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THE EFFECT OF AMMONIUM SULFATE ON THE ACTIVITY OF RIBONUCLEASE A

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SUMMARY

We have examined the effect of $(NH_4)_2SO_4$ upon (a) ribonuclease A activity at pH 5.5 at non-saturating concentrations of U–C, C–A, and 2′,3′-CMP and (b) the kinetic parameters for the hydrolysis of 2′,3′-CMP at pH 5.5.

The effects of $(NH_4)_2SO_4$ on transferase and hydrolytic activity at non-saturating substrate concentrations at pH 5.5 are similar. In contrast to results obtained previously at pH 7.0 [Winstead, J. A. and Wold, F. (1965) J. Biol. Chem. 240, PC 3694–3696], there is little inactivation at high $(NH_4)_2SO_4$ concentrations.

At both pH 5.5 and 7.0, the maximum K_m for 2′,3′-CMP occurs at intermediate $(NH_4)_2SO_4$ concentrations. At pH 5.5, V also decreases 10-fold as the $(NH_4)_2SO_4$ concentration increases. This effect is less pronounced at pH 7.0.

These results can be rationalized in terms of the catalytic mechanism without invoking conformational changes.

INTRODUCTION

Winstead and Wold [1] have reported that, while low concentrations of $(NH_4)_2SO_4$ and Na_2SO_4 inhibit the hydrolytic activity of ribonuclease A at pH 7.0, high concentrations of these salts activate the enzyme. These authors have suggested that the activation may reflect a change in conformation. This possibility is highly significant, since the crystal structure of ribonuclease S was determined in the presence of 3 M $(NH_4)_2SO_4$.

In order to rationalize these observations in terms of structure and catalytic mechanism, the effects upon K_m and V must be separated. Pittz et al [2] have reported that, at an unspecified pH, $(NH_4)_2SO_4$ and Na_2SO_4 affect only K_m, and have concluded that there is no evidence for a conformational change at high salt concentrations.

The crystal structure determination indicates that there is a sulfate-binding site between His-12 and His-119, the occupancy of which decreases as the pH is raised [3]. Hence, one would expect the effect of sulfate salts upon activity to be strongly pH-

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dependent. Since the crystal structure was determined at pH 5.5, it is particularly important that the effect at this pH be investigated. We have compared the effects of $(NH_4)_2SO_4$ at pH 5.5 and 7.0 and Cs_2SO_4 at pH 7.0 upon the kinetic parameters of the hydrolytic reaction, and have examined the effect of $(NH_4)_2SO_4$ at pH 5.5 on both transferase and hydrolytic activity.

MATERIALS AND METHODS

2′,3′-CMP (NH_4^+ salt) and ribonuclease A (Type II A) were obtained from Sigma Chemical Co., Tris and $(NH_4)_2SO_4$ (both enzyme grade) from Schwartz-Mann, and Cs_2SO_4 from Fisher. All other chemicals were reagent grade.

All solutions were prepared using glass-distilled water. The concentrations of solutions of ribonuclease A and 2′,3′-CMP were determined spectrophotometrically, using molar extinction coefficients of 9700 [4] and (at pH 2) $13 \cdot 10^6$ cm²/mole [5], respectively. 0.1 M ammonium acetate was used as the buffer at pH 5.5, 0.1 M Tris–acetate at pH 7.0.

Hydrolytic and transferase activity were assayed on a Carey 14 spectrophotometer using the assays of Crook et al [6] and Witzel and Barnard [7], respectively. A slit width of 2.6 mm was maintained throughout for the determinations of K_m and V. Several enzyme concentrations were tested under each set of conditions to verify that the observed velocity was proportional to enzyme concentration.

The molar difference extinction coefficients which were used to calculate turnover numbers in the hydrolytic reaction at pH 5.5 and 7.0 were $1.4 \cdot 10^6$ and $0.45 \cdot 10^6$ cm²/mole, respectively.

In order to compare assays at different sulfate concentrations, it is necessary to show that the molar difference extinction coefficients are independent of ionic strength. $(NH_4)_2SO_4$ alters the spectral properties of 2′,3′-CMP, U–C, C–A, and 3-CMP in a way which is consistent with a slight increase in the pK values of their amino groups. However, since the spectra of substrates and products are affected similarly, the molar difference extinction coefficients do not change [8].

RESULTS AND DISCUSSION

As shown in Fig. 1, the effects of $(NH_4)_2SO_4$ upon hydrolytic activity at low substrate concentrations are very different at pH 5.5 and 7.0; the inhibition of hydrolytic activity at low salt concentrations is much more pronounced at pH 5.5, and very little activation at high salt concentrations is observed. At pH 5.5, transferase activity shows the same dependence on salt concentration as hydrolytic activity. This is not surprising, since the interactions of cyclic phosphate and dinucleoside phosphate substrates with the enzyme are very similar [9]. However, in the light of these results, Winstead and Wold's observation, that the ability of ribonuclease A to hydrolyze RNA is completely eliminated in 2 M $(NH_4)_2SO_4$ [1] deserves further investigation. Quite possibly, this effect results from an interaction between sulfate and RNA, or an interference of sulfate with the assay system, rather than an interaction between sulfate and the enzyme.

The data shown in Fig. 2 indicate clearly that, at pH 5.5, the maximum value of K_m and the minimum value of V in the hydrolytic reaction occur at intermediate

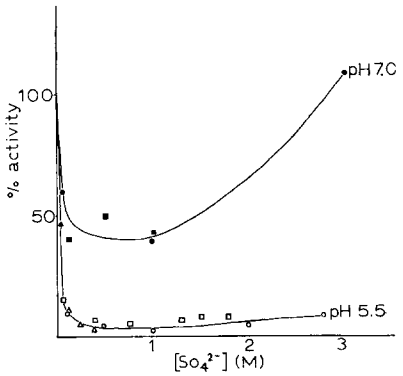

Fig. 1. The effect of sulfate upon the activity of ribonuclease A at pH 5.5 and 7.0 at non-saturating substrate concentrations. 2′,3′-CMP was used as substrate at pH 7.0, where points obtained in the presence of $(NH_4)_2SO_4$ and Cs_2SO_4 are indicated by the symbols ● and ■, respectively. The $(NH_4)_2SO_4$ data at pH 7.0 are taken from ref. 1. Only $(NH_4)_2SO_4$ was used at pH 5.5, and here the symbols refer to different substrates: 2′,3′-CMP (○), C–A (□) and U–C (△).

salt concentrations. K_m values and turnover numbers obtained by a least squares analysis of five sets of data are given in Table I. The overall variation in activity is less at pH 7.0 than at pH 5.5, making it difficult to resolve changes in K_m and V. However, the trends in these parameters appear to be similar at both pH values, although the variation in V is significantly more pronounced at pH 5.5. These effects appear to depend primarily upon SO_4^{2-}, since the effects of Cs_2SO_4 and $(NH_4)_2SO_4$ upon the kinetic parameters for the hydrolytic reaction at pH 7.0 are similar.

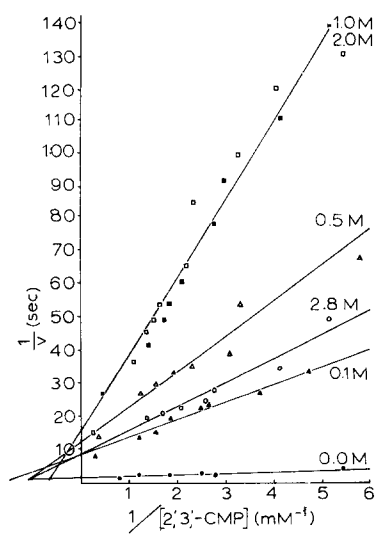

Fig. 2. Lineweaver–Burk plots of the ribonuclease A catalyzed hydrolysis of 2′,3′-CMP at various concentrations of $(NH_4)_2SO_4$ in 0.1 M ammonium acetate, pH 5.5. Enzyme concentration: $1.05 \cdot 10^{-7}$–$27.2 \cdot 10^{-7}$ M. $(NH_4)_2SO_4$ concentrations: ●, 0.0 M; ▲, 0.1 M; △, 0.5 M; ■, 1.0 M; □, 2.0 M; ○, 2.8 M.

TABLE I

KINETIC PARAMETERS FOR THE RIBONUCLEASE A-CATALYZED HYDROLYSIS OF 2′,3′-CMP AT pH 5.5 AT VARIOUS CONCENTRATIONS OF $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ concentration (M)	K_m (mM)	Turnover No. (s^{-1})
0.0	0.65 ± 0.25	1.1 ± 0.2
0.05	1.5 ± 1.0	0.4 ± 0.2
0.1	1.0 ± 0.9	0.16 ± 0.15
0.5	1.6 ± 0.8	0.09 ± 0.07
1.0	2.7 ± 1.0	0.05 ± 0.07
2.0	1.3 ± 0.3	0.05 ± 0.04
2.8	0.9 ± 0.5	0.12 ± 0.02

The difference in the magnitude of the effect of sulfate upon V at pH 5.5 and 7.0 is primarily responsible for the difference in the curves of hydrolytic activity at the two pH values given in Fig. 1. At pH 5.5, the 10-fold decrease in V almost swamps out the effect of the decrease in K_m at high $(NH_4)_2SO_4$ concentrations, while at pH 7.0, where there is no more than a 2-fold variation in V, the same decrease in K_m produces a substantial apparent activation.

The effects of SO_4^{2-} upon both K_m and V can be rationalized reasonably well in terms of the catalytic mechanism. The steady decrease in V with increasing salt concentrations can be attributed to the swamping out, with increasing ionic strengths, of the electrostatic interactions between the phosphate group of the substrate, the imidazole rings of His-12 and His-119, and the ε-amino group of Lys-41 [9]. The difference in the magnitude of the variation in V at pH 5.5 and 7.0 is consistent with the involvement of histidyl residues.

Since SO_4^{2-} binds at the active site at approximately the same position as the

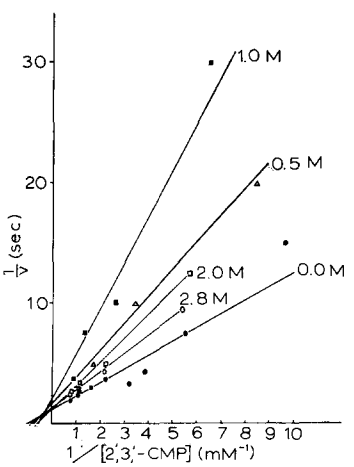

Fig. 3. Lineweaver–Burk plots of the ribonuclease A catalyzed hydrolysis of 2′,3′-CMP at various concentrations of $(NH_4)_2SO_4$ in 0.1 M Tris–acetate, pH 7.0. Enzyme concentration: $1.5 \cdot 10^{-7}$ M. $(NH_4)_2SO_4$ concentrations: ●, 0.0 M; △, 0.5 M; ■, 1.0 M; □, 2.0 M; ○, 2.8 M.

phosphate group of nucleotides [9], the increase in K_m with increasing salt concentration at low ionic strengths undoubtedly arises from the competition of SO_4^{2-} with substrates for the active site. The decrease at high salt concentrations indicates that, under these conditions, 2′,3′-CMP is able to compete more effectively with sulfate; i.e. high ionic strengths weaken the binding of sulfate much more than that of nucleotides. This is consistent with the crystallographic data which indicate that at pH 7.0 the sulfate binding site is only 25% saturated in the presence of 3 M $(NH_4)_2SO_4$ while under the same conditions, the dissociation constant for 3′-CMP is in the millimolar range [8]. High ionic strengths might be expected to weaken the binding of SO_4^{2-} more than that of nucleotides since the former involves only an electrostatic interaction while the latter also involves hydrogen bonds and nonpolar interactions [9].

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