Hydrolysis of Dehydrase Inactivated with 3-[1-^{14}C]Decynoyl-NAC. Lyophilized, inactivated dehydrase (4.3×10^5 dpm) was dissolved in 16 mL of 6 NHCl. After stirring for 3 h at room temperature, the suspension was transferred to a heavy-walled reaction tube, which was sealed under vacuum. The sample was heated at 110 °C for 20 h and then cooled to room temperature. After removal of HCl and H_2O, the sample (total recovered radioactivity, 4.12×10^5 dpm) was analyzed by HPLC: 33:67, MeOH/0.5% Et_3NHPO_4, pH 3.1; 250 × 4.6 mm Rainin Microsorb C-18 column; eluted at 1.0 mL/min, with effluent monitored at 210 nm (see Figure 2).

Hydrolysis of Model Adducts 4-6. Compounds **4-6** were hydrolyzed in the same way as were dehydrase and dehydrase inactivated with 3-decynoyl-NAC. The HPLC conditions were identical, as well.

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Proton Inventory of the Second Step of Ribonuclease Catalysis

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Abstract: The specificity ratio $k_{cat}/K_m = k_E$ has been measured for the bovine pancreatic ribonuclease A catalyzed hydrolysis of cytidine cyclic 2',3'-monophosphate at 25 °C, pH 7.47, and equivalent in H_2O–D_2O solutions containing a mole fraction n of D_2O. A graph of the partial kinetic solvent iostope effects $k_{E,n}/k_{E,0}$ vs. n is bowl shaped. The isotope effect data fit the equation $k_{E,n}/k_{E,0} = (1 - n + 0.57n)^2$. The transition state may involve two protons, each with an isotopic fractionation factor of 0.57. These results support previous studies that propose general-acid–general-base catalysis by the imidazole rings of histidine residues 12 and 119 as an essential feature of ribonuclease action.

The second step of ribonuclease (RNase) catalysis involves the hydrolysis of nucleoside cyclic 2',3'-phosphate esters to 3'-phosphates.[1] This reaction is believed to be catalyzed by donation of a proton to the 2' ester group and abstraction of a proton from water.[1,2] Although the chemical and physical evidence is complex,[1,2] the imidazole groups of histidine residues (12 and 119) are generally considered to be the proton donor and proton acceptor (Figure 1). We now report that a proton inventory[3,4] study of the bovine RNase A catalyzed hydrolysis of cytidine cyclic 2',3'-monophosphate (cCMP) supports this hypothesis. Our results indicate that, at about pH 7.5, two protons undergo a change of state upon activation of the dissociated enzyme and substrate to a single rate-controlling transition state.

Results

Partial kinetic solvent isotope effects (KSIEs) acquired for eight H_2O–D_2O mixtures on four occasions are given in Table I. The $k_{E,n}$ and $k_{E,0}$ are, respectively, the values of the specificity ratio k_{cat}/K_m in protium oxide containing a mole fraction n of deuterium oxide and in protium oxide. A graph of $k_{E,n}/k_{E,0}$ vs. n bulges downward, as shown in Figure 2. The dependence of the KSIEs on n was used with the Gross–Butler equation[3] (eq 1) to evaluate

Table I. Solvent Deuterium Isotope Effects on the Ribonuclease A Catalyzed Hydrolysis of Cytidine Cyclic 2',3'-Monophosphate[a]

n	$k_{E,n}/k_{E,0}$	average
0.117	0.927, 0.879, 0.932, 0.907	0.912 ± 0.024
0.234	0.801, 0.816, 0.793, 0.778	0.797 ± 0.016
0.352	0.688, 0.676, 0.737, 0.741	0.721 ± 0.024
0.469	0.627, 0.632, 0.645, 0.630	0.634 ± 0.008
0.586	0.536, 0.552, 0.562, 0.563	0.553 ± 0.013
0.703	0.513, 0.485, 0.500, 0.487	0.496 ± 0.013
0.820	0.447, 0.422, 0.438, 0.426	0.433 ± 0.011
0.935	0.364, 0.346, 0.354, 0.359	0.356 ± 0.008

[a] 25 °C; pH 7.47 in H_2O and its equivalent[3b,c] in H_2O–D_2O mixtures (0.10 M Tris buffer); pseudo-first-order conditions ($[E_0] \ll [S_0] \ll K_m$) throughout; average values of KSIEs are the mean ± 1 standard deviation of the KSIEs obtained at the same n in four separate proton inventories; average error in $k_{E,n}/k_{E,0}$ is 2.4%.

$$k_{E,n}/k_{E,0} = \frac{\overset{TS}{\prod}(1 - n + \phi^T_i n)}{\overset{TS}{\prod}(1 - n + \phi^R_j n)} \quad (1)$$

$$k_{E,n}/k_{E,0} = \overset{TS}{\prod}(1 - n + \phi^T_i n) \quad (2)$$

$$k_{E,n}/k_{E,0} = (1 - n + \phi^T n)^2 \quad (3)$$

the number of protons involved upon activation of the enzyme and substrate to the rate-controlling transition state or transition states. The ϕ^T_i and ϕ^R_j, respectively, represent the isotopic fractionation factors of the ith hydrogenic site of the transition state and jth hydrogenic site of the reactants. When $n = 1$, the overall KSIE is the product of all ϕ^T over the product of all ϕ^R. A reasonable initial assumption is that ϕ^R of the ionizable side-chain groups

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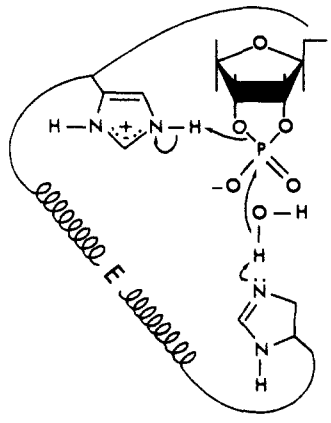

Figure 1. Proposed role of histidine residues as proton donor and proton acceptor at the transition state in the rate-controlling step of RNase-catalyzed hydrolysis of nucleoside cyclic 2′,3′-phosphate esters to 3′-phosphates.

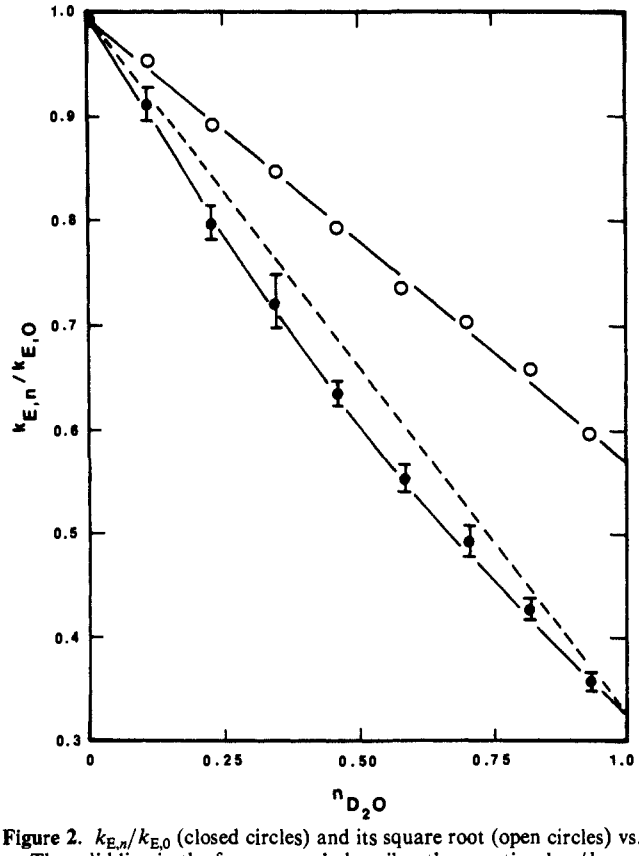

Figure 2. $k_{E,n}/k_{E,0}$ (closed circles) and its square root (open circles) vs. n. The solid line in the former graph describes the equation $k_{E,n}/k_{E,0} = (1 - n + 0.57n)^2$; error bars represent 1 standard deviation from the mean.

of the protein and the cyclic phosphate ester are unity,[3b,c] so that the KSIE is entirely generated by the ϕ^T (eq 2). The dependence of $k_{E,n}/k_{E,0}$ on n in eq 2 is linear for one hydrogenic site, quadratic for two equivalent sites, cubic for three, and so forth.

A least-squares fit[5] of the isotope effect data to the two-sites model represented by eq 3 gives $\phi^T = 0.571 \pm 0.004$ (F ratio 39 330). A plot of the square root of $k_{E,n}/k_{E,0}$ vs. n (Figure 2) is linear (correlation coefficient $r = 0.999$, F ratio 2899), as predicted for the quadratic dependence of the KSIE on n. Much poorer fits were obtained for one-site and infinite-sites[3c] ($k_{E,n}/k_{E,0} = \phi^n$) models. The results of proton inventory studies are never completely unambiguous, however. Some fortuitous combination of nonunitary ϕ^R and ϕ^T could be responsible for the shape of the proton inventory curve.[6] With unitary ϕ^R, additional ϕ^T might go undetected in the experimental error. For example, imprecision in the KSIEs makes it impossible to distinguish between transition states involving two or three equivalent protons. Least-squares analysis for the three-proton case gives $\phi^T = 0.697 \pm 0.003$ (F ratio 41 260).

(5) Curve fitting of models to the proton inventories employed a computerized iterative fit of the data by the Marquadt–Levenberg method (BBN Research Systems, Cambridge, MA).

Discussion

The simplest inference from our results is that the proton inventory data describe a single transition state in which each of two protons makes a normal contribution of $1/0.571 = 1.75$ to an overall $k_{E,0}/k_{E,1} = 3.07(1/0.571)^2$.

The magnitudes of the ϕ^T for the two-proton model are consistent with a hydrolytic mechanism that involves general catalysis by the imidazole rings of two histidine residues, being within the range of 0.50–0.60 typically obtained in the acylation and deacylation of serine proteases,[7] where histidine imidazole acts as a general catalyst. Evidently, the transition-state protons experience a weaker potential than that in bulk water ($\phi_w = 1.0$),[3] but the KSIEs they generate are smaller than expected for primary[8] (parallel)[9] effects. Thus, the second step of RNase action probably represents another case[3b,c,f] of solvation catalysis[10] (perpendicular effects[9]) in an enzymatic reaction.

The proposed model for protonic reorganization in step two of RNase catalysis does not exclude the possibility of additional interactions of the substrate with other protonated residues of the protein such as lysine-41.[1,2] Such interactions, if they exist, are not detected in the proton inventory. This situation could be analogous to the "oxyanion hole" of serine proteases, a hydrogen-bonding network that is thought to stabilize a tetrahedral-like transition state in the acylation and deacylation reactions.[11] Despite the presence of the oxyanion hole, proton inventory studies of hydrolyses of ester and amide substrates catalyzed by serine proteases are usually rationalized most simply by one- or two-proton models of protonic reorganization.[13-17]

Experimental Section

Bovine pancreatic RNase (Type III, Lot 44F-8155) and cytidine cyclic 2′,3′-monophosphate (sodium salt, Lot 63F-7075) were purchased from Sigma Chemical Co. and were used as received. The enzyme was a chromatographically purified product claimed by the manufacturer to contain less than 5% RNase B. Solutions of RNase (3.2 mg mL^{-1}) were prepared in the same buffer used to conduct kinetics experiments and kept on ice. The molar concentration of the enzyme was estimated spectrophotometrically at 277.5 nm, assuming a molar absorptivity of 9800 M^{-1} cm^{-1}.[18] The spectrophotometrically determined molarity was about 83% of the value expected from mass measurements, based on a molecular mass of 13 680 calculated from the amino acid sequence.[1a]

Solutions. Buffers were prepared from analytical-grade Tris and Tris-HCl. Deionized water was brought to a resistivity of >10 MΩ by passage through the activated carbon and deionizing cartridge of a Continental Water System. Deuterium oxide (KOR Stable Isotopes, Inc., 99.8 atom % D) was used as received.

Accurately weighed quantities of Tris and Tris-HCl needed to produce a 0.10 M total Tris buffer of pH 7.5 were dissolved in H_2O. The same

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amount of the buffer pair was dissolved in D_2O to produce the equivalent.[3b,c] Mixtures of H_2O–D_2O (L_2O) were prepared by volumetrically combining these solutions. Account was taken in computing final values of n of differences in the density at 25 °C between H_2O (d = 0.997 g/cm^3) and D_2O (d = 1.1044 g/cm^3),[19] as well as isotopic dilution of the L_2O solutions by protons released by the buffer salt and additions of enzyme and substrate in H_2O. The pH of the buffer in H_2O was determined to within ±0.01 unit on an Orion Model 701 A digital pH meter equipped with a Ross combination electrode.

Kinetics. Kinetics experiments were carried out by adding 0.1 mL of substrate solution followed by 0.1 mL of RNase solution (both in the Tris buffer, H_2O) to 3 mL of buffer in a cuvette in the thermostated cell compartment of a Variant Cary Model 219 spectrophotometer. Concentrations of the enzyme and substrate in the cuvette were approximately 6 μM and 0.33 mM, respectively. The absorbance increase owing to the hydrolysis of cCMP was continuously recorded at 284 nm.[20] In preliminary experiments, plots of log $(A_\infty - A_t)$ vs. time yielded straight lines through 90% reaction. For the experiments reported here, a Bascom–Turner data center was used to acquire and store 2000 evenly spaced voltages related to the absorbance increases through 9–10 half-lives. The stored data were treated according to the Guggenheim method to obtain pseudo-first-order rate coefficients k_{obsd}, which were divided by the RNase concentration to yield k_e. The Guggenheim treatment utilized data from at least 3 half-lives; the constant difference between readings taken at a series of times and a series of times later was selected to be approximately 2 half-lives. The mean value of $k_{E,0}$ taken from four proton inventories was 1180 ± 50 M^{-1} s^{-1}, in reasonably good agreement with the value of 1380 M^{-1} s^{-1} calculated from the expression

$$k_{E,0} = k_{lim}/(1 + H_a/K_a + K_b/H_b)$$

where k_{lim} = 7540 M^{-1} s^{-1}, pK_a = 5.4, and pK_b = 6.8 (25 °C, 0.1 M NaCl + 0.1 M Tris–acetate).[21]

A few five-point proton inventories were conducted in the usual way except the enzyme was dissolved in buffer constituted in D_2O rather than in H_2O. The values of $k_{E,n}$, the partial KSIEs, and the shapes of the proton inventory curves thus obtained were identical within the precision of the measurements with those found when the RNase was dissolved in the buffer constituted in H_2O. This suggests that slow exchange of solvent deuterons with protons of the protein during the time required to prepare the enzyme solution and conduct a complete proton inventory (8–10 h) does not produce conformational or other changes that might surreptitiously confound the reported results.

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Registry No. RNase, 9001-99-4; cCMP, 633-90-9; D_2, 7782-39-0; histidine, 71-00-1.

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Low-Temperature Magnetic Circular Dichroism Studies of Native Laccase: Confirmation of a Trinuclear Copper Active Site

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Abstract: Low-temperature magnetic circular dichroism (LTMCD), absorption, and EPR spectroscopies are used to determine the spectral features associated with the different types of copper in native laccase and to investigate N_3^- and F^- binding at this multicopper active site. This combination of techniques allows ligand field (d–d) and charge-transfer (CT) spectral features associated with the paramagnetic type 2 Cu(II) center to be differentiated from those of the antiferromagnetically coupled and therefore diamagnetic type 3 Cu(II) center. N_3^- binding with $K \sim 200$ M^{-1} ("low-affinity") to fully oxidized native laccase generates LTMCD and absorption features at 485 nm, assigned to $N_3^- \rightarrow$ type 2 Cu(II) CT, and an absorption feature at 400 nm with no corresponding LTMCD intensity, assigned to $N_3^- \rightarrow$ type 3 (coupled) Cu(II) CT. This indicates that N_3^- bridges between the type 2 and type 3 cupric centers. This type 2/type 3 bridging mode is strongly supported by spectral perturbations. The intensities of these two features decrease together with increasing pH. In addition, studies of the competitive binding of N_3^- and F^- show that the binding of a single F^- to the type 2 Cu(II) causes both the 400-nm absorption and 485-nm LTMCD bands to decrease by similar amounts. These correlations eliminate the possibility that two different N_3^-'s with similar binding constants bind separately to the type 2 and type 3 centers and strongly support a single bridging N_3^-. A second N_3^- binds with $K \geq 10^4$ M^{-1} ("high-affinity") and generates LTMCD and absorption features at 510 and 450 nm that are associated with N_3^- binding to the type 2 Cu(II) in a fraction (~25%) of the laccase molecules that contain a reduced type 3 copper center. Maximum intensity of these features is obtained with <1.0 protein equiv of N_3^- ([protein] ≅ 1.0 mM), and addition of small amounts of N_3^- to peroxide-oxidized laccase results in significantly decreased intensities of the 510- and 450-nm features. EPR studies of F^- binding to oxidized native laccase in 50% glycerol/phosphate buffer solutions show a clear superhyperfine doublet of the type 2 Cu(II) signal, indicating that one F^- binds to the type 2 Cu(II). The results of the N_3^-/F^- competition studies show that this F^- binds to the type 2 site at room and low temperatures. Ligand competition studies of high-affinity N_3^- with F^- indicate that F^- binds to the type 2 Cu(II) only when the type 3 coppers are oxidized. Investigation of the LTMCD features of native and peroxide-oxidized laccase, and their pH dependence, enables specific LTMCD features to be associated with the oxidized type 1 and type 2 centers in the presence of both reduced and oxidized type 3 centers (which do not contribute to the LTMCD spectrum). Changes in the type 2 d–d bands upon type 3 reduction demonstrate that the geometry of the type 2 center is strongly affected by the oxidation state of the type 3 center. The dependence of N_3^- and F^- affinity for the type 2 Cu(II) upon the oxidation state of the type 3 copper further demonstrates the strong interaction between these copper centers in anion binding. Finally, the presence of a type 2–type 3 trinuclear copper active site that is capable of binding and bridging at least the small molecule N_3^- suggests that a similar binding mode could contribute to the multielectron reduction of dioxygen to water at this site.

The multicopper oxidases,[1,2] laccase, ceruloplasmin, and ascorbate oxidase, catalyze the four-electron reduction of dioxygen to water, with concomitant one-electron oxidations of substrate. The active site of laccase is the simplest of these enzymes, con-

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