STUDIES ON THE ACTIVE SITE OF MIXED FUNCTION OXIDASES IN RABBIT LIVER MICROSOMES[+]

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SUMMARY

The pKs of free groups in the active centre of microsomal oxygenases from rabbit's liver catalyzing the N-oxygenation of aniline, N-ethylaniline and N,N-dimethylaniline were determined to be 7 and 7.4. Aniline is bound to the oxidized form of the oxygenase, whereas N-ethylaniline and N,N-dimethylaniline are bound to the reduced form of the N-oxygenating system. It is concluded that there are differences in the hydroxylases N-oxygenating the three substrates.

In previous experiments a close relationship between the N-oxygenation of N-alkylanilines and of N,N-dialkylanilines by rabbit liver microsomes has been demonstrated. Furthermore, the N-oxygenation of these substrates was found to differ from the N-hydroxylation of aniline in affinity for oxygen, sensitivity to carbon monoxide and other respects (1, 2). Recently Ziegler (3) reported on the isolation of an enzyme system from pork liver microsomes producing N,N-dimethylaniline-N-oxide from N,N-dimethylaniline. No cytochrome P-450 was detected in the purified enzyme preparations. These and other results hint at the existence of several oxygenating mechanisms in microsomes.

[+] The results of this study were briefly presented at the Meeting of the Deutsche Pharmakologische Gesellschaft in Mainz on March 15 to 18, 1970 (4)

This study reports on the influence of the hydrogen ion concentration on the Michaelis constants and maximum reaction velocities in the N-oxygenation of aniline, N-ethylaniline and N,N-dimethylaniline by rabbit liver microsomes.

MATERIALS AND METHODS

Microsomes from rabbit livers were prepared according to v. Jagow et al. (5). Hemoglobin was removed from microsomal suspensions by several washings with 0.15 M potassium chloride (6). For determining the rates of oxygenation at various pH values the microsomal pellets were suspended in the following buffers: 0.15 M phosphate pH 6.5 and 0.15 M tris-HCl pH 7 to 9, the ionic strength ranging from 0.02 to 0.3. Suspensions of microsomes containing about 3 mg microsomal protein per ml and 1.2×10^{-4} M NADP, 4.8×10^{-3} M glucose-6-phosphate, 1.2×10^{-2} M nicotinamide, 6×10^{-3} M magnesium chloride, 0.7 I.U. glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) per ml, and 10^{-4} to 10^{-3} M substrate (anilines) were incubated for 20 min under air at 37°. Phenylhydroxylamine was determined according to Herr and Kiese (7) after being oxidized to nitrosobenzene. Ziegler and Pettit's method (8) was used for determining N,N-dimethylaniline-N-oxide. Protein contents were measured according to Gornall et al. (9).

Michaelis constants and maximum reaction velocities were taken from Lineweaver-Burk plots. The effect of pH on these parameters was interpreted according to Dixon (10, 11).

RESULTS AND DISCUSSION

As illustrated by Figure 1 the N-hydroxylation of aniline

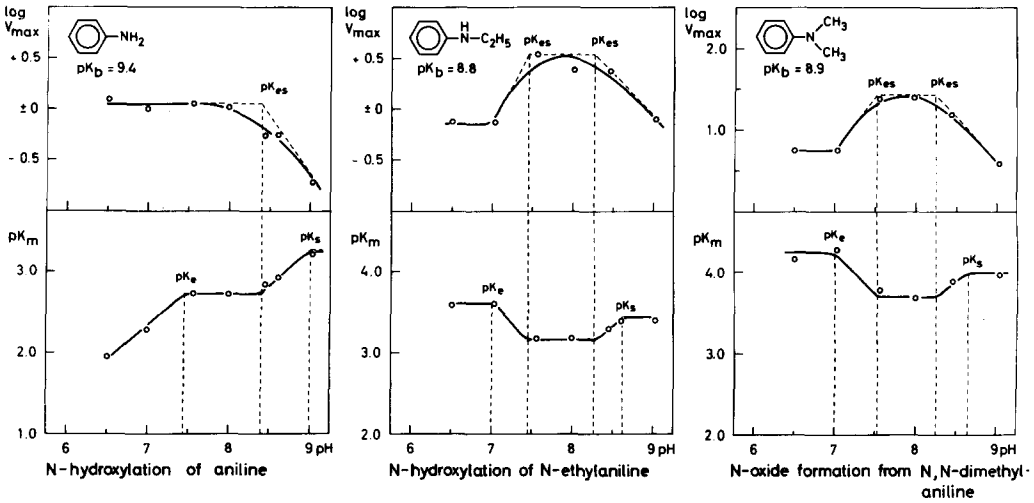

Figure 1

Effect of pH on logarithm of maximum velocity (V_{max}) and negative logarithm of the Michaelis constant (K_m) in the N-oxygenation of aniline, N-ethylaniline and N,N-dimethylaniline by rabbit liver microsomes. pK_e, pK_s and pK_{es} are constants due to ionization of the enzyme, the substrate and the enzym-substrate complex. The data are the means of three to five experiments.

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shows three pKs in the plot of pK_m versus pH. The pK near 9.1 indicates the presence of free aniline. The pK at 8.4 is evidently due to the ionization of the amino group of aniline bound to the enzyme. Binding to the active centre of the enzyme makes aniline less basic. Then the pK at 7.4 reflects the ionization of a group in the active site of the oxygenase.

Maximum velocity is affected by the ionization of the amino group of aniline bound to the enzyme. The pK of this

group is showing up at 8.4 when logarithm of maximum velocity is plotted versus pH. In the pH range between 6.5 and 7.5 a " + 1 unit slope " is observed. This means that the enzyme molecule gains one negative charge on aniline activation. Thus aniline seems to be bound to the oxidized enzyme. Since aniline is positively charged throughout this pH range, the data suggest a combination $E + S^+ + e^- \longrightarrow ES$.
The N-oxygenation of N-ethylaniline and N,N-dimethylaniline shows plots of pK_m versus pH very similar to each other. The pKs at 8.6 and 8.7 correspond with those of the free substrates. The pK at 8.3 is most likely to be due to the ionization of the N-substituted amino groups of N-ethyl- and N,N-dimethylaniline bound to the enzyme. Since neither substrate has a pK at 7.0, this pK belongs to a free functional group in the active site of the enzyme. The pK at 7.5 represents the ionization of the enzyme-substrate complex. Ionization of this complex affects maximum reaction velocity. The pK of the enzyme-substrate complex is showing up at 7.5 when logarithm of maximum velocity is plotted versus pH. In the alkaline range a " - 1 unit slope " is observed. This means that the enzyme loses one negative charge on substrate activation. Thus N-ethylaniline as well as N,N-dimethylaniline seems to be bound to the reduced form of the enzyme which catalyzes the N-oxygenation of these substrates. Since both substrates are positively charged at a pH around 7.0 combination of N-ethyl- and N,N-dimethylaniline with the oxygenase is represented by $E^= + S^+ - e^- \longrightarrow ES$.

These differences in the carbon monoxide sensitive N-hydroxylation of aniline (1, 12) on the one hand and the carbon monoxide insensitive N-oxygenation of N-ethyl- and N,N-dimethyl-

aniline (1, 2) on the other hand can easily be explained by assuming two different enzymes involved in these N-oxygenations. This view is supported by Ziegler's findings demonstrating N-oxide formation in the absence of cytochrome P-450 (3).

The pK values at 7.0 and 7.4, representing ionization of free functional groups, are closely related to the imidazole group of histidine, 5.6 to 7.0, or the N-terminal α-ammonium group of cystine, 6.5 to 8.5, as observed in proteins (13). Therefore a histidine or cystine residue is believed to be involved in the catalytic process. This is consistent with results of other authors (14, 15, 16) which hint at liganding of sulfhydryl or imidazole groups to the heme of cytochrome P-450.

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