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INHIBITION OF ALDEHYDE DEHYDROGENASES BY METHYLENE BLUE

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Abstract—The effect of the redox dye methylene blue on the stability of NADH and on the activity of the enzyme aldehyde dehydrogenase (ALDH; EC 1.2.1.3) was examined. NADH was measured by HPLC with fluorometric or spectrophotometric detection. The ALDH activity assays were carried out by following the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) from 3,4-dihydroxyphenylacetaldehyde (DOPAL) using HPLC and electrochemical detection. Incubation of NADH solutions in the presence of methylene blue resulted in a time-dependent direct oxidation of NADH. Methylene blue inhibited the human erythrocyte and leukocyte ALDHs and the rat liver mitochondrial low-K_m ALDH in a concentration-dependent manner. The inactivation was reversible by dilution, and kinetic analysis indicated that methylene blue inhibits the rat liver mitochondrial low-K_m and human erythrocyte ALDHs competitively with respect to DOPAL, while no effect of the NAD^+ concentration was apparent. For the rat liver low-K_m ALDH, a K_i of 8.4 ± 2.8 μM (mean ± SD; N = 5) was calculated. The inhibition of ALDH and the resulting decrease in the redox effect on the NAD system bound to alcohol dehydrogenase (EC 1.1.1.1) could explain the protective effect of methylene blue against metabolic redox effects of ethanol.

The reducible dye methylene blue has been used to study the mechanism behind the effects of ethanol on biological oxidations such as lipid [1] and carbohydrate [2, 3] metabolism. The basis for this is that methylene blue, as hydrogen acceptor, reduces the redox effects of ethanol by directly re-oxidizing NADH to NAD^+ [1, 2, 4]. Thus, the cause of those effects of ethanol metabolism, which can be reduced by methylene blue, may be accumulation of NADH.

In a recent study of ethanol metabolism in isolated hepatocytes [5], the decrease in redox effect caused by methylene blue was found to be accompanied by a marked increase in the rate of ethanol–acetaldehyde exchange. This could not be explained by increased re-oxidation of NADH, since this would decrease the rate of ethanol–acetaldehyde exchange. On the other hand, the results were similar to those obtained with the aldehyde dehydrogenase (ALDH‖; EC 1.2.1.3) inhibitor cyanamide, and it was proposed that methylene blue in fact acted as an ALDH inhibitor. Inhibition of ALDH would decrease the redox effects of ethanol, since these appear to be caused largely by rapid elimination of acetaldehyde from the redox couple ethanol–acetaldehyde which is in near-equilibrium with the redox couple NADH–NAD^+ bound to alcohol dehydrogenase (ADH; EC 1.1.1.1) [6]. In view of the implications for the mechanism of alcohol-induced diseases, and for the use of methylene blue in the treatment of methaemoglobinaemia [7], it was considered important to establish if methylene blue directly inhibits ALDHs.

Measurement of ALDH activity is usually carried out spectrophotometrically by following the reduction of NAD^+ to NADH at 340 nm. However, since methylene blue could interfere with the spectrophotometric measurements by directly affecting the concentration of NADH, assays were instead carried out with a method where the aldehyde corresponding to dopamine (3,4-dihydroxyphenylacetaldehyde; DOPAL) is used as substrate and the amount of acid product (3,4-dihydroxyphenylacetic acid; DOPAC) formed is analysed by HPLC with electrochemical detection [8]. It should be noted that both aromatic and aliphatic aldehydes are oxidized at the same active site of the enzyme molecule [9]. Experiments were performed with ALDHs from human erythrocytes and leukocytes and from rat liver.

MATERIALS AND METHODS

Chemicals. DOPAC, dopamine hydrochloride, methylene blue, NAD^+ and NADH were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The bisulphite form of DOPAL was prepared enzymatically from dopamine by use of partially purified rat liver monoamine oxidase (MAO; EC 1.4.3.4) [10]. All other chemicals were of analytical grade from Merck (Darmstadt, Germany). All solutions were prepared in deionized Milli-Q water (Millipore, Bedford, MA, U.S.A.).

Preparation of ALDHs. The mitochondrial low-K_m ALDH was purified from rat liver (Sprague–

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‖ Abbreviations: ALDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; MAO, monoamine oxidase; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPAC, 3,4-dihydroxyphenylacetic acid.

Dawley) by a modification of the procedure of Tottmar *et al.* [11] by including affinity chromatography on a 5′-AMP-Sepharose 4B column (Pharmacia, Uppsala, Sweden) to remove trace amounts of MAO activity. The column was equilibrated and eluted with 30 mM sodium phosphate buffer (pH 6.0), containing 0.1% (v/v) 2-mercaptoethanol and 1.0 mM EDTA, and the ALDH was then eluted with the same buffer containing 0.75 mM NAD^+. The eluate was concentrated 10-fold by ultrafiltration using an Immersible CX-30 filter unit (Millipore) giving a final purification of approx. 100 times compared to the ALDH activity of the original liver homogenate. The ALDH preparation was essentially free of MAO and ADH activity. All purification steps were carried out at 4°, and the enzyme was stored in 20% (v/v) glycerol solution at −80° until used. The protein concentration of the final enzyme solution was 0.7 mg/mL, as determined by the method of Lowry *et al.* [12].

Human erythrocytes and leukocytes were isolated from citrated buffy coats by Percoll® (Pharmacia) density gradient centrifugation technique according to Helander and Tottmar [8]. The single ALDH in human erythrocytes is identical to the liver cytosolic isozyme [13], while leukocytes contain both the cytosolic and mitochondrial forms [14, 15].

HPLC system. The HPLC system consisted of a Milton Roy Mod. 396 Minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a Rheodyne Mod. 7125 injector with a 20-μL sample loop (Rheodyne Inc., Cotati, CA, U.S.A.). The analytical column was a 3-μm Nucleosil 120 C_{18} reversed-phase column (75 × 4.0 mm i.d.) from Skandinaviska GeneTec (Kungsbacka, Sweden). Chromatography was carried out isocratically at a flow-rate of 1 mL/min at ambient temperature.

Direct effect of methylene blue on NADH. The ability of methylene blue to directly oxidize NADH *in vitro* was examined in NADH solutions to which methylene blue (dissolved in water) was added. Determination of NADH was performed by a modification of the procedure by Stocchi *et al.* [16]. Aliquots were collected over 20 min and mixed with an equal volume of ice-cold 0.5 mM potassium hydroxide and injected directly into the HPLC system. The direct injection was necessary, since the oxidation of NADH by methylene blue was found to continue even after treatment with potassium hydroxide. The concentration of NADH was determined with fluorometric or spectrophotometric detection using a Shimadzu Mod. RF-535 monitor (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths of 340 and 430 nm, respectively, or a SpectroMonitor III (Laboratory Data Control) at 254 nm. The mobile phase consisted of 0.1 mM potassium phosphate buffer (pH 6.0), containing 10% (v/v) methanol.

Assays of ALDH activity. Assay of rat liver mitochondrial low-K_m ALDH activity was carried out by a slight modification of the procedure by Tottmar [17]. In the standard assay, the reaction mixture contained 50 mM sodium pyrophosphate buffer (pH 8.8), 0.5 mM NAD^+, 20 μM DOPAL, 5 μL of ALDH (0.7 mg/mL) and 5 μL methylene blue (dissolved in water) in a final volume of 0.5 mL. The assays were performed under gentle shaking at 37° in a water bath. The reaction mixtures were pre-incubated for 5 min at 37° before addition of DOPAL (similar results were obtained with pre-incubation times of 0–15 min), and the reaction was terminated after 2 min by addition of 0.2 mL ice-cold 17% (w/v) perchloric acid containing 30 mM sodium bisulphite to trap excess aldehyde. The samples were centrifuged at 15,000 *g* for 5 min and stored on ice until analysed. Under the present conditions, the reaction was linear for at least 5 min.

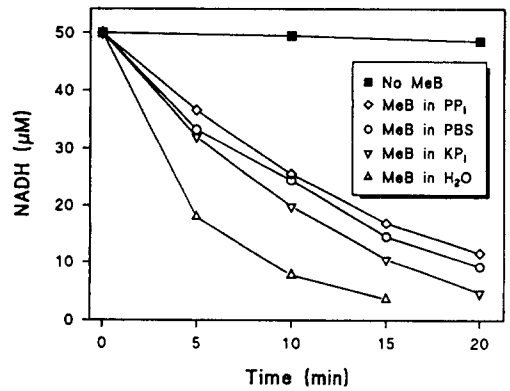

Fig. 1. Direct oxidation of NADH by methylene blue (MeB). The methylene blue (50 μM) was added to NADH solutions (50 μM) prepared in three different buffers and in water. Aliquots were collected over 20 min and the NADH concentration was determined by HPLC with fluorometric detection. Data represent mean values from three separate experiments. PP_i = 50 mM sodium pyrophosphate, pH 8.8; PBS = phosphate-buffered saline, pH 7.4; KP_i = 10 mM potassium phosphate, pH 7.5; H_2O = deionized water, pH 6.3.

Assays of human erythrocyte and leukocyte ALDH activities were carried out with both intact cells and cells disrupted by sonication according to the method by Helander and Tottmar [8]. The reaction mixtures were pre-incubated for 5 min in the presence of the methylene blue.

The ALDH activity was assessed from the amount of DOPAC formed, as determined by HPLC with electrochemical detection. A Mod. LC-3 electrochemical detector with a glassy carbon working electrode was used (Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.), and the potential was set at +0.75 V versus a Ag/AgCl reference electrode. The mobile phase consisted of 105 mM citric acid, 1.2% (v/v) methanol, 50 μM EDTA and 25 μM sodium octyl sulphate (pH 2.2). DOPAC was totally stable in the presence of methylene blue, while a slight decrease of the DOPAL peak was observed in the chromatogram. However, when corrected for, it was found not to affect the final analytical results.

RESULTS

Incubation of 50 μM NADH in the presence of

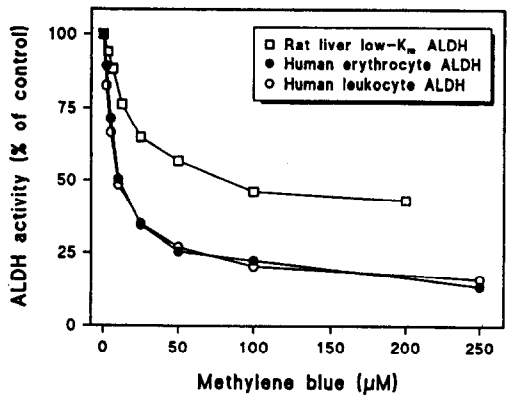

Fig. 2. Inactivation of ALDH from human erythrocytes and leukocytes and rat liver mitochondria (low-K_m form) by different concentrations of methylene blue. ALDH activity assays were carried out with 20 μM DOPAL as substrate and the amount of acid product (DOPAC) formed was determined by HPLC with electrochemical detection. Data represent mean values from three separate experiments.

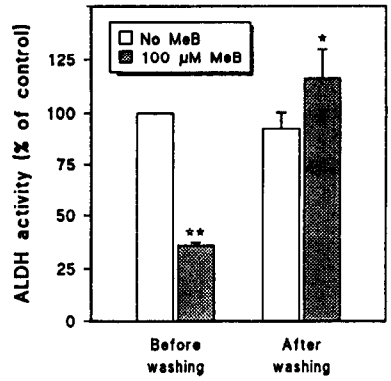

Fig. 3. Effect of washing of intact human erythrocytes treated with 100 μM methylene blue (MeB) or vehicle (no MeB) on the ALDH activity. Both methylene blue treated and untreated cells were subjected to 10 centrifugation–resuspension cycles in phosphate-buffered saline. Data represent mean values ± SD from four separate experiments. *$P < 0.05$; **$P < 0.01$ (Wilcoxon rank sum test).

50 μM methylene blue resulted in a time-dependent disappearance of NADH (Fig. 1). When spectrophotometric detection was used, the disappearance of NADH was shown to be accompanied by the formation of NAD^+ (data not shown). The oxidation rate was similar in the three different buffer systems examined, while it was accelerated in water (Fig. 1). The disappearance rate was unchanged when the experiments in potassium phosphate buffer were repeated anaerobically (i.e. in N_2-saturated buffer). NADH was stable when incubations in water or phosphate-buffered saline were carried out in the absence of methylene blue (96 ± 2% of control after 20 min; N = 4).

Methylene blue inhibited both the human erythrocyte and leukocyte ALDHs and the rat liver mitochondrial low-K_m ALDH in a concentration-dependent manner (Fig. 2). The ALDH activity of intact erythrocytes was regained after subsequent washing of cells treated with methylene blue (Fig. 3), indicating that the inactivation was reversible. However, the washing not only restored the original ALDH activity, but the activity obtained after the washing procedure was, in fact, significantly ($P < 0.05$; Wilcoxon rank sum test) higher than that of untreated control cells carried through the same procedure. The washing procedure also restored the original colour of the cells. Lineweaver–Burk (Fig. 4) and Eadie–Hofstee plots from assays with the rat liver low-K_m ALDH and human erythrocyte ALDH (data not shown) and 10, 50 or 100 μM methylene blue indicated that methylene blue inhibits ALDH competitively with respect to substrate. A similar degree of inhibition was observed with NAD^+ concentrations in the range 0.01–1.0 mM (the K_m for NAD^+ was about 50 μM). For the isolated rat liver ALDH, a K_i of 8.4 ± 2.8 μM (mean ± SD; N = 5) was calculated using ENZPACK 3.0 (Biosoft, Cambridge, U.K.).

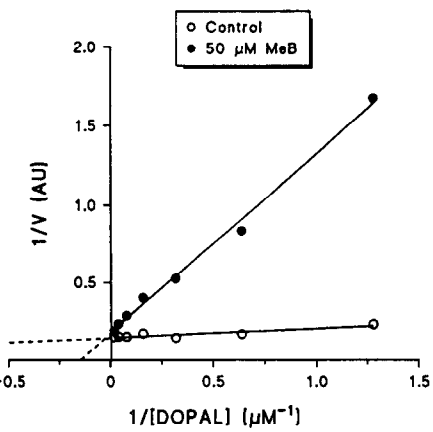

Fig. 4. Double-reciprocal plot for the inactivation of rat liver mitochondrial low-K_m ALDH by 50 μM methylene blue (MeB). Data are mean values from duplicate determinations of one representative experiment.

DISCUSSION

Direct oxidation of NADH by methylene blue as observed in the present study is in agreement with previous observations [4, 18]. Those studies were based on the fact that methylene blue acts as an artificial electron acceptor in various dehydrogenase systems [19]. In addition, the present study demonstrates that methylene blue is a potent inhibitor of both mitochondrial and cytosolic ALDH isozymes from human and rat. The inactivation was indicated to be reversible and competitive towards the substrate. The exact mechanism by which methylene blue inhibits ALDH is, however, still unknown.

When experiments were carried out with intact erythrocytes, the ALDH activity obtained after subsequent washing of cells treated with methylene blue was, in fact, higher than the control value. The cause of this finding is unknown, but it might possibly be explained by the direct re-oxidation of NADH by methylene blue, since the resulting decrease of free NADH within the cells would also decrease the concentration of enzyme-bound NADH, and, thus, increase the rate of aldehyde oxidation [20].

The inhibition of ALDH by methylene blue explains the increase in the rate of acetaldehyde reduction during the ethanol–acetaldehyde exchange that accompanies ethanol elimination in isolated hepatocytes [5]. It might also explain the protective effect of methylene blue against metabolic redox effects of ethanol [1, 2, 21, 22], since the effect of ethanol on the redox state of the NAD system appears to be dependent on rapid elimination of acetaldehyde [6]. Consequently, it might be necessary to revise the conclusions drawn from studies where methylene blue has been used to re-oxidize NADH formed during ethanol oxidation.

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