Influences of pH and Substrate Analogs on Ribonuclease T_1 Fluorescence*

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ABSTRACT: The binding of substrate analogs to ribonuclease T_1 results in a marked quenching of the tyrosyl and tryptophanyl fluorescence of ribonuclease T_1 over a wide pH range. The binding has been analyzed in terms of the saturation fraction of protein. The resulting modified Scatchard plots indicate 1:1 stoichiometry between ribonuclease T_1 and substrate analog. At pH 6.4 the apparent binding strengths of the substrate analogs were in the order guanosine 3′-monophosphate > adenosine 3′-monophosphate > cytidine 3′-monophosphate and guanosine 3′-monophosphate > guanosine $\simeq$ guanylyl-(2′–5′)-cytidine. The fluorescence data were interpreted to mean that the quenching is correlated to the specific binding of the substrate analogs in the active site of ribonuclease T_1.

The pH dependences of the tyrosyl and tryptophanyl fluorescence intensities of the free enzyme and the enzyme–guanosine 3′-monophosphate complex have been recorded. From the data it is concluded that the single tryptophan residue (Trp-59) and one or two tyrosine residues of ribonuclease T_1 are present in the active site of the enzyme, but are not directly involved in the binding of the inhibitor. Changes in the state of protonation of charged groups in the active site appear to have an effect on the tyrosyl and tryptophanyl fluorescence intensity.

The study of the fluorescence spectra of native and denatured ribonuclease T_1 (RNase T_1, EC 2.7.7.26) and of the solvent perturbation of the fluorescence of this enzyme showed, that the single tryptophan residue (Trp-59) of RNase T_1 is partially buried in the enzyme and that most of the tyrosine residues are not outside of the protein molecule (Pongs, 1970). From these studies it was suggested, that the tyrosine residues of RNase T_1 are involved mainly in two different interactions. Approximately two-thirds of the tyrosine residues interact with acidic amino acid residues, whereas approximately one-third interact with Trp-59. These results were supported by titration data, which showed that the pK values of seven tyrosine residues of RNase T_1 are perturbed and only two tyrosine residues titrate normally (Iida and Ooi, 1969). Further evidence was given by the fluorescence excitation spectra of RNase T_1 (Longworth, 1968).

In the amino acid sequence of RNase T_1 Trp-59 immediately follows Glu-58, an amino acid shown to be present in the active site of this enzyme (Takahashi *et al.*, 1967). Fluorescence spectroscopy should provide a suitable method to determine, if Trp-59 and also some of the tyrosine residues are part of the active site of RNase T_1 and hence are involved in the binding process of the substrates.

Besides solvent and environmental factors (Steiner *et al.*, 1964; Weber and Rosenheck, 1964), specific interactions also have a profound effect on the fluorescence intensities of tryptophan and tyrosine (Cowgill, 1966). Therefore, we used this method for the study of the interaction of RNase T_1 with substrate analogs as well as for the study of the pH dependences of the tyrosyl and tryptophenyl fluorescences of the free enzyme and one enzyme–inhibitor complex. Since RNase T_1 contains only one Trp residue, it was possible to use Trp-59 as an internal reporter in order to explore the immediate environment of the active site of RNase T_1.

Materials

Ribonuclease T_1 (Sankyo Co. Ltd., Lt. No. 8R20) was a generous gift of Dr. H. Ruterjans, University of Munster, Germany. The lyophilized and salt-free sample was used without further purification. Guanine, guanosine, 2′,3′-Gp,[1] 2′,3′-Ap, 2′,3′-Cp, and 2′–5′-GpC were supplied by Zellstoff-Fabrik Waldhof, Mannheim, Germany. All substances were checked for purity by chromatography on S and S Paper No. 2043b using the descending technique in the solvent system isopropyl alcohol–water–2 N NH_4OH (7:2:1). 3′-Cp and 3′-Ap were obtained from the mixtures of the 2′,3′ isomers by column chromatography on Dowex 1-X10 as described by Cohn and Khym (1957). From 2′:3′-Gp, which was prepared according to the method of Smith *et al.* (1958), 3′-Gp was obtained by enzymatic digestion with RNase T_1. All other chemicals used were reagent grade.

Experimental Procedures

The fluorescence spectra have been recorded with an Aminco-Bowman spectrofluorometer equipped with Corning filter, type 5330, for the emission light and an off-axis ellipsoidal–mirror condensing system for focusing the excitation

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[1] Abbreviations used are: nucleoside 2′,3′-monophosphates, 2′,3′-Cp, 2′,3′-Gp, 2′,3′-Ap; nucleoside 2′-monophosphates, 2′-Cp, 2′-Gp, 2′-Ap; nucleoside 3′-monophosphates, 3′-Cp, 3′-Gp, 3′-Ap; dinucleoside 3′–5′-monophosphates, 3′–5′-GpC, 3′–5′-GpG, 3′–5′-GpU, 3′–5′-GpA; dinucleoside 2′–5′-monophosphates, 2′–5′-GpC.

light. Spectral selectivity was controlled by sets of interchangeable slits of varying widths, which intercepted the activating and emitted beams. Appropriate slit sets have been described in detail by Price *et al.* (1962). All fluorescence intensities reported in this paper are relative values and are not corrected for wavelength variations in detector response. The excitation wavelength was 280 nm for tyrosine and tryptophan. The relative fluorescence intensities were measured at 295 and 360 nm. Before each measurement the instrument was calibrated with 10^{-4} M tryptophan solution in 0.1 M KCl. In all studies, the enzyme (0.1 mg/ml) was dissolved in 0.1 M KCl. A Radiometer pH meter Model 25 equipped with a combination electrode (Fa. Ingold, Frankfurt, Germany, Type No. 405-M-312-a) was used for the pH measurements. The accuracy of the meter readings was ±0.01 pH. The pH values were adjusted with 0.01 N NaOH or 0.01 N HCl. For the adjustment of pH values below 3 and above 10, 1 N HCl or 1 N NaOH was used. Where necessary, measured fluorescence intensities have been corrected for dilution caused by adjustment of the pH values. RNase T_1 concentration was calculated from the absorbance at 277 nm in a 0.1 M KCl solution of pH 6.5 using a molar extinction coefficient of 1.91×10^3 (Egami *et al.*, 1964). Concentrations of guanosine, 3′-Gp, 3′-Cp, and 3′-Ap were calculated from the absorbances in 0.1 M KCl solutions of pH 6.4 at the absorption maximum of each compound using molar extinction coefficients reported by Beaven *et al.* (1955). The concentrations of 2′–5′-GpC were calculated from the absorbance at 260 nm in a 0.1 M KCl solution of pH 6.4 using a molar extinction coefficient of 17.0×10^3. This is the sum of the molar extinction coefficients of 2′-Gp and cytidine at this wavelength, corrected for hypochromicity.

Results

The maxima of the fluorescence emission spectra of tyrosine and tryptophan are separated by about 50 nm. In proteins the tyrosyl peak is usually masked by the stronger emission of the tryptophanyl residue (Weber and Teale, 1965). However, it is possible under favorable circumstances to monitor the interactions of the protein by measurements of the fluorescence of both amino acid residues. If the changes of the fluorescence intensities at 295 and 360 nm are not in unison, the different contributions of the tyrosyl and tryptophanyl fluorescence to the overall fluorescence of the protein molecule can be monitored (Cuatrecasas *et al.*, 1967).

The fluorescence emission spectrum of RNase T_1 at neutral pH does not show a separate peak for the tyrosyl fluorescence. Flourescence excitation measurements have shown that the quantum yield of the fluorescence of the tyrosine residues of RNase T_1 is very low (Longworth, 1968). A separate peak for the tyrosyl fluorescence is seen first, when the enzyme has been denatured (Pongs, 1970). The tyrosyl and tryptophanyl peak are separated by about 55 nm in the fluorescence emission spectrum of denatured RNase T_1. A study of the influence of solvent perturbation and denaturing agents on the fluorescence of RNase T_1 has shown, that the fluorescence intensities at 295 and 360 nm do not change in unison. The contributions of the single tryptophan residue (Trp-59) and the tyrosine residues to the overall fluorescence of RNase T_1 have been distinguished unambiguously.

The influences of pH and substrate analogs on the fluorescence of RNase T_1, which are reported in this paper, also show that the fluorescence intensities at 295 and 360 nm do not change in unison. Therefore, all measurements of fluorescence intensity carried out at 295 nm, are assigned to the tyrosine residues and all measurements carried out at 360 nm, are assigned to the single tryptophan residue (Trp-59) of RNase T_1.

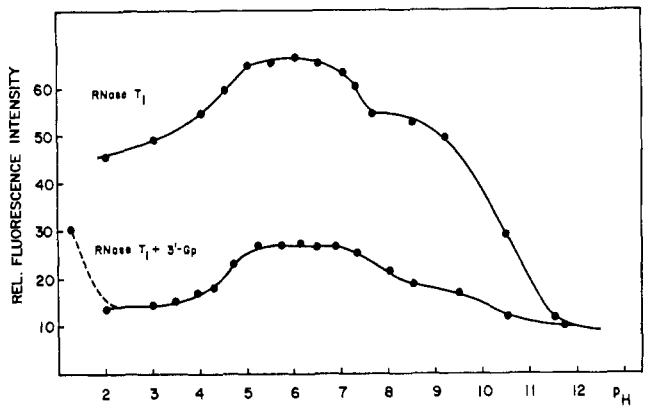

FIGURE 1: pH dependence of the tryptophanyl fluorescence intensity of RNase T_1 and the RNase T_1–3′-Gp complex. Solutions contained 10 μM RNase T_1 and 12 μM 3′-Gp in 0.1 M KCl. The wavelengths of excitation and emission were 280 and 360 nm, respectively. Dashed line: increase of fluorescence intensity of the RNase T_1–3′-Gp complex at acidic pH after 24 hr.

The pH dependence of the fluorescence intensity of Trp-59 at 360 nm is rather complex (Figure 1). It shows small changes between pH 3 and 8 and a large decrease of intensity at alkaline pH. The observed changes in fluorescence intensity are immediate and reversible between pH 2 and 10. Spectrophotometric investigations gave no evidence for a gross conformational change of RNase T_1 below pH 10.5 (Iida and Ooi, 1969). Current circular dichroism measurements also do not show a significant conformational change of RNase T_1 in this pH range (Sander *et al.*, unpublished data). Thus, the changes in fluorescence intensity between pH 3 and 10 are due to the change in the protonation of ionizable groups of the enzyme. These ionizable groups influence the fluorescence of RNase T_1. From the pH-dependent changes of the tryptophanyl fluorescence intensity apparent pK values can be estimated for these ionizable groups. The pK values are approximately 4.0, 7.2, and 10.0. By the addition of an equimolar amount of 3′-Gp to the enzyme solution, the fluorescence intensity at 360 nm is quenched to an extent of about 50% over a wide pH range (Figure 1). The apparent pK values of 4.0 and 7.2 for the free enzyme are shifted to 4.5 and 7.7. When an acidic solution of the mixture of RNase T_1 and 3′-Gp was allowed to stand over night at room temperature, the fluorescence intensity increased (dashed line in Figure 1) due to denaturation of the enzyme (Egami *et al.*, 1964).

A quite different pH dependence for the tyrosyl fluorescence intensity is found compared with that of the tryptophanyl fluorescence intensity (Figure 2). Since RNase T_1 does not undergo a gross conformational change between pH 3 and pH 10, apparent pK values can be estimated from the pH-dependent changes of the tyrosyl fluorescence intensity for ionizable groups, which cause these pH-dependent

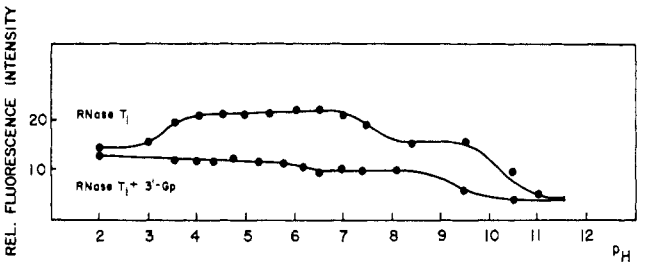

FIGURE 2: pH dependence of the tyrosyl fluorescence intensity of RNase T_1 and the RNase T_1–3′-Gp complex. Solutions contained 10 μM RNase T_1 and 12 μM 3′-Gp in 0.1 M KCl. The fluorescence intensity of tyrosine was measured with a 25 times higher amplification relative to that of tryptophan. The wavelengths of excitation and emission were 280 and 295 nm, respectively.

changes. The pK values are approximately 3.5, 7.5, and 10.0. The addition of 3′-Gp to the enzyme solution quenches the tyrosyl fluorescence intensity about 50% (Figure 2). The changes of tyrosyl fluorescence intensity at pH 3 and 7 are diminished.

From ultraviolet difference spectra of RNase T_1 with 2′-Gp at pH 5.0, gel filtration and ultracentrifugation experiments with the same system, it has been concluded that RNase T_1 forms a 1:1 complex with 2′-Gp at pH 5.0 (Sato and Egami, 1965). Kinetic experiments have shown that the RNase T_1 reaction follows Michaelis-Menten kinetics (Irie, 1968; Pongs, 1968). This suggested that the enzyme has only one active site. The enzyme is guanine specific. It splits RNA only at those places, where a guanine base is located in the 3′ position of the 3′–5′-phosphodiester linkages along the RNA chain (Egami *et al.*, 1964). Kinetic experiments have shown that the degree of inhibition of the RNase T_1 reaction decreases in the order Gp, Ap, and Cp (Irie, 1964; Pongs, 1968).

The influence of 3′-Gp, 3′-Ap, 3′-Cp, guanosine, and 2′–5′-GpC on the tyrosyl and tryptophanyl fluorescence of RNase T_1 was studied. The study was carried out at pH 6.4. Around this pH the binding of 3′-Gp to RNase T_1 is strongest and the hydrolysis of a dinucleoside monophosphate such as 3′–5′-GpC is fastest in this pH range according to kinetic experiments (Pongs, 1968). Varying concentrations of substrate analogs were added to solutions with a constant protein concentration (1.75×10^{-5} M).

The addition of 3′-Cp to a RNase T_1 solution at pH 6.4 does not significantly affect the fluorescence intensities at 295 and 360 nm (Figures 3 and 4). 3′-Cp is a pyrimidine nucleotide and has the least resemblance to the substrate of RNase T_1. The addition of 3′-Ap, a purine nucleotide, to a RNase T_1 solution at pH 6.4 quenches the tyrosyl and tryptophanyl fluorescence to a similar extent. A quenching of the tyrosyl and tryptophanyl fluorescence of about 10% was observed at the molar ratio of 1:1 between RNase T_1 and 3′-Ap (Figures 3 and 4). At this molar ratio 3′-Gp quenches the tyrosyl and tryptophanyl fluorescence at pH 6.4 about 50%. Its fluorescence titration curve clearly shows an inflection at the point, where the molar ratio is 1:1 between the enzyme and this substrate analog.

In comparison with 3′-Gp guanosine lacks the phosphate group. It is a weak inhibitor. In contrast to 3′-Gp it cannot

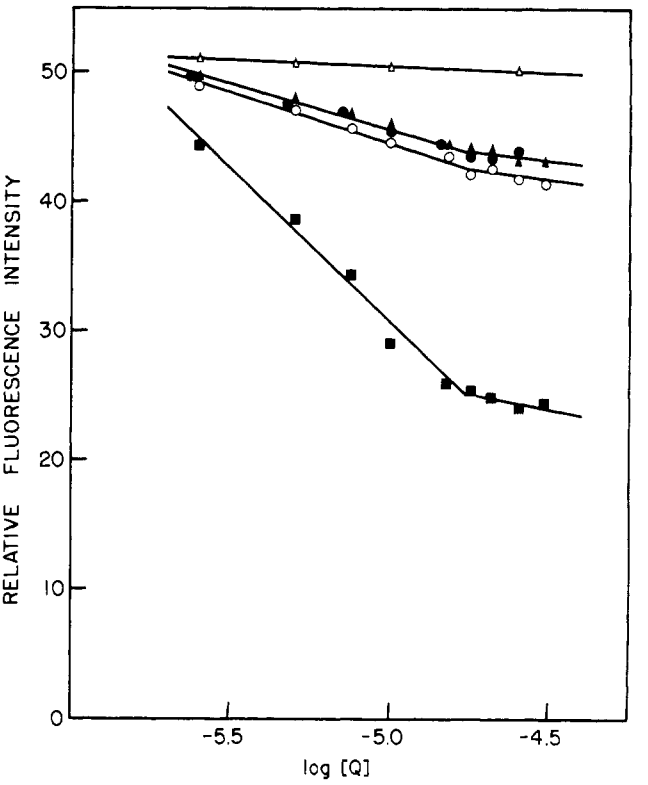

FIGURE 3: Fluorometric titration of RNase T_1 (17.5 mμmoles). Log (Q) denotes the log concentration of the following substrate analogs: guanosine (—○—), 3′-Gp (—■—), 2′–5′-GpC (—●—), 3′-Ap (—▲—), and 3′-Cp (—△—) Studies were performed in 0.1 M KCl solutions at pH 6.4. The wavelengths of excitation and emission were 280 and 360 nm.

protect RNase T_1 against inactivation by iodoacetate (Sato and Egami, 1965). At a molar ratio of 1:1 between RNase T_1 and guanosine the fluorescence intensities at 295 and and 360 nm are quenched about 10%. This decrease in fluorescence intensity is similar to the one caused by 3′-Ap.

2′–5′-GpC is an isomer of the RNase T_1 substrate 3′–5′-GpC. The addition of this compound to a RNase T_1 solution at pH 6.4 quenches the tryptophan fluorescence about 10% and the tyrosyl fluorescence about 20%, when the ratio between RNase T_1 and 2′–5′-GpC is 1:1.

The data given in Figures 3 and 4 can be further analyzed. Deranleau and Neurath (1966), have studied the combination of chymotrypsin and chymotrypsinogen with fluorescent substrates and inhibitors. The binding data have been represented by modified Scatchard plots. The binding has been described in terms of the saturation fraction of ligand. The saturation fraction of ligand could be experimentally determined from the fluorescence data. The fluorescence titration curves given in Figures 3 and 4 were treated in a similar manner.

The fluorescence intensity, I, of RNase T_1 decreases with decreasing number of free protein molecules. If no ligand is bound to the protein, the fluorescence intensity is I_{max}. Then I_{min} corresponds to the point, where all protein molecules are bound to ligand molecules. The binding can be described in terms of the saturation fraction of protein, s, the number of moles of protein bound per mole of protein. If the

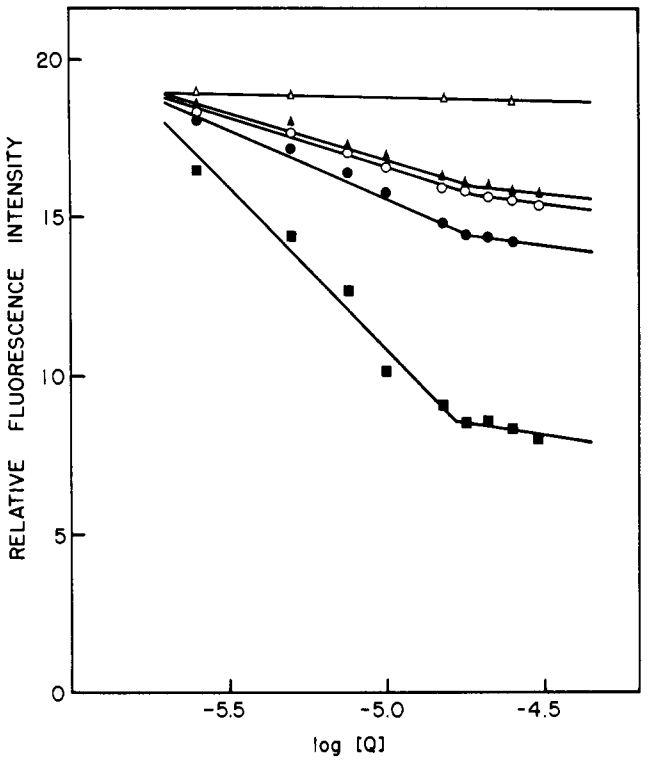

FIGURE 4: Fluorometric titration of RNase T_1 (17.5 mμmoles). Log (Q) denotes the log concentration of the following substrate analogs: guanosine (—○—), 3′-Gp (—■—), 2′–5′-GpC (—●—), 3′-Ap (—▲—), and 3′-Cp (—△—). Studies were performed in 0.1 M KCl solutions at pH 6.4. The fluorescence intensity of tyrosine was measured with a 25 times higher amplification relative to that of tryptophan. The wavelengths of excitation and emission were 280 and 295 nm.

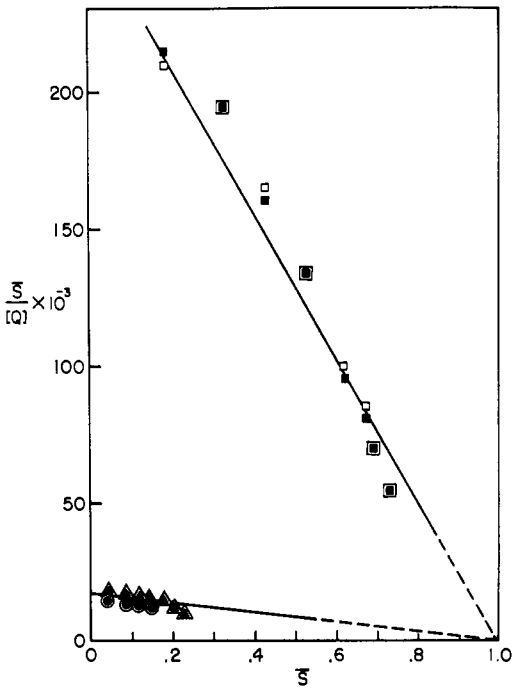

FIGURE 5: Modified Scatchard plots of the binding of 3′-Gp (■), 3′-Ap (▲), and 2′–5′-GpC (●) to RNase T_1 calculated from the data of Figure 3. The open symbols are calculated from the data of Figure 4. See text for full details.

quenching of the fluorescence of RNase T_1 is used as a measure of the saturation fraction of protein, then experimentally

$$s = (I_{max} - I)/(I_{max} - I_{min}) \qquad 0 \leqslant s \leqslant 1 \qquad (1)$$

In the case where there is only one ligand binding site per protein molecule, the binding constant, k, for the association reaction (E)+(Q) $\rightleftharpoons$ (EQ) can be obtained from the relation

$$s/(Q) = k(1 - s) \qquad (2)$$

where (Q) is the concentration of unbound quencher, *i.e.*, substrate analog. If $s/(Q)$ is plotted against s, the resulting plot is similar to a Scatchard plot.

Due to the weakness of the binding, experimental data for I_{min} are not available. I_{min} was obtained by extrapolation of the fluorescence titration curves in the following way. The inhibition of the transesterification of 3′–5′-GpC to 2′:3′-Gp and cytidine catalyzed by RNase T_1 has been measured (Pongs, 1968). From these kinetic experiments dissociation constants have been obtained for the binding of 3′-Gp (7.4×10^{-6} M) and 3′-Ap (1.4×10^{-4} M). For 2′–5′-GpC the same dissociation constant as for 3′-Ap was assumed, since both compounds quench the tryptophanyl fluorescence to a similar extent. These dissociation constants were used to calculate the saturation fraction s for the binding of each substrate analog at the highest substrate analog concentration used in the fluorescence experiments. With this information the I_{min} values have been obtained by extrapolation. If all the substrate analogs are bound at the same site of the protein, then it can be expected that in each case the same I_{min} value should be obtained. For the tryptophanyl fluorescence quenching this was found. For the tyrosyl fluorescence quenching the same I_{min} value was obtained for the binding of the mononucleotides 3′-Ap and 3′-Gp, whereas a different I_{min} value was obtained for the binding of the dinucleoside monophosphate 2′–5′-GpC.

With the extrapolated I_{min} values the saturation fractions s were calculated by means of eq 1. The concentrations of unbound quencher (Q) were calculated with the dissociation constants above. Figure 5 shows the data of Figures 3 and 4 plotted according to eq 2. In case of 3′-Gp the line passes through 1.0 on the s axis. This observation indicates that the original assumption of a single binding site was justified. Apparently this is also the case for the binding of 3′-Ap and 2′–5′-GpC, although the range of s values is too small to allow a definite statement (Deranleau, 1969). On the other hand, the kinetics of RNase T_1 reaction follow Michaelis–Menten kinetics (Irie, 1968; Pongs, 1968). Therefore, it is unlikely that for the binding of 3′-Ap and 2′–5′-GpC there exists more than one binding site. For the binding of guanosine data have been obtained which are similar to the data for the binding of 3′-Ap. The binding constants for 3′-Gp, 3′-Ap, and 2′–5′-Gpc are reflected in the slope (or $s/(Q)$ intercept) of the lines in Figure 5. A comparison of the slopes of these lines shows that the substrate of RNase T_1, *i.e.*, 3′-Gp, has the highest binding constant.

Discussion

Recently, we have suggested, that the tyrosine residues of RNase T_1 can be divided into two classes with respect to their environment: (1) tyrosine residues, which are not outside of the protein molecule and interact with acidic amino acid residues; (2) tyrosine residues, which are close to Trp-59, *i.e.*, exchange energy with it (Pongs, 1970). From the pH dependence of the tyrosyl and tryptophanyl fluorescence intensities it is possible to characterize the second class of tyrosine residues in more detail. Titration data of RNase T_1 show that only two out of nine tyrosine residues titrate normally and have a pK value of 9.7 (Iida and Ooi, 1969). The pH dependence of the tyrosyl fluorescence intensity reveals a pK value in this region, too. Therefore, it is concluded that the decrease of tyrosyl fluorescence intensity at alkaline pH is due to the ionization of the two free tyrosine residues of RNase T_1.

At alkaline pH the pH dependence of the tryptophanyl fluorescence intensity reveals a pK value very similar to the one of the pH dependence of the tyrosyl fluorescence intensity. Tryptophan itself does not ionize in this pH range. Therefore, the decrease of the tryptophanyl fluorescence intensity is presumably due to the change in protonation of amino acid residues, which are in the neighborhood of the tryptophan residue and can exchange energy with it. Data obtained by measurements of the fluorescence excitation spectrum of RNase T_1 have shown, that at neutral pH approximately one-third of the energy absorbed by the tyrosine residues is transferred to Trp-59 (Longworth, 1968). Thus, the decrease of the tryptophanyl fluorescence intensity would be due to the increasing ionization of free tyrosine residues, which leads to a quenching of the tryptophanyl fluorescence by a transfer of energy from Trp-59 to the ionized tyrosine residues (Forster, 1959; Steiner and Kolinski, 1968). The lysine residue of RNase T_1 has a pK value of 8.4 (Iida and Ooi, 1969). The deprotonation of this lysine residue should not influence the tryptophanyl fluorescence. From these data it is concluded that the two free tyrosine residues of RNase T_1 exchange energy with Trp-59.

It may be noted that the primary structure of RNase T_1 exhibits the sequence Tyr-Tyr-Glu-Trp enclosed by two prolines (Pro-55 and Pro-60) (Takahashi, 1965). Even though the tertiary structure of RNase T_1 is not known, from an examination of the CPK model of this sequence it is not unreasonable to suggest that these tyrosine residues are involved in the energy exchange with Trp-59.

Uv difference spectra of RNase T_1 with 2′-Gp at pH 5.0, gel filtration data and ultracentrifugation experiments suggested that this enzyme has only one binding site and forms with 2′-Gp a 1:1 complex (Sato and Egami, 1965). The data given in Figure 5 suggest the same conclusion. The modified Scatchard plots intercept the s axis at 1.0, which indicates that the complexes between the substrate analogs and RNase T_1 have 1:1 stoichiometry.

Of all compounds investigated in this study, 3′-Cp resembles least and 3′-Gp most the substrate of RNase T_1. Accordingly, 3′-Cp does not significantly affect the fluorescence intensities of tryptophan and tyrosine, whereas the binding of 3′-Gp to the enzyme produces a significant decrease in the tyrosyl and tryptophanyl fluorescence intensities. The extent of inhibition of the RNase T_1 reaction by 2′-Gp 2′-Ap, and 2′-Cp has been investigated at pH 7.0 (Irie, 1964). The degree of inhibition decreases in the order 2′-Gp > 2′-Ap > 2′-Cp. As indicated by the data in Figures 3, 4, and 5 the binding strength of the substrate analogs decreases in a similar order. This is consistent with the dissociation constants of these compounds determined by kinetic experiments (Pongs, 1968), which have been used to calculate the modified Scatchard plots in Figure 5. The extent of quenching is proportional to the binding strength of each inhibitor. Evidently, it is correlated to the specific binding of the substrate analogs in the active site of RNase T_1.

The complex between RNase T_1 and 2′–5′-GpC also has 1:1 stoichiometry, as indicated by the data in Figure 5. Compared with 3′-Gp and 3′-Ap, 2′–5′-GpC has an additional nucleoside, which is linked in 5′ position to the phosphate. Whereas the mononucleotides 3′-Gp and 3′-Ap as well as guanosine quench the tyrosyl and tryptophanyl fluorescence to a similar extent, the dinucleoside monophosphate 2′–5′-GpC quenches the tyrosyl fluorescence more (about 20%) than the tryptophanyl fluorescence (about 10%). A study of the rate of enzymatic cleavage of the dinucleoside monophosphates 3′–5′-GpC, 3′–5′-GpA, 3′–5′-GpG, and 3′-5′-GpU by RNase T_1 showed that the nucleoside in 5′ position of these compounds influences the reaction rate (Whitfeld and Witzel, 1963). This suggests that RNase T_1 is also interacting with the 5′-nucleoside of the dinucleoside monophosphates besides the predominant interaction with the 3′-nucleoside.

The observed decrease in fluorescence intensity by binding of 3′-Gp to the enzyme cannot result from the elimination of water from the environment of Trp-59 and the tyrosine residues involved. A decrease in the polarity of the environment of tryptophan and tyrosine *increases* the quantum yields of the tyrosyl and tryptophanyl fluorescence (Steiner *et al.*, 1964; Cuatrecasas *et al.*, 1967). This suggests that Trp-59 and the tyrosine residues are present in the active site of RNase T_1, but are not involved directly in the binding of the inhibitor. The binding process would bring these residues into closer contact with charged groups of the enzyme or the peptide backbone and thus result in the observed quenching. This concept may also explain, why a modification of the tyrosine or Trp-59 residues only decreases the activity of RNase T_1, but does not abolish it (Shiobara *et al.*, 1962; Terao and Ukita, 1969).

From kinetic experiments (Irie, 1968; Pongs and Witzel, 1968) and nuclear magnetic resonance investigations (Ruterjans *et al.*, 1969) it was concluded that two histidine and two acidic amino acid residues are present in the active site of RNase T_1. One of these acidic amino acid residues has been identified by carboxymethylation experiments as Glu-58 (Takahashi *et al.*, 1967). According to the amino acid sequence of RNase T_1 Glu-58 is next to Tyr-57 and Trp-59. From the nuclear magnetic resonance data it was further concluded that in the active site of RNase T_1 the carboxylate anions of the acidic amino acid residues are close to the histidine residues. They interact with each other, which leads to a perturbation of the pK values of these residues, *i.e.*, the pK value of histidine is shifted to higher values (7.0–7.5) and the pK value of the acidic amino acid is shifted to lower values (4.0–3.5). The fluorescence measurements show that Trp-59 as well as tyrosine residues are in or near the active site of RNase T_1. Since RNase T_1 undergoes no

conformational change between pH 2 and 10, the small changes of the fluorescence intensities between pH 3 and 8 would be due to ionized groups in the neighborhood of Trp-59 and the tyrosine residues involved. Thus, they can be understood on the basis that changes in the protonation of the histidine residues and the carboxylate groups of the acidic amino acid residues in the active site of RNase T_1 affect the interactions between these two amino acid residues as well as the interactions of these two amino acid residues with the tyrosine and tryptophan residues.

The fluorescence of tryptophan is quenched by the imidazolium of histidine (Shinitzky and Fridkin, 1969) as well as by carboxylate anions (Beaven, 1961). The tyrosyl fluorescence intensity is also affected by positively and negatively charged groups (Edelhoch *et al.*, 1968). Since the carboxylate and imidazolium ions in the active site of RNase T_1 interact with each other in the pH range 4–8, they would not be available for an interaction with the neighboring tyrosines and Trp-59. The protonation of the carboxylate anions in the active site at acidic pH yields imidazolium ions, which decrease the fluorescence intensities of tyrosines and tryptophan in their neighborhood. On the other hand, deprotonation of the imidazolium ions at alkaline pH yields carboxylate anions, which again decrease the tyrosyl and tryptophanyl fluorescence intensities in this pH range. From this it follows that the fluorescence intensities are highest in the pH range 4–8 as seen in Figures 1 and 2.

This concept is further supported by the data showing that the interaction of 3′-Gp with the enzyme affects the pH-dependent changes of the fluorescence intensities in the pH range 4–8. The pK values of 4.0 and 7.2, derived from the pH dependence of the tryptophanyl fluorescence intensity of the free enzyme, are shifted to 4.5 and 7.7. An analogous shift of the pK value of one histidine from 7.1 to 7.7 has been found by the nuclear magnetic resonance investigations. This histidine interacts with the phosphate moiety of 3′-Gp. The small pH-dependent changes of the tyrosyl fluorescence intensity of the free enzyme are not observed in the pH-dependence of the tyrosyl fluorescence intensity of the enzyme–inhibitor complex. This may indicate that the interaction of 3′-Gp with charged groups in the active site of RNase T_1 diminishes the influence of these charged groups on the tyrosyl fluorescence.

The interaction of 3′-Gp with RNase T_1 quenches the tyrosyl and tryptophanyl fluorescence over a wide pH range. The merging of the two curves for the pH dependence of the fluorescence intensities for the free enzyme and the enzyme–3′-Gp complex does not reflect the pH dependence of the enzyme–inhibitor complex as determined by kinetic experiments (Pongs, 1968). However, the data are comparable with the nuclear magnetic resonance data (Ruterjans *et al.*, 1969) and the titration data (Iida and Ooi, 1969).

In the kinetic experiments the inhibition of the RNase T_1 reaction by 3′-Gp has been measured, whereas in the fluorescence as well as the nuclear magnetic resonance and titration experiments the binding between RNase T_1 and 3′-Gp has been studied. The binding step and the actual reaction step should be distinguished. So the pH dependences of the enzyme–3′-Gp complex, which have been determined by a study of the binding step or the reaction step, are not directly comparable. Above pH 8 and below pH 5 the activity of RNase T_1 is very low but apparently binding still occurs. In this connection an observation of the nuclear magnetic resonance investigations is important. The histidine residue involved in the binding of G does not titrate normally in the pH range, which has been investigated in the nuclear magnetic resonance study (pH 2–8). The histidine residue located at the site where the reaction takes place, does titrate in this pH range.

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Water-Insoluble Enzymes. Synthesis of a New Carrier and Its Utilization for Preparation of Insoluble Derivatives of Papain, Trypsin, and Subtilopeptidase A*

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ABSTRACT: A new type of highly insoluble polyfunctional, diazotizable resins, (dialdehyde)–starch–methylenedianiline (S-MDA), could be prepared by the condensation of dialdehyde starch (a commercially available periodate oxidation product of starch) with *p,p'*-diaminodiphenylmethane (MDA) and the subsequent reduction of the Schiff's base-type polymeric product. The S-MDA resins following diazotization were coupled with papain and mercuripapain, subtilopeptidase A (subtilisin Carlsberg), and polytyrosyl trypsin, giving highly active water-insoluble derivatives of these enzymes. The S-MDA resins had diazotization capacities of 0.26–0.33 mequiv/g and a protein binding capacity of about 100 mg of protein/g. The water-insoluble S-MDA–protein conjugates had particulate form, were easily filtered and could be conveniently used in columns.

Amino acid analysis of acid hydrolysates of S-MDA derivatives of papain, subtilopeptidase A, and polytyrosyl trypsin showed that the tyrosine, arginine, and lysine contents of the insoluble derivatives were considerably lower than the respective values obtained for the native enzymes. It was assumed that the missing amino acids represented the amino acid residues through which the covalent bonds between the protein and the diazotized carrier were formed. The pH–activity profiles of S-MDA–papain, S-MDA–subtilopeptidase A, and S-MDA–polytyrosyl trypsin, acting on benzoyl-glycine ethyl ester, acetyl-L-tyrosine ethyl ester, and benzoyl-L-arginine ethyl ester, respectively, were displaced toward more alkaline pH values by one to two pH units, as compared with the native enzymes. This effect was found to be essentially independent of the ionic strength of the medium.

Enzyme derivatives in which the biologically active protein is covalently bound to a water-insoluble polymeric carrier may serve as easily removable reagents of considerably improved shelf-stability. Immobilized enzyme derivatives are well suited for repeated or continuous use. Immobilized derivatives of proteolytic enzymes have been successfully applied in the limited digestion of proteins (Goldstein and Katchalski, 1968), and in column form for the isolation and purification of specific enzyme inhibitors (Fritz *et al.*, 1968). The methods which have been utilized for the immobilization of proteins have been recently summarized in several reviews (Silman and Katchalski, 1966; Goldstein and Katchalski, 1968; Goldstein, 1969).

A method that has been widely used for both the preparation of water-insoluble enzyme derivatives and in the preparation of immunoadsorbents utilized the coupling reaction between a protein and the polydiazonium salt derived from a water-insoluble resin such as poly-*p*-aminostyrene (Grubhofer and Schleith, 1954), *p*-aminobenzylcellulose (Campbell *et al.*, 1951), and more recently a *p*-amino-DL-phenylalanine-L-leucine copolymer (Bar Eli and Katchalski, 1963). The recoveries of enzymic activity in the insoluble derivatives when diazotized poly-*p*-aminostyrene or *p*-aminobenzylcellulose were used as carriers were in general rather low. The diazotized *p*-amino-DL-phenylalanine–L-leucine copolymer has been successfully used for the preparation of highly active water-insoluble derivatives of polytyrosyl trypsin (Bar Eli and Katchalski, 1960, 1963), papain (Cebra *et al.*, 1961; Silman *et al.*, 1966), and urease (Riesel and Katchalski, 1964). The rather elaborate procedures involved in the preparation of the *p*-amino-DL-phenylalanine–L-leucine copolymer, however, precluded its wider application, despite its superior properties as a carrier.

In the present article, a new, readily synthesized, diazotizable resin, S-MDA,[1] is described. The S-MDA resins were prepared by the condensation of dialdehyde–starch, DAS[2] (a commercially available periodate-oxidation product of starch) with *p,p'*-diaminodiphenylmethane (bismethylenedianiline, MDA) and the subsequent reduction of the Schiff's base polymeric product (Scheme I). Diazotized S-MDA resins were employed for the preparation of water-insoluble derivatives of papain, trypsin, and subtilopeptidase A (subtilisin Carlsberg) of high enzymic activity. Preparations containing up to 10%

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[1] The name of the resin, S-MDA, is derived from the names of its chemical components: (dialdehyde)–starch–methylenedianiline (see Scheme I).

[2] Abbreviations used are: DAS, dialdehyde starch; MDA, *p,p'*-diaminodiphenylmethane (methylenedianiline); PAB-cellulose, *p*-aminobenzylcellulose; EMA, ethylene–maleic acid copolymer.