Inhibition of Ribonucleotide Reductase by Nitric Oxide Derived from Thionitrites: Reversible Modifications of Both Subunits

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ABSTRACT: Thionitrites are spontaneous nitric oxide (NO) donors in neutral aqueous solutions. Consequently, they inhibit ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis, from *Escherichia coli* and murine adenocarcinoma TA3 cells. They also inhibit tumor cell proliferation. Reaction of thionitrites with protein R1, the large subunit, results in the nitrosation of cysteines, as shown from the formation of a chromophore with a characteristic absorption at 340 nm. EPR spectroscopy both on whole murine R2-overexpressing L1210 cells and on the pure protein showed that the tyrosyl radical of protein R2, the small subunit, reversibly couples to the NO radical, presumably leading to nitrosotyrosine adducts. Both molecular events might be at the origin of the inhibition of ribonucleotide reductase by NO, since a number of cysteines and the tyrosyl radical are essential for catalysis. These results identify NO donors as a new class of inhibitors of ribonucleotide reductase with potential applications as anticancer or antiviral chemotherapy agents.

Activated macrophages cause a profound inhibition of DNA synthesis in tumor cells, bacteria, and intracellular parasites or viruses, thus contributing to the host immune defense against rapidly proliferative pathogens (Stuehr & Nathan, 1989; Nathan & Hibbs, 1991). The cytotoxic effects are partly due to the induction of a nitric oxide (NO) synthase and the large production of NO derived from enzymatic oxidation of L-arginine (Marletta, 1993). Citrulline is the other end product of this pathway. Nitric oxide is a short-lived molecule in aerated aqueous solutions and is rapidly transformed into nitrite (Ignarro et al., 1993). A variety of other cells (hepatocytes, vascular endothelial and smooth muscle cells, tumor cells) also have the ability to generate NO after induction of intracellular NO synthases (Marletta, 1993). Under such conditions, DNA synthesis in tumor cells is inhibited and proliferation ceases (Lepoivre et al., 1990).

Recent reports have implicated ribonucleotide reductase (RNR) as a possible target molecule of nitric oxide (Lepoivre et al., 1990, 1991, 1992, 1994; Kwon et al., 1991). Inhibition of ribonucleotide reductase by an inducible NO synthase has been clearly demonstrated in cell-free extracts of tumor cells. Ribonucleotide reductase catalyzes the reduction of ribonucleotides to their corresponding deoxyribonucleotides and thus provides a balanced supply of precursors for DNA synthesis (Thelander & Reichard, 1979). RNR is an attractive enzyme target in the chemotherapy of cancer. It is the rate-limiting step in the pathway leading to DNA synthesis and cell replication. Moreover, the RNR activity is intimately associated with the proliferative state of the cell (Takeda & Weber, 1981). There is a good correlation between RNR activity and tumor growth rate. Hydroxyurea is the only RNR inhibitor currently in clinical use as an anticancer drug, but its effectiveness both *in vitro* and *in vivo* is weak (Lammers & Follmann, 1983).

In all eukaryotic cells, as well as in *Escherichia coli*, the enzyme consists of two homodimeric proteins, denoted protein R1 and protein R2 (Stubbe, 1990; Fontecave et al., 1992). A one-to-one complex of R1 and R2 forms the catalytically active complex in which each protein contributes essential features for catalysis. The large subunit, protein R1, binds substrates and allosteric effectors and contains redox-active cysteines which provide the reducing equivalents required for the reaction. The small subunit, protein R2, contributes a stable tyrosyl free radical generated and stabilized by an adjacent binuclear μ-oxo-bridged non-heme-iron center. Destruction of this radical by hydroxyurea inactivates the enzyme and inhibits DNA synthesis. Hydroxyurea-resistant cells overexpress the radical-containing R2 subunit (Eriksson et al., 1984). Nitric oxide is also capable of inducing the disappearance of the R2-centered tyrosyl radical, as shown by EPR spectroscopy applied either to R2-overexpressing whole tumor cells or to the pure recombinant R2 subunit of the enzyme (Lepoivre et al., 1991, 1992, 1994).

In this paper we report that thionitrites, RSNO, a class of NO donors, have cytostatic properties, at least partly because of their inhibitory effects on ribonucleotide reductase. The detailed investigation of the reaction between thionitrite-derived NO and pure preparations of proteins R1 and R2 gives new insights into the mechanisms of NO-dependent inactivation of ribonucleotide reductase, which probably involves both the tyrosyl radical of R2 and the cysteines of R1. In particular, a new type of reaction of the tyrosyl radical is demonstrated in this study.

MATERIALS AND METHODS

Materials. CDP, ATP, dithiothreitol (DTT), dithioerythritol (DET), and *N*-acetyl-D,L-penicillamine were purchased

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from Sigma. *t*-BuONO (90% or 98%) was purchased from Aldrich. Cysteamine hydrochloride was from Fluka. Radioactive CDP (specific activity, 680 GBq/mmol) and thymidine (specific activity, 37 GBq/mmol) were obtained from Amersham, France. *S*-nitroso-*N*-acetylpenicillamine **4** was synthesized as previously described (Field et al., 1978). Thionitrites **1-3** were prepared by reacting the corresponding thiols with *tert*-butyl nitrite (unpublished results). Oxyhemoglobin was prepared from human methemoglobin (Sigma) according to Di Orio (Di Orio, 1981). The concentration was determined from the absorption coefficient: λ_{max}, nm (ϵ, mM^{-1} cm^{-1}) = 541 (13.5), 576 (14.6). Protein R1 and protein R2 from *E. coli* were purified from overproducing strains of *E. coli* as previously described (Larsson et al., 1988; Sjöberg et al., 1986). MetR2 was obtained from R2 by treatment with hydroxyurea (Barlow et al., 1983). Protein concentration was determined colorimetrically using a molar extinction coefficient of ϵ_{280} = 189 000 M^{-1} cm^{-1} for the R1 dimer. R1 mutants R1C225S, R1C462S, R1C439S, and R1C759S were kindly provided by Professor J. Stubbe (Department of Chemistry, M.I.T., Cambridge, MA). Thioredoxin and thioredoxin reductase were kindly given by Dr. J.-P. Jacquot (Université Paris-Sud, Orsay, France).

Methods. Cell Lines. The K562 human myelogenous leukemia, the L1210 murine lymphoblastic leukemia, and the R2-overproducing TA3H2 mutant derived from a murine adenocarcinoma and kindly provided by Pr. L. Thelander (University of Umeå, Umeå, Sweden) were maintained in RPMI 1640 medium supplemented with antibiotics, 5% heat-inactivated fetal calf serum (Gibco BRL Sarl, Cergy-Pontoise, France), and 25 mM HEPES, pH 7.4. This medium will be referred to as culture medium in the following sections.

Determination of [^{3}H]Thymidine Incorporation. Human K562 cells were seeded in 96-well microculture plates (Nunclon, Roskilde, Denmark) at 2 × 10^5 cells/well in 100 μL of culture medium. Thionitrites prepared extemporaneously in culture medium were added in 100 μL to the cultures that were incubated for 30 min or 4 h at 37 °C in a humidified 5% CO_2 incubator. Then cells were pulsed for 90 min with 37 kBq of [^{3}H]thymidine; afterward DNA was harvested and radioactivity counted as previously described (Lepoivre et al., 1990). Whether DNA synthesis is inhibited because of RNR inhibition can be concluded from the ability of exogenous deoxynucleosides to restore it. Thus in some experiments, deoxyadenosine and deoxyguanosine, or the corresponding ribonucleosides as controls, 200 μM each, were added during the last 90-min period.

Assay of Ribonucleotide Reductase Activity in Crude Cytosolic Preparations of TA3H2 Cells. Lysates from R2-overproducing TA3H2 cells were used as a crude source of murine RNR activity. Assays were performed with either low or high concentrations of the reducing agent DET, in the absence (controls) or presence of increasing concentrations of thionitrites. Conditions of high DET concentration were as previously described (Lepoivre et al., 1990). Ribonucleotide reductase activity was measured with 200 μM CDP, 74 kBq of [5-^{3}H]CDP as substrate, 5 mM ATP as effector, and 10 mM DET, in 100 mM HEPES, pH 7.6, and 15 mM magnesium acetate. Low-DET conditions were similar, except that DET concentration was reduced to 500 μM. Reducing equivalents for RNR activity were provided by a mixture of NADPH (500 μM), thioredoxin reductase (400 nM), and recombinant thioredoxin (25 μM) from *E. coli* in Tris·HCl 25 mM, pH 7.8. The incubation was carried out at 37 °C for 45 min in a final volume of 90 μL. Reaction was stopped by boiling for 2 min. Radiolabeled substrate and product were dephosphorylated by treatment with 10 μL of *Crotalus adamanteus* venom (200 mg/mL) and separated by reverse-phase HPLC on a 5-μm Zorbax ODS C18 column (SFCC Shandon, Eragny, France), connected to a Berthold LB 506 C-1 flow-through radioactivity monitor (Lepoivre et al., 1990).

Assay of Ribonucleotide Reductase Activity of Proteins R1 + R2. A typical assay mixture contained, in a final volume of 60 μL, 1.2 mM ATP, 8 mM $MgCl_2$, 25 mM Tris·HCl (pH 7.5), 6.8 mM DTT, 0.06 mg/mL thioredoxin, 1.2 mM CDP, 0.18 mM [5-^{3}H]CDP, 0.19 mg/mL R1 unless otherwise stated, 0.035 mg/mL R2, and variable concentrations of the inhibitors. After 10 min of incubation at 30 °C, 500 μL of 1 M $HClO_4$ and 50 μL of 5 mg/mL dCMP were added. The solution was then heated for 10 min in boiling water, neutralized at 4 °C with 4 N KOH, and centrifuged for 10 min at 3000 rpm. The supernatant solution was added to the top of a Dowex AG 50W-X8 column, 0.6 × 15 cm. CMP was eluted with 106 mL of 0.2 M acetic acid. Another 30 mL of 0.2 M acetic acid, now containing dCMP, was collected. From the ultraviolet absorbance of the carrier dCMP, it was found that the recovery of dCMP during chromatography ranged between 70% and 90%. The total amount of dCMP was determined from the radioactivity present in the dCMP fraction. Also, control experiments were performed with no inhibitor in the incubation mixture.

EPR Spectroscopy of Packed Cell Pellets. Murine L1210 cells were transfected by electroporation with a 13-kb *Hin*dIII fragment containing the entire mouse R2 gene, kindly provided by Pr. L. Thelander (Thelander & Thelander, 1989). Stable transfectants were selected for their increased resistance to the RNR inhibitor hydroxyurea over a 2-week period, and the procedure was repeated twice again (Lepoivre et al., 1994). The resulting mutant clone L1210R2, containing a 15-fold excess of R2 protein, as compared to the wild-type phenotype, was used for whole-cell EPR experiments. Concentration of R2-associated tyrosyl free radical in L1210R2 cells was determined according to Lepoivre et al., (1992). Briefly, two 14-cm culture dishes containing 40 × 10^6 cells each in 20 mL of culture medium were incubated for 30 min with increasing concentration of thionitrite. Then, cells were harvested by centrifugation, washed once in PBS, resuspended in 600 μL of PBS, and packed by centrifugation for 20 min at 500*g* in a 4-mm (o.d.) quartz tube (Varian, Sunnyvale, CA). The sample was analyzed at 77 K using a Varian E109 spectrometer, calibrated with the radical 1,1-diphenyl-2-picrylhydrazyl centered at g = 2.0036. In these experiments, the microwave power was 10 mW, the field modulation frequency was 9.18 GHz, and the modulation amplitude was 1 mT.

EPR Spectroscopy of Pure Protein R2. The reaction was carried out at 37 °C into an EPR tube containing R2 (1 mg/mL) and thionitrite **3** at various concentrations, in 150 μL of 50 mM Tris·HCl buffer, pH 7.5. At time intervals, the tube was frozen in liquid nitrogen, and the EPR spectrum of the solution was recorded at 110 K using a Varian E102 spectrometer. The amount of tyrosyl radical was determined from the comparison of the amplitude of the typical EPR signal at g = 2 to that of a pure sample of protein R2 (1

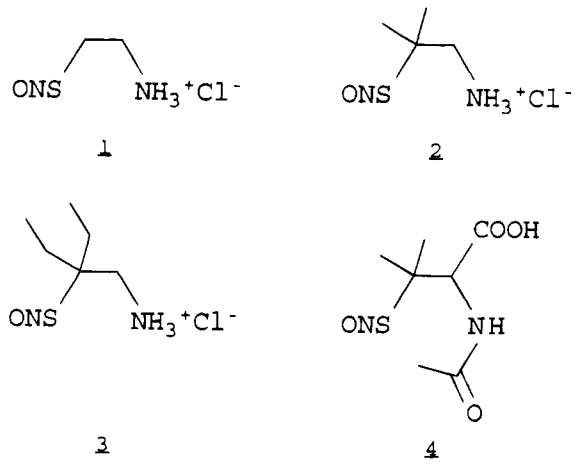


FIGURE 1: Structures of thionitrites **1**–**4**.

mg/mL). The microwave power was 1.5 mW, the field modulation frequency was 9.2 GHz, the modulation amplitude was 3.2 G, and the time constant was 0.25 s.

R1 Inactivation Time Course. DTT from R1 preparation was removed by using a Penefsky column (Penefsky, 1977). The reaction mixture contained 10 μM protein R1 in 34 mM HEPES buffer, pH 7.5. Thionitrite **1** was added to a final concentration of 50–250 μM; 5-μL aliquots were removed at various time points for dilution into 95 μL of an assay mixture containing the same buffer, 1.5 mM ATP, 0.4 mM NADPH, 1.5 mM CDP, 10 nM $MgCl_2$, 2.5 μg of thioredoxin reductase, 10 μg of thioredoxin, and 6 μM R2. The activity of R1 was determined from the rate of NADPH oxidation, monitored spectrophotometrically at 340 mM (ϵ_{340} = 6220 M^{-1} cm^{-1}). R1 specific activity is 1.5 μmol. min^{-1} mg^{-1}.

UV–Vis Spectroscopy of Pure Protein R1. UV–vis absorption spectra were recorded on a Uvikon 930 spectrophotometer. All experiments were at ambiant temperature in a microcuvette. Protein R1 and mutants were desalted anaerobically on a Centricon C30 instrument just before each experiment to remove DTT present in buffers. The cuvette was filled with 245 μL of 50 mM Tris·HCl buffer, pH 7.5, containing protein R1 at a final concentration of 2 mg/mL. The optical spectrum of the solution was recorded between 200 and 600 nm and stored in the memory of the spectrophotometer to be used as a reference (zero) spectrum for all subsequent readings. The reaction was started by addition of 5 μL of an aqueous solution of thionitrite **2**, final concentration 0.7 mM. Spectra between 200 and 600 nm were recorded at time intervals. The optical density at 340 nm is noted OD_1. In a second experiment made in the absence of protein R1, the optical density at 340 nm is noted OD_2. The nitrosated R1 protein was characterized by OD = $OD_1 - OD_2$, at each time point. Nitrosated R1 was desalted by cycles of dilution and centrifugation on a Centricon C30 instrument.

RESULTS

NO Generation from Thionitrites. The four water-soluble thionitrites (RSNO) used in this study are shown in Figure 1. They are unstable when dissolved in 50 mM Tris·HCl buffer, pH 7.5, and generate NO during decomposition together with the corresponding stable disulfide RSSR (Roy et al., 1994). The following half-lives have been measured spectrophotometrically by monitoring the loss of the characteristic absorption at 333 nm (**1**, 5 min) or 337 nm (**2** and **3**, 15 min; **4**, 5 h). Compound **4** is better known as a vasodilator named SNAP (*S*-nitroso-*N*-acetylpenicillamine).

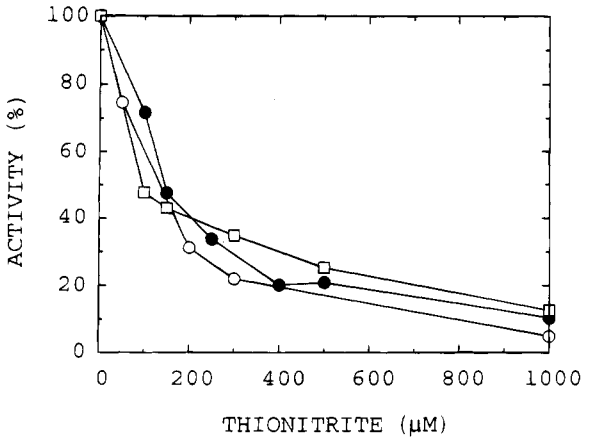


FIGURE 2: Inhibition of RNR activity by thionitrites **1** (●), **2** (□), and **4** (○). The assay was carried out with pure preparations of protein R1 and R2 from *E. coli* and increasing concentrations of inhibitors as described in the experimental procedure section.

The decomposition of RSNO follows first-order kinetics with k_1, the first-order rate constant (Roy et al., 1994). On the other hand, the fast reaction of NO with O_2, present in the reaction mixture, is second order in NO concentration and first order in O_2 concentration with $k'_2 = 6 \times 10^6$ M^{-2} s^{-1} the third-order rate constant (Kharitonov et al., 1994). The following equation may be used to calculate NO concentrations at time t:

$$d[NO]/dt = k_1[RSNO]_o\, e^{-k_1 t} - k'_2[NO]^2[O_2]$$

With $[RSNO]_o$, the initial concentration of thionitrite **3**, for example, in the range 0.1–1 mM, a peak of NO concentration is obtained after a few minutes ($[NO]_{max}$ = 10–30 μM). Then NO concentration decays rapidly ([NO] = 0.5–1 μM after 10 min). This shows that our chemical NO donor system gives NO concentration in the micromolar range, which is physiologically relevant. Under reducing conditions, i.e., during incubation in the presence of 6 mM DTT, the stability of SNAP is greatly decreased with a half-life value of about 4 min. DTT also greatly accelerated the decomposition of compounds **1**–**3**.

Inhibition of Ribonucleotide Reductase by Thionitrites. Ribonucleotide reductase activity of pure preparations of proteins R1 and R2 from *E. coli* was assayed during a 10-min reaction in the presence of increasing concentrations of compounds **1**, **2**, and **4** (Figure 2). The three inhibitors had similar IC_{50} values at approximately 150 μM in agreement with the similar fast release of NO from the three compounds, under the reducing conditions of the assay. At the highest concentrations tested (2 mM), the corresponding reduced and oxidized cysteamines were not active. In the assay, DTT served as the source of electrons which are transferred to R1 through catalytic amounts of thioredoxin. DTT had to be kept at low concentrations (1 mM) in the assay mixture since increased concentrations of DTT were found to protect the enzyme from inhibition by thionitrites (data not shown). As expected, oxyhemoglobin also protected the enzyme from inhibition by thionitrite-derived NO (data not shown).

Thionitrites were also tested against a murine RNR enzyme. The source of enzyme was a crude cytosolic extract from R2-overproducing TA3H2 cells. As shown in Figure

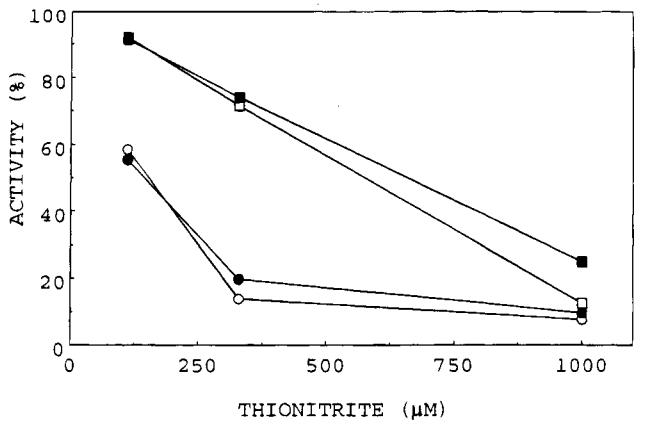


FIGURE 3: Inhibition of RNR activity by thionitrites. Ribonucleotide reductase present in crude extracts of TA3H2 cells was tested in the presence of **2** (open symbols) or **3** (closed symbols) and compared to controls without inhibitor. This experiment was performed with two different extract preparations, one containing 10 mM (squares) and the other 0.5 mM (circles) DET, as detailed in the experimental procedures section. Ribonucleotide reductase activity was 407 and 122 pmol/min/mg of protein for control samples in the absence of inhibitor, respectively.

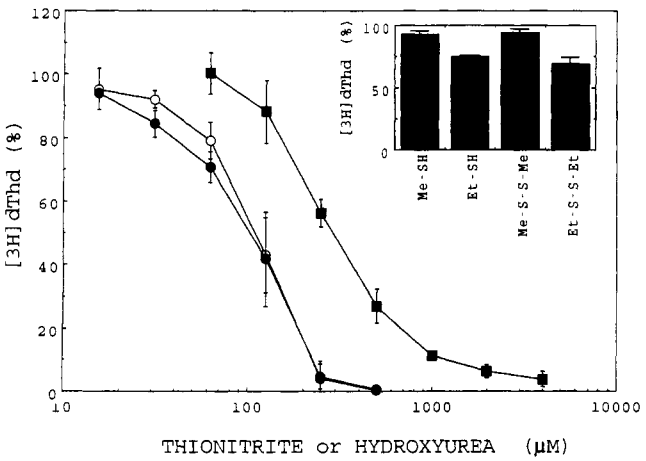


FIGURE 4: Inhibition of DNA synthesis in K562 cells by thionitrites. Thymidine incorporation by K562 cells was measured after 4 h in the presence of **2** (○), **3** (●), or hydroxyurea (■) and compared to untreated samples. Results are mean ± SE of two independent experiments. Inset: effect of reduced or oxidized cysteamine derivatives (Me-SH and Me-S-S-Me corresponding to **2**, Et-SH and ET-S-S-Et corresponding to **3**) on the proliferation of K562 cells. All substances were tested at a concentration of 250 μM as described above for thionitrites.

3, compounds **2** and **3** were potent inhibitors of RNR, with similar effectiveness over the concentration range tested. Also with this enzyme preparation, their inhibitory effect was increased when DET concentration was lowered from 10 mM to 500 μM. Reduced or oxidized cysteamines at 1 mM did not decrease RNR activity by more than 10% under conditions of high-DET concentration (data not shown).

Inhibition of Cell Proliferation by Thionitrites. In agreement with their inhibitory action against RNR activity, thionitrites markedly inhibited DNA synthesis in K562 target cells (Figure 4). Compounds **2** and **3** exhibited superimposable dose−response curves, with an IC_{50} close to 100 μM, which is 3 times lower than the IC_{50} of hydroxyurea, a specific inhibitor of RNR. At a concentration of 250 μM, thionitrites completely blocked DNA synthesis in K562 cells whereas the corresponding thiol and disulfide derivatives (denoted Me-SH and Me-S-S-Me from **2** and Et-SH and Et-S-S-Et from **3**) were not or only weakly active (Figure 4, inset).

The importance of RNR inhibition in thionitrite-induced cytostasis was investigated by suppplementing the culture medium with purine deoxyribonucleosides to compensate the defective synthesis of deoxyribonucleotides occurring during RNR inhibition. Exogenous deoxyribonucleosides, but not ribonucleosides, thus remarkably restored DNA synthesis in K562 cells cultured in the presence of 10 mM hydroxyurea, the selective inhibitor of RNR (data not shown). However, the same procedure failed to improve thymidine incorporation in cells incubated with 250 μM compound **3**. Also, resumption of DNA synthesis was not observed in one experiment where K562 cells were first incubated for 30 min with 250 μM compound **3**, and then washed and pulsed with [^{3}H]-thymidine for 90 min in fresh medium without inhibitor (percent [^{3}H]dThd uptake = 0.5 vs 1.7 without or with thionitrite, respectively). As a control, the same experiment was carried out with hydroxyurea. As expected, removing the drug from the medium gave back the cells the ability to synthesize DNA (percent [^{3}H]dThd uptake = 20 vs 1.8 without or with hydroxyurea, respectively). These results indicated that cytostasis caused by thionitrite cannot be simply attributed to RNR inhibition and exhibited a more complex pattern requiring further investigations.

Inactivation of Protein R1 by S-Nitrosocysteamine. Incubation of 10 μM DTT-free R1 with 50−250 μM compound **1** and then assaying for enzymatic activity by dilution into a solution containing a large excess of CDP and R2 resulted in a time-dependent inactivation. The double-reciprocal plot of pseudo-first-order rate constants of inactivation vs inhibitor concentration was linear (data not shown) and allowed the determination of the inactivation rate constant: $k_{inact} = 0.55\ min^{-1}$. The K_i value for compound **1** was 110 μM.

Reversible Nitrosation of Protein R1 by Thionitrites. The cysteines of protein R1 are likely targets of RSNO/NO. R1 contains 11 cysteines per monomer (Stubbe, 1990). Five are implicated in the electron transfers between R1, on one hand, and R2, thioredoxin, or the substrate, on the other. Consequently any mutation of these cysteines to serines modifies the activity of the protein drastically. A substitution of cysteine 439 by serine gives a totally inactive protein (Mao et al., 1992). We made the hypothesis that inactivation was due to cysteine nitrosation during reaction with thionitrites. The formation of a *S*-nitroso protein can be monitored by UV−vis spectroscopy since such groups, like small thionitrites, have a rather intense absorption band at around 340 nm (Stamler et al., 1992a,b). This is, to our knowledge, the only direct method to evidence it. Even though the above hypothesis is difficult to test experimentally since both reactant (low molecular mass thionitrite) and product (protein thionitrite) have similar spectroscopic features, we think that the data shown in Figure 5 qualitatively but clearly show that some cysteines of R1 have been nitrosated during reaction with thionitrites. Addition of R1, 2 mg/mL, to a solution of compound **2**, 0.7 mM, greatly slowed down the disappearance of the 340-nm absorption band. By correcting the absorbance at 340 nm for the spontaneous decomposition of the thionitrite **2** (Figure 5, open squares), it is possible to obtain the kinetics of R1 nitrosation during the first 50 min (Figure 5, closed circles). After 40 min of reaction, i.e., when the thionitrite **2** has been fully decomposed, the reaction mixture still contained a strong absorption band at 340 nm

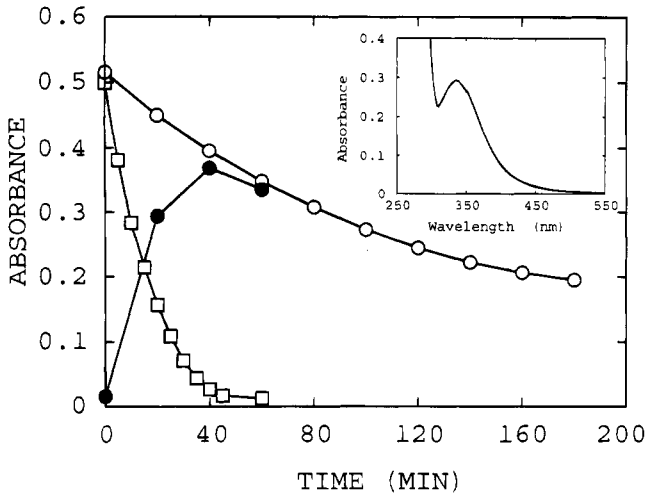


FIGURE 5: Time-dependent variations of the intensity of the 340-nm absorption band of a solution containing thionitrite **2** alone (□) or in the presence of protein R1 (○). The difference curve (●) reflects the nitrosation of R1 cysteines. Inset: optical spectrum of protein R1 after treatment with **2** for 60 min and desalting. The reaction results in formation of a new protein-bound chromophore absorbing maximally near 340 nm, assigned to *S*-nitrosocysteines of protein R1.

which persisted for hours. This band is conserved after desalting (Figure 5, inset), reflecting the presence of *S*-nitrosocysteines of protein R1. As already shown with other nitrosated proteins, the *S*-nitrosocysteines of R1 are remarkably stable (half-life estimated at about 2 h) when compared to free *S*-nitrosocysteine ($t_{1/2}$ = 1 min) (Myers et al., 1990). The decomposition of R1 *S*-nitrosocysteines follows first-order kinetics with a rate constant of $k = 5.3 \times 10^{-3}$ min^{-1}.

The experiment reported in Figure 5 was carried out with a R1 sample previously desalted under anaerobic conditions to remove DTT. Addition of DTT to nitrosated R1 resulted in faster decomposition of the *S*-nitrosocysteines and in enzyme reactivation. In one experiment DTT-free protein R1 was first treated with **2** during 15 min and then desalted to remove small molecules. Its activity in the presence of R2 then was greatly decreased when compared to a R1 sample treated under similar conditions but without **2** (15% activity). After incubation of that sample, at 37 °C, with 45 mM DTT, followed by desalting, the activity was partly restored (70% and 85% activity after 30- and 60-min incubation, respectively).

The extent of R1 nitrosation cannot be readily concluded from the intensity of the new absorption band because of uncertainties on the extinction coefficient and also because of the relative instability of that band. However, whether nitrosation takes place on any cysteine of R1 or only on a few ones, in particular among the redox-active ones, was tested by using site-directed R1 mutants to which the protocol described in Figure 5, designed to detect protein thionitrites spectrophotometrically, was applied. That less *S*-nitrosocysteines are formed due to the mutation could be detected by decreased absorptions at 340 nm at the end of the reaction. We chose the cysteine to serine mutants R1C225S, R1C462S, R1C439S, and R1C759S. Cysteines 225 and 462 are involved in the reduction of the ribonucleoside diphosphate, cysteine 439 in the electron transfer between R1 and R2, and cysteine 759 in the electron transfer from thioredoxin to R1. Unfortunately, a single mutation did not have a major effect on the absorbance at 340 nm, and variations cannot be accounted for less *S*-nitrosocysteines formed but rather

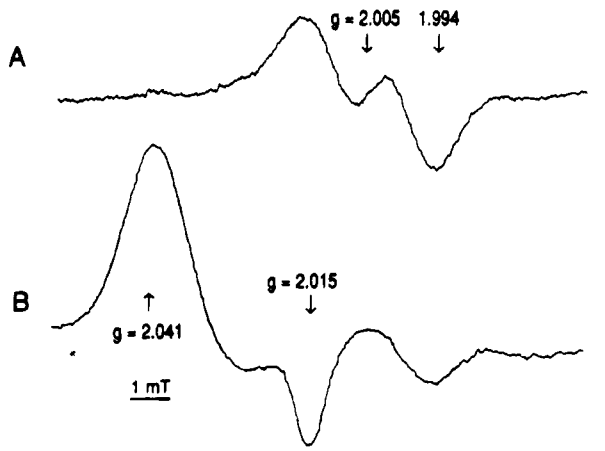


FIGURE 6: EPR spectra of L1210R2 cells during incubation with thionitrite **3**. A, before addition of **3**; B, after 30 min of incubation with 125 μM **3**. Intact cell pellets were analyzed by EPR spectroscopy at 77 K with a modulation amplitude of 1 mT. The tyrosyl radical EPR signal was quantitated from the amplitude of the g = 1.994 feature and the EPR signal of the dinitrosyl−iron−sulfur adduct from the amplitude of the g = 2.041 signal.

reflect experimental errors. In fact this instead shows that a large number of cysteines of R1 were nitrosated.

Effect of Thionitrite on R2-Centered Tyrosyl Free Radicals. Protein R2 and its radical are alternative targets of NO. Inactivation of RNR by thionitrites in mammalian cells was appreciated more directly by EPR analysis of the catalytically active tyrosyl free radical. The amount of tyrosyl radical can be determined from the intensity of the characteristic g = 2.00 EPR signal (Figure 6A). Nitric oxide has been previously shown to quench this radical efficiently in murine tumor cells induced to express NO synthase activity (Lepoivre et al., 1992). The same technique of whole-cell EPR spectroscopy was thus applied to R2-overexpressing L1210R2 cells cultured for 30 min in the presence of compound **3**, within the same concentration range that induced cytostasis. A dose-dependent decrease in the tyrosyl free radical content of L1210R2 cells was observed (Figure 7). Complete disappearance of the tyrosyl free radical EPR spectrum was noticed at 250 μM concentration of the drug. This concentration was not toxic for the cells (viability estimated 24 h later by trypan blue exclusion was ≥90%, compared to untreated cells). Interestingly, this effect was reversible since a significant fraction of the free radical reappeared when cells previously cultured in the presence of **3** were further incubated for 90 min in the absence of the NO donor (Figure 7, inset). Cycloheximide was added to prevent *de novo* R2 protein synthesis during the recovery period, suggesting that the chemical modifications at the tyrosine site induced by NO were not stable in a mammalian cell. As shown in Figure 6, the EPR spectrum of packed cells is in fact quite complex since it is composed of partially overlapping spectra from iron−nitrosyl complexes and R2 tyrosyl radical. The new EPR signal, with features at g = 2.041 and 2.015, is actually characteristic of nitrosyl adducts of intracellular iron-sulfur centers (Figure 6B) (Lepoivre et al., 1992, 1994; Drapier et al., 1991). Its intensity increased with increased concentration of thionitrite (data not shown). This confirms the presence of NO inside cells that have been treated by thionitrite.

In order to study the interaction between NO and the tyrosyl radical of protein R2 in greater detail, we investigated the reaction of 10 μM pure protein R2 from *E. coli* with

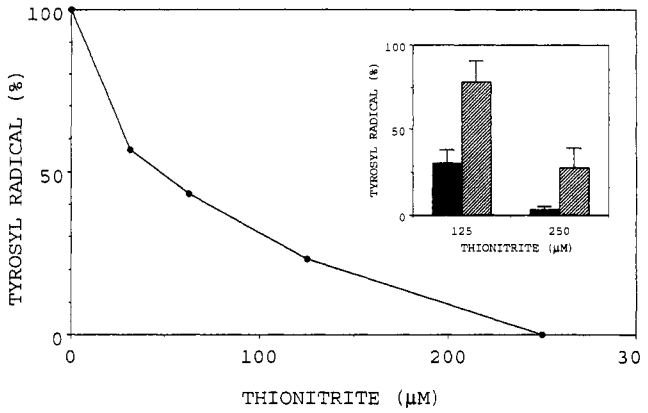


FIGURE 7: Dose-dependent decrease in the tyrosyl free radical content induced by **3** in murine tumor cells. Increasing concentrations of **3** were added to L1210R2 cells in culture medium. After 30 min at 37 °C, the concentration of R2-associated tyrosyl free radical in the cells packed in an EPR quartz tube was compared to a control incubated without thionitrite. During incubation an intracellular EPR-active iron−sulfur−NO species is accumulating. Since its EPR spectrum is partly overlapping that of the tyrosyl radical, in the $g = 2.00$ region, quantitation is made at $g = 2.041$ for the former and at $g = 1.994$ for the latter. These extreme features are not affected by the overlap. Inset: recovery of the R2-centered tyrosyl free radical from thionitrite effect. Cells cultured for 30 min with the indicated concentration of EtSNO were washed once and further incubated for 90 min in fresh culture medium containing 3 μg/mL cycloheximide. The concentration of the tyrosyl free radical in the cells before (black bars) or after (hatched bars) the recovery period was measured and expressed as described above. Results are mean ± SE of two independent experiments.

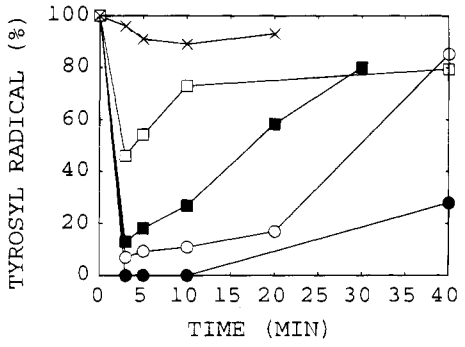


FIGURE 8: Reversible loss of the tyrosyl radical EPR signal during incubation of 10 μM protein R2 with thionitrite **3** (□, 20 μM; ■, 50 μM; ○, 100 μM; ●, 500 μM) in Tris·HCl (50 mM) buffer, pH 7.5. The reaction was carried out in a EPR tube. At time intervals the tube was frozen in liquid nitrogen and EPR spectra were recorded at 110 K. In one experiment, with 10 μM protein R2 and 180 μM compound **3**, 130 μM oxyhemoglobin was present in the reaction mixture (×).

increased concentrations of thionitrite **3** at neutral pH. As shown in Figure 8, the tyrosyl radical, quantitated by EPR spectroscopy, disappeared very rapidly during incubation. The reaction was remarkably efficient since, after 3 min, 50% of the tyrosyl radical was lost in the presence of only 20 μM thionitrite; 50 μM thionitrite led to a 90% loss of the radical. As a comparison, 1 mM hydroxyurea scavenged 33% radical after 5-min incubation. The corresponding thiol and disulfide derivatives had no effect on the EPR signal even at 1 mM.

Even more remarkable was the reappearance of the EPR signal after a short period of time indicating that the radical was regenerated. The rate of regeneration of the radical was dependent on the initial concentration of thionitrite (Figure 8). It was also dependent on the presence of oxygen since R2 remained EPR silent for a much longer period of time when the reaction was carried out under anaerobic conditions (data not shown). Moreover, recovery of the tyrosyl radical was much faster when the EPR tube was evacuated to remove NO (data not shown).

UV−vis spectroscopy is also useful for monitoring reactions at the level of the radical since the latter absorbs in the visible region at 410 nm. We found that after addition of the thionitrite to a pure solution of R2, the absorbance at 410 nm also rapidly dropped and then slowly increased. No modification of the absorption at 370 nm, characteristic of the iron center, could be observed, suggesting that the diiron center was maintained during the reaction. After a 60-min incubation of R2 with 100 μM **3** followed by desalting to remove low molecular mass species (thiols, disulfides, residual thionitrite, nitrite etc.), full activity and full radical content measured by both EPR and UV−vis spectroscopy were restored. Nitration of tyrosine probably did not occur since no band at 450 nm, characteristic of nitrotyrosine, could be detected (Knowles et al., 1974; Prütz et al., 1985); 10 μM nitrotyrosine would have led to an optical density at 450 nm of 0.04 and thus been detectable.

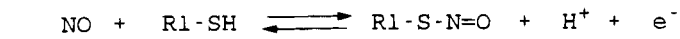
The strong protection of the tyrosyl radical by oxyhemoglobin (Figure 8) demonstrated that NO itself and not the thionitrite was the reactive species during scavenging of the radical. Moreover, when 100 μM SNAP was used as the NO source, no decrease of the intensity of the EPR signal could be observed, in good agreement with the extremely slow generation of NO from SNAP in the absence of reductant (data not shown).

The tyrosyl radical might have been transformed into tyrosine during the reaction. The intermediate EPR silent form of R2 might thus be the inactive form named metR2, which still contains the iron center but a normal tyrosine residue at the place of the tyrosyl radical. In *E. coli* metR2 is obtained during reaction of R2 with hydroxyurea (Barlow et al., 1983). metR2 can be transformed back to active R2 only when incubated with a strong reducing system (reduced methyl viologen, DTT at alkaline pH, or a NADPH:flavin oxidoreductase) aerobically (Sahlin et al., 1989; Fontecave et al., 1987, 1989, 1990). Clearly, thionitrites or their metabolites do not fulfill such conditions. Accordingly, no radical could be generated during reaction of 10 μM metR2 with thionitrite **3** or the corresponding thiol and disulfide, at any concentration and under prolonged incubation (data not shown). All these data exclude metR2 as the EPR silent intermediate species.

DISCUSSION

In this study we demonstrate that a variety of thionitrites, also named *S*-nitrosothiols, easily obtained by nitrosation of cysteamine derivatives, inhibited DNA synthesis in K562 human leukemia cells due to their ability to release NO. No effect was observed with the corresponding thiols and disulfides. Moreover, they are excellent inhibitors of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis, which provides the deoxyribonucleotides. The efficiency of these molecules is larger than that of hydroxyurea, a specific RNR inhibitor, used in clinics. However, inhibition of RNR is probably not sufficient to explain their antiproliferative properties since inhibition of DNA synthesis could not be reversed by addition of exogenous deoxyribonucleosides. That inhibition of RNR by thionitrites

Scheme 1



is mainly carried out by NO is consistent with (i) the demonstration that all thionitrites, including SNAP, release NO very rapidly, under the reducing conditions of the assay, (ii) the observed similarities of the drugs in terms of the efficiency of the inhibition, and (iii) the protection by oxyhemoglobin.

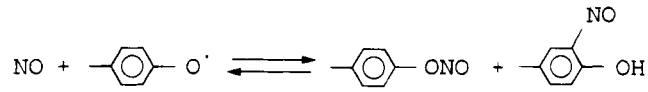
Several mechanisms by which NO interferes with RNR and thus inhibits enzyme activity have been investigated. The reduction of ribonucleotides requires the presence of active cysteines on protein R1 and a tyrosyl radical on protein R2 (Stubbe, 1990; Fontecave et al., 1992). We have shown that both cofactors are reversibly transformed by NO. The persistance of a characteristic absorption at around 340 nm, during several hours after reaction of thionitrites with a pure preparation of R1, clearly demonstrates that R1 cysteines were nitrosated. However, single site-directed mutations at the redox-active cysteines did not significantly change the intensity of the 340-nm absorption band. This shows either that many of the 22 cysteines were nitrosated or, more unlikely, that the redox ones were not. In fact, it is still uncertain whether the efficient time-dependent inactivation of R1 by thionitrites is specifically due to cysteine nitrosation, even though this is an attractive possibility. Further and more quantitative investigations are needed to clear up this point. While NO is not a nitrosating agent, it can, under aerobic conditions, transform a thiol into a thionitrite (Butler & Williams, 1993). The stability of the *S*-nitroso group in nitrosated R1 is remarkably larger than that of free *S*-nitrosocysteine, known to be exceedingly unstable under physiological conditions. Such an observation has been previously made with other nitrosated proteins (Stamler et al., 1992a,b). It thus seems that the stability of *S*-nitrosothiols greatly depends on their close environment, and in general, folded polypeptide chains protect them from decomposition.

On the other hand, this transformation of R1 is reversible since nitrosated R1 can be transformed back to R1, with active sulfhydryl centers, during incubation with a reducing agent such as DTT. This is in good agreement with the protection of RNR activity by increasing concentrations of DTT observed in cell extracts or on pure enzyme. There is probably an equilibrium between active R1 and nitrosated R1, which is controlled by NO concentration and the redox state of the solution (Scheme 1). Nitrosated R1 accumulates in the presence of a high NO generation, while as NO decreases, the intracellular reducing power progressively restores the reduced cysteines and the activity.

It is tempting to suggest that NO could play as a regulatory factor of RNR activity due to its ability to carry out a reversible nitrosation of essential cysteines of R1. This reaction may be one example of a general mechanism for the molecular control of protein function by NO. Another example is the NMDA receptor whose unique cysteine is reversibly nitrosated by NO, possibly providing a regulation of receptor activity (Lipton et al., 1993).

The other possible target of NO is the essential tyrosyl radical of protein R2. It has been previously shown that hydroxyurea-resistant tumor cells, exhibiting enhanced R2 expression, were also less sensitive than wild-type cells to NO produced by endogenous enzymatic oxidation of L-arginine. As expected from previous EPR experiments

Scheme 2



carried out on whole cells activated for NO generation (Lepoivre et al., 1992), the tyrosyl radical present in R2-overproducing tumor cells is progressively scavenged during incubation with thionitrites. However, these cells were able, after washing, to recover their tyrosyl radical in the absence of *de novo* R2 protein synthesis. There are several possible mechanisms by which the tyrosyl radical can be scavenged by NO and regenerated. First, one-electron reduction of the radical by NO could generate the inactive metR2 form in which there is still the iron center but not the radical. The regeneration of the radical requires intracellular reducing systems to carry out the reduction of the iron center. Reduced R2 then is transformed to the active radical-containing R2 form during reaction with molecular oxygen. Second, since the diferric center is required for the maintenance of the radical, disappearence of that radical in cells might originate from the mobilization of the iron center by NO. This is very likely to occur since the iron center of mammalian R2 is known to be highly unstable (Nyholm et al., 1993a,b). Regeneration of the radical would then occur by reconstitution of the iron center with ferrous iron and oxygen (Thelander et al., 1983). We now would like, on the basis of experiments carried out only with pure preparations of protein R2 from *E. coli* reported here, to propose another quite unexpected mechanism. Whether this mechanism is also valid for mammalian R2 remains to be shown. The remarkable observation we made was that the reappearance of the radical did not require the cell machinery but instead was an intrinsic property of the tyrosyl radical−NO system, since it was also observed with pure protein R2. metR2 and apoR2, the inactive iron and radical-free forms, cannot be the EPR silent intermediates since radicalization would require electrons and iron, respectively, not available in the purified system. Moreover, the iron center of *E. coli* R2 is very stable, and we have shown that it was not mobilized during incubation with thionitrites. We thus suggest that the tyrosyl radical carries out a radical−radical coupling reaction with NO, according to Scheme 2, when large amounts of NO are generated from thionitrites during the first minutes of the incubation. That NO itself is the scavenger is shown from the strong protection by oxyhemoglobin. This is the first time that a coupling reaction is shown for the tyrosyl radical of ribonucleotide reductase. On the other hand, there are very few examples in which NO couples to a free radical. One is the reaction with the superoxide radical $O_2^{\bullet-}$ giving rise to the peroxynitrite anion $ONOO^-$ (Koppenol et al., 1992). Whether nitration of the tyrosine, due to the coupling of the tyrosyl radical to $NO_2^{\bullet}$ (Prütz et al., 1985), occurs has been excluded since such a reaction is irreversible and should have given rise to an absorption at 450 nm.

Regeneration of the radical is possible because these NO adducts have the ability to slowly dissociate back to the tyrosyl radical, when NO progressively disappears from the solution by oxidation (Scheme 2). Accordingly, rates of radical regeneration are slower under anaerobic conditions and faster under reduced pressure. During the present work two papers appeared in the literature showing that such a behavior may be a general characteristic of phenoxyl radicals

reactivity since it was reported that NO couples reversibly to a variety of phenoxyl radicals, providing thus relevant model reactions of the R2−NO interaction (Janzen et al., 1993; Wilcox & Janzen, 1993). In these studies, it was shown that the intensity of the EPR spectra of stable model phenoxyl radicals was decreased in the presence of an excess of NO and could then be restored by bubbling nitrogen into the solution. NO thus could regulate RNR activity also through its unique reactivity toward the tyrosyl radical of R2.

The unique reaction between NO and the R2 radical reported here has another important implication. It may serve as a simple, sensitive, and highly specific assay for direct detection of NO by EPR spectroscopy *in vitro*. So far, no other molecule is able to reversibly couple to the tyrosyl radical, during a reaction that is inhibited by oxyhemoglobin. Micromolar amounts of NO can probably be detected by that way. The other spin trapping assays, currently used for detecting NO by EPR spectroscopy, i.e., with oxyhemoglobin or nitronyl nitroxides (Joseph et al., 1993), nitrones or nitroso compounds (Archer, 1993), and butadienes (Gabr et al., 1993) as spin traps, are less sensitive and not specific for NO, and their utilization is fraught with difficulties.

In conclusion, the data presented in this study show the potential of nitric oxide donors such as thionitrites as inhibitors of ribonucleotide reductase and DNA synthesis. This might provide a new strategy to design anticancer or antiviral agents and strongly invites to improve the efficiency and the selectivity of such drugs. In view of the variety of biological effects of NO, the last aspect is critical. Moreover, we have observed, for the first time, two reactions of RNR. One is the reversible nitrosation of cysteines, and the second is the reversible nitrosation of the tyrosyl radical. Both might be key events during the inhibition of the enzyme. Moreover, for their reversibility these reactions might provide a mechanism by which enzyme activity is regulated by NO concentrations. The new concepts reported here require further investigations in order to tell how important they are in the *in vivo* context.

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