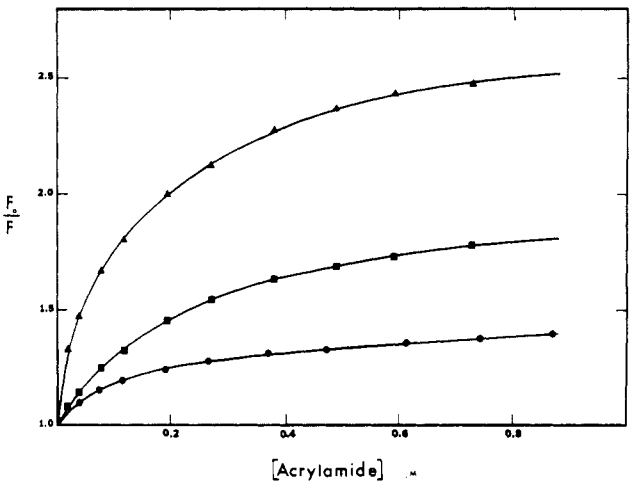

FIGURE 3: Stern-Volmer plots for the acrylamide quenching of LADH (●), the binary LADH–NAD^+ complex (■), and the ternary LADH–NAD^+–trifluoroethanol complex (▲). Excitation λ = 295 nm and emission λ = 323 nm. NAD^+ (250 μm) was added to form the binary complex; 0.032 M trifluoroethanol was added to form the ternary complex. All studies performed in 0.05 M bicine buffer. The protein concentration in these studies was approximately 8×10^{-7} M.

with excitation wavelength. The results are consistent with the buried residue, Trp-314, having an absorbance spectrum that is slightly red shifted as compared to the solvent-exposed Trp-15.

The above result is in disagreement with the claim of Barboy & Feitelson (1978), based on their I^- quenching studies, that Trp-314 can be selectively excited at an excitation wavelength of 300 nm; we find preferential excitation of Trp-314, but not complete selectivity. To clarify this point, we repeated the I^- quenching studies with LADH, as shown in Figure 2. A downward curving Stern–Volmer plot was observed with excitation at both 295 and 300 nm, and the f_{15} values at both wavelengths were found to be similar to those obtained with acrylamide.

Since both I^- and acrylamide quench primarily the fluorescence of Trp-15, this enabled us to perform a double-quenching experiment designed to provide a second estimate for the value of the acrylamide quenching constant of Trp-314. This was done by placing the protein in 0.45 M KI solution (pH 7.0, 0.033 M phosphate buffer). The concentration of acrylamide was then varied, and a nearly linear Stern–Volmer plot with a slope of 0.17 M^{-1} was obtained. At this [I^-], there will be a small residual fluorescence from Trp-15, amounting to ~5% of the remaining fluorescence at 323 nm. This residual Trp-15 fluorescent will be quenched by acrylamide, with a K of ~1.8 M^{-1}, reduced from the value in the absence of I^- by the factor $(1 + K_{15}^{I^-}[I])^{-1}$, where $K_{15}^{I^-}$ is the I^- quenching constant for Trp-15. When the quenching of residual Trp-15 is taken into consideration, a limit to the acrylamide quenching of Trp-314 can be set at $K_{314} \approx 0.12$ M^{-1}. This value is slightly larger than, but of the same order of magnitude as, the value of K_{314} determined above by the three parameter fit to the data shown in Figure 1. Since the presence of a high concentration of salt may slightly perturb the conformational stability of the protein, the slightly larger value for K_{314} found in the presence of KI is reasonable. The K_{314} value of from 0.04 to 0.12 M^{-1} is still one of the smallest acrylamide quenching constants that we have measured; only azurin, for which we observed no quenching at all at room temperature, has a smaller acrylamide K_{sv} (Eftink & Ghiron, 1976).

Acrylamide quenching studies were then performed on the binary and ternary complexes formed between LADH, NAD^+, and trifluoroethanol (TFE). In Figure 3 are shown the Stern–Volmer plots for the quenching of LADH fluorescence (323 nm emission λ) in the presence of 250 μM NAD^+ (95% saturation level at pH 9) and a TFE concentration of 0 and 0.032 M. For the LADH–NAD^+–TFE ternary complex, the quenching pattern shows an initial slope similar to that in the absence of the ligands, but approaches a higher limiting F_0/F ratio at high quencher concentration. Analysis of the data (assuming only one residue, Trp-15, is quenched) yields values of $K_{15} = 8.3$ M^{-1}, $V_{15} = 0.8$ M^{-1}, and $f_{15} = 0.51$. As will be discussed in the following section, the acrylamide quenching data are consistent with the fact that the formation of the ternary complex results in the decrease in the fluorescence yield of Trp-314 (Laws & Shore, 1978).

The Stern–Volmer plot for the acrylamide quenching of the LADH–NAD^+ binary complex is unusual, having a very large initial slope of ~20 M^{-1} and leveling off at an F_0/F value of ~2.6. The initial slope of a Stern–Volmer plot is approximately equal to $\sum f_i K_i$ for a heterogeneous system. The value of 20 M^{-1} obtained for the LADH–NAD^+ complex is a very large value for $\sum f_i K_i$. Assuming that $f_{15} \approx f_{314}$, this would require one of the K_i values (i.e., K_{15}) to be approximately 40, if the other K_i value (i.e., K_{314}) is approximately equal to zero. Since the dynamic quenching constant is the product of the quenching rate constant, k_q, times the fluorescent lifetime, τ_0, the value of $K_{15} \approx 40$ would correspond to a k_q value of approximately 5.5×10^9 M^{-1} s^{-1} [since τ_0 is 7.2 ns for Trp-15 (Ross et al., 1981)]. This k_q value is larger than that for any globular protein we have studied (Eftink & Ghiron, 1976). This fact, and the fact that the k_q for the quenching of Trp-15 in the apoprotein is only about 1.2×10^9 M^{-1} s^{-1}, argues that the acrylamide quenching of the LADH–NAD^+ complex involves a quenching process in addition to that normally found for proteins. The data suggests that acrylamide may bind to the protein in some fashion.

To test whether acrylamide interacts with the enzyme in the presence of NAD^+, we studied the effect of the quencher on the steady-state kinetics of LADH. We anticipated that acrylamide might bind at the ethanol binding site and thus act as a competitive inhibitor with respect to ethanol. However, as shown in Figure 4, when initial velocity measurements were made as a function of ethanol concentration at pH 9, acrylamide was found to be a noncompetitive inhibitor. Acrylamide was also found to be a noncompetitive inhibitor

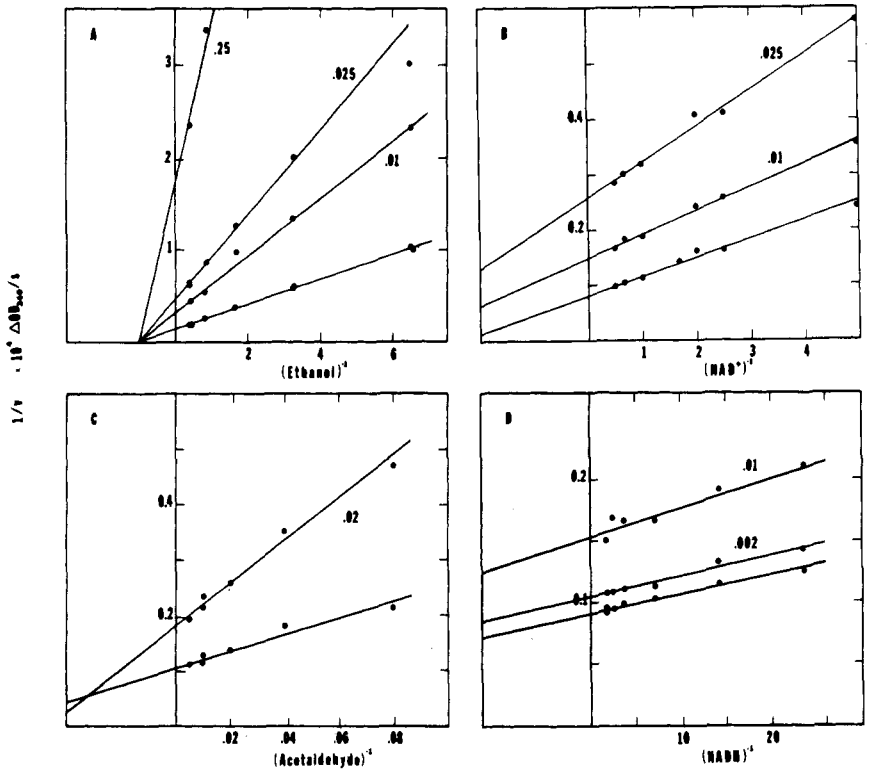

FIGURE 4: Lineweaver–Burk plots of studies of the inhibitory effect of acrylamide on the reactions catalyzed by LADH. All studies performed at pH 9.0 in 0.05 M bicine buffer, 25 °C. (A) In the presence of 1.2×10^{-3} M NAD^+. (B) In the presence of 1.8×10^{-3} M ethanol. (C) In the presence of 3.5×10^{-5} M NADH. (D) In the presence of 2.5×10^{-4} M acetaldehyde. The ordinates are all $\times 10^4$ M^{-1}. The concentration of added acrylamide is given adjacent to each line.

with respect to acetaldehyde and an uncompetitive (or mixed noncompetitive) inhibitor with respect to NAD^+ and NADH.

As we will argue under Discussion, the above studies suggest that acrylamide is able to bind to the enzyme, at least in the presence of coenzyme, to form a ternary complex. For further test of the possibility that acrylamide binds to the enzyme in the presence of either the oxidized or reduced form of the coenzyme, direct studies were made of the effect of added acrylamide on the binding of NAD^+ and NADH to the enzyme. The association constant for the coenzymes was determined by measuring the drop in the fluorescence of the protein as the ligands were added.

As shown in Figure 5, the affinity of NAD^+ and NADH for LADH is actually increased by the presence of acrylamide. In the presence of 0.2 M acrylamide, the association constant for NAD^+ is increased approximately 10-fold at pH 9.0. The dependence of the apparent binding constant for NAD^+ as a function of [acrylamide] is shown in the inset in Figure 5. Likewise, it was found that the binding constant for NADH is increased 2-fold in the presence of 0.01 M acrylamide at pH 9.0. Since the binding constant for NADH is so large under these conditions, it was difficult to accurately determine its value at higher acrylamide concentrations.

The acrylamide quenching of binary complexes formed with ADPR and NMN was also studied. Neither of these nucleotides significantly quenches the fluorescence of LADH upon binding (Theorell & Tatemoto, 1971). The acrylamide quenching pattern of both binary complexes was found to be the same as that for the apoenzyme (see Table I), suggesting that acrylamide does not form a ternary complex with these ligands.

Discussion

The present studies with acrylamide as quencher provide further evidence that Trp-15 of LADH is solvent exposed and Trp-314 is extensively buried from the solvent. Our analysis shows that the dynamic quenching constant for Trp-314 is probably not exactly equal to zero but is one of the smallest values found to date for a tryptophanyl residue in a globular protein. Trp-314 can be selectively excited at higher excitation wavelengths, as demonstrated by the increasing value of f_{314} (equal to $1 - f_{15}$ in Table I). Complete selectivity is not achieved, however, even at 303 nm, in contrast to the report by Barboy & Feitelson (1978).

Our studies of the quenching of LADH–coenzyme complexes reveal some interesting and complex quenching patterns. To begin with, the binding of NAD^+ to LADH results in the quenching of a large fraction (but not all; see below) of the fluorescence of Trp-314. As a result, there is a red shift of the fluorescence of the protein upon binding NAD^+ (Laws & Shore, 1978). The addition of a secondary ligand such as a trifluoroethanol causes a small additional drop in the fluorescence of the protein (Wolfe et al., 1977), again due primarily to the quenching of Trp-314 as indicated by the fluorescence difference spectrum. For the present study, the relative fluorescence levels (arbitrary 0–100 scale for fluorescence at 323 nm, λ_{ex} = 295 nm, pH 9.0) of the apoenzyme, binary complex, and ternary (TFE) complex are shown as the ordinate of Figure 6. The additional fluorescence drop of ~8 units on the binding of TFE to the binary complex will be due in part to the increased association of NAD^+ to the protein (i.e., the protein is only 95% saturated with NAD^+ in the absence of TFE); however, much of this drop appears to be due to a real quenching effect resulting from the binding of this secondary ligand. It should be noted that the small drop in fluorescence on the binding of a secondary ligand is not universal. We find that the formation of a ternary complex with pyrazole leads to a slight increase in fluorescence, above the level of the binary complex. One can only speculate as to the basis for the effect of these secondary ligands. It seems

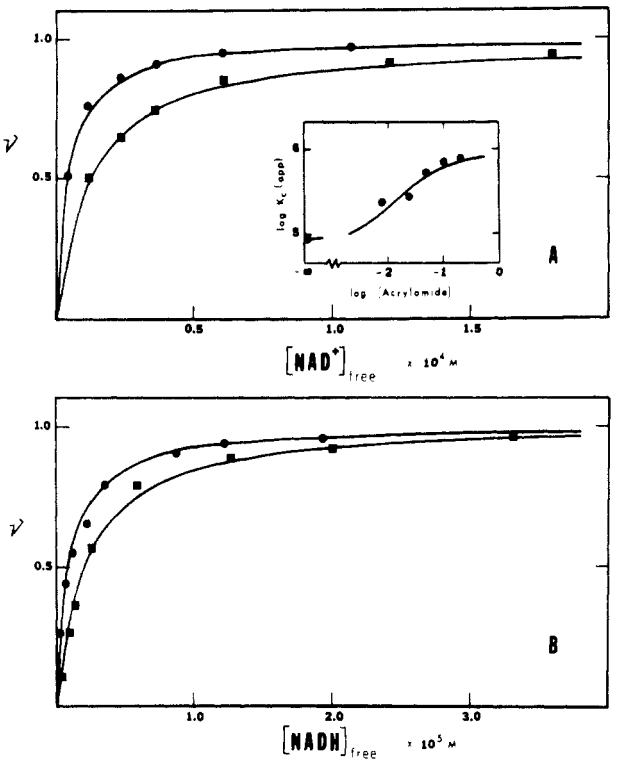

FIGURE 5: Binding isotherms for the interaction of NAD^+ (A) and NADH (B) with LADH. The circles represent binding studies in the presence of 0.008 M [in (A)] and 0.01 M [in (B)] acrylamide. All studies performed at pH 9.0 in 0.5 M bicine buffer. The binding constants determined for NAD^+ and NADH in the absence of acrylamide are 8.0×10^4 M^{-1} and 5.0×10^5 M^{-1}, respectively. [Inset in (A)] The dependence of the free-energy change for the binding of NAD^+ to LADH on the concentration of acrylamide. A fit of eq 4 to the data yields the following values: $K_C = 8.0 \times 10^4$ M^{-1}, $K_A = 16$ M^{-1}, and $K_{A,C} = 200$ M^{-1}.

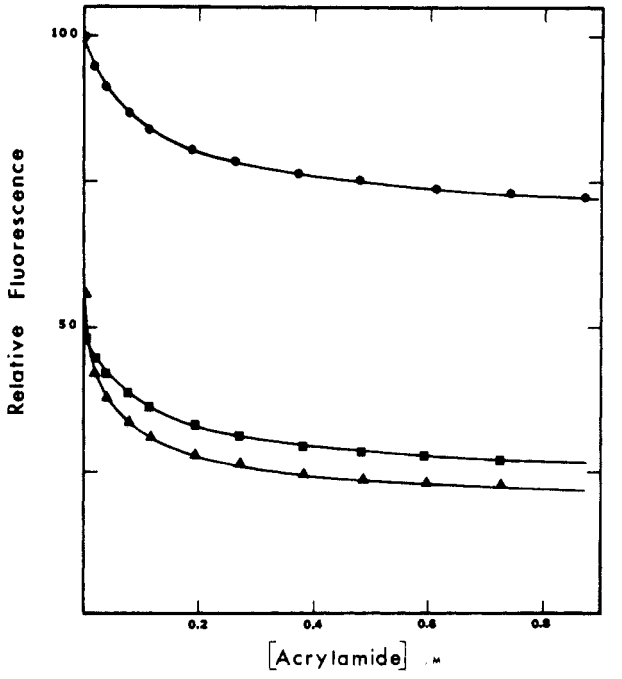

FIGURE 6: Relative fluorescence of LADH (●), the binary complex LADH–NAD^+ (▲), and the ternary complex (■), as a function of the concentration of added acrylamide.

likely, however, that their effect involves a modification of the mechanism by which the binding of NAD^+ quenching the protein's fluorescence.

On studying the acrylamide quenching of the LADH–NAD^+ binary complex, we found the initial K value to exceed the maximum expected value for dynamic quenching of a tryptophanyl residue in a globular protein and the F_0/F value to approach a plateau value of ~2.6 at [acrylamide] > 0.3 M. The large initial K suggests that quenching occurs through the binding of acrylamide to the LADH–NAD^+ binary complex.

This proposal is confirmed by studies showing acrylamide to be an inhibitor of the enzymatic reaction (see Figure 4)[3] and by studies that indicate that acrylamide binds to the protein with positive heterotropic cooperativity with respect to both NAD^+ and NADH.

The linked binding of acrylamide and coenzyme to the protein can be considered in terms of the following simple model, where A is acrylamide, E is LADH, and C is coenzyme.

$$\begin{array}{ccc} E + A + C & \overset{K_C}{\rightleftharpoons} & EC + A \\ \updownarrow K_A & & \updownarrow K_{A,C} \\ EA + C & \overset{K_{C,A}}{\rightleftharpoons} & ECA \end{array}$$

$$\log K_C(\text{app}) = \log K_C + \log\left(\frac{1 + K_{A,C}[A]}{1 + K_A[A]}\right) \quad (4)$$

The dependence of the apparent association constant for coenzyme on the concentration of acrylamide is given by eq 4. From the data presented in Figure 5, the association constant for NAD^+ is found to increase 10-fold in the presence of a saturating amount of acrylamide (free energy of interaction of −1.4 kcal/mol). Also from the data in Figure 5, $K_{A,C}$ the association constant for acrylamide to the LADH–NAD^+ binary complex, can be estimated to be approximately 200 M^{-1}, from which K_A would then be estimated to be ~16M^{-1}.

Although the formation of a ternary complex with NADH has not be studied as thoroughly, acrylamide, at a concentration of 0.01 M, has also been found to significantly increase the association constant for the reduced coenzyme.

The linked binding between acrylamide and the coenzymes is consistent with studies that show that several carboxylic acids, amides, mercaptans, and heterocyclic compounds as well as trifluoroethanol and hydroxylamine are capable of forming such ternary complexes (Winer & Theorell, 1960; Van Eys et al., 1958; Theorell & Yonetani, 1963; Kaplan et al., 1957). Isobutyramide, which is structurally quite similar to acrylamide, is often used to form a highly fluorescent (NADH fluorescence) ternary complex with NADH (Theorell & McKinney-McKee, 1961b). Most of the above ligands have been found to form a ternary complex with either NAD^+ or NADH. The finding that acrylamide forms a ternary complex with both states of the coenzyme makes this ligand somewhat unique. However, it should be noted that the free energy of coupling between acrylamide and NAD^+ (and presumably NADH also) is small, as compared to other ligands such as pyrazole or isobutyramide.

The fact that acrylamide forms a ternary complex with both the reduced and oxidized form of the coenzyme is further indicated by the enzyme inhibition patterns. Normally, the

[3] Although acrylamide can apparently bind weakly to LADH in the absence of coenzyme ($K_A \sim 16$ M^{-1}), there is no evidence to suggest that the formation of a LADH–acrylamide complex results in the quenching of the protein's fluorescence. Isobutyramide and trifluoroethanol both bind more strongly to the protein than does acrylamide, yet the addition of up to 0.2 M of these two ligands causes less than 1% quenching of the protein's fluorescence. Furthermore, the acrylamide quenching pattern is the same in the presence and absence of these two small ligands.

Table II: Model Describing LADH Fluorescence in Terms of Two Tryptophanyl Residues[a]

	Trp-15	Trp-314
emission λ_{max}	335 nm	323 nm
f_i at 323 nm, 295 λ_{ex}	0.28	0.72
τ[b]	7.2 ns	3.9 ns
k_q(acrylamide)	1.2×10^9 M^{-1} s^{-1}	0.01×10^9 M^{-1} s^{-1} [d]
$k_q(I^-)$	1.7×10^9 M^{-1} s^{-1}	~0
NAD^+ binding	quenches little[c]	quenches 55%
NAD^+ + TFE binding	quenches little[c]	quenches 66%
ionization of $pK_a = 9.8$ group	quenches little	quenches ~50%

[a] All values refer to an excitation wavelength of 295 nm and an emission wavelength of 323 nm as well as to the conditions of pH 9.0 and 25 °C. [b] Lifetime values taken from Ross et al. (1981) by interpolation from their values at other temperatures. M. R. Eftink and D. M. Jameson (unpublished results) have recently obtained similar lifetime values for the two residues using a cross-correlation phase fluorometer. [c] We estimate that the binding of NAD^+ plus TFE to LADH may result in the quenching of 15–20% of the fluorescence of Trp-15. A similar degree of quenching of Trp-15 probably also occurs when NAD^+ alone binds to the protein. See text for discussion. [d] The value for k_{314} should be taken only as an order of magnitude estimate.

rate of the LADH-catalyzed oxidation of ethanol is limited by the rate at which NADH is released from the enzyme. By binding to and thus stabilization of the LADH–NADH complex, acrylamide will further slow the rate of release of the coenzyme. This so-called "second phase" effect (Theorell & McKinney-McKee, 1961b) will result in a decrease in V_m. In addition, as demonstrated by the binding studies above, acrylamide interacts with the LADH–NAD^+ complex. If the binding of acrylamide to this complex competes with the binding of the substrate ethanol, first-phase competitive inhibition will also take place. As a result of both first- and second-phase effects, acrylamide will appear to be a noncompetitive inhibitor with respect to ethanol. From a similar set of arguments, the apparent uncompetitive (or mixed noncompetitive) inhibition found when [NAD^+] is varied (see Figure 4) can be interpreted as being due to second-phase inhibition combined with noncompetitive first-phase inhibition. The result is a kinetic pattern that appears to be uncompetitive inhibition. The acrylamide inhibition patterns for the reverse reaction, the reduction of acetaldehyde to ethanol, are similar to those for the forward reaction (compare Figure 4A,B with Figure 4C,D), consistent with the fact that acrylamide forms a ternary complex with LADH–NAD^+ as well as with LADH–NADH. While acrylamide is structurally similar to isobutyramide, the ability of the former to form a ternary complex with both states of the coenzyme leads to inhibition patterns that are different than those found by Theorell & McKinney-McKee (1961b) for isobutyramide.

The above studies demonstrate that in the presence of either NAD^+ or NADH, acrylamide binds to LADH, probably at approximately the same sight (to zinc metal ion) as does ethanol or acetaldehyde. No evidences is found, however, for the binding of acrylamide to the LADH–ADPR or LADH–NMN binary complexes.

The unusual acrylamide fluorescence quenching pattern for the LADH–NAD^+ complex must certainly be due, at least partially, to the ability of the quencher to bind to the protein. The following model, described by the information in Table II, is presented to account for the fluorescence quenching of the apoenzyme and the LADH–NAD^+ binary complex (all parameters for pH 9.0, 25 °C). The surface tryptophan, Trp-15, is proposed to have a λ_{max} of 335 nm and to contribute approximately 28% of the fluorescence (at 323 nm, excitation at 295 nm) of the apoenzyme. This residue is readily quenched by acrylamide ($k_q = 1.2 \times 10^9$ M^{-1} s^{-1}) and I^- ($k_q = 1.7 \times 10^9$ M^{-1} s^{-1}). The binding of NAD^+ has little effect on the fluorescence of this residue (Laws & Shore, 1978; Ross et al., 1981; see discussion below).

Trp-314, on the other hand, is extensively buried from the solvent, has a very small rate constant for acrylamide quenching, and has a fluorescence λ_{max} of 323 nm. Approximately 72% of the fluorescence of the apoenzyme (λ_{em} = 323 nm) is attributable to this residue. The binding of NAD^+ quenches approximately 55% of the fluorescence of Trp-314. The formation of the binary complex with NAD^+ thus leads to a red shift in the protein fluorescence (Laws & Shore, 1978), with the fluorescence of Trp-15 being relatively more pronounced in the complex. Secondary ligands, such as trifluoroethanol (or acrylamide; see further on), cause a small additional quenching of the fluorescence of Trp-314. The formation of the LADH–NAD^+–TFE ternary complex, for example, results in the quenching of approximately 66% of the fluorescence of Trp-314.

The acrylamide quenching of the apoenzyme is readily described by this model. So that the acrylamide quenching of the LADH–NAD^+ complex can be accounted for, the ability of acrylamide to participate in the formation of a ternary complex must be considered. If acrylamide mimics the effect of added trifluoroethanol, then in addition to the collisional and static quenching of Trp-15 and -314 (primarily the former residue), acrylamide also quenches an additional 5–10% of the protein's fluorescence as a result of the formation of a ternary complex, with part of the quenching being due to the increased saturation of NAD^+ binding sites in the presence of acrylamide. It should be pointed out that the addition of acrylamide may also lead to a small amount of quenching by promoting the binding to the protein of any NADH which happens to contaminate the solution. (As mentioned under Materials and Methods, a small amount of NAD^+ to NADH conversion was found to inevitably occur when the former is added to the enzyme.) Regardless, the addition of a relatively low concentration of acrylamide to the LADH–NAD^+ binary complex leads to the quenching of 5–10% of the protein's fluorescence as a result of the ability of acrylamide to interact to form a ternary complex.

For illustration of the way in which the above model describes the acrylamide quenching data, the data for the quenching of apoenzyme, binary, and ternary (TFE) complexes are replotted in Figure 6 on a relative scale. The addition of up to 0.8 M acrylamide results in the quenching of Trp-15 to the extent of 22–27 relative fluorescence units in each case. In addition, when acrylamide is added to the LADH–NAD^+ binary complex, an additional quenching of 5–10 units occurs due to the binding of acrylamide.

The quenching due to normal solute quenching by acrylamide, due to NAD^+ binding, and due to acrylamide binding all seem to be additive. Thus the acrylamide quenching patterns can be interpreted in a straightforward manner with the above model, despite the unusual shapes of the Stern–Volmer plots in Figure 3. A subtle feature of the above model is that a definite amount of fluorescence from Trp-314 remains even at high acrylamide concentration in the presence of NAD^+. The binding of coenzyme, therefore, does not lead to a significant increase in the exposure of Trp-314 to the solvent, as was mentioned as a possible consequence of NAD^+ binding (Wolfe et al., 1977). If an induced conformational

change in the protein did lead to the exposure of Trp-314, the fluorescence of the binary or ternary complexes would approach zero at high acrylamide concentration. The experiments with the LADH–ADPR and LADH–NMN binary complexes also indicate that such a conformational change is not induced by the binding of these ligands.

This study thus argues strongly against the first possibility mentioned in the introduction as an explanation for the mechanism of quenching of LADH fluorescence by the binding of NAD^+. Among the other possible explanations, Ross et al. (1981) have recently calculated that ~38% of the fluorescence of LADH can be quenched by radiationless energy transfer to bound NAD^+, with Trp-314 being selectively quenched due to its proximity to the coenzyme binding site. This amount of quenching corresponds to the drop in fluorescence lifetime they observed for Trp-314 on formation of a ternary LADH–NAD^+–pyrazole complex. Despite the difficulty in explaining the lack of energy-transfer quenching on the binding of ADPR to the protein (which may be due to a difference in the orientation of the adenine ring of the bound ligands), strong consideration must be given to this possible quenching mechanism.

As we discussed above, however, our acrylamide studies indicate that 66% of the fluorescence of Trp-314 is quenched on the formation of the LADH–NAD^+–TFE ternary complex [Laws & Shore (1978) have estimated an 80% quenching of Trp-314]. Thus while it is possible that energy transfer to NAD^+ accounts for some of the observed quenching, an additional quenching process appears to also take place involving either energy transfer to ionized tyrosine and/or a slight conformational reaarangement in the protein structure in the vicinity of Trp-314, bringing a quenching group into contact with the buried indole ring.

The question of whether the binding of NAD^+ induces the deprotonation of a tyrosine residue(s) has been investigated by UV difference spectroscopy. Laws & Shore (1979) have argued that the spectral change associated with forming the ternary (TFE) complex is due to the ionization of a tyrosine residue(s). More recently, Subramanian et al. (1981) have pointed out that the spectral changes can instead be due to perturbation of the absorption spectrum of the coenzyme. The latter workers also argued that their failure to observed tyrosinate phosphorescence by the ternary complex is inconsistent with the theory that the binding of NAD^+ induces a deprotonation of a tyrosine residue(s). The latter interpretation requires that the state of protonation of the tyrosine residue(s) remains unchanged at the low temperature employed for the phosphorescence measurements. Inspection of the crystallographic structure of LADH shows its four tyrosine residues to be solvent exposed (Eklund et al., 1976), making it difficult to visualize how the binding of coenzyme could be coupled to their deprotonation. In addition, recent studies of the degree of fluorescence quenching caused by NAD^+ binding as a function of pH makes the mechanism of energy transfer to ionized tyrosine seem less probable (J. Shore, personal communication).

Thus while mechanisms b, c, and d must still be considered as the basis for quenching due to NAD^+ binding, on balance the current evidence is, in our opinion, most consistent with mechanisms c (slight conformational change bringing quenching group closer to Trp-314) and d (energy transfer to bound NAD^+). For either of these mechamisms, it is easy to conceive that the binding of a secondary ligand such as TFE, acrylamide, or pyrazole could further affect the degree of quenching by either slightly altering the conformation of the protein or affecting the orientation of NAD^+ in the binding site.

Evidence is also provided by our acrylamide quenching studies that on formation of a ternary (TFE) complex that up to 15–20% of the fluorescence of Trp-15 may be quenched as well. This comes from the fact, as seen in Figure 6, that acrylamide (up to 0.8 M) can quench ~27 relative units of the apoenzyme but only 22 units of the LADH–NAD^+–TFE ternary complex. If, as we have argued above, acrylamide quenches primarily Trp-15, the above difference suggests that a small amount of Trp-15 fluorescence is quenched in forming the ternary complex. No drop in the lifetime of Trp 15 was observed by Ross et al. (1981) on ternary complex (with pyrazole) formation, however. The slight difference (27 vs. 22 units) may also be due to the fact that a small amount of acrylamide quenching of Trp-314 occurs for the apoenzyme ($K_{314} \sim 0.04\ M^{-1}$) but does not occur for the ternary complex. Further studies will be necessary to clarify this matter.

The present study demonstrates that solute quenchers such as acrylamide can, in some cases, interact specifically with a protein and that this can result in the quenching of the protein's fluorescence not only via the collisional quenching mechanism but also due to induced changes in the protein resulting from the binding of quencher. The possibility that quenchers may bind specifically to proteins must be kept in mind and experimentally tested. We have used acrylamide as a fluorescence quencher for a number of other proteins (Eftink & Ghiron, 1976), but only with LADH have we found acrylamide to significantly affect the catalytic or binding properties of the protein. Matko et al. (1980) have found acrylamide to be a noncompetitive inhibitor of phosphorylase *b*. They proposed that acrylamide inhibits this enzyme by binding in a nonspecific manner to cavities within the structure of this enzyme. In the present study with LADH, the fact that acrylamide binds with positive heterotropic cooperativity with respect to NAD^+ and NADH and that the inhibition patterns can be readily explained in terms of second-phase effects argues strongly that acrylamide binds in a specific fashion to the protein, thus lending no support to the model proposed by Matko et al. (1980) to explain the inhibitory effect of acrylamide in the case of phosphorylase *b*.

In conclusion, acrylamide quenching studies with LADH provide further support for the existence of two classes of tryptophanyl residues in this protein, with spectral characteristics as summarized in Table II. In the presence of NAD^+, acrylamide binds to the protein, leading to a small amount of quenching due to ternary complex formation. The formation of a binary (LADH–NAD^+) or ternary (LADH–NAD^+ plus trifluoroethanol or acrylamide) complex does not induce a conformation change that increases the exposure of Trp-314.

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Cation Binding Properties of the Multiple Subforms of RVV-X, the Coagulant Protein from *Vipera russelli*†

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ABSTRACT: The factor X activating enzyme from Russell's viper venom (RVV-X) has been shown to exist in multiple subforms, distinguished from each other by their isoelectric points. The differences in isoelectric points were due, at least in part, to dissimilarities in the respective sialic acid contents of the subforms. No functional difference was, however, discovered between any of the subforms. All of the subforms were found, by equilibrium ultrafiltration, to bind Ca^{2+} reversibly. At least two equivalent Ca^{2+} binding sites were observed on each protein molecule (M_r 79 000), with a K_D of 50 ± 15 μM at pH 7.4 and 25 °C. A new substrate for RVV-X, which does not bind Ca^{2+}, apoprotein AI from human high-density lipoprotein, was used to show that this reversibly bound Ca^{2+} was not essential for enzymic activity. All subforms have also been shown, by atomic absorption analysis, to contain nonexchangeable metal ions, to the extent of 1 mol of Ca^{2+} and 0.7 mol of Zn^{2+} per mol of protein. No Mn^{2+} or Mg^{2+} was detected. This nonexchangeable Ca^{2+} and Zn^{2+} could only be removed from the protein by incubation at pH 3.0 or by treatment with 6 M guanidine hydrochloride, conditions under which the protein lost activity irreversibly.

The venom from Russell's viper (RVV)[1] has long been known to possess a potent stimulatory effect toward blood coagulation (MacFarlane & Barnett, 1934). Williams & Esnouf (1962) purified a coagulant protein from RVV to apparent homogeneity, which they subsequently showed to be a potent activator of factor X (Esnouf & Williams, 1962): the protein with this activity is now designated RVV-X. Schiffman et al. (1969) showed that another coagulant protein could be separated from RVV, which functioned by stoichiometric acti-

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[1] Abbreviations: RVV, Russell's viper venom; RVV-X, the factor X activating enzyme from RVV; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; L-TAME, α-*N*-tosyl-L-arginine methyl ester; DEAE-cellulose, diethylaminoethylcellulose; QAE-Sephadex, quaternary aminoethyl-Sephadex; iPr_2PF, diisopropyl fluorophosphate.

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