Quenching of Protein Fluorescence by Transient Intermediates in the Liver Alcohol Dehydrogenase Reaction*

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SUMMARY

The addition of saturating concentrations of NAD+ and alcohol to liver alcohol dehydrogenase in a stopped flow fluorimeter results in a triphasic quenching of enzyme fluorescence. A rapid quenching occurs with a rate constant of 300 to 500 s⁻¹, followed by a slower reaction at 50 to 100 s⁻¹, and ultimately followed by a very slow reaction. The addition of NAD⁺ to enzyme in the absence of substrate causes a rapid quenching of enzyme fluorescence at 300 to 500 s⁻¹, with the same amplitude as the rapid phase in the presence of substrate. These studies demonstrate that NAD⁺ binding to liver alcohol dehydrogenase causes a conformational change at a rate compatible with the previously reported rate constant for proton release, indicating that proton release is probably coupled to the conformational change.

Previous studies using stopped flow spectrophotometry (1, 2) demonstrated that addition of saturating NAD⁺ and ethanol to liver alcohol dehydrogenase resulted in a transient formation of enzyme-bound NADH followed by steady state turnover. A substantial deuterium isotope effect on the transient phase indicated that the transient rate was at least partially limited by the hydride transfer with a rate constant of 130 s⁻¹ at pH 7.0 and 25°. A subsequent study (3) indicated that proton release preceded bound NADH formation, with a rate constant of 250 s⁻¹. Although the rates obtained were compatible with proton dissociation from a functional group on the enzyme, it was not possible to show whether the proton release was linked to a conformational change.

It has been established (4, 5) that the binding of NADH to liver alcohol dehydrogenase results in quenching of approximately 50% of the enzyme fluorescence. This quenching is probably due to singlet-singlet energy transfer from the tryptophan residues of the enzyme to the bound reduced coenzyme although some of the quenching may also be due to a conformational change resulting in an altered milieu of the tryptophan. It has also been reported (4, 5) that the binding of oxidized coenzyme results in quenching of approximately 20% of the fluorescence of liver alcohol dehydrogenase excited at 290 nm. The quenching by oxidized coenzyme could not be due to singlet-singlet energy transfer since the absorption peak of 290 nm precludes its acting as an acceptor. Since there are 2 tryptophan residues per subunit (6), collisional quenching would result in 50% of the fluorescence being quenched at saturating NAD⁺ if 1 of the 2 residues were at the active site, rather than the observed 20%. In support of this, the crystallographic structure of liver alcohol dehydrogenase indicates that tryptophan is not present at the coenzyme binding site (7). Since singlet-singlet energy transfer and Stern-Volmer mechanisms can be ruled out, the most probable cause for the quenching of liver alcohol dehydrogenase fluorescence by NAD⁺ is a conformational isomerization of the enzyme. Consequently, we decided to investigate the transient kinetics of liver alcohol dehydrogenase with ethanol and benzyl alcohol as substrates, monitoring the fluorescence of the enzyme. The liver alcohol dehydrogenase used in these studies was prepared by the method of Theorell et al. (8) and its concentration was determined by titration with NADH in the presence of isobutyramide (9). All experiments were performed at 20° in 0.1 M pH 7.0, phosphate buffer. NAD⁺ and NADH were products of Boehringer. Stopped flow spectrophotometry was performed on a split beam instrument, and stopped flow fluorimetry was performed on a ratio recording instrument. Both instruments were constructed at the University of Bristol and will be described in subsequent publications. The rate constants for transient production of bound NADH were calculated by the method of Guggenheim as explained in Ref. 10.

The results of addition of saturating NAD⁺ and ethanol to liver alcohol dehydrogenase at 20° in pH 7.0 phosphate buffer are presented in Fig. 1. The upper trace shows the change in absorbance at 325 nm resulting from the formation of enzyme-bound NADH followed by turnover of the enzyme. The calculated rate constant for the exponential phase was 70 s⁻¹. The lower trace shows the same reaction with a lower enzyme concentration, monitored by the quenching of enzyme fluorescence. This reaction is triphasic, with an initial rapid quenching with a rate constant of 300 to 500 s⁻¹, followed by a slower reaction with a rate constant approximating that of the spectrophotometric transient, and then by a very slow process. The slow phase corresponds to filling of the remaining sites on the enzyme by NADH produced during turnover. This is a slow process since the high NAD⁺ concentration in the reaction mixture results in a higher apparent dissociation constant for NADH.

Since the rate constant for hydride transfer is more than 1 order of magnitude faster than NADH dissociation, it might be expected that all of the enzyme would be in the form of a binary complex with NADH at the conclusion of the hydride transfer step and during the subsequent turnover. However, the balance between the rate constants for the reverse hydride transfer reaction and dissociation of aldehyde product determines the fraction of the enzyme remaining in the form of enzyme/NAD⁺/alcohol complex during turnover. Since the slow phase of the fluorescence quenching reaction accounts for 20 to 25% of the total quenching, it is reasonable to assume that this represents the fraction of enzyme in the form of a ternary complex with NAD⁺ and alcohol during turnover.

Similar experiments were performed with benzyl alcohol, which
gives a slower transient for the formation of enzyme-bound NADH, and the results are shown in Fig. 2. The upper trace demonstrates the transient bound NADH formation and subsequent steady state turnover measured spectrophotometrically at 325 nm (upper trace) and by protein fluorescence quenching (lower trace). Syringe 1, 50 mM benzyl alcohol and 2.1 mM NAD⁻; syringe 2, 29 µM enzyme (upper trace) and 4 µM enzyme (lower trace); pH 7.0, 0.1 mM sodium phosphate buffer, 20°, 0.1 ms time constant.

In order to clarify the origin of the initial rapid phase of the fluorescence quenching in Figs. 1 and 2, an additional experiment was performed. The rate of quenching of enzyme fluorescence due to addition of saturating concentrations of NAD⁺ was determined. Since the second order rate constant for NAD⁺ binding under our experimental conditions is 10⁸ M⁻¹ s⁻¹ (12), the pseudo-first order rate constant with 2 mM NAD⁺ should be 2000 s⁻¹, well beyond the resolution of our instrument. Fig. 3 shows the rate of quenching of enzyme fluorescence due to adding 2 mM NAD⁺, with a rate constant of 300 to 500 s⁻¹. This experiment provides an explanation for the rapid initial quenching observed in the experiments of Figs. 1 and 2 and indicates that this step represents a conformational isomerization of enzyme due to NAD⁺ binding. Furthermore, the rate constant for this isomerization is approximately equal to the rate constant for proton release due to NAD⁺ binding (3). Since proton release is due to perturbation of the pKᵣ of a functional group on the enzyme, it seems likely that this perturbation is linked to a conformational change.

The use of protein fluorescence quenching and stopped flow kinetics in these studies has demonstrated detection of at least one conformational isomerization during transient phases of the liver alcohol dehydrogenase reaction. Further studies, using alternate substrates, inhibitors, and modified reaction conditions, should enable characterization of these intermediates.

REFERENCES

Quenching of protein fluorescence by transient intermediates in the liver alcohol dehydrogenase reaction.
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