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SUBSTITUENT EFFECT ON RIBONUCLEASE T_1-CATALYZED TRANSPHOSPHORYLATION OF *para*-SUBSTITUTED BENZYL ESTERS OF GUANOSINE 3'-PHOSPHATE

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The maximal velocities have been measured for the ribonuclease T_1-catalyzed transphosphorylation of a series of substrates named in the title. Based on the present results together with other relevant data, it is concluded that the catalysis involves nucleophilic component as well as electrophilic character, and the breakdown of a pentacovalent intermediate is not rate-limiting. We have proposed a possible mechanism most pertinent to a description of the ribonuclease T_1 action.

Whereas much has been written about ribonucleases such as bovine pancreatic RNase A[1,2] and Taka-diastase RNase T_1[2c,3] no work has been made concerning the application of the linear free energy relationships[4] in RNase-catalyzed reactions. It was therefore decided to investigate how systematic perturbations in the structure of the substrate for RNase T_1 would affect the transphosphorylation, for the variation of V_{max} with substituent should provide some clue to the electronical situation at the transition state of the transphosphorylation in the ES complex.

The required substrates for RNase T_1 were prepared by the synthetic route using the *para*-substituted phenyl diazomethane and guanosine 3'-phosphate in pyridinium salt form. Thus were obtained in the ammonium salt form of Gp(3')$CH_2C_6H_5$, Gp(3')$CH_2C_6H_4$-*p*-CH_3, Gp(3')$CH_2C_6H_4$-*p*-Cl, and Gp(3')$CH_2C_6H_4$-*p*-NO_2, the structure of some of the products being based on NMR spectral evidence.

RNase T_1 catalyzes two separate steps, viz., transphosphorylation (or cyclization) of phosphodiester bonds in ribonucleate and hydrolysis of 2',3'-cyclic phosphate only when the base residue substituted in the ribose moiety is guanine[3a]. The overall scheme for RNase T_1-catalyzed reactions of the substituted benzyl esters[5] thus can be written as:

$$\text{Gp(3')}CH_2C_6H_4\text{-}p\text{-X} \xrightarrow{\text{RNase } T_1} \text{G(2',3')cyclic P} + HOCH_2C_6H_4\text{-}p\text{-X}$$

$$\text{G(2',3')cyclic P} \xrightarrow{\text{RNase } T_1} \text{Gp(3')}$$

Rate measurements were performed by measuring products and/or unreacted Gp(3')CH_2-C_6H_4-*p*-X. The experimental procedures are the same as outlined before[6]. In all kinetic runs the substrate was present in large excess of its K_m (approx. 25- to

100-fold), and thus the measured rates correspond to V_{max}. To avoid any detectable effect of product inhibition by Gp(3') and/or G(2',3')cyclic phosphate, the initial rates (less than 5% reaction) were used. A reasonable good fit to the Hammett equation[7] has been obtained when the constants, σ°, have been applied since the reaction center is insulated by methylene group, yielding at pH 7.5 ρ values of 0.64, 0.48, and 0.35 at 15°, 25°, and 35°, respectively. Hammett plots of V_{max} for $Gp(3')CH_2C_6H_4$-p-X are illustrated in Fig.1.

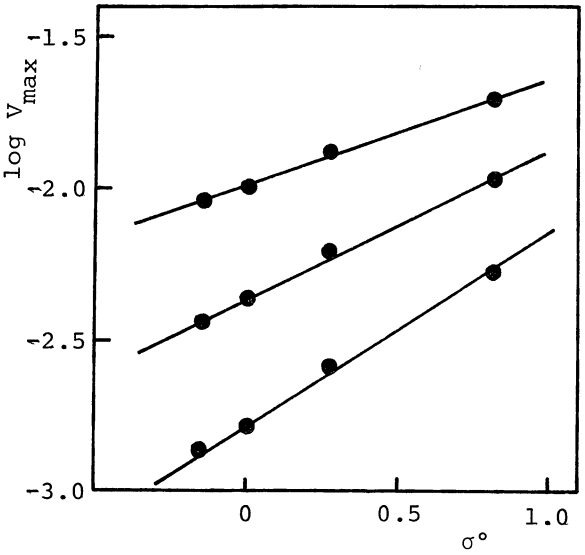


Fig. 1. Hammett plot for V_{max} for the RNase T_1-catalyzed transphosphorylation of $Gp(3')CH_2C_6H_4$-p-X at three different temperatures, pH 7.5, and 0.1M-ionic strength. V_{max} is in μM/min/20μg RNase T_1. Substrate concentration, 2×10^{-3}M∿8×10^{-3}M; enzyme concentration, 0.01mg/ml.

We have found that the nonenzymatic acid- and base-catalyzed transphosphorylation rate for a series of $Gp(3')CH_2C_6H_4$-p-X obeys a Hammett equation, being correlated with σ° with ρ-values of ±0.0 and +1.24, respectively[5]. The OH^--catalyzed reaction is known to proceed via the so-called "in-line" mechanism[8] whereas the H^+-catalyzed reaction takes place through pentacovalent addition intermediates[9]. The ρ value of ±0.0 for the H^+-catalyzed transphosphorylation indicates that the departure of the $HOCH_2C_6H_4$-p-X from the pentacovalent intermediate(s) is not the rate-determining step. It should be also noted that base-catalyzed hydrolysis of substituted benzyl acetates has a ρ value of 0.74 while the reaction catalyzed by H^+ has a value of -0.05[10]. Higher value of +1.24 compared with ρ = 0.74 may be attributed to the higher polarizability of the central ion at the transition state.

As regards RNase T_1-catalyzed reaction, it would be expected that the enzyme could supply general acid and general base catalysis[4b]. By analogy with the mechanism[8] already proposed for RNase A-catalyzed reaction, the essential features of RNase T_1-reactions consist of nucleophilic displacements about the phosphorus atom. The rate-determining step in transphosphorylation could also be either breakdown of the pentacovalent intermediate or addition of 2'-OH as a nucleophile concerted with elimination of the leaving group as $HOCH_2C_6H_4$-p-X. The leaving anion, $^-OCH_2C_6H_4$-p-X , is a strong base, so that a proton is partially transferred to the $OCH_2C_6H_4$-p-X group in the transition state. The maximal rate of transphosphorylation of $Gp(3')CH_2C_6H_4$-p-X by RNase T_1, as shown in Fig. 1, has been found to increase with electron-withdrawing substituents in the benzene ring. Therefore, a positive ρ value of +0.35 at 35° can be taken as a sign to indicate that the break-

down of an intermediate, EQ, is not rate-limiting, i.e., $k_b > k_{-f}$ in Fig. 3. Here it was decided to establish the pH-dependence of the transphosphorylation rate for $Gp(3')CH_2C_6H_5$, since the pH-dependence of the RNase T_1 activity had been previously determined for only RNA[11] on an arbitrary scale although Irie[12] reported the pH-rate profile over the rather limited pH range between 2.5 and 5.0 using a dinucleoside phosphate, guanylyl-3',5'-cytidine, as substrate which introduces some ambiguity in the interpretation of the results because the pK value of cytidine is about 4.2. The pH-dependence of V_{max} for $Gp(3')CH_2C_6H_5$ was determined and it was indicated that the amino acid residues having pK 3.0 and 7.9 are essential for RNase T_1 activity (Fig. 2). It seems, therefore, resonable to us to formulate a mechanism compatible with our results and those of others in terms of the scheme shown in Fig. 3 (For anti → syn conformational change with respect to the sugar-base torsion angle of Gp(3') on complexing with RNase T_1, see ref. 13.).

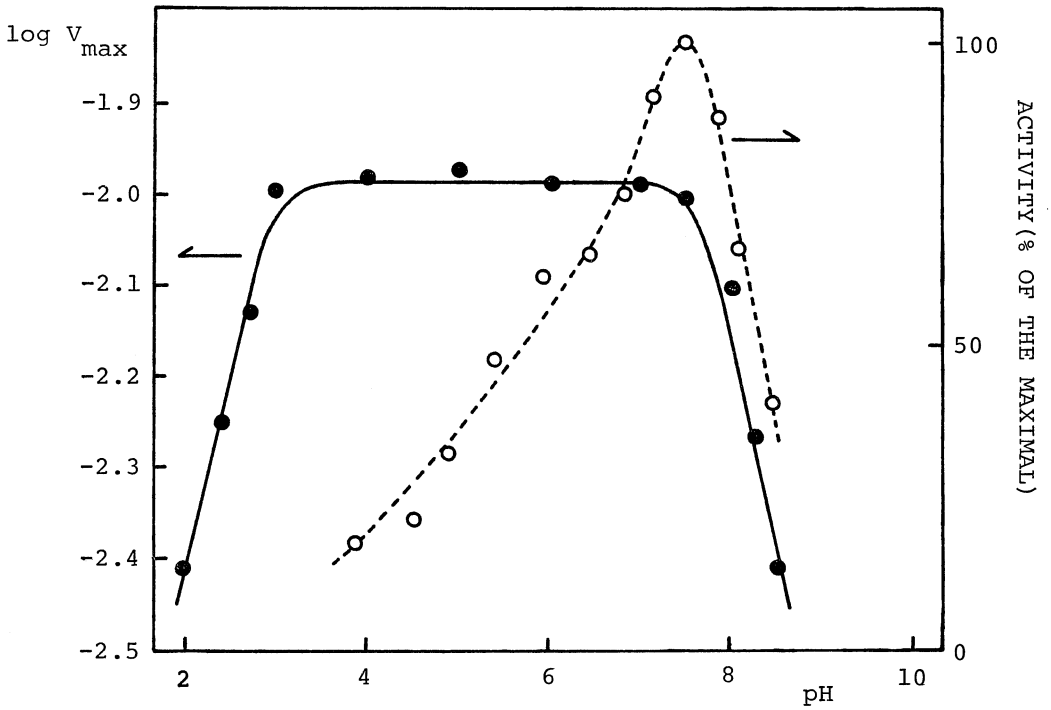
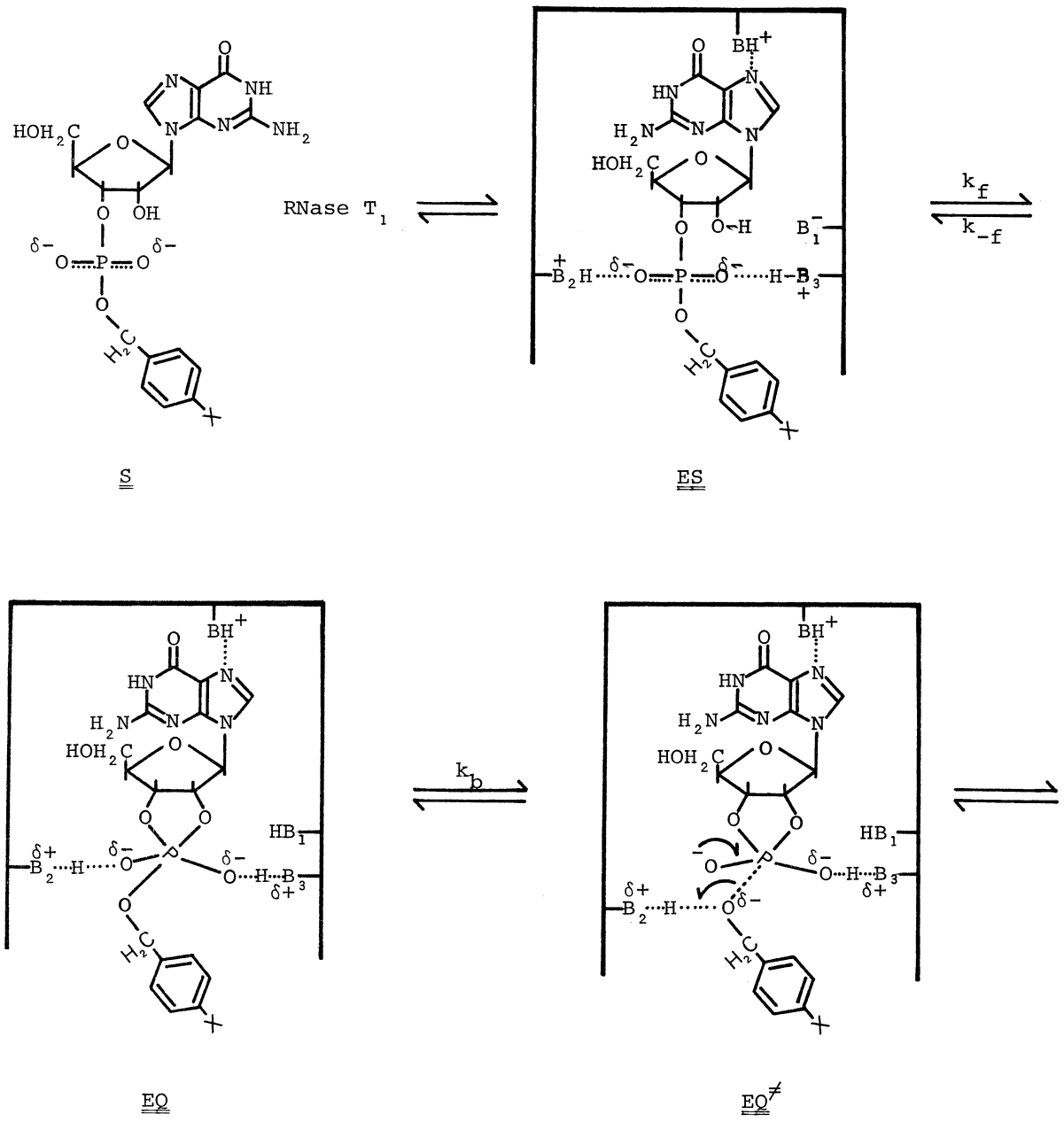


Fig. 2. Log V_{max} vs. pH for the RNase T_1-catalyzed transphosphorylation of $Gp(3')CH_2C_6H_5$ (—●—●—●—) at 35° and ionic strength, 0.1. The dashed line (-o----o----o-) is pH-dependence of the RNA digesting activity of RNase T_1 (Shobara, Takahashi, and Egami, 1962).

As illustrated in Fig. 3 we interpret the observed small Hammett sensitivity (ρ = +0.35 at 35°) for the overall rate to not only the nucleophilic contribution by B_1 but also the effective contribution of an electrophilic component in the transition state for k_f step through electrophilic assistance by general acid(s), B_2H^+ and/or B_3H^+ ——— partial proton donation to the two oxyanions ——— facilitating the formation and breakdown of a pentacovalent intermediate, EQ. Although the exact allocation of the catalytic amino acid residues is only conjecture, B_1, B_2H^+, and B_3H^+ are tentatively identified as the carboxylate group of Glu 58[14], His 92[15], and Arg 77[16], respectively, as judged from the results of extensive



G(2',3')cyclic P + $HOCH_2C_6H_4$-p-X + RNase T_1

Fig. 3. A proposed mechanism of the transphosphorylation of substituted benzyl esters of guanosine 3'-phosphate catalyzed by RNase T_1. EQ represents the suggested trigonal bipyramidal intermediate formed between enzyme and Gp(3')$CH_2C_6H_4$-p-X during catalysis. $EQ^{\neq}$ is the transition state for k_b step ($k_b > k_{-f}$).

chemical modification experiments by Takahashi together with the results of the pH-dependence study on the RNase T_1-reaction of $Gp(3')CH_2C_6H_5$.

The marked dependence of the Hammett sensitivity on temperature has been noticed and the temperature dependence of ρ is given by $\rho = -3.80 + \frac{1280}{T}$, indicating that ρ would change sign in the vicinity of 60°C, this being the isokinetic temperature[17] of this reaction series [RNase T_1 has been found to remain its activity at an elevated temperature as high as 60° in our kinetic study with $Gp(3')CH_2C_6H_5$as substrate[18].]. A possible explanation to account for such phenomena is that the expulsion of $HOCH_2C_6H_5$ which would contribute to a positive ρ might outweigh the negative effect of protonation to two oxyanions and the transition state in at least one step in the transphosphorylation would become progressively to have quite polar character as the reaction temperature is decreased. It is, therefore, expected that the reaction tends to proceed via a concerted direct displacement mechanism and the $P\text{-}OCH_2C_6H_4\text{-}p\text{-}X$ bond is significantly cleaved in the transition state at lower temperature.

References and Notes

1) Abbreviations used are: RNase A, ribonuclease A (bovine pancreatic), ribonucleate 3'-pyrimidino-oligonucleotidohydrolase (EC 3.1.4.22; formerly EC 2.7.7.16); RNase T_1, ribonuclease T_1 (Aspergillus oryzae), ribonucleate 3'-guanylo-oligonucleotidohydrolase (EC 3.1.4.8; formerly EC 2.7.7.26); G(2',3')cyclic P, cyclic 2',3'-guanosine monophosphate; Gp(3'), guanosine 3'-phosphate; K_m, Michaelis constant; V_{max}, maximal reaction velocity.

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(b) W. P. Jencks, Catalysis in Chemistry and Enzymology (McGraw-Hill, New York, N. Y., 1969).

5) Of the substituted benzyl esters studied, those having X = H, CH_3, Cl, and NO_2 as para substituents have been confirmed to undergo hydrolysis via transiently formed 2',3'-cyclic diester by only P-O cleavage both in enzymatic and non-enzymatic reactions, while for $Gp(3')CH_2C_6H_4\text{-}p\text{-}OCH_3$ we observed acid-catalyzed C-O cleavage. Details will be reported elsewhere.

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(c) idem., ibid., 69, 331 (1971).

16) K. Takahashi, J. Biol. Chem., 243, 6171 (1968).

17) Ref. (7) p.177.

18) Cf., The thermal transition of the enzyme has been reported to occur between 40° and 50° at pH 7.5 as measured by changes in absorption at 278 nm[19].

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