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Effects of the Ferrous Chelate of 4-Methyl-5-Amino-1-Formylisoquinoline Thiosemicarbazone (MAIQ-1) on the Kinetics of Reduction of CDP by Ribonucleotide Reductase of the Novikoff Tumor

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SUMMARY

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The inhibition of partially purified ribonucleotide reductase (EC 1.17.4.1) from rat tumor by the iron chelate of 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ-1) was studied. The chelate was a more effective inhibitor than free MAIQ-1 in the absence of added iron. MAIQ-1(Fe^{2+}) was kinetically noncompetitive with the nucleotide substrate and with Fe^{2+}; it was uncompetitive with thioredoxin, the protein dithiol substrate.

INTRODUCTION

The new antitumor agent 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (NSC 246112; MAIQ-1[2]) has been recommended by our laboratory (1) as a second generation compound of the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone series for possible clinical trial. The synthesis and rationale for the development of this drug, which, like other members of this class, acts by inhibiting the enzyme ribonucleotide reductase, and its activity against rodent neoplasms have been reported (1, 2).

Ribonucleotide reductase (ribonucleoside diphosphate reductase; EC 1.17.4.1) is responsible for the biosynthesis of DNA precursors by reduction of ribonucleotides. The small protein thioredoxin serves as the hydrogen carrier and the enzyme thioredoxin reductase catalyzes the regeneration of the reduced form of thioredoxin using NADPH as the hydrogen donor (3). It appears that ribonucleotide reduction may be a rate-limiting step in the pathway of DNA synthesis (4, 5); therefore, it would appear to be a logical target for a chemotherapeutic agent. The ribonucleotide reductase of the rat Novikoff tumor has been shown to consist of two protein components, assumed to be nonidentical subunits, referred to as P1 and P2 (3, 6).

Previous findings (7, 8) appear to indicate that the iron chelate of the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones is the active form of the inhibitor. For this reason, the iron chelates of MAIQ-1 were synthesized and tested for inhibitory activity against ribonucleotide reductase. This report describes these experiments and the kinetic effects of the ferrous chelate of MAIQ-1 on reductase activity.

MATERIALS AND METHODS

Nucleotides and dithiothreitol (DTT) were supplied by P-L Biochemicals (Milwaukee, Wis.). [α-^{32}P]CTP, obtained from either New England Nuclear Corp. (Boston, Mass.) or Amersham Searle (Arlington Heights, Ill.), was converted to CDP by nucleoside diphosphate kinase and purified by a modification of the chromatographic procedure of Hurlbert and Furlong (9). Chelex and Dowex-50 resins were obtained from Bio-Rad Laboratories (Richmond, Calif.), and DEAE-cellulose (DE-23) was from Whatman, Inc., (Clifton, N.J.). To eliminate exogenous iron from the reaction mixture, stock solutions of

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[2] Abbreviations used: MAIQ-1, 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone; MAIQ-1 (Fe), iron chelate containing two molecules of MAIQ-1 with one atom of iron; IQ-1, 1-formylisoquinoline thiosemicarbazone; DTT, dithiothreitol; ID_{50}, concentration required to inhibit enzyme activity by 50%; P1 and P2, separable proteins both of which are required for ribonucleotide reductase activity.

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the phosphate buffer, DTT, and ATP were passed through a 0.9 × 1-cm column of Chelex (Na^+).

MAIQ-1 was prepared as described previously (2). To prepare the chelates, a solution of $FeCl_2$ or $FeCl_3$ (1mM) in 20 ml of methanol was added slowly to a solution of MAIQ-1 (2 mM) in 150 ml of boiling methanol. After stirring at room temperature for 2 h, the volume was reduced to 25 ml by evaporation under vacuum. The Fe^{2+} or Fe^{3+} chelates of MAIQ-1 were filtered, washed with methanol, and dried. The elemental anaylsis of Fe^{2+} chelate as dihydrochloride salt was as follows: Calculated for $C_{24}H_{26}N_{10}Cl_2S_2Fe$: C, 44.65; H, 4.03; N, 21.71; Cl, 11.01. Found: C, 44.38; H, 4.35; N, 21.43; Cl, 10.83. We have expressed the concentrations of the inhibitors as nanograms per milliliter rather than in terms of molarity to simplify the comparison of free MAIQ-1 with the chelates, since 1 mol of chelate contains 2 mol of MAIQ-1 which constitute 80% of its weight.

Partially purified ribonucleotide reductase was prepared by the procedure previously described (3), except that 50 mM ATP (treated with Chelex resin) was used to elute the enzyme from the affinity column, or a modification was used in which precipitation at pH 5.2 was omitted, and adsorption to DEAE-cellulose was done by stirring the adsorbent with the enzyme solution. The specific activities of several preparations varied from approximately 40 to 100 nmol of CDP reduced/30 min/mg protein, as measured with either subunit limiting in the presence of an excess of the other. At the level used in these experiments giving 2 to 6 nmol/30 min/incubation tube, the apparent specific activity of the purified enzyme alone was about 0.7 of that measured with excess subunit. A few experiments were done with less purified preparations, which had not been through the affinity column step, of specific activity 10 to 20 nmol/30 min/mg. Ribonucleotide reductase from *Escherichia coli* B3 was used after DEAE-cellulose chromatography at a similar specific activity. Thioredoxin was prepared as previously described (10) and was estimated to be about 90% pure by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, by comparison with a pure preparation.

The separate protein subunits, P1 and P2, were partially purified as reported previously through the DEAE-cellulose and ammonium sulfate steps (3). The separate P1 and P2 preparations were used only in the experiment of Fig. 2 and in the measurement of the specific activity of the purified enzyme, in order to obtain linear rather than quadratic concentration curves. Fraction P1 was stored in 0.02 M Tris–HCl, pH 7, with added ATP (0.6 mM), and fraction P2 was stored in 0.1 M NaAc, pH 7, containing 20% glycerol to increase stability. The concentrations of these components in the final assay mixture, when they were present, were ATP, 0.125 mM, in addition to that present in the substrate mix; NaAc, 25 mM; and glycerol, 5%. NaAc does not inhibit at concentrations up to 80 mM; 5% glycerol may inhibit about 15%.

Ribonucleotide reductase was assayed by the procedure described previously (6) except that the substrate mixture was modified. Thioredoxin was used as the reducing substrate, and a low concentration of DTT (0.33 mM) was used to regenerate it nonenzymatically (11). At this concentration, DTT without thioredoxin gives little activity. The standard incubation mixture contained in a total volume of 0.12 ml, 8.3 mM potassium phosphate buffer, pH 7.0; 8.3 mM magnesium acetate; 0.33 mM DTT; 2.0 mM ATP; 0.17 mM [^{32}P]CDP (sp act, 1.2 to 2.3 μCi/μmol); 0.015 mM ferrous ammonium sulfate; 3 to 9 μg thioredoxin (approximately 90% pure); and 0.07 to 0.28 mg partially purified ribonucleotide reductase which contributed about 4 mM Tris–HCl and up to 0.1 mM ATP. The 30-min incubation was started by adding the enzyme and transferring the tubes to a water bath at 37°C. The reaction was terminated by adding perchloric acid, the product was hydrolyzed to dCMP and isolated by chromatography on Dowex-50 (6), and one-half of the material was counted by Cherenkoff radiation at an efficiency of approximately 40%; results were corrected for carrier recovery.

For the experiment of Table 3, the reaction mixture for the enzyme from *E. coli* contained 8.3 mM Tris, pH 8.5, 4.2 mM ATP, 10 mM $MgAc_2$, 2 mM dithiothreitol, and 3.6 μg of slightly purified thioredoxin from *E. coli*. The reaction mixture for the rat enzyme was as described above except that 2 mM dithiothreitol and thioredoxin from *E. coli* were used.

The inhibitors were dissolved in dimethylsulfoxide at a concentration of 1 mg/ml, diluted 1 to 200 in 10% dimethylsulfoxide, and further diluted in water to the desired concentration to be added to the assay mixtures. The concentration of dimethylsulfoxide in the reaction mixtures did not exceed 0.1%; concentrations up to 1% have been found not to inhibit significantly (8). Ferrous ammonium sulfate was dissolved in 0.12 M HCl immediately before use; for the iron concentration curves, it was diluted in 1 mM HCl to delay oxidation. No more than 0.18 mM HCl was added to the reaction mixture; no change in the pH or effect on the enzyme was detected.

The apparent Michaelis constants and maximum velocities were calculated by computer according to the method of Cleland (12), which gives an estimate of the standard error for each constant. In calculating the apparent K_m for CDP, the average CDP concentration during the reaction was used. Segal states that with this procedure the error is about 4% when half the substrate is consumed (13). To correct for nucleoside diphosphate kinase activity, we used the observed activity at 42 μM CDP and assumed that the K_m (CDP) of the kinase was 150 μM, based on the K_m (UDP) of 500 μM reported by M. L. Simons for the enzyme from Novikoff tumor (thesis, University of Texas Graduate School of Biomedical Sciences, 1972) and the ratio of CTP to UTP in Novikoff tumor cells. The 50% inhibitory concentrations of agents were determined graphically. Kinetic data were plotted according to the Hanes-Woolf linearization (14), which has the coordinates $[S]/v$ versus $[S]$. The slope is $1/V_{max}$; the y intercept is K_m/V_{max}, and the x intercept is $-K_m$.

Spectra were obtained with a Model 118 Cary spectrophotometer, operated at 0.2 absorbance full scale, with a 1-cm path length and 3-ml cuvettes. The scanning speed was 2 nm/s. Ferrous ammonium sulfate and the (nominally) ferrous chelate of MAIQ-1 were dissolved immediately before adding to the cuvettes.

RESULTS

The addition of freshly dissolved ferrous ammonium sulfate solution (50 μM) to a 13 μM solution of MAIQ-1 gave an immediate color change from pale yellow to green, and a peak at 640 nm appeared in the spectrum (Fig. 1). On standing the color changed rather quickly to reddish brown, similar to that resulting from the addition of a ferric salt (not shown). The two chelate preparations, prepared as described in Materials and Methods, stored dry, and dissolved in dimethylsulfoxide as described, both yielded reddish brown solutions with identical spectra (Fig. 1, curve D). Upon the addition of DTT (0.4 mM) the color immediately changed to green and a peak at 640 nm appeared (Fig. 1). These findings suggest that the ferrous chelate had become oxidized to the ferric state and that the addition of DTT (at a concentration similar to that in the enzyme assay mixture) reduced the iron in the chelate to the ferrous state. Therefore we have referred to the chelate as MAIQ-1(Fe) without specifying the oxidation state, but have assumed that in solution in the enzyme assay mixture it is in ferrous form.

The ribonucleotide reductase preparation used in this study had a specific activity about four times higher than that of the preparations used in our previous inhibitor studies (8, 15). The concentration of DTT in the reaction mix was decreased from 6 mM, used in our previous studies, to 0.3 mM in order to make the reaction dependent on thioredoxin, more closely approximating the condition occurring *in vivo*.

Under the standard assay conditions, MAIQ-1 was somewhat more potent as an inhibitor than its iron chelate. Thus, the ID_{50} values calculated from the pooled data of three experiments using two preparations of partially purified enzyme and the two chelate preparations were 1 ng/ml for MAIQ-1, 4 ng/ml for the "ferrous" chelate, and 5 ng/ml for the "ferric" chelate. Dixon plots ($1/v$ vs i) were linear for MAIQ-1(Fe), but not for MAIQ-1. When $Fe(NH_4)_2(SO_4)_2$ was omitted from the reaction mixture, however, the results were quite different. As shown in Table 1, the chelates were much more potent that free MAIQ-1 in the absence of added iron. Enzyme activity in the presence of MAIQ-1 decreased as the iron concentration was increased, rather than increasing, as did the control. The percentage inhibition of reductase activity by the iron chelate of MAIQ-1 decreased as the iron concentration in the assay mixture was increased. Calculation of the K_m and V_{max} for iron from these data, however, showed that both were changed; the K_m increased from 0.49 to 2.0 μM, and the V_{max} decreased 60%. This pattern is called "noncompetitive" by Cleland and "mixed" by Segel. These and other kinetic constants are summarized in Table 2.

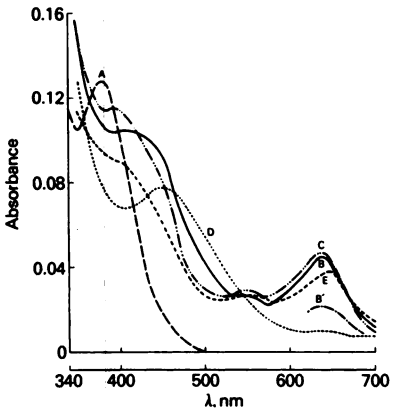


FIG. 1. *Spectra of solutions containing MAIQ-1 or its iron chelates in a simulated reaction mixture*

All solutions contained 2 mM potassium phosphate, pH 7, and 0.18% dimethylsulfoxide. MAIQ-1 and MAIQ-1(Fe) were added to give 3.3 μg/ml (12.9 μM MAIQ-1). DTT was added to give 0.4 mM, and $Fe(NH_4)_2(SO_4)_2$ to give 50 μM. A solution with all components except the thiosemicarbazone showed no absorbance in this wavelength range. Scanning rate was 2 nm/s. Curve A: MAIQ-1. Curve B: To A, $Fe(NH_4)_2(SO_4)_2$ was added and the spectrum was measured immediately. Curve B′: Same solution after 5 min. Curve C: To B′, DTT was added and the spectrum was measured immediately. After 15 min (not shown) the absorbance at 640 nm decreased about 10%. Curve D: Preformed ferric chelate of MAIQ-1. Curve E: To D, DTT was added and the spectrum was measured immediately.

TABLE 1

Effects of iron on inhibitor potency

The activity (nanomoles of CDP reduced in 30 min) of 200 μg of partially purified ribonucleotide reductase was determined by the procedure given in Materials and Methods, except that the concentration of $Fe(NH_4)_2(SO_4)_2$ was varied as indicated and inhibitors (2.1 ng/ml) were added as shown. The percentage inhibition is based on the activity at the same $[Fe^{2+}]$ in the absence of inhibitor.

Inhibitor	$[Fe^{2+}]$	Activity	Inhibition
	μM	*nmol/30 min*	%
None	0	2.67	
	5	3.34	
	15	3.45	
	25	3.51	
	40	3.70	
MAIQ-1	0	1.82	32
	5	1.35	60
	15	0.74	79
	25	0.58	83
	40	0.51	86
MAIQ-1(Fe)	0	0.53	80
	5	1.04	69
	15	1.27	63
	25	1.36	61
	40	1.38	63

TABLE 2

Summary of kinetic constants

The apparent constants were determined in three separate experiments with different enzyme levels, as described in Table 1, Fig. 2, and the text, and calculated by computer as described in Materials and Methods.

Substrate	MAIQ-1(Fe)	K_m	V_{max}	K/V
		μM	*nmol/30 min*	
CDP	0	3.6 ± 1.4	0.57 ± 0.04	6.3 ± 2.1
CDP	1 ng/ml	3.7 ± 0.8	0.37 ± 0.01	10.1 ± 1.7
Fe^{2+}	0	0.49 ± 0.24	3.64 ± 0.08	0.13 ± 0.06
Fe^{2+}	2.1 ng/ml	2.0 ± 0.13	1.45 ± 0.01	1.38 ± 0.08
Thioredoxin	0	0.42 ± 0.006	6.0 ± 0.05	0.070 ± 0.003
Thioredoxin	1 ng/ml	0.20 ± 0.014	2.7 ± 0.03	0.073 ± 0.006

That the inhibition is specific for the iron chelate was suggested by data obtained earlier with chelates of IQ-1 with various metals, which were presented orally (7) but have not yet been published. The iron chelate of IQ-1 was more effective than free IQ-1 when iron was omitted from the reaction mixture. The zinc chelate was less effective than free IQ-1, and the cobalt and platinum chelates had almost no effect, in the presence or absence of iron.

We had previously reported (8) that ribonucleotide reductase from *E. coli* was not inhibited by IQ-1. Since added iron does not stimulate the bacterial enzyme and is not included in the usual reaction mixture, we decided to test for inhibition of *E. coli* reductase by MAIQ-1(Fe). The results are shown in Table 3. The iron chelate did inhibit the bacterial enzyme, but only at a level more than two orders of magnitude higher than that which inhibits the rat enzyme.

To test for irreversible inhibition or enzyme titration, Ackerman–Potter (16) enzyme concentration curves were determined in the presence of MAIQ-1(Fe). Because the two-component enzyme gave a nonlinear response (activity roughly proportional to the square of enzyme concentration), the activities of the P1 and P2 subunits were tested separately. The amount of 50-fold purified ribonucleotide reductase was varied in the presence of an excess fixed amount of each slightly purified subunit; in this manner, the other subunit provided by the purified enzyme was limiting. Figure 2 is the resulting plot of activity versus enzyme amount. The findings show no evidence of enzyme titration. The slopes of the lines for both subunits were changed in the presence of the inhibitor, and neither crossed the horizontal axis to the right of the origin. The degree of inhibition by MAIQ-1(Fe) of the P2 subunit, measured in the presence of excess P1, was greater than that of the P1 unit, assayed with excess P2.

After most of these experiments had been completed, Cory (17) reported evidence for irreversible inhibition by MAIQ-1. We repeated one of his experiments with MAIQ-1(Fe), using an enzyme preparation of specific activity about 20, but did not confirm his results. The enzyme was allowed to stand in an ice bath for 1.5 h with

TABLE 3

Inhibition of reductase from E. coli

Slightly purified enzyme preparations were used. The uninhibited activities, without and with added iron (20 μM), were 7.18 and 7.48 nmol per 30 min for the *E. coli* reductase and 1.15 and 1.28 nmol per 30 min for the rat enzyme.

Source of enzyme	Inhibitor		Activity remaining	
			No Fe^{2+}	20 μM Fe^{2+}
	ng/ml		%	%
E. coli	0		100	100
	MAIQ-1	800	93	82
	MAIQ-1	5000	96	54
	MAIQ-1(Fe)	800	89	86
	MAIQ-1(Fe)	5000	66	77
Rat	0		100	100
	MAIQ-1	33	80	8
	MAIQ-1(Fe)	33	12	30

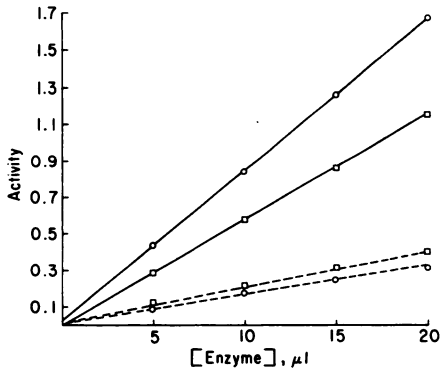


FIG. 2. *Ackerman–Potter plot of activity versus enzyme concentration*

Activity with excess P1 subunit is represented by ○, and with excess P2 by □. Solid lines, controls; dashed lines, with MAIQ-1(Fe), 2.1 ng/ml. The lines were generated by the method of least squares. Activity is expressed as nanomoles of CDP reduced in 30 min under standard conditions. The activity under the same conditions of the auxiliary P1 or P2 preparation alone (0.02 or 0.13 nmol) was subtracted as a blank.

0.03 μg/ml MAIQ-1(Fe), then passed through a gel filtration column of BioGel P-30 equilibrated with 0.02 M Tris–HCl, pH 7. The control was treated in the same way except that the 0.04% dimethylsulfoxide solvent replaced the inhibitor. The original and gel-filtered enzymes were assayed (without added subunits) at identical protein concentrations.

Before gel filtration, the enzyme was inhibited 80% as assayed without added iron and 55% with 17 μM $Fe(NH_4)_2(SO_4)_2$ added to the assay mixture. The specific activity of the control enzyme was decreased 35% after gel filtration. Compared to this control, however, the activity of the drug-treated, gel-filtered enzyme was 102% with iron and 83% without added iron. Both activities were higher than those of the inhibited enzyme before gel filtration.

The effect of thioredoxin on the inhibition of reductase activity by MAIQ-1(Fe) is shown in Fig. 3. The curves intersect on the $[S]/v$ axis, indicating that the inhibitor is uncompetitive. Since an uncompetitive inhibitor has the effect of changing both the K_m and the V_{max} values by the same factor, the value of K_m/V_{max} (the y intercept in the Hanes-Woolf plot) was unchanged. The K_m of the control was 0.42 μM. The K_m/V_{max} was 0.070 for the control and 0.073 in the presence of 1 ng/ml of MAIQ-1(Fe).

The effects of varying the concentration of CDP on the inhibition by the ferrous chelate were also studied. To ascertain the impact of possible incomplete removal of nucleoside diphosphate kinase during the purification of ribonucleotide reductase, samples of the incubation mixtures with the highest CDP concentration were chromatographed on thin-layer PEI cellulose before and after incubation with the enzyme; about 30% of the substrate was converted to CTP during the 30-min incubation period. Because of the very low K_m (3.6 μM) for CDP of the reductase, it was necessary to use relatively low concentrations in the experiment; at the lowest level, 30% of the substrate was reduced during the incubation. Calculations based on the average concentration during the reaction showed no marked changed in the apparent K_m

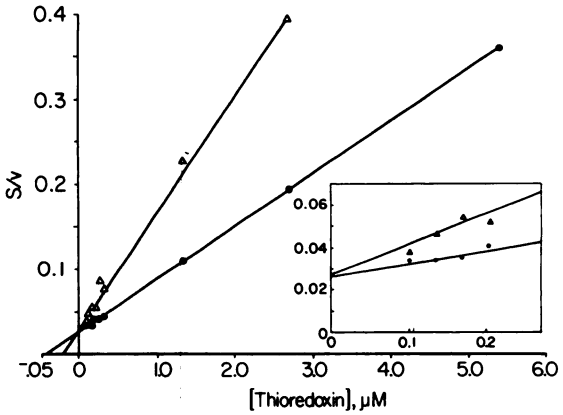


FIG. 3. *The effect of thiredoxin on the inhibition of ribonucleotide reductase by MAIQ-1(Fe)*

The thioredoxin concentrations are approximate, based on an estimate of 90% purity and on measurement of the protein concentration in the stock solution by the method of Kalb and Bernlohr (26). Controls are represented by ●——●; activity in the presence of MAIQ-1(Fe) (1 ng/ml), by △——△. The inset shows the lowest four points and the resultant intersection on the *y* axis.

for CDP in the presence of MAIQ-1(Fe) at 1 ng/ml. The values were 3.6 ± 1.4 μM for the control and 3.7 ± 0.8 μM in the presence of MAIQ-1(Fe). There was a change in the maximum velocity from 0.57 ± 0.04 to 0.37 ± 0.01. The percentage inhibition varied randomly from 33 to 37%. The apparent kinetic constants for thioredoxin, iron, and CDP are summarized in Table 2.

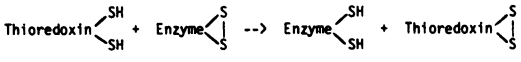
DISCUSSION

Although the heterocyclic carboxaldehyde thiosemicarbazone tumor inhibitors are all chelating agents, we concluded from our early experiments with IQ-1 (8) that chelation of free iron could not explain the inhibition of ribonucleotide reductase by these compounds. At one time we proposed that one molecule of the inhibitor might bind to iron which was bound to the enzyme active site. The data in Table 1, however, show an actual decrease in activity as iron was added to a reaction mixture containing free MAIQ-1. This indicates that iron promotes the inhibition as well as stimulating enzyme activity. We had obtained, but failed at first to emphasize, similar results with IQ-1 (5, 7, 8). That the inhibition is specific for iron is shown by the facts that the Co^{2+} chelate of IQ-1 was inactive and the Zn^{2+} chelate less active than free IQ-1. Very recently, Saryan *et al.* (18) also reported that the iron chelates of IQ-1 and MAIQ-1 were more effective inhibitors than the free drugs in the absence of added iron.

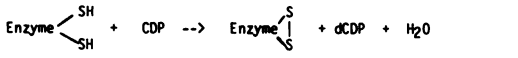
The ferric chelate of MAIQ-1 was rapidly reduced to the ferrous form, and free MAIQ-1 was rapidly complexed to Fe^{2+} under conditions similar to those in the incubation mix. Antholine *et al.* (19) reported the same results for the related inhibitor 2-formylpyridine thiosemicarbazone. These findings support the conclusion that the active form of the inhibitor is the ferrous chelate.

The small protein thioredoxin is a natural reducing substrate for ribonucleotide reductase. It is normally regenerated by NADPH and the flavoprotein thioredoxin reductase; to simplify the experiments described in this paper, we have used chemical reduction by DTT. Although DTT at relatively high concentrations (i.e., usually 6 to 10 mM) can replace thioredoxin in the reaction, as we have employed in much of our previous work (6, 8, 15), the concentration used in these experiments (0.33 mM) supported only 4% of the maximum activity in the absence of thioredoxin.

Figure 2 shows that the inhibition by MAIQ-1(Fe^{2+}) is uncompetitive with respect to thioredoxin. According to Segal (13), uncompetitive inhibition occurs when a dead-end inhibitor binds after the substrate or when an irreversible step, such as the release of product (at low product concentration), intervenes between binding of substrate and inhibitor. Thelander and co-workers (20, 21) have reported that the reaction mechanism of the ribonucleotide reductase from *Escherichia coli* is probably ping-pong (using Cleland's nomenclature), according to reactions I and II.



(I)



(II)

The data presented in Fig. 2 are consistent with the concept that this mechanism applies to the rat enzyme and that the inhibitor MAIQ-1(Fe) binds to the reduced enzyme or to a complex of enzyme and thioredoxin.

However, if the mechanism were exactly according to reactions I and II, and if MAIQ-1(Fe) bound the reduced enzyme, it would be expected to be competitive with CDP. We found, on the contrary, no evidence of a change in the K_m for CDP in the presence of MAIQ-1(Fe) or in the percentage inhibition over a four-fold range of CDP concentrations. Similar results were obtained with IQ-1 (5, 8). While the accuracy of the apparent K_m values is open to question, we would have expected to see some evidence of reversal if the inhibition was in fact competitive. One possible explanation for this apparent contradiction is that an additional step in the reaction sequence occurs, perhaps a change in enzyme conformation. Another is that the binding site for MAIQ-1 is distinct from and does not overlap that of CDP, although both sites must be affected in some way by the reduction of the active-site disulfide to dithiol. A third possibility is that MAIQ-1(Fe) actually binds to a complex of enzyme plus thioredoxin.

The interpretation of the iron concentration experiments with MAIQ-1(Fe) is not clear. Added iron decreased the degree of inhibition of enzyme activity by the iron chelate (Table 1) when assayed under the standard conditions. The reversal was incomplete, however, and kinetic analysis of the points (except zero iron) from Table 1 showed noncompetitive or mixed-type inhibition (Table 2).

The function of iron in the enzyme reaction is uncertain. Table 1 confirms stimulation of the uninhibited activity by added iron, which has been reported previously (5, 22, 23), but the activity without added iron (2.67 nmol/30 min)is about 70% of the calculated maximum velocity (3.64 nmol/30 min) in spite of all precautions to

exclude iron from the reaction mixture and enzyme preparation. Ribonucleotide reductase from *E. coli* contains tightly bound iron which seems to have the function of generating and stabilizing an organic free radical (24). If the free radical is lost, it can be regenerated by removing the iron (by dialysis against 8-hydroxyquinoline sulfonate) and replacing it via an iron–ascorbate complex. One possible interpretation of the partial iron stimulation shown in Table 1 is that such replacement of iron can occur in the rat enzyme under the conditions of the enzyme assay mixture and that some inactivation of free radical has occurred during the enzyme purification or storage. The results of Engström *et al.* (25) with calf thymus reductase are compatible with this suggestion. The inhibitor, then, might promote inactivation or interfere with reactivation. Such a mechanism could explain the insensitivity of the ribonucleotide reductase of *E. coli* to IQ-1 (8) and MAIQ-1, even in the presence of iron (Table 3), since it is much more difficult to inactivate or reactivate (24).

We have previously reported tentative evidence of partially irreversible inhibition of ribonucleotide reductase by IQ-1 (8), as shown by an increase in inhibition with time of incubation. Results of gel filtation and enzyme titration experiments were inconclusive, since at that time we did not take into account the two-component nature of the enzyme. Our present experiments give linear plots but show no evidence for titration of either component by MAIQ-1. They do suggest, however, slightly stronger inhibition when component P2 is limiting than when P1 is limiting. Cory and Fleischer (17) reported finding irreversible inhibition of the P2-like component of ribonucleotide reductase from mouse Ehrlich ascites tumor when it was incubated for 45 min at 4°C with MAIQ-1. We did not confirm the irreversibility in a similar experiment with unseparated enzyme, but as we did not have all the details of their procedure, there may have been differences in the conditions of inhibitor treatment or in the assay conditions which might relate to reactivation.

In conclusion, our data support the concept that the active form of MAIQ-1 is the ferrous chelate, that the inhibition is not totally irreversible under the conditions we have used, and that kinetically it appears uncompetitive with thioredoxin and noncompetitive or mixed type with CDP and ferrous ammonium sulfate. An explanation of the exact mechanism will require further experiments with a more purified enzyme.

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