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Published on Web 02/01/2003

Thiyl Radicals Abstract Hydrogen Atoms from the ᵅC−H Bonds in Model Peptides: Absolute Rate Constants and Effect of Amino Acid Structure

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Thiyl radicals ($RS^{\bullet}$) are important intermediates during biological conditions of oxidative stress.[1] In addition, several enzymes utilize thiyl radicals as reactive intermediates, for example, the ribonucleotide reductases.[2] Here, thiyl radicals selectively abstract H-atoms from the C3′ position of ribonucleotides as a primary step in the enzymatic synthesis of deoxyribonucleotides. Generally, H-transfer reactions between thiyl radicals and α-(hydroxy/alkoxy)-substituted C−H bonds (reaction 1) are reversible;[3,4] in this case, however, the irreversible elimination of water from the C3′ radical drives the reaction.

$$R_2CHR + RS^{\bullet} \rightarrow R_2C^{\bullet}R + RSH \qquad (1)$$

Radiation chemical studies have yielded $k_1 = 10^3-10^4\ M^{-1}\ s^{-1}$ for the H-abstraction from several small aliphatic model compounds for carbohydrates (alcohols and ethers).[4,5] These rate constants are small as compared to those for the reverse reaction, $k_{-1} = 10^7-10^8\ M^{-1}\ s^{-1}$.[6] Recently, we have optimized an NMR method to obtain absolute rate constants for the thiyl radical-mediated H-abstraction from carbohydrates, which are in the range of $(1-3) \times 10^4\ M^{-1}\ s^{-1}$.[7]

Usually, the C−H bond dissociation energies (BDEs) of α-(hydroxy/alkoxy)-substituted C−H bonds are on the order of 370−390 kJ/mol.[8] In contrast, the ᵅC−H bonds of model peptides show lower BDEs (<370 kJ/mol),[9,10] suggesting an even higher reactivity toward thiyl radicals than the C−H bonds of carbohydrates. For the ribonucleotide reductases, that could imply that the selectivity of the cysteine thiyl radical toward the C3′ group of ribonucleotides is controlled by conformational and dynamic rather than thermochemical parameters. Numerous proteins are regulated by cysteine oxidation.[11] Processes generating protein-bound thiyl radicals may trigger reactions with ᵅC−H bonds of nearby amino acids. The determination of absolute rate constants with the ᵅC−H bonds of amino acids, preferably in peptides, is key to the discussion of the reactivity and selectivity of thiyl radicals with protein components. These values can then be correlated with calculated BDEs to answer the question of whether these reactions are thermodynamically controlled and whether and to what extent other parameters, for example, conformational or polar effects,[12−14] play additional roles. In this paper, we have adapted our NMR method for measurements with small sample volume and substrate concentrations.

Rate constants were determined through competition kinetics at 37 °C in N_2-saturated D_2O, pD 3−3.6, containing $(1-2) \times 10^{-2}$ M 2,2′-azobis-(2-methyl-propionamidine)-dihydrochloride (AAPH), $(4-6) \times 10^{-3}$ M cysteamine (^+D_3N-CH_2CH_2-SD; CyaSD), $(5-20) \times 10^{-3}$ M 2-propanol,[15] and $(0.6-290) \times 10^{-3}$ M amino acid/peptide. We did *not* use cysteine as a source for thiyl radicals because it contains a reactive ᵅC−H bond itself. At 37 °C, AAPH yields carbon-centered radicals, $R_A^{\bullet}$, at a rate of $d[R_A^{\bullet}]/dt = 1.36 \times 10^{-6}$ [AAPH],[16] which will exclusively react with CyaSD to yield $CyaS^{\bullet}$ (Scheme 1).[7]

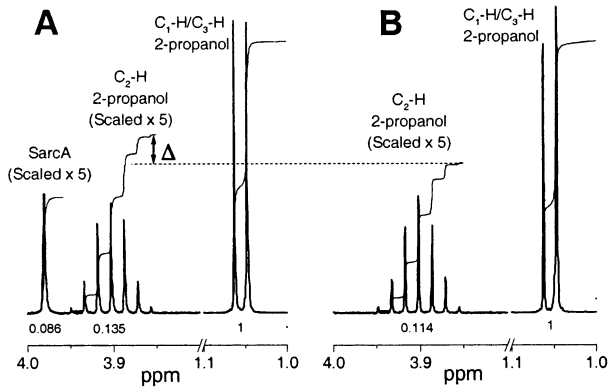
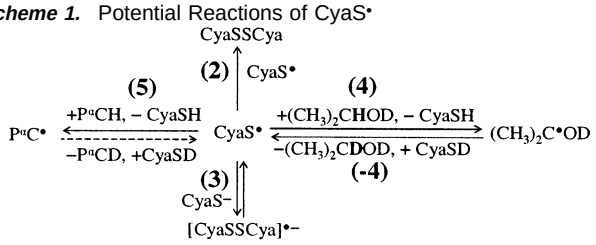


Figure 1. NMR spectra of N_2-saturated D_2O solutions of 19 mM 2-propanol, 5.5 mM CyaSD, and 14 mM AAPH at pD 3 after 44 h of reaction at 37 °C. Solution A contains 2.5 mM SarcA; solution B does not have any SarcA.

Scheme 1. Potential Reactions of $CyaS^{\bullet}$



A fraction of $CyaS^{\bullet}$ will dimerize via reaction 2 or reversibly associate with the thiolate $CyaS^-$ (reaction 3).[17] The remaining $CyaS^{\bullet}$ radicals are available for reactions 4 and 5. In reaction 4, $CyaS^{\bullet}$ abstracts the H-atom at C-2 from 2-propanol, yielding the 2-hydroxy-2-propanyl radical and CyaSH ($k_4^{37\ °C} = 4.0 \times 10^4\ M^{-1}\ s^{-1}$ [18]), which spontaneously undergoes H/D exchange. The 2-hydroxy-2-propyl radical can react again with CyaSD to yield 2-D-2-propanol (k_{-4} ca. $1.5 \times 10^7\ M^{-1}\ s^{-1}$ based on $k_H/k_D \approx 5$[5,7,19]). Thus, equilibrium 4 exchanges H for D at the C-2 of 2-propanol in a chain process, resulting in the loss of NMR intensity of C_2-H and coupling of C_2-H with the methyl protons of C-1 and C-3 (Figure 1). The length of the chain depends on the competition between equilibrium 4 and all termination reactions and increases with the 2-propanol concentration. Conversion of 2-propanol only took place in the presence of CyaSD, consistent with other reports on thiyl radical catalyzed isomerizations of organic alcohols and ethers.[12−14] Reactions of carbon-centered radicals with PᵅC−H are negligible ($k \approx 10^2\ M^{-1}\ s^{-1}$ [20]).

The addition of a peptide, PᵅC−H, to our system causes a concentration-dependent decrease of the conversion of 2-propanol to 2-D-2-propanol because of the competition between reactions 4 and 5. In a first approximation, reaction 5 is irreversible on the basis of the significant difference in the BDEs of S−H/S−D and PᵅC−H. We confirmed this by NMR for the direct reaction of thiyl

10.1021/ja0293599 CCC: $25.00

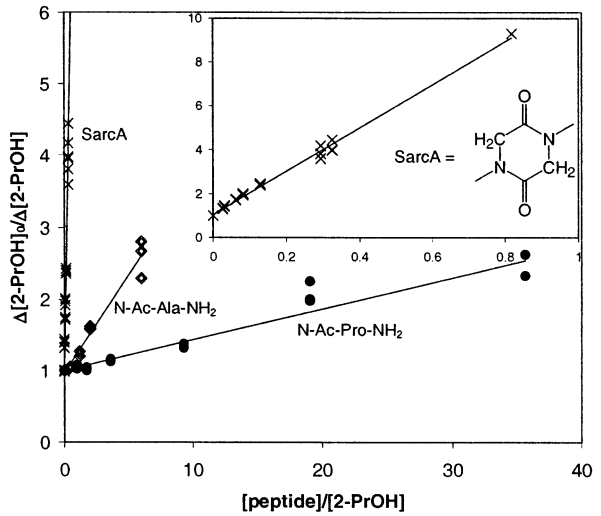


Figure 2. Concentration-dependent inhibition of 2-D-2-propanol formation for three model peptides. $\Delta[2\text{-PrOH}]_0$ and $\Delta[2\text{-PrOH}]$ represent the 2-propanol consumption at a given time in the absence and presence of the model peptide. Inset: Low ratios [SarcA]/[2-PrOH].

radicals with up to 0.4 M N-Ac-Ala-NH_2 in the absence of 2-propanol, where H-abstraction by CyaS$^\bullet$ selectively targets the P$^\alpha$C−H bond. Figure 1 shows representative NMR spectra of the reaction containing (A) 2.5×10^{-3} M sarcosine anhydride (SarcA) or (B) no SarcA. SarcA competes with 2-propanol and protects the 2-propanol C_2−H bond. The corresponding peak areas in Figure 1A are approximately 20% larger than those in Figure 1B. The combined integral of C_1−H and C_3−H does not change, but the relative peak intensities do because of H/D exchange at C-2. Figure 2 shows representative plots for SarcA, N-Ac-Ala-NH_2, and N-Ac-Pro-NH_2, from which the ratios k_5/k_4 can be calculated according to eq 2, where $\Delta[2\text{-PrOH}]_0$ and $\Delta[2\text{-PrOH}]$ represent the 2-propanol consumption (equivalent to the 2-D-2-propanol formation) at a given time in the absence and presence of the peptide substrate.

$$\frac{\Delta[2-\text{PrOH}]_0}{\Delta[2-\text{PrOH}]} = 1 + \frac{k_5}{k_4}\frac{[\text{substrate}]}{[2-\text{PrOH}]} \qquad (2)$$

This formula can be used independent of the chain length, provided that there is a constant steady-state concentration of radicals and a substrate conversion ≤20%. On the basis of $k_4{}^{37\ °C} = 4.0 \times 10^4\ M^{-1}\ s^{-1}$,[18] we obtain k_5's, which are summarized in Table 1.

Zhao et al.[21] had derived rate constants for H-abstraction by cysteine thiyl radicals at pH 10.5 from anionic glycine ($3.2 \times 10^5\ M^{-1}\ s^{-1}$) and alanine, respectively ($7.7 \times 10^5\ M^{-1}\ s^{-1}$). The deprotonated amino group ensures optimum captodative stabilization of the $^\alpha$C$^\bullet$ radical. However, deprotonated aliphatic amines are physiologically unrealistic. Our rate constants for model peptides are directly relevant for amino acids within proteins. The substrates in the table cover a broad range of $^\alpha$C−H bond energies. For example, in *N*-formyl-Asp-NH_2, the calculated BDE($^\alpha$C−H) = 332 kJ/mol, and in *N*-acetyl-Pro-NH_2, BDE($^\alpha$C−H) = 369 kJ/mol (*trans*-Pro) and 358 kJ/mol (*cis*-Pro). The rate constants k_5 vary by a factor of 235, but correlation with the BDEs of the $^\alpha$C−H bonds is poor. Thus, thermodynamics alone cannot rationalize our results. We do not expect large contributions of polar effects to the differences in k_5, as the individual peptide side chains are not suited for stabilizing polar transition states. Normalized to the number of $^\alpha$C−H bonds, the cyclic SarcA and glycine anhydride (GlyA) show a significantly higher reactivity than linear N-Ac-Gly-NH_2. The possibility of the peptide to support a planar, maximal captodatively stabilized $^\alpha$C$^\bullet$-radical seems to affect k_5. Future experiments with specifically selected secondary structures will test this hypothesis.

Table 1. Rate Constants for CyaS$^\bullet$ Reactions with Peptides at 37 °C

model peptide	k_5/k_4[a]	k_5, $10^4\ M^{-1}\ s^{-1}$[b]	k_5 per $^\alpha$C–H bond, $10^4\ M^{-1}\ s^{-1}$	BDE of $^\alpha$C–H, kJ/mol[c]
SarcA	10 ± 2	40 ± 8[d]	10	
GlyA	8 ± 4[e]	32 ± 16	8.0	350 (340[f])
N-Ac-Gly-NH_2	1.6 ± 0.7	6.4 ± 2.8	3.2	350
N-Ac-Ala-NH_2	0.26 ± 0.08	1.0 ± 0.3	1.0	345
N-Ac-Asp-NH_2	0.11 ± 0.04	0.44 ± 0.16	0.44	332
N-Ac-Gln-NH_2	0.047 ± 0.015	0.19 ± 0.06	0.19	334
N-Ac-Pro-NH_2	0.042 ± 0.015	0.18 ± 0.06	0.18	358 (cis) 369 (trans)

[a] No protolysis reaction is directly involved in reactions 4 and 5; therefore, no solvent isotope effects are expected.[22] [b] Taking k(CyaS$^\bullet$ + 2-propanol) = k(cysteine thiyl radical + 2-propanol) = $2.0 \times 10^4\ M^{-1}\ s^{-1}$ at 20 °C[23] and $k_4 = 4.0 \times 10^4\ M^{-1}\ s^{-1}$ at 37 °C.[18] [c] Ab initio calculated values.[9] Except for GlyA and N-Ac-ProNH$_2$, all values are for *N*-formyl amino acid amides. [d] This value compares well with $2.7 \times 10^5\ M^{-1}\ s^{-1}$ at room temperature, obtained by pulse radiolysis.[24] [e] For [GlyA]/[2-propanol] = 0.08−0.25 at [GlyA] < 2.5×10^{-3} M; at higher ratios, plots show slight curvature. [f] Experimental value.[10]

Acknowledgment. This work was supported by the NIH (2PO1AG12993).

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JA0293599