Differential Sensitivity of the Tyrosyl Radical of Mouse Ribonucleotide Reductase to Nitric Oxide and Peroxynitrite*

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Ribonucleotide reductase is essential for DNA synthesis in cycling cells. It has been previously shown that the catalytically competent tyrosyl free radical of its small R2 subunit (R2-Y) is scavenged in tumor cells co-cultured with macrophages expressing a nitric oxide synthase II activity. We now demonstrate a loss of R2-Y induced either by NO or peroxynitrite in vitro. The NO effect is reversible and followed by an increase in ferrie iron release from mouse protein R2. A similar increased iron lability in radical-free, dfferic metR2 protein suggests reciprocal stabilizing interactions between R2-Y and the diiron center in the mouse protein. Scavenging of R2-Y by peroxynitrite is irreversible and paralleled to an irreversible loss of R2 activity. Formation of nitrotyrosine and dihydroxyphenylalanine was also detected in peroxynitrite-modified protein R2. In R2-overexpressing tumor cells co-cultured with activated murine macrophages, scavenging of R2-Y following NO synthase II induction was fully reversible, even when endogenous production of peroxynitrite was induced by triggering NADPH oxidase activity with a phorbol ester. Our results did not support the involvement of peroxynitrite in R2-Y scavenging by macrophage NO synthase II activity. They confirmed the preponderant physiological role of NO in the process.

Nitric oxide is a cell-permeable small radical molecule synthesized in diverse organisms by NO synthase enzymes (NOS),1 which are P450 self-sufficient hemoproteins using L-arginine and dioxygen as substrates (reviewed in Ref. 1). In mammals, physiological functions have been attributed to NOS activities in cardiovascular, neural, gastrointestinal, genitourinary and immune systems (2, 3). So called “neuronal” NOS activities in cardiovascular, neural, gastrointestinal, genitourinary and immune systems (2, 3). So called “neuronal” NOS (NOS I) and “endothelial” NOS (NOS III), usually constitutively expressed, are transiently activated by elevation of intracellular Ca2+ concentration (4). A cytokine-inducible NOS (NOS II) is transcriptionally regulated, producing NO at basal Ca2+ levels for up to several days (4). Among cytotoxic and pathophysiological functions, NOS II was rapidly recognized to support a nonspecific antiproliferative activity capable of limiting the growth of invading agents, including viruses, bacteria, parasites, and tumor cells, consistent with the wide distribution of the isoform in different cell types (3, 4).

Cytostatic antitumor effector mechanisms of macrophages have been shown to rely on induction of NOS II activity, which severely alters energy production, iron metabolism, and DNA synthesis in tumor target cells (5, 6). There is still a debate about the identity of the cytotoxic nitrogen oxide(s) acting under physiological conditions. For instance, both NO and peroxynitrite ONOO− have been reported to inhibit mitochondrial respiratory chain, and to regulate iron regulatory protein 1 function, the keystone in cell iron homeostasis (5, 7–10). Ribonucleotide reductase (RR) inhibition by a NOS II product, probably NO, has been demonstrated in tumor cells co-cultured with macrophages (11, 12), or in tumor cells stimulated with cytokines for endogenous NOS II induction (13). Previous studies also indicated that RR inhibition supported an early, NOS-dependent inhibition of DNA synthesis in tumor cells (11, 12).

RR enzymes have been classified in at least four different groups (14, 15). They catalyze the reduction of ribonucleotides, providing the cells with deoxyribonucleotides required for DNA synthesis. The small homodimeric R2 subunit of class I RR in mammals and some prokaryotes like Escherichia coli harbors a free radical localized on a tyrosyl residue (R2-Y) (16–18). X-ray structures of mouse and E. coli protein R2 have been determined (19, 20). Production of R2-Y by a one-electron oxidation of Y122 (E. coli) or Y177 (mouse), and stability of the existing radical are dependent on a proximal nonheme diiron center which interacts electromagnetically with R2-Y (21). The radical/metal center of protein R2 is required for activity, since it triggers a radical-driven reduction of the substrate through a long-range electron transfer between protein R2 and protein R1, the other homodimeric subunit of RR containing binding sites for substrates and allosteric effectors (see Refs. 19 and 20, and references therein). In different models, R2-Y has been identified as a target for nitrogen oxides derived from NOS II activity (12, 22), but the chemical species involved have not been clearly identified. There is good evidence that NO participates in the reaction since HbO2, which binds NO, inhibits R2-Y quenching induced by NOS II activity or NO donors (22–24). Also, a reversible radical-radical coupling reaction of NO with R2-Y has been proposed to account for the disappearance of the tyrosyl free radical of E. coli protein R2 induced by thionitrites (24). However, so far, this experiment has not been reproduced with authentic NO gas under anaerobic conditions, to demonstrate unambiguously the role of NO in R2-Y scavenging. Moreover, little is known about the reactivity of other nitrogen oxides toward RR.

In the present study, we compared the effects of NO and ONOO− against murine protein R2 in vitro. We then used

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‡ The abbreviations used are: NOS, nitric oxide synthase; DHR, dihydroxyphenylalanine; RR, ribonucleotide reductase; DTT, dithiothreitol; TPA, 12-O-tetradecanoylphorbol-13-acetate.
these data in order to identify the nitrogen oxide resistance for R2-Y quenching in co-cultures of macrophages and tumor cells. Our results indicate that ONOO\textsuperscript{−} is not a physiological effector of the loss of R2-Y.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmasids—**NO gas was generated from Liquid Air (Moissy-Cramayel, France). Isopropyl-β-D-thiogalactopyranoside was from Eurogentec (Angers, France). [5-3H]CDP with a specific activity of 655 GBq/mmol was purchased from Amersham-France (Les Ulis). All other reagents were from Sigma (Ste Aubeix, France). 1-naphthol was used to estimate the concentration of protein R2-Y in cell samples, and the presence of a partially overlapping strong signal from nitroso iron complexes at g values of 2.041 and 2.015. Microwave power was 10 mW, field modulation frequency was 9.18 GHz, and modulation amplitude was 1 millistear.

**Measurement of RR Activity—**RR activity was assayed following the conversion of [\( ^{3}H \)]CDP into [\( ^{3}H \)]dCDP. Magnesium chloride (15 mM), 100 mM KCl, 10 mM dithiothreitol, 3 mM ATP, 200 μM CDP, 74 kBq of [5-\(^{3}\)H]CDP, and a 5-fold molar excess of protein R1 in 50 mM Tris-HCl buffer, pH 7.6, were mixed with 20 μl of protein R2 (0.5–2 μM) in a final volume of 90 μl. Samples were incubated at 37 °C for 10 min. Control experiments have shown that the reaction was linear over the 10-min assay. The reaction was stopped by heating for 2 min. CDP and dCDP were then dephosphorylated at 37 °C for 2 h with calf intestinal phosphatase from Croatalus adamanteus. After boiling and centrifugation, supernatants were analyzed by radio-high performance liquid chromatography as described elsewhere (13). Specific activity was expressed as nanomoles of dCDP formed per minute per mg of protein R2.

**Iron Release from Mouse Protein R2—**A volume of 500 μl of 20 μM protein R2 was deoxygenated with argon at room temperature in a quartz cuvette closed with a rubber septum. After 1 h, a UV visible absorption spectrum of the protein was recorded. Concentration of the R2-Y radical was determined from the absorbance at 417 nm (see above). NO or 4-propoxyphenol (used to obtain metR2) were introduced with an argon-degassed syringe, in a ratio [NO]:[R2-Y] of 8 or [4-propoxyphenol]:[R2-Y] of 1. Then, 10 μl of a deoxygenated solution of deferoxamine were added to a final concentration of 1 mM to monitor ferric iron release by the Fe\(^{3+}\)–deferoxamine complex absorbance at 430 nm (Δε = 2500 M\(^{-1}\) cm\(^{-1}\)), as previously reported (32). In these experiments, the free radical content of untreated control protein R2 decreased from 0.65 to approximately 0.5 radical per monomer after the 1-h flush with argon.

**Co-culture of Macrophages and Tumor Cells—**Portion macrophages were obtained by intraperitoneal injection of 50 mg of trehalose dimycolate to C57BL/6xDBA/2J mice, as previously obtained (33, 34). The R2-overexpressing L1210-R2 cell line was cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, as described previously (12). Macrophages (35 × 10\(^{6}\)) were allowed to adhere for 3 h in 14-cm culture dishes (Nunclon), then washed three times with phosphate-buffered saline. Lipopolysaccharide was added at 10 μg/ml, and a 10 mM of fresh culture medium to induce expression of NOS II activity. After 4 h, macrophages were washed again with phosphate-buffered saline and L1210-R2 cells were added to peritoneal macrophages in exponential growth phase were added in RPMI 1640 medium containing 5% fetal calf serum, at a macrophage:tumor cell ratio of 0.65. Cells were co-cultured for 5 h, with lipopolysaccharide. In some experiments, TPA (20 nM) was added with tumor target cells to trigger, \( \text{O}_2^- \), and \( \text{H}_2\text{O}_2 \) production by mitophagy NADPH oxidase activity (33). Nonadherent L1210-R2 cells were then gently harvested and packed by centrifugation into an EPR quartz tube (4 mm outer diameter), as previously mentioned (12). A first determination of R2-Y concentration in the cell pellet was obtained. Cells were then thawed and R2-Y was reactivated with a solution of 100 mM dithiobitol and 100 μM ammonium iron(II) sulfate added in a small volume, representing approximately 1/10 of the cell pellet, according to Ref 35. Cells were then washed and a 500 μg x 10 min and the reactivation procedure was repeated once, except that iron and the reductant were not added. Finally, cells were processed for a second determination of R2-Y content. In preliminary experiments, this procedure was shown to allow optimal reactivation of R2-Y.

**Detection of Dihydroyxylpallylalnine Residues—**Protein R2 from E. coli treated with various concentrations of peroxynitrite was first desalted on a Bio-Gel polyacrylamide column (Bio-Rad). The protein was then transferred onto a nitrocellulose membrane by dot-blotting. Detection of catechol structures was performed according to Ref. 36. The membrane was immersed in a 2 m potassium glycinate solution, pH 10,
Fig. 1. Quenching of the tyrosyl free radical of mouse protein R2 by NO and peroxynitrite. NO (42 μM) was added to protein R2 ([R2-Y] = 14 μM) in 50 mM Tris-HCl buffer, pH 7.6, under anaerobic conditions. Peroxynitrite (100 μM) was added to protein R2 ([R2-Y] = 3.7 μM) in the same buffer, but in the presence of oxygen. Samples were immediately frozen and analyzed for R2-Y content by EPR spectroscopy at 77 K. Spectrum b: R2 + NO; spectrum d: R2 + ONOO·. Spectra a and c: untreated controls of b and d, respectively. The gain for spectra c and d was 12.5 x 10^3. It was 2.5 x 10^2 for spectra a and b.

containing 0.24 mM nitro blue tetrazolium, and incubated in the dark for 45 min. Membranes were irreversibly stained by immersion at 4°C in 0.1 M sodium borate, pH 7.0.

Immunodetection of Nitrotyrosine—Mouse or E. coli protein R2 treated with NO or ONOO· was transferred onto a nitrocellulose membrane by dot blotting. The membrane was then blocked for 1 h with 3% skim milk in phosphate-buffered saline. A mouse monoclonal antinitrotyrosine (Upstate Biotechnologies, Lake Placid, NY) was added at a dilution of 1:2000, and the membrane was incubated overnight at 4°C in the blocking buffer. After three washings, a secondary goat anti-mouse IgG-horseradish peroxidase-linked antibody was added at a dilution of 1:5000 and incubated with the membrane for 90 min in the blocking buffer, at room temperature. Blots were revealed by enhanced chemiluminescence using a kit from Amersham.

Protein Determination—Protein concentration was measured using the Bradford protein assay (Bio-Rad). Bovine serum albumin was taken as a reference.

Nitrate and Citrulline Assays—Nitrate concentration in the co-culture medium of macrophages and L1210-R2 tumor cells was measured with the Griess reagent, as described previously (22). Citrulline production from l-[U-14C]arginine was measured over 5 h in macrophage cultures (1 x 10^6/ml/well), as reported elsewhere (13).

Fig. 2. Effect of NO on free radical content and activity of mouse protein R2. Protein R2 (0.8 μM) was added with increasing amounts of NO under anaerobic conditions in 50 mM Tris-HCl buffer, pH 7.6, and immediately tested for free radical content by EPR spectroscopy (filled squares). An aliquot of the solution was used to measure R2 activity in the presence of a 5-fold molar excess of protein R1, under aerobic conditions (filled circles), as described under “Experimental Procedures.” The remaining volume of the sample was gassed with argon for 45 min in ice, before measurement of tyrosyl free radical recovery (open squares).

Effects of NO on Mouse Protein R2—Previous studies have shown that the free radical of the small R2 subunit of RR was scavenged by chemical NO donors in the presence of oxygen, allowing NO autooxidation to higher nitrogen oxides. The present experiments were conducted under anaerobic conditions to study the effect of authentic NO gas. Other experiments with peroxynitrite were performed in the presence of oxygen, since identical results on R2-Y were obtained with or without oxygen in preliminary studies. The tyrosyl free radical of mouse protein R2 was quenched similarly by NO and peroxynitrite (Fig. 1). The EPR spectrum of partially quenched R2-Y recorded at 77 K in the presence of either nitrogen oxide did not reveal any modification of the radical profile, except its reduced amplitude. The disappearance of R2-Y induced by NO was concentration-dependent for both NO (Fig. 2) and protein R2 (not shown). The apparent stoichiometry of the reaction was clearly higher than 1:1. For instance, complete loss of the R2-Y EPR signal occurred at a ratio [NO]/[R2-Y] estimated to be 8.5 ± 1.0, from three independent experiments. The reaction was fast, going to completion within the time required to mixture R2 with NO and to transfer the solution into EPR tubes (about 30 s). The reaction was also reversible. If NO was purged from the solution with argon, R2-Y reappeared in samples kept carefully at 0°C to minimize iron loss (see below). With an 8-fold excess of NO, the recovery was nearly complete and reproducible (81.6 ± 6.0% of control, n = 3). The EPR profile of the recovered radical was identical to a native R2 control (not shown). Fig. 2 also shows that activity of protein R2 was not significantly reduced by NO. This result was unexpected, since the tyrosyl free radical quenched by NO is required for R2 activity. We therefore suspected that conditions used for assaying R2 activity (10 mM DTT, O2, and a 4.5-fold dilution of the sample) might have promoted the regeneration of R2-Y. In fact, we observed in one control experiment that, after it had been totally quenched by NO, R2-Y reappeared by 60% within 1 min under RR assay conditions.

Loss of R2-Y might have been a consequence of a direct effect of NO on the tyrosyl free radical, but might also have reflected the destruction of the iron center, which would have caused ipso facto the destruction of the free radical. To investigate this possibility, iron release from protein R2 was monitored spectrophotometrically in the presence of desferrioxamine, chelating fer-
MetR2 was then obtained by adding stoichiometric amounts of pH 7.6, was degassed for 1 h with argon in a closed quartz cuvette. squares release from metR2 and center was analyzed. As shown in Fig. 3, the time course of iron release from murine protein R2 (open squares), metR2 (open circles), or native protein in the presence of an 8-fold excess of NO (closed circles) was monitored at 430 nm by the formation of an iron complex with desferrioxamine, 1 mM, in anaerobiosis. Results are the mean ± S.E. of three independent experiments.

Increased ferric iron loss from murine protein R2 treated with NO. Native protein R2 (20 μM) in 50 mM Tris·HCl buffer, pH 7.6, was degassed for 1 h with argon in a closed quartz cuvette. MetR2 was then obtained by adding stoichiometric amounts of 4-propoxyphenol. Ferric iron release from active protein R2 (closed squares), metR2 (open squares), or native protein in the presence of an 8-fold excess of NO (closed circles) was monitored at 430 nm by the formation of an iron complex with desferrioxamine, 1 mM, in anaerobiosis. Results are the mean ± S.E. of three independent experiments.

FIG. 3.

Effects of ONOO\(^-\) on Mouse Protein R2—Peroxynitrite was added to protein R2 in the presence of oxygen. A concentration-dependent destruction of R2-Y by ONOO\(^-\) was observed between 100 and 400 μM (Fig. 5). On a molar basis, peroxynitrite was approximately 40 times less efficient than NO under anaerobiosis (IC\(_{50}\) = 124.7 ± 10.8, n = 4). The phenomenon was independent of the concentration of R2-Y in the range 0.5 to 4.0 μM (not shown), suggesting a reaction limited by peroxynitrite instability. Results from reverse-order experiments (addition of ONOO\(^-\) allowed to decompose before adding protein R2) confirmed that peroxynitrite was the effector (radical content after 400 μM ONOO\(^-\) was 79.3 ± 2.3% of control, n = 3). Attempts to regenerate R2-Y in ONOO\(^-\)-treated R2 protein were unsuccessful, even after addition of 10 mM DTT and 10 μM Fe\(^{2+}\) to reconstitute the radical/metal center (Fig. 5, inset). Furthermore, no regeneration of R2-Y was observed when a submaximal concentration of peroxynitrite (200 μM) was used. Thus, destruction of R2-Y by peroxynitrite was irreversible, contrasting with the reversible quenching of the radical by NO. Peroxynitrite also inactivated R2 activity. There was a good parallelism between loss of R2-Y and loss of activity, suggesting a causal relationship. However, treatment of radical-free, iron-free apoR2 protein with 400 μM peroxynitrite prevented subsequent formation of the metal/radical center (not shown).

Thus, R2-Y was not the only target for ONOO\(^-\) in the protein. Formation of active R2 protein was observed in control reverse-order experiments with apoR2 protein. Since peroxynitrite has been reported to induce nitration and hydroxylation of aromatic amino acids, we searched for the presence of nitrosylinosine and dihydroxyphenylalanine in peroxynitrite-modified R2 proteins. A concentration-dependent formation of nitrosylinosine was detected in mouse and E. coli R2 proteins incubated with inactivating concentrations of peroxynitrite (Fig. 6). The mouse subunit was less nitrated than the protein from E. coli. Reverse-order treatment was not effective, nor was incubation of protein R2 with a high concentration of NO ([NO]:[R2-Y] = 25:1). Dihydroxyphenylalanine structures were detected in bac-
terial R2 protein incubated with 100–400 μM (Fig. 7). The mouse protein R2 was not significantly colored using this technique.

**Quenching of R2-Y’ in Tumor Cells by NOS II Activity**—It was concluded from these experiments in vitro that effects of ‘NO and ONOO’ on mouse protein R2 could be clearly distinguished from each other on the basis of the reversibility of R2-Y’ quenching. Scavenging of the radical by ‘NO is spontaneously reversible and apoR2 protein that forms thereafter, upon prolonged incubation of protein R2 with ‘NO, can be easily regenerated into active R2 by reintroduction of the metal/radical center. By contrast, when incubated with peroxynitrite as active protein or apoprotein, the R2 subunit undergoes irreversible modifications that are not compatible with the existence of R2-Y’. This criteria of reversibility was therefore used in cell culture experiments as a probe to identify the nature of the nitrogen oxide derived from NOS II activity that induced R2-Y’ scavenging in tumor cells. Effector cells were murine peritoneal macrophages that were elicited by trehalose dimycolate in vivo and stimulated in vitro with LPS to induce NOS II expression (Fig. 8). TPA triggered in these cells a respiratory burst that lasts for 90 to 120 min. It was monitored by H2O2 production in arginine-free medium, to avoid trapping of O2− by NO. The activity was dependent on TPA concentration (Fig. 8, inset) so that we were able to adjust the production of reactive oxygen species to match NO synthesis, since highest yields of peroxynitrite are obtained when the two reactants NO and O2 are produced at similar rates (37, 38). In the absence of TPA, the tyrosyl free radical EPR signal of L1210-R2 cells co-cultured with macrophages was almost completely quenched within a few hours, in agreement with previous reports (Fig. 9 and Ref. 12). The tumor cell pellet was then thawed and supplemented with ferrous iron and DTT in a small volume and finally incubated for 10 min at 37 °C to allow the regeneration of the radical/metal center. Under these conditions, R2-Y’ was regenerated with a high efficiency (Figs. 9 and 10), supporting the previously proposed hypothesis that NO is mainly, if not exclusively involved in the reaction (12). In a second set of experiments, TPA was added at a low concentration to trigger a production of H2O2 equivalent to the production of nitrite (Fig. 8A). Since nitrite flux is approximately one-third to one-half of total NO synthesis in macrophage cultures and since H2O2 results from the dismutation of two molecules of O2−, it was considered that comparable rates of nitrite and H2O2 production should reflect comparable rates of NO and superoxide anion synthesis, favorable to peroxynitrite production during the first half of the co-culture period. Measurement of citrulline production, as a mirror of NO synthesis, was also performed in macrophage cultures incubated without target cells in 24-well culture plates. Citrulline synthesis was not modified by 2 to 8 nm TPA (not shown). However, the nitrite/citrulline ratio in the absence of TPA, equal to 0.31 ± 0.04, increased to 0.49 ± 0.1 upon TPA addition (mean ± S.E., n = 4). The reason for this increase in nitrite yield after TPA is obscure. It might be indicative of peroxynitrite production, since at physiological pH, peroxynitrite decay produces substantial amounts of nitrite (39). That peroxynitrite was effectively produced during the first 2 h was confirmed by TPA-dependent oxidation of DHR, inhibited by superoxide dismutase and aminoguanidine (Fig. 8B). Increasing superoxide dismutase concentration up to 2000 units/ml did not improve the inhibition of DHR oxidation, but combining superoxide dismutase (2000 units/ml) and aminoguanidine was about 20% more inhibitory than aminoguanidine alone (data not shown). The mean value for TPA-triggered superoxide dismutase and aminoguanidine-inhibited rhoda-
mine production from 500 μM DHR was 0.41 ± 0.06 nmol/10^6 macrophages (n = 3). A significant TPA-independent formation of rhodamine was also noticed, probably due to metal-catalyzed oxidation of DHR, or NADPH oxidase-independent production of O_2^- (Fig. 8B). Under similar experimental conditions, the mean yield of DHR (500 μM) oxidation by synthesized peroxynitrite added as a bolus at 10, 20, or 40 μM was 8.3 ± 1.6%. If the yield of the reaction in the presence of the continuous flux of peroxynitrite produced by the macrophage culture is not very different from this estimated value, it could indicate that macrophages might have produced approximately 5 nmol/10^6 cells of peroxynitrite during the first 2 h of the co-culture. When L1210-R2 tumor cells were co-cultured with macrophages stimulated with TPA, R2-Y' in tumor cells was quenched efficiently (Fig. 9). Regeneration of the metal/radical center with iron and DTT addition leads to a complete recovery of the free radical (Fig. 10). Thus, no irreversible loss of R2-Y' was observed in tumor target cells co-cultured with macrophages triggered for peroxynitrite production, indicating that macrophage-derived peroxynitrite was not efficient against the small R2 subunit of RR under our conditions.

**DISCUSSION**

Previous reports demonstrated a radical-radical addition reaction between 'NO and phenoxyl free radicals of diverse origins, including small model compounds, oxidized free l-tyrosine, N-acetyl-l-tyrosine, or tyrosine-containing peptides, and tyrosyl free radicals in prostaglandin H synthase, in the R2 subunit of bacterial RR, and in photosystem II (24, 40–44). A unique and surprising property of this radical-radical combination reaction is its reversibility. It occurred independently of a particular radical environment, like metal centers frequently located in the vicinity of the radical in proteins, as it has been observed using simple free molecules (40). Results from the present work, demonstrating a very fast and reversible scavenging of mouse R2-Y' by 'NO, are in good agreement with the existence of a coupling reaction between the two radicals. Contrast with previous studies (13, 24) these experiments were performed under anaerobiosis, precluding the formation of higher nitrogen oxides derived from 'NO autoxidation (NO_2, NO_3). Reduction of the free radical by NO is excluded, since, among other factors, the reintroduction of the free radical in protein metR2 would have required a reductant like DTT and oxygen (18). In a few models, further oxidation of the EPR-silent Tyr-NO adduct gave rise to iminoxy radicals and, eventually, stable nitrotyrosine (42, 44). Neither nitrotyrosine nor iminoxy radicals were detected after incubation of mouse R2 with 'NO (data not shown), suggesting that, under our experimental conditions, the Tyr-NO adduct is not further oxidized. A ratio NO:Ty' close to 8:1 was necessary to observe the complete scavenging of R2-Y'. This is significantly higher than the 1:1 ratio expected from the coupling reaction. Although we cannot exclude a nonspecific binding of 'NO to poorly defined sites, such as metal contaminants in the buffer or adsorbed onto the protein, it is conceivable that this apparent high stoichiometry may reflect a competition between the coupling reaction and the dissociation of the Tyr-NO adduct. The rate constant for the coupling reaction of 'NO with tyrosyl-centered radicals is very fast, of the order of 1 x 10^9 M^-1 s^-1 (41), but the rate of the reverse reaction has not been determined. Yet, the weakness of the Tyr-NO complex is illustrated by the very rapid reappearance of R2-Y' in experiments using a short-lived chemical precursor of 'NO (24). A very rapid dissociation of the coupled radicals is also supported by the absence of a significant decrease in R2 activity, measured immediately after addition of 'NO. Since R2-Y' is required for activity, its scavenging by 'NO was expected to inactivate the enzyme. A fast and spontaneous regeneration of R2-Y' within the first seconds of the assay is therefore suspected. It may have been further promoted by the 4.5-fold dilution of 'NO concentration before assaying R2 activity and also by the reaction of 'NO with oxygen during the test. Additional experiments are required to elucidate this important question.
starting iron release from mouse protein R2 do not react with NO (24, 45). In fact, we observed that iron was released mostly as Fe$^{3+}$ after NO treatment. It seemed therefore that the increased ferric iron lability of mouse protein R2 incubated with NO could be best rationalized as a direct consequence of NO coupling to R2-Y. The absorption spectrum of protein R2 was modified upon addition of NO. Especially, the absorption bands of the metal center at 330 and 370 nm were significantly reduced, indicating that scavenging of R2-Y by NO has modified the environment of the metal cofactor. A similar, featureless absorption spectrum is exhibited by a radical-free, diferric murine protein R2 (metR2), obtained by stoichiometric reduction of the tyrosyl free radical by propoxyphenol, in agreement with previously published data (47). Interestingly, the kinetics of ferric iron release by metR2 was much faster than iron loss from the native protein. The increased lability of the mouse metR2 iron site has been already described (48). Considering these results, we would like to propose the existence of unusual reciprocal interactions between R2-Y and the metal cofactor in mouse protein R2, each component stabilizing the other one. As a consequence, loss of R2-Y, either by reduction (metR2) or by coupling with NO, produces a radical-free protein with a very unstable metal center. Several arguments are in favor of this hypothesis. (i) Iron release is preceded by loss of R2-Y. (ii) Kinetics of ferric iron release by metR2 and by R2 treated with NO are very similar. (iii) Recent reports have shown that, if the dinuclear iron center stabilizes the radical in protein R2, conversely, the presence of the radical strongly influences the reactivity and the stability of the metal center (47–49). (iv) The diferric center of E. coli protein metR2 is stable and NO did not labilize iron in this protein (24). In mouse, and probably in other mammals, formation of apoR2 protein is therefore promoted by a slow release of iron from active R2 (35) and by a faster one from met- or pseudomet-R2 proteins like nitrosylated R2. These results are consistent with earlier observations showing that mouse RR activity required a continuous supply of iron and oxygen (50), in order to regenerate the metal/radical center in apoR2 protein. They may also help to better understand the link observed between protein R2 levels and expression of ferritin, the cell iron-storage protein (51).
Peroxynitrite has been proposed to exert a key role in several physiological and pathophysiological processes (reviewed in Ref. 52). Peroxynitrite is formed from the reaction of superoxide anion with 'NO. The rate of the reaction (k = 6.7 × 10^9 M^-1 s^-1) is fast enough to compete with the coupling of R2-Y with ONOO \(^{-}\) (41, 53). It was therefore of interest to evaluate the effects of peroxynitrite on R2, and especially on the small R2 subunit. Peroxynitrite induced the irreversible loss of R2-Y and inactivated protein R2 irreversibly. A similar dose-response curve for the two processes suggested that destruction of R2-Y might account for the loss of R2 activity. Nitrination of tyrosyl residues and hydroxylation of phenolic structures within E. coli and mouse protein R2 were detected, consistent with the known reactivity of ONOO \(^{-}\) (52). These covalent modifications might involve the amino acid carrying the free radical, thereby inhibiting R2 activity. This hypothesis is, however, probably too simplistic, considering the multifaceted reactivity of peroxynitrite. Other reactive residues might include the conserved Trp-48 and Tyr-356 (E. coli numbering), which are close to the surface and crucial for the long range electron transfer from R1 to R2-Y (19). Covalent modification of Tyr-307, located in the R2 surface area proposed to interact with the R1 subunit (19), might also inactivate the protein. Nitration of the conserved Phe-208 and Phe-212, close to the free radical and important for its stability (54), might also have led to the observed loss of R2-Y CYPR signal. However, these two hydrophobic amino acids, as well as R2-Y, are deeply buried within protein R2. They should be thus less accessible to ONOO \(^{-}\)/ONOONH than O2 \(^{•}\). It has been previously reported that the superoxide anion radical O2 \(^{•}\) irreversibly converted E. coli protein R2 into an inactive form lacking the tyrosyl free radical. But x-ray crystallographic studies established that R2-Y was not directly modified by O2 \(^{•}\), suggesting that oxidation at other site(s) might have caused both R2 inactivation and destruction of R2-Y (55). Similarly, loss of R2-Y induced by peroxynitrite might be more an index of a multisite reactivity of protein R2 rather than a marker of peroxynitrite action directed specifically against the free radical. Successful inhibition by peroxynitrite of apoR2 indicates that reactive sites other than R2-Y do exist in E. coli and mouse R2 proteins. The rank order of nitration staining (E. coli R2 > mouse R2), reflecting the higher tyrosyl amino acid content of the bacterial protein (16 in E. coli and only 9 in mouse R2), also supported a nonspecific inactivation of protein R2 by ONOO \(^{-}\)/ONOONH.

In our previous studies, R2-Y was shown to be quenched in R2-overexpressing tumor cells exposed to the products of NOS II activity (12, 22). This event has been linked to NO-induced inhibition of DNA synthesis and provided a molecular basis for the antiproliferative action of macrophages activated for tumor cytotoxicity (12). The cytotoxic action of macrophages has been first proposed to rely mainly on NO production (5, 6). However, accumulating evidence now suggests that peroxynitrite could also play a significant role (7, 8). Considering that both NO and ONOO \(^{-}\) can induce the disappearance of R2-Y in vitro, it was of importance to determine which of the two nitrogen oxides was the relevant radical scavenger in vivo. High, nonphysiological concentrations of peroxynitrite were required to quench R2-Y, but, owing to the very short half-life of the molecule, it was not possible to determine a priori whether lower but persistent levels of peroxynitrite generated from a long lasting biological source might not be as effective. Inasmuch as peroxynitrite irreversibly inhibited R2 activity, the full reversibility of R2-Y scavenging induced in L1210-R2 cells by murine peritoneal-activated macrophages, either stimulated or not with TPA, provided a clear evidence that ONOO \(^{-}\) was not, or marginally, involved. De novo synthesis of active R2 protein during the 3–4 h time interval between cessation of O2 \(^{•}\) production (and thus of peroxynitrite synthesis) and cell collection might have masked partially an inactivation of protein R2 by peroxynitrite. Since the half-life of mouse protein R2 is 3 h (56), about one-half of peroxynitrite-injured protein R2 still remained at the end of the experiment. Assuming a 10% error in EPR measurements, full recovery of R2-Y in L1210-R2 cells co-cultured with TPA-stimulated macrophages indicated that less than 20% of protein R2 is inactivated by peroxynitrite. This ratio is probably even much less in the absence of NADPH oxidase activation. In previous reports, indirect evidence indicated substantial formation of peroxynitrite by rodent macrophages, either activated with TPA or cultured in l-arginine-depleted medium (57, 58). In the present work, DHR oxidation testified to peroxynitrite production by TPA-stimulated macrophages. Autoenzymatic nitrination of intracellular proteins, or nitration of extracellular compounds by macrophage-derived peroxynitrite has been already described (57, 58). Under our conditions, irreversible loss of R2-Y in tumor cells was not observed. Western blots carried out with lysates of L1210-R2 cells or macrophages stimulated with 2 to 8 nm TPA were also negative for nitrotyrosine detection (not shown). These results show that, under our experimental conditions, peroxynitrite did not nitrate intracellular proteins, including protein R2, suggesting a protective effect of cellular antioxidant components.

We conclude from the present data that NO, and not peroxynitrite, is the main effector of the scavenging of R2-Y by cytotoxic macrophages. Since the modifications introduced by NO in protein R2 (i.e. coupling with the free radical and subsequent acceleration of iron release) are easily reversible in eukaryotic cells, persistent loss of the catalytically competent R2-Y is dependent on a sustained production of NO. The limitation of RR-dependent cytostatic activity to cells expressing NOS II, but not short-lasting NOS I and III, is in agreement with this conclusion.

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