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KINETIC STUDIES ON THE CERULOPLASMIN-CATALYZED OXIDATION OF PHENOTHIAZINE DERIVATIVES

ROLF A. LØVSTAD
Institute of Medical Biochemistry, University of Oslo, Karl Johans gt. 47, Oslo 1, Norway

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Abstract—The ceruloplasmin-catalyzed oxidation of several phenothiazine derivatives has been investigated. The results suggest that the activity decreases with increasing Hammett sigma value (para) for the substituent in the 2-position of the substrates, which have three carbon atoms between the nitrogen atom of the ring and the nitrogen atom in the side chain in the 10-position. The V_{max}-values for substrates with identical substituents in the 2-position and a three-carbon side chain in the 10-position do not vary much. However, at lower substrate concentrations compounds with a piperazinyl-propyl side chain are more rapidly oxidized than those with an aliphatic side chain, suggesting that the piperazinyl-propyl side chain enhances the enzyme affinity for the substrate molecule. Compounds with only two carbon atoms in the side chain in the 10-position (promethazine and diethazine) are more slowly oxidized by ceruloplasmin than analogs with a three-carbon side chain (promazine and alimemazine). Phenothiazine derivatives activate the ceruloplasmin-catalyzed oxidation of catecholamines. The substrates which are most rapidly oxidized by the enzyme also activate the oxidation of dopamine most effectively.

In a previous communication it was demonstrated that some phenothiazine derivatives (promazine, chlorpromazine, triflupromazine and levomepromazine), known to display good electron donor ability, were oxidized to free radicals by the copper-containing serum oxidase, ceruloplasmin [1]. Triflupromazine, having a rather strong electron-attracting substituent ($-CF_3$) [2] in the 2-position of the phenothiazine ring, was oxidized at a slower rate than the other derivatives mentioned. In the present kinetic study the effect of different substituents in the 2- and 10-position on the interaction of phenothiazine derivatives with ceruloplasmin has been investigated in order to obtain more detailed information on the substrate specificity of the enzyme.

MATERIALS AND METHODS

Human ceruloplasmin was obtained from AB Kabi and crystallized according to the method of Deutsch [3]. The purified enzyme had an absorbance ratio, A_{610}/A_{280}, of 0·044. Enzyme concentrations were calculated from the 610 nm absorption ($\epsilon = 10{,}900\ M^{-1}\ cm^{-1}$) [3]. Chlorpromazine and imipramine were obtained from A/S Dumex; perphenazine from Schering Corp.; prochlorperazine, trifluoperazine, alimemazine, promethazine, diethazine and periciazine from Pharma-Rhodia; triflupromazine and fluphenazine from E. R. Squibb & Sons; thioridazine from Sandoz AG; and promazine from AB Ferrosan. NADH and 3-hydroxytyramine (dopamine) were purchased from Sigma Chemical Co. All aqueous solutions were prepared in deionized, glass-distilled water.

The ceruloplasmin-catalyzed oxidation of the phenothiazine derivatives was measured by adding NADH to the reaction mixture and reading the change in absorption at 340 nm, due to the disappearance of NADH, which is immediately oxidized by the free radicals generated from the substrate [1]. The rate of aminochrome formation from dopamine in the presence of ceruloplasmin was measured spectrophotometrically at 480 nm [4]. An iron-chelating agent, 1,10-phenanthroline, was added in order to eliminate the activating effect of trace iron ions on the enzymic oxidation of dopamine [5]. Iron ions do not activate the ceruloplasmin-catalyzed oxidation of phenothiazine derivatives (R. A. Løvstad, unpublished results). A Beckman DK-1 recording spectrophotometer, equipped with a thermo cell, was used. The temperature was 30° in all experiments.

RESULTS AND DISCUSSION

Ceruloplasmin activity (V) towards phenothiazine derivatives was determined at several substrate concentrations. In the presence of NADH a linear correlation was obtained by plotting $1/V$ against $1/[S]$ (Figs. 1 and 2). The Michaelis constants (K_m) and maximum activities (V_{max}) were calculated from the plots and listed in Table 1. In a previous communication Levine and Peisach [6], studying the reaction between ceruloplasmin and several aryl polyamines and polyphenols, reported that the V_{max}-values decreased with increasing Hammett sigma values for the ring substituents. A similar tendency is observed in the present study of the ceruloplasmin-catalyzed oxidation of

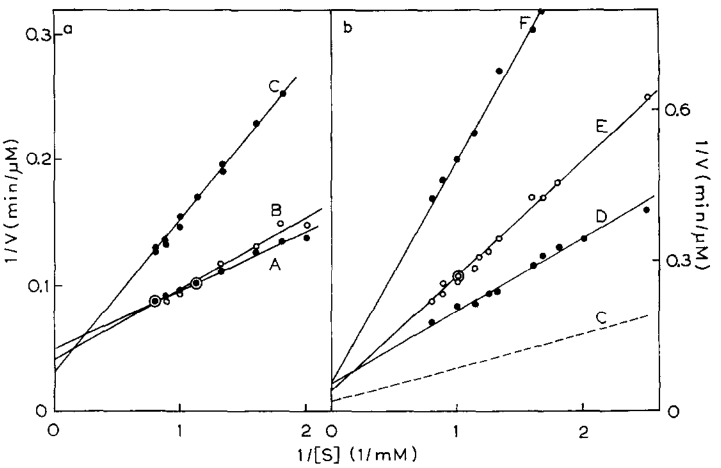


Fig. 1. Effect of prochlorperazine (A), perphenazine (B), chlorpromazine (C), trifluoperazine (D), fluphenazine (E) and triflupromazine (F) on the rate of NADH oxidation in the presence of ceruloplasmin. The reaction mixture contained ceruloplasmin [0·75 μM (1a) and 1·3 μM (1b)], phenothiazine derivative (0·40–1·25 mM), 0·17 mM NADH and 1·25 mM Cl^- in 0·25 M sodium-acetate buffer, pH 5·5.

phenothiazine derivatives with three carbon atoms between the ring nitrogen atom and the nitrogen atom of the side chain in the 10-position. This is shown in Fig. 3, where $V_{max}/[CP_0]$ (Table 1) is plotted against the Hammett sigma value (para) for the substituent in the 2-position of the phenothiazine ring. The Cl-substituted compounds are more rapidly oxidized than their CF_3-substituted analogs (Fig. 1). Periciazine, having the strong electron-attracting CN-group (σ_{para} = 0·628) [2] in the 2-position, is a poor substrate with a low V_{max}-value, while thioridazine, with $-SCH_3$ (σ_{para} = −0·047) [2] in the 2-position, is among the best substrates investigated (Fig. 2). The results indicate that an increased rate of product formation is associated with an increased ring electron density of the substrate. The electron donor ability of phenothiazine has been found to progressively fall as the Hammett sigma value (para) of the substituent in the 2-position rises [7]. The K_m-values for the CF_3-substituted compounds are higher than those obtained for the Cl-substituted analogs, suggesting that the enzyme has lower affinity for the CF_3-substituted derivatives.

As shown in Fig. 1 the V_{max}-values for different substrates with identical substituents in the 2-position do not vary much, indicating that the three-carbon side chain in the 10-position has little effect on the rate of

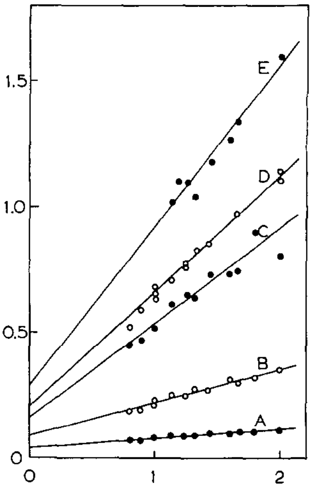


Fig. 2. Effect of thioridazine (A), alimenazine (B), diethazine (C), promethazine (D) and periciazine (E) on the rate of NADH oxidation in the presence of ceruloplasmin. The reaction mixture contained 0·75 μM ceruloplasmin, phenothiazine derivative (0·50–1·25 mM), 0·17 mM NADH and 1·25 mM Cl^- in 0·25 M sodium-acetate buffer, pH 5·5.

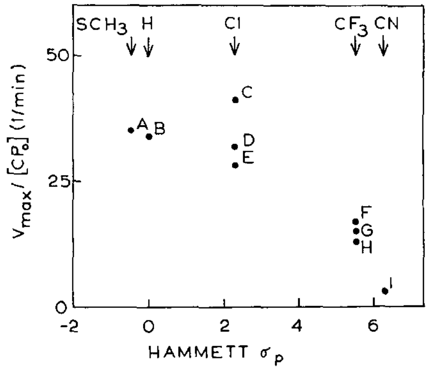
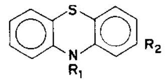
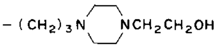
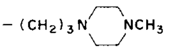
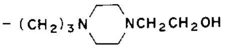
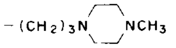
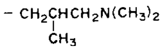
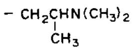
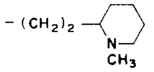
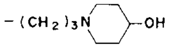


Fig. 3. The activity, expressed as $V_{max}/[CP_0]$, plotted against the Hammett sigma value (para) for the substituent in the 2-position of the phenothiazine ring. Thioridazine (A), promazine (B), chlorpromazine (C), perphenazine (D), prochlorperazine (E), fluphenazine (F), trifluoperazine (G), triflupromazine (H) and periciazine (I).

Table 1. List of kinetic parameters



Compound	R_1	R_2	K_m (mM)	$V_{max}/[CP_0]$* (min^{-1})
Chlorpromazine	$-(CH_2)_3N(CH_3)_2$	—Cl	3·5	41
Perphenazine		—Cl	1·3	32
Prochlorperazine		—Cl	0·9	28
Triflupromazine	$-(CH_2)_3N(CH_3)_2$	$—CF_3$	10·0	13
Fluphenazine		$—CF_3$	5·0	17
Trifluoperazine		$—CF_3$	2·8	15
Alimemazine	 $-CH_2\underset{CH_3}{CH}CH_2N(CH_3)_2$	—H	1·4	15
Promethazine	 $-CH_2\underset{CH_3}{CH}N(CH_3)_2$	—H	2·3	6·5
Diethazine	$-CH_2CH_2N(C_2H_5)_2$	—H	2·3	8
Thioridazine		$—SCH_3$	1·4	35
Periciazine		—CN	2·0	3

* Total ceruloplasmin concentration.

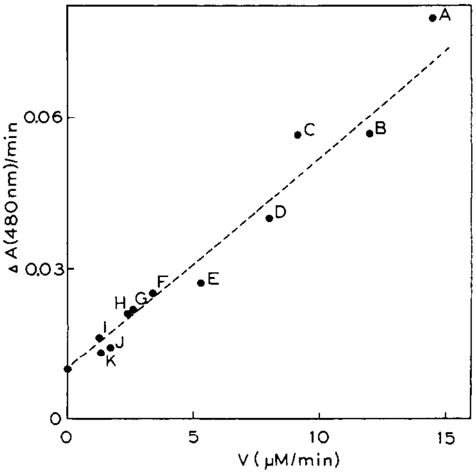


Fig. 4. The rate of dopaminochrome formation in the presence of ceruloplasmin and different phenothiazine derivatives plotted against the enzyme activity towards the phenothiazine derivatives. The reaction mixture for determining the rate of dopaminochrome formation contained 3·5 μM ceruloplasmin, 1·25 mM phenothiazine derivative, 2·5 mM dopamine, 25 μM 1,10-phenanthroline, and 3·75 mM Cl^- in 0·25 M sodium-acetate buffer, pH 5·5. The reaction mixture for determining the rate of phenothiazine oxidation contained 0·75 μM ceruloplasmin, 1·25 mM phenothiazine derivative, 0·17 mM NADH and 2·5 mM Cl^- in 0·25 M sodium-acetate buffer, pH 5·5. Thioridazine (A), prochlorperazine (B), promazine (C), chlorpromazine (D), alimemazine (E), trifluoperazine (F), fluphenazine (G), diethazine (H), periciazine (I), promethazine (J) and triflupromazine (K).

product formation. However, at lower substrate concentrations compounds with a piperazinyl-propyl side chain are more rapidly oxidized than those with an aliphatic side chain, suggesting that the enzyme has greater affinity for compounds with a piperazinyl-propyl side chain in the 10-position.

The ceruloplasmin activity towards promethazine and diethazine was rather low compared to activities obtained with other derivatives with a proton in the 2-position, such as promazine [1] and alimemazine (Fig. 2). Promethazine and diethazine have only two carbon atoms between the nitrogen atom of the ring and the nitrogen atom in the side chain (Table 1). A three-carbon side chain seems to be essential for a rapid enzymic oxidation. This is also a requirement for the psychotropic activity of phenothiazine derivatives [8, 9]. It is interesting that Barrass *et al.* [10], studying the ceruloplasmin-catalyzed oxidation of 3,4-dihydroxyphenylalkylamines, obtained maximum activity when three methylene groups were incorporated into the side chain (3,4-dihydroxyphenylpropylamine).

The present study demonstrates that the ceruloplasmin activity towards phenothiazine derivatives is markedly affected by the nature of the side chain in the

10-position. According to Mercier and Dumont [7] the electron donor ability of the phenothiazine ring is not related to the presence and the nature of this chain. It is therefore probable that the side chain in the 10-position plays a major role in the binding of the phenothiazine derivative to the active site on the ceruloplasmin molecule.

Barrass and Coult [11] found that phenothiazine derivatives increased the rate of the ceruloplasmin-catalyzed oxidation of catecholamines, probably by acting as a cycling intermediate between the enzyme and the catecholamine molecule [1]. It has been demonstrated that promazine free radicals spontaneously react with reducing compounds, such as dopamine, 3,4-dihydroxyphenylalanine (dopa), NADH, ascorbate and reduced glutathione [1]. In Fig. 4 the rate of aminochrome formation from dopamine in the presence of ceruloplasmin and different phenothiazine derivatives is plotted against the rate of the ceruloplasmin-catalyzed oxidation of the phenothiazine derivatives. The plot shows that the most reactive substrates are also the best activators of the enzymic oxidation of dopamine. Barrass and Coult [11] found no activating effect of promethazine and diethazine, while the present study shows that these relatively poor substrates do slightly activate the reaction. (Fig. 4). Imipramine had no effect on the rate of aminochrome formation in the presence of ceruloplasmin.

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