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Studies on Liver Alcohol Dehydrogenase Complexes

I. The Coenzyme-Binding Sites and Effects of Adenosine Diphosphate Ribose and *o*-Phenanthroline

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ADPR*, a potent inhibitor of LADH, competes strictly with NAD+ and NADH for LADH. The LADH–ADPR interaction is independent of substrates and PHL. LADH, ADPR, and PHL seem to form a mosaic complex (PhEA), in which ADPR and PHL are independently attached to LADH at their own binding sites of the enzyme. It is concluded that the ADPR moiety of the coenzyme is the site of the enzyme-coenzyme binding common to NAD+ and NADH, that Zn of LADH is not involved in the binding between LADH and the ADPR moiety of the coenzyme, and that the interaction of LADH with substrate-analogue-inhibitors and the dihydronicotinamide- or nicotiniumamide** -moiety of the coenzyme occurs at or near Zn.

Over the past decade, a number of studies have been reported in order to elucidate the mode of interaction between LADH and the coenzyme (NAD+ and NADH)[1–11]. It is generally agreed that the dissociation step of the enzyme-coenzyme complexes (EO and ER) is a rate-limiting one of the LADH reactions[1]. The formation of ER is characterized by a shift in the absorption maximum of NADH[2] and by an intensification and a band shift of the NADH-fluorescence[3]. NAD+ and NADH compete with one another for LADH[4]. These observations made it possible to determine the dissociation constants of ER ($K_{E,R}$) and EO ($K_{E,O}$) by spectrophotometric and spectrophotofluorometric methods[1,4,5]. Analyses of pH-dependence of $K_{E,R}$ and $K_{E,O}$ have revealed the nature of the ionic groups involved in the enzyme-coenzyme binding[4–6]. Studies of the inhibition of LADH by PHL[7–9] and of the complex formation between LADH and PHL[10,11] led to an assumption that Zn of LADH is the site of the enzyme-coenzyme binding[5,7–11].

* Abbreviations: LADH (E = ½ LADH), liver alcohol dehydrogenase; NAD+ (O), nicotiniumamide-adenine dinucleotide; NADH (R), dihydronicotinamide-adenine dinucleotide; ADPR (A), adenosine diphosphate ribose; PHL (Ph), *o*-phenanthroline; (I), isobutyramide; (Py), pyrazole; TN, turnover number. Letters in parentheses are simplified abbreviations used in expressing dissociation constants and forms of LADH complexes.

** 3-Carbamyl pyridinium is referred to as nicotiniumamide group[11a].

The marked protection of LADH by NADH and by NADH + isobutyramide against inactivation indicates that LADH and NADH interact at more than one site[12]. Inhibitors structurally analogous to the coenzyme will be useful in the study of the enzyme-coenzyme interaction. ADPR is one of potent LADH-inhibitors of this type[13,14].

This paper deals with a study of the interaction between LADH and ADPR. The competition between ADPR and the coenzyme for LADH as well as the interaction of LADH with ADPR and PHL have been studied by kinetic and equilibrium measurements.

EXPERIMENTAL

Materials. Crystalline LADH was prepared according to a modification of Dalziel's method[15]. The enzyme concentration was expressed as N, the normality of NADH-binding sites per l and was determined spectrophoto-fluorometrically[12]. The purity of the enzyme preparation was found to be 100 % on the basis of an absorbancy index at 280 mμ of 0.42 ml/mg × cm^{-1} [15] and a molecular weight = 84 000 per two binding sites[16]. NADH ($\varepsilon_{340\ m\mu} = 6.22\ mM^{-1} \times cm^{-1}$), ADPR ($\varepsilon_{259\ m\mu} = 16.0\ mM^{-1} \times cm^{-1}$), adenine, and adenosine were purchased from Pabst Laboratories. NAD+ ($\varepsilon_{259\ m\mu} = 18.0\ mM^{-1} \times cm^{-1}$), 1-methyl nicotiniumamide iodide ($\varepsilon_{265\ m\mu} = 4.2\ mM^{-1} \times cm^{-1}$), nicotiniumamide mononucleotide ($\varepsilon_{266\ m\mu} = 4.6\ mM^{-1} \times cm^{-1}$), AMP, ADP, and ATP were obtained from Sigma Chemical Co. Isobutyramide and *o*-phenanthroline were products of Eastman Organic Chemicals. Pyrazole was kindly supplied by Dr. B. Sjögren, AB. Astra, Sweden. Most of the experiments were performed in sodium phosphate buffer, pH 7.0, ionic strength 0.1. The pH-dependence of $K_{E,A}$ and K_i was determined in sodium phosphate buffer, ionic strength 0.1 (pH 6.0, 7.0, and 8.0) and in 0.1 M sodium glycine buffer (pH 9.0 and 10.0).

Methods. The inhibition of LADH by ADPR was studied fluorometrically by measuring the initial rates of the forward and reverse reactions of LADH with varied concentrations of NADH, acetyldehyde, NAD+, and ethanol at fixed concentrations of their reaction partners (Cases 1 to 4, respectively)[5,6] in the presence of ADPR. The competitive inhibitor constant of ADPR (K_i) was calculated from the slopes of Lineweaver-Burk plots[17]. The LADH-ADPR binding was studied spectrophotofluorometrically by titrating LADH with NADH[4,5] in the presence of ADPR. This is based on the strict competition between ADPR and NADH for the coenzyme-binding sites of LADH. The data from the titration were fed into a Wegematic 1000 digital computer which was programmed to calculate $K_{E,R}$ and apparent $K_{E,R}$ according to Theorell-Winer equations[4]. The dissociation constant of EA ($K_{E,A}$) was calculated from the apparent $K_{E,R}$ in the presence of ADPR and [ADPR] by analogy to the $K_{E,O}$ calculation[4,5]. No quenching of the NADH-fluorescence by ADPR was observed under the experimental conditions. The interaction between ADPR and LADH-complexes was studied by *double difference spectrophotometry*[18]. In this method, two pairs of cuvettes were used in order to subtract unnecessary light-absorption of the complexes and reductants and to observe small light-absorption changes in strongly light-absorbing backgrounds. A recording fluorometer[6], a recording spectrophotofluorometer[19], a Beckman DK-2 recording spectrophotometer, and a Radiometer pH-meter model 25 were used. All experiments were performed at 23.5°C.

RESULTS AND DISCUSSION

Lineweaver-Burk plots of initial rates of forward and reverse reactions of LADH in the presence and absence of ADPR revealed that ADPR is strictly competitive with both NAD+ and NADH, and is non- and un-competitive with ethanol and acetaldehyde, respectively. K_i values of ADPR are given in Table 1, together with $K_{E,A}$ values which were determined by the equilibrium titration in the absence of substrates. Three independently determined constants of ADPR, *i. e.*, K_i's for forward and reverse reactions and $K_{E,A}$, are in good agreement with each other at any pH values tested within the limits of experimental

Table 1. Competitive inhibitor constants (K_i) and dissociation constant ($K_{E,A}$) of ADPR.

	Kinetic constants						Equilibrium constants		
	NAD+ reduction [a]			NADH oxidation [b]					
pH	TN_{max} sec^{-1}	$K_{m(O)}$ [c] μM	K_i μM	TN_{max} sec^{-1}	$K_{m(R)}$ [c] μM	K_i μM	$K_{E,A}$ [d] μM	$K_{(E,R)}$ [e] μM	$K_{E,O}$ [e] μM
6.0	1.18	16	9.3	58	5.9	12	8.7	0.23	266
7.0	2.93	14	20	66	8.3	17	16	0.31	160
8.0	3.70	11	31	48	9.1	21	21	0.41	51
9.0	4.44	13	64	11	3.3	40	36	0.65	12
10.0	4.65	29	128	1.0	3.0	100	145	5.0 [f]	10 [f]

[a] 2.7×10^{-8} N LADH, 6.2 mM ethanol and $\pm$ 50μM ADPR with varied NAD+ (10, 20, 50, 100, 200, and 500 μM).
[b] 3.0×10^{-9} N LADH, 1.1 mM acetaldehyde and $\pm$ 50 μM ADPR with varied NADH (2, 5, 10, and 20 μM).
[c] In the absence of ADPR.
[d] 1.10 μN LADH was titrated with 8 successive additions of 0.487 μM NADH in the presence and absence of ADPR (5, 10, 20, 50, 100, 200, or 400 μM).
[e] From Theorell and McKee[5].
[f] From Theorell and Winer[4].

error. These observations indicate that the *LADH-ADPR interaction is independent of substrates,* and that *the ADPR moiety of the coenzyme is the site of the enzyme-coenzyme binding common to NAD+ and NADH.* This conclusion was further supported by the following experiments. The 325-mμ absorption maximum of ER was shifted toward 340 mμ upon an addition of excess ADPR. Similarly, the intensity of the 290-mμ absorption maximum of PyEO[20] decreased appreciably upon the addition of excess ADPR. Nicotinamide, 1-methyl nicotiniumamide iodide, nicotiniumamide mononucleotide, adenine, adenosine, AMP, ADP, and ATP were inactive as a potent inhibitor of LADH at pH 7.0. A combination of ADPR and each one of these substances did not affect the inhibitory effect of ADPR. Thus *the ADPR moiety seems to be a minimal structure required for the unique firm binding of the coenzyme with LADH.*

Although $K_{E,A}$ values are considerably higher than $K_{E,R}$ values, the effect of pH on $K_{E,A}$ and $K_{E,R}$ are remarkably similar: both are relatively pH-independent at pH 6 to pH 8 and rise suddenly above pH 9[4–6]. Therefore, *the general features of pH-dependence of $K_{E,R}$ should be attributed to the interaction between LADH and the ADPR moiety of NADH. The additional interaction between LADH and the dihydronicotinamide moiety of NADH may be responsible for the further stabilization of the fundamental binding between LADH and the ADPR moiety of the coenzyme.* The positively-charged nicotiniumamide moiety of NAD+, therefore, is responsible for the considerable difference in pH-dependence between $K_{E,A}$ and $K_{E,O}$. The fact that EO dissociates more at lower pH values suggests that *the ionic groups which become more positively-charged at lower pH values, such as Zn-OH^-, are involved in the interaction between LADH and the*

functional group of the coenzyme, i. e., the nicotiniumamide- or dihydronicotinamide-moiety, as suggested previously by Theorell *et al.*[4–6]

PHL, a metal-chelating agent, is a potent inhibitor of LADH, competing with the coenzyme[7–9] and with substrates[8,9], and acting on Zn of the enzyme[10,11]. A kinetic study of the LADH inhibition by a mixture of ADPR and PHL revealed that the inhibitory effects of ADPR and PHL are strictly independent of one another. Thus, these inhibitors seem to interact with LADH at different sites, though both are competitive with the same substance, the coenzyme.

Interactions of LADH with the coenzyme, substrate-analogue-inhibitors, ADPR, and PHL were studied by means of double difference spectrophotometry as described in details elsewhere[18]. The LADH-PHL interaction was previously studied by ordinary difference spectrophotometry[10,11], in which extra absorption maxima due to the Zn-PHL chelation were observed above strong absorption of the enzyme protein. Because of the interference of strong absorption at around 280 mμ due to aromatic amino acids, the 297-mμ absorption maximum of the Zn-PHL chelate was not observed in the case of Zn-containing enzymes[10]. By double difference spectrophotometry, however, extra absorption maxima at 297, 329, and 345 mμ due to the chelation between LADH-bound Zn and PHL were

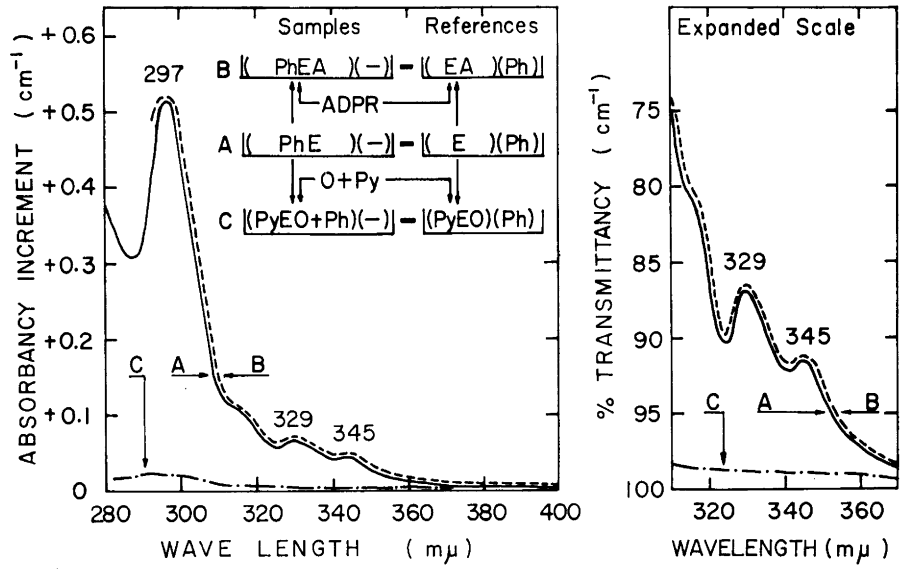

Fig. 1. Interactions of PHL-LADH complex with ADPR and with NAD+ + pyrazole. Measurements are performed at pH 7.0 with a Beckman DK-2 spectrophotometer which is set at 0–1.0 absorbancy or 75–125 % transmittancy scales. Reactant concentrations are 100 μN LADH (E), 100 μM PHL (Ph), 100 μM NAD+ (0), 500 μM ADPR (A), and 1 mM pyrazole (Py). A pair of sample cuvettes and a pair of reference cuvettes are used: the contents of each cuvette are indicated in parentheses in the figure. A, PhE (or Zn-PHL) absorption spectrum. B, PhEA absorption spectrum: ADPR has no effect on the Zn-PHL absorption. The spectrum below 295 mμ was not measurable because of too strong absorption of 500 μM ADPR. C, the Zn-PHL absorption disappears upon the addition of NAD+ + pyrazole. A similar effect is also observed with NADH + isobutyramide.

distinctly observable, because strong absorptions of both protein and PHL were simultaneously subtracted by the references (see details Fig. 1, A). Since values of $K_{E,A}$ and $K_{E,Ph}$ (the dissociation constant of PhE) are in the same order of magnitude, *i. e.*, $K_{E.A} = 16\ \mu M$ (pH 7.0, Table 1) and $K_{E,Ph} = 8.0\ \mu M$ (pH 7.0)[18], one can expect a partial disappearance of the Zn-PHL absorption upon additions of ADPR to PhE, provided ADPR and PHL compete for the same sites of LADH. However, upon the addition of ADPR, even in a large excess, to PhE, no change in the Zn-PHL absorption was observed (Fig. 1, B). This result further confirms the above-mentioned assumption that *ADPR and PHL independently interact with LADH at their own binding sites.* Judging from $K_{E,A}$ and $K_{E,Ph}$ values it seems likely that ADPR and PHL can attach simultaneously to the same molecule of LADH to form a ternary complex, PhEA, in which ADPR and PHL have no direct interaction between them. Thus this ternary complex, PhEA, is definitely different from ternary complexes of LADH, substrate-analogue-inhibitors, and the coenzyme, such as IER[5,12,21] and PyEO[20], in which three components are assumed to be bound with each other. *The complex, PhEA,* can be considered *to be a mosaic or hybrid complex of two binary complexes, PhE and EA.* Therefore, *the interaction between Zn of LADH and the adenine moiety of the coenzyme in the LADH-coenzyme complexes*[5,22,23] seems unlikely.

Because of the coenzyme-competitive inhibition[7–9] and the bound-Zn specific chelation[10,11] of PHL, Zn of LADH has been inductively assumed to be the site of the coenzyme-enzyme binding. Now it is possible to present more direct evidence for this assumption. Upon the addition of NADH, a partial disappearance of the Zn-PHL absorption of PhE was observed. The Zn-PHL absorption of PhE disappeared completely upon additions of either NADH + isobutyramide or NAD^+ + pyrazole (Fig. 1, C). This suggests that *the interaction of LADH with substrate-analogue-inhibitors and the nicotiniumamide- or dihydronicotinamide-moiety of the coenzyme occurs at or near Zn of the enzyme.* This is not only *direct evidence for the identification of Zn of LADH as the coenzyme-binding site,* but also provides *a convenient method to distinguish the enzymically essential Zn from extraneous Zn in LADH preparations.*

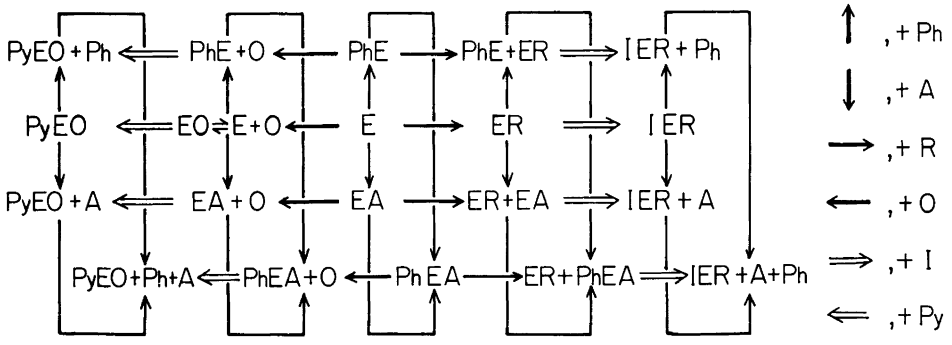

Fig. 2. Enzymically inactive equilibria of LADH (E), NAD+ (0), NADH (R) ADPR (A), PHL (Ph), pyrazole (Py), and isobutyramide (I). Although they are indicated by one-directioned arrows, all the reactions are fully reversible.

Further studies of interactions of LADH with coenzyme and inhibitors[18] have revealed that enzymically inactive equilibria, as shown in Fig. 2, exist among these multiple components. These equilibria are controlled by dissociation constants of the corresponding binary and ternary complexes. Since all of these constants have been determined by extensive kinetic- and equilibrium experiments[1,2,4–6,11,18–21], it is possible to calculate the amount of a complex present in a particular equilibrium mixture from the reactant concentrations.

In despite of many hypotheses[5,6,22–26], there has been no experimental evidence to show which part of the nicotiniumamide- or dihydronicotinamide-moiety of the coenzyme interacts with LADH and substrates at or near Zn of the enzyme. Further experiments are needed before presenting a convincing picture of the mode of their interaction. The nicotiniumamide modified analogues of the coenzyme, such as 3-acetyl-, 3-aldehyde-, and 3-thiocarbamyl-pyridinium-adenine dinucleotides[26], may be useful for this purpose.

The formation of the hypothetical mosaic-complex, PhEA, has been proved by the crystallization of PhEA as well as EA and PhE[18].

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REFERENCES

1. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.
2. Theorell, H. and Bonnichsen, R. *Acta Chem. Scand.* **5** (1951) 1105.
3. Boyer, P. D. and Theorell, H. *Acta Chem. Scand.* **10** (1955) 447.
4. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
5. Theorell, H. and McKinley McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1797, 1811, 1834.
6. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
7. Vallee, B. L., Williams, R. J. P. and Hoch, F. L. *J. Biol. Chem.* **234** (1959) 2621.
8. Plane, R. A. and Theorell, H. *Acta Chem. Scand.* **15** (1961) 1866.
9. Dalziel, K. *Nature* **197** (1963) 462.
10. Vallee, B. L., Coombs, T. L. and Williams, R. J. P. *J. Am. Chem. Soc.* **80** (1958) 397.
11. Vallee, B. L. and Coombs, T. L. *J. Biol. Chem.* **234** (1959) 2615.
11a. Yonetani, T. *Acta Chem. Scand.* **17** (1963) 855.
12. Yonetani, T. and Theorell, H. *Arch. Biochem. Biophys.* **99** (1962) 433.
13. Dalziel, K. *Nature* **191** (1961) 1098.
14. Dalziel, K. *J. Biol. Chem.* **237** (1963) 1538.
15. Dalziel, K. *Acta Chem. Scand.* **12** (1958) 459.
16. Ehrenberg, A. and Dalziel, K. *Acta Chem. Scand.* **12** (1958) 465.
17. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
18. Yonetani, T. *Biochem. Z.* (1963). *In press.*
19. Ehrenberg, A. and Theorell, H. In Florkin, M. and Stotz, E. H. *Comprehensive Biochemistry,* Elsevier Publishing Co., Amsterdam, 1962, Vol. III, p. 187.
20. Theorell, H. and Yonetani, T. *Biochem. Z.* (1963). *In press.*
21. Winer, A. D. and Theorell, H. *Acta Chem. Scand.* **14** (1960) 1729.
22. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 59.
23. Mahler, H. R. and Douglas, J. *J. Am. Chem. Soc.* **79** (1957) 1159.
24. Rabin, B. R. and Whitehead, E. P. *Nature* **196** (1962) 658.
25. Dixon, M. and Webb, E. C. *Enzymes,* Longmans Green Co., London, 1958, p. 351.
26. Kaplan, N. O. *The Enzymes,* 2nd Ed., Academic Press, New York, 1960, Vol III, p. 105.

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