Oxygen Activation by the Iron(II)-2-Mercaptobenzoic Acid Complex.
A Model for Microsomal Mixed Function Oxygenases

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Mixed function oxygenases catalyse the introduction of one oxygen atom of molecular oxygen into an organic substrate. The following stoichiometry has been established$^1$–$^3$:

\[
RH + DH_2 + 18O_2 = R^{18}OH + D + H_2O.
\]

The main problem in understanding the mechanism of these enzymes is the activation of the oxygen molecule. A direct investigation of this process is hampered by the lack of pure enzyme preparations. For several mammalian steroid hydroxylases and for the mixed function oxygenation system in liver microsomes it has been established that cytochrome P-450 is involved in the reduction and activation of molecular oxygen$^4$. Some spectral investigations have been made with respect to a possible oxygenated intermediate$^5$. However, an interpretation of any spectral data will only be meaningful if the chemistry of oxygen activation is fully understood.

Therefore, this problem has been extensively studied in chemical models systems for the activation of molecular oxygen$^6$–$^7$. A large number of hydroxylating systems have been described but, so far, none of them could be regarded as a true model simulating all characteristic features of the mixed function oxygenases. These primarily are concerned with the stoichiometry and introduction of molecular oxygen into the substrate. In addition, several chemical aspects of the mixed function oxygenation reactions have to be considered.

The broad substrate specificity of the microsomal mixed function oxygenation system turned out to be of great advantage for investigation of the chemical properties of enzymatically active oxygen. A large number of organic compounds have been studied as substrates, thus providing valuable information about the chemistry of mixed function oxygenases. Four reactions can be regarded as typical:

1. Hydroxylation of a CH-bond at a saturated carbon atom;
2. Dealkylation reactions at heteroatoms;
3. Hydroxylations of aromatic rings at positions of high electron density;
4. Epoxidation of double bonds.

Recently, special interest has been paid to the last reaction because epoxides probably are the common intermediates for different pathways of mixed function oxygenation as depicted in the following scheme:

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1. H. S. Mason, Advances in Enzymol. 19, 79 [1957].
mechanism of peracid oxidations which proceed in a concerted reaction with the OH\(^{\circ}\) ion (the protonated oxygen atom) as the attacking species. This mechanism, however, is hardly in accordance with the activation of molecular oxygen by cytochrome P 450 and does not seem feasible under physiological conditions.

Recently we have reported the existence of a new hydroxylation mechanism which is observed during the reduction of molecular oxygen by stannous phosphate complexes. The stoichiometry of the hydroxylation is described by the equation 14:

\[
\text{Sn}^{\circ\circ} + \text{RH} + {^18}\text{O}_2 + \text{H}^{\circ} \rightarrow \text{Sn}^{\circ\circ} + \text{R}^{18}\text{OH} + {^18}\text{OH}^{\circ}.
\]

By this reaction it was first shown that a two-electron reduction of the oxygen molecule can lead to a high-energy precursor of hydrogen peroxide able to hydroxylate aliphatic and aromatic compounds. With regard to oxygen activation this system represents the best model. However, the hydroxylation reactions of aliphatic and aromatic compounds are highly unselective and, therefore, lack the electrophilic properties of enzymatically active oxygen.

In order to approach more closely to the enzymatic systems it was desired to reduce the oxygen molecule by iron(II)-complexes instead of the stannous phosphate complex. It seemed probable that iron(III)-complexes could stabilize the active oxygen as in the peroxidase reactions 16, 17 and thereby lessen its reactivity.

Hydroxylating model systems with iron(II)-complexes are well known but they usually generate OH\(^{\circ}\) radicals by a sequence of three one-electron reduction steps 9, 18, 19. Since we know that oxygen reduction to an oxenoid species must proceed by a two-electron transfer, we have tried iron(II)-complexes with a thiol group as ligand in order to get a two-electron donating entity. In this paper, mixed function oxygenations by the iron(II)-2-mercaptobenzoic acid complex are investigated and compared with the corresponding reactions in liver microsomes.

Materials and Methods

2-Mercaptobenzoic acid (thiosalicylic acid) was obtained from Fluka AG, Buchs S.G., Switzerland. Dodecaderuterocyclohexane ("Uvasol" grade) was a product of Merck AG, Darmstadt, Germany.

The standard incubation mixture contained the following concentrations in an aqueous acetone medium: 2-mercaptobenzoic acid $10^{-1} \text{M}$, ferrous sulphate heptahydrate $10^{-2} \text{M}$, sodium hydroxide $5 \times 10^{-3} \text{M}$. The concentrations of substrates varied between 0.8 M and 2.0 M and are given in the legend to the tables. Ferrous sulphate and sodium hydroxide were added in water so that the total water content of the incubation mixture was 10 per cent. For the incubation in non-aqueous medium, anhydrous ferrous chloride* and sodium methylate were used.

The incubation was carried out at 25°C under vigorous shaking with air as the gas phase. After about 60 minutes the color of the mixture turns from blue to yellow indicating the end of the reaction. The isolation and identification of the products are given in the legend of each table. Concentrations were determined by means of calibration curves obtained by incubating the pure products with an oxidized substrate-free assay mixture. All values represent averages of 10 experiments.

Results

1. The hydroxylation of cyclohexane

Recently we have shown that cyclohexane is hydroxylated to cyclohexanol with high specific activity by the mixed function oxygenation system in liver microsomes 20. In principle the same hydroxylations at alicyclic carbon atoms occur with the various steroid hydroxylases. We, therefore, have studied the hydroxylation of cyclohexane as a model reaction for enzymatic hydroxylations at a saturated carbon atom 7,12. The results with the system Fe²⁺/2-mercaptobenzoic acid/O₂ are shown in Table 1. At the same time the existence of a kinetic isotope effect with dodecaderuterocyclohexane was investigated.

The system hydroxylates cyclohexane with good yields. As in the microsomal mixed function oxygenation of cyclohexane and dodecaderuterocyclohexane no isotope effect could be detected 20.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cyclohexanol [μMoles]</th>
<th>Cyclohexanone [μMoles]</th>
<th>kH/kD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexane</td>
<td>6.9</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Dodecaderuterocyclohexane</td>
<td>8.0</td>
<td>1.0</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Table 1. The hydroxylation of cyclohexane by the model system. Concentrations and incubation conditions are described under "Methods". Cyclohexane and dodecaderuterocyclohexane 2 M. Acetone was removed by evaporation and the residue was dissolved in 1 ml of 1 N NaOH. Cyclohexanol was extracted into 1.5 ml of ethyl acetate. 1.0 ml of the organic phase was concentrated to 0.1 ml and 3 μl aliquots were analysed by GLC (gas chromatograph F 6, Perkin-Elmer). 1st column: 15% trimethylolpropane trielargonate /Celite 545, 60—100 mesh, 200 cm. 2nd column: 15% polyethylene glycol 1500/Celite 545, 60—100 mesh, 200 cm. T = 115°C. Carrier gas: N₂, detector: FID. Retention times: cyclohexanol 12.8 min., cyclohexanol 16.0 minutes.

2. The O-dealkylation of N-acetylphenetidin (phenacetin)

Lipid soluble organic compounds with alkyl groups at heteroatoms like oxygen, nitrogen or sulphur, are readily dealkylated by the mixed function oxygenation system in liver microsomes 21—23. The alkyl group is found as the corresponding aldehyde in the reaction medium. It is generally accepted now that the main mechanism leading to dealkylation involves hydroxylation at the α-carbon atom of the alkyl group. In one case of a N-dealkylation this unstable intermediate could be identified 24. For N-acetylphenetidin, dealkylation is the major route of metabolism in the body 25. Hydroxylation at the aromatic ring occurs to a minor extent only.

OH-radicals, as well as the "oxenoid" mechanism in the system Sn²⁺/HPO₄²⁻/O₂ are also able to dealkylate phenacetin 26. The hydroxylation at the aromatic ring, however, is always favored. Table 2 contains the yields and ratios of the phenolic products obtained when phenacetin is used as a substrate in the Fe²⁺/2-mercaptopbenzoic acid/O₂ system.

The mixed function oxygenation of phenacetin in this system yields a higher ratio of dealkylation: hydroxylation than any other model system investigated.

* This compound was kindly supplied by Dr. H. J. Seifert, Institute for Inorganic and Analytical Chemistry, Gießen.

22 I. Axelrod, Biochem. J. 63, 634 [1956].
3. The hydroxylation of acetanilide and toluene

Aromatic compounds with one first order substituent (ortho-para directing) are hydroxylated by the unspecific liver microsomal system at positions of high electron density. This leads to a favored attack at the ortho- and para-positions, indicating that we were dealing with an electrophilic attacking species. In general the ortho-position always is subject to considerable steric effects so that the meta: para-ratio is a better parameter for expressing the selectivity of electrophilic properties of a substitution mechanism. In contrast, the active oxygen species in the system SnO2/HPO42−/O2 attacks the aromatic nucleus completely unselectively yielding meta: para ratios of about two which would be expected for a random substitution.

The distribution pattern of phenolic products obtained with acetanilide in the Fe(II)2-mercaptobenzoic acid/O2 system can be seen from Table 3.

Table 3. Hydroxylation of acetanilide in the model system. Acetanilide 1.25 mM. All conditions and procedures are the same as used for phenacetin (see legend to table 2).

<table>
<thead>
<tr>
<th>System</th>
<th>Yield [μMoles]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(II)2-Mercaptobenzoic Acid/O2</td>
<td>3.2</td>
</tr>
<tr>
<td>Rat Liver Microsomes/NADPH/O2</td>
<td>0.08</td>
</tr>
</tbody>
</table>

The low meta: para ratio of the isomers clearly points to an electrophilic and rather selective hydroxylation mechanism. The corresponding meta: para ratio for the hydroxylation of acetanilide by the microsomal mixed function oxygenation system was found to be around 0.01. When the incubation was carried out in a nonaqueous medium at conditions described under “Methods” about the same yields and an identical hydroxylation pattern are obtained.

In order to further establish the electrophilic properties of the active oxygen in microsomes and our model system we have determined the pattern of phenolic isomers obtained by the hydroxylation of toluene in both systems (Table 4).

Table 4. Ring hydroxylation of toluene in rat liver microsomes and the model system. Microsomes of phenobarbital-treated male rats (Wistar AF Han) were used. The incubation mixture contained in a total volume of 20.0 mg of tris buffer (0.05 M pH 7.5) the following concentrations: microsomal protein 2 mg/ml, NADPH 5 × 10−4 M, glucose-6-phosphate 2 × 10−3 M, glucose-6-phosphate dehydrogenase 10 U, toluene 10−5 M, added as 1 M solution in ethanol. The incubation was carried out for 30 min. at 25°C. Protein was removed by addition of uranium acetate, subsequent heating to 80°C, and centrifugation. The supernatant was extracted with 5 ml of ethylacetate 3 ml were concentrated to 0.1 ml and aliquots were separated by gaschromatography using two 200 cm columns (10% tri-2,3-xylyl phosphate). T = 140°C, carrier gas: N2 detector FID. Retention times: benzaldehyde 8.4 min., benzyl alcohol 30.4 min., ortho-cresol 71.5 min., para-cresol 91.5 min., meta-cresol 100.1 min. The same separation method was applied for the model hydroxylations. Toluene 2 M. The isolation of the products was the same as described for cyclohexanol (see legend to table 1).

It can be seen that an almost identical hydroxyl-
ation pattern is obtained in microsomes and the model system. In both systems the hydroxylation of
the side chain is considerable. The relative yields of
benzyl alcohol are not given because benzoic acid
as a main secondary oxidation product could not be
determined in the same test.

4. The mixed function oxygenation of naphthalene

Substrates containing double bonds are epoxidized
by the microsomal system and by mixed function
oxidases in general.\textsuperscript{1, 31, 32}

According to the scheme in Fig. 1 the epoxide
may undergo hydration to the dihydrodiol
compound.

Naphthalene can be regarded as an aromatic 10 π
electron system with a high degree of double bond
character in one ring. In liver microsomes in the
presence of NADPH and oxygen it is converted to
80% naphthalene-1,2-dihydrodiol and 20% π- and
β-naphthol.\textsuperscript{12} The trans-configuration of the two
hydroxyl groups\textsuperscript{31} and the fact that only one oxygen
atom derives from molecular oxygen\textsuperscript{33} are both
compatible with the assumption that the 1,2-epoxide
is the primary product of the attack by the enzymatically
active oxygen species. This was confirmed very
recently by the isolation and identification of
naphthalene epoxide from a microsomal system.\textsuperscript{34}

As the formation of an epoxide or a dihydrodiol
compound gives direct proof for the addition and
hence the electrophilic properties of an oxenoid spe-
cies, we have studied naphthalene as a substrate in
the model system (Table 5).

The main product obtained under the conditions
of the assay and isolation procedure was identified
as the dihydrodiol compound by its Rf-values, ir-
spectrum and nmr-spectrum. According to the nmr-
spectrum the hydroxyl groups have the trans-con-
figuration.

Discussion

The iron(II)-complex of 2-mercaptobenzoic acid
reduces molecular oxygen to an active oxygen spe-
cies which is capable of reacting with organic com-
pounds in a manner analogous to the microsomal
mixed function oxygenation system. The model
system can hydroxylate even in the complete ab-
sence of water indicating that the oxygen atom of
the hydroxyl group must derive from the oxygen
molecule of the gas phase.

Characteristics of this hydroxylation mechanism
are its electrophilic reaction towards substrates with
π-electron systems, and that it also can hydroxylate
aliphatic CH-bonds. The active oxygen, therefore,
is clearly distinct from the OH-radical which does not
form alcohols or the dihydrodiol compound of
naphthalene. On the other hand it is also different
from the oxene-mechanism in the stannous-phos-
hate/oxygen system in being selective and less re-
active.

<table>
<thead>
<tr>
<th>System</th>
<th>1-Naphthol (%)</th>
<th>2-Naphthol (%)</th>
<th>Naphthalene 1,2-dihydrodiol (%)</th>
<th>Yield [μMoles]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe\textsuperscript{2+}/2-Mercaptobenzoic Acid/O\textsubscript{2}</td>
<td>11</td>
<td>9</td>
<td>80</td>
<td>1.7</td>
</tr>
<tr>
<td>Rat Liver Microsomes/NADPH/O\textsubscript{2} \textsuperscript{12}</td>
<td></td>
<td></td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Mixed function oxygenation of naphthalene in the model system. The standard incubation mixture was used. Naphthalene 1 M. After completion of the reaction acetone was evaporated and 2.0 ml of 0.1 M NaOH were added. After neutralisation with solid ammonium acetate an excess of naphthalene was removed by extracting twice with 5.0 ml of petrol ether (b.p. 50–60 °C). The products were then extracted into 10 ml of ether. A 9.0 ml aliquot was evaporated and chromatographed by two-dimensional TLC. 1\textsuperscript{st} solvent: benzene 79, methanol 14, acetic acid 7 (v/v). 2\textsuperscript{nd} solvent: petrol ether 40, chloroform 55, 2-propanol 5, half-saturated with ammonia. The spots containing α- and β-naphthol, respectively, were collected in two-dimensional TLC. 1\textsuperscript{st} solvent: benzene 79, methanol 14, acetic acid 7 (v/v). The extinction was read at 578 mm in a photometer. Naphthalene-1,2-dihydrodiol was first converted into α-naphthol by treatment with 0.9 ml 0.1 M HCl for 60 min. at 100 °C. Then 0.1 ml 1 M NaOH was added and the blue color with the Folin-Reagen was determined as described before.

31 P. Sims, Biochem. J. 73, 389 [1959].
32 B. M. Bloom and G. M. Shull, J. Amer. chem. Soc. 77, 5767 [1955].
Most important is the finding of a system which forms the 1,2-dihydrodiol compound from naphthalene. This led us to suggest an oxenoid-species as the active oxygen. Further studies on the chemical properties of the mechanism are in progress in order to strengthen this hypothesis.

The sulfhydryl group was chosen as a ligand for ferrous ion in order to provide a donor for the second electron needed for the generation of an oxenoid species. It is not known at present, how the two-electron transfer to the oxygen molecule occurs at cytochrome P-450. However, one may speculate on the participation of a thiol group since cytochrome P-50 instantaneously loses its characteristic unusual spectral and chemical properties on treatment with iodine, N-bromosuccinimide or p-chloromercuribenzoate. At the same time a conversion from a low spin cytochrome to a high spin state is observed. The role of sulphur as a ligand of iron has been established in the case of a bacterial oxygenase.

The moderate reactivity of the oxenoid species in the system \( \text{Fe}^{2+}/2\text{-mercaptobenzoic acid}/O_2 \) compared to that of the system \( \text{Sn}^{2+}/\text{HPO}_4^{2-}/O_2 \) may be caused by complex formation thereby stabilizing the active oxygen at the ferric ion in a manner similar to the various oxygen complexes of iron-porphyrin enzymes.

In summary our results indicate that the reaction of the iron(II)-2-mercaptobenzoic acid complex with oxygen provides a suitable model for the activation of molecular oxygen by cytochrome P-450.

Further investigations are in progress with a more detailed study of the chemical and physical properties of this model system.

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