Short Communication

Oxidation of Some 2-Oximino-1,3-dioxo Compounds to Iminoxy Radicals by Horseradish Peroxidase and Hydrogen Peroxide Studied by Electron Spin Resonance (ESR) Spectroscopy

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The oxidation of oximino compounds (oximes) of the type $R^1R^2C{=}NOH$ (R^1,R^2 = alkyl or phenyl) by reagents such as $Pb(OAc)_4$ or potassium hexanitratocerate(IV) gives rise to the corresponding iminoxy radicals detectable by ESR spectroscopy.[1-5] Iminoxy radicals are considered to be σ radicals which are characterized, in this case, by a rather large coupling constant a_N, viz. between 26 and 32 G, and which are believed to be present as two geometric isomers, *syn* and *anti*, due to restricted rotation about the $>C{=}NO\cdot$ double bond ($R^1{\neq}R$). Many iminoxy radicals are rather unstable and can only be studied in flow systems with continuous mixing of the reagents. However, iminoxy radicals of the type $R^1COC(=NO\cdot)COR^2$ have been found to be relatively stable.[6] These radicals were produced by oxidation of the corresponding oximino compounds with potassium hexanitratocerate(IV), and also directly in the reaction between tetranitromethane and β-dioxo compounds, such as acetylacetone, benzoylacetone, dibenzoylmethane and β-oxo esters.

It has now been found that oximino compounds of the type $R^1COC(=NOH)COR^2$ are oxidized to the corresponding iminoxy radicals by horseradish peroxidase and hydrogen peroxide in high yields without using a flow system with continuous mixing of the reactants.

Materials and methods

Materials. 3-Oximino-2,4-dioxopentane and ethyl 2-oximino-3-oxobutanoate were prepared by standard methods.[7] 5-Isonitrosobarbituric acid (violuric acid) was obtained from Fluka AG. Horseradish peroxidase (EC 1.11.1.7) was from Sigma Chemical Company (P-8125). Potassium peroxylamine disulfate was obtained from Aldrich. Buffer substances and 30 % hydrogen peroxide were of analytical grade.

Preparation of iminoxy radicals. The oximino compounds were dissolved in phosphate buffer solutions of pH between 5.0 and 7.4 to a concentration between 10 and 10 mmol. Horseradish peroxidase was added to a concentration of about $1–2\cdot10^{-3}$ units per μl (one unit will form 1 mg of purpurogallin in 20 s from pyrogallol at pH 6.0 at 20 °C), together with hydrogen peroxide. The best results were obtained when the ratio of oxime to hydrogen peroxide was about 5:1.

Electron spin resonance measurements. The ESR spectra were recorded using a Varian E-9 spectrometer at 20 °C with the samples contained in a flat cell. The spectra were recorded with a microwave power of 2 mW and a 100 kHz modulation amplitude of 0.1 G. Hyperfine splitting constants

Table 1. Coupling constants (in G) for the iminoxy radicals formed from the corresponding oximino compounds (oximes) by oxidation with horseradish peroxidase and hydrogen peroxide.

Radical	a_N	Secondary splittings
3-Iminoxy-2,4-dioxopentane	28.7	a_H = 0.40 quartet
5-Iminoxy-barbituric acid[a]	31.2	a_N = 2.40 triplet a_H = 1.79 doublet
	30.2	–
Ethyl 2-iminoxy-3-oxobutanoate[b]	31.6	Unresolved structures
	30.0[c]	

[a]Two isomers above pH 5.7. [b]Two isomers. [c]Quantitatively dominant isomer.

were measured by comparison with the splittings of Fremy's radical (a_N = 13.0 G).

Results and discussion

The results with 3-oximino-2,4-dioxopentane, violuric acid (5-isonitrosobarbituric acid) and ethyl 2-oximino-3-oxobutanoate are described. These substances were selected as representatives for the 2-oximino-1,3-dioxo compounds which can be oxidized to iminoxy radicals by horseradish peroxidase and hydrogen peroxide. For the preparation of the radicals, it is essential to avoid an excess of hydrogen peroxide relative to 3-oximino-2,4-dioxopentane and ethyl 2-oximino-3-oxobutanoate. The radicals derived from violuric acid could also be detected after using an excess of hydrogen peroxide. No radicals could be observed in reaction mixtures containing the oximino compound, and peroxidase alone or hydrogen peroxide alone.

The ESR spectra of the radicals are dominated by a large triplet splitting due to the interaction of the unpaired electron with one ^{14}N nucleus. The coupling constants, a_N, varied between 28 and 31 G. Secondary splittings due to the interaction with other magnetic nuclei of the radical molecules were in general rather narrow. The radicals are evidently iminoxy radicals, identical with those described earlier.[6] The radicals were formed in the pH range 5.0 to 7.4. The coupling constants are given in Table 1.

Fig. 1 shows the ESR spectrum of 3-iminoxy-2,4-dioxopentane obtained at pH 5.8. One single species is present in this case, since $R^1=R^2=CH_3$ in $R^1COC(=NO\cdot)COR^2$. The secondary quartet splittings arise from the interaction of the unpaired electron with the hydrogen

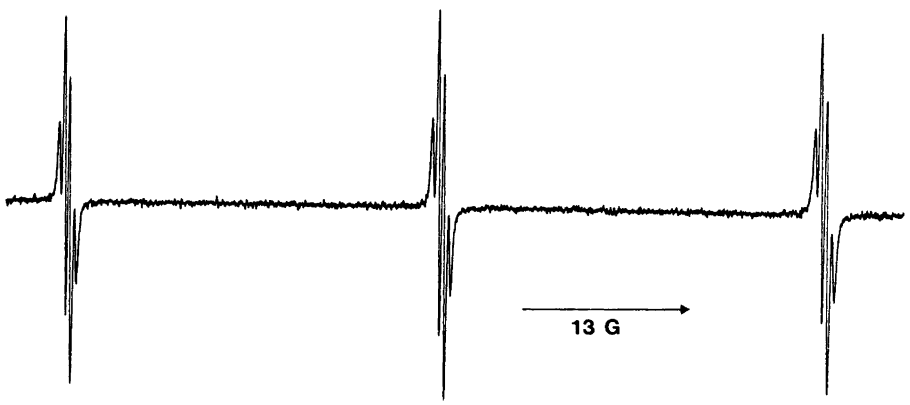

Fig. 1. ESR spectrum of the iminoxy radical $CH_3COC(N=O\cdot)COCH_3$ formed by oxidation of $CH_3COC(N=OH)COCH_3$ with horseradish peroxidase and H_2O_2 at pH 5.8.

atoms of the methyl group *syn* to the >C=NO· group.[8] The splittings from the methyl group in the *anti* position could not be resolved in this spectrum (cf. Ref. 6). Evidently, the radicals are present in the keto form.

Violuric acid gives rise to a single radical species below pH 5.7 due to the symmetry of the parent compound, barbituric acid. The secondary splittings show interactions with one ^{14}N nucleus and one hydrogen atom, which indicates that the radical is present in the keto form. Above pH 5.7 the ESR spectra exhibited an overlapping large triplet splitting from an additional isomer. The appearance of this radical species may be connected with rearrangement to an enol form and/or dissociation of a proton.

Ethyl 2-iminoxy-3-oxobutanoate is an unsymmetrical radical which is present in two isomeric forms; $R^1COC(=NO\cdot)COR^2$, $R^1 \neq R^2$. The ESR spectra of these species have been described earlier in more detail.[6]

The results seem to be in conformity with the reaction mechanism generally valid for horseradish peroxidase/H_2O_2 systems,[9–12] which can be expressed in this case as follows:

Peroxidase + H_2O_2 → Compound I

Compound I + oxime → Compound II + iminoxy radical

Compound II + oxime → Peroxidase + iminoxy radical.

In this sequence, Compounds I and II are intermediate forms of the enzyme.[9,10]

It should be pointed out that the oxidation of the oximes to iminoxy radicals by the iron-containing horseradish peroxidase/H_2O_2 system may be enhanced by strong binding of substrate molecules to the enzyme, since oximes such as violuric acid and 3-oximino-2,4-dioxopentane are known to be chelators of cations such as Fe(II) and Fe(III).[13]

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