The involvement of the imidazole ring of His 12 in the catalytic event mediated by bovine pancreatic ribonuclease has been documented by numerous investigations (3). Photolysis of RNase-A(4) or the isolated components of RNase-S(8) (RNase-S(1-20) and RNase-S(21-124)) resulted in modification of His 12 and 119 with loss of activity (8, 9). Furthermore, alkylation by α-haloacids at pH 5.5 has been shown to result in the modification of these 2 histidine residues with concomitant loss of activity (10-14). Also, moniodination of His 12 in RNase-S(1-20) has been reported to eliminate the potential activity obtained upon recombination with RNase-S(21-124) as well as to reduce the binding constant for this association (9).

Finn and Hofmann (15) have examined the importance to activity of the histidinyl residue at position 12 with various synthetic analogues. It was initially shown that a peptide consisting of residues 1 to 13, as well as a peptide of residues 1 to 12 with a COOH-terminal amide, produced full activity when combined with RNase-S(21-124). However, a peptide of residues 1 to 11, lacking His 12, was unable to elicit activity. Furthermore, substitution of a pyrazolyl group for the imidazole ring in position 12 of a 1 to 14 peptide completely eliminated activity, although the association of RNase-S(21-124) was unaffected (16). Finn et al. (17), guided by the inactivation of RNase by iodoacetate, incorporated 3-Cm-His into the 1 to 14 sequence at position 12. This derivative, while inactive, was bound to RNase-S(21-124) at least as well as normal 1 to 14 (17-19). These studies serve to establish the critical catalytic requirement for an unperturbed imidazole ring at position 12 in ribonuclease (20).

† The abbreviations used are: RNase-A, bovine pancreatic ribonuclease A; RNase-S, ribonuclease S; RNase-S', ribonuclease S'; 2' CMP, cytidine 2' monophosphate; cyclic CMP and cCMP, cyclic cytidine 2',3'-monophosphate; cyclic UMP, cyclic uridine 2',3'-monophosphate; DCC, N,N'-dicyclohexylcarbodiimide; t-Boc, t-butyloxycarbonyl; Boc, benzoyloxy carbonyl; 4-F-His, 4-fluoro-L-histidine; DMF, dimethylformamide.

1 The native and synthetic peptide fragments are named as described previously (6, 7). The prototype is "trivial name-(x-y)," where the trivial name denotes the origin and x and y denote the NH, and COOH-terminal amino acid residues, respectively.
The kinetic studies of Herries et al. (21) for a Step 2 substrate (cyclic CMP) and del Rosario and Hammes (cyclic UMP) (22) revealed kinetically apparent ionization constants in the range 5.4 to 7.5. Mechanisms consistent with the observed ionization constants may be written utilizing the histidine residues as general acid-general base catalysts (3, 23).

The involvement of His 12 and 119 was strongly supported by the structures of the active site that have emerged from the x-ray crystallographic work of Kartha et al. (24) on RNase-A and Wyckoff et al. (25) on RNase-S. The structure of a stable dinucleotide (UpcA) complexed with RNase-S is given in Ref. 3 and discussed in detail with respect to the implications for the catalytic mechanism.

Since the involvement of His 12 in the ribonuclease mechanism is so well established and its location in the folded structure well defined, this residue provides an ideal site for investigation of the structural and functional effect of minimal modification of catalytic side chains. As an example of this approach, we have synthetically incorporated 4-fluoro-histidine (27) into position 12 of the 1 to 15 sequence of RNase-S. If, as suggested, the mechanism of reaction of hydrolytic enzymes represented by RNase-A involves general acid-general base processes, then re-duction of the pKᵢ for His 12 by this substitution (27) should elicit altered kinetic properties.

This paper details the synthesis, describes the functional purification employed to obtain purified analogue peptide, and delineates the properties of this species in terms of its interaction with RNase-S-(21-124).

**EXPERIMENTAL PROCEDURES**

RNase-A (Worthington Biochemical Corp.) in phosphate buffer was dialyzed exhaustively against distilled water and lyophilized before use. RNase-S fragments were obtained by a procedure described previously (28).

Ribonuclease activity assays were carried out spectrophotometrically against cCMP in 0.05 M Tris-HCl, pH 7.13, containing 55 mm NaCl (29). Assays for activity on UpA were performed using the procedure described by Hammes and Walsh (30). Concentrations of cCMP solutions were determined spectrophotometrically using an Eₓₐ₅₃₃ at 268 nm of 9.57 (29). Concentrations of peptide and protein solutions were based on recovery of amino acids after acid hydrolysis.

Amino acid compositions were obtained as described (28) unless otherwise noted. 4-F-His was prepared as reported earlier (27). N⁺⁺⁻-Boc-4-F-His was prepared by established procedures (28). The N⁺⁻-Boc-4-F-His was converted to the N⁺⁻-Cbz derivative by reaction with Cbz chloride in sodium bicarbonate solution.

To test the effectiveness of the coupling of N⁺⁻-Boc-4-F-His, an N⁺⁻-Boc-prolyl Merrifield resin was prepared. The aminoacyl resin was deprotected, neutralized, and washed with chloroform, methylene chloride, and DMF and then coupled with N⁺⁻-Boc-4-fluorohistidine in 50% DMP-methylene chloride overnight. After washing, a sample of the resin was hydrolyzed with a mixture of 0.56 mm of 6 N HCl and 0.56 mm of proline acid in a sealed, evacuated tube for 2 hours at 130°C (31). Based on the recovery of proline and 4-F-His on the amino acid analyzer, the coupling was 89.5% complete after one coupling step.

Chemical synthesis of [4-F-His]₁₋₁₅ synthetic-(1-15), the peptide corresponding to the NH₁-terminal pentadecapeptide sequence of RNase-A with 4-F-His at position 12, was performed utilizing the Merrifield solid phase procedure with cleavage and deprotection by anhydrous HF and piperidine-urea essentially as described previously (28). After coupling N⁺⁺⁻-Boc-4-F-His to NH⁺⁺⁻-Met-Asp-Ser-resin a sample was hydrolyzed in 0.5 ml of dioxane plus 0.5 ml of concentrated HCl for 16 hours at 110°C. Based on the recovery of aspartic acid and 4-F-His, the coupling was 49.5% complete. At this point a second coupling was performed with N⁺⁻⁻⁻-Boc-N⁺⁺⁻⁻-CBZ-4-F-His. The amino acid composition of this sample showed no improvement in the apparent coupling yield. Enzymatic activities of crude or purified synthetic-(1-15) peptide or RNase-S-(1-124) were obtained by incubation of constant amounts of RNase-S-(1-124) (2 x 10⁻⁶ m) with variable amounts of peptide in 0.1 M Tris buffer (pH 7.13) (25 to 100 μl total volume) followed by duplicate assays of 10- to 20-μl aliquots from this mixture.

Amino acid analysis of crude or purified synthetic-(1-15) was obtained by the following scheme. Twenty milligrams of crude, deblocked synthetic peptide were mixed with 40 mg of purified RNase-S-(21-124) in 3 ml of 0.1 M sodium phosphate buffer at pH 6.5. This sample was incubated at 5°C for 1 hour and then applied to a sulfoethyl-Sephadex column (2.3 X 140 cm) and eluted with 0.1 M sodium phosphate at pH 6.5. After the appearance of a peak of ultraviolet-absorbing material, the eluant was changed to 0.2 M sodium phosphate at the same pH. Elution was continued until all ultraviolet-positive material came off the column and the eluate had the same conductivity as 0.2 M phosphate. The elution profile is shown in Fig. 1 and discussed in “Results.” The fractions containing the 4-fluorohistidinyl peptide plus RNase-S-(21-124) (the semisynthetic complex) were pooled, lyophilized, and then redissolved in 8 ml of 0.05 M ammonium bicarbonate and applied to a Sephadex G-25 column (1.8 X 60 cm). The phosphate-free peak of protein was pooled and lyophilized. The yield at this point was roughly 10 mg of complex. The material was dissolved in 200 μl of 50% acetic acid-water (v/v) and applied to a column (9.5 x 50-cm) of Sephadex G-75 in 50% acetic acid. The sample was eluted with 50% acetic acid and the fractions were assayed by ninhydrin reaction after alkaline hydrolysis. The amino acid compositions of aliquots from peak tubes were obtained and the RNase-S-(21-124) and [4-F-His]₁₋₁₅ synthetic-(1-15) were separately pooled and lyophilized to remove traces of acetic acid. The yield of purified synthetic peptide based on amino acid recovery was 0.5 mg. This was taken up in 1.0 ml of deionized water, providing the stock solution which was used for the experiments described below.

Difference spectra were obtained in the Cary 15 spectrophotometer using split cell cuvettes. Solutions of RNase-S-(21-124) and...
either RNase-S-(1-20) or [4-F-His 12]synthetic-(1-15) in 0.05 M Tris buffer, pH 7.11, were pipetted into separate sides of the blank and reference cuvettes. The base-line was recorded from 340 to 250 nm. The sample cuvette was then mixed by inversion and the difference spectrum was recorded. Additions of peptide to the sample cuvette and to the peptide side of the reference cuvette were then made. An addition of buffer equal to 1/4 volume of the peptide solution added to the sample cell was added to the protein side of the reference cuvette. The sample cuvette was then re-mixed and the spectrum was recorded. Additions were continued until no further change in absorbance was observed.

Thermal melting curves were obtained using stopped cuvettes in a Gilford 240 spectrophotometer equipped with thermospacers and a Lauda constant temperature water bath. All peptide and protein samples were first passed through a 0.22-μm Millipore filter to remove nondissolved material.

Subtilopeptidase digestion at 5°C was accomplished by incubating 250-μl samples of RNase-S-(21-124), RNase-S, and semisynthetic complex at a concentration of 3 × 10⁻⁴ M in the cold room. Four micrograms of subtilopeptidase A (Sigma) was added to each incubation and RNase activity was determined as a function of time.

**RESULTS**

After deblocking of the crude synthetic 4-F-His peptide and fractionation on Sephadex G-25, no activity could be detected when mixed in 0.1 M Tris buffer, pH 7.13, with RNase-S-(21-124) in a synthetic peptide to native protein ratio of 20:1. By inhibition of RNase-S activity, the ratio of crude [4-F-His 12]synthetic-(1-15) necessary to yield 50% inhibition was found to be 17.

Since the inhibition of RNase S activity by [4-F-His 12]synthetic-(1-15) implies binding of the synthetic fragment to RNase-S-(21-124), we used this capacity to purify the synthetic material. The synthetic analogue peptide was incubated with native RNase-S-(21-124) as described under "Experimental Procedures." As illustrated by Fig. 1, ion exchange chromatography of this mixture is sufficient to resolve the resulting semisynthetic complex (Peak 2) containing 4-F-His at position 12 from both excess synthetic peptide with no affinity for RNase-S-(21-124) (Peak 1) and the excess RNase-S-(21-124) (Peak 3). Identification of the peaks was established in the following way. Peak 1 had an ultraviolet spectrum with only a shoulder in the 270 to 280 nm region and strong absorbance at shorter wavelengths, while Peaks 2 and 3 exhibited typical protein spectra with well defined maxima at 277 to 278 nm. As shown in the inset of Fig. 1, addition of native RNase-S-(1-20) to aliquots from tubes of Peaks 2 and 3 regenerates RNase-S as measured by hydrolysis of cCMP. The greater ratio of RNase-S-(1-20) to RNase-S-(21-124) needed to elicit full activity in aliquots from Peak 2 (lower curve of inset) is due to the inhibition by the presence of the [4-F-His 12]synthetic-(1-15). The identification of the components in Fig. 1 are further confirmed by amino acid compositions obtained from aliquots of Peaks 1, 2, and 3. The ratio of tyrosine to phenylalanine is 3 for Peak 3 and 2 for Peak 2, consistent with the assignment of these peaks with RNase-S-(21-124) and semisynthetic complex, respectively. The isolated complex was also devoid of activity at this point.

The semisynthetic complex in Peak 2, after desalting, was separated into RNase-S-(21-124) and synthetic peptide on a Sephadex G-75 column. Fig. 2 illustrates the separation obtained. Peak B contains the synthetic peptide as determined by amino acid analysis. Peak A of Fig. 2 is RNase-S-(21-124) and Peak C is ammonia arising from the buffer used in the previous column. Table I gives the composition of Peak B obtained after acid hydrolysis.

Up to a 10-fold excess of the isolated synthetic peptide was mixed with 2 × 10⁻⁴ moles of RNase-S-(21-124) and then aliquots were assayed for activity against either cCMP or UpA. In both cases the measured activity was no greater than background levels (RNase-S-(21-124) alone).

The inhibitory activity of the synthetic peptide was exploited to measure the binding constant to RNase-S-(21-124) as suggested by Kenkare and Richards (9). Fig. 3 gives the activity

![FIG. 2. Absorbance at 570 nm obtained from alkaline hydrolysis followed by ninhydrin reaction of 20-μl aliquots from fractions of G-75 chromatography of purified semisynthetic complex.](http://www.jbc.org/)

![TABLE I](http://www.jbc.org/)

| Amino acid analysis | Crude peptide | Purified peptide | Theory |
|--------------------|---------------|-----------------|--------|
| Lysine             | 2.41          | 1.91            | 2      |
| Arginine           | 0.55          | 1.00            | 1      |
| Aspartic Acid      | 1.12          | 1.12            | 1      |
| Threonine          | 1.09          | 1.11            | 1      |
| Serine             | 0.97          | 1.01            | 1      |
| Glutamic Acid      | 2.53          | 3.02            | 3      |
| Alanine            | 3.35          | 2.93            | 3      |
| 4-fluoro-His       | 0.39          | 0.81            | 1      |
| Methionine         | -             | 1.00            | 1      |
| Phenylalanine      | 0.94          | 1.00            | 1      |

*G-75 Fraction B

![FIG. 3. RNase-S activity in cCMP hydrolysis measured as initial rate of increase in absorbance at 287 nm as a function of the ratio (α) of added RNase-S-(1-20) to a constant amount (5 × 10⁻⁴ moles) of RNase-S-(21-124). The points represent RNase-S-(21-124) ( ), RNase-S-(21-124) plus 1.29 eq of [4-F-His 12]synthetic-(1-15) ( □), and RNase-S-(21-124) plus 5.16 eq of [4-F-His 12]synthetic-(1-15) ( ●).](http://www.jbc.org/)

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(pH 7.13 Tris buffer) in cCMP hydrolysis titration curves for addition of increasing amounts of RNase-S-(1-20) to RNase-S-(21-124) (constant at $2 \times 10^{-3}$ μmoles) both alone (upper curve) and in the presence of two concentrations of [4-F-His 12]synthetic-(1-15). Equation 1 gives the appropriate expression for the calculation of $K_a$, the equilibrium constant for binding of RNase-S-(1-20) to RNase-S-(21-124), where $r_a$ is the [RNase-S-(1-20)];[RNase-S-(21-124)] ratio, $P_T$ is total RNase-S-(21-124) concentration, and $a$ is the ratio of activity at a given $r_a$ to activity at saturation. Equation 2, derived from Equation 1 in Ref. 9, allows calculation of $K_i$, the inhibition constant for binding of synthetic inhibitory peptide to RNase-S-(21-124), with $r_i$ the ratio of synthetic peptide to RNase-S-(21-124). Table II gives the results of these calculations. It may be seen that $K_i$ for the inhibitory peptide is within a factor of three of $K_a$ for native RNase-S-(1-20).

Table II

| Inhibitor/ S-protein | S-peptide/ S-protein | $K_a$       | $K_i$       |
|----------------------|----------------------|-------------|-------------|
| 0                    | 0.30 - 3.03          | 5.5 $\times 10^7$ | 2.27 $\times 10^7$ ± 0.8 $\times 10^7$ |
| 1.29                 | 0.30 - 3.78          | 2.27 $\times 10^7$ ± 0.8 $\times 10^7$ | 2.13 $\times 10^7$ ± 0.1 $\times 10^7$ |
| 5.16                 | 0.38 - 3.78          | 2.13 $\times 10^7$ ± 0.1 $\times 10^7$ |             |

Fig. 4 shows ultraviolet difference spectra for addition of RNase-S-(1-20) and the synthetic peptide to RNase-S-(21-124).

![Fig. 4. Ultraviolet difference spectra obtained on mixing either RNase-S-(1-20) (upper set of spectra) or [4-F-His 12]synthetic-(1-15) (lower set of spectra) to RNase-S-(21-124). The peptide to protein ratio is indicated for each curve. The amounts of RNase-S-(21-124) in the upper set of spectra is roughly twice that in the lower set of spectra.](http://www.jbc.org/)

Absorbance maxima at 280 and 287 nm are present in both cases, with isosbestic points at 275 and 293 nm. In Fig. 5 a plot of the corrected absorbance difference versus RNase-S-(1-20) concentration is given.

The values of $T_m$, the temperature corresponding to the midpoint of the thermal transition curve measured by ultraviolet spectral change at 287 nm, were obtained for the semisynthetic complex and related native species. These data are given in Table III.

In Fig. 6, plots are presented for the natural logarithm of the per cent of initial potential RNase-S activity remaining for analogue and RNase-S complexes versus the hours after addition of subtilopeptidase A. As indicated, the half-life of the decay of S-protein increases upon adding [4-F-His 12]synthetic-(1-15) to form the semisynthetic complex. Furthermore, addition of 2'-CMP to this complex provides a further resistance to proteolysis. The uppermost line refers to the degradation of RNase-S.

**DISCUSSION**

Solid phase peptide synthesis, since its introduction by Merrifield (32, 33), has proven extremely valuable in probing questions of the function of amino acid residues of natural polypeptides. One major drawback to this synthetic tool, however, is the heterogeneity of the products formed due to truncation and deletion errors (34), or to side reactions during synthesis and deblocking steps. Several procedures have been developed for achieving purification of biologically active peptides derived from solid phase synthesis. Several of these techniques are functionally based, depending for their success on the specific binding of the
serine at position 12 gave a peptide with a 7-fold weaker binding 
tute for histidine at this locus. However, the substitution of 
association to RNase-S-(1-20), fl-pyrazolylalanine could substi-
residues at several positions (see Ref. 20 for a complete discus-
sion). For example, it was demonstrated that any large NH*-
terminal truncation of RNase-S-(1-20) leads to weaker binding
alteration in the nature of the side chain at position 12 is tolerated
study however is the finding of Hofmann et al. (19) that some
Hofmann and Scoffone and their associates has defined the
properties of the complex. In the case of RNase-S, the work of
proteins of correct amino acid sequence to complementary 
templates (35). In the case of the RNase-S-(1-20) peptide, the
these which form stable noncovalent complexes under this set of conditions
(5°, 0.1 to 0.2 m phosphate buffer, pH 6.5). Since the extent of
binding of RNase-S-(1-20) to RNase-S-(21-124) decreases as
the temperature is increased (36), and since phosphate ion also
in stabilization of the complex, variation in temperature and
phosphate concentration allow some flexibility in this separa-
Thus one might achieve an even more stringent selection
by changing these conditions to disrupt the weaker complexes.
In other experiments it has been observed that the position
of elution of the complex in the ion exchange chromatography
depends to some extent on the sequence of the synthetic peptide. In
The complex containing [4-F-His 12]synthetic-(1-15) elutes in
the same position as a complex containing synthetic-(1-15) of
normal sequence.

After removal of phosphate by gel filtration, the complex was
disrupted in 50% acetic acid and the fragments were isolated by
Sephadex G-75 molecular sieving (Fig. 2). The amino acid
composition of the recovered synthetic fragment is given in
Table 1 and serves to identify this species as [4-F-His 12]syn-
thetic-(1-15). The presence of fluorine in the imidazole ring gives
4-F-His an elution position on the amino acid analyzer very
different from that of histidine, such that 4-F-His appears on the
long column in about the same position as valine. Since fluorine
reduces the pK of the imidazole ring from 6 to 2.5, the marked
change in behavior is not surprising.

Despite this large change in the pK of the side chain at position
12, this peptide is still capable of binding quite firmly to RNase-
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polypeptides of correct amino acid sequence to complementary 
templates (35). In the case of the RNase-S-(1-20) peptide, the
template suitable for achieving this binding is the naturally
derived RNase-S-(21-124) fragment. Selective association
provides a basis for removing from the mixture of synthetic
peptides those which form stable noncovalent adducts. By
virtue of its much higher molecular weight, different ionic charac-
ter and shape, the complex is readily separable from smaller
fragments in the mixture. Peptides which form weak complexes
with the protein template will be discriminated against if the
separation procedure is selective for more stable complexes.

Any change in the structure of the amino acid added at a given
position in the peptide may disturb the association of the peptide
with the template so that the selective purification described
above will not be possible. Indeed, for the method to be suc-
successful, truncations or deletions in the native peptide sequence
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tute for histidine at this locus. However, the substitution of
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the aromatic character of the imidazole, would be expected to
yield a derivative with binding properties similar to those of the
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In fact, the inhibition of activity observed when crude [4-F-
H|s 12]synthetic-(1-15) is added to RNase-S' indicates that some
component of the synthetic mixture is binding to RNase-S-
(21-124) and preventing productive binding of native RNase-S-
(1-20).
The synthetic material that is capable of binding to RNase-
S-(21-124) was allowed to form the noncovalent complex and
then purified by ion exchange chromatography on sulfoethyl-
Sephadex. The improvements in the isolation procedure are
reported under “Experimental Procedures” and “Results.”
In this separation we have selected out those peptides which
form stable noncovalent complexes under this set of conditions
(5°, 0.1 to 0.2 m phosphate buffer, pH 6.5). Since the extent of
binding of RNase-S-(1-20) to RNase-S-(21-124) decreases as
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Changes in the active site geometry of a nature sufficient to
 disrupt catalytic function cannot be defined in detail by any
technique short of X-ray diffraction. On the other hand, the
strong binding of [4-F-His 12]synthetic peptide to RNase-S-

*B. M. Dunn and I. M. Chaiken, unpublished results.
XRXY program of Feldmann et al. (37) on an ADAGE graphics unit interfaced to a PDP-10 computer. The circles centered on UpcAmRNaseS complex from Wyckoff et al. (25), within the In, i). This picture was generated from the coordinates of the His 12 with a fluorine atom substituted in position 4 (solid ball size.

Asn 44, Thr 45, and Phe 120. The uridine 3'.phosphate portion of the inhibitor UpcA is labeled L. A, space-filling representation with atoms keyed by type. B, stereo pair of view A with reduced ball size.

The difference spectra produced on mixing either RNase-S-(1-20) or [4-F-His 12]synthetic-(1-15) with RNase-S-(21-124) are virtually identical. Lack of large amounts of purified synthetic peptide prevented titration to saturation in that case. The spectral change produced by a given amount of added peptide is, on a molecular basis, virtually the same in both cases. This is in agreement with the similarity in binding constant obtained from inhibition measurements. It is worthwhile noting that this good binding stands in contrast to the result obtained by Kenkare and Richards for RNase-S-(1-20) iodinated at His 12 (9). This enzymatically inactive derivative, presumably iodinated at either the 2 or 4 position (39), was bound to RNase-S-(21-124) 3200 times less strongly than unmodified RNase-S-(1-20). The difference between the binding of the iodohistidine and fluorohistidine derivatives could arise from the different properties of the two substituted imidazole rings—from the different positions of substitution (4-fluoro versus 2-iodo, if this is the predominant product), or from alterations in other amino acid side chains in the iodination procedure. Because of the uncertainty of the position of iodination in the imidazole ring, the lack of activity in the latter case is more difficult to attribute to alteration in the functional character of the histidine.

An attempt was made to use the difference spectra obtained to define the binding constants of [4-F-His 12]synthetic-(1-15) and RNase-S-(1-20) to RNase-S-(21-124). Under the conditions employed in this study, the ultraviolet absorbance difference spectrum increases linearly with each addition of RNase-S-(1-20) to a constant amount of RNase-S-(21-124) up to roughly 1.1 eq. After this point further addition of peptide produces no further spectral changes. This is illustrated in Fig. 5 where the solid line through the points makes an abrupt shift at high RNase-S-(1-20) concentration. These spectral data clearly indicate that the binding is too tight to allow calculation of an association constant.

Changes in substrate binding would explain a decrease in activity, but, as illustrated in Fig. 6, the competitive inhibitor 2'CMP stabilizes the semisynthetic complex against digestion by subtilopeptidase while it has no stabilizing effect on RNase S-(21-124) alone. This result indicates binding of the substrate-like molecule to the “active site region” of the complex; such binding is known to stabilize the RNase conformation (38). The orientation of the substrate or inhibitor when bound to the complex might be altered such that the binding is nonproductive, but, due to the minimal modification of the active site by the fluorine substitution, this is considered unlikely as well.

Finally, we must consider the altered properties of the catalytic system. As mentioned above, the fluorine atom alters the acidic character of the imidazole ring, reducing the pKₐ to 2.5. Since the evidence implicates a basic group with a pKₐ of 5.4 (or 6.4) in the RNase reaction mechanism and since the x-ray structure places His 12 in a very favorable location to act as a proton acceptor, the most reasonable mechanism for ribonuclease action involves the histidine acting as a general base. The alteration of the pKₐ of this histidine makes the derivative a much weaker base. Thus the lack of activity of the [4-F-His 12]synthetic-(1-15) complex with native RNase-S-(21-124) finds its most acceptable explanation in an alteration in the effectiveness of the catalytic function.

The difference spectra obtained by Finn (40), when compared to data obtained from activity assays, lead to the conclusion that the binding constants are roughly two orders of magnitude lower, or about 5 x 10⁶ M⁻¹ in the absence of substrate than in its presence. In deed, earlier studies by Finn and Hofmann (15) on the properties of synthetic analogues of RNase give data showing that the association of synthetic-RNase-S-(1-13) to RNase-S-(21-124) increases as the substrate is varied from cUMP to cCMP and is
nearly stoichiometric when RNA is the substrate. The experiments in Fig. 6 would also lend support to this concept, since the effect of adding the inhibitor to the semisynthetic complex is undoubtedly to shift the association equilibrium toward complex. In spite of the consistency of these arguments, it is clear from our data (obtained at pH 7.11) that the concentrations of RNase-S (21-124) needed to observe difference spectra, generally about $2 \times 10^{-5} \text{ M}$, are large enough with respect to the proposed association constant of the complex even in the absence of substrate (about $5 \times 10^{-5} \text{ M}$), to lead to stoichiometric binding. Under these conditions the extrapolation procedure used previously (41) is not valid and one cannot obtain a binding constant for direct, protein associated is increased to 5 x 10^{-7} \text{ M}.

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