Biochemical Pharmacology, Vol. 44, No. 12, pp. 2403–2408, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

EFFECT OF GALLIUM ON THE TYROSYL RADICAL OF THE IRON-DEPENDENT M2 SUBUNIT OF RIBONUCLEOTIDE REDUCTASE

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(*Received* 18 *May* 1992; *accepted* 6 *August* 1992)

Abstract—Gallium, a pharmacologically important metal which resembles iron, was shown in previous studies to inhibit ribonucleotide reductase. To better understand its mechanism of action, we have examined the interaction of gallium with the iron-dependent M2 subunit of ribonucleotide reductase. In its active form, M2 contains an iron center and a tyrosyl free radical which is detectable by ESR spectroscopy. In the present study, cytoplasmic extracts prepared from murine leukemic L1210 cells after an 18-hr incubation with 960 μM gallium nitrate displayed a > 60% inhibition in their M2 tyrosyl radical ESR signal. However, this signal was restored within 15 min to levels greater than that of controls by the addition of increasing concentrations of ferrous ammonium sulfate. Gallium citrate added directly to cytoplasmic extracts from control cells also decreased the tyrosyl radical signal, an effect which could be reversed by iron. Immunoblot analysis revealed that incubation with gallium did not diminish the amount of M2 protein in cells, thus indicating that the decrease in the tyrosyl radical signal was not due to a decrease in cellular M2 content. In immunoprecipitation studies of ^{59}Fe-labeled M2, gallium displaced 55–60% of the ^{59}Fe incorporated into M2. Our studies suggest that gallium displaces iron from the M2 subunit of ribonucleotide reductase, resulting in a loss of the tyrosyl radical and an accumulation of inactive M2 within the cell.

Gallium, a group IIIA metal, has gained clinical importance recently as a pharmacologic agent for the treatment of hypercalcemia and certain malignancies [1, 2]. Although gallium is not a transition element, many aspects of its chemistry resemble those of iron. The ionization potentials, ionic radii, and the coordination number of the aqueous +3 cations are similar for both the metals. The remarkable similarity between gallium and iron allows for the interaction of gallium with various iron-containing proteins. It is known that gallium avidly binds to the iron transport protein transferrin, resulting in stable transferrin–gallium complexes [3], which can be incorporated into cells via transferrin receptor-mediated endocytosis [4, 5]. The cytotoxicity of gallium can be enhanced significantly when cells are exposed to gallium as transferrin–gallium rather than gallium nitrate [6]. In addition, exposure of cells to transferrin–gallium results in a decrease in iron uptake, an increase in cellular transferrin receptors and a decrease in ferritin content, findings consistent with cellular iron deprivation [6].

The importance of iron in DNA synthesis is related in part to the activity of the enzyme ribonucleotide reductase which catalyzes the conversion of ribonucleotides to deoxyribonucleotides. Mammalian ribonucleotide reductase consists of two non-identical subunits termed M1 and M2 [7]. The M1 subunit contains the substrate and the effector binding sites, while the M2 subunit contains non-heme bridged iron atoms and a tyrosyl free radical which gives a characteristic signal on ESR spectroscopy [8]. The activity of ribonucleotide reductase is linked closely to the tyrosyl radical and the presence of iron. Removal of this iron results in the loss of both the tyrosyl radical ESR signal and enzyme activity [8, 9].

In prior studies, we have shown that exposure of HL60 cells to transferrin–gallium results in a decrease in deoxyribonucleotide pools and a loss of the tyrosyl radical ESR signal. This inhibited ESR signal can be restored by co-incubation of cells with hemin [10]. More recently, gallium was found to directly inhibit the synthesis of dCDP and dADP [11]. To obtain a better insight into the mechanism of action of gallium, we have extended our earlier investigations to examine the direct effect of gallium on the tyrosyl radical in cell-free preparations and show that this effect can be modulated by the addition of exogenous iron. Furthermore, immunoprecipitation studies of ^{59}Fe-labeled M2 protein from a hydroxyurea-resistant, ribonucleotide reductase over-expressing cell line suggest that gallium inhibits the M2 subunit of ribonucleotide reductase by displacing iron from the subunit.

MATERIALS AND METHODS

Materials. Gallium nitrate was obtained from Alfa Products (Danvers, MA). Human transferrin (Tf)§, dithiothreitol (DTT), ferrous ammonium sulfate,

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§ Abbreviations: Tf, transferrin; DTT, dithiothreitol; MoAb, monoclonal antibody; and SATCH, salicylaldehyde thiocarbohydrazone.

thiocarbohydrazide and goat anti-rat IgG alkaline phosphatase conjugate were purchased from the Sigma Chemical Co. (St. Louis, MO). Standard gallium (1010 μg/mL), sodium thiosulfate, potassium hydrogen phthalate and salicylaldehyde were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Sodium fluoride, and sodium phosphate (mono and dibasic) were purchased from the Fisher Scientific Co. (Fair Lawn, NJ). Tachisorb rat IgG immunoadsorbent (goat antibody to rat IgG conjugated to Pansorbin *Staphylococcus aureus* cells) was obtained from Calbiochem (La Jolla, CA). Rat monoclonal antibody (MoAb) JB4 against the M2 subunit of mouse ribonucleotide reductase [12] was provided by Dr. Lars Thelander (University of Umeå, Sweden). $^{59}FeCl_3$ was obtained from New England Nuclear (Boston, MA) and ^{59}Fe-Tf was prepared as previously described [6].

Cell culture and preparation of cell extracts. Murine leukemic L1210 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in an atmosphere of 6% CO_2 at 37° in RPMI 1640 medium containing 10% horse serum. For ESR studies, large volumes of cells were required and cells were plated in 500 mL Spinner flasks in the absence (control) or presence of 960 μM gallium nitrate. After an 18-hr incubation, cells (5×10^8/flask) were harvested and washed twice with ice-cold phosphate-buffered saline (PBS), and cell homogenates were prepared as previously described for assay of ribonucleotide reductase enzyme activity in cell-free extracts [11]. The final cytosolic sample containing ribonucleotide reductase was dialyzed against Tris–DTT buffer (0.02 M Tris–Cl, pH 7.4, containing 1 mM DTT) and concentrated using a Centricon 30 microconcentrator (Amicon Corp., Danvers, MA). For studies involving labeling of the ribonucleotide reductase M2 subunit with ^{59}Fe and immunoprecipitation (described below), we used a hydroxyurea-resistant L1210 subline which was developed by growth of L1210 cells in medium in which hydroxyurea was increased in a step-wise fashion from 0 to 250 μM over a 6-month period. Hydroxyurea-resistant cells were routinely maintained in medium containing 250 μM hydroxyurea, displayed growth kinetics similar to the wild type cells, and were approximately 16-fold more resistant to growth inhibition by hydroxyurea. When compared with the parent cell line, these hydroxyurea-resistant cells contained more M2 protein and over-expressed the M2 tyrosyl radical ESR signal by at least an order of magnitude. These findings are consistent with drug resistance to hydroxyurea, as reported by others [13–15].

ESR spectroscopy studies. ESR studies of the tyrosyl free radical of ribonucleotide reductase were performed at the National Biomedical ESR Center of the Medical College of Wisconsin. X-band ESR spectra were obtained by using a standard Century series Varian E-100 spectrometer operating at X-band (9 to 9.5 GHz) with 100 kHz field modulation. ESR measurements were made on frozen cytosolic preparations (4 mm diameter × 3 cm long icicles) at −196° in quartz finger Dewar flasks. After obtaining initial spectra of cytosolic preparations from control and gallium-treated cells, the samples were thawed and ferrous ammonium sulfate/gallium citrate was added to each sample. Following a 15-min incubation at 37° in air, the samples were frozen again and a second series of ESR measurements were made. Each of the ESR spectra was recorded 9–16 times and averaged with a computer program called Viking. The absolute concentration of the tyrosyl radical was determined by comparison of the areas under the double integral of the ESR signal to a 1 mM copper perchlorate standard.

Measurement of gallium content in cells. Gallium content in cells was determined using a modification of a previously described fluorometric method [16], which is based on the reaction of gallium with salicylaldehyde thiocarbohydrazone (SATCH). The reagent SATCH was synthesized by the condensation of salicylaldehyde with thiocarbohydrazide and a 1 mM SATCH solution was prepared in 100% ethanol [17]. Following incubation with gallium nitrate, cells (1×10^8 cells) were washed with ice-cold PBS (10 mM sodium phosphate, pH 7.5, 150 mM NaCl) and centrifuged at 1000 rpm for 10 min. Concentrated HNO_3 (5 mL) was added to the pellet and the sample was digested in a round bottom flask in a reflux apparatus for 45 min with drop-wise addition of 5 mL of 30% H_2O_2. After digestion, the samples were evaporated to a small volume (<0.5 mL), cooled, neutralized with sodium hydroxide, and diluted to 10 mL with deionized water.

For determination of gallium content, various concentrations of gallium standard (atomic absorption grade, 10 μg/mL) and cell digests were used. Sodium thiosulfate (0.3 mL of a 0.1% solution) and sodium fluoride (25 μL of a 0.1% solution) were added to 250 μL sample (cell digest or gallium standard) and the mixture was incubated for 5 min at room temperature. The following reagents were then added: 1.6 mL of 0.1 M potassium hydrogen phthalate buffer, pH 2.3, 4 mL of 100% ethanol, 1.2 mL of SATCH and deionized water to yield a final volume of 10 mL in each assay tube. The reaction mixture was incubated at room temperature for 30 min. The fluorescent intensity of each sample was then measured with a SPF-500 ratio spectrofluorometer (American Instrument Co., Silver Spring, MD) using an excitation wavelength of 395 nm and an emission wavelength of 445 nm. A standard curve was generated from the fluorescent intensities of samples containing known concentrations of gallium, and the gallium content in cell samples was determined from this curve. Cell digests from control cells (grown without gallium) were used as background blanks.

Detection of M2 protein by immunoblotting. Cytosolic extracts from cells incubated without or with 960 μM gallium were analyzed for M2 protein content by immunoblotting using a commercially available chromogenic immunoblotting assay (Immun-Blot Assay kit, Bio-Rad, Richmond, CA). Previous studies have established the use of MoAb JB-4 for detection of M2 protein by this method [18]. Fifteen micrograms of total protein from the extracts was loaded onto BA83 nitrocellulose membrane using a 96-well Minifold apparatus (Schleicher & Schuell Inc., Keene, NH). After

incubation of the membrane in blocking buffer (3% gelatin in 50 mM Tris, 100 mM NaCl, pH 7.5), M2 protein was detected by probing the membrane with buffer (1% gelatin, 50 mM Tris, 100 mM NaCl, pH 7.5) containing JB4 MoAb against the M2 protein, followed by incubation in buffer containing goat anti-rat IgG alkaline phosphatase conjugate. The membrane was then incubated in color development reagent according to recommendations of the manufacturer and the intensity of the color of the dots was compared. No color reaction was detected in control dots loaded with 50 μg bovine serum albumin.

The findings on dot blotting were verified further by Western blot analysis of the cytoplasmic extracts. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of extracts from cells incubated without or with 960 μM gallium lysates was performed according to the method of Laemmli [19]. Proteins were transferred from the gel onto nitrocellulose membrane as described by Towbin *et al.* [20], using a Transblot system (Bio-Rad). As in the dot blot method described above, immunoreactive M2 protein was detected using anti-M2 MoAb as the probe.

Immunoprecipitation of ^{59}Fe*-labeled M2 protein.* To determine the interaction of gallium with the iron center of the M2 subunit of ribonucleotide reductase, cells or cell lysates were labeled with ^{59}Fe and the ^{59}Fe-M2 protein was immunoprecipitated using an approach similar to that described by McClarty *et al.* [21]. To obtain sufficient ^{59}Fe cpm incorporation into the M2 protein, a hydroxyurea-resistant subline of L1210 cells possessing increased ribonucleotide reductase activity was used for these studies. Cells were labeled with ^{59}Fe under two conditions: (a) hydroxyurea-resistant L1210 cells were incubated for 18 hr with ^{59}Fe-Tf in growth medium containing 1% horse serum. Cells were washed and homogenized, and the soluble cytosolic fraction containing ^{59}Fe-labeled proteins was used for immunoprecipitation; (b) unlabeled lysates from control cells were incubated with $^{59}FeCl_3$ for 15 min at 37° in air, after which immunoprecipitation was carried out. Immunoprecipitation of the ^{59}Fe-labeled M2 protein was performed using a double-antibody method. Samples were incubated without (control) or with 5 μg JB4 MoAb to mouse M2 protein for 90 min at 37°. Then 1.1 vol. of Tachisorb (goat antibody to rat IgG) was added and the incubation continued for an additional 45 min at room temperature. Following this, the tubes were centrifuged and the radioactivity in the pellet (representing ^{59}Fe-labeled M2 protein) was counted. Specific incorporation of ^{59}Fe into M2 was calculated by subtracting the ^{59}Fe cpm precipitated in control samples from the cpm immunoprecipitated by anti-M2 antibody. To examine the effect of gallium on ^{59}Fe bound to M2 protein, samples were incubated with 1–3 mM gallium citrate for 30 min at 37° prior to immunoprecipitation. In separate experiments, it was determined that 3 mM gallium citrate did not interfere with the binding of the antibody to the M2 protein.

RESULTS

ESR studies on cytoplasmic extracts of cells incubated with gallium nitrate. Following an 18-hr incubation of L1210 cells with 960 μM gallium nitrate, cytosolic extracts were prepared and analyzed by ESR spectroscopy. Figure 1 shows that the M2 subunit tyrosyl radical signal in the cytosol from cells incubated with gallium nitrate was decreased by > 60%. Upon incubation of cytosols with increasing concentrations of ferrous ammonium sulfate for 15 min at 37°, there was a progressive increase in the ESR signals from control and gallium-treated samples. Interestingly, the addition of ferrous ammonium sulfate resulted in a larger increase in the intensity of the tyrosyl radical signal in the cytosolic extract from gallium-treated cells than in the control. Figure 2 shows the changes in the tyrosyl radical ESR spectra in cytosolic extracts following the addition of ferrous ammonium sulfate and/or gallium citrate. The addition of 10 μM ferrous ammonium sulfate to cytosolic extracts from gallium-treated cells resulted in recovery of the tyrosyl radical ESR signal (compare spectra A with B in Fig. 2). The addition of gallium citrate to the same sample resulted in a decrease in the amplitude of the signal (spectra C and D, Fig. 2); however, the ESR signal could again be restored to its prior level with further addition of ferrous ammonium sulfate (spectrum E).

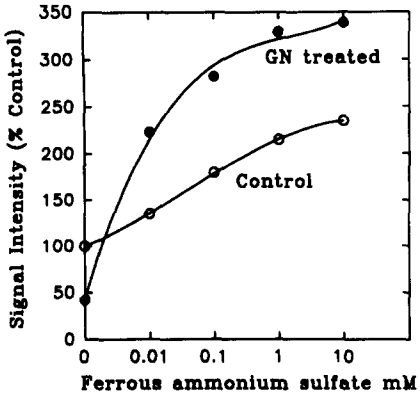

Fig. 1. Effect of iron on the relative intensity of the M2 tyrosyl radical ESR signal from control and gallium-treated cells. ESR studies were performed on cytoplasmic extracts prepared from cells incubated for 18 hr without (control) or with 960 μM gallium nitrate (GN). After obtaining initial ESR spectra, samples were thawed and incubated with ferrous ammonium sulfate for 15 min at 37° in air. Data shown represent ESR spectra obtained on the same samples following the consecutive addition of increasing concentrations of ferrous ammonium sulfate. The relative amplitudes of the ESR signals are in arbitrary units. The amplitude of the ESR signal of control cells prior to the addition of iron is expressed as 100% and all other values shown are relative to this.

M2 protein content following incubation of cells with gallium nitrate. Since the decrease in the tyrosyl radical signal after incubation of intact cells with gallium could result from a decrease in M2 protein content, cytosolic extracts from cells were analyzed for M2 protein by immunoblotting. As shown in Fig. 3A, the amount of immunoreactive M2 protein

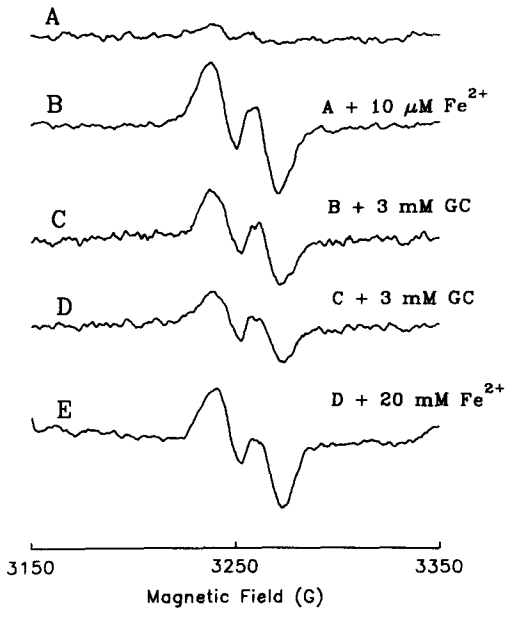

Fig. 2. Effect of sequential addition of iron and gallium on the M2 tyrosyl radical ESR signal. Cytoplasmic extracts were prepared from cells incubated for 18 hr with 960 μM gallium nitrate. A, B, C, D and E represent spectra obtained from the same sample. Key: (A) initial spectrum; (B) following the addition of ferrous ammonium sulfate; (C) and (D) following the addition of gallium citrate (GC); and (E) following the addition ferrous ammonium sulfate. Note that the spectrum shown in A represents a signal which has been inhibited markedly by incubation of cells with gallium nitrate.

content in gallium-treated cells, as analyzed by dot blotting, remained comparable to that of controls over the 18-hr incubation. This finding was confirmed by Western blotting shown in Fig. 3B which revealed two immunoreactive bands of approximately 44 and 88 kDa, consistent with the monomeric and dimeric forms of M2 protein (the 88 kDa band most likely represents unreduced M2). Again, comparable amounts of M2 protein were detected in both control and gallium-treated cells. These studies clearly indicate that the decrease in the tyrosyl radical signal following incubation with gallium was not the result of a decrease in M2 protein in cells.

ESR studies on cell-free extracts from control cells. The studies described above were performed on cytosolic extracts from cells that had been incubated with gallium nitrate for 18 hr and displayed a baseline decrease in the ESR signal. To examine the effect of gallium on the tyrosyl radical signal from cells not previously exposed to gallium, increasing concentrations of gallium citrate were added sequentially to the cytosolic extract of L1210 cells. As shown in Fig. 4, a progressive diminution in the intensity of the M2 tyrosyl radical signal was seen with the addition of gallium citrate. A 60% inhibition of the ESR signal was seen with 16 mM gallium citrate (not shown). Figure 4 also shows that the inhibitory effect of 9 mM gallium on the tyrosyl radical was reversed completely with the addition of 20 mM ferrous ammonium sulfate to the sample. These studies, utilizing cytosolic extracts from both gallium-treated and control cells, suggest that the inhibitory effect of gallium on the M2 subunit tyrosyl radical involves interaction with the iron center of the M2 protein.

Cellular concentration of gallium. From the above

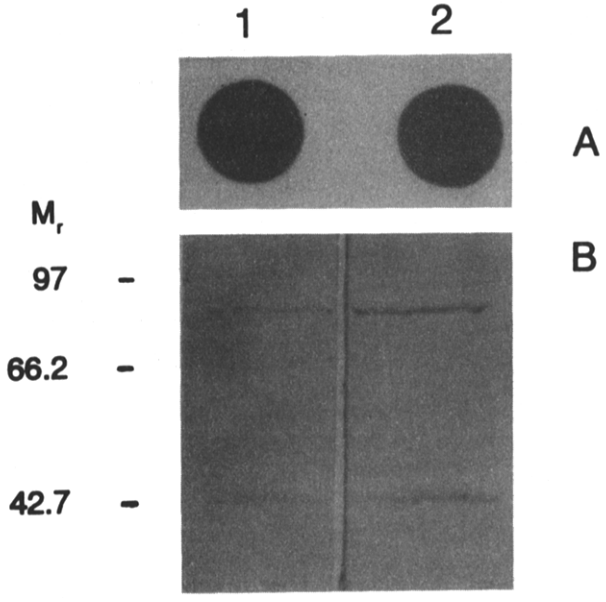

Fig. 3. Comparison of M2 protein content of control and gallium-treated cells. Cytoplasmic extracts were prepared from cells incubated for 18 hr without or with 960 μM gallium nitrate. M2 protein was detected by immunoblotting using an anti-M2 MoAb as the probe. Key: (A) Dot blot analysis of cell extracts; 30 μg protein was loaded in each well. (B) Western blot analysis of cellular extracts. Lane 1, control; lane 2, gallium-treated cells.

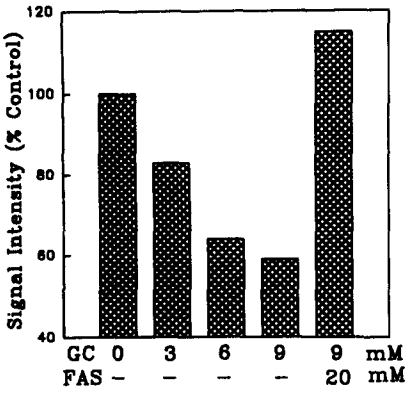

Fig. 4. Effect of gallium on the M2 tyrosyl radical ESR signal from cytoplasmic extracts of L1210 cells. Cytoplasmic extracts were prepared from cells grown in the absence of gallium nitrate. As in the previous experiments, increasing concentrations of gallium citrate (GC) were added to the same sample. The relative amplitudes of the ESR signals are in arbitrary units. Note that 100% signal intensity (control) represents the amplitude of the tyrosyl radical signal from cytoplasmic extracts of control L1210 cells grown without iron or gallium added to the medium. FAS, ferrous ammonium sulfate.

studies it can be seen that millimolar amounts of exogenous gallium citrate were required for inhibition of the ribonucleotide reductase M2 tyrosyl radical signal. Since the ESR studies were performed on concentrated cytosolic extracts representing ribonucleotide reductase from 5×10^8 cells, it was important to determine whether the amount of gallium used in these studies correlated with the amount of gallium actually incorporated into cells. Measurement of the gallium content of packed cells (5×10^8 cells) after 18 hr of incubation with 960 μM gallium nitrate revealed a gallium concentration of 2.1 ± 0.45 mM (mean $\pm$ SEM).

Effect of gallium on ^{59}Fe-labeled M2 subunit of ribonucleotide reductase. To examine the effect of gallium on the iron center of the M2 protein, ^{59}Fe-labeled M2 protein was immunoprecipitated using a specific MoAb. M2 protein was labeled with ^{59}Fe by pulsing cytosolic extracts with ^{59}Fe in air at 37° for 30 min (thus allowing for ^{59}Fe incorporation into the M2 subunit during activation of the tyrosyl radical) or by incubating ^{59}Fe with cells for 18 hr. The addition of gallium nitrate to samples containing ^{59}Fe-M2 resulted in a decrease in the amount of ^{59}Fe immunoprecipitated, thus indicating displacement of ^{59}Fe from M2 by gallium. In samples in which ^{59}Fe had been incorporated into M2 protein over an 18-hr incubation, 1 mM gallium citrate displaced 55% of the ^{59}Fe from M2 over 135 min (the duration of the immunoprecipitation experiment). Similarly, in samples in which ^{59}Fe had been incorporated into M2 protein by incubation with cellular cytosolic extracts, 3 mM gallium citrate displaced 60% of ^{59}Fe from M2.

DISCUSSION

Because of its important role in the synthesis of DNA precursors, ribonucleotide reductase is considered to be an attractive target enzyme for anti-neoplastic and anti-viral drugs. Functionally active ribonucleotide reductase is composed of a large and a small dimeric subunit felt to exist in 1:1 stoichiometry. Studies of the enzyme in *Escherichia coli* have shown that each monomer of the smaller subunit (termed B2 in bacteria) contains an iron center which is made up of 2 iron atoms linked by a μ-oxo bridge. The tyrosyl free radical is located at Tyr 122, 5.3 Å from the nearest iron [22]. Titration experiments under anaerobic conditions have suggested that the B2 subunit can exist as apoB2 which lacks the iron center and contains a nonradical tyrosine 122. With the addition of ferrous iron, apoB2 is converted to reduced B2 which, in the presence of oxygen, is converted to the fully active B2 containing ferric iron and an ESR detectable tyrosyl radical. Reduction of active B2 results in the formation of metB2 which contains ferric iron but lacks the tyrosyl radical. MetB2, in turn, may either be reduced further or may lose its iron to yield reduced B2 or apoB2, respectively [23]. Likewise, titration studies using substoichiometric amounts of Fe(II) added to recombinant mouse apoR2 protein under anaerobic conditions followed by exposure to air have shown that the formation of each dimeric Fe(III) center produces a tyrosyl free radical [24]. It is clear from numerous studies by different investigators that the iron center of the B2/M2 subunit plays a critical role in the overall activity of the enzyme [9, 23–26].

Because gallium resembles iron in many respects, the present study was undertaken to specifically examine the interaction of gallium with the iron-dependent M2 subunit of ribonucleotide reductase. We show that the activity of the M2 tyrosyl radical was inhibited after exposure to gallium. Although maximum inhibition was seen after an 18-hr incubation of cells with gallium, direct addition of gallium to cytoplasmic extracts also led to inhibition of the tyrosyl radical ESR signal. In both situations, the radical signal could be restored by the addition of exogenous ferrous iron.

The direct addition of gallium citrate to cytoplasmic extracts containing an active tyrosyl radical also resulted in a reduction in the intensity of the radical ESR signal. While the amount of gallium used to inhibit the M2 tyrosyl radical may appear to be high (millimolar concentrations), it should be appreciated that the ESR studies were performed on lysates prepared from 5×10^8 cells and that the concentration of gallium needed to directly inhibit the tyrosyl radical signal was close to the amount actually taken up by an equivalent number of cells. The slightly higher concentration of gallium needed to directly inhibit the tyrosyl radical in cytoplasmic extracts may be due to the shorter duration of incubation of cytosolic extracts with gallium and the interaction of this metal with macromolecules other than the M2 subunit. With the addition of iron, the tyrosyl radical signal was restored to its previous level, thus indicating that the inhibitory effect of gallium involved an interaction with the iron center of M2. This conclusion was further confirmed by the immunoprecipitation studies which demonstrated

that gallium was able to displace ^{59}Fe from radiolabeled M2.

The present studies therefore suggest strongly that the M2 protein present in cells after incubation with gallium exists mainly as inactive apoM2 or as an inactive form containing gallium in place of iron (or a combination of both). Either situation would result in a loss of the tyrosyl radical ESR signal and an inhibition of overall enzyme activity.

In conclusion, our studies have further elucidated the interaction of gallium with the iron-dependent M2 subunit of ribonucleotide reductase and have provided an additional insight into the mechanism by which gallium inhibits DNA synthesis. It is likely that gallium targets other intracellular iron-dependent processes and studies are in progress to examine these interactions.

Acknowledgements—We are grateful to Dr. Lars Thelander for providing us with monoclonal antibody against the M2 subunit of murine ribonucleotide reductase. This work was supported by USPHS Grants RO1 CA41740 (C.R.C.) and RR01008.

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