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Inhibition of alcohol dehydrogenases by thiol compounds

Li-Yao Cheng and Lee-Hua Lek

Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Republic of Singapore

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2-Mercaptoethanol is a strong inhibitor of LADH. The inhibitory effect is likely due to the binding of the SH group to the enzymatic zinc ion. Various thiol compounds do not inhibit YADH and it is suggested that the zinc atoms involved in the catalytic mechanism of LADH and YADH may have different structural arrangements and that these zinc atoms in YADH may not be blocked by thiol compounds. Thiol compounds also quench the enhanced fluorescence of LADH–NADH in a pH-dependent manner. At pH 9.2, the binding of coenzyme to LADH is replaced by 2-mercaptoethanol, whilst at pH 7.3, it further quenches the fluorescence of NADH–LADH. This quenching of fluorescence is likely attributed to a conformational change and energy transfer upon binding of 2-mercaptoethanol to the LADH-NADH complex. Complete reversal of the inhibitory effect of thiol compounds on LADH can be obtained by dialysis.

Alcohol dehydrogenase; Inhibition; Mercaptan; Fluorescence quenching

1. INTRODUCTION

Alcohol dehydrogenase (Alcohol-NAD$^+$ oxidoreductase, EC 1.1.1.1) from horse liver (LADH) and yeast (YADH) are inhibited by a variety of compounds which are capable of binding either to the enzymatic zinc atoms [1–6] or the sulphydryl groups [1–3,7,8]. Also, the fluorescence intensity of the binary complex of LADH–NADH is quenched by thyroxine and related compounds [9]. These thyroid hormones appear to inhibit the enzyme by blocking the normal coenzyme-binding mechanism.

We report here the effect of thiol compounds on the activity of both LADH and YADH, and on the fluorescence emission spectrum of the binary LADH–NADH complex. Thiol compounds are of interest in the light of the previous reports that several mercaptans are competitive inhibitors of LADH [10–14]. 2-Mercaptoethanol has also demonstrated to be a weak substrate of LADH [10,11] and to promote the reassociation of LADH subunits after dissociation in urea, guanidine-HCl, sodium dodecyl sulfate and extremes of pH [15–20].

2. MATERIALS AND METHODS

2.1. *Materials*

LADH and YADH were purchased from Boehringer (Mannheim, Germany), and NAD$^+$, NADH and various thiol compounds from Sigma Chemical Co. (St. Louis, U.S.A.) Other chemicals were of reagent grade.

Correspondence address: Li-Yao Cheng, Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Republic of Singapore. Fax: (65) 779 1453.

2.2. *Enzyme assay*

Concentrations of enzymes and coenzymes were determined spectrophotometrically, ($E^{1\%}_{cm}$: 4.55 and 12.60 for LADH [21] and YADH [22] at 280 nm, respectively, and 17.8 mm^{-1}·cm^{-1} for NAD$^+$ at 260 nm and 6.22 mm^{-1}·cm^{-1} for NADH at 340 nm). Molar concentrations are based on molecular weights of 80 kDa for LADH [21] and 150 kDa for YADH [22]. The activity of LADH and YADH was determined by measuring the change in absorbance at 340 nm on a Gilford Response TM Spectrophotometer. The 3.0 ml assay solution was made of 8.7 mM ethanol, 0.42 mM NAD$^+$ and 0.05 M glycine- NaOH, pH 10 for LADH and 0.1 M ethanol, 0.42 mM NAD$^+$ and 0.05 M glycine-NaOH, pH 8.5 for YADH.

2.3. *Inhibition experiments*

Inhibition experiments are described in the figure captions.

2.4. *Fluorescence emission spectra and intentsy measurements*

These were made at 25°C with a Perkin-Elmer Luminescence Spectrometer, Model LS5.

3. RESULTS

3.1. *Effect of thiol compounds on the activity of LADH and YADH*

The effect of 2-mercaptoethanol on the activity of both LADH and YADH was examined. 2.1 μM YADH was pre-incubated with concentrations of up to 0.15 M 2-mercaptoethanol at pH 7.3 and 25°C, aliquots were then withdrawn and assayed for the residual activity. It was found that the catalytic activity of YADH was not significantly affected under these conditions. However, LADH was profoundly inhibited by 2-mercaptoethanol (Fig. 1), and the extent of inhibition was complete within a few seconds and there was no further loss of enzymatic activity over a period of more than 24 h. The degree of inhibition of enzymatic actitivy also depended upon the concentration of 2-mercaptoethanol. In the presence of 7 mM 2-mercaptoethanol, the remaining

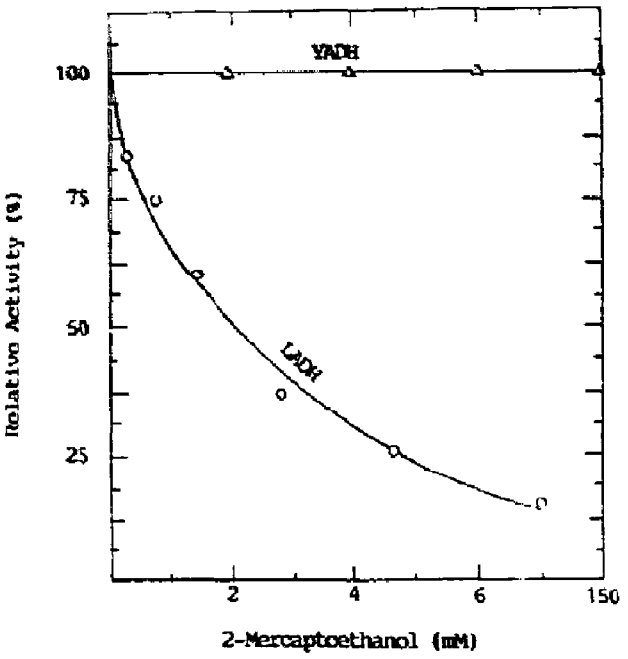

Fig. 1. Effect of 2-mercaptoethanol on the activity of LADH and YADH. 1.57 μM LADH or 2.1 μM YADH was preincubated at 25°C with various concentrations of 2-mercaptoethanol in 0.01 M phosphate buffer, pH 7.3. 50 μl aliqots were then withdrawn and introduced to assay solution for measuring the residual activity.

residual activity was found to be only 12% of the control. In contrast, both mercaptoacetic acid and 3-mercaptopropionic acid inhibited the activity of LADH, but 3-mercaptopropionic acid showed a stronger inhibitory effect than mercaptoacetic acid. The rate of inhibition of LADH by 3-mercaptopropionic acid was rapid, whereas mercaptoacetic acid was time-dependent. In the latter case, the loss of enzymatic activity was complete in about 15 minutes, and was constant thereafter. Table I summarises the concentrations of various thiol compounds required to inhibit 50% of the activity of LADH.

3.2. *Reversal of the inhibitory effect*

Complete reversal of the loss of LADH activity by these thiol compounds could be obtained by removal of the inhibitors from the enzyme solution by dialysis against 2 changes of 0.01 M phosphate buffer, pH 7.3, at 4°C over a period of about 20 h.

3.3. *Effect of thiol compounds on LADH–NADH fluorescence*

When irradiated by light at 350 nm, NADH exhibits fluorescence with an emission maximum at 450 nm. The presence of LADH, the intensity of NADH fluorescence was enhanced [2] and the emission maximum was shifted to 445 nm (Fig. 2A). The fluorescence of the binary LADH–NADH complex was rapidly quenched by adding 2-mercaptoethanol, but it did not affect the fluorescence of free NADH. The quenching of fluorescence depended on both pH and the concentration of

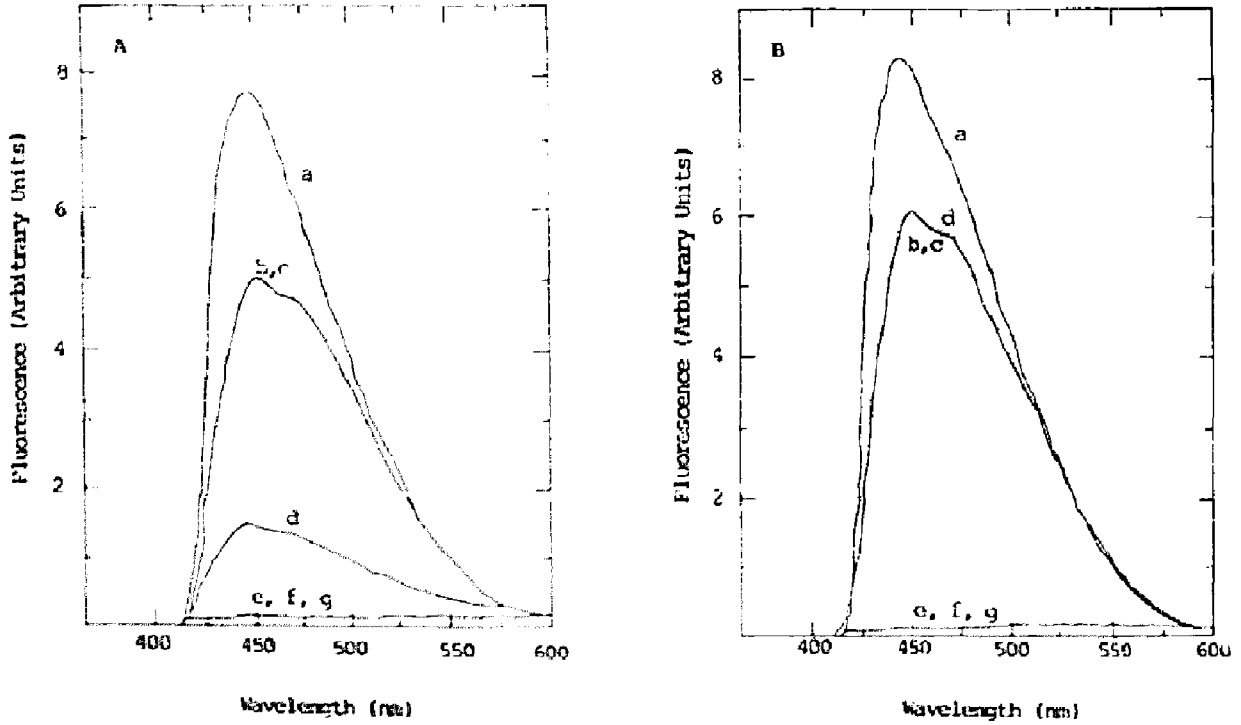

Fig. 2. (A) Fluorescence emission spectra for NADH, LADH and 2-mercaptoethanol. Measurements were carried out at 25°C with 3.0 ml solution containing 0.01 M phosphate buffer, pH 7.3 and (a) E+R, (b) R, (c) R+I, (d) E+R+I, (e) none, (f) E, (g) E+I. (B) The same conditions as for (A) except 0.01 M phosphate buffer was replaced by 0.05 M glycine-NaOH, pH 9.2. The wavelength of the excitation light was 350 nm. E=0.92 μM LADH, R=6.8 μM NADH, I=17.9 mM 2-mercaptoethanol.

thiol compounds. At pH 7.3, 2-mercaptoethanol quenced the enhanced fluorescence of LADH–NADH to lower than that of free NADH (Fig. 2A), whilst at pH 9.2–9.7, the fluorescence emission spectrum returned to that characteristic of free NADH (Fig. 2B). Once the maximum quenching of the fluorescence was attained, further addition of thiol compounds did not produce more quenching of the fluorescence. However, the data obtained showed a discrepancy in stoichiometry with the number of coenzyme molecules binding to the enzyme.

4. DISCUSSION

LADH is profoundly inhibited by thiol compounds, but these compounds exert no effect on YADH, indicating that the catalytic region in both enzymes may have different structural arrangements. The inhibitory effect of thiol compounds on LADH may be due to the binding ot the SH group to enzymatic zinc ions, since the locus of imidazole binding has been suggested to be enzymatic zinc ions [1–3], and it protects LADH from thiol compounds inactivation (data not shown). This suggestion is further supported by the finding that the SH group favours the linkage with zinc atoms [23]. Thiol compounds do not inhibit YADH, suggesting that the zinc atoms which participate in the catalytic mechanism of YADH may not be blocked by thiol compounds. Preferential substrate and competitive inhibitors binding to a hydrophobic region of LADH has been suggested by Winer and Theorell [24], Sigman [13] and Miwa et al. [14]. Since the inhibitory effect of 3-mercaptopropionic acid on LADH is stronger than that of mercaptoacetic acid, it is suggested that the binding of thiol compounds to LADH is not only to the Zn^{2+} ions in the active site of LADH but probably also stabilized by neighboring hydrophobic binding site of the enzyme. The presence of an OH group in the thiol compound causes stronger inhibition than does the COOH group, which suggests that the more negative nature of the latter compound has a low affinity for LADH.

Thiol compounds do not affect the fluorescence emission spectrum of free NADH, but at pH 9.2, the addition of 2-mercaptoethanol, the enhanced fluorescence emission spectrum of LADH–NADH returns to that characteristics of free NADH. These results suggest that 2-mercaptoethanol replaces the binding of the coenzyme for LADH. However, there is also a possibility that the formation of a ternary complex of LADH–NADH–mercaptoethanol in which the nicotinamide moiety of the bound NADH shows no enhanced fluorescence. At pH 7.3, the fluorescence of LADH–NADH is further quenched by 2-mercaptoethanol. The quenching of LADH–NADH fluorescence is likely attributed to the binding of 2-mercaptoethanol to the binary LADH–NADH complex which causes a conformational change and results in energy transfer. This energy transfer is pH-dependent and requires the presence of LADH.

Table I

Concentrations of inhibitors required to inhibit 50% of the activity of LADH at pH 7.3 and 25°C

Inhibitor	Concentration of inhibitor required for 50% inhibition, (mM)
2-Mercaptoethanol	2.2
3-Mercaptopropionic acid	44.5
Mercaptoacetic acid	107.0*

*1.57 μM LADH was preincubated in mercaptoacetic acid for 25 min before 50 μl aliquots were withdrawn for measuring the residual activity. Other details were as in Fig. 1.

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