Proton Equilibria and Kinetics in the Liver Alcohol Dehydrogenase Reaction Mechanism†

J. D. Shore,* H. Gutfreund, R. L. Brooks, D. Santiago, and P. Santiago

ABSTRACT: The liberation of protons during turnover of liver alcohol dehydrogenase was studied using transient and inhibition kinetics and direct titrimetric determinations. Proton release occurred prior to, and uncoupled from, the catalytic hydrogen transferring step. The addition of saturating concentrations of NAD^+ to the enzyme resulted in a pH-dependent release of protons, with 0.5 proton/equiv liberated at pH 7.6. Formation of a ternary complex of enzyme, NAD^+, and trifluoroethanol resulted in liberation of 1 proton/equiv of enzyme in the pH range 5.5–8.5. Trifluoroethanol binding to binary complex was dependent on an enzyme functional group with a pK_a of 7.6, with tight binding to the unprotonated form. Caprate binding showed the reverse pH dependence but with the same pK_a, and caused uptake of the protons liberated due to NAD^+ binding. The rate constants for proton release in burst reactions and from formation of ternary enzyme–NAD^+–trifluoroethanol complex, 250–280 sec^{-1}, were compatible with a functional group with a pK_a of 7.6. A scheme was developed indicating that protons were released from the enzyme during turnover as a result of perturbation of the pK_a of a functional group on the enzyme from 9.6 to 7.6, and direct binding of the alcoholic hydroxyl group to the basic form of the perturbed functional group. This scheme accounts for the required stoichiometry of proton liberation and is compatible with a concerted hydride transfer mechanism of catalysis.

Many previous studies have indicated that the reaction mechanism of liver alcohol dehydrogenase (EC 1.1.1.1.) is sequentially ordered, with coenzyme binding prior to substrate (Theorell and Chance, 1951; Dalziel, 1963; Wratten and Cleland, 1963). Transient kinetics (Shore and Gutfreund, 1970) demonstrated that the addition of saturating concentrations of NAD^+ and ethanol to the enzyme resulted in an exponential burst of bound NADH[1] formation followed by steady-state turnover. A substantial deuterium isotope effect on the rate constant for the exponential phase indicated that it was limited by the rate of transfer of hydrogen from alcohol to NAD^+. Further studies demonstrated that the rate of this step was very dependent on substrate structure (Brooks and Shore, 1971) and required the unprotonated form of an enzyme group with a pK_a of 6.4 (Brooks *et al.*, 1972).

The stoichiometry of the overall reaction of liver alcohol dehydrogenase indicates that for every NADH molecule formed a proton must be liberated. The present study was undertaken to determine when in the reaction sequence the proton was released and where it originated—the enzyme or the alcohol hydroxyl group. The combined use of transient and steady-state kinetics, in conjunction with direct observation of proton liberation, provided answers to these questions and supplied interesting inferences regarding the catalytic mechanism.

Materials and Methods

Liver alcohol dehydrogenase was prepared from frozen horse livers by the method of Theorell *et al.* (1966). The concentration of enzyme was determined by assay (Dalziel, 1957) and by fluorimetric titration with NADH in the presence of isobutyramide (Theorell and McKinley-McKee, 1961a). NAD^+ was purchased from Sigma Chemical Corp. and purified by dilute sulfuric acid elution from Dowex-1 by the method of Stinson and Holbrook (1973). Trifluoroethanol was purchased from Aldrich Chemical Corp., and deuterioethanol-d_6 from International Chemical and Nuclear Co.

Steady-state kinetics were performed with an Eppendorf fluorimeter. All rate curves were done in triplicate and inhibition constants were determined by the method of Dixon (1953) with a least-squares analysis of the data. Sodium phosphate buffer, 0.1 μ, was used in all experiments with the addition of 5 mM succinate at pH 5.5 and 3.5 mM glycine at pH 8.5, 9, and 10. Stopped-flow kinetics were performed in a Durrum-Gibson spectrophotometer which had a dead time of 2.5 msec and a cuvet path length of 2.0 cm. All studies were performed at pH 7.6 in the presence of 0.5 mM phosphate, 0.1 M sodium sulfate, and approximately 10 μM Phenol Red. The pH of all solutions was adjusted to 7.6 with a Radiometer titrigraph immediately prior to use and absorbance at 560 nm was determined in a Zeiss PMQ spectrophotometer. Solutions differing by more than 0.02 in absorbance were readjusted before use. The concentration of protons produced in burst reactions was estimated by calibrating the absorbance change at 560 nm during the steady-state turnover phase against the production of free NADH measured at 330 nm. Since hydrogen ions are released concomitant with NADH during turnover, it was possible to relate changes in absorbance at 560 nm to proton concentration. This relationship, and an extrapolation to account for the 2.5 msec dead time of the instrument, provided an estimation of the size of the proton burst. The rate constants of exponential burst reactions of 560 and 330 nm were calculated by the method of Guggenheim (Frost and Pearson, 1965).

Proton liberation due to binary and ternary complex for-

† From the Edsel B. Ford Institute, Detroit, Michigan 48202, and the Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, England. *Received April 26, 1974.* This work was supported in part by National Science Foundation Grant GB-18027A and the Science Research Council (U. K.).

[1] Abbreviations used are: NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; E or enzyme, horse liver alcohol dehydrogenase.

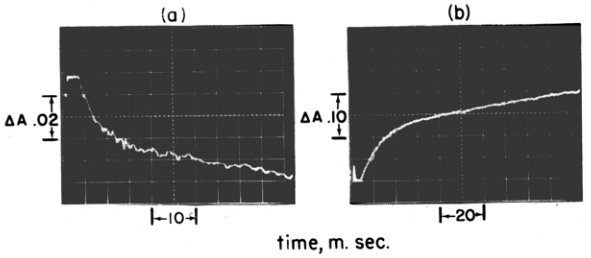


FIGURE 1: Proton and bound NADH burst rates with ethanol substrate, pH 7.6: (a) 560 nm; (b) 330 nm. Syringe 1, 44 μN enzyme, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red; Syringe 2, 3.92 mM NAD^+, 42 mM ethanol, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red.

mation was followed in a Radiometer TTT-1 titrigraph. The experiments were performed in 0.5 mM phosphate in the presence of 0.1 M sodium sulfate. The sodium hydroxide used for titration was freshly made using boiled distilled water, and proton release due to binary and ternary complex formation was calibrated by addition of a known amount of HCl subsequent to complex formation.

Results

In order to determine when protons are released in the reaction sequence, the exponential burst resulting from the addition of saturating concentrations of NAD^+ and ethanol to the enzyme was studied at two wavelengths in weakly buffered solutions containing Phenol Red. At 330 nm, the formation of enzyme-bound NADH was observed while at 560 nm the release of protons was monitored. Figure 1 shows oscilloscope traces of the reactions, in which it can be seen that proton liberation was more rapid than bound NADH formation. The rate constant for the 560-nm reaction was 270 sec^{-1} while the value for the formation of bound NADH at 330 nm was 150 sec^{-1}. To ascertain whether the release of protons was related to the production of bound NADH or uncoupled from it, deuterioethanol was used as substrate. As evidenced by Figure 2, the reaction measured at 330 nm was considerably slower, 35 sec^{-1}, while the proton release occurred with approximately the same rate constant as when ethanol was the substrate, 260 sec^{-1}. With both ethanol and deuterioethanol, however, a full proton burst occurred, with the amount of hydrogen ions released being equal to the concentration of enzyme active sites.

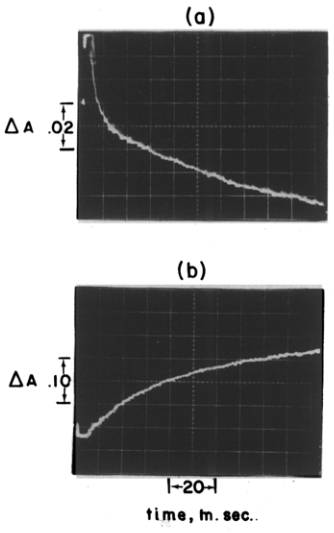


FIGURE 2: Proton and bound NADH burst rates with deuterioethanol substrate, pH 7.6: (a) 560 nm; (b) 330 nm. Syringe 1, 52 μN enzyme, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red. Syringe 2, 3.92 mM NAD^+, 42 mM ethanol-d_5, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red.

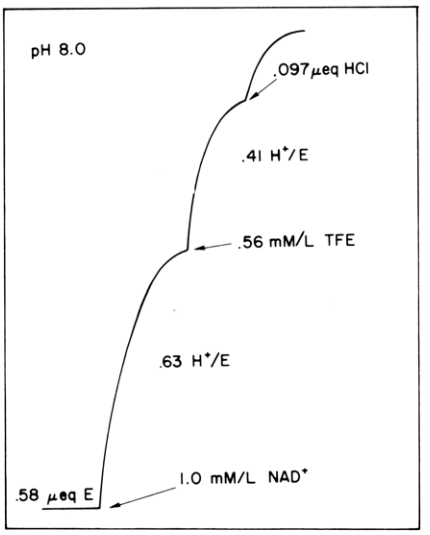


FIGURE 3: Titrimetric determination of proton release due to binary and ternary complex formation at pH 8.0. The initial volume was 3.0 ml, containing the enzyme in 0.05 M phosphate and 0.1 M Na_2SO_4. After additions of NAD^+, trifluoroethanol, HCl, and the titrated NaOH, the final volume was 3.95 ml.

Since proton liberation precedes the reduction of NAD^+, a reasonable assumption was that it was related to binary or ternary complex formation. An invaluable aid to this work was the previous steady-state investigation to Dalziel (1963), who reported that the formation of binary enzyme–NAD^+ complex was dependent on an enzyme functional group with a pK_a of 9.6, with preferential binding of NAD^+ to the enzyme form in which this group was protonated. The rate constant for dissociation of the binary complex, however, was dependent on a group with a pK_a of approximately 8.0. These results were interpreted to indicate that binding of NAD^+ resulted in perturbation of the pK_a of that functional group from 9.6 to 8.0.

If the binding of oxidized coenzyme to horse liver alcohol dehydrogenase results in a pK_a perturbation, it should be possible, at the proper pH, to observe directly proton release resulting from binary complex formation. Figure 3 shows a typical experiment in which saturating NAD^+ was added to enzyme in a Radiometer titrigraph at pH 8.0. It can be seen

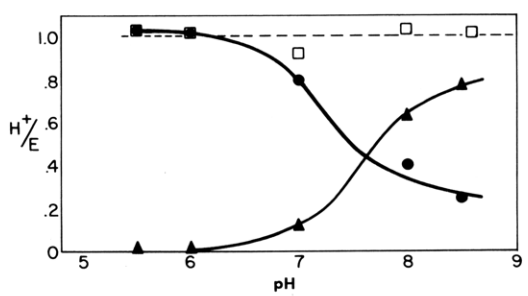


FIGURE 4: pH dependence of proton liberation. Concentrations of enzyme, NAD^+, and trifluoroethanol were 100 μN, 2 mM, and 0.5 mM, respectively, at pH values of 7.0–8.5 and 100 μN, 5 mM, and 10 mM at pH values below 7. (▲) proton release due to NAD^+ binding; (●) additional proton release due to trifluoroethanol binding; (□) additive protons due to ternary complex formation.

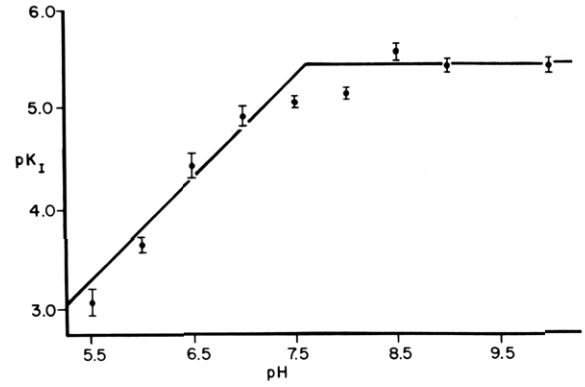


FIGURE 5: The pH dependence of inhibition by trifluoroethanol. The NAD^+ concentration was 2 mM at pH 7.5–10 and 5 mM at pH 5.5–7.0.

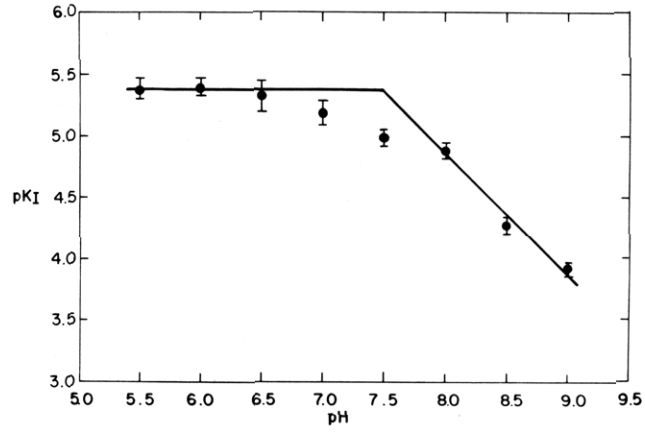


FIGURE 7: The pH dependence of inhibition by caprate. The NAD^+ concentration was 2 mM at pH 7.5–9.0 and 5 mM at pH 5.5–7.0.

that substantial proton liberation occurred due to NAD^+ binding, with further release of protons due to formation of a ternary complex with trifluoroethanol, an inhibitor competitive with ethanol. The release of protons due to binary and ternary complex formation was studied in the pH range 5.5–8.5 (Figure 4). The amount of protons released due to addition of saturating concentrations of NAD^+ was pH dependent, with 0.5 proton released/equiv of enzyme at a pH of 7.6 ± 0.2.

The formation of a ternary complex with trifluoroethanol always resulted in the liberation of sufficient additional protons to result in 1 proton/equivalent of enzyme, with 0.5 proton released due to trifluoroethanol addition at pH 7.6 ± 0.2. To clarify the mechanism by which trifluoroethanol binding results in proton release, the variation of its binding constant to binary enzyme–NAD^+ complex with pH was determined. A plot of the K_I as an inhibitor competitive with ethanol *vs.* pH (Figure 5) indicated that trifluoroethanol was selectively binding to the unprotonated form of a functional group of the enzyme–NAD^+ complex with a pK_a of 7.6 ± 0.2. To elucidate further the proton release mechanism, caprate, an inhibitor which is also competitive with ethanol (Theorell and McKinley-McKee, 1961b), was studied. The addition of saturating concentrations of caprate to binary enzyme–NAD^+ complex resulted in uptake of the same amount of protons liberated due to formation of the binary complex (Figure 6). The inhibition constant for caprate showed the reverse pH dependency of the inhibition constant for trifluoroethanol, with tight binding to the protonated form of a functional group of the enzyme–NAD^+ complex with a pK_a of 7.6 ± 0.2 (Figure 7).

The rates of proton release due to binary and ternary complex formation were determined at pH 7.6. Figure 8 shows a comparison of the rates of proton liberation due to addition of saturating NAD^+ to the enzyme in the presence and absence of saturating concentrations of trifluoroethanol. In the absence of trifluoroethanol the rate constant for proton release was approximately 500 sec^{-1}. In the presence of trifluoroethanol, the amplitude of the reaction was doubled and the rate constant was 280 sec^{-1}. These results were expected since the reaction at pH 7.6 proceeds to an equilibrium in the absence of inhibitor, with only 0.5 proton produced/equiv of enzyme.

Since the data accumulated indicated that at pH 7.6 half the proton liberation results from NAD^+ binding with the remainder from ternary complex formation, an experiment was designed to demonstrate this with ethanol as substrate. By using a low concentration of ethanol in the presence of isobutyramide to inhibit turnover, it was possible to generate a biphasic proton burst (Figure 9a) at pH 7.6. The initial rapid proton release was due to NAD^+ binding and occurred with a rate constant of 270 sec^{-1}. It was followed by a slower proton liberation with a rate constant of 28 sec^{-1} corresponding to formation of ternary enzyme–NAD^+–ethanol complex, which occurred slowly due to the low ethanol concentration. At 330 nm (Figure 9b) the formation of enzyme-bound NADH was followed and showed only one phase with a rate constant of 26 sec^{-1}. The homogeneous

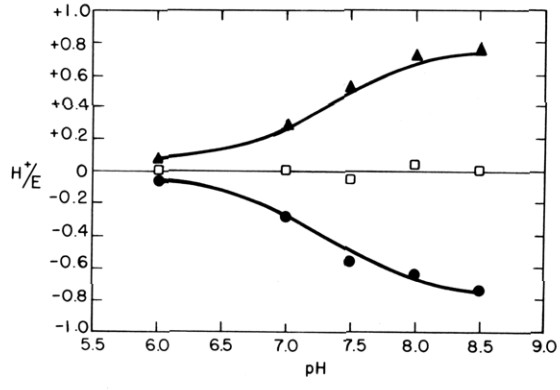


FIGURE 6. pH dependence of proton release by NAD^+ binding (▲) and uptake due to caprate binding (●). (□) Net proton change due to ternary complex formation. The concentrations of enzyme, NAD^+, and caprate used were 100 μN, 2 mM, and 5 mM, respectively, at pH 7.0–8.5 and 100 μN, 5 mM, and 1 mM, respectively, at pH values below 7.0.

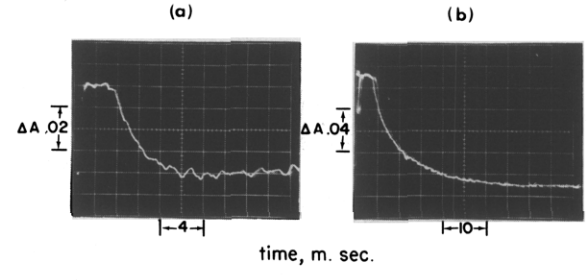


FIGURE 8: Rates of proton release due to binary and ternary complex formation, pH 7.6, 560 nm. (a) Syringe 1, 79.5 μN enzyme, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red; syringe 2, 4.05 mM NAD^+, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red. (b) Syringe 1, 79.5 μN enzyme, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red; syringe 2, 4.05 mM NAD^+, 1.8 mM trifluoroethanol, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red.

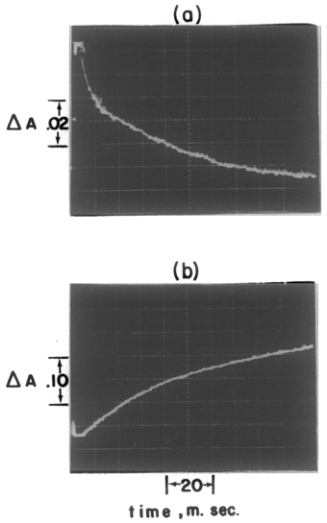


FIGURE 9: Proton burst rates at pH 7.6 with low ethanol at (a) 560 nm and (b) 330 nm. Syringe 1, 66 μN enzyme, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red. Syringe 2, 3.92 mM NAD^+, 2.08 mM ethanol, 12 mM isobutyramide, 0.1 M Na_2SO_4, 0.5 mM phosphate, and Phenol Red.

reaction with the same rate constant as the slower phase of proton release was anticipated since only ternary complex could result in bound NADH formation.

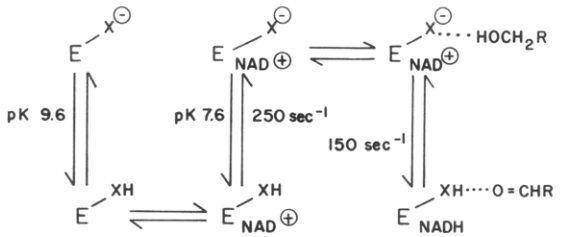
Discussion

The comparison of rates of proton release with rates of enzyme-bound NADH formation, shown in Figures 1 and 2 with ethanol and deuterioethanol as substrates, establishes that proton release precedes NAD^+ reduction at neutral pH values. Furthermore, since the liberation of protons occurred at the same rate with substrates in which the rates of bound NADH formation were very different, proton release appears to be uncoupled from the hydride transfer step. The release of 1 full proton/equiv of enzyme in the exponential burst phase accounts for the proton which must be produced stoichiometrically with NADH production during turnover of the enzyme.

The release of protons due to binding of NAD^+ confirms the previous proposal of Dalziel (1963) that binary complex formation results in perturbation of the pK_a of a group on the enzyme from 9.6 to a lower value. The value we obtained for the perturbed pK_a, 7.6 ± 0.2, is reasonably close to the pK_a of approximately 8 determined by steady-state kinetics. The pH dependence of trifluoroethanol binding, with selective binding to the unprotonated form of a group with a pK_a of 7.6, provides an explanation for the release of 1 proton/equiv of enzyme due to ternary complex formation. Depending on the pH, part of the protons result from perturbation of the pK_a of an enzyme functional group and the remainder are liberated by the binding of alcohol to the unprotonated form of the group.

The results obtained in this study can be explained by Scheme I, in which the functional group involved in proton liberation is designated by X. This group has a pK_a of 9.6 in free enzyme (Dalziel, 1963) and NAD^+ binds preferentially to the enzyme form in which group X is protonated. The binding of coenzyme perturbs the pK_a by 2 pH units to 7.6. Since NAD^+ binding perturbs the pK_a of the group, it is not bound directly to it but most probably close to it enabling the positively charged nicotinamide ring to affect the pK_a. The pH dependency of the NAD^+ binding rate, indicating preferential binding to an enzyme form in which group X is protonated, may be a manifestation of a pH-dependent conformational change. If the acidic form of the enzyme possessed much greater affinity for oxidized coenzyme, it is possible to reconcile the pH dependency of binding kinetics with perturbation of the pK_a of group X rather than direct binding to it.

SCHEME I



Subsequent to binary complex formation, the pK_a of group X is 7.6 and some protons will have been liberated depending on the pH. Our results are consistent with the alcoholic hydroxyl group being hydrogen bonded to the basic form of the perturbed functional group of the enzyme. The pK_a of 7.6 for trifluoroethanol binding, with tight binding to the basic form, supports this interpretation. Furthermore, the biphasic proton burst of approximately equal amplitudes (Figure 9a) obtained at pH 7.6 at low ethanol concentrations demonstrates consistency between the results with ethanol and trifluoroethanol. The direct binding of the alcoholic hydroxyl group to the basic form of group X explains the stoichiometric release of 1 proton/enzyme equivalent prior to the formation of enzyme-bound NADH. It also provides an explanation for the release of 1 proton/enzyme equivalent at all pH values due to formation of ternary enzyme–NAD^+–trifluoroethanol complex. These results indicate that the proton is released from a functional group of the enzyme during turnover rather than directly from the hydroxyl group of the substrate.

After ternary complex formation, the catalytic step results in formation of enzyme–NADH–aldehyde complex. Concomitant with, or subsequent to, this step the pK_a of group X would become unperturbed due to loss of the positive charge of the nicotinamide ring and revert to a pK_a of 9.6. The carbonyl group of the aldehyde is hydrogen bonded to the protonated form of this group. Preliminary studies in our laboratory indicate that isobutyramide, an inhibitor competitive with aldehyde, binds to the protonated form of a group with a pK_a of 9.5–10. This would support Scheme I, although further studies will be required to fully elucidate the aldehyde binding interaction.

It has recently been reported (Dunn, 1974) that in the reverse reaction at pH 8.8 with azoaldehyde as substrate, proton uptake occurred subsequent to hydride transfer with a rate which was limited by dissociation of alcohol from the ternary enzyme–NAD^+–alcohol complex. The displacement of NAD^+ from binary enzyme–NAD^+ complex by NADH addition at pH 8.8 resulted in proton uptake. These results are compatible with Scheme I, which would predict proton uptake due to NAD^+ dissociation from binary complex at pH 8.8. Since the dissociation of alcohol from ternary complex in the azoaldehyde system is almost one order of magnitude slower than NAD^+ dissociation, the rate constant for proton uptake was limited by the rate of desorption of alcohol rather than NAD^+.

The rates of proton release due to binary and ternary complex formation are also compatible with Scheme I. A

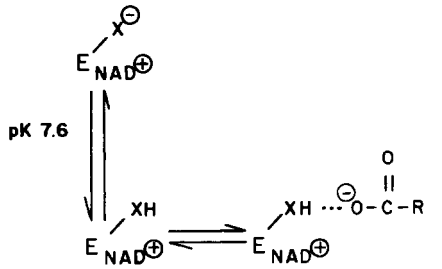
pK_a of 7.6 corresponds to an acid equilibrium constant of 2.5×10^{-8} M. Assuming a value of 10^{10} M^{-1} sec^{-1} for the diffusion limited binding of a proton (Eigen and DeMaeyer, 1963), the rate of dissociation of the proton would be 251 sec^{-1} from a group with a pK_a of 7.6. The value of 280 sec^{-1} obtained due to binding NAD^+ to enzyme in the presence of trifluoroethanol is therefore in reasonable agreement with the expected rate constant for proton dissociation. In the absence of trifluoroethanol, at pH 7.6, the proton release due to NAD^+ binding proceeds to an equilibrium in which group X has yielded half of its protons. The observed rate constant for proton release should be the sum of the forward and reverse rate constants. Since the diffusion limited rate constant for protonation is 10^{10} M^{-1} sec^{-1}, and the hydrogen ion concentration at pH 7.6 is 2.51×10^{-8}, the pseudo-first-order rate constant for protonation would be 251 sec^{-1}. The observed rate of proton liberation due to NAD^+ binding at pH 7.6 should then be 251 sec^{-1} plus 251 sec^{-1}, or 502 sec^{-1}. The value obtained of approximately 500 sec^{-1}, and the relative magnitudes of the reactions in the presence and absence of trifluoroethanol, are in good agreement with theoretical expectations. The pseudo-first-order rate constant for NAD^+ binding, considering the concentration used, should be of the order of 2000 sec^{-1} (Dalziel, 1963), and therefore would not be responsible for the observed rate constants for proton release.

In addition to supplying support for Scheme I, the compatibility of proton release rates with chemical theory provides evidence that a change of enzyme conformation is not required to explain the observed rates. In fact, if a conformational change occurs due to binary or ternary complex formation it must be at least as fast as the rate constant for deprotonation, approximately 250 sec^{-1}. The proton liberation rates also provide indirect evidence for the binding of the alcoholic hydroxyl group directly to the basic form of group X. Ternary complex formation could result in stoichiometric proton release by further perturbing the pK_a of group X to a lower value. This value would have to be lower than 4.5, since a full proton was liberated due to ternary complex formation at pH 5.5 (Figure 4). If the pK_a of group X was 4.5 in the ternary complex, the rate constant for proton release would be approximately 3×10^6 sec^{-1} in the presence of trifluoroethanol and would not be observable by stopped-flow techniques. Instead, the observed rate constant was slower when NAD^+ and trifluoroethanol were mixed with enzyme than when NAD^+ was added in the absence of trifluoroethanol. This obviates the possibility of further pK_a perturbation of group X in the ternary complex, making the direct binding of alcohol to the basic form of the group the most probable explanation of our results.

The biphasic proton burst obtained at pH 7.6 with low ethanol concentration (Figure 9a) further supports Scheme I and indicates that our concept of alcohol binding, based on results with trifluoroethanol, is valid for ethanol. The rapid phase of proton release is due to perturbation of the pK_a of group X to 7.6 while the slow phase is due to ternary complex formation, which occurs slowly at low alcohol concentrations. The same rate constant for the slow proton release and the formation of bound NADH was expected, since only ternary complex could result in formation of NADH.

The somewhat unusual results obtained due to caprate binding can also be attributed to the existence of group X. The uptake of protons due to caprate binding, equal to the protons which had been liberated as a result of NAD^+ binding, can be explained in two ways. Binding of the negatively charged dissociated acid could result in neutralization of the charge of the oxidized nicotinamide ring, with the pK_a of group X no longer perturbed and reverting to its original value of 9.6. Alternatively, the caprate could bind to the protonated form of group X, which would also result in uptake of those protons which had been liberated from group X by the binding of NAD^+. The latter explanation, demonstrated in Scheme II, is the true one as proven by the pH dependence of caprate binding (Figure 7). The hydroxyl group of caprate is dissociated at neutral pH while the hydroxyl groups of ethanol and trifluoroethanol are not. Consequently, the alcohols bind to the unprotonated form of group X, while acids bind to the protonated form. The tight binding of caprate to the protonated form of a group with a pK_a of 7.6 (Figure 7), compared with trifluoroethanol which shows the reverse pH dependence, substantiates this explanation.

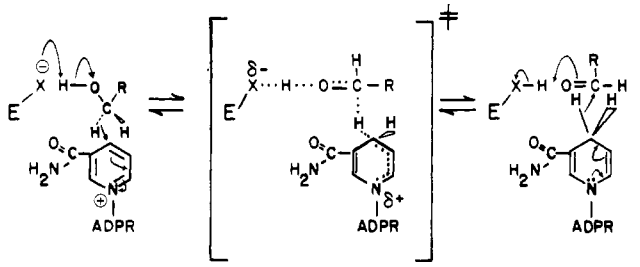
SCHEME II



The elucidation of Scheme I, and the experimental evidence supporting it, provides an answer to the origin of the proton released stoichiometrically with NADH. The proton comes from the enzyme rather than directly from the alcohol hydroxyl group. One aspect which deserves some consideration is whether the protons are liberated from a specific functional group, as delineated in Scheme I, or as the result of conformational changes caused by binary and ternary complex formation. The stoichiometry and patterns of proton release, slope of 1 for the pK_I *vs.* pH curves for trifluoroethanol and caprate, rates of proton release, and reverse pH dependency of caprate binding with the same pK_a all supply compelling evidence for the involvement of only one functional group rather than nonspecific proton production due to conformational changes. This does not contradict previous reports of a different conformation of the enzyme in binary and ternary complexes (Zeppezauer *et al.*, 1967). It merely means that if conformational isomerizations occur and are on the main catalytic pathway, they must be at least as fast as the proton release rate of approximately 250 sec^{-1}.

The involvement of a specific functional group on the enzyme in substrate binding must be compatible with a role for the group in the approach to the transition state. Scheme III shows the involvement of group X in a concerted hydride transfer mechanism, with a possible transition-state structure. The role of other functional groups and the zinc ion believed to be involved in catalysis (Vallee and Hoch, 1957) have not been included, but a likely role for zinc would be to increase the acidity of the alcohol hydroxyl group, facilitating hydride transfer by a concerted mechanism. The other noteworthy feature of Scheme III is acid catalysis of alcohol oxidation and base catalysis of aldehyde reduction, which may be a general feature of dehydrogenase catalysis.

SCHEME III



It has been reported (Theorell and McKinley-McKee, 1961c, Taniguchi *et al.*, 1967) that the pK_a of a functional group on the enzyme is lower by 2 pH units in the binary enzyme-NAD^+ complex, in qualitative agreement with the present results. The group to which this is attributed is a water molecule bound to the zinc atom at the active center. Studies of ternary complexes involving imidazole and caprate resulted in the proposal that substrates and inhibitors bind directly to zinc of the binary enzyme-NAD^+ complex by displacing either a water molecule or a hydroxide ion, depending on the pH. The present results indicating release of protons due to trifluoroethanol binding at low pH, and the rate and extent of proton release due to ternary complex formation, would not be compatible with the displacement of water by substrate. It is feasible, however, that group X is a zinc bound water molecule rather than an amino acid functional group of the enzyme.

Substrate binding to group X may also explain one of the kinetic anomalies of the liver alcohol dehydrogenase reaction. Although the initial proposal of a sequentially ordered mechanism (Theorell and Chance, 1951) seems valid under conditions of low and moderate alcohol concentration, there have been indications that at high alcohol concentrations the mechanism becomes partially random (Silverstein and Boyer, 1964; Wratten and Cleland; 1963, Dalziel and Dickinson, 1966). It has also been shown, using bipyridyl (Sigman, 1967), that ethanol can bind to free enzyme with a dissociation constant of 50 mM at pH 7.0. If an interaction with the unprotonated form of group X is an important aspect of the affinity of alcohol for the enzyme, at neutral pH the alcohol would be bound much more rapidly and tightly to the enzyme-NAD^+ complex than to free enzyme. This is due to the pK_a of 7.6 for group X in the binary complex resulting in a large concentration of molecules with the group dissociated. Free enzyme, with a pK_a 2 pH units higher, would provide a negligible population of molecules able to bind alcohol but at very high substrate concentrations some randomization of the mechanism could occur.

The complexity of their mechanisms and paucity of specific data precludes extension of Scheme I to cover many of the other dehydrogenases. Two enzymes in which protons have been studied extensively are H_4 and M_4 lactic dehydrogenase (Novoa and Schwert, 1961; Holbrook and Ingram, 1973; Holbrook and Gutfreund, 1973). These enzymes do not follow the same proton release mechanism as liver alcohol dehydrogenase does, since NAD^+ binding is independent of pH. The proton is liberated together with or subsequent to pyruvate dissociation from the ternary enzyme-NADH-pyruvate complex, and comes from the histidine-195 residue with a pK_a of 6.8. One feature which does seem to be common to lactate and alcohol dehydrogenases is acid catalysis in the direction of NADH oxidation and base catalysis in the NAD^+ reduction reaction. Lactate is bound to the unprotonated form of histidine-195 while pyruvate binds to the protonated form.

It should be emphasized that these studies have resulted in elucidation of only part of the reaction mechanism of liver alcohol dehydrogenase. The involvement of zinc in ternary complexes and the transition-state structure, and the role of the group with a pK_a of 6.4 which must be unprotonated for maximum rates of hydride transfer (Brooks *et al.*, 1972), have not yet been clarified. It is possible that the group with a pK_a of 6.4 is related to a rapid preequilibrium step involving a conformational isomerization of ternary enzyme-NAD^+-alcohol complex, which is currently being investigated. The identity of group X is also currently in the realm of speculation, but it is anticipated that either affinity labeling or high-resolution crystallography will resolve the question.

Acknowledgments

The authors acknowledge the technical assistance of Mr. Otto Urschel and Miss Carolyn Huetter in these studies.

References

Brooks, R. L., and Shore, J. D. (1971), *Biochemistry 10,* 3855.

Brooks, R. L., Shore, J. D., and Gutfreund, H. (1972), *J. Biol. Chem. 247,* 2382.

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

Dalziel, K. (1963), *J. Biol. Chem. 238,* 2850.

Dalziel, K., and Dickinson, F. M. (1966), *Biochem. J. 100,* 34.

Dixon, M. (1953), *Biochem. J. 55,* 170.

Dunn, M. (1974), *Biochemistry 13,* 1146.

Eigen, M., and DeMaeyer, L. (1963), *Tech. Org. Chem. 8,* 895.

Frost, A. A., and Pearson, R. G. (1965), Kinetics and Mechanism, New York, N. Y., Wiley, p 49.

Holbrook, J. J., and Gutfreund, H. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 31,* 157.

Holbrook, J. J., and Ingram, V. A. (1973), *Biochem. J. 131,* 729.

Novoa, W. B., and Schwert, G. W. (1961), *J. Biol. Chem. 236,* 2150.

Shore, J. D., and Gutfreund, H. (1970), *Biochemistry 9,* 4655.

Sigman, D. S. (1967), *J. Biol. Chem. 242,* 3815.

Silverstein, E., and Boyer, P. D. (1964), *J. Biol. Chem. 239,* 3908.

Stinson, R., and Holbrook, J. J. (1973), *Biochem. J. 131,* 719.

Taniguchi, S., Theorell, H., and Åkeson, Å. (1967), *Acta Chem. Scand. 21,* 1903.

Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5,* 1127.

Theorell, H., and McKinley-McKee, J. S. (1961a), *Acta Chem. Scand. 15,* 1797.

Theorell, H., and McKinley-McKee, J. S. (1961b), *Acta Chem. Scand. 15,* 1834.

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

Theorell, H., Taniguchi, S., Åkeson, Å., and Skursky, L.

(1966), *Biochem. Biophys. Res. Commun. 24,* 603.
Vallee, B. L., and Hoch, F. L. (1957), *J. Biol. Chem. 225,* 185.
Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2,* 935.
Zeppezauer, E., Soderberg, B. O., Branden, C. I., Åkeson, Å., and Theorell, H. (1967), *Acta Chem. Scand. 21,* 1099.

pH-Induced Cold Lability of Rabbit Skeletal Muscle Phosphofructokinase†

Paul E. Bock‡ and Carl Frieden*

ABSTRACT: In phosphate buffers below pH 7, rabbit skeletal muscle phosphofructokinase loses activity as a function of time. The inactivation is reversible and becomes more extensive at lower temperature, lower pH values, and lower enzyme concentrations. It is correlated with changes in the molecular weight of the enzyme as measured by light scattering. The effect of temperature between pH 6 and 7 shows the characteristics of enzyme systems described as cold labile. In general the loss of activity is biphasic but the phases do not appear to be directly correlated with individual molecular events. A mechanism consistent with the inactivation data as a function of enzyme concentration involves three reversible steps: dissociation of the active enzyme to an inactive form which can either repolymerize to an inactive form or isomerize to a different inactive form. ATP addition early in the inactivation process results in rapid partial inactivation (and depolymerization). ATP addition late in the inactivation process results in partial reactivation (and repolymerization). The results may be explained in terms of ATP binding with different affinities to the different forms of the enzyme arising in the inactivation process.

Phosphofructokinase is a complex regulatory enzyme of primary importance in the control of glycolysis in many tissues. The enzyme from rabbit skeletal muscle has been well studied and shown to exhibit a number of properties which are pH dependent. For example, at pH values below about 7–7.5, ATP is not only a substrate, but also an inhibitor of the reaction with the degree of inhibition increasing at lower fructose 6-phosphate levels. At inhibitory levels of ATP, sigmoidal plots of initial velocity *vs.* fructose 6-phosphate concentration are observed with the degree of sigmoidicity a function of the ATP level. Similarly, the effect of allosteric effectors like cAMP, AMP, and ADP is greater at the lower pH values. It has been shown that the overall activity of the enzyme is influenced by a pH-dependent conversion of active to inactive enzyme and it would be of interest to know if this property of the enzyme is related to its kinetic and regulatory behavior in terms of the ability of different ligands to affect this interconversion.

At low pH values, inactivation has been correlated with the conversion of the active enzyme to an inactive form of lower molecular weight for either the rabbit muscle (Paetkau and Lardy, 1967; Hofer and Pette, 1968) or sheep heart enzyme (Mansour, 1965). For the muscle enzyme in phosphate buffer, the process appears to involve dissociation to an inactive form of the enzyme containing two subunits and is cooperative with respect to the hydrogen ion concentration (Hofer and Pette, 1968; Pavelich and Hammes, 1973; Aaronson and Frieden, 1972). It has also been shown that this process can be reversed by raising the pH (Frieden, 1968; Hofer and Pette, 1968) and by the addition of ligands (Aaronson, 1971; Alpers *et al.,* 1971; Lad *et al.,* 1973), again with correlation between the increase in activity and the formation of the active enzyme of higher molecular weight.

It appears possible that the association–dissociation reaction involving active and inactive enzyme may be related to the role of various allosteric effectors in controlling enzyme activity. For example, Lad *et al.* (1973) have shown that citrate inhibition at pH 7 appears to be correlated with the ability of this compound to dissociate the enzyme.

The importance of this association–dissociation process in the regulation of the enzyme activity has therefore led us to a more careful study of the effect of pH as well as temperature on the rate of this process and its possible mechanism.

Materials and Methods

Phosphofructokinase. Crystalline rabbit muscle phosphofructokinase was obtained as an ammonium sulfate suspension in 1 mM ATP from the Sigma Chemical Co. (lot 102C-8720). Most preparations of the enzyme used in these experiments had a specific activity of 170 units/mg measured by the rate of NADH oxidation coupled to fructose 1,6-bisphosphate formation by aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase, where one enzyme unit is defined as the formation of 1 μmol of fructose 1,6-bisphosphate/min at 24° and pH 8 in the assay described below.

The enzyme was routinely charcoal treated to remove bound ATP by the following procedure: crystalline enzyme was centrifuged and the crystals were dissolved in 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA and

† From the Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63110. *Received May 28, 1974.* This investigation was supported in part by U. S. Public Health Service Grant AM-13332. A preliminary report of this work has appeared (Bock and Frieden, 1974).
‡ Predoctoral trainee supported by Training Grant GM-01311.