*STEREOCHEMICAL ANALYSIS OF THE SPECIFICITY OF PANCREATIC RNASE WITH POLYFORMYCIN AS SUBSTRATE: DIFFERENTIATION OF THE TRANSPHOSPHORYLATION AND HYDROLYSIS REACTIONS**

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Abstract.—A stereochemical analysis of the substrate and inhibitor specificities of bovine pancreatic ribonuclease A is presented. A scheme is proposed in which the binding specificity for this protein–nucleic acid interaction is rationalized in terms of a simple system of H-bonds. The functional groups that govern substrate binding for transphosphorylation and hydrolysis, respectively, are considered and differentiated, and predictions are offered concerning the interaction of presumptive substrates with RNase.

In this paper we outline proposals to account for the substrate specificity and for some of the catalytic properties of bovine pancreatic ribonuclease A.

It has previously been reported[1] that formycin residues (Fig. 1) in polyribonucleotides are susceptible to attack by low concentrations of pure RNase. For example, poly F‡ is degraded quantitatively to F > p; the enzymatic digest contains no 3′-FMP. The depolymerization of F-containing polymers is not attributable to trace contaminants in the enzyme preparation, nor is it in any way comparable to the feeble and questionable RNase susceptibility of poly A. Poly F is degraded by minute concentrations of RNase; the rate of degradation is one third that of poly U and equal to that of poly C under identical conditions (Fig. 2). Moreover, poly F competes with poly C for degradation by RNase. Finally, RNase derivatives specifically carboxymethylated at histidine-119 or dinitrophenylated at lysine-41 are inactive with respect to poly F just as they are with poly C and poly U. Since poly F is converted exclusively to F > p, which is immune to subsequent enzymatic hydrolysis, the use of poly F as substrate clearly differentiates between the two characteristic activities of RNase—transphosphorylation and hydrolysis.

Witzel has stated, on the basis of investigations dealing mainly with the hydrolytic reaction, that the interaction of pyrimidine nucleotides and polynucleotides with RNase is nonspecific with respect to the base, and that susceptibility to catalytic action depends solely on the presence of a keto function in the base at a position α to the glycosyl bond.[2,3] These specifications are inconsistent with our observations, which demonstrate that no oxygen in the aglycone is required for the transphosphorylating action of RNase.

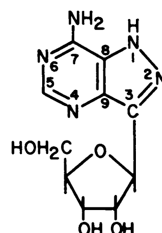

Fig. 1.—Structure of formycin, 7-amino-3-(β-D-ribofuranosyl) pyrazolo-(4,3-d)pyrimidine.

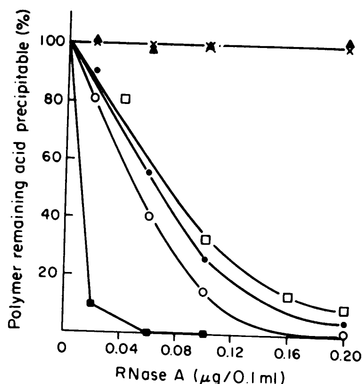

FIG. 2.—Degradation of synthetic polyribonucleotides by pancreatic RNase: poly U (■), poly C (○), poly F (●), poly L (□), poly A (▲), and poly G (×). All reactions were incubated for 10 min at 37°C in 0.1 *M* sodium acetate, pH 5.1, with the indicated enzyme concentration. The polymer concentrations (μM) used were: poly U, 260; poly C, 256; poly F, 270; poly L, 125; poly A, 265; and poly G, 278. The extinction coefficients of poly F and poly L were 7.6 and 7.0, respectively.

The Transphosphorylation Reaction.—In assessing the significance of our observations, we have been guided by the following assumptions and tentative proposals.

(1) The two characteristic reactions of RNase—transphosphorylation to, and hydrolysis of, the cyclic 2′,3′ phosphate—may have different substrate specificities and may perhaps even proceed by fundamentally different catalytic mechanisms.

(2) The interaction of nucleotides and polynucleotides with the enzyme requires simultaneous binding of a phosphoryl group and some functional groups in the base. This is suggested by the fact that polyribose phosphate[5] and nucleosides[6] bind RNase less efficiently than RNA or nucleotides.

(3) In both reactions, the interaction of the base with the enzyme is *specific;* i.e., a combination of individual, defined functional groups in the enzyme and substrate interact in a stereospecific manner.

(4) The same active site is responsible for the depolymerization (transphosphorylation) of poly F, poly U, and poly C. Any model which purports to account for the substrate specificity of RNase should rationalize the susceptibility of these polymers in terms of reasonable stereochemical similarities.

At first sight, CMP and UMP seem to bear little if any resemblance to FMP. However, an examination of the three-dimensional structure of these nucleotides demonstrates that, as far as the distribution of H-bonding groups is concerned, FMP can be considered a hybrid of CMP and UMP. This is a consequence of the fact that, in neutral aqueous solution, the individual bases of poly U and poly C, like the nucleosides[7] and double-stranded DNA,[8] appear to have the *anti* conformation; in contrast, the F residues of poly F possess the *syn* conformation.[1] In Figure 3, 3′-FMP (*syn*) is compared with a hybrid pyrimidine structure containing the H-bond donor groups of both CMP and UMP (*anti*). In these comparisons, the 3′-phosphoryl groups of the three nucleotides have been superimposed. Under these conditions the donor NH_2 of F is close to that of C and the donor N^1-H of F to the N^3-H of U. FMP contains no functional group homologous with the 2-keto group of CMP and UMP. We propose, therefore, that the F residue interacts with RNase through two H-bonds, in which both the C^7-NH_2 and the N^1-H of F act as donors. Although the coincidence between the H-bonding groups in F and those in U and C is not so exact as that between groups

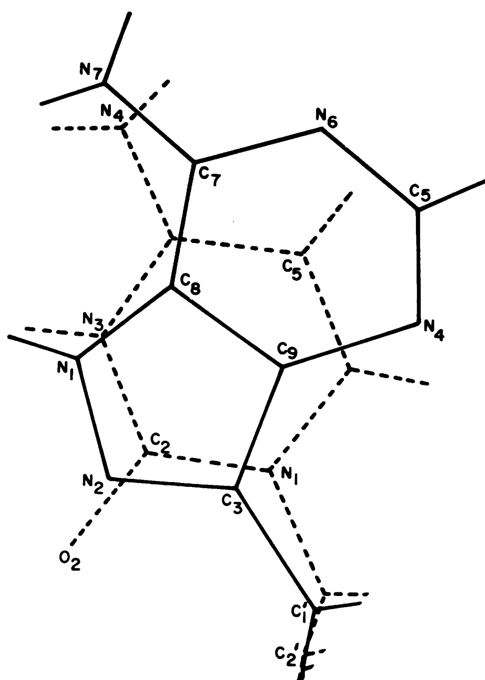

FIG. 3.—Stereochemical comparison of 3'-FMP (*syn*) (*solid line*) and an H-bonding hybrid of 3'-CMP and 3'-UMP (*anti*) (*dashed line*). The phosphorus atoms of both nucleotides have been superimposed but only the bases and glycosyl bonds are illustrated. The torsion angles about the glycosyl bonds differ by 180° in the two nucleotides. All other torsion angles are identical and are like those in ribose nucleotides of helical RNA. This permits a comparison of atomic positions using torsion angles which are stereochemically acceptable. The identity in the H-bonding groups (N^7 and N^1 of F with the N^4 of C and N^3 of U) can be improved although the 3'-phosphate groups are then no longer in exactly the same positions.

in C and U, we can be sure that this model is stereochemically reasonable by consideration of the base pairing between A and U, with the use of the Watson-Crick[9] and Hoogsteen[10] pairing schemes. In both cases, the same donor and acceptor sites are used on U, but those in A are different. From inspection of Figures 1 and 3, it can be seen that the H-bond groups used when C or U are substrates for RNase make the bonding like that in a Watson-Crick pair, while for F the pattern is like that in the Hoogsteen pairing of A and U.

The importance of the conformational requirements outlined above can be illustrated by the stepwise degradation of the alternating copolymer r(F-U). When r(F-U) is exposed to RNase, it is first degraded to FpUp and then to F > p and Up. In the two-stranded molecule the F residues have the normal *anti* conformation.[1] As such they are *not* acceptable substrates for RNase and the polymer is cleaved only at the U residues to yield FpUp. Once they are freed from the constraints imposed by the polymer structure, the F residues in these dinucleotides can assume the *syn* conformation, whereupon they are attacked by the enzyme.

If the above scheme is correct, it is possible to formulate predictions concerning other presumptive RNase substrates. Our stereochemical reasoning leads to the prediction that polymers of laurusin (in which the NH_2 group of F is replaced by a keto group) should be substrates for RNase, provided that the L residues exist in the *syn* conformation. We have synthesized poly L and find that its ORD spectrum qualitatively resembles that of poly F,[1] suggesting that the individual nucleotide units do indeed maintain the *syn* conformation. As predicted, poly L is readily degraded by RNase (Fig. 2). It may be noted once again that the

ketonic oxygen of LMP is sterically homologous with the C^4-keto, but not with the C^2-keto, of UMP.

Additional support for the above proposals is provided by the behavior of poly azaG. With individual residues in *syn*, poly azaG is an H-bonding hybrid of poly C and poly U, but in contrast to poly F, in which both relevant H-bonding positions are donors, those in poly azaG (N^7 and C^6=O) are both acceptors. From the work of Levin,[11] who showed that azaG residues in polynucleotides are susceptible to attack by RNase, it appears likely that poly azaG, like poly F, is degraded solely to the level of the cyclic phosphate and is therefore a substrate *only* for transphosphorylation. The restricted degradation of poly F is, therefore, not simply a reflection of the C-ribosyl structure. The following conclusions are drawn (see also Table 1):

(1) F (*syn*) is an H-bonding hybrid of C + U (*anti*), and is recognized as such by RNase. However, F (*anti*) is also an H-bonding analog of A (*anti*) in replication reactions which require base pairing of the Watson-Crick type.

(2) Laurusin (*syn*) is an H-bonding analog of U (*anti*) for RNase, and laurusin (*anti*) is an H-bonding analog of G (*anti*) in replication reactions.[12]

(3) The substrate specificity for binding and catalysis in the transphosphorylating action of RNase is based on a stereochemically defined disposition of functional groups. These include, interchangeably, the H-bonding groups at positions 3 and 4 of U or C, or positions 1 and 7 of F or L, *and* a phosphoryl group with a suitable and specific spatial orientation in relation to the H-bonding groups. The sufficiency of two H-bonds for transphosphorylation does not exclude the possibility that three H-bonds may be formed normally by pyrimidine substrates. Indeed, as discussed below, the nature of substituents at the 2-position of pyrimidines (and homologous positions in F and in purines) may be critical for enzyme function in both reactions. Another possibility consistent with our data is that any two of the three H-bonds potentially formed by pyrimidines may suffice for transphosphorylation.

(4) The presence of an oxygen function α to the glycosyl bond is not required for binding or for enzyme-catalyzed transphosphorylation.

These findings, insofar as they concern base specificity, may be related to the

TABLE 1. *Relationship of nucleotide conformation and H-bonding homologies to the substrate specificity of pancreatic RNase.*

Nucleotide substrate	Conformation	Nucleotide Binding Sites*				Enzyme Binding Sites†			
		S_1	S_2	S_3	S_4	E_1	E_2	E_3	E_4
Cytidine	*Anti*	C^4—NH_2	N^3:	C^2=O	$(PO_4)^-$	A	D	D	I
Uridine	*Anti*	C^4=O	N^3—H	C^2=O	$(PO_4)^-$	D	A	D	I
Formycin	*Syn*	C^7—NH_2	N^1—H	—	$(PO_4)^-$	A	A	—	I
Laurusin	*Syn*	C^7=O	N^1—H	—	$(PO_4)^-$	D	A	—	I
8-Ketoguanosine	*Syn*	C^6=O	N^7—H	C^8=O	$(PO_4)^-$	D	A	D	I
Uric acid	*Syn*	C^6=O	N^7—H	C^8=O	$(PO_4)^-$	D	A	D	I
8-Azaguanosine	*Syn*	C^6=O	N^7:	—	$(PO_4)^-$	D	D	—	I

* S_1, S_2, and S_4 are sufficient for transphosphorylation; S_3 and S_4 appear to be the determinants for hydrolysis (see text).

† A, H-bonding acceptor; D, H-bonding donor; I, ionic—the nature of the phosphate group interaction is not necessarily identical in the transphosphorylation and hydrolytic steps (see text). E_1 interacts with S_1, E_2 with S_2, etc.

enzyme structure. Taken together, the RNase susceptibility of poly F, poly L, poly C, and poly U shows that the presence of either H-bond donor or acceptor groups at positions 3 and 4 of the pyrimidine ring (or homologous positions in F and L) render substrates equally acceptable to the enzyme for transphosphorylation. This implies that the base-binding region of the protein also possesses functional groups capable of acting interchangeably as H-bond donors or acceptors. Indeed, the crystallographic studies of RNase[13] demonstrate this to be the case. Richards and co-workers[14] have observed that the hydroxyls of threonine-45 (and possibly that of serine-123) are capable of forming H-bonds with the 3 and 4 positions, respectively, of the inhibitors 2′ (3′) (5-iodo-UMP) (*anti*). Our findings are in accord with these observations and are reassuring because they suggest that the location of the inhibitor is actually at the substrate binding site (transphosphorylation). In addition, the X-ray studies suggest that a third H-bond is formed between the 2-keto oxygen of the pyrimidine inhibitor and a protein N-H group (threonine-45).[14]

The above formulations are entirely consistent with the known consequences for enzyme susceptibility of substitutions in the pyrimidine ring. For example, the failure of N^3-substituted uridylate derivatives to function in either transphosphorylation[15] or hydrolysis[2] is readily understandable: an essential H-bond group is unavailable in the first instance, whereas steric repulsion due to space-filling obstruction by the 3-substituent prohibits binding nonspecifically in both instances. The RNase resistance of helical polynucleotide complexes is a reflection of the fact that the very positions required for RNase action are those already involved in the H-bonding system of the helical polymer and therefore unavailable for binding to the enzyme.

The Hydrolytic Reaction.—The above suggestions apply so far only to the transphosphorylase activity of RNase. However, the behavior of poly F provides insight also into substrate specificity for the hydrolytic action of the enzyme. There are reasons for believing that these two reactions are dissimilar. It is well established that the hydrolytic reaction proceeds at a much slower rate than transphosphorylation,[16] and that the enzyme shows a much lower affinity for 2′,3′ cyclic phosphates than for RNA.[16] Moreover, the steric orientation of the phosphoryl group and of its negative charge differ significantly in cyclic phosphates as compared with dinucleoside phosphates. Irrespective of the precise catalytic mechanism in either case, the phosphate acceptors in the two reactions are different, the 2′-OH being the acceptor in transphosphorylation and one of the elements of water being the acceptor in hydrolysis. Finally, transphosphorylation proceeds without a change in ionization of the phosphoryl group, whereas the contrary is true of hydrolysis. All the above facts can be taken to suggest that the states of the enzyme may differ in the two cases. Since it is known that most structural modifications of the protein impair both reactions,[17] it seems likely that the active sites for the two have many structural features in common and are largely overlapping. The differences between them would then be the result of conformational changes in a single active site, one consequence of which is to alter the immediate environment and therefore the chemical properties of the catalytically active amino acid residues. The lower affinity for substrate and the

lower velocity of hydrolysis both suggest that the "hydrolytic" conformation is a less probable one and more exacting with respect to the environmental conditions. Other alternatives, which cannot be excluded on the basis of present data, are (1) that the substrates for transphosphorylation and hydrolysis bind to a single active site in different ways, or (2) that the conformation of the substrates in the two reactions differs at the glycosyl bond. Each of these hypotheses would imply some constancy in the ratio of transphosphorylation/hydrolysis as a function of changing pH, but data that could convincingly test this possibility are not now available.

Initially, the results of Gassen and Witzel[3] suggest that a keto group α to the glycosyl bond is a necessary and sufficient requirement for hydrolysis. The properties of poly F lend support to this suggestion. Poly F is rapidly degraded by RNase and is quantitatively converted to F > p. However, the resulting F > p is totally inert with respect to RNase: it is not converted to 3′-FMP and is therefore resistant to hydrolysis; it does not act as an inhibitor for the degradation of poly C; it does not participate in the synthetic reactions catalyzed by RNase. In short, F > p does not bind to RNase. Since phosphodiesters such as FpF > p bind to RNase and act as substrates, we may conclude that the functional groups which mediate binding of dinucleotides to RNase do not suffice to promote an interaction of the homologous cyclic phosphate.

However, there are other possibilities which cannot be excluded by these studies. For example, all the compounds tested by Gassen and Witzel[3] which did not act as substrates possess an H at the position α to the glycosyl bond. It is well established from crystal structure analysis that an H-bond donor group must not be obstructed from forming an H-bond.[19] The presence of the H at positions homologous with $C^2{=}O$ of pyrimidines could produce steric repulsion with a component of the protein and thereby prevent a compound from acting as a substrate. No comparable H is present in F or in azaG. Thus, polymers with N at position 2 of the pyrimidine ring would provide a more stringent test for the requirement of the 2-keto group in hydrolysis. In our model, polymers of 4-keto-1-ribosyl-1,2,3-triazine should be subject to transphosphorylation. The susceptibility, if any, to hydrolysis of the corresponding 2′,3′ cyclic phosphate or the 2′,3′ cyclic phosphate of 4-keto-1-ribosyl-1,2-pyridazine would more firmly identify the role of the 2-keto group in hydrolysis. Likewise, if the presence at C^2 of H-bond donors, or even a single H, is sterically unacceptable to the enzyme, then poly 4-keto pyrimidine, poly iso C, and analogous structures might be resistant to transphosphorylation.

Furthermore, F (*syn*) is deficient as an analog for U or C (*anti*), since in addition to lacking a homolog of the pyrimidine 2-keto group, the glycosyl linkage (and hence parts of the sugar and phosphate group) is not in an exactly equivalent position to that of pyrimidines. This discrepancy is due to the different relative position of the glycosyl bond and H-bonding functions in F and U (or C), and also to the C-glycosyl in F. Only the former difference is relevant to azaG (*syn*) and it may therefore be significant that 8-keto G > p is a substrate for hydrolysis[3] whereas azaG > p is probably not.[11] From these considerations the substrate specificity of the hydrolytic reaction still remains uncertain, although it seems

likely that a keto group α to the glycosyl bond is indeed the important determinant. In any case, poly F and poly azaG show unambiguously that the substrate requirements for transphosphorylation and hydrolysis are different. The RNase I of plants[18] (which cleaves all the diester bonds of RNA but hydrolyzes only purine 2′,3′ cyclic phosphates) represents an analogous situation.

Inhibitors.—The stereochemical analysis outlined above can be used in an attempt to rationalize the binding of certain inhibitors of RNase. For example, the phosphomonoesters of adenosine and guanosine are effective competitive inhibitors of the enzyme,[20] whereas the corresponding 2′,3′ cyclic phosphates are not;[6] nor are poly A and poly G significantly degraded by RNase. By analogy with FMP, AMP and GMP (both in *syn*) can be visualized as H-bonding analogs, respectively, of CMP and of UMP + CMP (in *anti*). The purine 2′,3′ cyclic phosphates, lacking the equivalent of a pyrimidine 2-keto group, fail to bind to the enzyme and are therefore (like F > p) neither substrates nor inhibitors. If we assume that the purine mononucleotides may undergo an *anti-syn* conversion with relative ease,[21] these inhibitors, in the *syn* conformation, can then bind to the enzyme. In contrast, the individual residues in poly A and poly G are confined in *anti* and are thus immune to enzymatic attack.

Although these comparisons are attractive, several uncertainties remain. For example, the active site region of RNase contains functional groups which could mediate the binding of purine nucleotides in *anti*. Furthermore, if the normal interaction with pyrimidines during transphosphorylation is through three H-bonds, A and G residues in *syn* might not bind like F (*syn*) because the presence of the C^8-H in both could sterically hinder the enzyme structure which normally interacts with the pyrimidine 2-keto group. An examination of the inhibitory properties of the available purine derivatives modified at N^1, N^7, and C^8 could resolve whether or not such inhibitors bind to the enzyme in *syn* or *anti* conformation.

Predictions.—Extension of this stereochemical reasoning leads to specific predictions, the verification of which should serve as tests of our proposal for transphosphorylation and perhaps clarify aspects of hydrolysis and inhibition. Methylation of poly F or poly L at N^1 or dimethylation of the amino group of poly F should abolish susceptibility to RNase. Poly F and poly L methylated at N^6 should be degraded. Full discussion of additional predictions and a more detailed description of current work, including results obtained with other polynucleotides, will be presented elsewhere.

Materials and Methods.—The chemical synthesis of nucleotides, the preparation of enzymes and polymers, and the assay of polymer susceptibility to nucleases have been described in previous publications.[1,4] Laurusin was generously provided by Dr. R. K. Robins of the University of Utah. Radioactive (^{3}H) laurusin was prepared by deamination of (^{3}H) formycin with $NaNO_2$ at pH 3. F > p was differentiated from 2′,3′ or 5′-FMP by (1) the resistance of F > p to dephosphorylation by *E. coli* alkaline phosphatase and (2) the difference in mobility in paper electrophoresis at pH 3.5 and 7.5. F > p is differentiated from the 3′,5′ cyclic phosphate by the susceptibility of the former to RNase T_2, which converts F > p to a product that is dephosphorylated by alkaline phosphatase but not by snake venom 5′-nucleotidase.

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‡ *Abbreviations:* A, C, U, G, F, L, azaG—adenosine, cytidine, uridine, guanosine, formycin, laurusin, 8-azaguanosine. Poly—polynucleotide derivative of one of the preceding nucleosides. >p—2′,3′ cyclic phosphate. -MP—5′ monophosphate. r(F-U)—alternating copolymer of FMP and UMP. RNase—unless otherwise indicated, bovine pancreatic ribonuclease A; Up-3′UMP.

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