Aniline Derivatives as Substrates for Ceruloplasmin

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Ceruloplasmin is known to catalyze the oxidation of a number of aromatic compounds with at least two electron-supplying groups, such as aromatic diamines, aminophenols and catecholamines.[1] Curzon and Speyer have pointed out that some related compounds with single electron-supplying groups (*e.g.* phenol and aniline) neither are substrates nor inhibitors of the enzyme, and suggested that at least two electron-supplying groups are necessary for complex formation at substrate-binding sites, either because the complex is formed through these groups or because its formation involves charge-transfer and hence is dependent upon the electron-donor properties of the substrate molecule as a whole.[2] An obvious way to distinguish between these possibilities is to investigate whether or not ceruoplasmin catalyzes the oxidation of aromatic compounds which are strong electron-donors but only contain a single electron-supplying group.

The energy of the highest occupied molecular orbital (EHOMO) is known to be an appropriate measure of electron-donor properties in charge-transfer interactions,[3] and it was recently pointed out that aromatic substrates for ceruloplasmin are characterized by exceptionally high EHOMO values (>0.6 β, where $\beta<0$ stands for the resonance integral for neighbouring carbon atoms).[4] While aniline itself is a fairly poor electron donor (EHOMO ≈ 0.7 β), alkylation of the amino group would be expected to increase the EHOMO value considerably. Results of molecular orbital calculations for some *N*-alkylated aniline derivatives are given in Table 1, and show that such compounds have sufficiently elevated energy levels to characterize them as substrates for ceruloplasmin in view of the above criterion. On the other hand, none of the compounds listed in Table 1 could be oxidized by the enzyme if substrate-binding must take place through two electron-supplying substituents in the aromatic nucleus.

Table 1. Energy of the highest occupied molecular orbital (EHOMO) of aniline and some *N*-alkylated derivatives, given in units of the resonance integral β for neighbouring carbon atoms.

Compound	EHOMO
Aniline	0.681 β
N-Methylaniline	0.555 β
N,*N*-Dimethylaniline	0.393 β
N,*N*-Diethylaniline	0.385 β

The experiments described below clearly show that ceruloplasmin catalyzes the oxidation of *N*-alkylated aniline derivatives, which apparently are oxidized by an initial step of one-electron transfer as are other substrates for the enzyme.[5,6] The main course of the reaction with *N*,*N*-dimethylaniline, for example, was found to be identical with the one established for non-enzymatic [7–9] as well as peroxidase catalyzed [10] oxidations of this compound (see Fig. 1).

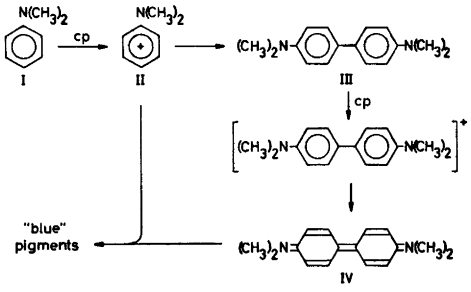

Fig. 1. Established sequence of reactions in a number of enzymatic and non-enzymatic oxidations of *N*,*N*-dimethylaniline. Reaction steps shown to be catalyzed by ceruloplasmin are indicated by cp.

The kinetics of the reaction with *N*,*N*-dimethyl- and *N*,*N*-diethylaniline were thoroughly studied, but the results obtained did not seem to provide any new information on the function of the enzyme and no quantitative data will be reported here.

The fact that *N*-alkylated aniline derivatives are readily oxidized by ceruloplasmin

definitely eliminates the possibility that two electron-supplying substituents in the nucleus of aromatic substrates are necessary for complex-formation at substrate-binding sites.

Experimental. Methods. Molecular orbital data were calculated as described previously.[11] Enzymatic assays were carried out at 25° in 0.1 M acetate buffer solutions, pH 5.5, containing 100 μM EDTA and 0.1 – 20 mM aniline derivative tested. Reactions were started by addition of ceruloplasmin (about 5 μM) and were followed spectrophotometrically. Human ceruloplasmin (about 70 % pure) was obtained from KABI, Stockholm, and was used without further purification. Apoceruloplasmin was prepared by the method of Morell and Scheinberg.[12]

Identification of oxidation products. When ceruloplasmin was added to a 20 mM solution of *N,N*-dimethylaniline the reaction mixture became transiently yellow and quickly changed to intense green. The green colour gradually changed to blue-green and finally to blue-purple. With *N*-methylaniline as the substrate the formation of blue pigments was more rapid, while the yellow colour obtained in reaction solutions containing *N,N*-diethyl-, -dipropyl-, and -dibutylaniline persisted for several hours.

The above colour reactions have previously been observed in both non-enzymatic [7–9] and enzymatic [10] oxidations of *N*-alkylated aniline derivatives and, in the case of dimethylaniline (I), indicate the rapid formation of the yellow quinone diimine (IV) corresponding to *N,N,N',N'*-tetramethylbenzidine (III), followed by a gradual conversion into polymeric blue dyes; the formation of III and polymeric dyes appears to take place through the dimethylanilinium cation radical (II) as indicated in Fig. 1.

The green solution obtained on oxidation of dimethylaniline with ceruloplasmin was passed through a column of Sephadex G-25, when blue pigments remained essentially at the top of the column and a yellow pigment was collected in the initial fractions containing low-molecular weight compounds. The optical absorption spectrum of the yellow pigment (λ_{max} = 445 nm) was indistinguishable from that of *N,N,N',N'*-tetramethylbenzidine quinone diimine. The yellow fractions from several experiments were combined, decolourized by addition of sodium dithionite, and extracted with light petroleum. On subsequent concentration white needles of *N,N,N'N'*-tetramethylbenzidine separated, m.p. and mixed m.p. 194°.

N,N,N',N'-Tetramethylbenzidine was isolated and identified in a similar manner from the yellow solutions (λ_{max} = 470 nm) obtained with diethylaniline, and the yellow oxidation products of dipropyl- and dibutylaniline (λ_{max} = 478 and 482 nm, respectively) undoubtedly also are of the benzidine quinone diimine type; solutions obtained by iodosobenzene acetate oxidation [7] of the corresponding *N*-alkylated benzidines showed the same absorption maxima.

On addition of ceruloplasmin to solutions of *N,N*-dimethyl- and *N,N*-diethylbenzidine the corresponding yellow quinone diimines were rapidly formed. No formation of blue pigments took place in this case. None of the aniline or benzidine derivatives tested was oxidized at a significant rate in absence of ceruloplasmin or in presence of apoceruloplasmin.

In view of the above experiments it appears that the ceruloplasmin catalyzed oxidation processes mainly conform to the established reaction path for enzymatic and non-enzymatic oxidations of *N*-alkylated aniline derivatives (Fig. 1).

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