Effect of iron and hemoproteins on hydrogen peroxide-supported styrene oxidation to styrene oxide[1]

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Summary. Fe^{2+}, Fe^{3+} and their complexes with EDTA and hemin, methemalbumin and methemoglobin were active catalyzers of H_2O_2 supported styrene oxidation to styrene oxide. Methemoglobin was the most active compound; its peroxidative activity was comparable to that of cytochrome P-450 in liver microsomes of phenobarbital-treated rats. Cumene hydroperoxide supported styrene oxidation with methemoglobin and microsomal hemoproteins and was found to be more efficient than H_2O_2.

Iron salts, in the presence of H_2O_2, can catalyze the oxidation of different chemicals by dealkylation and hydroxylation[2] and the interaction between O_2^- and H_2O_2 after chelation with EDTA, with production of more reactive O_2 intermediates[3,4].

The catalytic power of iron greatly increases when it is coordinated with the ring of protoporphyrin IX[5] and particularly when the resulting heme molecule functions as the prosthetic group of hemoproteins such as cytochrome P-450. This cytochrome is present in mammalian tissues, mainly the liver, and catalyzes the metabolism of a wide variety of substrates in the presence of NADPH and molecular oxygen[6]. Moreover it has been shown that cytochrome P-450 can function as a peroxidase[7,8] and that it is able to catalyze hydroxylation supported by various organic hydroperoxides as well as H_2O_2 as cosubstrate[9-11].

These findings suggested that it would be interesting to study the extent of metabolism supported by peroxides, particularly H_2O_2, in different iron environments which could affect its catalytic activity. Styrene, a widely used industrial product which is activated metabolically to the potentially toxic and mutagenic styrene oxide[12,13], was chosen as model compound.

Materials and methods. Hemin, methemoglobin and albumin were obtained from Sigma (St. Louis, MO, USA). Horseradish peroxidase and NADPH were purchased from Boehringer (Mannheim, FRG). Methemalbumin was prepared as described by Tenhunen et al.[14].

Liver microsomes were prepared from Charles River CD male rats (b.wt 150–180 g) according to the method of Kato and Takayanaghi[15], utilizing for liver homogenization a solution consisting of phosphate buffer 0.05 M, pH 7.4, containing saccharose 0.25 M; the cytochrome P-450 content was measured according to Omura and Sato[16]. Animals were induced by pretreatment with phenobarbital dissolved in 0.9% NaCl with a dose of 80 mg/kg i.p. daily for 3 days or with 3-methyl-cholanthrene, dissolved in corn oil, with a single dose of 40 mg/kg i.p. After the last treatment rats were fasted for 16 h before being killed.

The standard incubation mixture consisted of 1 ml of 0.2 M phosphate buffer, pH 7.4; concentrations of the different chemicals and enzymes are indicated in the tables.

Samples were incubated for 10 min at 37 °C with shaking. The amount of styrene oxide formed during incubation was measured by a method previously described[17-19]. In this procedure, at the end of incubation the styrene oxide formed is quantitatively hydrated chemically by overnight incubation with H_2SO_4 (0.6 N) to the glycol, which is more suitable for gas chromatographic analysis. Styrene glycol is then quantitatively determined by a sensitive gas chromatographic procedure using an electron capture detector as previously described[20].

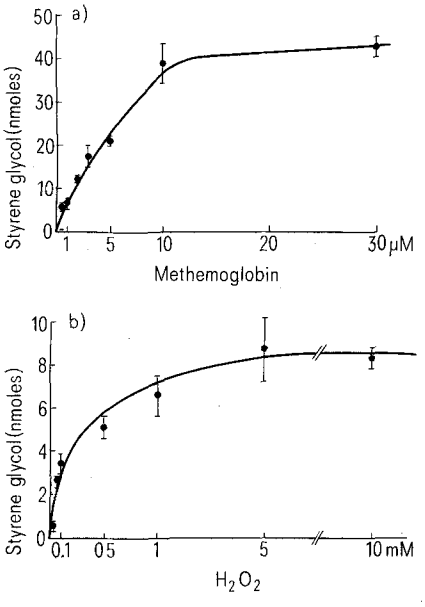
Results. H_2O_2 increased the formation of styrene oxide catalyzed by Fe^{2+} and Fe^{3+} (table 1). H_2O_2 had a similar effect with iron ions in the presence of albumin or chelated with EDTA. The activity of hemin, although expressed in table 1 per nmole heme to permit comparisons with the activity of hemoproteins, can also be directly compared with that of iron and iron complexes, expressed per μmole Fe since hemin contains 1 mole of iron per mole of heme. This comparison shows that hemin was as active as Fe^{2+} and Fe^{3+} EDTA complexes at an 8 times lower iron

Table 1. Hydrogen peroxide-supported styrene oxidation to styrene oxide

System	Styrene glycol (nmoles/μmole Fe) $-H_2O_2$	$+H_2O_2$
A) Effect of iron and iron complexes		
Fe^{2+}	2.80 ± 0.09	7.31 ± 0.41
Fe^{3+}	n.d.	7.83 ± 0.26
Fe^{2+} + EDTA (0.70 mM)	2.27 ± 0.32	75.80 ± 8.50
Fe^{3+} + EDTA (0.70 mM)	n.d.	39.90 ± 2.90
Fe^{2+} + albumin (11 μM)	2.40 ± 0.19	8.18 ± 0.68
Fe^{3+} + albumin (11 μM)	n.d.	5.04 ± 0.55

System	Styrene glycol (nmoles/nmole heme) $+H_2O_2$
B) Effect of hemin and hemoproteins	
Hemin (50 μM)	0.026 ± 0.003
Methemalbumin (50 μM)	0.140 ± 0.010
Methemoglobin (1 μM)	1.30 ± 0.070
Horseradish peroxidase (50 μM)	0.022 ± 0.001

Fe^{2+} and Fe^{3+} concentration was 0.42 mM; styrene and H_2O_2 concentrations were 50 mM and 1 mM respectively. Styrene glycol formed in blank samples with no iron ions was subtracted. Values are the mean ± SE (replication No. 6); n.d., not detectable.



Styrene oxide formation with different methemoglobin concentrations (H_2O_2 1 mM) (a), and with different H_2O_2 concentrations (methemoglobin 1 μM) (b). Samples were incubated for 10 min with 50 mM styrene.

concentration and its catalytic capacity increased 5 times when the heme was non-specifically complexed with albumin to obtain methemalbumin (table 1). With methemoglobin, the activity expressed per nmole of heme is higher than hemin and methemalbumin, indicating that the structure of this hemoprotein is able per se to increase the catalytic activity.

However the catalytic activity of horseradish peroxidase, a hemoprotein with peroxidative activity, was comparable to hemin (table 1). The production of styrene oxide as an intermediate in the formation of styrene glycol was confirmed for the Fe^{2+}-EDTA complex and methemoglobin by a much smaller amount of glycol in samples extracted after addition of NaOH in place of H_2SO_4 (data not shown).

Styrene oxidation catalyzed by methemoglobin was studied in detail and showed concentration dependent kinetics with different amounts of either the hemoprotein or H_2O_2 (fig.). In these kinetics the non-variables were present in non-saturating amounts in order to avoid H_2O_2 mediated heme destruction[21] and styrene precipitation of methemoglobin which are more likely when saturating concentrations of H_2O_2 and methemoglobin are used. This reaction was compared to that of cytochrome P-450 present in liver microsomes of control (CO), phenobarbital (PB)-and 3-methylchloranthrene (3-MC)-treated rats (table 2). Results with 2 other cofactors, NADPH and cumene hydroperoxide, are reported in the same table.

The activity of methemoglobin for the H_2O_2-supported reaction was comparable to that of PB-induced microsomes, but 10 times higher than that of CO and 3-MC microsomes. This hemoprotein was inactive in the presence of NADPH and showed about 4 times greater activity in the presence of cumene hydroperoxide compared to that with H_2O_2. However the peroxidative activity of methemoglobin was lower than all microsomal preparations with cumene hydroperoxide.

The peroxidative activity of microsomes differed depending on the pretreatment. PB microsomes were the most active for H_2O_2-supported styrene oxidation but had the lowest activity with cumene hydroperoxide, while 3-MC-induced microsomes showed the opposite pattern.

Discussion. H_2O_2, like O_2^- and $^{\cdot}OH$, is one of the reactive O_2 intermediates formed in several biological processes such as lipid peroxidation and the bactericidal action of phagocytes[22,23]. These reactive species may be important in the activation of xenobiotics to potentially toxic derivatives.

Table 2. Effect of methemoglobin and rat liver microsomes on hydrogen peroxide and cumene hydroperoxide-supported styrene oxidation to styrene oxide

System	Styrene glycol (nmoles/nmole hemoprotein)		
	NADPH	H_2O_2	Cumene hydroperoxide
Methemoglobin	n.d.	6.42 ± 0.73	24.3 ± 0.4
CO	46.0 ± 1.6	0.80 ± 0.17**	123.0 ± 0.7**
PB	32.1 ± 6.6	5.37 ± 1.13	61.5 ± 1.7*
3-MC	18.9 ± 3.3	0.62 ± 0.21**	117.8 ± 3.5**

Samples were incubated with styrene (50 mM); H_2O_2, cumene hydroperoxide and NADPH concentration was 1 mM. Methemoglobin concentration was 1 μM and for rat liver microsomes from control (CO), phenobarbital (PB)- and 3-methylcholanthrene (3-MC)-treated animals an amount of protein corresponding to a final concentration of 1 μM cytochrome P-450 was used. Styrene glycol formed in blank samples consisting of buffer and cofactors was subtracted. Values are the mean ± SE, (replication No.4); n.d., not detectable. * $p < 0.05$ compared to methemoglobin; ** $p < 0.01$ compared to methemoglobin.

The present study shows that reactive O_2 intermediates produced in the interaction of H_2O_2 with iron ions and hemoproteins are able to support styrene oxidation to styrene oxide. Whether the differences in the extent of formation of styrene oxide with iron ions and hemoproteins are due to the greater catalytic activity of the hemoproteins or to the formation of different oxygenating intermediates remains to be established. Three different hemoproteins were tested: methemoglobin, horseradish peroxidase and cytochrome P-450 in microsomal preparations. Methemoglobin was reported to have peroxidative activity and to be converted to oxyhemoglobin[24] in the presence of H_2O_2. Probably this oxygenated form of hemoglobin is responsible for styrene oxidation. In fact human erythrocytes are able to support styrene oxidation to styrene oxide in the absence of H_2O_2 and this reaction is inhibited by CO[25].

Horseradish peroxidase was less active than the other hemoproteins and similar results were obtained by Nordbloom et al.[11] who found no activity in H_2O_2-supported benzphetamine demethylation catalyzed by this enzyme. H_2O_2 and cumene hydroperoxide have been shown to catalyze oxidation of xenobiotics with microsomal preparations and purified forms of cytochrome P-450[9-11]. In the latter case other microsomal electron carriers are not required in peroxide supported reactions[11]. NADPH and hydroperoxidesupported monooxygenases reactions have often been compared in attempts to clarify the mechanism by which cytochrome P-450 activates molecular oxygen catalyzing the xenobiotic metabolism[10,26,27]. It has been proposed that the active O_2 intermediates might be similar in the 2 types of reactions[11]. However, pronounced differences have been found in cytochrome P-450 dependent metabolism of benzo(a)pyrene supported by NADPH and cumene hydroperoxide and differences were also observed in catalytic activity with either cytochrome P-450 or P-448 for the cumene hydroperoxide supported reaction[27,28]. Our data show that there is no parallelism between NADPH and hydroperoxides with the different types of microsomes for styrene oxidation.

These results suggest that in the series of events which lead to the formation of the reactive O_2 intermediate(s), the apoprotein structure of the hemoproteins could play an important role.

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Preparation and properties of hexitol-lysyl conjugates[1]

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Summary. The facile preparation of hexitol-lysyl derivatives is reported. Some of the properties of these compounds, particularly those relevant to protein structural studies, are described.

The occurrence in several proteins of glycosyl derivatives in which the linkage is an aldimine bond between a reducing sugar and an amino group (either the N-terminal α-amino or the ε-amino of a lysyl residue) of the protein has recently been reported. Proteins that are substrates for this nonenzymatic derivatization include albumin[4-6], haemoglobin[7,8], collagen[9-12], and crystallins[13]. The function of these is unknown but changes in their concentrations have been reported in pathological conditions, most notably diabetes[4,8,11,12]. The failure to detect these earlier and the lack of knowledge concerning their function is probably the result of their instability under normal protein hydrolysis conditions.

One method of detection is the conversion of the carbohydrate moiety to the 5-hydroxymethylfurfural derivative and the colorimetric assay of this with thiobarbituric acid[7]. The disadvantage of this assay is that it does not distinguish the isomeric form of the sugar attached to the lysyl or hydroxylysyl residue. An alternative technique is the reduction of the aldimine linkage, so forming the more stable hexitol-lysyl. The use of these derivatives in quantifying Schiff bases in foodstuffs has been described[14]. It is the preparation of N^{ε}-hexitol-lysyls and some of their characteristics, particularly under conditions used in protein structural studies, that is described in this communication.

Although both lysine and hydroxylysine have been successfully conjugated to galactose, glucose and mannose, the combination of galactose with these 2 amino acids will be presented here as an example of the procedure. The amino acid (10 µmoles) and the hexose (20 µmoles) are each dissolved in buffer and mixed (final volume of 4 ml) and stirred at room temperature for 1 h. 140 µl of freshly prepared sodium borohydride (50 mg/ml) is added and stirred for another h. The reduction reaction is then stopped by lowering the pH to approximately 3.5 with acetic acid and the mixture lyophilized. After redissolving, the reactants and products are separated by preparative high voltage paper electrophoresis at pH 2.1 and 3000 V. After ninhydrin and/or radioactivity scanning of markers, the hexitol-lysine can be eluted with 2% acetic acid.

The coupling and reduction can be performed in non-amino containing volatile buffers such as pyridine-acetate with yields of up to 50% for the lysyl and 70% for the hydroxylysyl derivatives. The use of such buffers is advantageous for the electrophoretic step. The pH optimum is between pH 6.0 and 6.5 and the number of aliquots in which the sodium borohydride is added does not significantly influence the yield. That the Schiff base does involve the ε-amino rather than the α-amino group of the amino acid is confirmed by performing the synthesis using α-tBOC-lysine[14] (Fluka) and ε-tBOC-lysine (Sigma). After the reduction, the blocking group is removed with 98% formic acid[15]. Only the α-tBOC-lysine yields the same hexitol lysine as when lysine is the substrate.

Frequently these hexitol-lysyl products have to be examined in protein hydrolyzates. So their behavior when subjected to hydrolysis in constant boiling hydrochloric acid in vacuo at 110 °C for 24 h and in 2N sodium hydroxide at 110 °C for 24 h was examined. The figure illustrates the multiple peaks that appear in amino acid chromatograms of acid hydrolyzed hexitol-lysines. There is a close similarity to the pattern reported by Robins and Bailey[9] although their hydrolyses were only for 12 h. They suggested that the formation of acid anhydrides was the cause of the multiplicity of peaks. This increase in the number of ninhydrin-positive species is confirmed by high voltage paper elctrophoresis. The alkaline hydrolyzates yield no ninhydrin-positive material that is detectable either on the amino acid analyzer or by paper chromatography or electrophoresis.

The relative mobilities of these products with respect to serine when electrophoresed at pH 2.1 in a Savant high electrophoresis enclosure are presented in the table. As might be expected at pH 6.4, these behave as neutral amino acids. R_f-values for descending paper chromatography in butanol-acetic acid-water solvents (with or without pyridine) are all less than 0.1 as are lysine and hydroxylysine. However, in phenol-water solvents, better separations are achieved and are detailed in the table. The sugar isomers are not separable by these paper techniques, but can be identified by amino acid analysis.

Thus, although the detection of Schiff bases involving amino groups of the protein and reducing moieties of sugar is difficult due to the lability of these bonds, stabilization