The Rates of Binding of Reduced Nicotinamide–Adenine Dinucleotide Analogs to Liver Alcohol Dehydrogenase*

Joseph D. Shore

ABSTRACT: The kinetics of the binding of reduced nicotinamide–adenine dinucleotide, reduced deamino nicotinamide–adenine dinucleotide, and reduced 3-acetylpyridine–adenine dinucleotide to liver alcohol dehydrogenase have been studied. The "on" and "off" velocities of the two analogs were compared with the values for reduced nicotinamide–adenine dinucleotide. The "on" velocity for reduced deamino nicotinamide–adenine dinucleotide was much slower while its "off" velocity was the same as for reduced nicotinamide–adenine dinucleotide. The "on" velocity of reduced 3-acetylpyridine–adenine dinucleotide was the same as for reduced nicotinamide–adenine dinucleotide but its "off" velocity was much faster. This indicates that the adenine ring is not bound subsequent to the nicotinamide ring and that the amide group binding is independent of the spectral shift due to pyridine ring binding. The possible sequential nature of the binding reaction and its mechanistic implications are discussed.

It has been demonstrated (Yonetani and Theorell, 1964; Yonetani, 1963) that NADH is bound to horse liver alcohol dehydrogenase (EC 1.1.1.1) by at least two points of attachment, since *o*-phenanthroline and adenosine diphosphoribose, inhibitors competitive with NADH, could be bound to horse liver alcohol dehydrogenase simultaneously and independently. It was recently suggested (Theorell *et al.*, 1967) that the binding reaction was a two-step process, with the adenosine diphosphoribose moiety bound before the nicotinamide ring. A subsequent study (Geraci and Gibson, 1967) demonstrated that no time lag occurred in the rate of binding of the nicotinamide ring of NADH to horse liver alcohol dehydrogenase, indicating that it is not possible to distinguish experimentally between simultaneous and separate binding of both rings of NADH using the stopped-flow technique.

Since there are at least two points of attachment of the NADH to horse liver alcohol dehydrogenase, it is not unreasonable to assume that the binding reaction is two step but that the second step is so fast that no difference would be seen by stopped-flow methods. Another possibility would be that the adenine ring is bound subsequent to the nicotinamide ring. In order to investigate these possibilities, the rates of binding of two analogs of NADH were studied: deamino-NADH in which the adenine ring is modified, and 3-APADH in which the nicotinamide ring has been changed. Both analogs are active with horse liver alcohol dehydrogenase but bound much less tightly. It therefore seemed probable that the "on" and "off" velocities of the analogs and the possible appearance of a lag time would provide some information regarding the mechanism of the binding reaction.

Experimental Procedure

Crystalline horse liver alcohol dehydrogenase was prepared from horse liver by the method of Theorell *et al.* (1966). The concentration of horse liver alcohol dehydrogenase was determined by the assay method of Dalziel (1957) using an A_{280} of 0.455 ml/(mg cm^{-1}) for pure enzyme, and by titration with NADH in the presence of isobutyramide (Theorell and McKinley-McKee, 1961). Both methods gave comparable results. NADH and 1,10-phenanthroline were obtained from Sigma Chemical Co. and 3-APADH and deNADH were obtained from P-L Biochemicals, Inc.

Double-difference spectrophotometry (Theorell and Yonetani, 1964) of the binary complexes was performed on a Cary Model 11 spectrophotometer, using 1.0-cm cuvets in tandem with the 0–0.1 optical density scale. All spectra were in 0.1 μ phosphate buffer (pH 7.0). Dissociation constants of the binary complexes were determined at pH 7.0 by the method of Theorell and Winer (1959) using a Farrand spectrophotofluorometer. The results were calculated using an IBM 360 computer.

Stopped-flow spectrophotometry was performed using a Durrum-Gibson instrument at the wavelength of the trough due to the shift in absorbance caused by binding of the nicotinamide ring. A 1.7-mm slit was used and all experiments were performed at 8°, using a time constant of 0.1 msec for the "on" velocity determinations and 0.1- or 1.0-msec time constants for the "off" velocity determinations. All solutions were in 0.1 μ phosphate buffer (pH 7.0). The dead time of the instrument was 3 msec. The "on" velocity, k_1, was calculated from half-times using eq 1 for a reversible second-order reaction with equal concentrations of reactants (Laidler, 1958).

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TABLE I: Rate and Dissociation Constants for the Binding of NADH and NADH Analogs to Horse Liver Alcohol Dehydrogenase.

	k_1 (μM^{-1} sec^{-1})	k_{-1} (sec^{-1})	(k_{-1}/k_1) (μM)	K_D (μM)
NADH	6.9	1.7	0.25	0.33
Reduced deamino nicotinamide-adenine dinucleotide	1.3	2.6	2.0	3.1
Reduced 3-acetylpyridine-adenine dinucleotide	5.6	34.8	6.2	5.4

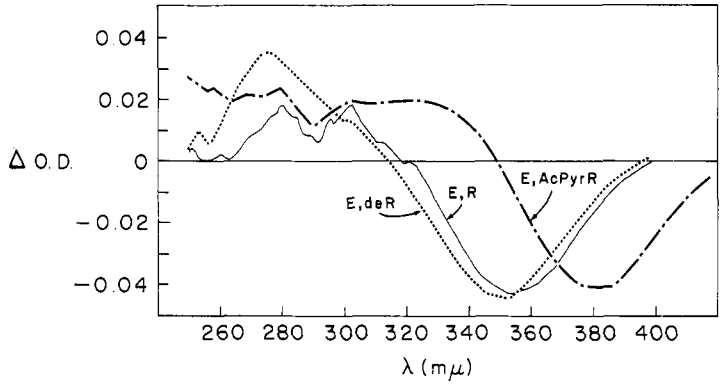

FIGURE 1: Double-difference spectra for binary complexes of horse liver alcohol dehydrogenase and NADH analogs. (——) 15 μN horse liver alcohol dehydrogenase and 15 μN NADH; (···) 15 μN horse liver alcohol dehydrogenase and 35 μN reduced deamino nicotinamide-adenine dinucleotide; (—·—·—) 15 μN horse liver alcohol dehydrogenase and 22 μN reduced 3-acetylpyridine-adenine dinucleotide.

$$k_1 = \frac{(ER)_{equil}}{t[(E)^2 - (ER)^2_{equil}]} \times \ln\left(\frac{(ER)_{equil}[(E)^2 - (ER)(ER)_{equil}]}{(E)^2[(ER)_{equil} - (ER)]}\right) \quad (1)$$

The k_{-1} values were determined by adding 1,10-phenanthroline to the binary complexes and following the decrease in % T at the trough wavelengths. The rate of binding of 1,10-phenanthroline to free horse liver alcohol dehydrogenase was measured at 297 mμ and the rate constant was 9×10^{-3} μM^{-1} sec^{-1}, which results in a psuedo-first-order rate constant of 59 sec^{-1} at 6.6 mM 1,10-phenanthroline. This rate was apparently sufficient to result in competitive displacement of enzyme from the enzyme-coenzyme complex since the half-times for the dissociation of the enzyme-coenzyme complexes were the same at 5.0 and 6.6 mM *o*-phenanthroline. The half-times of the enzyme-coenzyme dissociation reactions in the presence of 6.6 mM *o*-phenanthroline were used to determine k_{-1} for each analog.

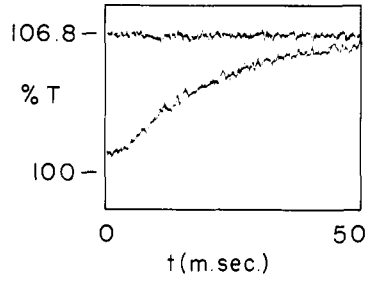

FIGURE 2: Measurement of the rate of binding of NADH to horse liver alcohol dehydrogenase at 355 mμ. The concentrations of NADH and horse liver alcohol dehydrogenase after mixing were 8.0 μN and the cuvet path length was 2.0 cm.

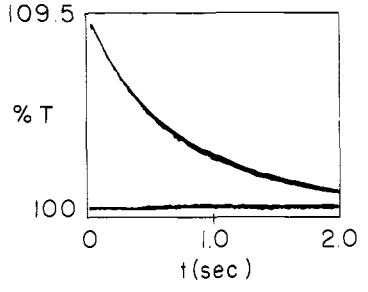

FIGURE 3: Measurement of the rate of dissociation of NADH from the liver alcohol dehydrogenase-NADH complex by addition of 1,10-phenanthroline. The concentrations after mixing were: horse liver alcohol dehydrogenase 10.4 μN and NADH 8.1 μN; 1,10-phenanthroline 6.6 mM.

Results

The double-difference spectra for binary complexes of horse liver alcohol dehydrogenase with NADH, deamino-NADH, and reduced 3-acetylpyridine-adenine dinucleotide can be seen in Figure 1. The wavelengths chosen for stopped-flow studies were therefore 355 mμ for NADH and deNADH and 380 mμ for 3-APADH.

Figure 2 shows a typical example of a curve used for measuring k_1, the rate of binding of coenzyme to horse liver alcohol dehydrogenase. The slight lag seen at the beginning of the reaction represents the lag between activation of the oscilloscope and the stop of flow. The deviation of the lag time level from 100% T is due to the instrument dead time. No true lag time was observed in any of the binding reactions with NADH or the analogs. An example of measurement of k_{-1}, the "off" velocity, can be seen in Figure 3.

The results of determinations of k_1 and k_{-1} for NADH and the two analogs are presented in Table I. The values for binary complex dissociation constants, K_D, determined independently by spectrophotofluorometric titration, are also listed. These values can be compared with the k_{-1}/k_1 ratios obtained from stopped-flow kinetics.

A comparison of the values for NADH and deNADH shows that the k_{-1} values are of the same order of magnitude and that the higher dissociation constant for the horse liver alcohol dehydrogenase–deNADH complex is due to a greatly diminished "on" velocity. The studies with 3-APADH, however, indicate the opposite effect. In this case, the k_1 values are of the same order of magnitude and the k_{-1} is very much higher, which accounts for the higher dissociation constant.

Discussion

The general validity of the velocity constants can be estimated from the correlation between the K_D values and k_{-1}/k_1 ratios. Considering the possible errors in both stopped-flow measurements and the fluorometric dissociation constant determinations, the correlation is as close as could be expected. If it were possible to form a horse liver alcohol dehydrogenase–NADH–*o*-phenanthroline complex in which only the adenosine diphosphoribose moiety of the NADH is bound, one would expect a much larger k_{-1} value for NADH by the method used, but this did not occur. Since adenosine diphosphoribose is very much less tightly bound than NADH, it is probable that once the nicotinamide ring is displaced the adenosine diphosphoribose part of the molecule also dissociates. The value of 6.9 μM^{-1} sec^{-1} for k_1 with NADH at 8° is within the expected range since values of 5.0 at 3° (Geraci and Gibson, 1967) and 17 at 23.5° (Theorell *et al.*, 1967) have been reported.

The data obtained with the deNADH indicate that the k_{-1} value is the same as for NADH, and that only k_1, the "on" velocity, is changed. Since in our velocity measurements we are measuring the rate of the spectral shift of the nicotinamide ring upon binding, this rate would not be affected by modification of the adenine ring if the nicotinamide ring were bound first. Therefore, it can be concluded that the nicotinamide ring is not bound prior to the adenine ring. There are two other possibilities: that both rings are bound simultaneously, or that the adenine is bound first. Considering the separate binding sites, the assumption that both rings are not bound exactly simultaneously would seem reasonable and it is probable that the nicotinamide ring is bound very soon after the adenine ring.

The results obtained with the 3-APADH were the reverse of those with deNADH. In this case k_1, the "on" velocity, was the same as with NADH, and only k_{-1}, the "off" velocity, was extensively changed. If the binding of the amide group was responsible for the spectral shift which occurs when NADH is bound to horse liver alcohol dehydrogenase, it could be expected that changing this group to an acetyl group would modify the "on" velocity of the coenzyme. This did not happen, which provides evidence that the functional group is bound independently and not prior to the pyridine ring and that some other interaction of the pyridine ring is responsible for the spectral shift. A feasible explanation would be that the amide group is bound subsequent to the pyridine ring and that replacement of it with an acetyl group results in an increased "off" velocity of the pyridine ring. The binding of the amide group would poise the coenzyme for the next step, the hydride transfer. This would be supported by the steady-state kinetics with 3-APADH, which demonstrated that the reciprocal ϕ_2 value was much slower than with NADH (Shore and Theorell, 1967).

The approach used in this study is applicable to all enzymes for which catalytically active substrate analogs are available. From a knowledge of K_m, V_{max}, and k_1 determined by presteady-state kinetics (Gutfreund, 1955) it should be possible to determine the "on" and "off" velocity of each analog. This would provide evidence regarding the mechanism of substrate binding.

References

Dalziel, K. (1957), *Acta Chem. Scand. 11*, 397.

Geraci, G., and Gibson, Q. H. (1967), *J. Biol. Chem. 242*, 4275.

Gutfreund, H. (1955), *Disc. Faraday Soc. 20*, 167.

Laidler, K. (1958), *in* The Chemical Kinetics of Enzyme Action, London, Oxford University, p 28.

Shore, J. D., and Theorell, H. (1967), *European J. Biochem. 2*, 32.

Theorell, H., Ehrenberg, A., and de Zalinski, C. (1967), *Biochem. Biophys. Res. Commun. 27*, 309.

Theorell, H., and McKinley-McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1811.

Theorell, H., Taniguchi, S., Åkeson, Å., and Skurský, L. (1966), *Biochem. Biophys. Res. Commun. 24*, 603.

Theorell, H., and Winer, A. (1959), *Arch. Biochem. Biophys. 83*, 291.

Theorell, H., and Yonetani, T. (1964), *Arch. Biochem. Biophys. 106*, 252.

Yonetani, T. (1963), *Biochem. Z. 338*, 300.

Yonetani, T., and Theorell, H. (1964), *Arch. Biochem. Biophys. 106*, 243.