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CATALYTIC ACTIVITY OF N^{τ}-CARBOXYMETHYLHISTIDINE-12 RIBONUCLEASE: pH DEPENDENCE

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Summary

The pH-dependence of RNAase A and of N^{τ}-carboxymethylhistidine-12-RNAase (ribonucleate 3′-pyrimidino-oligonucleotidohydrolase) catalysis was studied. Apparent acid dissociation constants were obtained by least squares analysis of the kinetics data. These dissociation constants were compared with pK_a values of model imidazole compounds, and with pK_a values of histidine residues 12 and 119 on the protein.

The shapes of the k_{cat} versus pH profiles for RNAase A and its carboxymethyl derivative are very similar, from which it is concluded that the mechanism of catalysis is closely similar in the two proteins. Apparent pK_a values obtained from the kinetic data are higher for the carboxymethylated protein than for RNAase A, as are the pK_a values of residues 12 and 119. The similar shifts are consistent with the conclusions that both these residues are functionally significant in native and modified enzyme, and that an unblocked τ-nitrogen on histidine-12 is not essential for activity.

From the enzyme's catalytic dependence on pH, and the NMR determined pK_a values we propose that histidine 12 and 119 function catalytically in their basic and acidic forms respectively.

On the basis of extensive experimentation in many laboratories, the τ-nitrogen of histidine-12 has been assigned an acid/base function in the catalytic mechanism of bovine pancreatic RNAase A [1]. In the X-ray crystallographic

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The abbreviations used are: 12-CMRNAase, N^{τ}-carboxymethylhistidine-12 RNAase; CMhistidine, N^{τ}-carboxymethylhistidine. The nomenclature of carboxymethylhistidine is that recommended in the IUPAC-IUB CBN recommendations [28]. In this nomenclature the imidazole nitrogen nearest the side chain methylene is designated π, the other τ. 2′,3′-cyclic UMP, uridine 2′ : 3′-cyclic monophosphate; 2′,3′-cyclic CMP, cytidine 2′ : 3′-cyclic monophosphate; Tris, tris(hydroxymethyl)-aminomethane.

structure the position of the imidazole side chain is fixed, with the τ-nitrogen facing into the active site, by a hydrogen bond between threonine-45 and the second, π nitrogen of the ring [2]. This structure leads to the prediction that a covalent substitution on the τ-nitrogen should abolish enzymatic activity. In apparent contradiction, N^{τ}-carboxymethylhistidine-12-RNAase (12-CMRNAase has significant, albeit reduced activity against both RNA and 2′,3′-cyclic UMP [3,4]. This observation suggests at first that histidine-12 has no catalytic function; but the residue's position at the active site, and its enhanced reactivity make this conclusion intuitively unacceptable. At least two additional explanations for the activity of 12-CMRNAase can be formulated. First, histidine-12 may serve as a catalytically important acid/base in the native, but not in the modified enzyme; i.e. carboxymethylation alters the reaction mechanism, and residual activity is due to a less efficient reaction pathway. Second, histidine-12 may have an important catalytic function in both native and modified enzymes, but one which does not depend on the τ-nitrogen. For example, the π- rather than τ-nitrogen may be the functional position, or one or the other may serve as the acid/base locus under the proper conditions; or histidine-12 might not function as an acid/base, but rather as a center of positive charge.

In both these postulates a catalytic function is assigned to histidine-12 in RNAase A. In order to differentiate between them, and thereby to narrow the number of possible functional assignments for histidine-12, we have compared the activities of RNAase A and 12-CMRNAase over a wide range of pH. It has been believed generally that the k_{cat} vs. pH profile of RNAase A is bell-shaped [6—8], leading to the acceptance of Scheme I (presented in the Discussion). However, del Rossario and Hammes [8] pointed out a possible deviation from the bell shape at low pH with 2′,3′-cyclic CMP as substrate, and recently Rübsamen et al. [9] presented data obtained with 2′,3′-cyclic UMP, and 3′,5′-UpA which support this observation. The results we shall present indicate deviations both at low pH, and on the basic leg of the pH profile.

From an analysis of the kinetic results we can assign pK_a values to one acid-functional and one base-functional group on both RNAase A and the carboxymethylated derivative. These kinetically derived dissociation constants are compared with pK_a values obtained directly by proton NMR. While Meadows et al. [10] have already noted changes in the C-2 proton resonances of two imidazoles on the modified enzyme, they did not assign these resonances to individual residues. This assignment is reported here. Finally, we present a comparison between the NMR titration of the 12-carboxymethyl histidine residue on the protein and the potentiometric titrations of small histidine derivatives.

Materials and Methods

The synthesis and purification of 12-CMRNAase, the RNAase S protein and the substrate 2′,3′-cyclic UMP has been described previously [4]. N^{τ}-carboxymethylhistidine was synthesized and isolated by the procedure of Crestfield et al. [3]. Histidine was acetylated using a modification of the procedure of Kolb and Toennies [11]. Carboxymethylimidazole was synthesized by the method of Birkofer et al. [12].

Enzyme-catalyzed hydrolysis of 2′,3′-cyclic UMP was monitored at 286 nm

[8] using a Cary 16 spectrophotometer with a cell holder thermostatically maintained at 25.0 ± 0.1°C. Significant non-enzymatic hydrolysis was not observed under our experimental conditions. The pH range of 3.5—10.5 was covered using the following series of solutions, (0.1 M in buffer and 0.1 M in NaCl): 3.5—4.0, citrate; 4.0—6.0, acetate; 5.0—9.0, Tris/cacodylate; 8.75—10.5, glycinate. When passing from one buffer to the next, activity was measured at one or more identical pH using both buffer solutions. No significant buffer effects were seen. The difference between the extinction coefficients of product and substrate at 286 nm was determined from the total absorbance change obtained upon addition of RNAase A to known amounts of substrate. $\Delta\epsilon_{286}$ is pH-dependent, the curve resembling a titration (Fig. 1). However, a best fit calculated on the assumption of a single ionizing species does not adequately represent the observed results. No attempt was made to determine a more suitable model. All reaction velocities were calculated with the experimental values of $\Delta\epsilon_{286}$ given in Table I. The concentrations of RNAase A and 12-CMRNAase were determined from solution absorbances at 278 nm, using the extinction coefficient of 9800 $M^{-1} \cdot cm^{-1}$ [13].

Initial reaction rates were determined from the initial, linear portion of the absorbance trace. Apparent k_{cat} and K_m values were calculated at each pH using the gradient expansion algorithm [14] to fit the Michaelis-Menten equation. These parameters were used only for comparison purposes. The initial velocity of an enzyme catalyzed reaction can with the proper assumptions be expressed explicitly as a function of both substrate and H^+ concentrations [15]. The various acid equilibrium and reaction rate constants which occur in this explicit formulation can then be determined by fitting all the individual data points to the derived equation. The experimental data triplets v_i, (H^+), and (S) were fit to

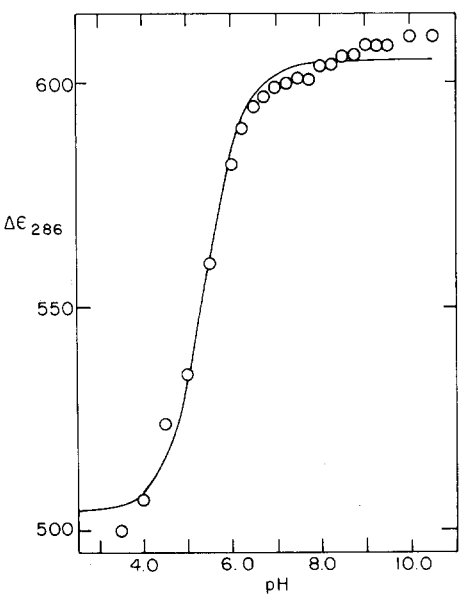


Fig. 1. $\Delta\epsilon_{286}$ for the hydrolysis of 2',3'-cyclic UMP as a function of pH. The reaction conditions are: 25°C, pH maintained by the buffer mixtures described in the methods section. The solid line represents the best fit curve assuming two species in equilibrium with one another in a single proton dissociation.

TABLE I

2',3'-CYCLIC UMP HYDROLYSIS

$\Delta\epsilon_{286}$ as a function of pH. The calculated values were obtained with the polynomial:

$$\Delta\epsilon = \sum_{i=0}^{5} a_i x^i$$

The coefficients a_i, which have no theoretical significance, were obtained by a linear least squares regressio fit to the data and are: $a_0 = 2196.6$; $a_1 = -1442.2$; $a_2 = 443.68$; $a_3 = -64.360$; $a_4 = 4.4556$; $a_5 = -0.11927$.

pH	$\Delta\epsilon_{obs}$ ($M^{-1} \cdot cm^{-1}$)	$\Delta\epsilon_{calc}$ ($M^{-1} \cdot cm^{-1}$)	pH	$\Delta\epsilon_{obs}$ ($M^{-1} \cdot cm^{-1}$)	$\Delta\epsilon_{calc}$ ($M^{-1} \cdot cm^{-1}$)
3.50	500	500	7.00	599	599
3.75	—	501	7.25	600	601
4.00	507	506	7.50	601	602
4.25	—	514	7.75	601	603
4.50	524	523	8.00	604	604
4.75	—	534	8.25	604	604
5.00	535	545	8.50	606	605
5.25	—	555	8.75	606	606
5.50	560	565	9.00	608	606
5.75	—	574	9.25	608	608
6.00	582	581	9.50	608	609
6.25	590	587	10.00	610	611
6.50	595	592	10.25	—	611
6.75	597	596	10.50	610	610

a number of different equations using either a grid-searching technique, or the method of steepest descent [14]. To ensure that the search routines did not settle into false minima, the initial parameter estimates were varied widely.

The titrations of imidazole, histidine, and their derivatives were performed potentiometrically at 25°C, in 0.1 M NaCl using a Corning model 12 pH meter, and a Thomas No. 4094-L15 combination electrode; the solution was maintained under a nitrogen atmosphere to minimize CO_2 contamination. The experimental data was fit by linear regression [14] to the equation:

$$y = \sum_{i=0}^{n} a_i x^i \tag{1a}$$

where x is the pH, and y equivalents of base or acid added. The value of n generally was not allowed to exceed 5. The pK_a was determined by a Newton-Raphson solution for the real root of the derivative equation:

$$y' = 0 = \sum_{i=0}^{n} i a_i x^{i-1} \tag{1b}$$

This is mathematically equivalent to setting the pK_a as the pH at which the titration inflection occurs.

Titration curves for the histidines of the protein were determined from the pH dependences of the proton NMR shifts [10.16]. Spectra were obtained with a Bruker B-90-C using 1000 pulses in the Fourier transform mode. The solutions contained 10% protein in ^2H_2O with the p^{2}H adjustments made with ^{2}HCl or NaO^{2}H. The temperature of the sample was approximately 27°C. Chemical shifts were measured relative to HO^{2}H. The acid dissociation constants for the

individual histidine residues were determined from the shift versus pH data using the same polynomial fitting technique just described.

Data analyses were performed either on a Nova 1220 minicomputer, or for the bigger programs on an IBM 370. The various fitting programs employed were written in this laboratory, but were in general based upon the subroutines described by Bevington [14].

Bovine pancreatic RNAase was purchased from Worthington Biochemical Corporation (Freehold, N.J.) as a phosphate free powder, and used with no further purification. Subtilopeptidase A was obtained from Sigma Chemical Corporation, and all other chemicals were purchased from commercial sources.

Results

The hydrolysis of 2′,3′-cyclic UMP catalyzed by both RNAase A and the carboxymethylated derivative, 12-CMRNAase was measured at 25°C varying both the substrate concentration, and the pH. The Michaelis-Menten parameters, k_{cat} and K_m, were calculated from approximately 15 (v_i,S_0) pairs at each pH (Table II). The pH vs. rate profiles of both enzymes are not bell-shaped as seen from

TABLE II

MICHAELIS-MENTEN PARAMETERS FOR THE HYDROLYSIS OF 2′,3′-CYCLIC UMP

pH	Buffer	RNAase A		12-CMRNAase	
		k_{cat} (min^{-1})	K_M (mM)	k_{cat} (min^{-1})	K_M (mM)
3.50	Citrate	0.105	2.89		
4.00	Citrate	0.147	2.25		
4.00	Acetate	0.157	2.40	0.00175	1.99
4.50	Acetate	0.265	1.54	0.00259	1.59
5.00	Acetate	0.442	1.07		
5.00	Tris/cacodylate	0.480	1.11	0.00537	1.19
5.50	Tris/cacodylate	1.55	0.759	0.0207	1.19
5.50	Acetate	1.68	0.736		
6.00	Acetate	2.24	1.36		
6.00	Tris/cacodylate			0.0510	1.35
6.50	Tris/cacodylate	3.91	3.76	0.154	2.70
6.75	Tris/cacodylate	4.19	4.54	0.177	3.55
7.00	Tris/cacodylate	3.54	4.98	0.193	8.78
7.25	Tris/cacodylate	3.42	10.1	0.204	4.78
7.50	Tris/cacodylate	2.20	13.2	0.126	13.5
7.75	Tris/cacodylate	1.74	14.4	0.148	12.4
8.00	Tris/cacodylate	1.30	16.4	0.0927	12.7
8.25	Tris/cacodylate	0.748	16.6	0.0748	17.0
8.50	Tris/cacodylate	0.396	17.2	0.0528	17.6
8.75	Tris/cacodylate			0.0326	17.9
8.75	Glycinate	0.237	16.9		
9.00	Glycinate	0.117	17.1	0.0134	17.4
9.00	Tris/cacodylate	0.103	19.0		
9.25	Glycinate	0.0467	17.7	0.00763	18.1
9.50	Glycinate	0.0156	18.0	0.00312	17.5
9.75	Glycinate	0.0124	18.4	0.00201	17.6
10.0	Glycinate	0.00559	18.2	0.000927	17.5
10.50	Glycinate	0.0014	17.8		

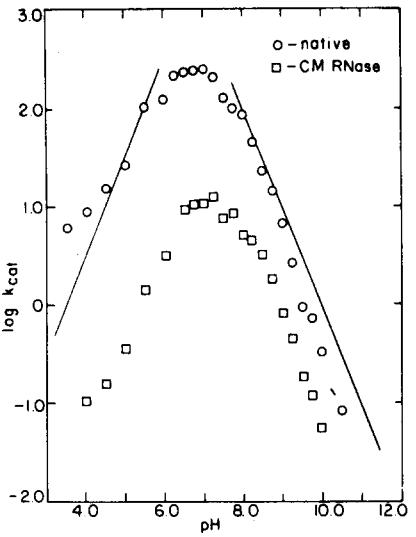


Fig. 2. pH-rate profiles for the RNAase A and 12-CMRNAase catalyzed hydrolysis of 2′,3′-cUMP. Reaction conditions are those described under Methods and Materials. The straight solid lines have no significance other than to indicate slopes of +1 and −1.

the plot of log k_{cat} versus pH (Fig. 2). In the acidic leg the points deviate upward from a line of slope 1, the theoretical slope for the bell shape. Near pH 8 there is an apparently small, but nonetheless significant inflection. These deviations introduce difficulties for quantitative interpretations of the data as will be discussed below. They are, however, important for a qualitative discussion since their presence emphasizes the striking similarity of the two curves in Fig. 2. Although the profile obtained with 12-CMRNAase is lower in magnitude, and is shifted by approximately 0.5 pH units towards basic pH, its shape is identical to that obtained with RNAase A, except for a slight difference at low pH. On the basis of this close similarity we conclude that reactions catalyzed by RNAase A and 12-CMRNAase proceed along the same pathways.

The basic shift of the 12-CMRNAase rate profile relative to RNAase arises from a pK_a shift in one or more of the groups responsible for the shape of the curve. Since histidine-12 is the site of the chemical modification, we investigated the acid dissociations of the small, model compounds *N*-carboxymethylimidazole, and N^{τ}-carboxymethylhistidine. The measured acid dissociation constants are presented in Table III together with results reported by Hapner [17]. The substitution of either a methyl or carboxymethyl group onto the τ-nitrogen of histidine decreases the pK_a of the imidazole side chain by 0.2—0.4 units. The apparent dissociation constant of imidazole is 0.3 units lower than its micro-pK_a, since there are two identical sites capable of releasing a proton. When this statistical factor of 2 is taken into account, we see that *N*-carboxymethylimidazole is also a stronger acid than its parent compound. The pK_a shifts observed in these model compounds is thus opposite to that suggested by kinetic data. To determine whether pK_a of the proteins's carboxymethylated histidine-12 shifts when it is carboxymethylated in the basic (kinetic results) or acid (model compound results) direction, we undertook the proton NMR determi-

TABLE III

pK_a OF IMIDAZOLE, HISTIDINE, AND VARIOUS DERIVATIVES

Compound	pK_a (this report) *	pK_a (lit) **
Imidazole	7.24	
N-carboxymethylimidazole	7.24	
L-histidine	6.24	6.13
N^{τ}-carboxymethyl-L-histidine	5.87	5.74
N^{π}-carboxymethyl-L-histidine		6.33
N^{τ}-methyl-L-histidine		5.97
N-acetyl-L-histidine	7.35	
N-acetyl-N^{τ}-carboxymethyl-L-histidine	7.19	

* All data were collected in 0.1 M NaCl, 25° C. The concentrations of the compounds were in the range 8—15 mM.

** Results obtained from ref. 17.

nation of the histidine pK_a values for 12-CMRNAase.

Three of the resonance peaks previously assigned to the C-2 protons of the imidazole rings [10,16] were located in the spectra. The chemical shifts are pH-dependent, and the shape of the shift versus pH curves indicates single proton ionization in each case (Fig. 3). That the curve assigned to peak 3 crosses the curve of peak 2 at low pH, and of peak 1 at high pH, was determined from the rates of the C-2 proton exchanges in the pH regions about the crossovers. The C-2 proton resonance peaks disappear slowly as deuterium from the solution replaces the proton. At pH 6.0 the relative rates of exchange are ordered peak 1 > peak 2 ≫ peak 3. We assumed the same relative rates occurred at pH 5.0 and 7.5, and

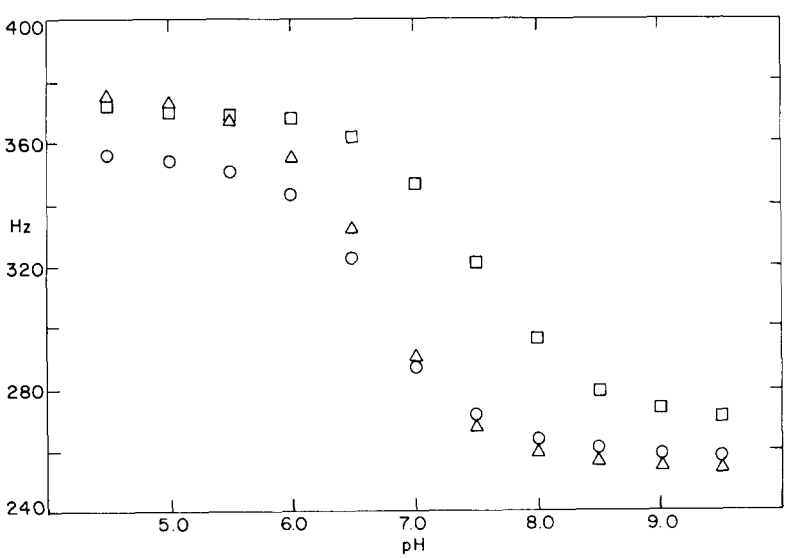


Fig. 3. Chemical shifts as a function of pH for the observable C-2 protons of the imidazoles in 12-CMRNAase. Experimental conditions are described in the text. ○, peak No. 1; □, peak No. 2; △, peak No. 3.

TABLE IV

CHEMICAL SHIFTS OF THE C-2 PROTONS OF 12-CMhisRNAase AND 12-CMhisRNAase S AT p^2H 6.50

Protein	Activity (units/mg) *	Chemical shifts (Hz)		
		peak 1	peak 2	peak 3
12-CMRNAase	118	322	362	332
12-CMRNAase S **	114	291	331	301
Exchanged 12-CMRNAse S ***	116	291	—	301

All chemical shifts are reported relative to HO^2H at 27°C.

* Activity determined with *Torula* RNA as substrate [19].

** Prepared as an equimolar mixture of 12-CMhis S peptide, and S protein in ^2H_2O at $p^2H = 6.50$.

*** Prepared as an equimolar mixture of 12-CMhis S peptide previously exchanged in ^2H_2O at 30°C, pH 8.5, for 4 days, and S protein in ^2H_2O at $p^2H = 6.50$.

made assignments accordingly. Were this assumption incorrect, then the errors introduced into the calculated pK_a values would be small due to the nature of the data, as can be seen in Fig. 3. The calculated acid dissociation constants at 27°C, not corrected for the effect of ^2H_2O on the electrodes or the imidazolium ionization *, are: peak 1, 6.75; peak 2, 7.54; peak 3, 6.73. We now turn to the residue assignments.

In RNAase A solutions containing no acetate the C-2 proton resonance of histidine-48 is not observed [18]. Since acetate was absent from our samples, we assume that the three peaks are associated with the three remaining imidazole side chains of histidines 105 and 119, and CMhistidine-12. The spectra of 12-CMRNAase contain an additional pH dependent resonance with a shift falling in the range 190—240 Hz. A very similar peak in the spectrum of RNAase A has been assigned to the C-4 proton of histidine-105 [10]. This peak obtained with 12-CMRNAase displays a pK_a of 6.78, similar to the pK_a of the corresponding RNAase A resonance, 6.73 at 27°C (estimated from the data of Roberts et al. [18]). We conclude that this peak in the 12-CMRNAase spectrum is that of the C-4 proton in histidine-105. The pK_a values associated with peaks 1 and 3 are 6.75 and 6.73. Thus, either one of these peaks most probably is due to the C-2 proton of histidine 105. The remaining peak 2 must arise from either histidine-119 or CMhistidine-12.

The assignment of peak 2 was made by the procedure of Meadows et al. [10]. 12-CMRNAase was converted to 12-CMRNAase S by subtilopeptidase-catalyzed hydrolytic cleavage between residues 20 and 21. The carboxymethyl-S-peptide was separated from S-protein by gel exclusion chromatography [4]. After exchange of the N^τ-carboxymethylhistidine C-2 proton with deuterium, the peptide was mixed with S-protein to regenerate 12-CMRNAase S.

Comparison of 12-CMRNAase S spectra before and after this treatment reveals a missing resonance which is assigned to N^τ-carboxymethylhistidine-12.

* While the pH meter reading should be corrected by 0.4 pH units to account for the deuterium effect on the glass electrode, there is a compensatory correction which may be almost as large due to the effect of ^2H_2O on the ionization of imidazole [10]. We therefore assume that the uncorrected pK_a is similar to the true pK_a with the protein dissolved in water.

The chemical shifts were determined at $p^2H = 6.5$ where the three peaks are well separated. The results are presented in Table IV. In comparing 12-CMRNAase and 12-CMRNAase S we see a displacement of all three peaks by approximately 30 Hz. Patel et al. [20] have reported similar differences between the spectra of RNAase A and RNAase S, and have shown that the relative shifts between the three remain unchanged. When the C-2 proton of the CMhistidine-12 residue is exchanged against deuterium, peak 2 disappears. We therefore, assign this peak to residue 12, and conclude that the pK_a of the N^τ-carboxymethylhistidine-12 side chain is 7.5 *. Peaks 1 and 3, therefore, arise from histidines 105 and 119. No further assignments of these peaks are required; the two pK_a values associated with these residues are sufficiently close to allow us to state that the pK_a values of histidines 105 and 119 are approximately 6.7—6.8 under the conditions of these experiments.

Discussion

We have previously reported residual activity in a preparation of 12-CMRNAase, which was shown to be free of a number of potential contaminants, among them unmodified RNAase A [4]. This raised a serious question regarding the catalytic role of histidine-12. If the N^τ position were a catalytically functional acid/base, as commonly believed, then substitution at this position should abolish activity. On further reflection additional explanations, presented in the Introduction, were conceived, and an acid/base function for the τ-nitrogen of histidine-12 could be accomodated to the observation of residual activity. In order to eliminate a number of these interpretations, and in the hope that a definitive assignment of function could be made for histidine-12, we have studied the pH-dependence of the 12-CMRNAase catalysis. While some interpretations have been eliminated, we have not achieved the definitive assignment of function we sought for reasons to be discussed here.

The kinetic results lead directly to two conclusions. The first is that substrate K_m values are very similar for both RNAase A and 12-CMRNAase (Table II). Because of the crystal structure [1,2], the negatively charged carboxymethyl group would be expected to lie near the phosphate of the substrate. The close similarity of K_m values is, therefore, surprising. Presently we have no well formulated explanation, although a proposal we shall present below is consistent with this observation.

The second conclusion is that the shapes of the k_{cat} versus pH curves of RNAase A and 12-CMRNAase are strikingly similar (Fig. 2). There is only one plausible interpretation; the catalytic pathway(s) are either identical or very similar for the two enzymes. Any postulate which requires a gross mechanistic change to explain the residual activity of 12-CMRNAase can be ignored, and, therefore, a free τ-nitrogen on histidine-12 is not essential to enzymatic activity. This still leaves at least three arguments in explanation of the residual activity: (i) histidine-12 has no catalytic function ** in either RNAase A or 12-CMRNAase; (ii) histidine-12 has a catalytic function in both proteins, but not

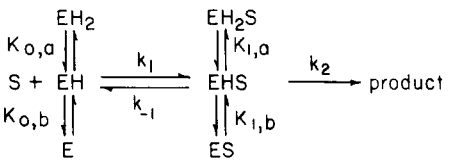
* See footnote p. 533.

** We include in this term a possible structural role the residue might have in maintaining the proper geometric relationships between enzyme and substrate during a rate-limiting step.

as an acid/base; (iii) histidine-12 has a catalytic function in both proteins as an acid/base, but with the proper orientation either nitrogen of the imidazole side chain can be utilized.

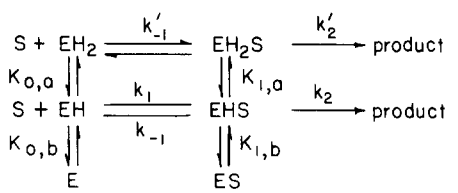
The pH-dependence of an enzyme's catalytic properties can, upon making certain assumptions, be analyzed to obtain the acid dissociation constant(s) of the group(s) responsible for the observed dependence [15]. It has been commonly believed that the pH vs. turnover profile of RNAase A is bell-shaped, and can be explained by the mechanism of Scheme I. Our results suggest that Scheme I is an inadequate model. As first observed by del Rosario and Hammes [8], there is an upward deviation from the bell shape at low pH (Fig. 2; the

Scheme I.



slope of the log k_{cat} versus pH curve is 0.5 between 4 and 5), and K_m passes through a minimum (Fig. 4) in the same region. The simplest kinetic explanation of these two observations is an additional acid pathway (Scheme II). Approximately 400 individual experimental triplets obtained with RNAase A, v_i, (S), and (H^+) were fit to the equation derived from Scheme II using both a grid search and a steepest descent procedure [14]. While Scheme II may be the

Scheme II.



simplest kinetic proposal in an attempt to explain the low pH results, it is not the only valid one. For example, similar results might be expected from substrate ionization on the enzyme; or from the presence of an additional protein residue which dissociates in this pH region, and modulates the activity of the EH and EHS enzyme forms. These and other more complicated mechanisms are either isokinetic with Scheme II, or indistinguishable from it due to experimental limitations. Thus, Scheme II must be considered as an empirical framework for data analysis, and the constants extracted on this basis may contain a collection of true microconstants. As a result conclusions reached with the help of these calculated parameters must be viewed cautiously.

Although Scheme II offers a better explanation of the data at low pH, it is not an adequate basis for an explanation of all the experimental results. From a non-linear regression analysis of v_i, (S) pairs at individual pH, K_m and k_{cat} values were calculated (Table II). These were called "observed" results. The

TABLE V

EQUATIONS FOR FITTING EXPERIMENTAL KINETIC DATA

The solutions for the steady-state kinetic equations corresponding to Schemes I—III of the text were obtained as described by Alberty and Bloomfield [15]. Each solution has the general form:

$k_{cat} = \bar{k}_2$

$K_M = \bar{k}_2/\bar{k}_1 + (k_{-1}/k_1)(f_0/f_1)$

The specific formulations for the various parameters are given below. Numbering is the same as that used in the text.

	Scheme I	Scheme II	Scheme III
f_0	$1 + (H^+)/K_{0,a} + K_{0,b}/(H^+)$	$1 + (H^+)/K_{0,a} + K_{0,b}/(H^+)$	$1 + (H^+)/K_{0,a} + K_{0,b}/(H^+) + K_{0,b}K_{0,c}/(H^+)^2$
f_1	$1 + (H^+)/K_{1,a} + K_{1,b}/(H^+)$	$1 + (H^+)/K_{1,a} + K_{1,b}/(H^+)$	$1 + (H^+)/K_{1,a} + K_{1,b}/(H^+) + K_{0,b}K_{0,c}/(H^+)^2$
$\bar{k}_1$	k_1/f_0	$[k_1 + k_1'(H^+)/K_{0,a}]/f_0$	$[k_1 + k_1'(H^+)/K_{0,a} + k_1''K_{0,b}/(H^+)]/f_0$
$\bar{k}_2$	k_2/f_1	$[k_2 + k_2'(H^+)/K_{1,a}]/f_1$	$[k_2 + k_2'(H^+)/K_{1,a} + k_2''K_{1,b}/(H^+)]/f_1$

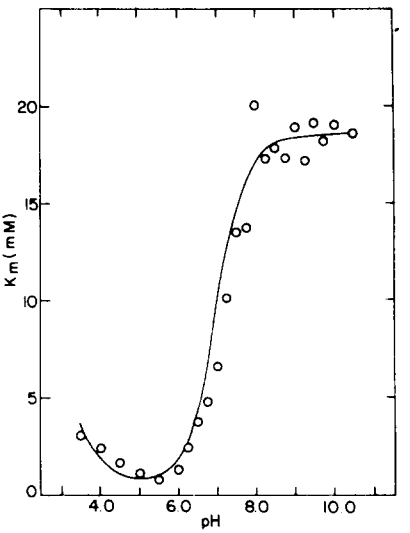
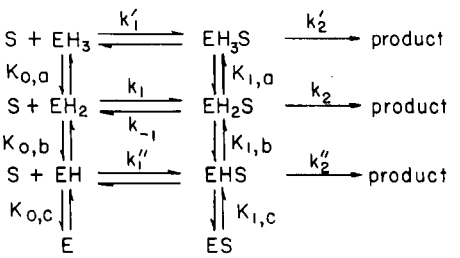


Fig. 4. pH vs. K_m profile for the RNAase A catalyzed hydrolysis of 2',3'-cyclic UMP. Reaction conditions are identical with those of Fig. 2. The solid line is calculated on the basis of Scheme II using best fit constants.

same parameters were calculated from the constants obtained by fitting the equations of Table V to the data. Calculated and "observed" results were then compared by plotting the residual ratios, (observed-calculated)/calculated versus pH. The non-random distribution of the k_{cat} residuals above pH 8 indicates that Scheme II is inadequate (Fig. 5II). This poor fit is obviously related to the inflection on the basic leg of the k_{cat} vs. pH profile (Fig. 2). Once again while it need not be valid, the simplest kinetic explanation is an additional reactive pathway (Scheme III). Fitting the RNAase A data triples to this model by the

Scheme III.



method of steepest descent yields the residual ratio plot of Fig. 5III. The use of Scheme III does result in a qualitatively better fit. However, it should be recognized that improved fits can be achieved by the introduction of enough additional parameters without any greater understanding of underlying mechanism. Pragmatically, it is of no concern which of the two, Scheme II or Scheme III, is more appropriate, since the calculated parameters of interest to us are similar in both (Table VI).

The initial velocity data obtained with 12-CMRNAase (approximately 300

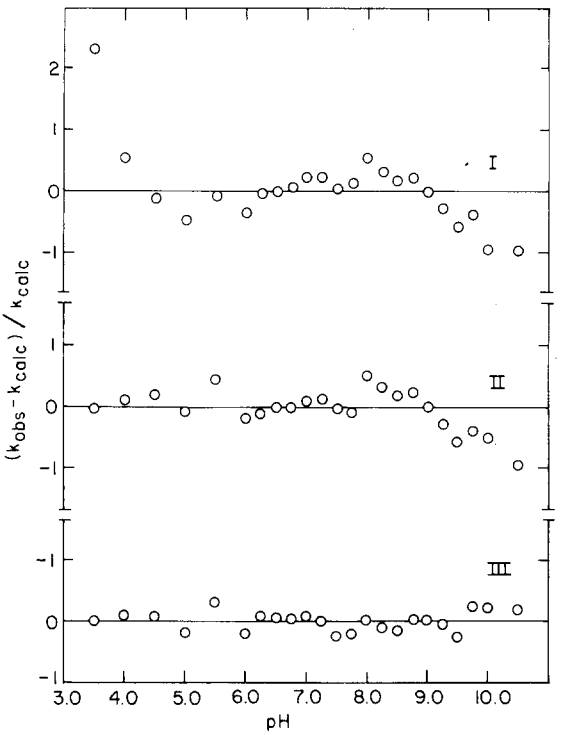


Fig. 5. Adequacy of reaction mechanisms: fitting analysis using observed and calculated k_{cat} as a function of pH. Observed values of k_{cat} were determined at each pH using the Michaelis-Menten equation. Calculated values of k_{cat} were obtained with the equations presented in Table III, and the constants computed by least squares non-linear regression analysis. The comparisons are given for Schemes I, II and III as indicated in the panels.

points) were also fit to Schemes II and III. The pK_a values of the functional acid/base groups on the free enzyme, as calculated from the kinetic results, are collected in Table VI. We note first that the two computational procedures, steepest descent and grid search, yield constants sufficiently close to eliminate the probability of bias introduced by the method of calculation. Second; the apparent dissociation constants p$K_{o,a}$ and p$K_{o,b}$ are very similar for both Schemes II and III. Third; comparing RNAase A and 12-CMRNAase results shows that the kinetically derived pK_a values of the functional groups on the free enzyme appear to be higher for the carboxymethylated protein. In contrast, N^{τ}-carboxymethylhistidine, and N-carboxymethylimidazole are both stronger acids than the parent compounds (Table III). If the apparent dissociation constants determined by kinetic analysis are a reasonable reflection of the pK_a values for two catalytically functional acid/base residues on the protein, then the disparity in the pK_a shifts observed between enzyme and model compounds would suggest that histidine-12 is not catalytically functional. On the other hand, the properties of the model compounds are most probably not an adequate representation for histidine-12. As described in the section on results, the pK_a of carboxymethylated histidine-12 was measured using proton NMR. The results presented in Table VI show that carboxymethylation at histidine-12

TABLE VI

ACID DISSOCIATION CONSTANTS FOR RNAase A AND 12-CMhis RNAase

	Kinetic dissociation constants [a]		
	$pK_{0,a}$	$pK_{0,b}$	$pK_{0,c}$
RNAase A			
Scheme II, steepest descent	5.78	6.08	—
Scheme II, grid search	5.76	6.17	—
Scheme III, steepest descent	5.71	6.26	8.97
12-CHhisRNAase			
Scheme II, steepest descent	6.34	6.74	—
Scheme II, grid search	6.25	6.86	—
Scheme III, steepest descent	6.33	6.76	9.34

	NMR dissociation constants [b]		
	Ref.	histidine-12	histidine-119
RNAase A			
	18 [c]	5.8	6.3
	21 [c]	5.8	6.1
	22	5.8	6.2
	23	6.2	6.5
	24 [c]	5.7	
	25 [c]	6.0	6.2
12-CMRNAase A	10 [d]	7.6	6.7
	This paper	7.5	6.7—6.8

[a] Kinetic assignments of pK_a values are those shown in Schemes II and III.

[b] Constants were chosen for approximately similar experimental conditions. Where possible, the pK_a in the table was estimated for 27°C from the data presented in the reference. Where not possible, the nearest temperature was used.

[c] Peak assignments used for these results are those of Markley [22] and Patel et al. [20].

[d] Peak assignments were not made by Meadows et al. [10], but are ours.

increases the pK_a values of both residues 12 and 119. We conclude that the apparent pK_a changes observed in the kinetic properties of the enzyme at least parallel the acid dissociation alterations of the pertinent histidine residues.

Digressing for a moment, let us consider possible interpretations of the acid weakening induced by carboxymethylation of RNAase A in contrast to the acid strengthening observed in the model systems. The X-ray crystallographic structure of RNAase indicates a number of positively charged groups about the active site [1,2]. The introduction of a negative carboxymethyl moiety might reduce electrostatic 'strain', thereby making the two active site histidines weaker acids. The large pK_a shift observed for residue 12 may have an additional, and perhaps more appropriate explanation. Both crystallographic [2] and NMR [26,27] data suggest a hydrogen bond involving the π-nitrogen of histidine-12 and the oxygen of threonine-45. Since the π-nitrogen is the obligatory site of protonation for an N^{τ}-carboxymethylhistidine residue, a potential hydrogen bond involving this site would stabilize the acid form of the residue. Thus, it is reasonable that CMhistidine-12 is a weaker acid than histidine-12.

That the basic shifts observed in the histidine pK_a values by NMR titration of 12-CMRNAase are paralleled by similar shifts in the apparent pK_a values ob-

tained from the kinetic experiments, strongly supports a catalytic function for both histidine-12 and histidine-119. As mentioned above Schemes II and III are only the simplest kinetic models we can formulate to fit the experimental results; and as is generally true for any system characterized by multiple equilibria, the derived parameters $pK_{o,a}$ and $pK_{o,b}$ may be collections of elementary constants rather than true dissociation constants. Therefore, we must introduce the assumption that these are, or approximate true equilibrium constants in order to compare kinetic and NMR results. This assumption renders the remaining discussion conjectural. The results in Table VI suggest that in the native enzyme the groups associated with $pK_{o,a}$ and $pK_{o,b}$ are histidines 12 and 119 respectively, i.e., histidine-12 is functional in its basic form, histidine-119 in its acid form. In the case of 12-CMRNAase $pK_{o,b}$ compares favorably with the measured pK_a of histidine-119, but $pK_{o,a}$ is more than 1 unit lower than the NMR derived parameter for CMhistidine-12. Two simple interpretations may be invoked which do not require a major change in the reaction mechanism between native and modified enzymes.

We propose that histidine-119 is functional in its acid form for both native and modified enzymes, and that the functional base is histidine-12 in RNAase A, and CMhistidine-12 in 12-CMRNAase. Either the newly inserted carboxyl group, or the unblocked π-nitrogen of CMhistidine-12 assume the role assigned to the free τ-nitrogen of the native enzyme. If the carboxyl group functions as the base, then the kinetic $pK_{o,a}$ would be assigned to it, necessitating an explanation for its relatively high value. The results of Patel et al. [26] suggest that the π-nitrogen of CMhistidine-12 remains hydrogen bonded to threonine-45, in which conformation the free nitrogen would not be accessible to the substrate. If, however, some small fraction of the modified enzyme exists with the N^{τ}-carboxymethylhistidine side chain rotated such that the π-nitrogen faces into the active site and is no longer hydrogen-bonded, then this nitrogen could act catalytically, and the low apparent $pK_{o,a}$ would be a reasonable consequence. Either of these possibilities are intriguing, since they would suggest some flexibility in the requirements for the catalytic geometry between protein and substrate.

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