Acceleration of the NAD–Cyanide Adduct Reaction by Lactate Dehydrogenase: The Equilibrium Binding Effect as a Measure of the Activation of Bound NAD†

John W. Burgner, II,* and William J. Ray, Jr.

ABSTRACT: The binary complex of NAD and lactate dehydrogenase reacts reversibly with cyanide to produce a complex (E·NAD–CN) whose noncovalent interactions are similar to those in the E·NADH complex (where E is one-fourth of the tetrameric dehydrogenase). The reaction apparently is a simple bimolecular nucleophilic addition at the 4 position of the bound nicotinamide ring; viz., cyanide does not bind to the enzyme prior to reaction. The value of the dissociation constant for E·NAD–CN is about 1×10^{-6} M and is independent of pH over the range of 6–8. The equilibrium constant for the reaction of cyanide with E·NAD is about 400-fold larger than that for the nonenzymic process after a statistical correction. This increment in K_e is accounted for by a 220-fold increase in the rate of the forward enzymic reaction (20 M^{-1} s^{-1}) as compared with an approximately 2-fold decrease for the reverse process (9×10^{-5} s^{-1}). Thus, the increased value of the rate constant for bond formation in the enzymic reaction is attributed to an equilibrium binding effect that is translated almost entirely into a rate effect on that step (bond formation). Since the nonenzymic reaction is sensitive to solvent composition, this equilibrium binding effect likely is produced by environmental effects at the nicotinamide/dehydronicotinamide part of the coenzyme binding site on the enzyme.

Much of the catalytic power of enzymes arises from noncovalent interactions that facilitate changes in covalent bonding, as is reflected in the concept of transition-state binding (Wolfenden, 1972, 1978; Lienhard, 1973), and a variety of possible ways for using noncovalent interactions to promote changes in covalent bonding have been discussed [cf. Jencks (1975, 1980)]. Because representations of noncovalent interactions, either pictorially or by means of molecular models, do not provide even a semiquantitative insight into their strengths, an assessment of the relative importance of various types of noncovalent interactions in a given enzyme-catalyzed reaction is exceedingly difficult, even when the structure of the enzyme is available from crystallographic data. For example, formulating a *quantitative* rationale for the known difference in binding of NAD[1] and NADH to LDH in terms of the structure of the enzyme is difficult, even though the oil–water–histidine model (Shore et al., 1975; Parker & Holbrook, 1977) provides a reasonable qualitative explanation. However, in some cases, semiquantitative information about the importance of various types of noncovalent interactions that occur during the catalytic step can be obtained by evaluating the interactions of an enzyme with component parts of its substrate [cf. Ray & Long (1976) and Moore & Jencks (1982)]. An initial assumption in this approach is that the overall effect of noncovalent interactions is the sum of simpler, discrete effects such as reactant immobilization, environmental effects, etc., although in the final analysis a linear combination of simpler effects may not suffice; e.g., the extent of reactant immobilization may depend on the size of the environmental effects and etc. In fact, this paper is the first of three papers that probe the question of the extent that additive effects can provide a rationale for the bond making/breaking efficiency of the LDH reaction [cf. Burgner & Ray (1983b)] and that describe the extent that noncovalent interactions between LDH and bound NAD alter the suceptibility of the nicotinamide ring toward nucleophilic attack at the 4 position. To obtain at least a minimal estimate of the size of this effect, a nonbinding nucleophile, cyanide, is used to assess the reactivities of free NAD and enzyme-bound NAD. In addition, the enzymic binding of the reactant, NAD, and the product, the NAD–CN adduct, is compared; the effect of the enzyme on the rate of decomposition of the bound NAD–CN adduct also is determined.

Materials and Methods

Materials. "Grade III" lithium lactate, sodium pyruvate, and 1-methylnicotinamide chloride (Sigma) and "chromatopure" NAD and NADH (P-L Biochemicals) were used without further purification. The best available grades of other chemicals were used. Solutions of substrates and reactants were prepared and adjusted to the designated pH just before use.

Enzymes. Dogfish M_4 LDH was prepared by methods described previously (Burgner & Ray, 1974, 1978); pig H_4 LDH originally was purchased from Sigma but more recently was prepared by the following procedure. Fresh or frozen pig hearts were extracted and the extracts passed through an oxamate–Sepharose affinity column (1 × 10 cm) according to the procedure of O'Carra & Barry (1972). The column was washed first with 50 mL of 0.05 M sodium phosphate, pH 7.0, containing 0.5 M NaCl and 0.2 mM NADH and then with 100 mL of the same phosphate buffer containing 0.2 mM NADH and 10 mM potassium oxamate. The eluted enzyme

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received November 9, 1983.* This investigation was supported by a research grant from the National Science Foundation (PCM8012576).

[1] Abbreviations: NAD, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase (either the dogfish A_4 or the pig B_4 isozymes); E, one-fourth of the LDH tetramer; E·X, a complex of LDH with X; Pyr, pyruvate; NAD–CN, the cyanide adduct of NAD, viz., the product of the covalent addition of cyanide at the 4 position of the nicotinamide ring of NAD; K_X, the dissociation constant of the E·X complex; $K_{eq,x}$, $k_{f,x}$, and $k_{r,x}$, the equilibrium constant and rate constants for the forward and reverse directions, respectively, for the process identified by x, where x is e for enzymic reaction and ne for nonenzymic reaction. The formal charge is omitted from NAD and H.

0006-2960/84/0423-3620$01.50/0

was treated with an amount of pyruvate equivalent to twice the NADH present, rapidly diluted 3-fold, and immediately placed on a (Whatman) DE-52 column, 1 × 5 cm, which had been equilibrated with 0.05 M sodium phosphate, pH 7.0. After the column was washed with 100 mL more of the equilibrating buffer, the enzyme was eluted with a gradient of 0–0.3 M NaCl in 0.5 M sodium phosphate, pH 7.0. The enzyme was concentrated by ultrafiltration against the appropriate buffer and either was used immediately or was precipitated with $(NH_4)_2SO_4$ for storage (at 4 °C). The stored enzyme was readied for use by procedures described by Burgner & Ray (1978).

Syntheses. 1-Methyl-1,4-dihydronicotinamide was prepared by the method of Karrer & Blumer (1947); its identity was verified and its purity checked by NMR spectroscopy. The cyanide adducts of NAD or 1-methylnicotinamide were prepared by dissolving the reactant in 0.1 M KCN, in either H_2O or methanol. (A total of 2 NAD equiv of NaOH was added in the case of the coenzyme.)

Methods. The rate and equilibrium point for both the enzymic and nonenzymic reactions of NAD and cyanide were measured at 327 nm in a Model 575 Perkin-Elmer spectrophotometer equipped with an electronic temperature controller. The reactions typically were initiated by addition of a small aliquant of 1 M potassium cyanide. (Cyanide solutions, especially after they were partially neutralized, were kept on ice and discarded after a few hours.) The temperature was maintained at 15 °C except where indicated, and pH values for the various reaction mixtures were measured at this temperature. The reverse, nonenzymic reaction was initiated by diluting the above reaction mixture by 100-fold (initial pH 10.5) into a solution containing 0.3 M imidazole chloride, pH 7.0; the disappearance of the absorbance at 327 nm was followed. The reverse enzymic reaction was examined after either (a) addition of a 2-fold excess of NAD–CN to the enzyme at pH 7.0 or (b) removal of excess reactants (NAD and cyanide) and unbound product (NAD–CN) from an equilibrium mixture by rapid molecular sieving at 4 °C (Penefsky, 1977). In the latter procedure, small Plexiglas tubes (0.5 × 6 cm) with a nylon screen glued to the bottom were filled with a Bio-Gel P-6 (50–150 mesh) slurry and preequilibrated with the reaction buffer by centrifugation at full speed in a clinical centrifuge (International Model CL) for 2 min at 4 °C. The sample (0.5 mL) was added to the top of the gel, which again was centrifuged as above but for 1 min. The effluent was added immediately to a spectrophotometer cuvette precooled to 15 °C. In either case, the disappearance of the E·NAD–CN complex was followed by repeatedly scanning through its absorbance maximum at 327 nm. In both cases, the concentration of enzyme (subunits) was maintained at a value at least 100 times greater than that of the dissociation constant for E·NAD–CN.

The binding of NAD–CN to the enzyme was quantitated by measuring the quenching of enzymic fluorescence as a function of added NAD–CN, by procedures described previously (Burgner & Ray, 1978). The cyanide adduct was prepared (see above) so that its final concentration was about 10^4-fold greater than that of the enzymic subunits, about 0.1 μN, and a given enzyme solution was used for only two additions of the adduct. Immediately prior to the addition, an aliquant of the stock adduct was diluted in ice-cold water so that 1–5 μL would give the desired concentration when added to the 1-mL assay mixture. Corrections of less than 10% for inner filter effects at high adduct concentrations ($>5 \times 10^{-6}$ M) were obtained by titrating a protein that does not bind the adduct in this concentration range: rabbit muscle phosphoglucomutase.[2]

Table I: Values for the Kinetic and Equilibrium Constants in Scheme I[a]

constant	nonenzymic	pig B_4	dogfish M_4
$k_{f,ne}$ (M^{-1} s^{-1})	4.5×10^{-2} [b]		
$k_{r,ne}$ (s^{-1})	1.9×10^{-4}		
$K_{eq,ne}$ (M^{-1})	200 (240)[b,c]		
$k_{f,e}$ (M^{-1} s^{-1})		9.7	6.9
$k_{r,e}$ (s^{-1})		9×10^{-5}	1.4×10^{-4} [e]
$K_{eq,e}$ (M^{-1})		1×10^{5} [c]	5×10^{-4} [e]
K_{NAD} (M)		3×10^{-4} [d]	1.0×10^{-3} [d]
K_{NAD-CN} (M)		1×10^{-6}	8×10^{-6}

[a] Values obtained from the plots in Figures 1–3 (see also Results). [b] Statistically corrected—see Results. [c] Value in parentheses is obtained from k_f/k_r; the other value is from direct measurement. [d] From Burgner & Ray (1978). [e] Calculated from product of equilibrium constants in Scheme I.

Results

Nonenzymic Reaction of NAD and Cyanide. The nonenzymic reaction of NAD and cyanide is a bimolecular process involving nucleophilic attack by the anion at the 4 position of the nicotinamide ring of NAD (Wallenfels & Schuly, 1959); both A- and B-side addition to the ring occur (Arnold & Kaplan, 1974). Since evidence presented below indicates that cyanide attacks only one side of NAD in the E·NAD complex, an allowance is made for this difference when comparisons between the enzymic and nonenzymic reactions are made. This allowance is made by multiplying the measured values of the forward rate constant for the nonenzymic reaction, $k_{f,ne}$, as well as the overall equilibrium constant for the nonenzymic reaction by a factor of 0.5. All values that are so altered are refered to as "statistically corrected".

Under conditions similar to those used previously for the addition of pyruvate to E·NAD (Burgner & Ray, 1978), conversion of E·NAD to the corresponding cyanide adduct complex is incomplete, even in the presence of excess cyanide where the appearance of product is first order in E·NAD. Only at pH values greater than the pK_a for HCN (9.5; Izate et al., 1962) can complete conversion to products be observed at the concentrations of cyanide employed in these studies. Equation 1 describes the relationships among the observed first-order

$$k_{obsd} = \frac{k_{f,ne}[CN_T]}{1 + [H]/K_a} + k_{r,ne} \quad (1)$$

rate constant, k_{obsd}, for approach to equilibrium, the true bimolecular rate constant for the forward reaction, $k_{f,ne}$, and the corresponding first-order constant for the reverse reaction, $k_{r,ne}$. Here, $[CN_T] = [CN^-] + [HCN]$, and K_a is the ionization constant for HCN. Estimates for both $k_{f,ne}$ and $k_{r,ne}$ are obtained from linear plots (not shown) of k_{obsd} against $[CN_T]$ and appear in Table I. A more precise estimate of $k_{r,ne}$ was obtained by converting an equimolar mixture of NAD and CN^- entirely to NAD–CN at high pH (11.0) and subsequently following the reverse of the adduct reaction after lowering the pH to 7. The disappearance of NAD–CN under these conditions is a first-order process: $k_{r,ne} = 2 \times 10^{-4}$ s^{-1}. This value agrees with that obtained from studies on the forward reaction, 1.9×10^{-4} s^{-1} (Table I).

[2] In such an experiment, the decrease in protein fluorescence with increasing adduct concentration is caused by inner filter effects, since NAD and NADH do not bind at the active site of phosphoglucomutase in the 10–20 μM concentration range (W. J. Ray, Jr., unpublished results).

Table II: Environmental Effects on the Cyanide Adduct Reaction[a]

substrate	medium	k_f (M^{-1} s^{-1})	k_r (s^{-1})	K_{eq}[b]
NAD	pig B_4 LDH	9.7	9×10^{-5}	10^5
NAD	dogfish A_4 LDH	6.9	1×10^4	5×10^4[c]
NAD	H_2O	0.045	2×10^{-4}	200[c]
NAD	CH_3OH	0.43	1.2×10^{-4}	3500
1-methylnicotinamide	H_2O	0.02	1.2×10^{-2}	2
1-methylnicotinamide	CH_3OH	0.3	6×10^{-4}	500

[a] Values obtained as described under Results. [b] Calculated from k_f/k_r, except as noted, and statistically corrected where necessary (see Results). [c] Measured directly.

The statistically corrected equilibrium constant for the cyanide reaction, K_{eq}, is defined as [NAD–CN]/(2[NAD]-[CN_T]) and is evaluated both from the ratio $k_{f,ne}/k_{r,ne}$ (240 M^{-1}) and by direct measurement of the amount of NAD–CN remaining at equilibrium (200 M^{-1}). Several values for the statistically corrected equilibrium constant in methanol also are given in Table II. Both the values of rate and equilibrium constants as well as the thermodynamic parameters given below are in substantial agreement with those obtained elsewhere under similar conditions (Gerlach et al., 1965; Lindquist & Cordes, 1968; Baumrucker et al., 1972; Okubo & Ise, 1974) and will be compared in a subsequent section with the corresponding values for the enzymic process.

The effect of temperature on the rate and equilibrium constants for the cyanide addition reaction also was investigated by means of the techniques described above. In these experiments, the change in concentration of CN^- produced by small temperature-dependent variations in the reaction pH was taken into account after interpolation of data on the pK_a of HCN from Izatt et al. (1962) to the appropriate temperature. In Figure 1, plots of log K_{eq}, log $k_{f,ne}$, or log $k_{r,ne}$ against $1/T$ are linear over the temperature range 15–40 °C. From the slopes of the lines, $\Delta H°$ is −10 kcal/mol, and $\Delta H^‡$ for the forward and reverse reactions is 12 kcal/mol and 22 kcal/mol, respectively.

Enzymic Reaction of NAD and Cyanide. The rate of the enzyme-catalyzed reaction between bound coenzyme and cyanide was measured under conditions similar to those used for the nonenzymic reaction. Under these conditions, neither NAD nor the NAD–CN adduct were bound stoichiometrically to the enzyme, and rapid equilibration between both bound and unbound reactants and product occurred (see below). The problem of measuring the rate constant for the enzymic reaction in the absence of stoichiometric binding and in the presence of the slower, competing nonenzymic process was minimized by measuring the *initial* velocity of the adduct reaction as a function of enzyme concentration at [E_T] ≪ [CN^-] and [NAD]. Under these conditions, the overall rate equation is

$$\lim (dp/dt)_{t\to 0} = k_{f,ne}[NAD][CN_T] + k_{f,e}[E_T][CN_T][NAD]/(K_{NAD} + [NAD]) \quad (2)$$

The plots of initial velocities in Figure 2 for the cyanide adduct reaction with both pig heart and dogfish muscle LDH are linear functions of the total enzyme concentration, as expected from eq 2, and the slope and intercept values of the plots for both enzymes were estimated by linear least-squares analysis. The estimates of $k_{f,e}$ and $k_{f,ne}$ for both enzymes in Table I were calculated from these slope and intercept values plus the appropriate value of K_{NAD} for the two enzymes in this table. The statistically corrected value obtained for $k_{f,ne}$, 0.05 M^{-1} s^{-1}, is in good agreement with that obtained by direct measure-

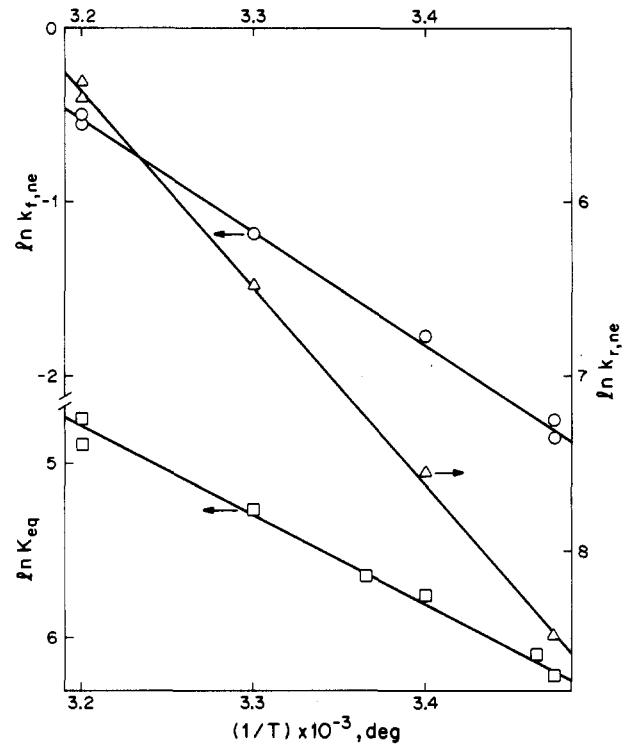


FIGURE 1: Effect of temperature on the kinetics and equilibrium of the nonenzymic cyanide reaction. The initial concentrations of reactants were 1.6×10^{-4} M for NAD and 3.5×10^{-3} M for total cyanide at pH 9.4. The buffer was 0.05 M pyrophosphate. (See text for other details.) The lines were drawn by eye; $\Delta H°$ is −10 kcal, and $\Delta H^‡$ is 12 kcal and 22 kcal for the forward and reverse reactions, respectively.

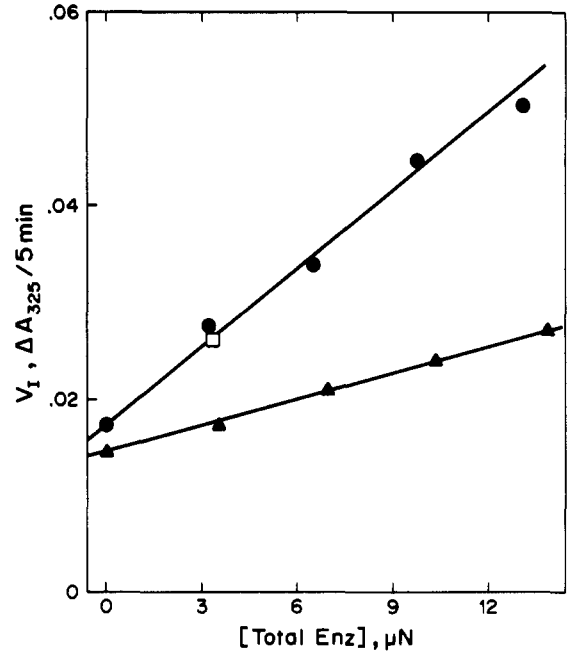
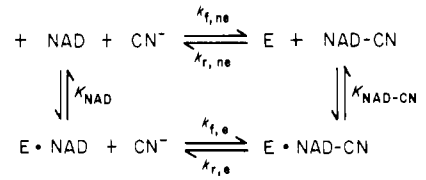


FIGURE 2: Effect of total enzyme concentration on the initial velocity of the reaction between NAD and cyanide. A plot of the *initial* rate of change in A_{327} against the total concentration of enzyme sites at pH 7.0 is shown for pig heart (●) and dogfish muscle (▲) LDH in 0.3 imidazole chloride. The initial concentrations of NAD and KCN were 0.3 mM and 0.1 M, respectively; a data point also was obtained at an initial cyanide concentration of 0.05 M (□). The solid lines were drawn from linear least-squares estimates of the values for the intercept and slope; the values for $k_{f,e}$ in Table 1 were calculated from the slope term by taking into consideration the ratio [CN^-]/[CN_T].

Scheme I: Cyanide Adduct Reaction



ment, 0.045 M^{-1} s^{-1} (see above). The ratio of the enzymic and nonenzymic rates indicates that the pig and dogfish enzymes can accelerate the cyanide adduct reaction by approximately 220- and 150-fold, respectively; a similar value (400-fold) was obtained previously at pH 7.0 and 25 °C for the pig heart enzyme (Gerlach et al., 1965).[3]

To measure the rate of the reverse reaction (E·NAD–CN → E·NAD + CN^-; see Scheme I), the enzyme is either incubated with reactants or treated with 2 equiv of the cyanide adduct and rapidly separated from the reactants plus unbound adduct by centrifugation through a molecular sieve column (see Methods). Because the initial concentration of E·NAD–CN is much greater (~50-fold) than the dissociation constant for this complex (see below), the alternative route for decomposition of E·NAD–CN, via E + NAD–CN and subsequent nonenzymic decomposition of the dissociated NAD–CN, is not significant. The disappearance of E·NAD–CN in solutions produced by either of the above procedures is a first-order process described by the rate constant $k_{r,e}$, which equals 9×10^{-5} s^{-1}. This constant is about half that observed for the corresponding nonenzymic reaction: 1.9×10^{-4} s^{-1} (Table I). Thus, the enzyme slightly stabilizes the bound adduct relative to the unbound adduct. In addition, an estimate of 1×10^5 M for the equilibrium constant of the enzymic adduct reaction is calculated from the ratio $k_{f,e}/k_{r,e}$. These values will be considered further under Discussion.

Dissociation Constant for E·NAD–CN. A titration of the binding sites for NAD–CN in pig heart LDH can be conducted even though NAD–CN is not a thermodynamically stable entity, because both E·NAD–CN and NAD–CN are kinetically stable under the conditions used. Bound NAD–CN quenches the fluorescence of enzymic tryptophanes, and the fractional saturation, as a function of NAD–CN concentration, is described by the linear relationship

$$\frac{1}{1 - F_i^{1/4}} = \frac{1}{1 - X} + \frac{K_{NAD-CN}}{(1 - X)[NAD-CN]}$$

where F_i is the ratio of the fluorescence in the presence of added NAD–CN to the initial fluorescence after correction of the former for inner filter effects (see Methods) and X is the one-fourth power of the fluorescence rato at infinite NAD–CN (again, after correction for inner filter effects). The one-fourth power of the fluorescence ratio takes into consideration the geometric quenching of enzymic fluorescence by NAD–CN. This type of quenching relationship was observed previously for NADH (Holbrook, 1972) and for the NAD–Pyr and NAD–SO_3^- adducts (Burgner & Ray, 1978; Parker et al., 1978). A plot (Figure 3) of $(1 - F_i^{1/4})^{-1}$ against initial NAD–CN concentration is linear over the range of 28–96% of saturation when [E·NAD–CN] is insignificant with respect to total [NAD–CN]. To ensure that insignificant decomposition of NAD–CN occurs during the titration, only two additions of NAD–CN were made per aliquant of protein solution, and both measurements were made within 1 min. Since the concentration of NAD that was added along with the NAD–CN always was much less than K_{NAD}, competitive effects by NAD can be ignored. The value, 1.9×10^{-6} M, estimated from the slope–intercept ratio of the line at pH 7.0 in Figure 3 represents the apparent dissociation constant of the E·NAD–CN complex. Because the NAD–CN used in this titration was prepared nonenzymically, both the A and B isomers of the dihydropyridine ring are present (at a distribution of 60:40 in favor of the A isomer; Arnold & Kaplan, 1974). Since only the A isomer binds (see below), the true value for K_{NAD-CN} (at pH 7) must be about 1×10^{-6} M (instead of 1.9×10^{-6} M), which is similar to that obtained for the dissociation constant of E·NADH by Stinson & Holbrook (1973), $(0.5–0.9) \times 10^{-6}$ M. Thus, noncovalent interactions between the enzyme and the nicotinamide ring and/or the added cyano group must increase as the reaction proceeds from bound NAD to bound NAD–CN, since the ratio of K_{NAD} to K_{NAD-CN} is about 300-fold.

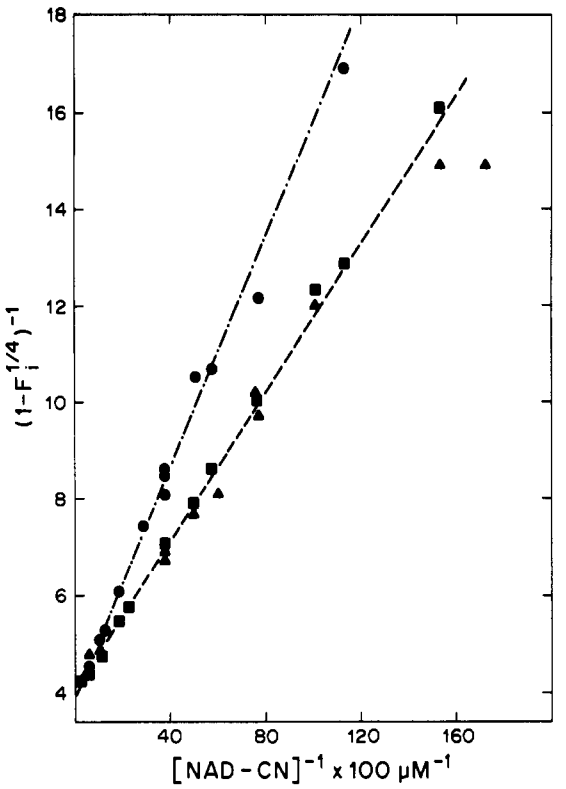


FIGURE 3: Effect of pH on the binding of NAD–CN to LDH. A double-reciprocal plot of fluorescence quenching and unbound NAD–CN concentration is shown. The fluorescence observed at each ligand concentration is corrected for nonlinear quenching and for inner filter effects (see Methods). The assay mixture initially contained 0.3 M imidazole chloride at pH 6 (– –), 7 (– –), and 8 (–·–) as well as 1.7×10^{-7} N pig heart LDH. The lines in the figure are drawn from the numerical estimates (see Methods) of the dissociation constants and intercept terms: 2.1 μM and 0.26 at pH 6; 1.9 μM and 0.24 at pH 7; 3.1 μM and 0.25 at pH 8.

Interactions between the Enzyme and the Cyanide Moiety. The enzyme discriminates between addition to the A and B sides of the bound coenzyme ring. This is demonstrated by titrating the enzyme (at pH 7.0 under conditions where [E] ≫ K_{NAD-CN}) with nonenzymically prepared NAD–CN and measuring the formation of the E·NAD–CN complex by using the fluorescence-quenching procedure described earlier. A plot (not shown) of $1 - (F_i/F_0)^{1/4}$, which is directly proportional to [E·NAD–CN], against total [NAD–CN] added is linear for the first portion of the curve, and the linear phase extrapolates to an equivalence point of 2 mol of added NAD–

[3] An estimate of 250 for the rate of enhancement of the cyanide reaction induced by pig heart LDH was provided by J. J. Holbrook (personal communication).

Table III: Effect of pH on the Values of the Enzymic Kinetic and Equilibrium Constants in Scheme I[a]

pH	$k_{f,e}$ (M^{-1} s^{-1})[b,c]	$k_{f,ne}$ (M^{-1} s^{-1})[d]	K_{NAD-CN} (M)[d]	K_{NADH} (M)[e]
6.2	10.0	0.06	$(1.07 \pm 0.05) \times 10^{-6}$	1×10^{-6}
7.0	12.0	0.05	$(0.94 \pm 0.05) \times 10^{-6}$	1×10^{-6}
8.0	11.0	0.05	$(1.5 \pm 0.1) \times 10^{-6}$	1×10^{-6}

[a] Values obtained at 15 °C with pig heart LDH: see Results. [b] Calculated using eq 2. [c] Corrected for minor changes in K_{NAD} with values obtained from Stinson & Holbrook (1973). [d] Statistically corrected. [e] From Stinson & Holbrook (1973).

CN/mol of active sites.[4] Hence, one of the NAD–CN isomers is discriminated against, presumably because the enzyme shields one side of the bound nicotinamide ring—at least at the 4 position. By analogy with the pyruvate adduct reaction where only A-side attack occurs (Arnold & Kaplan, 1978), the CN^- attack also is presumed to occur on the A side.

The active site histidine appears closest to the CN^- group during its approach to NAD [cf. Holbrook et al. (1975)]. Hence, both the dissociation constant for E·NAD–CN and the rate constant for its formation were determined at pH 6 and 8 (by the procedures described above), where the active site histidine changes from largely charged to largely uncharged, on the basis of the pK_a value of 6.8 estimated for this group by Holbrook & Gulfreund (1973), in both the presence and absence of bound NAD. The observed values for the dissociation constant of E·NAD–CN are given in Table III. Also given in Table III are values for the rate constants of the forward enzymic ($k_{f,e}$) and nonenzymic ($k_{f,ne}$) reactions—those for $k_{f,ne}$ are statistically corrected. These values are obtained from plots of initial velocity against enzyme concentration for the cyanide reaction (cf. Figure 2). Clearly, both $k_{f,e}$ and K_{NAD-CN} are essentially independent of pH within the above range. Hence, the ionization of the active site histidine does not influence either the rate or extent of the cyanide reaction.

Discussion

The results in Table I show (a) that the rate of the reaction of CN^- with E·NAD is some 200-fold faster than the nonenzymic NAD + CN^- reaction, (b) that a similar increase in noncovalent binding interactions, about 300-fold, accompanies the enzymic process (viz., $K_{NAD-CN} = 300$), and (c) that the enzyme actually retards the decomposition of bound NAD–CN by about 2-fold. Before we consider how the cyanide adduct reaction might relate to the normal LDH reaction, the effect of LDH on the equilibrium and rate constants of this addcuct reaction will be considered.[5] Since we shall compare the rate constants for the enzymic and nonenzymic adduct reactions as a measure of the extent that LDH "activates" bound NAD, some attention is given to showing that no significant interactions occur between the enzyme and the attacking CN^-. For instance, the pseudo-first-order rate constant for the forward cyanide adduct reaction catalyzed by the enzyme is linearly dependent on the [CN^-] over a 10-fold concentration range (up to about 10 mM CN^-). In addition, the rate of the reverse reaction is independent of cyanide concentration over the same concentration range. Hence, the enzymic reaction is bimolecular under the conditions used. Whether a transitory complex between E·NAD and cyanide forms prior to the transition state cannot be evaluated by the above approach. But the enzyme does not seem to exert a substantial influence on the CN^- component of the reaction. For example, both the rate constant for the reaction of cyanide with E·NAD and the dissociation constant for the E·NAD–CN complex (Table II) are independent of pH over the range 6–8 where the active site histidine (His-195) changes from predominantly charged to predominantly uncharged (Holbrook et al., 1975). Thus, the charge on the enzymic group closest to the reaction site [cf. Burgner & Ray (1984b)] does not affect significantly either the rate or the extent of the cyanide adduct reaction.[6] In addition, the next closest anion binding site Arg-171, where the carboxylate of pyruvate binds [cf. Grau et al. (1981)], probably is too far from the nicotinamide ring for binding of CN^- at this site to affect directly the adduct reaction. [The weak binding of Cl^- that is competitive with pyruvate probably occurs at this more distant site—see Burgner & Ray (1984a).] Finally, the similarity in the binding constants for NADH and NAD–CN (between pH 6 and 8, see Table III) suggests that interactions between the enzyme and the cyano group in the product E·NAD–CN also are insignificant. The proposed mechanistic similarity between the enzymic and nonenzymic process, then, is both reasonable and consistent with the avaiable data.

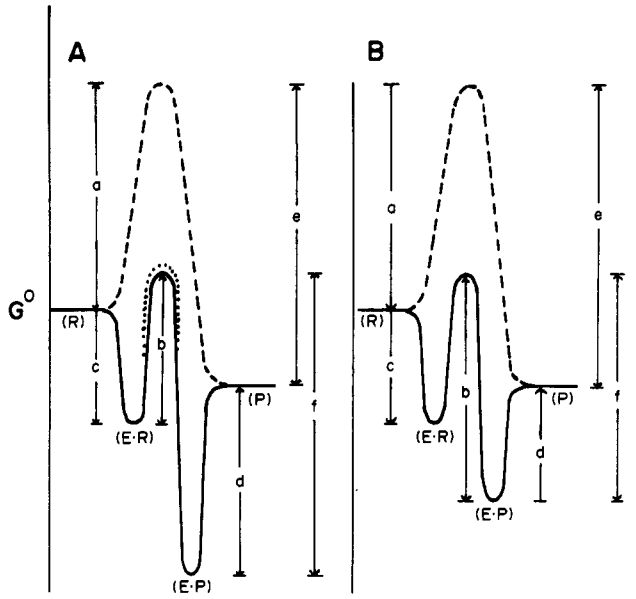
The enzyme alters the equilibrium constant for the cyanide adduct reaction by an extent equal to the ratio of the binding constants for NAD and NAD–CN, $K_{eq,e}/K_{eq,ne}$, as required by Scheme I. The observed effect, 400-fold, also approximately equals the ratio of binding constants for NAD and NADH, 300-fold (Stinson & Holbrook, 1973). In simple systems, an increase in the equilibrium constant for a reaction could be caused, in theory, by a change in a single rate constant; i.e., either the forward rate constant is increased (reactant destabilization), or the reverse rate constant is decreased (product stabilization). But usually both rate constants are altered since increasing the chemical potential of the reactant can increase the height of the transition state for the reverse reaction and decreasing the chemical potential of the product can decrease the height of the transition state in the forward reaction (Hammond, 1955). The opposite extreme is also possible—especially in enzymic reactions—where, again in theory, the rates of both the forward and reverse reactions are altered equally by transition-state binding effects (Wolfenden, 1969, 1972; Lienhard, 1973; Jencks, 1975) leaving the equilibrium constant for interconversion of bound reactants and products unaltered, i.e., unchanged from the solution process. These extremes are illustrated by the hypothetical Gibbs energy profiles in Scheme II, where the dashed curve refers to the nonenzymic reaction and where only the magnitudes of the binding constants and of $k_{r,e}$ differ in A and B of Scheme II. In Scheme IIA, the increase in binding interactions between the product complex and the reactant complex (d – c) completely accounts for the enzyme-induced rate enhancement (a – b); i.e., only the rate constant for the forward reaction increases. We refer to this type of rate enhancement as an "equlibrium binding effect". Note that

[4] Studies on the reverse enzymic reaction also indicate that one of the NAD–CN isomers is excluded. For instance, when the reverse enzymic reaction is initiated by adding 1 equiv of nonenzymically prepared NAD–CN to the enzyme, [E] $\gg K_{NAD-CN}$, an initial burst is observed with a magnitude of approximately 30% of the total NAD–CN added. The values for $K_{eq,ne}$ and $k_{r,ne}$, estimated numerically, are in good agreement with those obtained above.

[5] To a first approximation, all conclusions apply to either enzyme; hence, quantitative results are given only for the pig heart isozyme.

[6] The lack of variation of the rate constant for the cyanide adduct reaction over the pH range 6–8 as calculated for CN^- also indicates that HCN is not an important reactant in the enzymic adduct reaction (the observed rate actually increases by a factor of 100-fold over this range, whereas the concentration of HCN decreases).

Scheme II: Gibbs Energy Profiles Comparing Extreme Examples of Rate and Equilibrium Binding Effects[a]



[a] In part A, $a - b = d - c$ (forward rate effect = equilibrium effect); $e = f$ (reverse rate constants equal). In part B, $a - b > d - c = 0$ (rate effect with no equilibrium effect); $e = f$ (rate effect same as in forward reaction).

in a reaction proceeding along the dotted line, the increase in the forward rate constant is smaller than the equilibrium binding effect, but the rate effect still can be viewed reasonably as induced by an equilibrium binding effect. In Scheme IIB, no increase in the binding interactions occurs in the product complex (relative to the reactant complex) since c = d, and both the forward and reverse enzymic reactions are accelerated by identical extents (a − b = e − f), i.e., a transition-state binding effect. In most reactions catalyzed by enzymes, we expect that some combination of the extremes in A and B of Scheme II will apply. Thus, both the normal redox reaction and the pyruvate adduct reaction catalyzed by LDH probably use a combination of these extremes (see the third paper in this series). But, in the cyanide reaction, only the first case (A) applies, since nearly all of the increase in K_e produced by binding interactions in E·NAD appears in the rate constant for the bond-forming process. Although we cannot specify how an equilibrium binding increase produces an essentially equivalent rate effect,[7] we will refer to the above rate increase as arising from an equilibrium binding effect and thus will consider ways that the enzyme might use binding interactions to alter the equilibrium constant for this reaction.

One way that the enzyme might increase the *equilibrium* constant for the cyanide adduct reaction involves binding the nicotinamide ring in a geometrically distorted way that is relieved in the dehydronicotinamide-like product. Unfortunately, although the pyridinium ring of NAD is known to be planar, the geometry of the dihydro ring is less clear. Thus, Kosower (1962) has argued for a planar 1,4-dihydronicotinamide ring from chemical considerations, and 1-benzyl-1,4-dihydronicotinamide and 1,4-dihydrobenzyl alcohol are planar in the crystal phase and in solution, respectively (Paschah & Rabideau, 1974). But, more recent NMR studies of free NADH in its folded conformation (Oppenheimer et al., 1971, 1978) and bound NADH (Lee et al., 1974) were interpreted in terms of a puckering of the dihydronicotinamide ring.[8] Although the importance of geometric destablization on the equilibrium constant for the cyanide adduct reaction cannot be entirely discounted, environmental destabilization, which is discussed below, seems more likely because of the paucity of *directional* interactions between the bound nicotinamide ring and LDH. For instance, only three *directional* interactions are possible between the nicotinamide ring, the adjacent ribose, and the enzyme (Grau et al., 1981; Eventoff et al., 1977). This scarcely seems sufficient to produce a geometric destabilization. Solvent effects provide a simple rationale for an equilibrium binding effect on a reaction where the reactant is charged and the product neutral, and as expected, the identity of the solvent (methanol or water) does affect the equilibrium constant for the nonenzymic cyanide adduct reaction involving either 1-methylnicotinamide or NAD (cf. Table II). Furthermore, solvation effects alter both the forward and the reverse rate constants. In the case of bound NAD, the solvation of the nicotinamide ring is quite different than that in a homogeneous solution, because in the enzymic system the ring is bound so that one side makes contact with an oily array of residues (Grau et al., 1981; Evantoff et al., 1977). Whether this serves to destabilize the reactant NAD (by producing distortionless strain; Fehrst, 1974, 1977) or stabilize the NADH-like product is not significant here. Nevertheless, a similar chemical change does occur in the normal redox reaction (viz., NAD → NADH), and by analogy, we argue that the equilibrium effect that manifests itself almost entirely in one direction in the cyanide adduct reaction probably does so also in the normal redox reaction catalyzed by LDH. And while we tend to view this as a substrate destabilization effect, subsequently, we will refer to it simply as an equilibrium binding effect that produces essentially a unidirectional rate effect (Burgner & Ray, 1984b).

[7] In bi-bi reactions, equilibrium binding effects could be caused by either reactant destabilization that is relieved in the transition state or transition-state binding that is retained in the product complex.

[8] It also should be pointed out that Cook et al. (1981) have interpreted an ^{15}N isotope effect on both the forward and reverse redox reactions catalyzed by alcohol dehydrogenase as evidence that geometric distortion of the nicotinamide ring does occur in the transition state.

References

Arnold, L. J., Jr., & Kaplan, N. O. (1974) *J. Biol. Chem. 249*, 652.

Baumrucker, J., Calzadilla, M., Centeno, M., Lehrmann, G., Urdaneta, M., Lindquist, P., Dunham, D., Price, M., Sears, B., & Cordes, E. M. (1972) *J. Am. Chem. Soc. 94*, 8164.

Burgner, J. W., II, & Ray, W. J., Jr. (1974) *Biochemistry 13*, 4229.

Burgner, J. W., II, & Ray, W. J., Jr. (1978) *Biochemistry 17*, 1654.

Burgner, J. W., II, & Ray, W. J., Jr. (1984a) *Biochemistry* (second paper of three in this issue).

Burgner, J. W., II, & Ray, W. J., Jr. (1984b) *Biochemistry* (third paper of three in this issue).

Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817.

Eventoff, W., Rossmann, M. G., Taylor, S. S., Torff, H. J., Meyer, H., Keil, W., & Kiltz, H. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2677–2681.

Fersht, A. (1974) *Proc. R. Soc. London, Ser. B 1877*, 397.

Fersht, A. (1977) *Enzyme Structure and Function*, p 270, W. H. Freeman, San Francisco.

Gerlach, D., Pfleiderer, G., & Holbrook, J. J. (1965) *Biochem. Z. 343*, 354.

Grau, U., Trommer, W., & Rossmall, M. G. (1981) *J. Mol. Biol. 151*, 289.

Hammond, G. S. (1955) *J. Am. Chem. Soc. 77*, 334.
Hinz, H.-J., Schmid, R., Scheurmann, W., & Jaenicke, R. (1977) *Eur. J. Biochem. 80*, 543.
Holbrook, J. J. (1972) *Biochem. J. 128*, 921.
Holbrook, J. J., Lijas, H., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes, 3rd Ed. 2*, p 191–292.
Izatt, R. M., Christensen, J. J., Pack, R. T., & Bench, R. (1962) *Inorg. Chem. 1*, 828.
Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219.
Jencks, W. P. (1980) *Mol. Biol., Biochem. Biophys. 32*, 3–25.
Karrer, P., & Blumer, F. (1947) *Helv. Chim. Acta 30*, 1157.
Kosower, E. H. (1962) *Molecular Biochemistry*, pp 198–199, McGraw-Hill, New York.
Lee, C.-Y., Oppenheimer, N. J., & Kaplan, N. O. (1974) *Biochem. Biophys. Res. Commun. 60*, 838.
Lienhard, G. E. (1973) *Science (Washington, D.C.) 180*, 149.
Lindquist, E. H., & Cordes, E. H. (1968) *J. Am. Chem. Soc. 90*, 1269.
Moore, S. A., & Jencks, W. P. (1982) *J. Biol. Chem. 257*, 10893.
O'Carra, P., & Barry, S. (1972) *FEBS Lett. 25*, 234.
Okubo, T., & Ise, N. (1974) *J. Biol. Chem. 249*, 3563.
Oppenheimer, N. J., Arnold, L. J., Jr., & Kaplan, N. O. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200.
Oppenheimer, N. J., Arnold, L. J., Jr., & Kaplan, N. O. (1978) *Biochemistry 17*, 2613.
Parker, D. M., & Holbrook, J. J. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 485–501, De Gruyter, New York.
Parker, D. M., Lodola, A., & Holbrook, J. J. (1978) *Biochem. J. 173*, 959.
Paschah, J. W., & Rabideau, P. W. (1974) *J. Am. Chem. Soc. 96*, 272.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891.
Ray, W. J., Jr., & Long, J. W. (1976) *Biochemistry 15*, 4018.
Shore, J. D., Weidig, C., Lodola, A., Parker, D. M., & Holbrook, J. J. (1975) *Proc. FEBS Meet. 40*, 63.
Stinson, R. A., & Holbrook, J. J. (1973) *Biochem. J. 131*, 719.
Wolfenden, R. (1969) *Nature (London) 233*, 704.
Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10.
Wolfenden, R. (1978) in *Transition States* of *Biochemical Processes* (Gandour, R., & Schowen, R., Eds.) pp 553–577, Plenum, New York.

The Lactate Dehydrogenase Catalyzed Pyruvate Adduct Reaction: Simultaneous General Acid–Base Catalysis Involving an Enzyme and an External Catalyst†

John W. Burgner, II,* and William J. Ray, Jr.

ABSTRACT: The pH dependence of the reaction catalyzed by lactate dehydrogenase, where pyruvate adds covalently to NAD to yield a NAD–Pyr adduct, together with published data on the pH dependence of parameters in the *normal* redox reaction suggests similar binding modes for enolpyruvate and lactate in their complexes with E·NAD (where E is one-fourth of the tetramer), for ketopyruvate in its complexes with the protonated species, E·H·NAD and E·H·NADH, and for the NAD–Pyr adduct and NADH plus pyruvate in their complexes with E·H. These similarities, together with previous data, suggest a reaction scheme for the formation of the enzyme–adduct complex that includes the relevant proton-transfer steps. Seven different amine chloride buffers were used in a study of the reverse adduct reaction, i.e., the decomposition of E·H·NAD–Pyr. These act with varying efficiencies as *external* general acid catalysts; the enzyme apparently acts as a (internal) general base. The involvement of the amine chloride buffers as external general catalysts is supported by the concentration dependence of the buffer effect, by a Brönsted plot, and by solvent deuterium isotope effects. The involvement of the enzyme as an internal general catalyst is inferred from the pH dependence of the reaction and the identities of the nearby groups in the E·H·NAD–Pyr complex (from crystallographic studies). The dependence of the adduct reaction on chloride concentration indicates the presence of dead-end inhibitor complexes of E·H·Cl and E·H·NAD·Cl. Chloride also accelerates the decomposition of the adduct in the complex E·H·NAD–Pyr by binding to this complex.

In the previous paper of this series (Burgner & Ray, 1984a), we compare the nucleophilic addition of cyanide to both free NAD[1] and the complex of NAD with lactate dehydrogenase, E·NAD. Cyanide was used in that comparison, because it is a small anion. The object of that study was to assess the extent that binding of NAD to LDH increases the susceptibility of the nicotinamide ring toward nucleophilic attack at its 4 position.

Pyruvate also acts as a nucleophile toward free NAD, and the analogous adduct reaction[2] with bound NAD also is catalyzed by LDH. But, both the enzymic and the nonenzymic adduct reactions involving pyruvate are more complex than

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received November 9, 1983.* This investigation was supported by a research grant from the National Science Foundation (PCM8012576).

[1] Abbreviations: NAD, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase (the dogfish A_4 enzyme unless otherwise specified); E, a subunit of an LDH tetramer; Pyr, pyruvate; Pyr_K and Pyr_E, the enol and keto forms of pyruvate, respectively; NAD–Pyr or adduct, the product from a covalent addition of the β-carbon of pyruvate enol at the 4 position of the nicotinamide ring of NAD; E·adduct, E·H·adduct, or E·NAD–Pyr, the adduct complex. Equilibrium dissociation constants are designated by K, with appropriate subscripts. k_r^{obsd} is equivalent to k_{obsd}^r used in a previous paper (Burgner & Ray, 1978). In this paper, the formal charges on NAD, H, and BH are omitted.

[2] The adduct reaction refers to the enzymically catalyzed *formation* of the adduct complex (E·H·adduct) from NAD and Pyr; the "reverse" reaction refers to the process E·H·adduct → $E{\cdot}NAD{\cdot}Pyr_E$ → E·NAD + Pyr.

0006-2960/84/0423-3626$01.50/0