Biochemical Pharmacology, Vol. 39, No. 4, pp. 639–646, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

HERPES SIMPLEX VIRUS TYPE 1 RIBONUCLEOTIDE REDUCTASE: SELECTIVE AND SYNERGISTIC INACTIVATION BY A1110U AND ITS IRON COMPLEX

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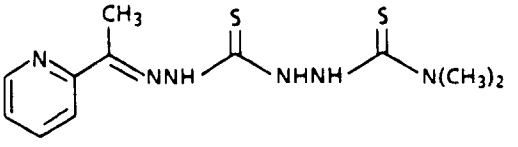
(*Received* 23 *February* 1989; *accepted* 2 *August* 1989)

Abstract—2-Acetylpyridine-5-[(dimethylamino)thiocarbonyl]thiocarbonohydrazone (A1110U) inactivated herpes simplex virus Type 1 ribonucleotide reductase (EC 1.17.4.1) by a first-order process (k_{inact}) which had a maximum value ($^{M}k_{inact}$) of 8 hr^{-1} and a K_d that was less than 1 μM. The stable complex between iron and A1110U, $(A1110U)_2Fe^{+i}$, inactivated this enzyme with a $^{M}k_{inact}$ of 7 hr^{-1} and a K_d of 7 μM. Free A1110U and its iron-complex synergized as inactivators of the enzyme. For example, the k_{inact} for the combination of 2 μM A1110U and 1 μM $(A1110U)_2Fe^{+i}$ as independent inactivators was calculated to be about 9 hr^{-1}, while the observed value was 32 hr^{-1}. The bimolecular rate constant for inactivation of the viral enzyme by $(A1110U)_2Fe^{+i}$ in the presence of a saturating concentration of A1110U was 2.5 10^7 M^{-1} hr^{-1} at 30°. Human ribonucleotide reductase was less sensitive to the inhibitory effects of A1110U and its iron-complex. This enzyme was neither inhibited nor inactivated by A1110U and was weakly inhibited by $(A1110U)_2Fe^{+i}$. Furthermore, inactivation required the combination of A1110U and $(A1110U)_2Fe^{+i}$. The bimolecular rate constant for inactivation of human ribonucleotide reductase by $(A1110U)_2Fe^{+i}$ in the presence of a saturating concentration of A1110U was considerably smaller (3.8 10^6 $M^{-1}hr^{-1}$ at 37°) than the analogous constant for the viral enzyme. Several iron-chelating reagents with unrelated structures substituted for free A1110U in its various roles with both enzymes. However, the iron complexes of these alternative chelators did not substitute for $(A1110U)_2Fe^{+i}$. The rates of inactivation of both enzymes were independent of the oxidation state of iron in $(A1110U)_2Fe^{+i}$ and of CDP concentration. The inactivated enzymes were reactivated rapidly by $FeSO_4$, but were not reactivated by $CoCl_2$, $CuSO_4$, or $NiCl_2$. $MnCl_2$ inhibited reactivation of the viral enzyme by $FeSO_4$.

The thiocarbonohydrazone derivative A1110U† (I) inactivates HSV-1, HSV-2 and varicella-zoster ribonucleotide reductase and potentiates the *in vitro* antiherpetic action of acyclovir [1]. This observation led to the development of A1110U and acyclovir as synergistically therapeutic reagents in a topical treatment of HSV-infected animals.‡§ A mechanism for this synergy was suggested by the observations that addition of A1110U to HSV-1 infected cells, which had been treated with acyclovir, causes an 11-fold decrease in dGTP levels and a 9-fold increase in acyclovir triphosphate levels [1]. These perturbations in nucleotide triphosphate levels resulted in acyclovir triphosphate competing more effectively for the dGTP binding site on viral DNA polymerase. As a result, chain termination became a more probable event.



(I)

The decrease in dGTP found after treatment of HSV-1 infected cells with A1110U was readily explained by the time-dependent "inactivation" of viral ribonucleotide reductases [1]. Thiosemicarbazone derivatives, in general, are potent inhibitors of ribonucleotide reductases and a mechanism of their action has been proposed from work with 1-formylisoquinoline thiosemicarbazone [2–8]. In this case, the iron complex of the thiosemicarbazone inhibited mammalian ribonucleotide reductase via a redox reaction in which an essential tyrosine radical was destroyed by a one-electron reduction [9]. Since these results suggested an obligatory role for metal ions in the inhibition of mammalian ribonucleotide

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† Abbreviations: A1110U, 2 - acetylpyridine - 5 - [(dimethylamino) thiocarbonyl] thiocarbonohydrazone; $(A1110U)_2Fe^{+i}$, the metal complex between A1110U and iron in which the valence state of iron is not specified. If the valence of iron is specified the value of i is 2 or 3; HSV-1, herpes simplex virus type 1; desferal or desferrioxamine, desferrioxamine B; desferal-Fe^{+i}, iron complex of desferrioxamine; and Hepes, *N*-(2-hydroxyethyl)piperazine.

‡ Ellis MN, Lobe DC and Spector T, Combination for treatment of HSV-1 infection in athymic mice. *26th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abst. No. 625, 1986.

§ Lobe DC, Spector T and Ellis MN, Synergistic antiviral chemotherapy for HSV-1 orofacial infection of athymic mice. *27th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abst. No. 1157, 1987.

reductases by thiosemicarbozones, we investigated the effects of metal ions on the time-dependent "inactivation" of HSV-1 ribonucleotide reductase by A1110U. A parallel study with human ribonucleotide reductase was conducted to determine the basis for the selectivity of A1110U for the viral enzyme.

MATERIALS AND METHODS

Materials. Glucose oxidase (*Aspergillus niger*), catalase (bovine liver), EDTA, bathophenanthroline disulfonic acid, 2,2-bipyridyl and phenanthroline were Sigma products (St Louis, MO). Desferal mesylate (Lot No. 30315) was a CIBA Pharmaceutical Co. product (Summit, NJ). These reagents were used without further purification. 2-Acetylpyridine-5-[(dimethylamino)thiocarbonyl]thiocarbonohydrazone (A1110U) was synthesized from 4,4-dimethylthiosemicarbazide and acetylpyridine [1]. Metal complexes of A1110U were generated by addition of $FeSO_4$ or $CoCl_2$ to A1110U (30–100 μM in deionized H_2O) in a 1 to 2 molar ratio and were used immediately. [U-^{14}C]CDP (499 mCi/mmol) was from New England Nuclear (Wilmington, DE) and was purified further by ion exchange chromatography [10]. ATP (Lot No. 61002) was a Pharmacia Biochemicals product (Piscataway, NJ). Human (isolated from Detroit 98 cells) and HSV-1 (Type 1) ribonucleotide reductases were purified chromatographically [10, 11].

Methods. Ribonucleotide reductases were assayed in temperature equilibrated glass reaction vessels by following the reduction of CDP to dCDP. The reactions were terminated by addition of hydroxyurea and EDTA, which quenched the reaction and decreased the blank rates to an insignificant level. The product was dephosphorylated and isolated as the nucleoside by chromatography on a Dowex-borate column [10, 12]. Human ribonucleotide reductase was assayed at 37° in 100 mM Hepes (Na^+), 5 mM dithiothreitol, 12 μM CDP with a specific activity of 75 mCi/mmol, 5 mM ATP (iron free by the method described below) and 6 mM $MgCl_2$ at pH 7.4 (buffer A). Viral ribonucleotide reductase was assayed in 200 mM Hepes (K^+) at pH 7.7 with 10 mM dithiothreitol and 2 μM CDP with a specific activity of 499 mCi/mmol (buffer B). The Hepes and dithiothreitol solutions were stored with Chelex 100 to minimize iron contamination. Since the viral enzyme was more stable at 30° than it was at 37°, this temperature was used for the inactivation experiments with the viral enzyme. One unit of these enzymes was that amount of enzyme which catalyzed the formation of 1 pmol of dCDP in 1 min.

Solutions of ATP (2 mL of approximately 200 mM) were rendered metal-ion free by passage through a 1 5 cm Chelex 100 column. Prior to loading the sample onto the column, the pH of the solution was adjusted to pH 7.6 with 0.1 N NaOH, and 10 mM dithiothreitol was added. Dithiothreitol was required for the removal of iron from ATP which suggested that iron was displaced from the ATP–iron complex in the ferrous form. Iron remaining in the purified ATP preparations was quantitated by the shift in the spectrum of A1110U at 310 nm (see Fig. 1) which occurred upon formation of $(A1110U)_2Fe^{+i}$ when an ATP sample was added to a solution of 20 μM A1110U, 6 mM $MgCl_2$ and 10 mM dithiothreitol. Prior to treatment with Chelex 100, a 5 mM ATP solution was contaminated with 2–4 μM iron. A similar ATP solution after treatment with Chelex 100 was contaminated with less than 0.5 μM iron.

Assay solutions were depleted of O_2 either by purging with argon (99.999%) or catalytically reducing O_2 with glucose and glucose oxidase which was quantitated spectrophotometrically at 450 nm [13]. In the latter case, the typical assay buffer with 4 μM glucose oxidase, 2 mM glucose and 1.7 μM catalase was anaerobic within 10 sec.

The oxidation of $(A1110U)_2Fe^{2+}$ and reduction of $(A1110U)_2Fe^{3+}$ were followed by the absorbance changes of the complexes at 590 nm (see Fig. 1).

The inactivator induced decelerating time courses for ribonucleotide reductase catalyzed formation of dCDP were fit to equation 1.

$$[\text{dCDP}] = A + B \cdot e^{-C \cdot t} \tag{1}$$

where C (hr^{-1}) is k_{inact} and $-B \cdot C$ (pmol/hr) is the initial velocity of the reaction.

When k_{inact} was measured as a function of the concentration of a single inactivator (I), the data were fit to equation 2

$$k_{inact} = \frac{[I] \cdot ({}^{M}k_{inact})}{[I] + K_d} \tag{2}$$

where ${}^{M}k_{inact}$ (hr^{-1}) is the maximum rate of inactivation and K_d (μM) the concentration of inactivator that yielded a k_{inact} equal to one-half of ${}^{M}k_{inact}$.

When the concentration of $(A1110U)_2Fe^{+i}$ was varied and the concentration of A1110U was fixed, the rate data were modeled to a straight line (equation 3)

$$k_{inact} = k_0 + k_2[(A1110U)_2Fe^{+i}] \tag{3}$$

where k_0 (hr^{-1}) is the first-order rate constant for inactivation in the presence of a saturating concentration of A1110U and k_2 the bimolecular rate constant ($M^{-1}\,hr^{-1}$) for inactivation of the enzyme by $(A1110U)_2Fe^{+i}$. The parameters for equations 1 and 2 were determined by an iterative non-linear least squares fitting of the data to these equations [14]. The values for k_{inact} were independent of enzyme concentration over a 5-fold range.

RESULTS

Metal complexes of A1110U. Thiosemicarbazones bind transition metal ions tightly with characteristic changes in their UV spectrum [15]. Similar changes occurred when A1110U complexed iron (Fig. 1). On the basis of these spectral changes, A1110U was titrated with $FeSO_4$. The stoichiometry of the reaction was 1.7 mol of A1110U for each mol of $FeSO_4$ (inset of Fig. 1) which suggested a structural formula for the complex of $(A1110U)_2Fe^{2+}$. Since the analogous complex between A1110U and Fe^{3+} formed slowly at pH 7.6, it was generated *in situ* by the oxidation of $(A1110U)_2Fe^{2+}$ with O_2 (Fig. 1).

The stabilities of $(A1110U)_2Fe^{2+}$ and $(A1110U)_2Fe^{3+}$were estimated from the absorbance at 310 nm that would increase if these complexes lost

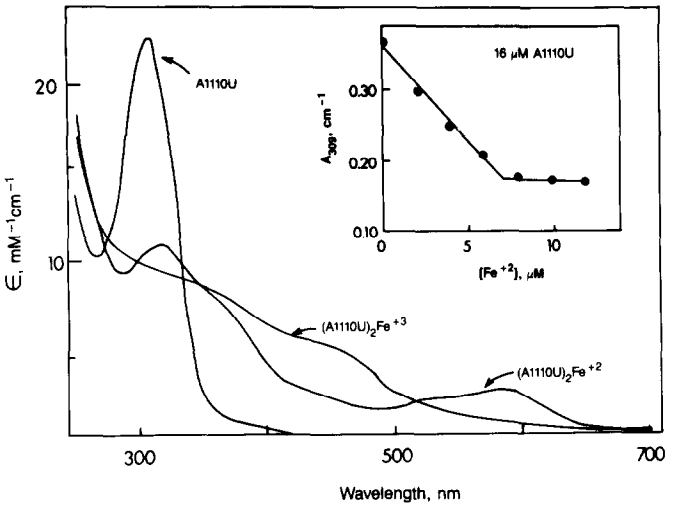

Fig. 1. Spectral properties of A1110U, $(A1110U)_2Fe^{2+}$ and $(A1110U)_2Fe^{3+}$. The spectrum of $(A1110U)_2Fe^{2+}$ (19 μM) was recorded in 0.1 M Hepes–Na^+ (pH 7.6) at ambient temperature after anaerobic addition of 20 μM $FeSO_4$ to 38 μM A1110U. The spectrum of $(A1110U)_2Fe^{3+}$ was recorded after aerobic oxidation of $(A1110U)_2Fe^{2+}$. The stoichiometry between A1110U and Fe^{2+} was determined by anaerobic titration of A1110U with $FeSO_4$ (inset).

iron and formed free A1110U. When 100 μM EDTA was used to sequester the free iron that would form upon dissociation of either complex in a solution at pH 7.6, there was no significant absorbance increase at 310 nm over 30 min. This demonstrated that these complexes were relatively stable under these conditions. Complexes of Ni^{2+}, Co^{2+}, and Cu^{2+} with A1110U were also detected spectrally. These complexes were sufficiently stable that they did not lose metal when treated with 100 μM EDTA (3.3-fold excess) for 10 min. In contrast, the Mn^{2+} complex was dissociated by addition of EDTA. Mg^{2+} was not complexed by A1110U ($K_d > 50$ mM).

The valence of iron in the complex between A1110U and iron was dependent on the composition of the buffer. $(A1110U)_2Fe^{2+}$ was oxidized at pH 7.6 by 200 μM O_2 to $(A1110U)_2Fe^{3+}$ with a first-order rate constant of $24 \pm 2\ hr^{-1}$ and $(A1110U)_2Fe^{3+}$ was reduced to the ferrous complex by 4 mM dithiothreitol with a first-order rate constant of $100 \pm 5\ hr^{-1}$. As a result, the steady-state concentrations of $(A1110U)_2Fe^{2+}$ and $(A1110U)_2Fe^{3+}$ in the typical ribonucleotide reductase assay were a function of the concentrations of O_2 (200 μM) and dithiothreitol (5 mM). This mixture of complexes was designated $(A1110U)_2Fe^{+i}$.

Inactivation of ribonucleotide reductase by A1110U and $(A1110U)_2Fe^{+i}$. As previously observed with reactions catalyzed by viral ribonucleotide reductase [1], A1110U caused the rate of product formation to decelerate to zero within a few minutes. This type of progressive inhibition will be referred to as inactivation. A non-progressive decrease in the initial velocity will be referred to as inhibition. Rate constants for inactivation of the viral enzyme were determined at six concentrations of A1110U from which $^{M}k_{inact}$ was estimated to be $8.3 \pm 0.4\ hr^{-1}$ with a K_d that was less than 1 μM. Since treatment of the assay buffer with Chelex 100 or 1 μM EDTA to eliminate trace iron did not change the rate of inactivation, it was unlikely that iron as $(A1110U)_2Fe^{+i}$ contributed to the inactivation process. Human ribonucleotide reductase was neither inhibited nor inactivated by A1110U (Figs. 2 and 3).

$(A1110U)_2Fe^{+i}$ also appeared to be a time-dependent inactivator of HSV-1 ribonucleotide reductase. The rate of inactivation was measured at eleven concentrations of $(A1110U)_2Fe^{+i}$ (1–20 μM) to give a $^{M}k_{inact}$ of $7 \pm 1\ hr^{-1}$ and a K_d of 7 ± 3 μM.* In contrast, $(A1110U)_2Fe^{+i}$ was a weak inhibitor of human ribonucleotide reductase that did not inactivate the enzyme (Fig. 2).

HSV-1 and human ribonucleotide reductases were synergistically inactivated by A1110U and $(A1110U)_2Fe^{+i}$. In the presence of 1 μM $(A1110U)_2Fe^{+i}$, the k_{inact} for the viral enzyme was nearly hyperbolically dependent on A1110U concentration (inset of Fig. 3). In contrast, the dependence of k_{inact} on A1110U concentration for the human enzyme was clearly non-hyperbolic in the presence of 3 μM $(A1110U)_2Fe^{+i}$ (inset of Fig. 3). In this case, the lag phase could be attributed to residual iron that persists in the ATP even after treatment with Chelex 100. Upon depletion of contaminating iron by A1110U, free A1110U accumulated during the course of the titration, which together with $(A1110U)_2Fe^{+i}$ rapidly inactivated the enzyme.

The rates of inactivation of human and viral enzyme ribonucleotide reductases were linearly dependent upon the concentration of $(A1110U)_2Fe^{+i}$ when measured in the presence of a saturating concentration of A1110U (Fig. 3). The bimolecular rate constant for inactivation of viral ribonucleotide

* The concentration of free A1110U in this preparation of $(A1110U)_2Fe^{+i}$ was minimized by adding a 5% molar excess of $FeSO_4$.

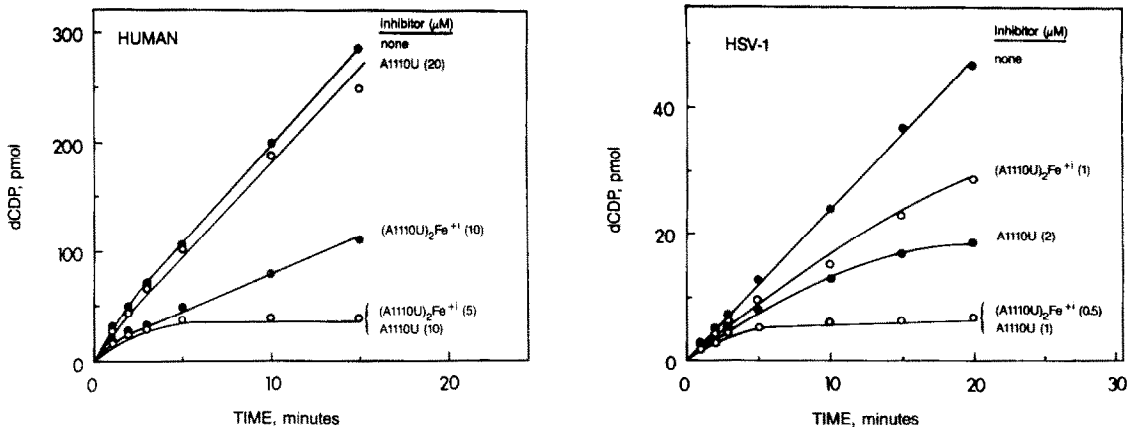

Fig. 2. Inactivation of human and HSV-1 ribonucleotide reductases by A1110U and $(A1110U)_2Fe^{+i}$. Assay buffer A (left panel) and assay buffer B (right panel) were modified by the additions noted. Reaction of the enzyme with $(A1110U)_2Fe^{+i}$ alone contained a 5% molar excess of free iron. The total concentrations of A1110U in the inhibited reactions (i.e. [A1110U] and $2[(A1110U)_2Fe^{+i}]$) were 20 μM with the human enzyme and 2 μM with the viral enzyme. After initiation of the reactions with 18 units of human ribonucleotide reductase (left panel) and 2.4 units of HSV-1 ribonucleotide reductase (right panel), the reaction mixtures (250 μL) were sampled (35 μL) at the indicated times for assay of dCDP.

reductase by $(A1110U)_2Fe^{+i}$ was $2.5 \times 10^7\ M^{-1}\ hr^{-1}$ at 30°. For human ribonucleotide reductase, the analogous bimolecular constant was $3.8 \times 10^6\ M^{-1}\ hr^{-1}$ at 37°. If the human enzyme was assayed at 30° in the HSV-1 assay buffer supplemented with 12 μM CDP, 5 mM ATP and 6 mM $MgCl_2$, the bimolecular rate constant for inactivation was decreased marginally to $2.7 \times 10^6\ M^{-1}\ hr^{-1}$. Moreover, the rates of inactivation of the viral enzyme were the same in the presence of 6 mM $MgCl_2$ and 5 mM ATP, which were in the human reductase assay buffer. Thus, these second-order rate constants were properties of the respective enzymes and were not a function of the slightly different assay conditions used.

The enhancement of the rate of inactivation of both ribonucleotide reductases by the metal complex of A1110U appeared to be specific for iron. Neither the cobalt nor the nickel complex of A1110U could substitute for the iron complex of A1110U (data not shown).

Effect of substrate on k_{inact}. Since the experimental protocols for determining k_{inact} included high concentrations of CDP (relative to the K_m), A1110U and $(A1110U)_2Fe^{+i}$ were inactivating enzyme that was complexed with either substrate or product. To differentiate between the reactivity of the enzyme–substrate complexes and free enzyme with A1110U and $(A1110U)_2Fe^{+i}$, k_{inact} was measured at a high concentration of CDP ($S/K_m > 5$) and at a low concentration of CDP ($S/K_m < 0.8$). In the former case, k_{inact} reflected the reactivity of the enzyme–substrate complexes with the combination of A1110U and

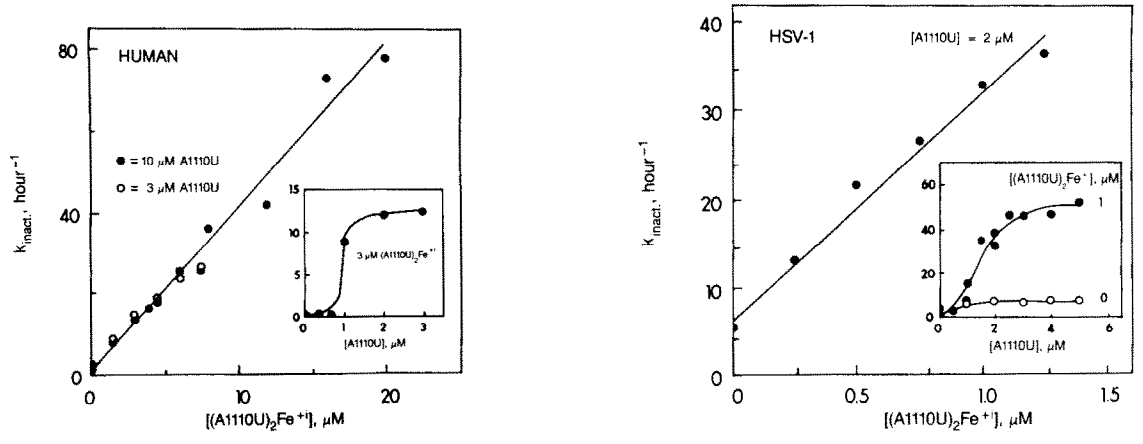

Fig. 3. Dependence of k_{inact} on the concentrations of A1110U and $(A1110U)_2Fe^{+i}$. The k_{inact} for human (left panel) or HSV-1 (right panel) ribonucleotide reductase was measured at the indicated concentrations of $(A1110U)_2Fe^{+i}$ with a fixed concentration of A1110U. At a fixed concentration of $(A1110U)_2Fe^{+i}$, k_{inact} for human or HSV-1 ribonucleotide reductase was measured at the indicated concentrations of A1110U (inset). Other reaction conditions are detailed in the legend to Fig. 2.

$(A1110U)_2Fe^{+i}$, and in the latter case k_{inact} was a weighted average of the reactivity of free enzyme and the enzyme–substrate complexes with the inactivator. The viral enzyme was inactivated by 1 μM A1110U and 0.5 μM $(A1110U)_2Fe^{+i}$ with a k_{inact} of $20 \pm 2\ hr^{-1}$ at 15 μM CDP (K_m = 0.65 μM [11]) and with a k_{inact} of $15 \pm 1\ hr^{-1}$ at 0.5 μM CDP. If this difference between the k_{inact} measured at the two substrate concentrations were considered significant, the rate constants for inactivation of free enzyme and the enzyme–substrate complex by this combination of A1110U and $(A1110U)_2Fe^{+i}$ were calculated from these data and the K_m of the enzyme for CDP to be 11 and $20\ hr^{-1}$ respectively. The rate of inactivation of the human enzyme with 10 μM A1110U and 3μM $(A1110U)_2Fe^{+i}$ was $14 \pm 1\ hr^{-1}$ at 12 μM CDP and $15 \pm 1\ hr^{-1}$ at 2 μM CDP (K_m = 2.4 μM [16]).

Dependence of k_{inact} *on the oxidation state of iron in* $(A1110U)_2Fe^{+i}$. The valence of iron in $(A1110U)_2Fe^{+i}$ depended upon the relative concentrations of O_2 and dithiothreitol in the assay buffer. In the absence of oxygen, $(A1110U)_2Fe^{+i}$ was essentially all $(A1110U)_2Fe^{2+}$. In the presence of oxygen and dithiothreitol $(A1110U)_2Fe^{+i}$ was a mixture of valence states. The valence state of iron in the complex that participated in the inactivation process was determined by measuring the rate of aerobic and anaerobic inactivation of the enzyme. The data of Table 1 show that the values of k_{inact} for both ribonucleotide reductases were independent of the presence of O_2 (<0.3 to 200 μM) in the assay buffer. Thus, the oxidation state of iron in the $(A1110U)_2Fe^{+i}$ complex did not affect the reactivity of $(A1110U)_2Fe^{+i}$ with the enzyme. Furthermore, these data and the observation that neither catalase nor superoxide dismutase affected k_{inact} (data not shown) show that O_2 and reactive oxygen-derived intermediates were not participates in the inactivation process.

Inactivation by iron-chelating agents which are structurally unrelated to A1110U. Viral enzyme was not stimulated by addition of iron, but was inactivated by several different types of chelating reagents. For instance, *l*-mimosine, desferrioxamine and 1,10-phenanthroline increased the value of k_{inact} from an inherently small value that was $<1\ hr^{-1}$ to a value that ranged from 4.1 to $4.7\ hr^{-1}$ which is almost as rapid as the $7.6\ hr^{-1}$ constant observed with A1110U.

In contrast, the activity of human ribonucleotide reductase was found to double after addition of 4 or 20 μM $FeSO_4$ to the standard assay medium. Similar results have been reported for mammalian ribonucleotide reductases from Novikoff tumor and rabbit bone marrow [7, 17]. Iron chelators, such as desferrioxamine or 1,10-phenanthroline (20 μM), decreased the enzymatic activity in the iron-supplemented medium to that observed in the iron-free assay medium. These chelators neither inactivated nor inhibited this enzyme in the iron-free assay buffer.

Several iron-chelating agents and their iron complexes were tested as substitutes for free A1110U or $(A1110U)_2Fe^{+i}$ in the inactivation reaction. The iron complex of desferrioxamine did not inactivate viral enzyme (Fig. 4). The combination of A1110U or desferrioxamine with the iron complex of desferrioxamine (10 μM) inactivated the enzyme at the same rate as A1110U or desferrioxamine alone. However, 5 μM desferrioxamine in combination with 0.5 μM $(A1110U)_2Fe^{+i}$ rapidly inactivated the enzyme with a k_{inact} of $18\ hr^{-1}$ (Fig. 4), which was similar to the value for 1 μM A1110U and 0.5 μM $(A1110U)_2Fe^{+i}$ ($20\ hr^{-1}$). Similar results were observed with 1,10-phenanthroline (5 μM) and $(A1110U)_2Fe^{+i}$.

Human ribonucleotide reductase was not inactivated by 10 μM desferrioxamine, 10 μM A1110U or 7.5 μM $(A1110U)_2Fe^{+i}$. Furthermore, the com-

Table 1. Inactivation of ribonucleotide reductase by $(A1110U)_2Fe^{2+}$ and $(A1110U)_2Fe^{3+}$

	% $(A1110U)_2Fe^{2+}$*	K_{inact}† (hr^{-1})
Human ribonucleotide reductase		
Aerobic	20	7.1 ± 0.7
Anaerobic‡	>90	7.5 ± 0.8
HSV-1 ribonucleotide reductase		
Aerobic	50	33 ± 3
Anaerobic‡	>90	37 ± 4

* The percent of $(A1110U)_2Fe^{+i}$ as $(A1110U)_2Fe^{2+}$ was assumed to be linearly related to absorbance of the solution at 590 nm. The absorbance at 590 nm in the presence of dithionite was equated to 100% $(A1110U)_2Fe^{2+}$. The absorbance at 590 nm which remained after the oxidation of $(A1110U)_2Fe^{2+}$ by O_2 was equated to 100% $(A1110U)_2Fe^{3+}$.

† Human ribonucleotide reductase was inactivated with 10 μM A1110U and 1.5 μM $(A1110U)_2Fe^{+i}$. HSV-1 ribonucleotide reductase was inactivated with 2 μM A1110U and 1 μM $(A1110U)_2Fe^{+i}$. Other reaction conditions were similar to those given in the legend to Fig. 2.

‡ O_2 was eliminated from the assay medium with 4 μM glucose oxidase, 2 mM glucose and 1.7 μM catalase. Glucose and glucose oxidase inhibited the activity of the human ribonucleotide reductase by 30% but did not affect the activity of HSV-1 ribonucleotide reductase. The steady-state concentration of O_2 in the reaction vessel was estimated to be 0.3 μM (Porter DJT, unpublished method).

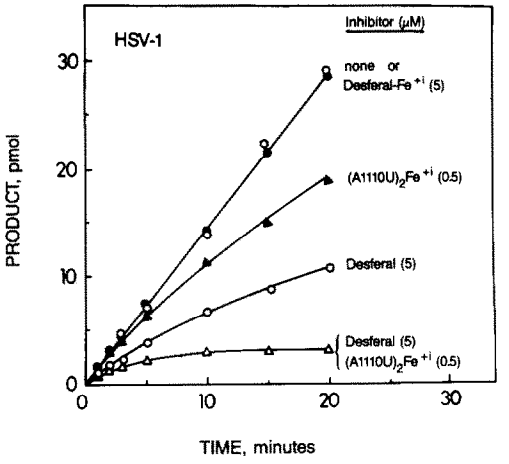

Fig. 4. Desferrioxamine enhancement of the rate of inactivation of HSV-1 ribonucleotide reductase by $(A1110U)_2Fe^{+i}$. Assay buffer B was modified by the additions noted. The reactions (250 μL) were initiated with 1.5 units of HSV-1 reductase and were sampled (30 μL) at the indicated times for assay of dCDP.

bination of 8 μM desferrioxamine–iron complex and 10 μM A1110U did not inactivate this enzyme. However, the combination of 10 μM desferrioxamine and 7.5 μM $(A1110U)_2Fe^{+i}$ inactivated the enzyme with a k_{inact} of 31 hr^{-1}, which was similar to the value for the combination of 10 μM A1110U and 7.5 μM $(A1110U)_2Fe^{+i}$ (28 hr^{-1}). It is apparent that even though the iron complexes of several chelating agents were unable to substitute for $(A1110U)_2Fe^{+i}$ in combination with A1110U to produce rapid inactivation of these enzymes, these chelators in their metal-free form were effective substitutes for free A1110U in this combination.

Reversal of inactivation by reconstitution with iron. Since it was not possible to dialyze or gel filtrate these enzymes in an iron-free environment without the addition of iron chelators, which by themselves inactivated the viral enzyme, indirect methods were used to remove the inhibitor to demonstrate the reversibility of the inactivation process. One of the inactivators, A1110U, could be trapped as $(A1110U)_2Fe^{+i}$ with Fe^{2+}. Addition of excess $FeSO_4$ to inactivated human or viral ribonucleotide reductase partially reactivated the enzyme. The fraction of enzymatic activity that was not regenerated by this treatment was due to inhibition by the remaining $(A1110U)_2Fe^{+i}$ (Fig. 5). The recovery of enzymatic activity could be attributed either to the trapping of free A1110U as $(A1110U)_2Fe^{+i}$ and/or to the presence of free excess Fe^{2+}. To distinguish between these possibilities, cobalt was substituted for iron as a sequestering agent for free A1110U. Under these conditions, inactivated viral ribonucleotide reductase was not reactivated by $CoCl_2$ unless $FeSO_4$ was added also (Fig. 6). Thus, excess iron was an essential component for reactivation.

The analogous experiment with the human enzyme was not feasible, because A1110U was not complexed by $CoCl_2$ to form the spectrally distinct $(A1110U)_2Fe^{2+}$ complex in human ribonucleotide reductase assay buffer (buffer A). The ATP (5 mM) present in this buffer probably competed with A1110U for Co^{2+} which rendered Co^{2+} an ineffective trap for A1110U.

The A1110U and $(A1110U)_2Fe^{+i}$ concentrations in an inactivation reaction mixture were also reduced by dilution. If viral enzyme was inactivated by 1 μM A1110U and 1 μM $(A1110U)_2Fe^{+i}$ and then diluted 20-fold into the standard assay supplemented with 1 μM $FeSO_4$, 70–100% of the enzymatic activity was recovered (10-min assay). Alternatively, inactivated enzyme diluted into an iron-free assay buffer (containing 1 μM EDTA) regained less than 5% of its activity (Fig. 7).

$CuSO_4$, $CoCl_2$ and $NiCl_2$ did not substitute for iron

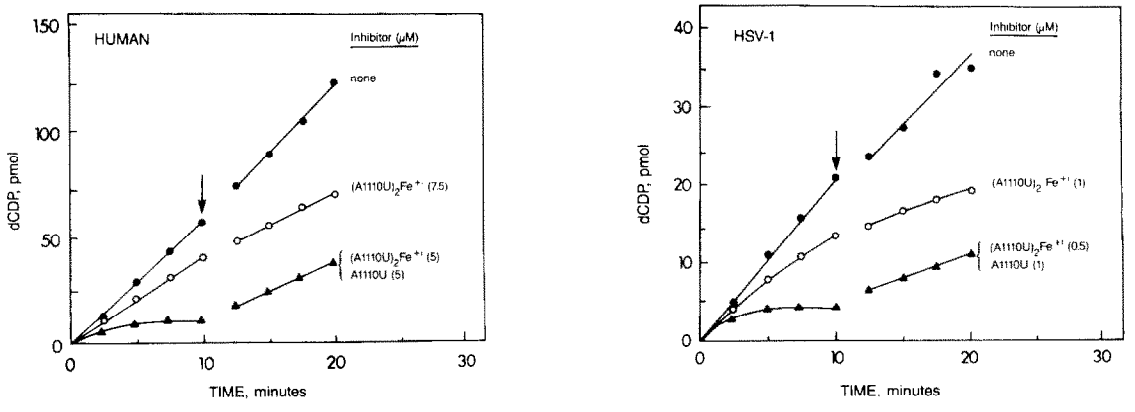

Fig. 5. Reactivation of inactivated ribonucleotide reductase by $FeSO_4$. Assay buffer A and assay buffer B were modified by the additions indicated (the final volume was 250 μL). The reactions were initiated with 5.9 units of human ribonucleotide reductase (left panel) or 2.1 units of HSV-1 ribonucleotide reductase (right panel). At the time indicated by the arrow, $FeSO_4$ was added to the reactions (<5% dilution) to give the final concentrations of 5 μM Fe^{2+} (--●--) or 5 μM Fe^{2+} and 7.5 μM $(A1110U)_2Fe^{+i}$ (--○--, --▲--) in the human ribonucleotide reductase assay and 5 μM Fe^{2+} (--●--) or 5 μM Fe^{2+} and 1 μM $(A1110U)_2Fe^{+i}$ (--○--, --▲--) in the HSV-1 ribonucleotide reductase assay. Samples (25 μL) were removed at the indicated times from the respective assay mixtures (250 μL) and were assayed for dCDP.

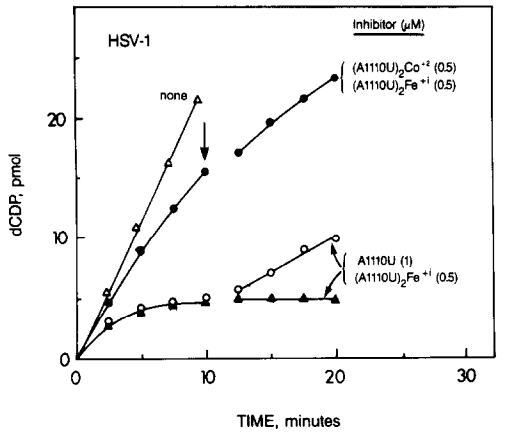

Fig. 6. Effect of the removal of A1110U as $(A1110U)_2Fe^{2+}$ on inactivated HSV-1 ribonucleotide reductase. Assay buffer B was modified with the inhibitors indicated, and the reactions were initiated with 2.1 units of HSV-1 ribonucleotide reductase. At the time indicated by the arrow, $CoCl_2$ was added to the assay medium to convert all free A1110U to 0.5 μM $(A1110U)_2Co^{2+}$ plus 2 μM excess $CoCl_2$ for reactions (--○--) and (--▲--), and to give 2 μM excess $CoCl_2$ for reaction (--●--). At this point, all reactions except the control contained 0.5 μM $(A1110U)_2Fe^{+i}$, 0.5 μM $(A1110U)_2Co^{2+}$ plus 2 μM excess $CoCl_2$. $FeSO_4$ was then added to reaction (--○--) 30 sec after the addition of $CoCl_2$ to also provide 2 μM excess $FeSO_4$. Other reaction conditions are detailed in the legend to Fig. 2. For a more detailed explanation see the text.

in the reactivation experiments and did not inhibit the reactivation of the enzyme by iron. However, 10 μM $MnCl_2$ inhibited the reactivation of inactivated viral enzyme by 0.1 μM $FeSO_4$. If inactivated enzyme was treated with 0.1 μM $FeSO_4$ for 2 min prior to addition of $MnCl_2$, the enzyme was reactivated rapidly. Alternatively, if 2 μM $FeSO_4$ was added subsequent to dilution of inactive enzyme into assay buffer that contained 10 μM $MnCl_2$, the enzyme regained activity in a time-dependent manner with a rate constant of 13 hr^{-1}. These results suggested that Mn^{2+} bound to the iron site of inactivated enzyme in a manner that did not support catalysis. Fe^{2+} reactivated the enzyme by slowly displacing the bound Mn^{2+}.

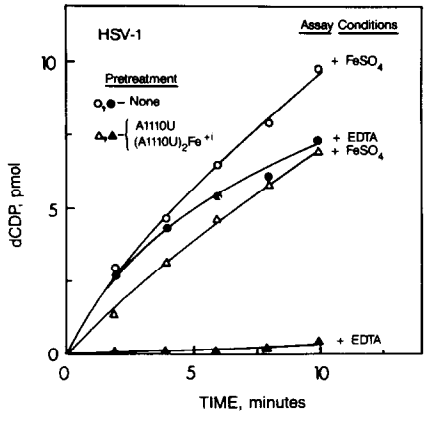

Fig. 7. Reactivation of inactivated HSV-1 ribonucleotide reductase by iron. HSV-1 ribonucleotide reductase (1.5 units) was inactivated by an 8-min incubation at 30° with 1 μM A1110U plus 1 μM $(A1110U)_2Fe^{+i}$ in 0.1 M Hepes–Na^+ at pH 8.1 with 10 mM dithiothreitol. A1110U and $(A1110U)_2Fe^{+i}$ were omitted from the control reaction mixture. The treated enzyme was diluted (1:20) into assay buffer B (200 μL) that contained either 1 μM EDTA or 1 μM $FeSO_4$. Samples (35 μL) were removed at the indicated times and assayed for dCDP formation.

Viral ribonucleotide reductase was inactivated [80% relative to control enzyme which was treated the same except for $(A1110U)_2Fe^{+i}$] by 13 μM $(A1110U)_2Fe^{+i}$ (in the absence of A1110U and substrate) after 30 min at 30°. Unlike enzyme inactivated by treatment with a combination of A1110U and $(A1110U)_2Fe^{+i}$, this enzyme was not reactivated by dilution into an assay buffer supplemented with 10 μM $FeSO_4$.

DISCUSSION

Viral ribonucleotide reductase was inactivated by A1110U, $(A1110U)_2Fe^{+i}$ or the combination of the two. These inactivators favored the viral enzyme over the human enzyme, for three reasons. First, A1110U was a specific inactivator of the viral enzyme. It neither inhibited nor inactivated the human enzyme. Second, $(A1110U)_2Fe^{+i}$ inactivated the viral enzyme but only inhibited the human enzyme weakly. Finally, even though both enzymes were synergistically inactivated by the combination of A1110U and $(A1110U)_2Fe^{+i}$, the second-order rate constant for inactivation of viral ribonucleotide reductase was significantly larger than that for the human enzyme [$25 \times 10^6\ M^{-1}\ hr^{-1}$ (30°) versus $3.7 \times 10^6\ M^{-1}\ hr^{-1}$ (37°)].

The present study has attempted to characterize the inactivation of viral and human ribonucleotide reductases by the synergistic interaction between $(A1110U)_2Fe^{+i}$ and a chelating agent. The salient characteristics of this process were: (1) $(A1110U)_2Fe^{+i}$ rapidly inactivated HSV-1 and human ribonucleotide reductases in the presence of iron-chelating agents such as A1110U, desferrioxamine and 1,10-phenanthroline, (2) addition of excess of $FeSO_4$ reversed the inactivation process, (3) the rates of inactivation were essentially independent of substrate concentration and the oxidation state of iron in $(A1110U)_2Fe^{+i}$.

The iron complex of 1-formylisoquinoline thiosemicarbazone, which is structurally analogous to $(A1110U)_2Fe^{+i}$, was reported to potently inhibit mammalian ribonucleotide reductase by reduction of a tyrosyl radical essential for enzymatic activity [8]. Two observations suggest that A1110U and $(A1110U)_2Fe^{+i}$ do not inactivate viral and human ribonucleotide reductases by an analogous mechanism. First, this mechanism predicts that the rate of onset of inactivation with non-saturating concentrations of $(A1110U)_2Fe^{+i}$ should depend upon the fraction of the complex in the ferrous state when, in fact, the rates of inactivation of these enzymes are independent of the oxidation state of iron in the complex. Second, this mechanism does not account

for the synergy between $(A1110U)_2Fe^{+i}$ and iron-chelating agents.

Since excess iron reverses the inactivation of the enzyme by the combination of A1110U and its iron complex, it can be argued that inactivation involves removal of a catalytically essential iron. To explain the synergy between $(A1110U)_2Fe^{+i}$ and A1110U (or another suitable metal-free chelator such as 1,10-phenanthroline or desferrioxamine), it is proposed that $(A1110U)_2Fe^{+i}$ forms a complex with the enzyme which readily loses a catalytically essential iron to the metal-free chelator. Since the rate of inactivation of the enzyme in the presence of a saturating concentration of A1110U was linearly dependent on the concentration of $(A1110U)_2Fe^{+i}$ (Fig. 3), it is not possible to determine the K_d for the initial complex between $(A1110U)_2Fe^{+i}$ and the enzyme under these conditions. The observation that $MnCl_2$ inhibited reactivation of the enzyme by iron is additional support for the hypothesis that iron is removed from the enzyme during inactivation. The simplest explanation for this observation is that iron and manganese compete for the same site on inactivated enzyme and manganese does not support catalysis.

A degree of controversy exists as to whether the thiosemicarbazone type of inhibitors causes inactivation of mammalian ribonucleotide reductase. Several investigators reported that they do inactivate while others report that they do not [1, 3, 7, 18–20]. It is clear from the present data that the ratio of iron to the thiosemicarbonazone is critical for the detection of inactivation. If all the inactivator were converted to the metal complex, it is quite possible that inactivation would not be observed. In fact, no inhibition would be detected if iron were totally absent from the assay medium. However, since iron was found to be a contaminant of ATP, a combination of free and iron-complexed inhibitor could be generated and rapid inactivation would be expected provided that excess iron was not added to the reaction mixtures.

Since either A1110U or its iron-complex caused inactivation of herpes ribonucleotide reductase at comparable rates, the total absence or the presence of excess iron, as expected, did not affect significantly the rates of inactivation by two thiosemicarbazones [3, 4]. However, the very rapid inactivation requires the careful titration of the inactivator to produce the synergistic combination of free and iron-complexed inhibitor.

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