J. RADIAT. RES., 339-345 (1973)

Rate Constants for the Reactions of OH Radicals with the Enzyme Proteins as Determined by the p-Nitrosodimethylaniline Method

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(Received Apr. 24, 1973)

ABSTRACT

Rate constants for the reactions of OH radicals with commonly occuring amino acids and several enzyme proteins were determined by the competition kinetics using p-nitrosodimethylaniline. From the assumption that the reaction of OH radicals with an amino acid residue is restricted to the surface region of enzyme molecule, an estimation of rate constant for the reaction of OH radicals with enzyme was attempted. The calculated values for cytochrome c and lysozyme were in good agreement with those obtained experimentally.

INTRODUCTION

The radiation-induced inactivation and degradation of enzymes in dilute aqueous solutions are known to be caused by the active species produced in the radiolysis of water. A contribution of OH radicals for the inactivation and degradation is considered to be significant[1, 2], although the detailed mechanism of the process is still unclear and even the initial reaction of OH radicals with proteins are not understood in terms of radiation chemistry.

The method to measure the rate constants for the reactions of OH radicals under the continuous irradiation includes an ambiguousness in the reaction mechanism. A convenient method using p-nitrosodimethylaniline (PNDA) as a standard substance in the competition kinetics, proposed by Kraljić and Trumbore[3, 4], was criticized by Dainton and Wiseal[5] from the results obtained in the pulse radiolysis experiment. However, the method yielded the reasonable values for rate constants of the considerable numbers of compounds[3, 4, 6, 7]. Recently Willson and his coworkers[8] examined the several methods determining the rate constant for the reaction of OH radicals using the pulse radiolysis technique and concluded that the PNDA method may give the consistent results when the rate constant for the reaction of OH radicals with p-nitrosodimethylaniline is taken as 1.25×10^{10} M^{-1} sec^{-1}.

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In the present investigation, we made an attempt to obtain the rate constants for the reaction of OH radicals with the enzyme proteins and with the commonly occuring amino acids according to the method of Kraljić and Trumbore and then compared them with the values obtained by the other methods. Furthermore, the reactivity of OH radicals with the enzymes is discussed by assuming that the reaction of OH radicals with amino acid residues is restricted to the surface region of enzyme molecules.

MATERIALS AND METHODS

The enzymes except cytochrome c were purchased from Worthington Biochem. Corp. as the following preparations: Three times crystallized, salt-free, lyophilized powder for bovine pancreatic α-chymotrypsin; two times crystallized, salt-free, lyophilized powder for egg white lysozyme; the purest grade, lyophilized powder for beef pancreatic ribonuclease; two times crystallized, salt-free, dry powder for bovine pancreatic trypsin. Cytochrome c was prepared from the horse heart muscle according to the method of Hagihara *et al*[9]. Amino acids were purchased from the Ajinomoto Co. Ltd.

The samples for irradiation were prepared with the triply distilled water and adjusted to neutral pH with NaOH. The gamma rays with dose rate of 1.92×10^{16} eV g^{-1} min^{-1} were delivered to the air-saturated samples from a ^{137}Cs source.

Relative rate constants were determined by the kinetic treatment of the competitive reaction as described by Kralijić and Trumbore[3] and the absolute values were calculated by taking 1.25×10^{10} M^{-1} sec^{-1} as the rate constant for PNDA.

RESULTS AND DISCUSSION

The rate constants for the reaction of OH radicals with the commonly occuring amino acids are summarized in Table 1. The values obtained by Scholes and his coworkers[10] are cited as a reference. The values are corrected for the recent rate constants of thymine and thiocyanate ion by taking 5.0×10^{9} M^{-1} sec^{-1} as k (OH + thymine)[8] and 1.1×10^{10} M^{-1} sec^{-1} as k ($OH+CNS^{-}$)[1].

The rate constants for aliphatic amino acids obtained by the other two methods are in good agreement with those estimated in the present work.

Higher reactivity was generally found for aromatic amino acids. Armstrong and Swallow[11] determined the rate constant for tryptophan as 1.25×10^{10} M^{-1} sec^{-1} by pulse radiolysis. Thymine and PNDA methods seem to give a lower value for tryptophan. Although the radiation chemistry of PNDA solution is not yet understood in detail, partial recovery of PNDA was observed by pulse radiolysis[5, 20]. If the intermediate radicals derived from tryptophan hinder the recovery in the same manner as halogen ions, the lower rate constant of tryptophan will be expected for PNDA method. There might be the same situation for thymine method.

In case of S-containing amino acids, the electron transfer which proceeds fairly fast is likely to occur. The rate constant for cystine appears to include some

Table 1. Rate constants for the reactions of OH radicals with amino acids.

Amino acid	PNDA method		Thymine* method		CNS^-* method	
	pH	k	pH	k	pH	k
		$M^{-1}sec^{-1}$		$M^{-1}sec^{-1}$		$M^{-1}sec^{-1}$
Glycine	6.7	1.7×10^7	2.8	7.3×10^6	5.8～6.0	1.7×10^7
Alanine	6.8	7.9×10^7	2.0～2.2	7.4×10^7	5.5～6.0	7.7×10^7
Valine	6.6	6.6×10^8	〃	6.7×10^8		
Leucine	6.9	1.8×10^9	〃	1.9×10^9	5.5～6.0	1.6×10^9
Isoleucine	6.6	1.7×10^9	〃	1.7×10^9		
Serine	6.6	2.3×10^8	〃	2.7×10^8	5.5～6.0	3.2×10^8
Threonine	6.6	5.1×10^8	〃	3.6×10^8		
Proline	6.8	6.5×10^8	〃	2.8×10^8		
Hydroxyproline	6.8	3.2×10^8	〃	3.3×10^8		
Aspartic acid	6.5	4.9×10^7	〃	3.1×10^7	6.8～7.0	7.5×10^7
Asparagine	6.6	4.9×10^7				
Glutamic acid	6.5	2.3×10^8	2.0～2.2	1.27×10^8		
Glutamine	6.0	5.4×10^8				
Lysine	6.6	3.5×10^8	2.0～2.2	6.0×10^8		
Arginine	6.7	5.7×10^8	〃	7.3×10^8	6.5～7.0	3.5×10^9
Phenylalanine	6.9	7.2×10^9	〃	7.1×10^9	5.5～6.0	5.8×10^9
Tyrosine	6.5	1.05×10^{10}	〃	9.4×10^9		
Tryptophan	6.2	7.8×10^9	〃	7.1×10^9	6.1～6.3	1.4×10^{10}
Histidine	6.7	4.3×10^9			6.0～7.0	5.0×10^9
Cystine	6.5	2.1×10^9	2.0～2.2	5.2×10^9		
Methionine	6.6	6.5×10^9	〃	6.0×10^9	5.5～5.7	8.2×10^9

* Reference 10.

Table 2. Rate constants for the reactions of OH radicals with several enzyme proteins.

Enzyme	PNDA method		Reference		
	pH	k	pH	k	method
		$M^{-1}sec^{-1}$		$M^{-1}sec^{-1}$	
Lysozyme (egg white)	6.4	4.2×10^{10}	7.4	4.9×10^{10}	Direct (p. r.)[a]
			7.4	3.25×10^{10}	t-BuOH (p. r.)[a]
			5.6	5.0×10^{10}	CNS^- (p. r.)[b]
Trypsin (bovine pancreas)	6.3	3.9×10^{10}	7.4	8.2×10^{10}	Direct (p. r.)[c]
			7.4	8.2×10^{10}	D-Glucose (p. r.)[c]
Cytochrome c (horse heart)	6.3	5.1×10^{10}	7.0	4.2×10^{10}	Tl^+ (γ)[d]
α-chymotrypsin (bovine pancreas)	6.6	3.5×10^{10}			
Ribonuclease A (beef pancreas)	6.5	2.5×10^{10}		1.9×10^{10}[e]	

a. Reference 1). b. Reference 18). c. Reference 12). d. Reference 13). e. Reference 19).

ambiguity, because of its poor solubility.

Table 2 shows the rate constants for the reactions of OH radicals with several enzymes, including those obtained by the other methods. The reaction of lysozyme with OH radicals was well investigated by Adams and his coworkers, and the rate constant was given 3.25×10^{10} to 4.9×10^{10} M^{-1} sec^{-1} [11]. Our result is consistent with the above values, although the direct method provided a little larger value.

For trypsin, there is a significant difference between the rate constants obtained by PNDA method and by pulse radiolysis[12]. In pulse radiolysis experiment, the rate constant was directly estimated from a buildup of absorbance at 330 nm, which was attributed to the OH-adduct of tryptophan residues in trypsin molecule. The same value was estimated by competition with glucose[12]. If the value obtained in the previous investigation includes some errors, an assignment that the absorption at 330 nm is attributed only to OH-adduct of tryptophan residues might be one of the causes.

If the absorption at 330 nm consists of two components, that is, OH-adduct and H-adduct, the result from the pulse radiolysis data should be corrected as follows. For the direct determination, the following rate law will be applied,

$$\frac{d\,OD_{330}}{dt}=\left\{1+\frac{\varepsilon_H k_H(H)}{\varepsilon_{OH}k_{OH}(OH)}\right\}\varepsilon_{OH}k_{OH}(try)\ (OH) \qquad (1)$$

where k_{OH} and k_H are the rate constants for the reactions of trypsin with OH radicals and H atoms, respectively. ε_{OH} and ε_H represent molar extinction coefficients of OH-adduct and H-adduct of trypsin at 330 nm. The first term enclosed with braces should be more than unity. Consequently k_{OH} becomes smaller than that originally reported.

For the competition kinetics, the following expression will be applied,

$$\frac{OD_{OH}}{OD-OD_H}=1+\frac{k_{glu}(glucose)}{k_{try}(trypsin)} \qquad (2)$$

where OD_{OH} and OD_H represent the maximum absorbance at 330 nm of OH-adduct and H-adduct of trypsin and OD is an observed absorbance at 330 nm for a given concentration of glucose. k_{glu} and k_{try} represent the rate constants for the reactions of OH radicals with glucose and trypsin respectively. Comparing the expression (2) with that used in the previous report[12], the present one may give the smaller value for the rate constant of trypsin towards OH radicals. If all tryptophyl residues locate on the surface of trypsin molecule, and all OH radicals are consumed in the reaction with tryptophyl residues, the PNDA method will provide a lower reactivity. In fact, the present work has shown that PNDA method yields the lower rate constant for free tryptophan than the direct method. Four tryptophyl residues contained in trypsin, however, can afford only about five eighth of the value obtained in the previous investigation, although the rate constant estimated by the direct method was 1.25×10^{10} M^{-1} sec^{-1}. True value may be found in the midst of them.

In the case of cytochrome c, a correction was made on absorbance at 440 nm

since a change in absorbance at 440 nm brought about by reduction of cytochrome c was observed. The reactivity of ferricytochrome c towards OH radicals was determined previously by competition kinetics using thallous ions under the continuous irradiation[13]. In the previous work, the rate constant for the reaction of OH radicals with thallous ions was taken as 1.0×10^{10} M^{-1} sec^{-1} [14], but the pulse radiolysis provided a new value, 7.6×10^{9} M^{-1} sec^{-1} [15]. The corrected value is shown in Table 2. Although the present value is a little higher than that previously determined, the argument in the previous paper[13] will be still relevant and the present result may offer a support on the assumption that there is an electron transfer pathway in a cytochrome c molecule.

Abstraction and addition reactions of OH radicals seem to be restricted on the surface region of the protein molecule. When a free amino acid reacts with OH radical, the molecule can be attacked from whole directions by the radical. For amino acid residue located on the surface of the protein molecule, the attack will be restricted to the direction from an open space where no other residue exists. As a rough approximation, a cube is applied to the space occupied by an amino acid residue (Fig. 1). If an amino acid residue spreads freely to an open space from the surface of protein molecule, an attack of OH radical is possible from the directions of five surfaces of a cube and the rate constant is deduced to five sixth of that for free amino acid. When two surfaces of the cube are blocked by some other residues, the rate constant is deduced to four sixth of that for free amino acid.

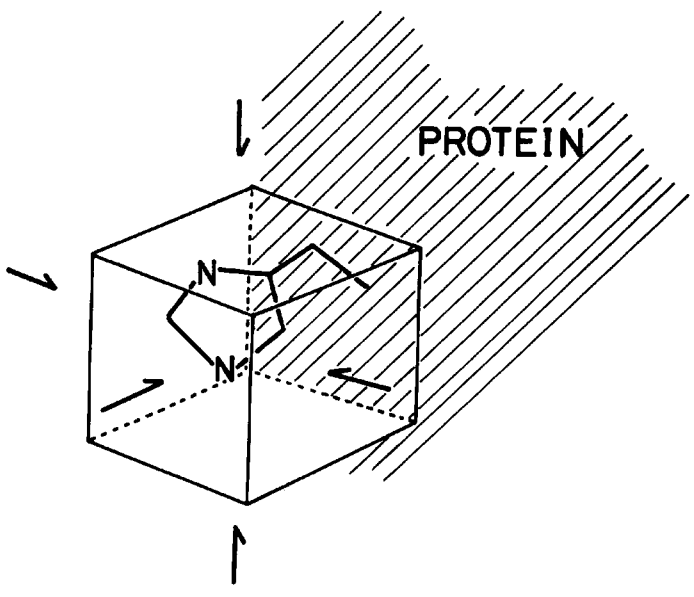

Fig. 1. A cubic model for the estimation of rate constant for the reaction of OH radicals with an amino acid residue located on the surface of enzyme molecule.

Histidyl residue is illustrated as an example. Arrows show the derections of the attack of OH radicals.

Based on the above assumption, a rate constant for the reaction of OH radicals with an enzyme protein is given by the following expression.

$$k=\sum_i a_i k_i \qquad (3)$$

$$a_i=\sum_j f_j \qquad (4)$$

f_j is a correction factor for a given amino acid residue j on the surface of protein and a_i is a summation of the correction factors for i kind of amino acid residue. k_i is the rate constant for the reaction of OH radicals with i kind of amino acid.

A correction factor for a given amino acid residue was

determined by the observation of stereophotograph of enzyme molecule models[16, 17]. The calculated values of rate constants based on the above assumption are 4.71×10^{10} M^{-1} sec^{-1} for lysozyme and 4.46×10^{10} M^{-1} sec^{-1} for cytochrome c respectively. These values appear to be in fairly well agreement with the experimental ones shown in Table 2. Calculation for α-chymotrypsin, trypsin, or ribonuclease was not carried out, because the sufficient informations of the state of surface could not be obtained for these enzymes. The above result suggests that the summation rule is relevant for the estimation of the reactivity of enzyme protein toward OH radicals.

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