Interaction of Cytidine 3′-Monophosphate and Uridine 3′-Monophosphate with Ribonuclease *a* at the Denaturation Temperature

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ABSTRACT: Differential scanning calorimetry (DSC) measurements were performed on the thermal denaturation of ribonuclease *a* and ribonuclease *a* complexed with an inhibitor, cytidine or uridine 3′-monophosphate, in sodium acetate buffered solutions. Thermal denaturation of the complex results in dissociation of the complex into denatured ribonuclease *a* and free inhibitor. Binding constants of the inhibitor to ribonuclease *a* were determined from the increase in the denaturation temperature of ribonuclease *a* in the complexed form and from the denaturation enthalpy of the complex. Binding enthalpies of the inhibitor to ribonuclease *a* were determined from the increase in the denaturation enthalpy of ribonuclease *a* complexed with the inhibitor. For the cytidine inhibitor in 0.2 M sodium acetate buffered solutions, the binding constants increase from 87 ± 8 M^{-1} (pH 7.0) to 1410 ± 54 M^{-1} (pH 5.0), while the binding enthalpies increase from 17 ± 13 kJ mol^{-1} (pH 4.7) to 79 ± 15 kJ mol^{-1} (pH 5.5). For the uridine inhibitor in 0.2 M sodium acetate buffered solutions, the binding constants increase from 104 ± 1 M^{-1} (pH 7.0) to 402 ± 7 M^{-1} (pH 5.5), while the binding enthalpies increase from 16 ± 5 kJ mol^{-1} (pH 6.0) to 37 ± 4 kJ mol^{-1} (pH 7.0). The binding constants and enthalpies of the cytidine inhibitor in 0.05 M sodium acetate buffered solutions increase respectively from 328 ± 37 M^{-1} (pH 6.5) to 2200 ± 364 M^{-1} (pH 5.5) and from 22 kJ mol^{-1} (pH 5.5) to 45 ± 7 kJ mol^{-1} (pH 6.5). The denaturation transition cooperativities of the uncomplexed and complexed ribonuclease *a* were close to unity, indicating that the transition is two state with a stoichiometry of 1.

The determination of equilibrium binding constants of inhibitors to enzymes is important in evaluating the effect of an inhibitor on enzymatic activity. Equilibrium constants determined at one temperature can be used to calculate equilibrium constants at other temperatures, provided that the enthalpy and heat capacity change of the binding reaction are accurately known. This is particularly important for enzymes that exhibit large heat capacity changes upon binding of ligands to the enzyme (Sturtevant, 1974; Eftink et al., 1983). The aim of this investigation is to determine the binding constants and the enthalpies of binding of inhibitors to enzymes at the denaturation temperature of the enzyme by using DSC. The systems studied were the binding of the inhibitors cytidine 3′-monophosphate and uridine 3′-monophosphate to ribonuclease *a* in sodium acetate buffered solutions. These systems have been studied extensively at room temperature by difference spectrophotometry (Hammes & Schimmel, 1965; Anderson et al., 1968) and isothermal flow calorimetry (Flogel & Biltonen, 1975; Bolen et al., 1971). Eftink et al. (1983) have determined the enthalpies of binding of the cytidine inhibitor to ribonuclease *a* up to 317.2 K, about 20 K below the denaturation temperature, using batch calorimetry.

To determine the equilibrium constant and enthalpy of the binding reaction at the denaturation temperature, DSC measurements were performed on solutions of ribonuclease *a* and ribonuclease *a* complexed with the cytidine or uridine inhibitor. Analysis of the DSC measurements yielded the temperature ($T_{0,c}$), the enthalpy (ΔH), the cooperativity of the denaturation transition of ribonuclease *a* in the uncomplexed and complexed form, and the change in the heat capacity of the solution upon denaturation (ΔC_p). Equilibrium dialysis measurements were performed, and they demonstrated that ribonuclease *a*, R, binds in a 1:1 ratio with the inhibitor, I, only in the native state. Therefore, at the denaturation temperature

$$\mathrm{R} + \mathrm{I} = \mathrm{RI} \qquad \Delta H_b(T_c),\ \Delta C_{pb} \qquad (1)$$

and

$$\mathrm{RI} = \mathrm{D} + \mathrm{I} \qquad \Delta H_c(T_c),\ \Delta C_{pc} \qquad (2)$$

where D is the denatured state. The binding constants (K_b)

$$K_b = [\mathrm{RI}]/[\mathrm{R}][\mathrm{I}] \qquad (3)$$

were determined at the denaturation temperature by an equation derived by Schellman (1975)

$$K_b = (\exp[(T_c - T_0)\Delta H_c(T_c)/RT_cT_0] - 1.00)/[\mathrm{I}] \qquad (4)$$

which has been used to study the binding of the substrate tri-*N*-acetylglucosamine to lysozyme (Pace & McGrath, 1980). T_0 is the denaturation temperature of the uncomplexed ribonuclease *a*, $R = 8.314$ J mol^{-1} K^{-1}, and [I] is the free inhibitor concentration. The derivation of eq 4 assumes that changes in the denaturation entropy and enthalpy are small over the temperature range T_0 to T_c (Schellman, 1975). The binding enthalpies at the denaturation temperature were calculated at inhibitor concentrations where the ribonuclease *a* exists exclusively in the complexed form from

$$\Delta H_b(T_c) = \Delta H(T_c) - \Delta H_c(T_c) \qquad (5)$$

where $\Delta H(T_c)$ is the denaturation enthalpy of the uncomplexed ribonuclease *a* at T_c. Fukada et al. (1983) employed this method to determine the heat of binding of arabinose to l-arabinose binding protein from DSC measurements. Similarly, the heat capacity change for reaction 1 was calculated from

$$\Delta C_{pb} = \Delta C_p - \Delta C_{pc} \qquad (6)$$

where ΔC_p is the heat capacity change of the uncomplexed ribonuclease *a* solution upon denaturation. Since the binding constants and enthalpies depend on the pH and ionic strength of the solution (Hammes & Schimmel, 1965; Anderson et al., 1968; Flogel & Biltonen, 1975; Bolen et al., 1971), they were determined at the denaturation temperature as a function of pH in 0.2 M sodium acetate buffered solutions for the cytidine and uridine inhibitors and also in 0.05 M sodium acetate

buffered solutions for the cytidine inhibitor. Comparisons were also made between the binding constants determined at room temperature and room temperature values from the literature.

In addition to thermodynamic information on the binding reaction at the denaturation temperature, DSC may provide, through changes in the cooperativity of the denaturation transition, information on structural changes that occur in the enzyme upon complexation with an inhibitor. Fukada et al. (1983) observed a change from 1.3 to 0.9 in the cooperativity of the denaturation transition of l-arabinose protein upon complexation with l-arabinose. This change was attributed to a decrease in the number of intermediate states participating in the denaturation of the complex. Privalov et al. (1981) observed a change from 1.7 to 0.85 in the cooperativity of the first denaturation transition peak of pepsin upon binding with the inhibitor pepstatin. This change was attributed to the merging of two structural domains of the pepsin into one domain via binding of the pepstatin to the pepsin. To determine any structural changes induced by binding of the inhibitor, the cooperativities of the uncomplexed and complexed ribonuclease *a* transitions were compared.

Experimental Procedures[1]

Materials. The bovine pancreatic ribonuclease *a* had a stated purity of 90%. A sodium dodecyl sulfate gel electrophoresis analysis (Weber & Osborn, 1969) of the ribonuclease *a* showed an intense band at 14 kDa (M_r of ribonuclease *a* 13 700) and a very weak band at 18 kDa. The free acid form of the cytidine 3′-monophosphate and the sodium salt of the uridine 3′-monophosphate were used, and both inhibitors were 98% pure. Acetic acid and sodium acetate were of reagent quality.

Preparation of Solutions. Millimolar solutions of ribonuclease *a* were prepared by dissolving ribonuclease *a* in 0.20 or 0.05 M sodium acetate–acetic acid solutions buffered in the pH range 4.0–7.0. The pH of the solutions was measured with an Orion 811 pH meter equipped with a Corning EX-L glass electrode. Since the pH of sodium acetate buffered solutions increases by only 2% as the temperature increases from 298.2 to 333.2 K (Perrin & Dempsey, 1974), the pH at room temperature was recorded as the pH at the denaturation temperature. The solutions were dialyzed and analyzed spectroscopically as described previously (Schwarz & Kirchhoff, 1988). Enzymatic analysis using the method of Crook et al. (1960) of some of the solutions indicated that the enzyme concentration was within 4% of the concentration determined by the spectroscopic analysis. Solutions of the inhibitor–ribonuclease *a* complex were prepared by dissolving a known mass of inhibitor in a sample of the dialyzed ribonuclease *a* solution and adjusting the pH to the desired value. A spectroscopic analysis of diluted aliquots of the 0.04 M cytidine 3′-monophosphate complexed solutions [ϵ_{265nm} = 7600 cm^{-1} M^{-1} at pH 7.0 (Beaven et al., 1955)] agreed within 5% of the inhibitor concentration calculated from the mass of inhibitor added to the ribonuclease *a* solution and the total final mass of the solution.

Equilibrium Dialysis Measurements. To determine if the stoichiometry of the ribonuclease *a*–inhibitor complex is 1:1 and if the inhibitor binds to the denatured state of ribonuclease *a*, spectroscopic measurements were performed on solutions of ribonuclease *a* and denatured ribonuclease *a* in equilibrium with cytidine inhibitor solutions. In three separate experiments, samples of a 0.90 mM ribonuclease *a* solution contained in a dialysis tube were dialyzed against aliquots of a 3.0 mM cytidine 3′-monophosphate solution in 0.2 M sodium acetate buffer (pH 6.0). At pH 6.0, the ribonuclease *a*–inhibitor binds tightly to ribonuclease *a* (Anderson et al., 1968), and the complex is irreversibly denatured by heating the solution to 363 K for 1 h. Samples of the ribonuclease *a* solution were 0.8 mL dialyzed against 3 mL of the inhibitor solution, 1.02 mL dialyzed against 3 mL of the inhibitor solution, and 2.1 mL dialyzed against 4 mL of the inhibitor solution. After the solutions were allowed to equilibrate overnight, a 0.1-mL aliquot of the cytidine solution was removed and analyzed spectroscopically to determine the cytidine concentration. The solutions were heated to 363 K for several hours to ensure at least 90% irreversible denaturation of the ribonuclease *a* and then allowed to reequilibrate overnight at room temperature. The concentration of the inhibitor was redetermined and compared to the original concentration in equilibrium with the undenatured ribonuclease *a*.

DSC Measurements. DSC measurements were performed with a Hart 7707 DSC heat conduction scanning microcalorimeter as described previously by Schwarz (1986) and Schwarz and Kirchhoff (1988). The DSC was operated at a scan rate of 18 K h^{-1} from 303 to 363 K with sample masses of approximately 0.5 g. The power input from a thermal scan of buffer vs buffer was subtracted from the power input of each solution vs buffer scan to obtain the excess power input for the ribonuclease *a* transition.

All the excess power thermal scans were corrected for the thermal lag of the DSC by the Tian equation and converted to excess heat capacity vs temperature scans by dividing by the scan rate as described in Schwarz and Kirchhoff (1988). A sigmoidal base line, C_{ps}, was extrapolated under the transition curve from least-squares fits of the pre- and posttransitional base lines to linear equations in T and the fractional area under the transition curve at T, α, by the relationship

$$C_{ps} = a + b(T - T_{0,c}) + [\Delta a + \Delta b(T - T_{0,c})]\alpha \quad (7)$$

where $a + b(T - T_{0,c})$ is the linear fit of the pretransitional base line to T, and $\Delta a + \Delta b(T - T_{0,c})$ is the difference between linear extrapolations of the pre- and posttransitional base lines at T. An initial estimate of α was determined by using the pre- and posttransitional base line fits extrapolated to the midpoint of the transition curve as the transitional base line, and then C_{ps} was calculated by successive approximations. The transition enthalpy $\Delta H_{,c}$ was determined from the area under the transition curve and from the number of moles of ribonuclease *a* in the cell while the transition temperature $T_{0,c}$ was recorded as the temperature where $\alpha = {}^1/_2$. The change in the heat capacity upon denaturation, ΔC_p, was determined from the difference between the extrapolated pre- and posttransitional base lines at $T_{0,c}$ and the number of moles of enzyme in the cell. The cooperativity η, which is the ratio of the transition enthalpy to the van't Hoff enthalpy, was determined by the approximate relation

$$\eta = \Delta H_{,c}^2/(4RT_{0,c}^2C_m) \quad (8)$$

where C_m is the height of the transition peak per mole of enzyme at the transition temperature.

Results and Discussion

DSC Measurements. A typical excess heat capacity scan of the denaturation transition of ribonuclease *a* in a sodium

[1] Certain commercial equipment, instruments, and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Bureau of Standards nor does it imply that the material, instrument, or equipment identified is necessarily the best available for the purpose.

Table I: Results of DSC Measurements of the Denaturation Transition of Ribonuclease *a* in Sodium Acetate Buffered Solutions[a]

pH	sample concn (mM)	T_0 (K)	ΔC_p (kJ mol^{-1} K^{-1})	η	ΔH (kJ mol^{-1})
			In 0.2 M Sodium Acetate Buffer		
4.0	1.49	329.0	3.11 ± 0.70	0.900	395 ± 23
4.0	1.36	327.5	1.80 ± 1.08	0.856 ± 0.011	361 ± 5
4.0	1.52	327.6	0.38 ± 0.10	0.930 ± 0.043	394 ± 11
4.1	0.744	329.0	5.49 ± 1.65	0.851 ± 0.002	389 ± 3
4.3	1.31	331.5	0.86 ± 0.15	0.856 ± 0.004	400 ± 2
4.4	1.55	332.0	2.94 ± 0.24	0.873 ± 0.042	401 ± 7
4.5	0.766	332.7	5.41	0.844	419
4.7	1.47	332.8	7.02 ± 0.24	0.954 ± 0.048	425 ± 3
5.0	1.47	334.6	5.67	0.825	401
5.5	1.47	335.7	3.13	0.934	444
5.5	0.70	336.2	4.92	0.995	472
5.5	1.34	336.2	2.47 ± 0.38	0.854 ± 0.001	419 ± 2
5.9	1.47	335.8	5.40 ± 1.26	0.944 ± 0.004	463
6.0	1.43	336.2	4.03 ± 0.56	0.857 ± 0.043	425 ± 12
6.0	1.53	336.0	4.36 ± 1.21	0.951 ± 0.018	459 ± 3
6.5	0.766	336.0	7.02 ± 0.68	0.907 ± 0.016	453
6.5	1.47	336.1	2.23 ± 0.69	0.947 ± 0.032	480 ± 9
average			3.78 ± 2.18	0.899 ± 0.053	
			In 0.05 M Sodium Acetate Buffer		
4.5	0.890	328.6	10.0 ± 2.9	1.000 ± 0.015	373 ± 2
5.1	1.07	333.8	4.31	0.986	424
5.9	1.01	335.0	4.96	0.942	438
5.6	1.02	335.4	7.09	0.901	423
6.6	0.875	335.6	7.06	0.989	452
average			7.24 ± 3.03	0.970 ± 0.041	

[a] The sample size was approximately 0.5 g, and the scan rate was 18 K h^{-1}. The error in the ΔC_p, η, and ΔH values is the average deviation resulting from two determinations of the value, while the average deviation in T_0 is ±0.1 K.

acetate buffered solution is shown in Figure 1. For comparison, a scan of the same ribonuclease *a* solution (1.43 mM) containing 0.04 M cytidine 3′-monophosphate is also shown in Figure 1. Both scans exhibit a single symmetrical peak for the denaturation transition and a positive increase in the heat capacity of the solution upon denaturation. However, the denaturation temperature and enthalpy are respectively 3.7 K higher and 17% larger for the transition of ribonuclease *a* complexed with the cytidine inhibitor. Repeated scans of the uncomplexed and complexed solutions showed that the transitions of both the complexed and uncomplexed ribonuclease *a* are reversible, although the degree of irreversibility depends on the upper temperature limit of the thermal scan.

The results obtained from analysis of the DSC scans of the uncomplexed ribonuclease *a* in sodium acetate buffered solutions are summarized in Table I. Since there is no correlation between the pH and the heat capacity change of the solution and the cooperativity of the transition in Table I, average values of these properties are also presented in the table. Similar results on the denaturation transition of ribonuclease *a* complexed with cytidine 3′-monophosphate and with uridine 3′-monophosphate in 0.2 M sodium acetate buffered solutions are presented respectively in Tables II and III. Table IV contains results on the denaturation transition of ribonuclease *a* complexed with cytidine 3′-monophosphate in 0.05 M buffered sodium acetate solutions. Average values of the transition cooperativities and heat capacity changes of the complexed solutions at high inhibitor concentrations are also presented in Tables II–IV.

From comparisons of the results between Table I and Tables II–IV, thermodynamic information was obtained on the binding of the cytidine and uridine derivatives to ribonuclease *a* at the denaturation temperature. At each pH, the transition temperatures are higher in the inhibitor-containing solutions than in the inhibitor-free ribonuclease *a* solutions, indicating ribonuclease *a*–inhibitor complex formation from pH 4 to pH 7. Also presented in Tables II–IV are the initial inhibitor

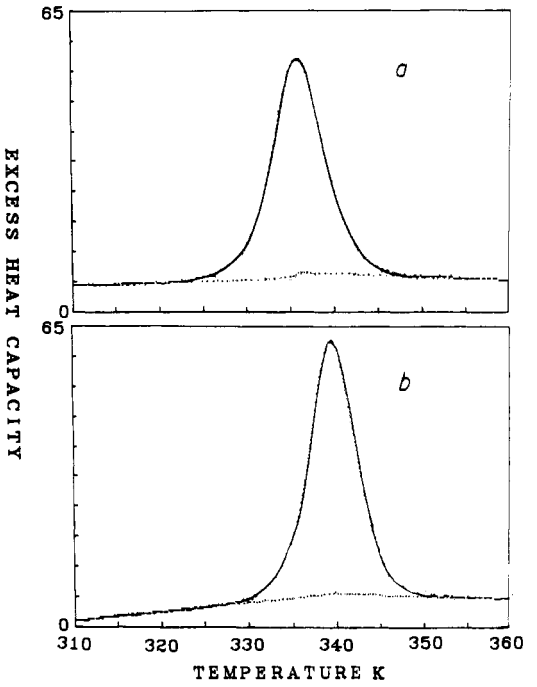

FIGURE 1: (a) DSC thermal scan of a 0.5-g sample of 1.43 mM ribonuclease *a* in 0.2 M sodium acetate buffer at pH 6.0 from Table I. (b) DSC thermal scan of a 0.5-g sample of 1.43 mM ribonuclease *a* and 41.7 mM cytidine 3′-monophosphate in 0.2 M sodium acetate buffer at pH 6.0 from Table II.

concentrations (I_0), the binding constants (K_b) calculated from eq 4, the enthalpy calculated from the temperature dependence of the enthalpy of the uncomplexed ribonuclease *a* transition as described below (ΔH), and the difference between the calculated uncomplexed and complexed transition enthalpies at T_c. The initial ribonuclease *a* concentrations were calculated from the spectroscopically determined concentration of the original enzyme solution and the known mass of inhibitor added to the solution. The thermodynamic properties of the

Table II: Results of DSC Measurements of the Denaturation Transition of Ribonuclease *a* Complexed with Cytidine 3′-Monophosphate in 0.2 M Sodium Acetate Buffered Solutions

pH	initial concn (mM) rib.	inh	T_c (K)	ΔC_p (kJ mol^{-1} K^{-1})	η	ΔH_c (kJ mol^{-1})	K_b (M^{-1})	ΔH (kJ mol^{-1})	$\Delta H_c - \Delta H$ (kJ mol^{-1})
4.0	1.34	17.8	331.4	3.95 ± 1.89	0.825 ± 0.045	403 ± 5	267 ± 2	408	0
4.0	1.39	24.4	333.0	5.98 ± 0.89	0.876 ± 0.015	406 ± 3	442 ± 2	442	
4.5	1.53	47.1	337.0	4.52	0.937	462	160	457	6
4.7	1.31	37.1	339.0	4.72 ± 0.21	0.949 ± 0.032	491 ± 13	739 ± 88	474	17 ± 13
5.0	1.39	40.9	340.7	4.67 ± 2.07	0.968 ± 0.024	525 ± 10	1030 ± 104	489	36 ± 10
5.0	1.32	41.2	341.2	2.60 ± 1.03	1.071 ± 0.024	566 ± 4	1790 ± 46	493	73 ± 5
5.5	1.34	43.3	341.9	5.24 ± 1.18	1.022 ± 0.024	583 ± 11	840 ± 56	499	85 ± 11
5.5	1.36	44.8	342.0	1.05	1.033	557	770	500	57
5.5	1.45	48.3	341.9	5.99 ± 0.13	1.042 ± 0.003	582 ± 3	747 ± 15	499	84 ± 3
6.0	1.43	4.1	338.5	6.07 ± 0.93	0.916 ± 0.037	474 ± 4	738 ± 10	469	5 ± 4
6.0	1.18	10.4	334.0	3.27 ± 1.51	0.855 ± 0.012	470 ± 3	638 ± 44	482	
6.0	1.14	14.3	340.4	5.03 ± 3.37	0.879 ± 0.005	471	557 ± 17	485	
6.0	1.21	18.0	340.5	1.97 ± 0.08	0.831 ± 0.021	475 ± 4	502 ± 25	487	
6.0	1.12	30.6	341.0	6.06 ± 0.38	1.034 ± 0.040	568 ± 14	626 ± 50	491	77 ± 14
6.0	1.31	37.2	341.4	2.67 ± 0.20	0.964 ± 0.016	559 ± 5	601 ± 2	494	65 ± 5
6.0	1.36	38.6	341.2	10.5 ± 2.39	0.948 ± 0.006	544 ± 4	502 ± 3	493	51 ± 5
6.0	1.43	41.7	341.3	6.10 ± 1.35	0.987 ± 0.023	581 ± 1	594 ± 2	493	88 ± 1
6.0	1.37	46.5	341.6	4.50 ± 1.27	1.034 ± 0.034	585	635 ± 20	496	90
6.0	1.45	41.3	341.4	4.42 ± 0.94	0.997 ± 0.004	572 ± 2	585 ± 26	494	78 ± 2
6.5	0.97	40.1	340.1	2.99 ± 0.10	0.923 ± 0.018	525 ± 5	218 ± 5	483	42 ± 5
6.5	1.38	40.9	339.8	5.88 ± 1.74	0.998 ± 0.023	560 ± 6	210 ± 6	481	80 ± 6
6.5	1.33	46.3	340.0	3.17 ± 1.95	0.859 ± 0.040	540 ± 9	185 ± 13	482	58 ± 8
7.0	1.52	46.7	339.0	0.57	0.790	535	90.4	473	62
7.0	1.23	47.1	338.8	3.10 ± 0.45	1.006 ± 0.004	551 ± 2	91.6 ± 4	472	79 ± 2
7.0	1.04	47.2	338.9	1.95 ± 0.72	0.900 ± 0.007	537 ± 2	89.6 ± 1	473	64 ± 2
average[b]				3.74 ± 1.87	0.971 ± 0.065				

[a] Entries with error limits are the average results from two deteriminations. The sample size was about 0.5 g, and the scan rate was 18 K h^{-1}. The error in ΔC_p, ΔH_c, η, K_b, and $\Delta H_c - \Delta H$ is the average deviation, and the average deviation in T_c is ± 0.1 K. $K_b = (\exp[(T_c - T_0)\Delta H_c/RT_cT_0] - 1.00)/([I_0] - [R_0]/2)$, where T_0 = 327.5 K at pH 4.0, 332.7 K at pH 4.5 and 4.7, 334.0 at pH 5.0, and 336.0 K at pH 5.5, 6.0, 6.5, and 7.0; R = 8.314 J mol^{-1} K^{-1}; $[I_0]$ is the initial inhibitor concentration; and $[R_0]$ is the initial ribonuclease *a* concentration. [b] The average values were averaged over all the determinations with inhibitor concentrations >30 mM.

Table III: Results of DSC Measurements of the Denaturation Transition of Ribonuclease *a* Complexed with Uridine 3′-Monophosphate in 0.2 M Sodium Acetate Buffered Solutions[a]

pH	initial concn (mM) rib.	inh	T_c (K)	ΔC_p (kJ mol^{-1} K^{-1})	η	ΔH_c (kJ mol^{-1})	K_b (M^{-1})	ΔH (kJ mol^{-1})	$\Delta H_c - \Delta H$ (kJ mol^{-1})
5.5	1.44	45.5	341.8	2.15 ± 0.47	0.859 ± 0.010	485 ± 1	402 ± 7	498	
6.0	1.53	47.2	341.6	5.99 ± 1.61	0.890 ± 0.004	508 ± 2	392 ± 6	496	12 ± 2
6.0	1.44	47.5	341.2	3.42 ± 1.29	0.872 ± 0.003	512 ± 1	327 ± 16	493	19 ± 2
6.5	1.57	45.9	340.4	6.25 ± 2.21	0.884 ± 0.001	512 ± 2	209 ± 4	485	27 ± 3
6.5	1.45	46.6	340.2	3.17 ± 1.90	0.930 ± 0.048	519 ± 13	190 ± 18	484	36 ± 13
7.0	1.44	47.7	339.3	2.98	0.920	510	104	476	34
7.0	0.98	48.1	339.3	1.03 ± 0.21	0.882 ± 0.051	515 ± 2	105 ± 1	476	39 ± 2
average				3.76 ± 2.42	0.889 ± 0.038				

[a] Each entry with the exception of 1.44 mM enzyme at pH 7.0 is the average of two determinations. The sample size was about 0.5 g, and the scan rate was 18 K h^{-1}. The error in ΔC_p, η, K_b, and $\Delta H_c - \Delta H$ is the average deviation, and the average deviation in T_c is ±0.1 K. $K_b = (\exp[(T_c - T_0)\Delta H_c/RT_cT_0] - 1.00)/([I_0] - [R_0]/2)$, where T_0 = 336.0 K at pH 5.5, 6.0, 6.5, and 7.0; R = 8.314 J mol^{-1} K^{-1}; $[I_0]$ is the initial inhibitor concentration; and $[R_0]$ is the initial ribonuclease *a* concentration.

denaturation transition of the complexed and uncomplexed ribonuclease *a* are discussed in more detail below.

Cooperativity of the Denaturation Transition. In Tables I–IV, the transition cooperativities are 0.899 ± 0.053 for the ribonuclease *a* in 0.2 M sodium acetate buffer, 0.971 ± 0.065 for the cytidine-complexed ribonuclease *a* in 0.2 M sodium acetate buffer at $[I_0] > 30$ mM, 0.889 ± 0.038 for the uridine-complexed ribonuclease *a*, 0.970 ± 0.041 for the ribonuclease *a* in 0.05 M buffer, and 0.969 ± 0.039 for the cytidine-complexed ribonuclease *a* in 0.05 M sodium acetate buffer at $[I_0] > 13$ mM. The cooperativities of the cytidine complexes and of ribonuclease *a* in 0.05 M sodium acetate buffer are within experimental error of one, which indicates that the denaturation transition in these solutions is a two-state transition with a stoichiometry of 1. Since the cooperativities of the uncomplexed ribonuclease *a* in the 0.2 M buffer and the uridine complex solutions are within experimental error of the cooperativities of the other solutions, the stoichiometry of the two-state transitions occurring in these solutions is assumed to be also 1 in the discussion to follow.

The lack of a change in the transition cooperativity upon binding of the inhibitor to the ribonuclease *a* is at variance with the results of kinetic studies on the same system. Kinetic studies, using the temperature jump method, indicate that binding of the cytidine or uridine inhibitor to the ribonuclease *a* involves an isomerization of the ribonuclease *a*–inhibitor complex (Hammes & Walz, 1969). An increase in the cooperativity would arise from isomerization of ribonuclease *a* back to its inhibitor-free state as the inhibitor breaks away from the enzyme during denaturation. A possible explanation is that the isomerization involves essentially no enthalpy change and is, thus, undetectable by DSC.

Table IV: Results of DSC Measurements of the Denaturation Transition of Ribonuclease *a* Complexed with Cytidine 3′-Monophosphate in 0.05 M Sodium Acetate Buffered Solutions[a]

pH	initial concn (mM) rib.	inh	T_c (K)	ΔC_p (kJ mol^{-1} K^{-1})	η	ΔH_c (kJ mol^{-1})	K_b (M^{-1})	ΔH (kJ mol^{-1})	$\Delta H_c - \Delta H$ (kJ mol^{-1})
5.0	1.01	50.4	340.8	5.46 ± 2.58	1.024 ± 0.045	499 ± 16	1440 ± 305	490	9 ± 14
5.5	1.01	6.8	339.2	11.38	0.859	453	2080	475	
5.5	1.00	13.5	340.4	11.29 ± 1.78	0.945 ± 0.042	469 ± 11	2020 ± 209	486	
5.5	0.861	27.1	341.5	5.98	0.962	520	2680	498	22
6.0	0.861	18.9	340.5	5.28 ± 0.74	0.971 ± 4	351	1300 ± 41	488	42
6.0	0.795	21.4	340.4	4.03 ± 2.39	0.946 ± 0.013	534	1020 ± 110	487	47
6.5	0.927	11.8	338.4	13.7	0.951	499	299	467	31
6.5	0.839	14.9	339.0	6.86 ± 1.73	0.960 ± 0.013	516 ± 5	360 ± 4	473	43 ± 5
6.5	0.744	18.0	338.9	4.82	0.969	522	294	472	49
average[b]				6.39 ± 3.08	0.969 ± 0.039				

[a] Entries with error limits are averaged over two determinations. The sample size was about 0.5 g, and the scan rate was 18 K h^{-1}. The error in ΔC_p, ΔH_c, η, K_b, and $\Delta H_c - \Delta H$ is the average deviation, and the average deviation in T_c is ±0.1 K. $K_b = (\exp[(T_c - T_0)\Delta H_c/RT_cT_0] - 1.00)/([I_0] - [R_0]/2)$, where T_0 = 332.7 K at pH 5.0, 333.7 K at pH 5.5, 334.8 K at pH 6.0, and 335.6 K at pH 6.5; R = 8.314 J mol^{-1} K^{-1}; $[I_0]$ is the initial inhibitor concentration; and $[R_0]$ is the initial ribonuclease *a* concentration. [b] $I_0 > 13$ mM.

Heat Capacity Change of the Solution upon Denaturation. Upon denaturation, the average heat capacity increases in the 0.2 M sodium acetate buffer are as follows: 3.78 ± 2.18 kJ mol^{-1} K^{-1} for the uncomplexed ribonuclease *a* solutions, 3.74 ± 1.87 kJ mol^{-1} K^{-1} for the cytidine-complexed ribonuclease *a* solutions at inhibitor concentrations (Table II), and 3.76 ± 2.42 kJ mol^{-1} K^{-1} for the uridine-complexed ribonuclease *a* solutions (Table III). The heat capacity change for the binding reaction from eq 6 is, thus, within a probable error of 2.87 kJ mol^{-1} K^{-1} $[(2.18^2 + 1.87^2)^{1/2}]$ for the cytidine inhibitor and 3.26 kJ mol^{-1} K^{-1} for the uridine inhibitor. The heat capacity increases are larger, however, in the 0.05 M sodium acetate buffer, 7.24 ± 3.03 kJ mol^{-1} K^{-1} for the uncomplexed ribonuclease *a* solutions and 6.39 ± 3.08 kJ mol^{-1} K^{-1} for the cytidine-complexed ribonuclease *a* solutions at $[I_0] > 13$ mM. For the 0.05 M sodium acetate buffered solutions, the heat capacity change resulting from binding of the cytidine inhibitor to the enzyme is within a probable error of 4.24 kJ mol^{-1} K^{-1} in eq 6. Eftink et al. (1983) determined a heat capacity change for the binding of the cytidine inhibitor to ribonuclease *a* of −0.732 ± 0.063 kJ mol^{-1} K^{-1} at μ = 0.2 and of −0.942 ± 0.063 kJ mol^{-1} K^{-1} at μ = 0.05. Both values are well within the probable error of the binding heat capacity change determined by eq 6.

From DSC measurements of the heat capacity increases of four globular protein solutions upon denaturation, Privalov et al. (1974) found that the increase is proportional to the number of contacts between elementary nonpolar groups in the folded state of the protein. It was concluded that the increase in the heat capacity of the solution results essentially from breakage of the nonpolar contacts and interaction of the nonpolar groups with water as the protein unfolds. Any reduction in the number of contacts between the nonpolar groups of ribonuclease *a* such as through weak binding of the acetate anions to the protein would reduce the change in the heat capacity upon denaturation. Closer scrutiny of Table I at pH 4.5 and pH 5.9 shows that the denaturation temperature is higher by respectively 4.3 and 1 K in the 0.2 M buffer solutions, which can be attributed to weak association between the acetate ions and the positive charge centers of ribonuclease *a*. This weak association would account for the smaller heat capacity changes in the heat capacity of the 0.2 M sodium acetate buffered solutions.

Determination of the Binding Constants at the Transition Temperature. The application of eq 4 to the determination of the binding constants can yield meaningful results only if the inhibitor does not bind to the denatured state of ribonuclease *a*. To determine if this is the case and if the stoichiometry of the inhibitor–ribonuclease *a* complex is 1:1, a series of equilibrium dialysis measurements were performed with cytidine 3′-monophosphate in the presence of ribonuclease *a* in the native and denatured state. The results of the measurements are presented in Table V where the molar decrease in the original inhibitor concentration is equal to the molar concentration of ribonuclease *a* concentration in the sample. This equality shows that the inhibitor binds in a 1:1 ratio with ribonuclease *a* in solution, which was also demonstrated by Anderson et al. (1968) for binding of the inhibitors to ribonuclease *a* in buffered 0.05 M Tris, 0.05 M sodium acetate, and 0.1 M KNO_3 solutions. Following irreversible denaturation of the cytidine-complexed ribonuclease *a*, the equilibrium concentration of the cytidine inhibitor is the same as the original inhibitor concentration. This can only occur if the cytidine does not bind to the irreversible form of the denatured state, and thus, it is assumed that the inhibitor does not bind to the reversible form of the denatured state. Since the uridine inhibitor is similar in structure to the cytidine, it is also assumed that the uridine inhibitor does not bind to the reversible form of the denatured state of ribonuclease *a*.

Table V: Results of Equilibrium Dialysis Experiments on Ribonuclease *a* Equilibrated with Cytidine 3′-Monophosphate in 0.2 M Sodium Acetate Buffer at pH 6.0

initial concn of inhibitor (mM)	equil concn of inhibitor (mM)	difference between initial and equil concn	ribonuclease *a* concn (mM)	concn of inhibitor following denaturation
2.38 ± 0.02	2.20 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	2.34 ± 0.01
1.96 ± 0.02	1.65 ± 0.01	0.30 ± 0.02	0.31 ± 0.01	2.01 ± 0.01
2.35 ± 0.02	2.16 ± 0.01	0.19 ± 0.02	0.19 ± 0.01	2.37 ± 0.01

Since the free-inhibitor concentration I in eq 4 is not known, but the binding constants are high (Anderson et al., 1968), the free-inhibitor concentration is approximated by

$$I \simeq I_0 - R_0/2 \quad (9)$$

at initial inhibitor concentrations, I_0, greater than the initial ribonuclease *a* concentration, R_0. At the denaturation temperature where ΔG(denaturation transition) = 0, the native ribonuclease *a* concentration is half the original concentration. Equation 4, thus, becomes

$$K_b(T_c) = [\exp\{(T_c - T_0)\Delta H_c/RT_cT_0\} - 1.00]/([I_0] - [R_0]/2) \quad (10)$$

The binding constants calculated from eq 10 are presented in Tables II–IV. Values for T_0 at each pH are the best average

Table VI: Thermodynamic Properties of Ribonuclease *a* Complexed with Cytidine 3′-Monophosphate[a]

pH	max T_c (K)	$K_b(T_c)$ (M^{-1})	$-\Delta H_b(T_c)$ (kJ mol^{-1})	lit. $-\Delta H_b$ (kJ mol^{-1}) 317.2 K	lit. $-\Delta H_b$ (kJ mol^{-1}) 298.2 K	K_b, 298.15 K ($\times 10^3$ M^{-1})	lit. K_b, 298.2 K ($\times 10^3$ M^{-1})	
			In 0.2 M Sodium Acetate Buffer					
4.0	333.0	355 ± 124						
4.7	339.0	739 ± 88	17 ± 13					
5.0	341.0 ± 0.4	1410 ± 54	55 ± 23		16 ± 4	3.1–32.1		7.53
5.5	342.0 ± 0.1	786 ± 48	79 ± 15	52	20 ± 5	9.1–42.8	26.8*	12.1
6.0	341.3 ± 0.2	598 ± 70	73 ± 16		54 ± 14	6.8–24.2		9.68
6.5	339.9 ± 0.2	205 ± 18	60 ± 18		67 ± 17	0.75–4.45	9.80*	5.23
7.0	338.8 ± 0.2	91 ± 3	70 ± 10		48 ± 12	0.82–2.0		1.61
			In 0.05 M Sodium Acetate Buffer					
5.0	340.8	1970 ± 156	9 ± 14		31 ± 3			
5.5	341.5	3905 ± 60	22	59	36 ± 4	4.05	39	
6.0	340.5 ± 0.1	1505 ± 216	45 ± 3		32 ± 3			
6.5	338.9	394 ± 61	45 ± 7		41 ± 4			

[a] The two literature values of ΔH_b at 317.2 K are from Eftink et al. (1983). The literature values of ΔH_b at 298.2 K for the 0.2 M sodium acetate buffered solutions are for solutions of ionic strength 0.2 (Hammes & Schimmel, 1965). An error of 25% was assumed for the literature values since they were based on equilibrium constants determined with an estimated error of 25%. The literature values of ΔH_b for the 0.05 M sodium acetate buffered solutions are from Table I in Flogel et al. (1975). The literature values of K_b at 298.2 K for the 0.2 M sodium acetate buffered solutions are for buffered solutions of ionic strength 0.2 consisting of 0.05 M Tris, 0.05 M sodium acetate, and either 0.1 M KCl (denoted by *) or 0.1 M KNO_3 (Anderson et al., 1968), while the one value for the 0.05 M sodium acetate buffered solution is from Bolen et al. (1971).

Table VII: Summary of the Thermodynamic Properties of Ribonuclease *a* Complexed with Uridine 3′-Monophosphate in 0.2 M Sodium Acetate Buffered Solutions[a]

pH	max T_c (K)	$K_b(T_c)$ (M^{-1})	$-\Delta H_b(T_c)$ (kJ mol^{-1})	lit. K_b, 298.2 K ($\times 10^3$ M^{-1})
5.5	341.8	485 ± 1		
6.0	341.4 ± 0.2	360 ± 40	16 ± 5	12.2
6.5	340.3 ± 0.1	200 ± 19	32 ± 12	6.06
7.0	339.3 ± 0.1	104 ± 1	37 ± 4	2.27

[a] The literature values were determined in ionic strength 0.2 buffered solutions consisting of 0.05 M Tris, 0.05 sodium acetate, and 0.1 M KCl (Anderson et al., 1968).

values of the transition temperatures presented in Table I. For the 0.2 M sodium acetate buffered solutions, the average values of T_0 were 337.5 K at pH 4.0, 332.7 K at pH 4.5 and 4.7; 334.0 at pH 5.0, and 336.0 K at pH 5.5, 6.0, 6.5, and 7.0. For the 0.05 M sodium acetate buffered solutions, the average values of T_0 were 332.7 K at pH 5.0, 333.7 K at pH 5.5, 334.8 K at pH 6.0, and 335.6 K at pH 6.5.

The average binding constants at each pH level are summarized in Table VI for the cytidine inhibitor solutions and in Table VII for the uridine inhibitor solutions. The error in the binding constants ranges from 0.2% for the uridine inhibitor at pH 5.5 to 33% (pH 4.0 in the 0.2 M sodium acetate buffer) in Tables VI and VII. For the 0.2 M cytidine complex solutions, the binding constants were also determined as a function of the initial inhibitor concentration at pH 6.0. Despite an increase in the initial inhibitor concentration by more than an order of magnitude, all the binding constants are within 23% of the average binding constant at this pH. The calculation of K_b from eq 10 depends more critically on the choice of T_0. For example, the binding constant for the 0.2 M sodium acetate buffered solutions at pH 5.5 is 2657 ± 387 M^{-1} for T_0 = 334 K and 86 ± 48 M^{-1} for T_0 = 336 K.

In Table VI, the binding constants increase from 355 ± 124 M^{-1} at pH 4.0 and 87 ± 8 M^{-1} at pH 7.0 to a maximum of 1410 ± 54 M^{-1} at pH 5.0 in the 0.2 M sodium acetate buffered solutions. A maximum in the binding constant also occurs at 298.15 K but at pH 5.5 (Hammes & Schimmel, 1965; Anderson et al., 1968). This increase in the pH of the maximum with decrease in temperature continues to 280.15 K where the maximum occurs at pH 6.0 (Hammes & Schimmel, 1965). This pH shift is attributed to the temperature dependence of the pK of an imidazole side chain involved in the binding process. The K_b values summarized in Table VII for binding of the uridine inhibitor to ribonuclease *a* in the 0.2 M sodium acetate buffered solutions are close to the corresponding values of K_b for the cytidine derivative. This has also been observed at room temperature (Anderson et al., 1968) and is attributed to the involvement of the same two groups having pK values of approximately 5 and 6.5 on ribonuclease *a* in the binding of the inhibitor to the enzyme.

In Table VI, the K_b values summarized for the 0.05 M sodium acetate buffered solutions increase from 1440 ± 305 M^{-1} at pH 5.0 and 328 ± 37 M^{-1} at pH 6.5 to a maximum value of 2200 ± 364 M^{-1} at pH 5.5. The K_b values in the 0.05 M sodium acetate buffered solutions are at least a factor of 2 greater than the corresponding values at 0.2 M sodium acetate buffer concentration. This is consistent with the increase of K_b from 5.3 × 10^3 M^{-1} at 0.50 M sodium acetate buffer concentration to 36 × 10^3 M^{-1} at 0.05 M sodium acetate buffer concentration observed at 298.15 K (Bolen et al., 1971). Bolen et al. (1971) attributed this increase in K_b to either ionic strength effects on the pK_a of the ionizable groups involved in the binding reaction or to a decrease in counterion shielding of the electrostatic interaction between the phosphate group of the inhibitor and the histidine residues on ribonuclease *a*.

Determination of the Enthalpy of Binding at the Denaturation Temperature. The high binding constants in Tables VI and VII indicate that more than 90% of the native ribonuclease *a* at T_c is in the complexed form at the highest inhibitor concentrations in the pH range from 4.0 to 6.5. Since the inhibitor does not bind to the denatured state, denaturation of the complex results in cleavage of the ribonuclease *a*–inhibitor bonds. The denaturation enthalpy must, thus, include a contribution from the inhibitor binding enthalpy. The binding enthalpy (eq 1) was determined from eq 2 and 5 and the denaturation enthalpy of the uncomplexed ribonuclease *a*.

$$R = D \qquad \Delta H(T_c) \qquad (11)$$

The denaturation transition of the uncomplexed ribonuclease *a* occurs, however, only at lower temperatures, T_0.

To determine ΔH at the denaturation temperature of the complex $\Delta H(T_c)$ from $\Delta H(T_0)$, the transition enthalpies of the uncomplexed ribonuclease *a* presented in Table I were plotted as a function of the transition temperatures. Least-

squares fits of the enthalpy data to the transition temperatures for the denaturation of the uncomplexed ribonuclease *a* are best represented by the following linear equations:

$$\Delta H(T_0) = 417 \pm 4 + (9.71 \pm 1.3)(T_0 - 333.2) \text{ kJ mol}^{-1} \quad (12)$$

for the 0.05 M buffer solutions and

$$\Delta H(T_0) = 424 \pm 4 + (8.53 \pm 1.1)(T_0 - 333.2) \text{ kJ mol}^{-1} \quad (13)$$

for the 0.2 M buffer solutions. At a maximum temperature of 341.9 K, the error in the fit of $\Delta H(T_0)$ to T_0 is 3.7% in eq 12 and 2.6% in eq 13. For the unfolding of globular proteins, the derivative $d(\Delta H)/dT_0$ was observed to be the same as the change in the heat capacity of the solution upon denaturation (Privalov & Khechinashvili, 1974). This is also observed in the denaturation of the complexes where $d(\Delta H)/dT_c = 9.71 \pm 1.3$ kJ mol^{-1} in eq 12 is within experimental error of $\Delta C_p = 7.24 \pm 3.03$ kJ mol^{-1} K^{-1} for the 0.05 M sodium acetate buffered solutions and $d(\Delta H)/dT_c = 8.53 \pm 1.1$ kJ mol^{-1} K^{-1} in eq 12 is near the upper limit of $\Delta C_p = 3.78 \pm 2.18$ kJ mol^{-1} K^{-1} for the 0.2 M sodium acetate buffered solutions.

The binding enthalpies determined from eq 5 are presented in Tables II–IV. Average values of the binding enthalpies are summarized as a function of pH in Tables VI and VII. Enthalpy differences, $\Delta H_c - \Delta H$, which are within the calculated error of eq 13 such as at initial inhibitor concentrations <30 mM at pH 6.0 in Table II and at pH 5.5 in Table III were not averaged with the other values at the same pH level since apparently not all the ribonuclease *a* is complexed with the inhibitor at these low concentrations. Similarly, the enthalpy differences at initial inhibitor concentrations below 14 mM in the 0.05 M sodium acetate solutions were also not included in the averages. Literature values of the binding enthalpies are also presented in Table VI for comparison. The room temperature literature values at 0.2 M buffer concentration were determined from measurements of the ribonuclease *a*–cytidine-binding constant in solutions of ionic strength 0.2 at 280.1 and 298.1 K (Hammes & Schimmel, 1965). Since the error in the binding constants was estimated to be 25% (Hammes & Schimmel, 1965), an error of 25% is assumed for the literature values of ΔH_b. For the 0.05 M buffer solutions, the literature values at room temperature were determined from mixing cytidine solutions with ribonuclease *a* solutions in a flow microcalorimeter and are the binding enthalpies of cytidine to ribonuclease *a* from Table I in Flogel et al. (1975). The literature values at 317.2 K and at $\mu = 0.05$ and 0.2 were determined from batch calorimetry measurements (Eftink et al., 1983). In Table VI, the DSC-determined binding enthalpies for the 0.2 M buffered solutions are within experimental error of the room temperature literature values at pH ≥6.0 and the literature value of 52 kJ mol^{-1} at pH 5.0 and 317.2 K. The DSC-determined binding enthalpy at pH 6.5 is within experimental error of the room temperature literature value for the 0.05 M buffered solutions. Assuming that the decrease of -0.732 ± 0.063 mJ mol^{-1} K^{-1} in the heat capacity of the binding reaction of the cytidine inhibitor to ribonuclease *a* at pH 5.0 and $\mu = 0.2$ determined by Eftink et al. (1983) is the same up to pH 7.0, then the enthalpy should decrease by −32 kJ mol^{-1} from 298.2 to 342.2 K. In Table VI, a decrease in the average enthalpy values is observed over this temperature range for the 0.2 M buffered solutions at pH 5.0, 5.5, 6.0, and 7.0, and the magnitude of this decrease is close to −32 kJ mol^{-1} when the error of the DSC and literature values are taken into consideration. For the 0.05 M buffered solutions, a corresponding decrease in the binding enthalpies at pH 6.0 and 6.5 is, however, less than a calculated value of −41 kJ mol^{-1} based on a binding heat capacity change of -0.942 ± 0.063 kJ mol^{-1} at $\mu = 0.05$ and pH 5.0 from Eftink et al. (1983). An increase in the binding heat capacity change at pH 6.0 and 6.5 in the low ionic strength solutions would reduce the calculated decrease of −41 kJ mol^{-1}.

In Table VII, the binding enthalpies of uridine 3′-monophosphate are greater than the binding enthalpies of cytidine 3′-monophosphate by a factor of ~2 at pH 5.5–7.0 in the 0.2 M sodium acetate buffered solutions. However, the binding constants of the uridine inhibitor are close to the binding constants of the cytidine inhibitor at pH ≥6.0 and at T_c and 298.2 K. Apparently, the higher binding enthalpies of the uridine inhibitor are compensated by higher binding entropies of the uridine inhibitor. This enthalpy–entropy compensation has been discussed in detail by Eftink et al. (1983) for protein–liquid interactions. The binding enthalpies of the larger purine inhibitor adenosine 5′-monophosphate are also greater than the binding enthalpies of the cytidine inhibitor by almost a factor of 3 in the pH range 6.5–7.0 (Myer & Schellman, 1962). However, the binding constants from pH 6.5 to 7.0 are lower than those of the cytidine and uridine inhibitors, suggesting a different interaction mechanism with ribonuclease *a*.

Determination of the Binding Constants at 298.2 K. In Table VI, the binding constants were calculated at 298.2 K with the DSC-determined binding enthalpies and constants from the equation:

$$\ln [K_b(T)/K_b(T_c)] = [(-\Delta H_b(T_c) + \Delta C_{pb} T_c) \times (1/T - 1/T_c) + \Delta C_{pb} \ln (T/T_c)]/R \quad (14)$$

where $T = 298.2$ K and values for ΔC_{pb} were determined at pH 5.0 by Eftink et al. (1983). Since the error in the $\Delta H_b(T_c)$ determinations is quite large, a range of calculated K_b values is presented in Table VI. The literature values from Anderson et al. (1968) exhibit a 2-fold increase in the cytidine-binding constant upon substituting 0.1 M KCl for 0.1 M KNO_3 in the 0.2 ionic strength solutions at pH 5.5 and 6.5. Since Bolen et al. (1971) found that the ionic strength effect on the magnitude of the cytidine inhibitor binding constant was the same for sodium acetate and KCl at $\mu = 0.05$–0.50, the calculated DSC-binding constants at 298.2 K should be compared to the literature values measured in the presence of 0.1 M KCl in Table VI. If a 2-fold increase in the binding constant is assumed for the literature values at pH 5.0, 6.0, and 7.0 in the 0.2 M buffered solutions, then the literature values at pH 5.0–6.0 are within range of the calculated DSC K_b at 298.2 K. The discrepancy between the literature values and the range of DSC values at 298.2 K at pH 6.5 and 7.0 may arise from the pH dependence of the binding heat capacity change (Eftink et al., 1983). The calculated DSC-binding constant at 298.2 K and pH 5.5 in the 0.05 M buffer solution is a factor of 10 less than the literature value. However, the calculated DSC-binding constant is based on one experimental determination of ΔH_b, and if the literature value for ΔH_b at 317.2 K is used, a binding constant (26×10^3 M^{-1}) close to the literature value is obtained. Calculations of the binding constants of the uridine inhibitor at 298.2 K were not performed since the binding heat capacity change for the uridine inhibitor is not known.

Conclusions

The DSC results show that the interaction of ribonuclease *a* with cytidine 3′-monophosphate at the denaturation temperature is essentially the same as the interaction at room

temperature in the 0.2 M sodium acetate buffered solutions. The binding enthalpies and constants at the denaturation temperature can be calculated from the binding constants, the binding enthalpies, and the heat capacity change of the binding reaction determined at room temperature. This appears to be true for the interaction at the lower ionic strength of 0.05, although measurements of the binding constant as a function of pH at 298.2 K and ΔC_{pb} as a function of pH are needed to verify this. Although the binding constants of uridine 3'-monophosphate to ribonuclease *a* are close to those of the cytidine inhibitor at the denaturation temperature and at room temperature, it is difficult to conclude that the interaction of the uridine inhibitor at the denaturation temperature is the same as at room temperature since the binding enthalpies and the heat capacity change accompanying this interaction are unavailable in the literature. Isomerization of the ribonuclease *a*-inhibitor complex following an initial association interaction between the inhibitor and the enzyme has not been observed in the DSC cooperativity determinations. Any enthalpy change resulting from the isomerization may be too small to be detected by the DSC.

Registry No. Cytidine 3'-monophosphate, 84-52-6; uridine 3'-monophosphate, 84-53-7; ribonuclease, 9001-99-4.

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Inhibition of Glucocorticoid Receptor Transformation, Subunit Dissociation, and Temperature-Dependent Inactivation by Various N-Substituted Maleimides†

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ABSTRACT: A series of N-substituted maleimides were synthesized, and their effect on the activation to the DNA binding state of the rat liver glucocorticoid receptor was studied. Unactivated (preincubated at 0 °C) cytosolic [^{3}H]triamcinolone acetonide–receptor complexes were pretreated with various *N*-alkylmaleimides at 0 °C and then heated at 25 °C and assayed for DNA–cellulose binding. No inhibition of the DNA binding activity was observed with either *N*-ethylmaleimide or N-substituted maleimides bearing an ionizable substituent, like *N*-(ω-carboxyalkyl)maleimides and *N*-[2-(trimethylammonio)ethyl]maleimide. On the contrary, treatment with long-chain alkylmaleimides like *N*-heptylmaleimide resulted in significant inhibition. The highest inhibition was obtained with *N*-benzylmaleimide and, to a lesser extent, *N*-(ethylphenyl)-maleimide, whereas *N*-benzylsuccinimide was ineffective. Treatment of cytosol containing unactivated glucocorticoid complexes at 3 °C with *N*-benzylmaleimide also prevents the temperature-mediated conversion of 8S receptor to 4S. Moreover, *N*-benzylmaleimide was able to inhibit the inactivation of the receptor steroid-binding activity caused by heat. *N*-Benzylmaleimide shares with molybdate ions the ability to inhibit glucocorticoid receptor activation, dissociation, and inactivation. However, their respective mechanisms of action are probably distinct, since their effects on receptor inactivation appear additive. It is suggested from the comparison of the various maleimides tested that the sulfhydryl groups essential for receptor activation and dissociation lie in a rather nonpolar environment including aromatic amino acid(s).

Sulhydryl groups are involved in two very important functions of the glucocorticoid receptor, i.e., steroid binding and subsequent acquisition of DNA binding activity by the steroid–receptor complex (Rousseau, 1984). Treatment of unliganded receptors with sulfhydryl-modifying reagents prevents subsequent binding of glucocorticoid (Rees & Bell, 1975; Young et al., 1975). Moreover, the same reagents, when added to preformed steroid–receptor complexes, impede their

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