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A kinetic study on the phenothiazine dependent oxidation of NADH by bovine ceruloplasmin

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Abstract

Tranquillizing drugs of the phenothiazine class form charge-transfer complexes with a ceruloplasmin-Cu(II) ion [De Mol NJ. 1985 *Biochim Pharmacol* **34**, 2605–2609], the interaction resulting in a stimulatory effect on the ceruloplasmin catalyzed oxidation of catecholamines and NADH; the latter used as substrate in the present study. A good correlation between stability of the enzyme–drug complex and electron donor ability of the phenothiazine molecule was obtained for drugs with an aliphatic propyl side chain in 10-position (promazine > chlorpromazine > triflupromazine). The hydrofobic methyl group in the side chain of levomepromazine appeared to reduce the stability. A simple correlation between specific efficiency of the enzyme–drug complex and electron donor ability was not obtained (chlorpromazine > promazine = levomepromazine > triflupromazine). The K_m-values, characterizing the reaction between NADH and the different enzyme–drug complexes, were estimated. The data suggest that the enzyme–chlorpromazine complex has the best affinity for NADH. The stimulatory effect of levomepromazine closely followed that of promazine.

Introduction

Ceruloplasmin (ferroxidase) is a copper containing α_2-plasma glycoprotein produced in the hepatocytes. However, cloning of liver mRNA led to the discovery that other cells could express it; Sertoli cells (Skinner & Griswold 1983, Onada & Djakiew 1990), uterus (Aldred *et al.* 1987, Thomas & Schreiber 1989), and the chorionid plexus, producing cerebrospinal fluid (Schreiber 1987, Thomas *et al.* 1989). Ceruloplasmin is also detected in milk, suggesting the possibility that the protein is produced in the mammary gland (Cerveza *et al.* 2000).

Human and bovine ceruloplasmin contain six copper atoms as an integral part of the native enzyme (Zgirski & Frieden 1990); two of these (Type-1 Cu(II)) are responsible for the blue colour of the protein. Ceruloplasmin catalytically oxidizes ferrous ions, certain diamines and diphenols, including catecholamines (for a review, see Gutteridge & Stocks 1981). It is proposed to function as a ferroxidase *in vivo* (Osaki *et al.* 1966, Carver *et al.* 1982, Harris *et al.* 1995).

Phenothiazine drugs constitute a large group of structurally related neuroleptic agents. Barrass & Coult (1972) reported that several drugs of the phenothiazine class markedly stimulated the ceruloplasmin catalyzed oxidation of catecholamines and serotonin. The drugs also caused ceruloplasmin to catalytically oxidize ascorbate and NADH (Løvstad 1974); compounds that otherwise do not function as substrate for the enzyme. A study by De Mol (1985) concluded that the activating effect of phenothiazine derivatives was due to formation of a charge-transfer complex between the electron donating drug and

ceruloplasmin-Cu(II), suggesting that these complexes might be a model for such interactions with copper or suitable metal containing (receptor) proteins. The present NADH-coupled kinetic study, based on this activation mechanism, was undertaken in order to obtain more information about the interaction of phenothiazine derivatives with ceruloplasmin, and their effect on the enzyme catalyzed oxidation of NADH.

Materials and methods

Bovine serum ceruloplasmin (EC 1.16.3.1), NADH, promazine, chlorpromazine, and triflupromazine were purchased from Sigma Chemical Company (St. Louis, Missouri, USA), levomepromazine from AB Mekos (Helsingborg, Sweden), and EDTA from E. Merck AG (Darmstadt, Germany). The bovine ceruloplasmin concentration was calculated from the 610 nm absorption band ($\varepsilon = 9.37\ mM^{-1}\ cm^{-1}$; Zgirski & Frieden 1990).

The rate of NADH oxidation by the drug activated ceruloplasmin molecule was followed spectrophotometrically at 360 nm ($\varepsilon = 3.96\ mM^{-1}\ cm^{-1}$). EDTA was added to the reaction solutions in order to prevent the activating effect of trace iron on ceruloplasmin catalyzed oxidations (McDermott *et al.* 1968). All aqueous solutions were prepared in deionized, glass-distilled water. Spectrophotometric measurements were carried out in a Helios γ instrument.

Results and discussion

The time course curves in Figure 1 show the effect of 2 mM phenothiazine derivative (promazine, chlorpromazine, triflupromazine, levomepromazine) on the oxidation of NADH in the presence of bovine ceruloplasmin. The NADH concentration was found to decrease linearly with time, initially. The enzyme activity was determined from this part of the curve. Chlorpromazine increased the reaction more than promazine and levomepromazine, while triflupromazine was less effective. In the absence of phenothiazine no significant oxidation of NADH by the enzyme was observed (Figure 1).

The relationship between ceruloplasmin concentration and initial rate of NADH oxidation, in the presence of activating drugs, was tested. In all cases a linear correlation between activity and enzyme concentration was obtained, as shown in Figure 2.

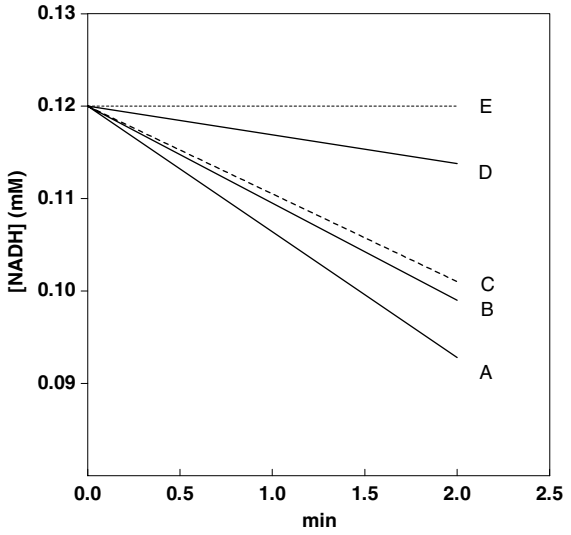

Figure 1. Effect of phenothiazine derivative on the oxidation of NADH in the presence of ceruloplasmin. The reaction solutions contained 2 mM phenothiazine, 0.12 mM NADH, 50 μM EDTA and 0.44 μM ceruloplasmin in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30$ °C). A, Chlorpromazine; B, promazine; C, levomepromazine; D, triflupromazine; E, control.

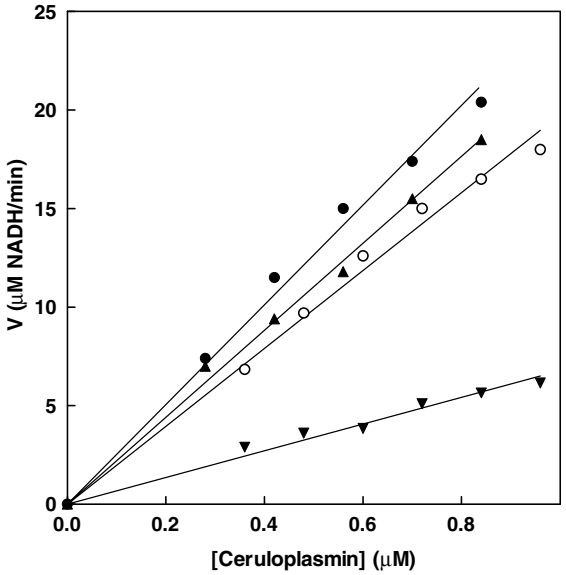

Figure 2. Effect of ceruloplasmin concentration on the chlorpromazine (●), promazine (▲), levomepromazine (●) and triflupromazine (▼) stimulated oxidation of NADH. The reaction solutions contained 1 mM phenothiazine, 0.12 mM NADH, 50 μM EDTA and ceruloplasmin (0.28–0.96 μM) in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30$ °C).

The following reaction mechanism is proposed:

$$D + E \overset{K_d}{\leftrightarrow} D - E$$

$$D - ECu(II) + S \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} [Complex] \overset{k_2}{\rightarrow} D - ECu(I) + Ox.prod$$

$$D - ECu(I)_4 + O_2 + 4H^+ \overset{k_3}{\rightarrow} D - ECu(II)_4 + 2H_2O$$

A charge-transfer complex (D–E) between drug (D) and enzyme (E) is formed (De Mol 1985); the complex catalyzing the oxidation of NADH (S) to NAD^+ (Løvstad 1974), as well as oxidation of catecholamines (De Mol 1985), which form coloured aminochrome products. During the reaction enzyme-bound cupric ions are reduced to the cuprous state. The enzyme is spontaneously reoxidized by molecular oxygen, which is reduced to water in the process, accepting four electrons from ceruloplasmin.

The rate of NADH oxidation, as a result of phenothiazine effect on ceruloplasmin oxidase activity, was determined at various concentrations of the drugs (Figure 3). The hyperbolic curves obtained can be explained in terms of a gradual 'saturation' of the enzyme by the phenothiazine derivative, the activity finally reaching a maximum, V_{max}. Since the activity is proportional to the concentration of the enzyme–drug complex, and since the total drug concentration, $[D_o]$, is much higher than the total enzyme concentration, $[E_o]$, the equation for the hyperbolic curves is $[V = V_{max}[D_o]/(K_d + [D_o]]$. By means of a computer program published by Cleland (1967) the dissociation constants (K_d) and the V_{max}-values, characterizing the different drugs studied, were calculated and listed in Table 1, which also shows

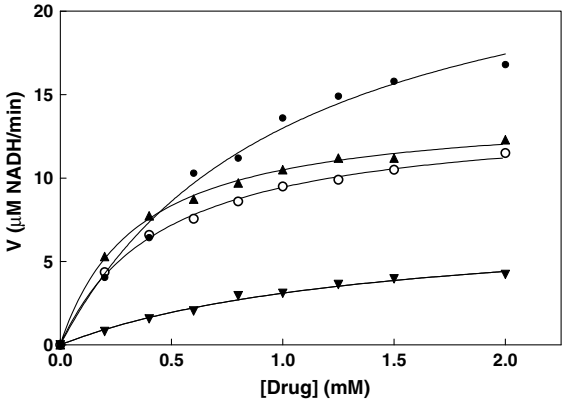

Figure 3. Effect of phenothiazine derivative concentration on the initial rate of NADH oxidation in the presence of ceruloplasmin. The reaction solutions contained drug (0.2–2 mM), 0.12 mM NADH, 50 μM EDTA and 0.44 μM ceruloplasmin in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30$ °C). (●) Chlorpromazine; (▲) promazine; (●) levomepromazine; (▼) triflupromazine. The curves were drawn by the computer, using the kinetic constants in Table 1.

the Hammet σ_{para}-value for the substituent in 2-position of the phenothiazine ring system. It has been reported that the electron donor ability of the drug progressively falls as the Hammett σ_{para}-value increases, and that the side chain in 10-position does not significantly influence the electron donor ability (Mercier & Dumont 1972). In a previous communication it was demonstrated that the rate of phenothiazine dependent reduction of ceruloplasmin Type-1 (blue) Cu(II), in the presence of NADH, increased with electron donor ability of the drug (promazine > chlorpromazine > triflupromazine) (Løvstad 1977). Figure 4 shows that the stability of the enzyme–drug complex also increases with electron donor ability of these drugs. The K_d-value obtained with levomepromazine, characterized by the lowest σ_{para}-value was, however, of the same order of magnitude as that of promazine (Table 1), indicating that the hydrophobic methyl group in the 10-side chain affects the interaction of drug with enzyme. The activity

Table 1. List of kinetic parameters.

Compound	R_2	R_{10}	K_d ± SE mM	V_{max} ± SE μM/min	σ_p-value
Promazine	H	$(CH_2)_3$–$N(CH_3)_2$	0.35 ± 0.03	14.1 ± 0.3	0
Chlorpromazine	Cl	$(CH_2)_3$–$N(CH_3)_2$	0.97 ± 0.12	25.8 ± 1.5	0.23
Trifluprazine	CF_3	$(CH_2)_3$–$N(CH_3)_2$	1.44 ± 0.27	7.6 ± 0.8	0.55
Levomepromazine	CH_3O	CH_2CHCH_2–$N(CH_3)_2CH_3$	0.46 ± 0.04	13.8 ± 0.3	−0.27

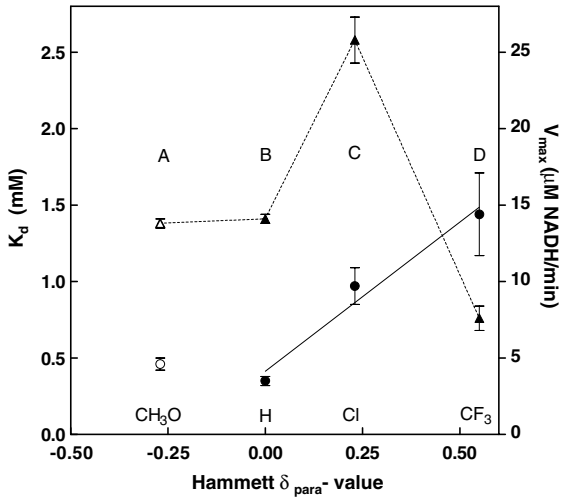

Figure 4. The K_d- (circles) and V_{max}-values (triangles) plotted against the Hammett σ-value (para) for the substituent in 2-position of the phenothiazine ring system. A, Levomepromazine; B, promazine; C, chlorpromazine; D, triflupromazine.

estimated, when all enzyme molecules were bound to phenothiazine (V_{max}), shows that the activating efficiency of the enzyme–drug complex does not correlate with the electron donor ability of the drug (Figure 4). Although triflupromazine, a poor donor, was the least effective activator, repeated experiments showed that the V_{max}-value obtained with chlorpromazine, was higher than the one obtained with promazine, a better electron donor. The observation suggests that the rate of product formation from the ternary enzyme–drug–NADH complex, characterized by the k_2-value, is increased when Cl is the substituent in 2-position of the phenothiazine ring system.

The rate of enzyme–drug catalyzed oxidation of NADH was measured at various NADH concentrations, keeping the concentration of the different phenothiazines constant. As shown in Figure 5 hyperbolic curves were obtained, the activity approaching a maximum value; NADH gradually 'saturating' the catalytic enzyme–drug molecules. From the experimental points in Figure 5 the $K_m = ((k_{-1} + k_2)/k_1)$ for NADH with the different enzyme–drug complexes were estimated by means of a computer (Cleland 1967) and listed in Table 2. The activating effect of levomepromazine paralleled that of promazine, as also shown in Figure 3. The observation that the enzyme–chlorpromazine catalyzed reaction, characterized by a high k_2-value, gave the same K_m-value as those found with enzyme bound promazine and levomepromazine, suggests that this complex has a higher affinity ($K_a = k_1/k_{-1}$) for NADH than the enzyme–promazine and enzyme–levomepromazine complexes. In the case of triflupromazine a low k_2-value would contribute to the lower K_m obtained with this activator (Table 2).

Phenothiazine derivatives have also been shown to interact with the iron-containing enzymes, lactoperoxidase and horseradish peroxidase, stimulating their catalytic oxidation of catecholamines to aminochrome products; intermediates in the synthesis of melanin. In this case a

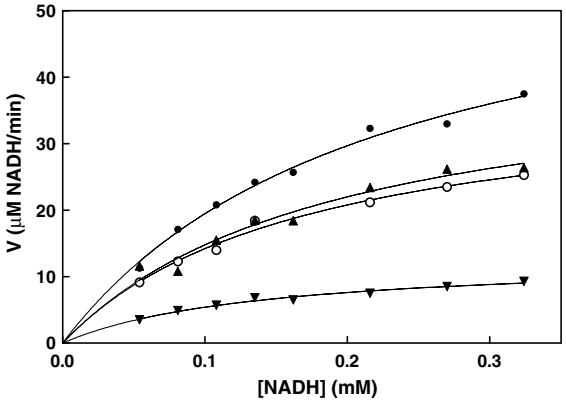

Figure 5. Effect of NADH on the initial rate of NADH oxidation in the presence of various enzyme–drug complexes. The reaction solutions contained 2 mM phenothiazine derivative, NADH (0.054–0.32 mM), 50 μM EDTA and 0.56 μM ceruloplasmin in 0.1 M sodium acetate buffer, pH 6.0 ($T = 30$ °C). (●) Chlorpromazine; (▲) promazine; (○) levomepromazine; (▼) triflupromazine. The curves were drawn by a computer, using the kinetic constants in Table 2.

Table 2. List of K_m-values.

Complex	$K_m \pm$ SE mM
Enzyme–promazine	0.23 ± 0.053
Enzyme–chlorpromazine	0.22 ± 0.027
Enzyme–triflupromazine	0.14 ± 0.015
Enzyme–levomepromazine	0.21 ± 0.046

good correlation between activity and electron donor ability of the substituent in 2-position was obtained for drugs with identical side chain in 10-position (Løvstad 1980). Interestingly, schizophrenic patients on prolonged chlorpromazine therapy frequently accumulate melanin in the brain, liver, skin, kidney and lungs (Greiner & Nicolson 1965, Satanove 1965, Nicolson *et al.* 1966). Phenothiazine treatment can also result in Parkinsonian conditions (Hornykiewicz 1973).

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