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Dissociation Constants of Bovine Heart Lactate Dehydrogenase-Reduced Coenzyme Compounds*

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Dissociation constants of LDH · $NADH_2$*** over a wide range of pH and experimental conditions were determined by fluorescence titration equilibrium measurements. Fair agreement was observed over the pH range 6 to about 8 in Tris-KCl buffer, $\mu = 0.2$, 28.5°C with constants calculated from kinetic parameters assuming a compulsory ternary compound mechanism. At higher pH values, the fluorimetrically determined constants were 3 – 4 times smaller than the kinetically determined constants. The binary compound LDH · NAD as well as the ternary inactive compound LDH · NAD · pyruvate have been observed by fluorescence and absorbancy measurements and it is suggested that the latter compound contributes significantly to the reaction mechanism, particularly in alkaline solution.

The evaluation of enzyme-coenzyme dissociation constants by the fluorescence equilibrium method, first exploited by Theorell and co-workers[1,2] with horse liver alcohol dehydrogenase, permits a comparison of these constants with those calculated from kinetic parameters as derived from any proposed reaction mechanism. Discrepancies between the kinetically determined and directly determined dissociation constants indicate either that the proposed reaction

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*** Abbreviations:
LDH, bovine heart lactate dehydrogenase; E, ¼ LDH (since each molecule of LDH has 4 independent binding sites per 135 000 g[3]); ADH, horse liver alcohol dehydrogenase; $NADH_2$ (R), reduced nicotinamide adenine dinucleotide; NAD (O), oxidized nicotinamide adenine dinucleotide; APNAD, oxidized 3-acetyl pyridine analog of NAD; $K_{E,R}$, apparent dissociation constant of the binary compound, LDH · $NADH_2$; $K_{E,O}$, apparent dissociation constant of the binary compound, LDH · NAD; *p*MB, *p*-mercuribenzoate (written to indicate the uncertainty of the associated anion in solutions of the mercurial); Tris, tris (hydroxymethyl) aminomethane; EDTA, ethylenediaminetetraacetate.

mechanism needs further consideration, that fluorescence measurements do not represent quantities which can be compared to the specifically defined dissociation constants, or that the rate measurements are in error. In the case of liver alcohol dehydrogenase, Theorell *et al.*[1,2] found good agreement between the values for the dissociation constants of the binary compounds ER and EO ($K_{E,R}$ and $K_{E,O}$) as determined from fluorescence equilibrium measurements in absence of substrate, and those calculated from the relation between the kinetic "off" and "on" rate constants, as determined in kinetic studies in the presence of substrate. In these calculations, the Theorell-Chance mechanism, in which only binary compounds of EO and ER are kinetically significant, was assumed valid over the pH range investigated.

For bovine heart LDH, a reaction sequence was formulated in 1956 by Takenaka and Schwert[3] in which there is a compulsory pathway such that substrates can combine only with the enzyme-coenzyme compounds to form one or more kinetically indistinguishable but significant ternary compounds. Kinetic data obtained over a wide range of pH and experimental conditions, coupled with ultracentrifuge equilibrium binding experiments[4] have supported the ternary compound mechanism. Similar mechanisms in which ternary compounds have kinetic significance have been suggested for other dehydrogenases such as ribitol[5], pig heart malic[6], yeast alcohol[7], rabbit muscle lactate[8] and rat liver lactate[9]. Recently, Alberty and co-workers[10] and Baker[11] have suggested that even for liver ADH the Theorell-Chance mechanism is unlikely to provide a satisfactory description of the reaction mechanism as a result of the omission of kinetically significant ternary compounds.

Since kinetic data on bovine heart LDH was available over a wide range of pH[12] it was considered of interest to compare the apparent dissociation constants of the LDH · $NADH_2$ compound obtained fluorimetrically in the absence of substrate with those constants calculated from kinetic parameters assuming the intermediary ternary compound mechanism to be valid under the experimental conditions used. This paper reports on $K_{E,R}$ at various pH values and experimental conditions. A comparison of these constants with the dissociation constants as derived from kinetic measurements indicates fairly good agreement over the pH range 6–8 but differs considerably at higher pH values, perhaps indicating that the reaction mechanism needs further consideration. It is suggested that the presence of the inactive compound LDH · NAD · pyruvate, the formation of which is favored in alkaline solution, contributes significantly to the reaction mechanism. Preliminary spectrophotometric and fluorimetric evidence for the formation of the compounds LDH · NAD and LDH · NAD · pyruvate is also presented.

EXPERIMENTAL

Enzyme. Bovine heart LDH was prepared according to the method of Schwert *et al.*[13] and component "A" isolated by column chromatography on hydroxyapatite. The chromatographed enzyme was recrystallized from ammonium sulfate 2–8 times and all final titrations with coenzyme were performed with 8× recrystallized enzyme. To ensure that no L-lactate was present on the enzyme, solutions of LDH in 0.05 M phosphate, pH 6.9 + 0.001 M EDTA were treated with a few mg of NAD/ml of enzyme and the solution dialyzed 4–5 times (2–3 days) against 0.05 M phosphate buffer, pH 6.9. However, treatment of the enzyme in this way

gave identical fluorescent titration curves with $NADH_2$ as did untreated enzyme. Diluted enzyme solutions from either the crystals in 0.6 saturated ammonium sulfate or from the NAD treated enzyme, were prepared fresh daily. Activity measurements were made before and after each titration in a number of experiments and no loss of activity was found during this time over the pH range 6 to 10. The enzyme was assayed either in the presence of excess L-lactate and NAD at pH 10 in glycine-phosphate buffer[13] or by determining the concentration of binding sites by titration with $NADH_2$ in the presence of excess oxalate (0.3 M) as was done previously with liver ADH, $NADH_2$ and isobutyramide[2]. During the early phase of this work, protein concentration was estimated from absorbancy measurements at 280 mμ. A value of 19.3×10^4 cm^{-1} M^{-1} was used as the absorbancy index of the crystalline enzyme[3] and the molecular weight was taken as 135 000. The enzyme was assumed to be pure fraction "A" when stock solutions had an E_{280} which in relation to the activity determined either from rate measurements or from titration in the presence of oxalate, had an absorbancy index of $19 \pm 0.5 \times 10^4$ cm^{-1} M^{-1}.

Coenzymes and reagents. β-$NADH_2$ was purchased from the Sigma Chemical Co. and assayed with yeast alcohol dehydrogenase. The β-$NADH_2$ used was 72 % by weight using an absorbancy index of 6.22×10^3 cm^{-1} M^{-1} at 340 mμ. The residual fluorescence after enzymatic oxidation was 5 % which was corrected for in all calculations. Concentrations of β-$NADH_2$ were used which showed no fluorescence quenching as described previously[14]. Stock solutions of $NADH_2$ were prepared in Tris-KCl buffer, ionic strength 0.2, pH 10 and working solutions made in the desired buffer directly before use and stored in the dark at 3°C. β-NAD was purchased from the Sigma Chemical Co. and was assayed according to the method of Dalziel[15]. It was found to be 97 % by weight using an absorbancy index at 260 mμ of 18.3×10^3 cm^{-1} M^{-1}. The 3-acetyl pyridine analog of NAD (APNAD) (from Sigma) was 95 % by weight assuming an absorbancy index at 260 mμ of 16.4×10^3 cm^{-1} M^{-1}. Stock solutions of coenzymes were prepared in water and titrated to pH 6 with sodium hydroxide. These solutions were used directly in both the fluorescence titrations and absorbancy measurements and were stable for days in the cold at 3°C. All buffers contained 0.001 M EDTA and were prepared in 2× quartz distilled water. Potassium pyruvate, L-lactate and oxalate were prepared as described earlier[3].

Methods. Fluorescence measurements were performed on a Farrand Model A recording fluorimeter equipped with a water jacketted cell compartment. A Corning primary filter No. c. s. 7–60 was used at the excitation entrance and a secondary filter No. c. s. 3–74 at the fluorescence emission exit. All fluorescence measurements were made at 28.5°C in either 0.2 ionic strength phosphate or Tris-KCl buffers unless otherwise stated. The temperature in the cells was measured with a probe electronic thermometer and pH measurements were made with a Beckman combination probe electrode. The excitation wave length was 340 mμ and the fluorescence intensity measured at 420 mμ. Corrections were made on both the activation and emission spectra. Although the apparent maximum fluorescence emission of $NADH_2$ is at 465–470 mμ on this instrument and at the settings employed, 420 mμ was used for recording fluorescence intensity since the deflection ratio of bound to free coenzyme is considerably higher at these wave length settings *i. e.* Q (see Ref. 1) is 7.6 and independent of pH from 6 to 10. A final volume of 2.00 ml in 1×1 cm quartz cuvettes was used in all titrations with addition of the coenzymes from a microtitrator. Spectrophotometric measurements were made on a Cary Model 11 equipped with a constant temperature sample cell compartment.*

Evaluation of $K_{E,R}$. As has been reported with liver ADH[1], the apparent dissociation constants of EDH · $NADH_2$ were evaluated either by single additions of $NADH_2$ to a constant enzyme concentration (about twice $K_{E,R}$) or in experiments where Q is to be determined, additions of enzyme to a constant amount of $NADH_2$.

The first method leads to the compound E · $(NADH_2)_4$ at infinite $NADH_2$ concentration and the second method leads to the formation of the compound E · $NADH_2$ at infinite enzyme concentration. Q was independently determined over the whole pH range investigated and found to be 7.6 ± 0.5. Values of $K_{E,R}$ as determined by titration were averaged from 5–6 points and the absence of any systematic drift gives credence to the equivalence of the four coenzyme binding sites per 135 000 g of protein.

* Constructed by Mr. Thomas Orr of the Research Machine Shop.

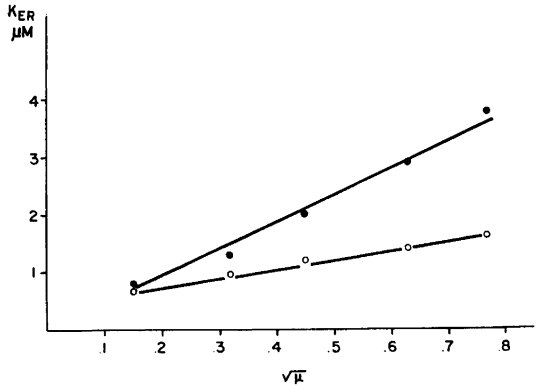

Fig. 1. Variation of $K_{E,R}$ with ionic strength of phosphate (O) and Tris-KCl (●) buffer, at 28.5°C.

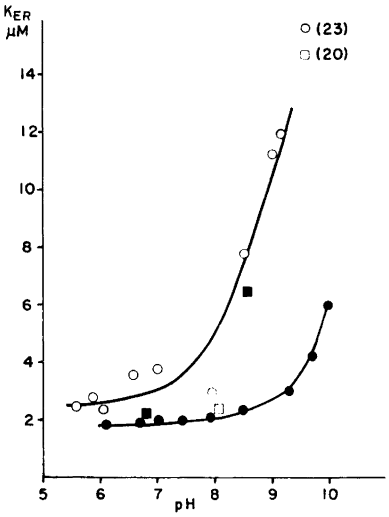

Fig. 2. Comparison of $K_{E,R}$ with pH in Tris KCl buffer, $\mu = 0.2$, at 28.5°C as determined fluorometrically (●), kinetically from K_{RP}/K_P (O), from *p*MB inhibition experiments (□), and from competitive inhibition experiments (■). For definitions of the Michaelis and rate constants see Ref[12].

RESULTS

It has been reported that the bovine heart LDH system is very susceptible to anion effects[16]* and the magnitude of the kinetic constants depends markedly on the buffer system used. For example, the Michaelis constants are approximately ten times greater in Tris-KCl buffer than in phosphate buffer of identical pH and ionic strength. Earlier kinetic studies[17] had indicated that $K_{E,R}$ in 0.1 M phosphate buffer was about half as large as it was later shown to be in Tris-KCl buffer of the same concentration[12]. The dependence of $K_{E,R}$ on the ionic strength of phosphate and Tris-KCl buffers is shown in Fig. 1. The value of $K_{E,R}$ at an ionic strength of 0.2 is about twice as great in Tris-KCl as in phosphae, and at an ionic strength of 0.125, the values of $K_{E,R}$ approach one another to about 0.8 μM. As seen in the lower curve of Fig. 2 values of $K_{E,R}$ as determined in the present fluorescence studies range from 2.0 μM at pH 6 to 6.3 μM at pH 10. The upper curve shows the variation of $K_{E,R}$ with pH as calculated from a variety of kinetic studies, as defined in the legend, and the values range from 2.2 μM at pH 5.8 to

* Buffers such as Tris-acetate have been used to evaluate $K_{E,R}$[18] and these values are undoubtedly low since acetate (I), as an inhibitor, would have the effect of lowering the apparent dissociation constant, thus $K_{ER,I} \ll K_{E,R}$.

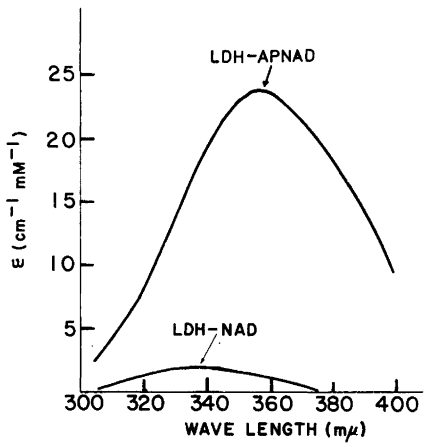

Fig. 3. Difference spectra of the LDH·NAD and LDH·APNAD compounds in Tris-KCl buffer, pH 8.15, μ=0.2, 28.5°C. The spectra have been corrected for absorbancy due only to enzyme or to coenzyme and represent enzyme saturated with coenzyme.

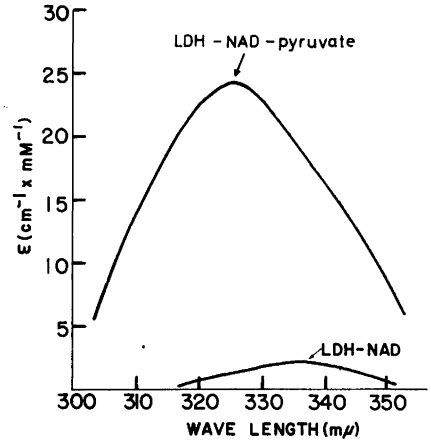

Fig. 4. Difference spectra of the LDH·NAD and LDH·NAD·pyruvate compounds in Tris-KCl buffer, pH 8.15, μ=0.2, 28.5°C. The spectra have been corrected for absorbancy due only to enzyme, pyruvate or NAD and represent enzyme saturated with NAD or NAD and pyruvate.

23 at pH 9.8. Independent determinations of $K_{E,R}$ from kinetic experiments in which LDH is protected from *p*MB inactivation in the presence of $NADH_2$ gave values of 2.2 μM at pH 8.03 and 20 μM at pH 10.02, while values obtained from experiments in which NAD and $NADH_2$ compete for the same site on the enzyme were 2.2 μM at pH 6.8 and 6.3 μM at pH 8.5.

When an attempt was made to determine $K_{E,O}$ by the addition of NAD to the enzyme prior to $NADH_2$ additions, as described earlier with alcohol dehydrogenase[1], it was observed that solutions of enzyme and oxidized coenzyme, when activated by 340 mμ light, exhibit a fluorescence emission spectrum with a maximum at about 450 mμ*. A spectrophotometric maximum was also observed at 335 mμ as was the compound LDH · APNAD with a wave length maximum of 355 mμ as shown in Fig. 3. The millimolar absorbancy index of the LDH · APNAD compound is about 10 times greater than that of the LDH · NAD compound. Formation of both binary compounds is pH dependent, and, at equilibrium, a greater amount of fluorescence or absorbancy is observed at higher than at lower pH values. Determination of the apparent dissociation constants from the concentrations of added coenzyme required to obtain half maximal fluorescence or optical density gave values in Tris-KCl buffer, pH 8.15 of 8.0×10^{-5} M for LDH · NAD and 0.9×10^{-5} M for LDH · APNAD. Titrations with APNAD, in which the equilibrium is very much more favorable than NAD, indicated that 3.7 moles of coenzyme analog were bound per mole of enzyme. When pyruvate

* A complete report on the properties of the binary LDH · NAD and ternary LDH · NAD · pyruvate compounds is in preparation and will be reported elsewhere.

is added to the LDH · NAD compound, a new absorbancy maximum is seen at 325 mμ as shown in Fig. 4. The millimolar absorbancy index of the LDH · NAD · pyruvate compound is 24 and the apparent dissociation constant 5×10^{-4} M under the conditions of the experiment as indicated in the legend of Fig. 4. Formation of the LDH · NAD · pyruvate compound at equilibrium is pH dependent and favored in alkaline solution. This compound is probably identical to the one recently reported by Fromm[19] with rabbit muscle LDH.

DISCUSSION

The fluorometric titration measurements of the apparent dissociation constants of LDH · $NADH_2$ from pH 6 to 8 in 0.2 ionic strength Tris-KCl buffer appear to be in fairly good agreement with those constants calculated from kinetic parameters as derived from a reaction mechanism including significant ternary compounds. That such equilibrium constants do indeed represent quantities which can be compared to the specifically defined dissociation constants may be questioned. The fact that these constants show rather good agreement from pH 6 to 8 with the independent kinetic evaluations of $K_{E,R}$ from *p*MB or competitive coenzyme inhibition experiments may be fortuitous. However, the stoichiometry of coenzyme binding by fluorescence techniques is in agreement with the stoichiometry as determined by ultracentrifuge binding studies[3]. Also oxamate and oxalate, inhibitors of the enzymatic reaction, in that they form ternary compounds E · NADH · oxamate and LDH · NAD · oxalate, respectively, show similar dissociation constants whether estimated from fluorescence[20] or ultracentrifuge binding data[4]. The existence of the LDH · NAD compound has previously been assumed from competitive binding experiments[12] and ultracentrifuge separation data[3] but this is the first direct fluorometric and spectrophotometric demonstration of this binary compound. The single value of $K_{E,O}$ determined by absorbancy measurements is about 2× lower than the corresponding value as calculated from kinetic parameters[12]. A more complete determination of $K_{E,O}$ over a wide range of pH will permit a comparison of these constants with those calculated from kinetic parameters and this work is currently in progress.

The fluorometric detection of the ternary compound LDH · NADH · L-lactate has been reported at low pH values[21] and the compound LDH · NAD · pyruvate with rabbit muscle enzyme has recently been reported at pH 7.6 in Tris-chloride buffer by Fromm[19] using difference spectrophotometry. The concentration of pyruvate used to detect this compound, however, was sufficient to produce complete inhibition of the enzymatic reaction at the pH employed[12]. The concentrations of pyruvate used in the present studies were of the order of the Michaelis constant and the equilibrium constant for the dissociation of the LDH · NAD · pyruvate compound to give pyruvate and LDH · NAD is also of the order of the Michaelis constant for pyruvate.

Recently, the kinetic importance of ternary compounds in the mechanism of dehydrogenases was shown experimentally by the product inhibition method first proposed by Alberty[22]. Fromm and co-workers[5,7] have found inhibition data for ribitol and muscle lactate dehydrogenases to be consistent with the formation of inactive ternary compounds between enzyme, coenzyme, and the wrong substrate, but only when relatively high concentrations of the product inhibitor

were employed. However, whether the formation of the inactive compound of LDH · NAD · pyruvate can account fully for the difference in $K_{E,R}$ observed fluorimetrically and kinetically in the present studies at higher pH values cannot be answered at this time.

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REFERENCES

1. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
2. Theorell, H. and McKinley-McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1811.
3. Takenaka, Y. and Schwert, G. W. *J. Biol. Chem.* **223** (1956) 157.
4. Novoa, W. B. and Schwert, G. W. *J. Biol. Chem.* **236** (1961) 2150.
5. Fromm, H. J. and Nelson, D. R. *J. Biol. Chem.* **237** (1962) 215.
6. Raval, D. N. and Wolfe, R. G. *Biochemistry* **1** (1962) 215.
7. Nygaard, A. P. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1300.
8. Zewe, V. and Fromm, H. J. *J. Biol. Chem.* **237** (1962) 1668.
9. Vestling, C. S. *Methods of Biochem. Analysis,* Interscience, New York, 1962, Vol. X, p. 137.
10. Bloomfield, V., Peller, L. and Alberty, R. A. *J. Am. Chem. Soc.* **84** (1962) 4367.
11. Baker, R. H., Jr. *Biochemistry* **1** (1962) 41.
12. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **231** (1958) 1065.
13. Schwert, G. W., Millar, D. B. S. and Takenaka, Y. *J. Biol. Chem.* **237** (1962) 2131.
14. Winer, A. D., Schwert, G. W. and Millar, D. B. S. *J. Biol. Chem.* **234** (1959) 1149.
15. Dalziel, K. *Biochem. J.* **84** (1962) 244.
16. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1155.
17. Hakala, M. T., Glaid, A. J. and Schwert, G. W. *J. Biol. Chem.* **221** (1956) 191.
18. Velick, S. F. *J. Biol. Chem.* **233** (1958) 1455.
19. Fromm, H. J. *Biochim. Biophys. Acta* **52** (1961) 199.
20. Novoa, W. B., Winer, A. D., Glaid, A. J. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1143.
21. Winer, A. D., Novoa, W. B. and Schwert, G. W. *J. Am. Chem. Soc.* **79** (1957) 6571.
22. Alberty, R. A. *J. Am. Chem. Soc.* **80** (1958) 1777.

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