EVIDENCE FOR TWO DIFFERENT COMPLEXES OF LIVER ALCOHOL DEHYDROGENASE WITH IMIDAZOLE

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1. Introduction

Alcohol dehydrogenase (EC 1.1.1.1.) from horse liver forms highly-fluorescent complexes with NADH and imidazole [1]. Enhancement of fluorescence of enzyme-bound NADH was used to estimate dissociation constants of binary and ternary complexes, at pH values of 6, 7, 8 and 9. Imidazole also stimulates inactivation of the enzyme by iodoacetate [2, 3]; dissociation constants of the binary enzyme–imidazole complex were calculated at pH 7.2 and 9.0, and 7.4 and 10.0, respectively. These two methods gave similar values in the pH region 7 – 8, but the discrepancy at pH 9 was considerable. This prompted an examination by fluorescence of the binding properties at pH 10, reported here; a discrepancy was also found. This indicates that the different methods are probably measuring different complexes of imidazole with the enzyme.

2. Materials and methods

The source, assay and purity of horse liver alcohol dehydrogenase were as described previously [4]. Imidazole [1] and other materials [3] were as used previously. Fluorescence measurements were made using a Farrand spectrophotofluorimeter (Farrand Optical Co. Inc., New York) with recorder. The method was similar to that used previously [1]; experimental details are to be published [5, 6].

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3. Results

The results of fluorescence titrations of enzyme with NADH at pH 10, in the presence and absence of imidazole, are shown in fig. 1. This graphical method of presenting results was used by McKay and Kaplan [7]; the negative of the gradient gives the apparent dissociation constant of NADH from the enzyme. The concentration of imidazole used (90 mM) was sufficient to produce greater than 95% saturation of the enzyme. Titration of fixed amounts of enzyme and NADH with aliquots of imidazole is shown in fig. 2. During this titration, the amount of enzyme-bound NADH decreased slightly, because imidazole weakens the binding of NADH to the enzyme. However, due to the very high fluorescence of the enzyme–NADH–imidazole ternary complex, this has a negligible effect on the estimated dissociation constant of imidazole from the ternary complex.

Since $K_{E,I} = \frac{K_{E,R}}{K_{EI,R}} \times K_{ER,I}$, $K_{E,I}$ can be calculated.

(The notation is as used previously [1]).

The calculated values, together with those from earlier work, are given in table 1. The values calculated from stimulation of inactivation at pH 7.2 and 9.0 [2] are probably slightly too high, because of reversible binding by iodoacetate [3].

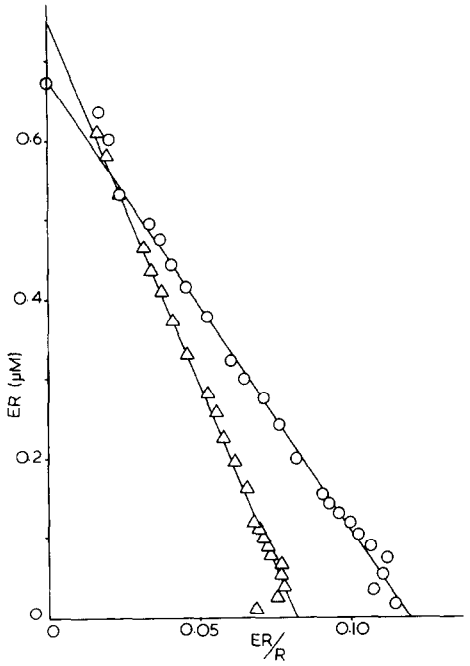

Fig. 1. Binding of NADH to liver alcohol dehydrogenase at pH 10.0. (o–o–o): Absence of imidazole; (Δ–Δ–Δ): presence of 90 mM imidazole. ER represents the concentration of enzyme-bound NADH, and R the concentration of free NADH, determined by fluorescence enhancement. Excitation, 325 nm; emission, 410 nm. Buffer was glycine, 20 mM NaOH and disodium phosphate, ionic strength 0.10, at 23.5°.

4. Discussion

The results in table 1, particularly at pH 9 and 10, indicate that the two different methods are probably measuring different imidazole–enzyme complexes. The possibility of more than one binding-site for imidazole was raised in connection with inactivation kinetic data [3]. Temperature-jump experiments [8] indicated more than one type of ternary complex of imidazole, enzyme and NADH.

Liver alcohol dehydrogenase has 2 zinc atoms per subunit [9]. Bidentate ligands such as orthophenanthroline can only bind to one of these metal atoms, the "functional" zinc [10], and they are displaced by monodentate ligands such as imidazole [3, 11]. Presumably, it would be possible for imidazole to occupy either of the 2 ligand sites used by bidentate chelating agents. Another possibility is that imidazole might be able to bind to the other ("structural") zinc atom in the enzyme, which is inaccessible to bidentate chelating agents. Interaction with the "structural" metal atom could perhaps be detected spectrally by using hydrid zinc–cobalt enzyme [12]. It is also possible that imidazole may bind elsewhere than to a metal atom in the enzyme.

The dissociation constant of NADH from the enzyme is now clearly seen not to become pH-independent in the presence of imidazole. This is as expected from the binding of adenine nucleotides [3]. The apparent invariance of $K_{ER,I}$ with pH indicates that the ionisation (or ionisations) which affect the binding of imidazole are altered considerably by the binding of NADH.

In earlier work [1–3], imidazole was considered to replace a zinc-bound water-molecule; the latter would ionise at high pH, and a pH effect would be suppressed by imidazole. While not invalidating the earlier work, the results presented here indicate that

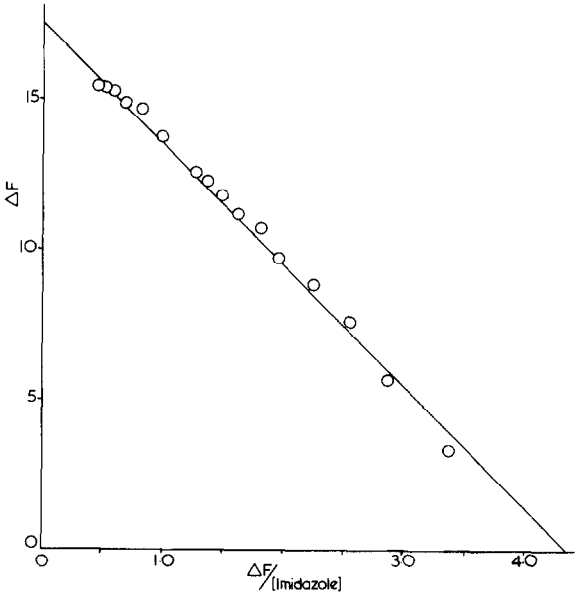

Fig. 2. The effect of imidazole on fluorescence of enzyme-bound NADH at pH 10.0. ΔF represents increase in fluorescence (arbitrary units), and the imidazole concentration is in millimolar units. Enzyme binding-sites, 3.3 μM, and NADH 7.6 μM. Conditions as in fig. 1.

Table 1

Dissociation constants.

Method	pH	$K_{E,R}$ (μM)	$K_{EI,R}$ (μM)	$K_{ER,I}$ (mM)	$K_{E,I}$ (mM)	Reference
Stimulation of inactivation	7.2	–	–	–	0.7	[2]
	9.0	–	–	–	5.4	
Stimulation of inactivation	7.4	–	–	–	0.86	[3]
	10.0				5.9	
Fluorescence enhancement	6.0	0.23	2.01	4.2	0.47	[1]
	7.0	0.31	2.03	3.5	0.55	
	8.0	0.41	2.20	3.6	0.68	
	9.0	0.65	2.91	3.0	0.67	
Fluorescence enhancement	10.0	5.5	9.4	4.0	2.36	This work.

R represents NADH, and I imidazole (base form).

caution in interpreting the effect of imidazole is to be advised.

Acknowledgement

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