Reduction of the 610 nm Absorption Band of Ceruloplasmin by Phenothiazine Derivatives

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Ceruloplasmin (ferroxidase, coeruloplasmin, EC 1.16.3.1) is a blue serum protein, which catalyzes the oxidation of phenothiazine derivatives to free radicals.[1,2] The protein molecule contains paramagnetic Type-1 and Type-2 Cu(II) as well as diamagnetic copper.[3] The 610 nm absorption band of the enzyme is due to the Type-1 Cu(II), which is involved in the catalytic process. Experiments suggest that electrons are initially transferred from substrate to the blue Type-1 copper.[3–7] The reduction of the 610 nm chromophore by ascorbate and certain aryl diamines and diphenols exhibited a second order dependence on the substrate concentration, and differences in the rate of reduction correlated to differences in the ionization potential of the reducing substrates.[8]

In the present communication the transient reduction of the 610 nm chromophore by various phenothiazine derivatives has been studied in order to find out if a correlation exists between the rate of reduction and the electron donor ability of the phenothiazines. Fig. 1 shows some typical progress curves

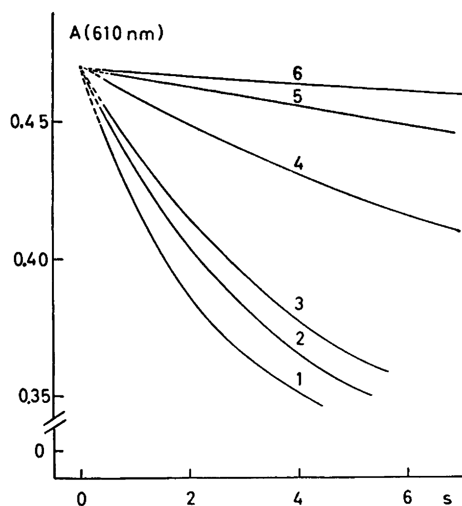

Fig. 1. Time course of the 610 nm absorbance change during the reduction of ceruloplasmin by phenothiazine derivative. 1, 1.25 mM promazine; 2, 1 mM promazine; 3, 0.63 mM promazine; 4, 1.25 mM alimemazine; 5, 2.5 mM diethazine; 6, 1.25 mM promethazine.

obtained on mixing ceruloplasmin with phenothiazine derivatives. The rate of reduction was found to increase with increasing substrate concentration, the reaction initially exhibiting a second order dependence on the substrate concentration. A rate constant, k, was calculated according to the equation, $k=\ln (A_0/A)/[S]t$, where A_0 represents the 610 nm absorption at zero time and [S] the substrate concentration. Three different concentrations of each substrate were used for the determination of the k-value (five in the case of chlorpromazine). The average k-values are listed in Table 1 together with the Hammett σ_{para}-values for the substituents in the 2-position of the phenothiazine ring.[9] It has been reported that the electron donor ability of the phenothiazines progressively falls as the σ_{para}-value increases,[10] and that the nature of the side chain in 10-position of the phenothiazine ring does not significantly influence the electron donor ability. In Fig. 2

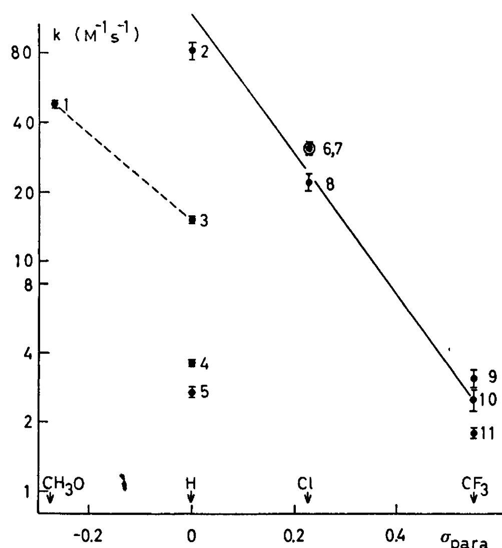

Fig. 2. The rate constant, k, plotted against the Hammett σ_{para}-value for the substituent in the 2-position of the phenothiazine ring. 1, Levomepromazine; 2, promazine; 3, alimemazine; 4, diethazine; 5, promethazine; 6, chlorpromazine; 7, perphenazine; 8, prochlorperazine; 9, trifluoperazine; 10, fluphenazine; 11, triflupromazine. Standard deviations are indicated on the figure.

the rate constant, k, is plotted semilogarithmically against the Hammett σ_{para}-value for the phenothiazines studied. A simple correlation between the rate of reduction of the 610 nm chromophore and the electron donor ability of the substrate could not be obtained. Promazine, alimemazine, promethazine, and di-

Table 1. Second order rate constants and Hammett σ_{para}-values for the phenothiazine derivatives.

Compound	R^2	R^{10}	k/ M^{-1} s^{-1}	σ_{para}-values
Levomepromazine	CH_3O	$CH_2CH(CH_3)CH_2N(CH_3)_2$	48	−0.27
Alimemazine	H	$CH_2CH(CH_3)CH_2N(CH_3)_2$	15	0.00
Promazine	H	$(CH_2)_3N(CH_3)_2$	82	0.00
Promethazine	H	$CH_2CH(CH_3)N(CH_3)_2$	2.7	0.00
Diethazine	H	$(CH_2)_2N(C_2H_5)_2$	3.6	0.00
Chlorpromazine	Cl	$(CH_2)_3N(CH_3)_2$	31	0.23
Prochlorpromazine	Cl	$(CH_2)_3N$ (piperazine) NCH_3	22	0.23
Perphenazine	Cl	$(CH_2)_3N$ (piperazine) NCH_2CH_2OH	31	0.23
Triflupromazine	CF_3	$(CH_2)_3N(CH_3)_2$	1.8	0.55
Trifluoperazine	CF_3	$(CH_2)_3N$ (piperazine) NCH_3	3.1	0.55
Fluphenazine	CF_3	$(CH_2)_3N$ (piperazine) NCH_2CH_2OH	2.5	0.55

ethazine, having a proton in 2-position and hence equal electron donor ability,[10] gave different k-values. Phenothiazines with three carbon atoms between the ring nitrogen and the side chain nitrogen atom (promazine and alimemazine) reacted faster than those with only two (promethazine and diethazine). The former were also more rapidly oxidized by ceruloplasmin.[2] The present experiments demonstrate that the reaction between Type-1 Cu(II) and substrate is influenced by the nature of the side chain in 10-position.

Among the phenothiazines with a propyl side chain (promazine, chlorpromazine, perphenazine, prochlorperazine, triflupromazine, fluphenazine, and trifluoperazine) the rate of decolourization was found to decrease with decreasing electron donor ability of the substrate as shown in Fig. 2 ($\log k \simeq -3.3\ \sigma_{para} + 2.2$). Similarly, levomepromazine, characterized by a methylated three carbon side chain (Table 1), reacted faster than its analog, alimemazine, displaying a lower electron donor ability. Alimemazine had a lower k-value than promazine, indicating that the carbon bound methyl group lowers the rate of reaction between the enzyme and phenothiazine.

Previous results, based on steady state kinetics, suggested that ceruloplasmin had greater affinity for chlorphenothiazines than for trifluphenothiazines.[2] The present observation that chlorphenothiazines react faster with Type-1 copper than the triflupheno-thiazines (Fig. 2) is in accordance with this suggestion.

Materials. Human ceruloplasmin was purchased from AB Kabi and crystallized according to the method of Deutsch.[11] The purified enzyme had an absorbance ratio, A_{610}/A_{280}, of 0.038. Enzyme concentrations were calculated from the 610 nm absorption ($\varepsilon = 10.9$ mM^{-1} cm^{-1}).[11] Promazine was obtained from AB Ferrosan, chlorpromazine from A/S Dumex, levomepromazine from AB Mekos, triflupromazine and fluphenazine from E. R. Squibb & Sons, perphenazine from Schering Corp., alimemazine,

promethazine, diethazine, prochlorperazine and trifluoperazine from Pharma Rhodia, NADH from Sigma Chem. Co. and 2,2′-dipyridyl from E. Merck AG. Aqueous solutions were prepared in deionized, glass-distilled water.

Methods. Kinetic experiments were performed in a Beckman DK-1 recording spectrophotometer. A solution of phenothiazine derivative, NADH and 2,2′-dipyridyl was rapidly mixed with ceruloplasmin, using a stopped flow apparatus. The reaction mixture contained 43 μM ceruloplasmin, 0.25 mM NADH, phenothiazine derivative (0.63 – 5 mM) and 62 μM 2,2′-dipyridyl in 0.4 M sodium acetate buffer, pH 5.5, at 20 °C. Phenothiazine derivatives are oxidized to coloured radicals by ceruloplasmin.[1] Addition of NADH, which spontaneously reduces the radicals generated,[1] effectively prevents accumulation of these compounds. The rate of 610 nm chromophore reduction by phenothiazines was independent of the NADH concentration. NADH alone did not reduce the blue colour of the enzyme. 2,2′-Dipyridyl was added in order to prevent trace iron from activating the reaction.[8,12] Preliminary experiments established that the variation of the 2,2′-dipyridyl concentration between 62 μM and 0.38 mM had no effect on reaction velocities observed.

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