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Kinetics of Amide and Peptide Cleavage by Alkaline Hydrogen Peroxide

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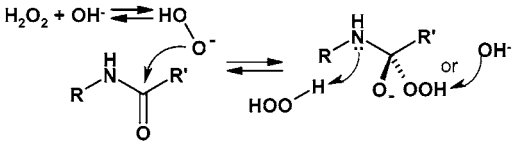
ABSTRACT



The hydroperoxide anion cleaves unactivated amides and peptides although it is completely unreactive toward ethyl esters. The cleavage by HO_2^- proceeds faster than by OH^- and involves additional routes with general acid assistance by H_2O_2 and general base assistance by OH^- and HO_2^-. Cleavage of polypeptides occurs at the N-terminal peptide bond.

There is much current interest in the development of new reagents and/or catalysts for the hydrolytic cleavage of peptides and proteins.[1−3] The most active system reported so far is the iron chelate mediated cleavage achieved with peptide-attached iron(II) complexes in combination with H_2O_2 and ascorbic acid.[4,5] The proposed reaction mechanism involves the nucleophilic attack of the peptide bond by the metal-bound HO_2^- anion producing the tetrahedral addition intermediate, which undergoes further decomposition to the final hydrolytic products. However, a serious problem with this mechanism is that the HO_2^- anion is not expected to be able to afford the nucleophilic substitution of unactivated amide bonds. Indeed, the well-known α-nucleophilic character of HO_2^-, i.e., its strongly enhanced reactivity over that expected from its basicity, is manifested only in reactions with substrates possessing highly activated leaving groups, such as aryl esters,[6] phosphonates,[7] or phosphates.[8] Already ethyl esters do not react with HO_2^- at all.[9] The reason for this lack of reactivity is that HO_2^- is not sufficiently basic to throw out more basic ethoxide anion from the tetrahedral intermediate. The situation should be apparently even worse for amides and peptides possessing even more basic leaving groups. This paper demonstrates, however, that the α-effect is observed for HO_2^- in reactions with unactivated amides and peptides and also gives preliminary results on the selectivity of the peptide cleavage by HO_2^-.

The compounds studied as substrates (acetamide, glycinamide, *N*-acetylglycine, glycylglycine, and other peptides) were purchased from Sigma and used without further purification. Reagent-grade inorganic salts and hydrogen peroxide from Aldrich were used as supplied. UV spectra were obtained with a Hewlett-Packard 8452A spectrophotometer. ^{1}H NMR spectra were recorded on a 300 MHz Varian Unity INOVA spectrometer.

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Kinetics of the cleavage reactions were followed by ^{1}H NMR at 37 °C in D_2O containing 0.3−3% of H_2O introduced with H_2O_2 solution. Typically, 0.02−0.05 M solutions of amides or peptides, 0.1−0.5 M NaOD, and 0.1−1.0 M H_2O_2 were employed.

Figure 1 illustrates a typical kinetic experiment with GlyGly as a substrate. The signal at 3.0 ppm belongs to the

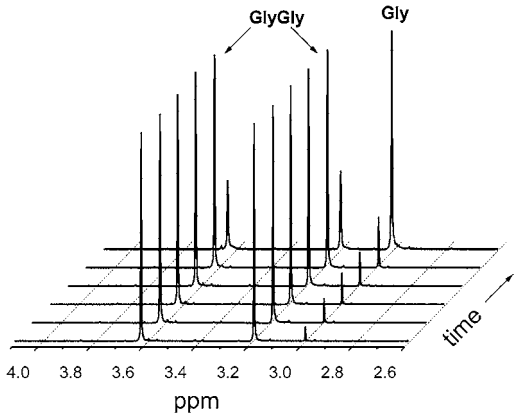

Figure 1. ^{1}H NMR spectra of 0.04 M GlyGly in D_2O in the presence of 0.5 M NaOD and 0.1 M H_2O_2 recorded after 13, 26, 38, 50, and 60 min and ca. 10 h at 37 °C.

glycinate anion, and its intensity grows with increase in the incubation time. In this and other experiments, only signals of the substrate and of the hydrolysis product(s) were observed. Apparently, the peroxy acids expected as primary reaction products are either too unstable under the reaction conditions or give the NMR signals indistinguishable with those of respective acids. The cleavage reactions were followed up to ca. 10−20% substrate conversion, and the initial rates were calculated from the plots of the product concentration obtained by the integration of signals vs the incubation time. The observed first-order rate constants (k_{obs}) were then calculated by dividing the initial rates by the initial substrate concentration.

Figure 2 shows the effect of added H_2O_2 on the rate of acetamide cleavage in the presence of 0.1 M NaOD (solid triangles) and the effect of added NaOD at a constant 0.2 M H_2O_2 (open triangles). Mixing of H_2O_2 with NaOH leads to a nearly quantitative deprotonation of H_2O_2. Therefore, an increase in k_{obs} on addition of H_2O_2 means that HO_2^- is a stronger nucleophile than OH^-. Additions of higher concentrations of H_2O_2 when it already is in excess over NaOH does not produce more HO_2^- anions, but the reaction rate is obviously going higher, thus indicating that the reaction with HO_2^- is catalyzed by neutral H_2O_2. On the other hand, additions of increasing amounts of NaOD in the presence of a fixed concentration of H_2O_2 in excess of the latter also lead to a significant acceleration, indicating that the reaction with HO_2^- is catalyzed also by OH^-.

To analyze the results quantitatively we determined the equilibrium constant K for the reaction 1 at 37 °C by

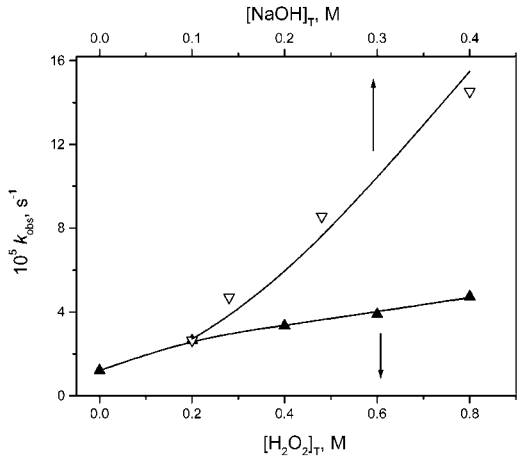

Figure 2. Observed first-order rate constants for the cleavage of 0.04 M $AcNH_2$ vs total H_2O_2 concentration in the presence of 0.1 M NaOD (lower axis) and vs total NaOD concentration in the presence of 0.2 M H_2O_2 (upper axis) at 37 °C. Solid lines are theoretical profiles calculated in accordance with eq 2 and rate constants given in Table 1.

spectrophotometric titration of H_2O_2 with NaOD in D_2O as described in ref 8. The value of $K = 276 \pm 2$ M^{-1} in D_2O agrees well with those reported in the literature for aqueous solutions[8,10] and indicates a small positive solvent isotope effect of ca. 1.5.

$$H_2O_2 + OH^- \leftrightarrows HO_2^- \quad (1)$$

With this equilibrium constant, concentrations of free OH^-, HO_2^-, and H_2O_2 at each given mixture of known total NaOH and H_2O_2 concentrations were calculated, and the results in Figure 2 were analyzed in terms of the following kinetic equation

$$k_{obs} = k_{OH}[OH^-] + k_{HO2}[HO_2^-] + k_{GA}[HO_2^-][H_2O_2] + k_{GB}^{OH}[HO_2^-][OH^-] \quad (2)$$

where k_{OH} and k_{HO2} are the second-order rate constants for the cleavage of the substrate by OH^- and HO_2^- respectively, k_{GA} and k_{GB}^{OH} are the third-order rate constants for the cleavage by HO_2^- assisted by general-acid and general-base mechanisms, respectively. The k_{OH} was measured in the absence of H_2O_2 and appeared to be very close to the value reported in the literature in H_2O.[11] Then the values of k_{obs} were corrected for the contribution of the alkaline hydrolysis and the eq 2 was rearranged as follows:

$$(k_{obs}\text{-}k_{OH}[OH^-])/[HO_2^-] = k_{2obs} = k_{HO2} + k_{GA}[H_2O_2] + k_{GB}^{OH}[OH^-] \quad (3)$$

Parameters of the eq 3 were then calculated by multiple regression of k_{2obs} as a linear function of two independent

Table 1. Rate Constants for Different Paths of the Amide and Peptide Cleavage by Alkaline Hydrogen Peroxide at 37 °C[a]

substrate	k_{OH}, M^{-1} s^{-1}	k_{HO_2}, M^{-1} s^{-1}	k_{GA}, M^{-2} s^{-1}	k_{GB}^{OH}, [c] M^{-2} s^{-1}	$k_{GB}^{HO_2}$, M^{-2} s^{-1}
$AcNH_2$	1.2×10^{-4}	2.4×10^{-4}	3.4×10^{-4}	2.1×10^{-3}	
GlyGly	3.8×10^{-5}	6.8×10^{-5}	3.9×10^{-4}	4.8×10^{-4}	1.9×10^{-4}

[a] Relative error ±10%.

variables $[H_2O_2]$ and $[OH^-]$. The resulting rate constants are given in Table 1.

The cleavage of a simplest peptide substrate GlyGly was studied in more details. Figure 3 shows the results obtained

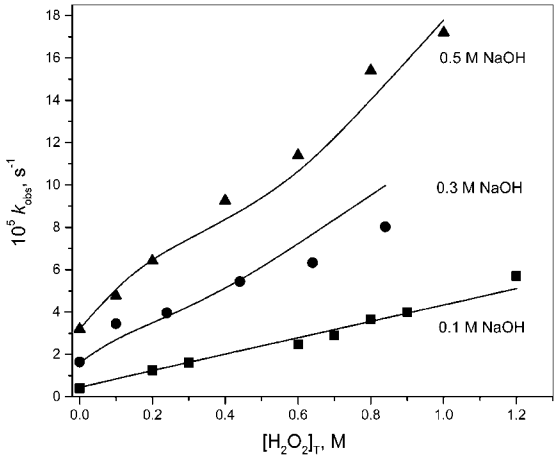

Figure 3. Observed first-order rate constants for the cleavage of 0.04 M GlyGly vs total H_2O_2 concentration in the presence of 0.1, 0.3, and 0.5 M NaOD at 37 °C. Solid lines are theoretical profiles calculated in accordance with eqs 4 and 5 and rate constants given in Table 1.

by variations in total hydrogen peroxide concentration in the presence of three different fixed total sodium hydroxide concentrations. General tendencies are similar to those for acetamide, but the reaction kinetics is more complicated in this case. Under conditions employed, GlyGly is a mono-anion and is ca. 1 order of magnitude less reactive than acetamide.

The rate of the alkaline hydrolysis of GlyGly was not a linear function of $[OD^-]$: the second-order rate constant k_{OH} increased on increase in $[OD^-]$ in accordance with the empirical eq 4

$$k_{OH} = k_{OH}^{1} + k_{OH}^{2}[OD^-] \quad (4)$$

where $k_{OH}^1 = 3.8 \times 10^{-5}$ $M^{-1}s^{-1}$ and $k_{OH}^2 = 5.1 \times 10^{-5}$ $M^{-2}s^{-1}$. This may be partially due to a positive salt effect produced by increased concentration of NaOD on the reaction between two anions, but most probably reflects the general-base catalysis by the second hydroxide anion reported previously for the hydrolysis of anilides.[12,13] The analysis of the results in terms of the eq 3 required also an additional term proportional to the concentration of free HO_2^-, so the results were fitted to the eq 5.

$$k_{2obs} = k_{HO2} + k_{GA}[H_2O_2] + k_{GB}^{OH}[OH^-] + k_{GB}^{HO2}[HO_2^-] \quad (5)$$

The numerical values of all rate constants for the cleavage of GlyGly are given in Table 1. Lower values of these constants, as compared to those for the cleavage of acetamide, can be attributed to steric effects and the negative charge of the peptide. The latter explains the decreased relative contribution of the general-base-catalyzed path, which involves an additional anionic species in comparison with the general-acid path involving neutral hydrogen peroxide.

Several other glycine derivatives were studied as substrates under similar conditions for comparative purposes. The results are collected in Table 2. As expected, glycinamide

Table 2. Selected First-Order Rate Constants for the Amide and Peptide Cleavage by Alkaline Hydrogen Peroxide at 37 °C[a]

substrate	k_{obs}, s^{-1}	$[NaOD]_T$, M	$[H_2O_2]_T$, M
$AcNH_2$	3.9×10^{-5}	0.1	0.6
$GlyNH_2$	2.5×10^{-4}	0.1	0.6
N-AcGly	1.9×10^{-5}	0.3	0.8
N-$AcGlyNH_2$[a]	4.4×10^{-4}	0.3	0.6
GlyGly	8.0×10^{-5}	0.3	0.8
GlyGlyGlyGly[b]	6.3×10^{-5}	0.3	0.7
GlyGlyAla[c]	4.7×10^{-5}	0.3	0.6

[a] Cleavage to *N*-AcGly and NH_3. [b] Ceavage to Gly and GlyGlyGly. [c] Cleavage to Gly and GlyAla.

and *N*-acetylglycine are more and less reactive than acetamide, respectively, due to a positive inductive effect of the α-amino group for the former and the negative charge for the latter. In agreement with this, the hydrolysis of *N*-acetylglycinamide proceeds with formation of *N*-acetylglycine and NH_3. The cleavage of GlyGly is faster than that of *N*-acetylglycine due to the presence of α-amino group.

To see the exo/endo selectivity of the peptide cleavage a tetra- and a tripeptide substrates were employed. In the case

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of tetraglycine during first 2 h only formation of Gly and GlyGlyGly was observed with the rate constant similar to that for the GlyGly cleavage (Table 2). Only after accumulation of ca. 25% of GlyGlyGly did the appearance of GlyGly became detectable. These results clearly indicate the cleavage of one of the terminal peptide bonds. To distinguish between N- and C-terminal bonds, a tripeptide with different terminal amino acids GlyGlyAla was used. Only the cleavage of Gly-Gly bond was observed with the rate constant again similar to that for GlyGly (Table 2). Thus, the preferable reaction path is the cleavage of N-terminal peptide bond.

There is a certain similarity in the kinetics of peptide cleavage by HO_2^- and by NH_2OH. Studies of the hydroxylaminolysis of similar substrates showed the same reaction paths as indicated in the eq 5, the only difference being that for NH_2OH the general-acid path was always strongly predominant over other reaction paths.[14] This is explicable by much the higher acidity of NH_3OH^+ ($pK_a = 6.0$) as compared to H_2O_2 ($pK_a = 11.5$), which makes the former a more powerful catalyst. On the other hand, the absolute reactivity of HO_2^- assisted by H_2O_2 is substantially higher than that of NH_2OH assisted by NH_3OH^+: the third-order rate constant for the latter with GlyGly anion equals 2.9×10^{-4} M^{-2} s^{-1} at 60 °C [14] while for the former $k_{GA} = 3.9 \times 10^{-4}$ at 37 °C (Table 1).

No rate constants were reported for the iron chelate mediated protein cleavage, but since the reaction is typically complete in 10 s [4] one may expect the k_{obs} value to be about 1 s^{-1}. In accordance with the proposed mechanism (see Introduction) this rate constant refers to the intramolecular attack (k_{intra}) of the peptide bond by metal-bound HO_2^-. Therefore, one may estimate the "effective molarity" (EM)[15] of the system by using the k_{HO_2} value for GlyGly as the rate constant for the intermolecular reaction (k_{inter}) and to obtain $EM = k_{intra}/k_{inter} = 1/(6.8 \times 10^{-5}) = 1.4 \times 10^4$ M, a value which is within the range typical for nucleophilic substitution reactions.[15] Thus, the results presented here indirectly confirm the validity of the hydrolytic mechanism proposed for the iron chelate mediated protein cleavage.

The HO_2^- anion shows a modest α-effect in the cleavage of amide and peptide substartes: the ratio k_{HO2}/k_{OH} equals ca. 2 for $AcNH_2$ and GlyGly (Table 1). Since the rate-determining step in the amide hydrolysis is the decomposition of the tetrahedral addition intermediate to final products, this ratio should be closer to the "equilibrium" α-effect observed for nucleophile addition to carbonyl compounds [16] rather than to the "kinetic" α-effect observed for the cleavage of substrates with good leaving groups. In the case of hydrogen peroxide both types of effects are similar and are of the order of 10^2-10^3.[16] However, for substrates with poor highly basic leaving groups, e.g., with ethyl acetate, the low basicity of HO_2^- makes it unreactive because the tetrahedral intermediate decomposes principally to the starting materials. Apparently the protonation of the leaving RNH^- anion, postulated as a necessary step in the amide hydrolysis,[13,17] reduces its basicity sufficiently and makes possible the partition of the tetrahedral intermediate toward the reaction products rather than to starting materials.

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