DIVALENT METAL ION CATALYSIS OF THE OXIDATION OF RIFAMYCIN SV TO RIFAMYCIN S

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1. Introduction

The antibiotic, rifamycin SV, has been characterized as a potent inhibitor of the initiation of RNA synthesis by certain prokaryotic RNA polymerases [1]. The characteristics of inhibition by rifamycin SV resemble those described for the chelating agent, 1:10-phenanthroline [2], and binding studies indicate that both these inhibitors specifically prevent the low affinity interaction of prokaryotic RNA polymerase with purine nucleotides which occurs in the absence of added divalent metal ions [3]. Since prokaryotic RNA polymerase has been shown to be a zinc metalloenzyme [2], it seemed possible that inhibition by both rifamycin SV and 1:10-phenanthroline might result from interaction with the bound metal ion. During EPR studies designed to measure the affinity of rifamycin SV for Mn^{2+} it was noted that the colour of the test solutions changed from yellow to deep red during the period of observation. Since this colour change appeared dependent on addition of Mn^{2+}, further studies were performed to elucidate the nature of the effect. These studies have shown that certain divalent metal ions are effective catalysts of the oxidation of rifamycin SV to rifamycin S. The effect may explain the previous report that rifamycin SV is unstable in solution and undergoes spontaneous oxidation to rifamycin S [4].

2. Materials and methods

Rifamycin SV was obtained from Schwartz-Mann Inc. and 'Spec-pure' salts of the various metal ions from Johnson-Matthey Inc.

Spectra and difference spectra were taken on a Cary 14 double beam spectrophotometer. Rate measurements were made using a Gilford Model 240 spectrophotometer equipped in some cases with a Model 2445 Spectrospin accessory. The reaction rate was obtained either from the initial rate of the increase in absorbance at 536 nm or of the decrease in absorbance at 444 nm.

All other chemicals were A.R. grade reagents obtained from a reputable supply house.

3. Results and discussion

When the spectrum observed for a system containing 0.1 mM rifamycin SV + 0.1 mM $MnCl_2$ is compared using tandem cells with that of the individual components, a time-dependent appearance of a visible and ultraviolet difference spectrum is observed as illustrated in fig.1. The difference spectrum exhibits maxima at 266 nm, 320 nm, 358 nm and 536 nm and minima at 236 nm, 296 nm, 345 nm and 444 nm, while the final product of the reaction exhibits a spectrum with maxima at 313 nm and 520 nm. Isosbestic points are observed at 249 nm, 382 nm and 494 nm (fig.1). Several lines of evidence indicate that these spectral changes induced by addition of Mn^{2+} are due to oxidation of rifamycin SV to rifamycin S. First, the visible and ultraviolet spectrum of the product is identical to that previously reported for rifamycin S [4]. Second, analysis of the reaction mixture by paper chromatography during and after completion of the spectral change shows the progressive disappearance of the rifamycin SV spot and the appearance of a spot having an R_F similar to

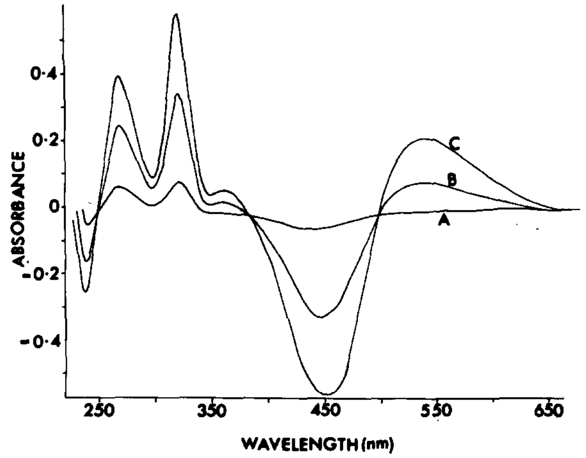

Fig.1. Difference spectrum generated by incubation of rifamycin SV with Mn^{2+}. The incubation system contained, in 1.0 ml, 200 mM Na^+ Hepes, pH 7.8, 0.1 mM rifamycin SV and 0.1 mM $MnCl_2$. The experiment was performed in tandem cells in which the compartments contained respectively buffer, buffer + rifamycin SV, buffer + $MnCl_2$ and the complete system. The spectrum was scanned at 50 nm/min starting at 700 nm in a Cary 14 double beam recording spectrophotometer after initiation of the reaction by addition of $MnCl_2$. The spectra shown were taken at 0–9 min (A), 12–21 min (B) and 40–49 min (C). In the latter case the reaction was complete and no further change in absorbance was detected on repeating the scan. A similar difference spectrum was generated if 100 mM $MgCl_2$ was used in place of $MnCl_2$.

that predicted for rifamycin S [5]. And three, gassing of the solutions with N_2 prevents the appearance of the difference spectrum in a system containing Mn^{2+} whereas gassing with O_2 somewhat accelerates this change suggesing that O_2 may be required (table 1). Similar results are obtained using Mg^{2+} as the divalent cation (table 1). In order to achieve the effect of N_2 it is necessary to flush the solutions thoroughly with this gas before initating the reaction. Both this observation and the finding that the reaction rate rapidly increases when the N_2 flow is discontinued in a stirred system suggest that the system has a high affinity for O_2.

The requirement for divalent metal ion in acceleration of the conversion of rifamycin SV to rifamycin S is indicated by the finding that chelating agents such as EDTA and oxalate are effective inhibitors of this reaction when Mn^{2+} is the added metal ion. In contrast succinate which has a much lower affinity for Mn^{2+} has little effect (table 2). Similar results were obtained when Mg^{2+} is added in place of Mn^{2+}. Examination of the specificity of the divalent metal ion effect shows that Mn^{2+} is by far the most effective of the species which catalyse the conversion. However, other metal ions, e.g., Co^{2+}, Ni^{2+}, Ca^{2+} and Mg^{2+} are effective although the concentrations required are generally greater, and/or the reaction rates lower, than those observed for Mn^{2+} (table 2). The observation of sig-

Table 1
Requirement for O_2 in conversion of rifamycin SV to rifamycin S

Additions	Rate (ΔA_{536} nm/min)
pH 8.5 Rifamycin SV (0.3 mM) ± O_2	0.0005
+ $MnSO_4$ (1 mM)	0.083
+ N_2	0.00
+ O_2	0.159
pH 9.0 Rifamycin SV (0.3 mM) ± O_2	0.0010
+ $MgSO_4$ (9 mM)	0.0095
+ N_2	0.00
+ O_2	0.014

The assay system contained, in 3.0 ml, 100 mM Na^+ Hepes at the pH indicated and the additions as described above. The solution in the cuvette was stirred in a Gilford Model 240 spectrophotometer using a Model 2445 spectrostir accessory. O_2 or N_2 was introduced through a fine tube located in one corner of the cuvette and threaded through a channel in the lid to the cuvette chamber.

Table 2
Requirement and specificity for divalent metal ions in conversion of rifamycin SV to rifamycin S

Additions	Rate (ΔA_{536} nm/min)
Rifamycin SV (0.5 mM)	0.0005
+ 8 mM EDTA, 10 mM oxalate or, 10 mM succinate	0.0005
+ 2 mM MnSO$_4$	0.850
+ 8 mM EDTA	0.015
+ 10 mM succinate	0.620
+ 2 mM Co(NO$_3$)$_2$	0.23
+ 2 mM NiCl$_2$	0.06
+ 10 mM } MgCl$_2$ (pH 9.0)	0.02
120 mM	0.99
+ 100 mM } CaCl$_2$ (pH 9.0)	0.03
500 mM	0.71

The assay system contained, in 3.0 ml, 100 mM Na$^+$ Hepes, pH 8.2 (or pH 9.0 where indicated) and the additions as indicated. Initial rates of increase in absorbance at 536 nm were measured in a stirred system as described for table 1.

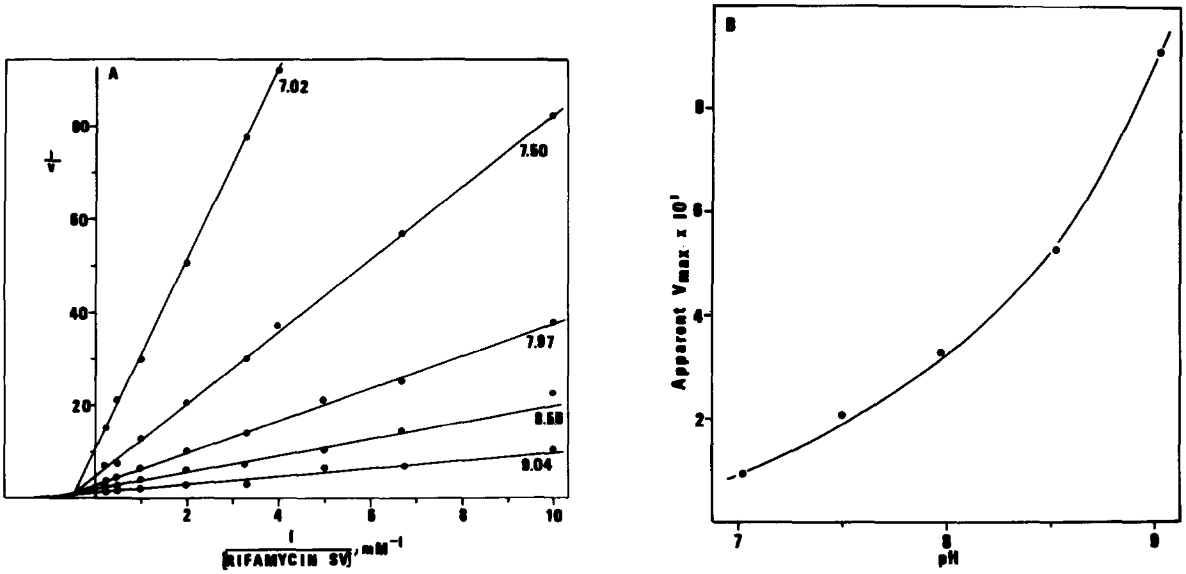

Fig.2. Effect of pH on the oxidation of rifamycin SV catalysed by Mn^{2+}. The incubation system contained, in 0.1 ml, 200 mM Na$^+$ Hepes of the appropriate pH, 2 mM MnCl$_2$ and the concentrations of rifamycin SV as indicated. The reaction was initiated by addition of MnCl$_2$ and the initial rate obtained from the decrease in absorbance at 445 nm. In (A) the dependence of reciprocal initial rate (Expressed as $1/\Delta A_{445}$nm/min) on reciprocal rifamycin SV concentration is shown for a series of pH values as indicated, which were those observed for the complete assay medium. In (B) the apparent V_{max} for the reaction is plotted as a function of pH. Curves similar in shape to that shown in (B) are generated when reaction is measured as a function of pH in the presence of Mg^{2+}, Co^{2+} and Ni^{2+}. The rates shown are corrected for the minimal change in absorbance observed in the absence of added metal ion.

nificant activation by Mg^{2+} and Ca^{2+} is of considerable importance in regard to the role proposed for the metal ions in this reaction.

When the initial rate of reaction is measured as a function of the concentration of rifamycin SV at a constant Mn^{2+} concentration, a hyperbolic relationship is observed indicating the formation of a rifamycin–Mn^{2+} complex (fig.2A). When such studies are conducted at a series of different pH values in the range pH 7–9, the 'K_m' for rifamycin SV is found to vary little with H^+ concentration over this range decreasing from a value of 1.8 mM, at pH 7.0, to 1.1 mM, at pH 9.2. Similar studies performed at a fixed pH (8.2) and a series of changing fixed concentrations of Mn^{2+} in the range 1–40 mM revealed that changes in metal ion concentration did not alter the nature of the dependence of initial rate on rifamycin SV concentration and did not significantly alter the apparent K_m for rifamycin SV.

In contrast measurement of the initial rate of oxidation as a function of metal ion concentration at a fixed concentration of rifamycin approximating the K_m reveals a more complex relationship if such studies are performed over a wide range of metal ion concentration. As shown in fig.3A the initial rate shows a clear biphasic dependence on the metal ion concentration for certain of the effective metal ions (Mn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}) although for Mg^{2+} and Ca^{2+} the initial phase is much less marked than is the case for Mn^{2+} (fig.3A). When Ni^{2+} is the added metal ion the relationship approximates to a rectangular hyperbola over the range tested (1–100 mM) and no evidence was obtained for a further increase in rate at higher Ni^{2+} concentrations (fig.3B). It is notable that the maximal rate of oxidation obtained with Ni^{2+} is an order of magnitude lower than that observed for Mn^{2+} (fig.3) in accord with observations made at non-saturating metal ion concentrations (table 2). Thus the primary site responsible for catalysis of the oxidation of rifamycin SV may not be accessible to occupancy by Ni^{2+}.

The data presented permit several conclusions regarding catalysis of the oxidation of rifamycin SV to rifamycin S. This conversion can be effected by a wide range of oxidising agents including for example H_2O_2 and lead tetracetate, and also occurs when rifamycin SV is exposed in air over a long period [6]. It seems clear that such instability is attributable to metal ion catalysis of oxidation by O_2 since in our hands solutions of rifamycin SV at pH 7 are stable if precautions are taken to exclude metal ions. Participation of the metal ion as electron carrier in the oxidation is excluded since Ca^{2+} and Mg^{2+}, which are effective catalysts at high concentration (table 2), cannot exist in more than one oxidation state. The marked increase in rate which is observed at alkaline pH for all effective metal ions suggest that a metal–hydroxide ion (*M*–OH) complex may be the active

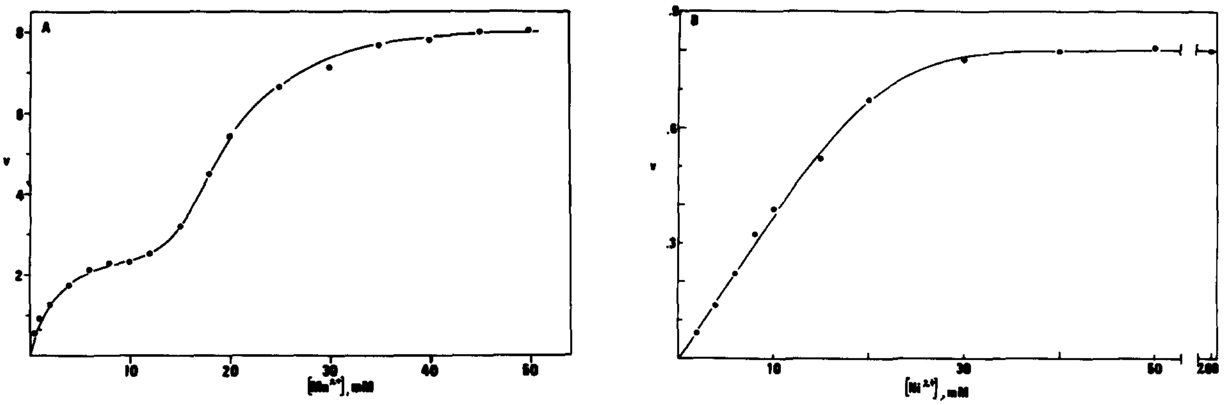

Fig.3. Concentration dependence of the effect of $MnCl_2$ (A) and $NiCl_2$ (B) on the oxidation of rifamycin SV. The incubation system contained, in 1.0 ml, 200 mM Na^+ Hepes, pH 8.0, 2 mM rifamycin SV and the concentration of metal ion as indicated. The reaction was initiated by addition of metal ion and the initial rate obtained from the increase in absorbance at 536 nm. Rates (v) are expressed as ΔA_{536} nm/min..

species. The initial EPR studies failed to detect formation of a rifamycin SV–Mn^{2+} complex having a dissociation constant in the mM range, but a weaker interaction could not have been detected and is in fact demonstrated by isolation of crystalline magnesium and calcium salts of rifamycin SV [4]. The salts form as a result of ionisation of the phenolic hydroxyl on the ring adjacent to the hydroquininoid system [6] which might be expected to labilise the hydroquinone system and hence promote its oxidation. Although ionisation of the phenolic hydroxyl might be expected to occur over the pH range examined here, the failure to observe an effect of pH on the apparent 'K_m' for rifamycin SV (fig.2) does not seem consistent with the involvement of this process in the rate-determining step of the oxidation.

Acknowledgement

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