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Reactions of Aromatic and Sulfur Amino Acids in Ribonuclease with Hydrogen Atoms in Water Solution

Abstract. *Neutral hydrogen atoms are simple, one-electron-equivalent reagents and their reactions are of fundamental interest for chemical kinetics. Functional correlation between aromatic and divalent-sulfur amino acids in ribonuclease is shown in the reaction of ribonuclease with hydrogen atoms in aqueous solution. Products of the reaction are partially characterized.*

Rather unexpected functional correlation between amino acids which contain divalent sulfur and others which contain aromatic rings was indicated in studies of the interaction of hydrogen atoms—a one-electron equivalent reagent—with enzyme proteins in aqueous solution (*1*). The reactions of atomic hydrogen with inorganic and organic solutes in water may be studied quantitatively; the rate constants and reaction mechanisms can be determined by a procedure developed in recent years (*2*).

As a result of studies of the rates and mechanisms of the reaction of H atoms with simple inorganic solutes (*2, 3*), amino acids (including aromatic and sulfur-containing ones) (*4*), the tripeptide glutathione (which contains divalent sulfur but no aromatic residues) (*5*), and the metal-enzyme cytochrome c (*6*), the action of H atoms on the hydrolytic enzymes chymotrypsin (*7*) and trypsin (*8*) was investigated. Chymotrypsin showed effective specific inactivation, but apparently without the formation of partially damaged, enzymatically still somewhat active molecules. Investigation of the reaction of H atom with trypsin yielded similar results. In addition, changes in the absorption spectrum and fluorescence, as well as the concurrent evolution of H_2S, indicated that tyrosine, tryptophan, and divalent-sulfur-containing amino acids participated in the reaction. Quantitatively, there was a definite relation between the number of enzyme molecules inactivated and the number of aromatic amino acids and amino acids containing divalent sulfur. Ribonuclease was chosen for the next investigation (*9*), because of its well-established tertiary structure (*10*) and because it does not contain tryptophan. Results similar to those obtained for trypsin showed specific inactivation and damage to aromatic and divalent-sulfur-containing amino acids. Total amino acid analysis of the treated sample proved that of all the amino acids present, only cystine and methionine (containing sulfur) and tyrosine and phenylalanine (containing aromatic rings) were appreciably attacked. The results also indicated that the uptake of a few H atoms, possibly only one by an enzyme molecule, might have produced these extensive changes in the protein.

In order to substantiate these results and to ascertain the mechanism of interaction of H atoms with ribonuclease, we studied the reaction with improved techniques of separation for the analysis of the reaction products. Pancreatic ribonuclease A (Sigma or Worthington)—used in the previous investigation —was purified (*11*) and dissolved in triple distilled water (0.5 mg/ml). The solutions were thus about 3.5×10^{-5} mole/liter for every experiment. The total dose of hydrogen atoms delivered to the solution was determined by the ferricyanide method (*12*). The total dose varied in experiments between about 10 and 100×10^{-5} mole/liter. Ribonuclease competes for H atoms with their recombination reaction.

For a macromolecule such as ribonuclease, it is difficult to determine the exact percentage of H atoms that reacted with it, but it is possible to estimate that not more than 10 percent reacted with the enzyme at the concentrations and doses used. In our experiments at different total doses there were from less than one to about three H atoms available per ribonuclease molecule in the solution. The percentage of inactivation (0 to 50 percent) is roughly proportional to the dose over the range 0 to 80×10^{-5} mole/liter. The extent of experimental scatter, in two discrete series of experiments at eight different total doses, was consistent with the 5 percent error in the dose rate. The enzyme solution was 50 percent inactivated by 80×10^{-5} *M* H (atom). The results are consistent with the assumption that the uptake of only one H atom is sufficient to cause inactivation of the enzyme.

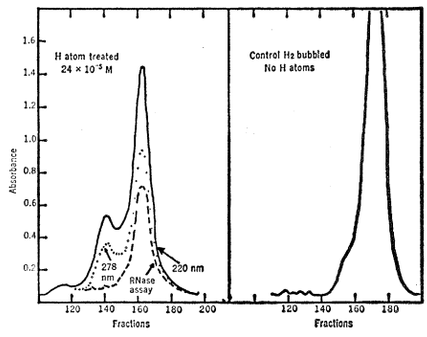


Fig. 1. Separation on Sephadex G 75 in 0.01*N* HCl of ribonuclease treated with H atom and untreated controls. Treated sample: dose $24 \times 10^{-5}M$ H (atoms), $8 \times 10^{-5}M$ per minute for 3 minutes. Untreated sample: bubbled with H_2 gas for 3 minutes. Ribonuclease activity of treated sample was 85 percent of untreated. Column volume 850 ml, length 135 cm, flow rate 24 ml/hr, unit fraction 3.85 ml for treated sample, 3.70 for untreated control. Solid line, absorbance at 220 nm; dotted line, absorbance at 278 nm; and dashed line, ribonuclease activity determined according to Shapira (*11*). Absorbance and enzyme activity was determined on every unit fraction.

The behavior of untreated and treated samples on a Sephadex G75 column is shown in Fig. 1. The untreated sample is uniform and contains no fractions other than the fully active one. After treatment with H atoms the partially inactivated sample separates into two main components: (i) fully active, unaffected enzyme, and (ii) totally inactive, affected one. With increasing dosage of H atom, this latter increases at the expense of the first proportionally, without the appearance of other intermediate or additional fractions.

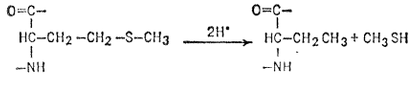
When the treated sample is permitted to stay at room temperature for several days after H atom treatment, some slight recovery of enzyme activity is observed and three fractions are recovered after Sephadex treatment: unaffected, partially affected, and totally inactive. There is, however, no far-reaching recovery of activity on standing. Separation of the freshly treated material does not yield products of aggregation obtained, for example, in samples inactivated by ionizing radiations (*13*).

Total amino acid analysis was carried out on the totally inactive fraction separated on Sephadex. A Beckman-Spinco model 120C analyzer with resins PA-35 and UR-30 was used in an accelerated system (*14*). The buffer change on the long column was delayed 8 minutes to

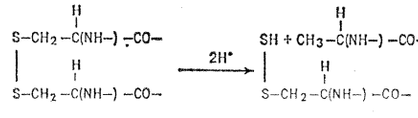
Table 1. Total amino acid analysis of ribonuclease after treatment with $28.5 \times 10^{-5} M$ H atoms. Percent inactivation after treatment was 18. Number of unchanged amino acids: lysine, 10; histidine, 4; arginine, 4; aspartic acid, 15; threonine, 10; serine, 15; glutamic acid, 12; proline, 4; glycine, 3; valine, 9; isoleucine, 3; leucine, 2.

Changed amino acids	No. of units in molecule				
	Theoretical	Found by analysis			
		Starting material	Sephadex fraction		
			Active	Inactive	
Half cystine	8	7.1	7.0	4.1	Decreased
Methionine	4	3.9	3.9	2.5	
Tyrosine	6	5.3	5.3	3.4	
Phenylalanine	3	3.0	3.0	2.0	
Alanine	12	12.0	12.0	14.5	Increased
α-Aminobutyric acid	0	0	0	1.5	

allow the α-aminobutyric acid to elute before the buffer change. The results were compared with those obtained for the untreated control, the unseparated treated sample, and the enzymatically active fraction obtained after Sephadex gel filtration (Table 1). Only the amino acids containing divalent sulfur, cystine and methionine, and the aromatic amino acids tyrosine and phenylalanine have decreased significantly in number. There are, however, additional products, as shown by the increase in alanine and by the appearance of some entirely new products, one of which has been identified as α-aminobutyric acid. We may assume therefore that methionine reacts according to:



Thus CH_3SH could be a product. We have not identified it. For cystine, one possible reaction would lead to alanine, according to:



Alanine is a major, but possibly not the only, product in the reaction. The fate of the sulfur-containing fragment has not been followed in detail, but H_2S was identified in the previous work as a major product (*9*).

The results show that there are up to ten amino acids affected in each enzyme molecule inactivated on the uptake of a small number, possibly only one H atom. One possible assumption is (*1*, *9*) that we are dealing with a linked process, in which the addition of one H atom forms a free radical, for example, on one of the sulfur-containing amino acids. The free radical now received another reduction equivalent from an aromatic amino acid which should yield oxidized, possibly quinonoid products, containing carbonyl groups, in a linked oxidation-reduction process. Tests for such products were carried out using the reaction with dinitrophenylhydrazine in sulphuric acid medium, followed by the addition of KOH to alkaline *p*H. Untreated samples gave no reaction; treated samples gave positive color reaction for $-C{=}O$ groups with this reagent, with absorption maxima at 540 and 420 nm. These results support the assumption of an oxidative mechanism on the aromatic residues, but do not fully prove it as yet.

To ascertain the site of attack on the ribonuclease protein, full peptide analysis was carried out on controls and samples treated with H atom, after pepsin digestion in 5 percent formic acid and 2 percent (by weight) of pepsin in relation to ribonuclease at 37°C for 18 hours. We used the Beckman-Spinco model 120C stream-splitting analyzer with PA35 resins and a pyridine-acetate gradient (*15*). Comparison of the results from control and treated samples showed that six of the peptides present in the control decreased in number in the H-treated sample; in the treated samples there were eight new peptides not present in the control, which gave altogether 24 peptides. The peptides thus investigated were low in divalent-sulfur-containing and aromatic amino acids. From their composition it could be deduced that some arose from the neighborhood of two of the disulfide bridges, those at I-VI (amino acids 26 and 84) and IV-V (65 and 72).

The present results support the view that, on addition of H atoms to the molecule of ribonuclease, an efficient process of intramolecular radical transfer takes place. As a result, specific damage occurs at sensitive sites, selectively, rather than indiscriminately, leading to effective inactivation of the enzyme and modification of the molecule. The results emphasize the difference between (i) the action of the one-electron-equivalent reducing agent (atomic H), which functions via compulsory free radical intermediate formation, and (ii) the possibility of reversible reductive opening of all four disulfide linkages in ribonuclease when other reagents operate without the intermediate formation of free radicals, for example, by simultaneous two-electron equivalent transfer (*1*). Our results are related to the action of ionizing radiations on aqueous solutions of ribonuclease that is due to hydrated electrons, but are likely to differ from those due to oxidizing radicals, particularly the more reactive OH radicals, which are likely to lead to peripheral, indiscriminate damage.

After H atom treatment there is another small fraction even more highly damaged than the one described in Table 1. After Sephadex separation, the total recovery from the sum of all fractions was only 85 to 90 percent of the starting material. The amino acid analysis of the treated sample before Sephadex separation showed a greater decrease in the four affected components than the sum of the recovered fractions.

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Staphylococcal Nuclease: Size and Specificity of the Active Site

Abstract. *The dissociation constants and standard free energies of complex formation determined with staphylococcal nuclease and a series of 5′-phosphoryloligothymidyl derivatives of increasing chain length suggest that maximum stability is reached with an oligonucleotide containing three nucleotide units. A proposed model of the active site that contains other knowledge of the specificity and the catalytic mechanism of this enzyme postulates the existence of three nonequivalent phosphate binding subsites and a closely related phosphodiester hydrolytic subsite.*

The affinities of enzymes for substrates or inhibitory analogs reflect the number, strength, and spatial distribution of interacting sites in the enzymically active centers. By studying the effects of a number of peptides on affinity and rate constants, it was suggested that the active site of papain extends for approximately 25 Å and accommodates seven amino acids of the substrate molecule (*1*). On the same basis, the active site of carboxypeptidase A covers five "subsites" over 18 Å (*2*). The activity of the exonuclease of Ehrlich ascites tumor cells is progressively more strongly inhibited by a series of 3′-phosphoryloligoadenyl derivatives, $(Ap)_x$, of increasing chain length up to $x = 6$, suggesting that a hexanucleotide sequence is the substrate unit best accommodated in the active site of this enzyme (*3*). Using a similar approach, we have examined the length and orientation of oligonucleotide sequences recognized by the active site of staphylococcal nuclease.

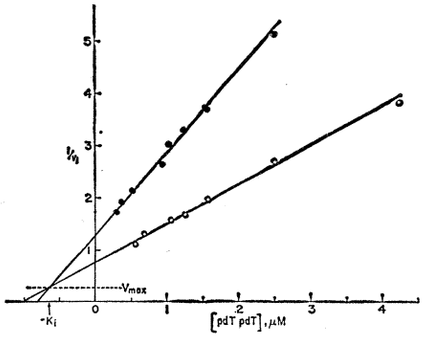

Fig. 1. Dixon plot (*11*) of inhibition of staphylococcal nuclease activity by $(pdT)_2$. Enzyme (20 μg) was added to a solution containing 0.05*M* borate buffer (*p*H 8.8), 0.01*M* $CaCl_2$, 10 μ*M* (●) or 20 μ*M* (○) synthetic substrate, deoxythymidine 3′,5′-di-*p*-nitrophenylphosphate (*5*, *10*), and the amount of oligonucleotide indicated.

Staphylococcal nuclease, which hydrolyzes DNA and RNA to yield 3′-phosphomononucleosides, is inhibited competitively by 5′-nucleotides and derivatives bearing a 5′-terminal phosphate (*4*, *5*). Oligonucleotides bearing 5′-phosphomonoester end groups are very resistant to hydrolysis (*6*, *7*). We have therefore determined the dissociation constants (K_i) of a series of 5′-phosphoryloligothymidyl derivatives, $(pdT)_x$, ranging in length from one to six nucleotide residues.

Staphylococcal nuclease (Foggi strain) was obtained by a modification (*8*) of techniques described by Fuchs *et al.* (*9*); thymidine-3′,5′-diphosphate (pdTp) and pdT were purchased from Calbiochem, and $(pdT)_4$ was a gift of Dr. H. G. Khorana; the other $(pdT)_x$ derivatives were provided by Dr. M. Nirenberg. Deoxythymidine 3′,5′-di-*p*-nitrophenylphosphate (NP-pdTp-NP) was synthesized from pdTp, and deoxythymidine 5′-phosphate-3′-*p*-nitrophenylphosphate (pdTp-NP) was obtained from the latter by hydrolysis with snake venom diesterase (*10*).

Dissociation constants (K_i) were obtained from Dixon plots (*11*) of the inhibition of the enzymatic release of *p*-nitrophenylphosphate from NP-pdTp-NP (*5*, *10*) (Fig. 1). At least two substrate concentrations were used for each study, and the results were confirmed by independent measurements of V_{max} (Fig. 1). The oligonucleotide concentration was determined spectrophotometrically after hydrolysis in 0.3*M* NaOH for 18 hours at 37°C with E_{1l} (267 nm) $= 9.6 \times 10^{-3}$ (*12*).

The dissociation constants (K_i) and standard free energies ($\Delta F°$) of $(pdT)_x$-nuclease complex formation indicate that maximum stability of the complex is reached with an oligonucleotide containing three nucleotide units (Table 1 and Fig. 2). As the chain length increases to six, there is progressively less inhibition. This is probably due to the slow hydrolysis that can occur with oligonucleotides of chain length greater than three (*6*), although some steric interference with binding cannot be excluded. As the nucleotide chain increases in length, regions of its sequence that are susceptible to the principal, endonucleolytic action of the enzyme may compete with the inhibitory 5′-phosphoryl end, until very large oligonucleotides become excellent substrates.

Table 1. Binding constants of oligonucleotide-nuclease complexes.

Nucleotide	Dissociation constant* (molar)	$\Delta F°$† (cal/mole)
pdT	1.9×10^{-4}	−4,400
$(pdT)_2$	6.3×10^{-7}	−8,500
$(pdT)_3$	9.3×10^{-8}	−10,000
$(pdT)_4$	1.1×10^{-7}	−9,500
$(pdT)_5$	1.4×10^{-7}	−9,400
$(pdT)_6$	6.0×10^{-7}	−8,500
pdTp	2.0×10^{-7}	−9,100
pdTp-NP ‡	1.1×10^{-6}	−8,100
NP-pdTp-NP ‡	1.7×10^{-5}	−6,500

* K_i, obtained by Dixon plots (Fig. 1); the value for NP-pdTp-NP is K_m (approx.), obtained from Lineweaver-Burk plots under identical experimental conditions. † $\Delta F° = RT \ln K_i$. ‡ *p*-Nitrophenyl phosphate (NP) esters.

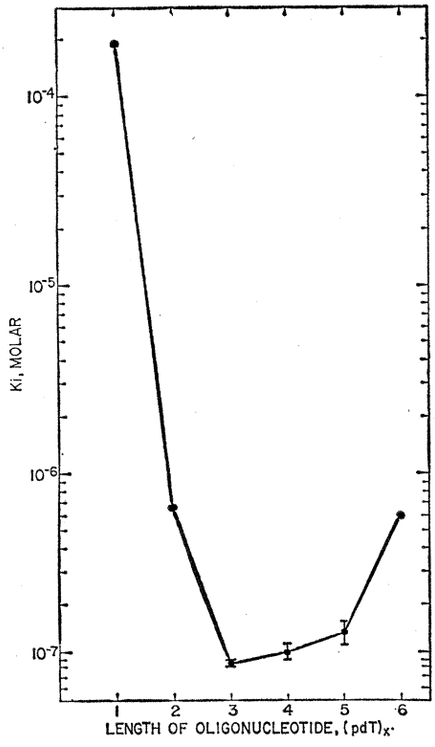

Fig. 2. Semilogarithmic plot of the dissociation constant (K_i) of $(pdT)_x$ as a function of the length of the oligonucleotide (x). The range of experimental values observed in the case of the tetra- and pentanucleotides is indicated.