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IMPORTANCE OF THIOLS IN THE REDUCTIVE BINDING OF 2-NITROIMIDAZOLES TO MACROMOLECULES

JAMES A. RALEIGH*† and CAMERON J. KOCH‡

*Radiation Oncology Department, University of North Carolina Hospitals, Chapel Hill, NC 27599-7512; and ‡Radiation Oncology Department, University of Pennsyvania, Philadelphia, PA 19104, U.S.A.

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Abstract—Reductive activation of 2-nitroimidazoles in the presence of bovine serum albumin (BSA) led to binding of the nitroheterocycles to the protein. The binding was most efficient to BSA in which protein disulfides had been reduced to thiol groups. Protein thiols were at least twenty times more efficient than other protein, RNA or DNA nucleophiles in binding the reductively-activated nitroheterocycles. This result is of practical importance in the development of immunoassays for 2-nitroimidazoles as hypoxia markers in normal and tumor tissue.

The bioreduction of nitroheterocyclic compounds such as nitrothiazoles, nitrofurans and nitroimidazoles results in reactive intermediates which bind to cellular molecules [1–5]. Such reductive activation has been associated with nitroheterocyclic cytotoxicity and mutagenesis [6–8]. In mammalian cells, the binding is selective for hypoxic cells [3, 9–13]. In addition to the possible significance of this for nitroheterocyclic toxicity, it has been suggested that the binding of labeled nitroheterocyclic compounds at non-toxic concentrations could be used to detect tumor hypoxia [11, 12]. The idea has been developed along a number of lines differing only in the label and method of detection (for review see Ref. 13).

A hypoxia marker approach based on immunohistochemistry was reported recently in which a 2-nitroimidazole bound to a protein under reducing conditions served as the immunogen for antibody production [14, 15]. During an investigation of the mechanism and optimal conditions for the reductive binding of 2-nitroimidazoles to proteins, we discovered that the binding to thiol rich proteins was very efficient compared to the binding of reductively-activated 2-nitroimidazoles to other cellular macromolecules. Not only is this of practical importance to hypoxia marker development but it is also of possible interest in connection with the metabolism of 2-nitroimidazoles.

Nitroheterocyclic radiosensitizers were developed originally to reverse the radiation resistance of hypoxic cells, with the central concept that drug consumption would be much less for these compounds than for oxygen. The reported extent of binding of reductively-activated 2-nitroimidazoles to a variety of macromolecules (often less than 1% of the nitroheterocycle reduced [16–18]) is inconsistent with this concept because, if the known, overall binding rates to hypoxic cells represented only 1% of the 2-nitroimidazoles metabolized, then radiosensitizer consumption would be a substantial problem for *in vivo* radiosensitization—which it is not [19]. This dilemma might very well be resolved by the observations reported here that thiol rich proteins efficiently bind reductively-activated 2-nitroimidazoles. The thiol content of intracellular proteins in tumor cells is typically 0.06 to 0.15 μmol/mg (10 to 30 mM) of water soluble protein [20], and it may be that thiol rich proteins such as reduced bovine serum albumin with a thiol content of up to 0.5 μmol/mg protein [21] are good models for studies of the binding reactions which occur in cellular systems.

MATERIALS AND METHODS

Misonidazole (**Ia**, see Scheme 1) was a gift of the Drug Synthesis and Chemistry Branch of the NCI (Bethesda, MD). The hexafluorinated compound 1-[2-hydroxy-3(1,1,1,3,3,3-hexafluoroisopropoxy)-propyl] 2-nitroimidazole (CCI-103F, **Ib**) was synthesized as described previously [22]. Tritium-labeled misonidazole and CCI-103F were synthesized according to a procedure described by Born and Smith [23]. The specific activities of the labeled 2-nitroimidazoles were 469 and 227 μCi/mg respectively. Bovine serum albumin Fraction V powder (BSA), bovine pancreatic ribonuclease (RNAase, type XII-A), deoxyribonucleic acid (DNA, type I), ribonucleic acid (RNA, type III), bovine liver superoxide dismutase, and bovine red blood cell catalase were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. All other chemicals were reagent grade and were obtained from local suppliers.

The reductive activation of the 2-nitroimidazoles was achieved by radiation chemical means [24, 25]. The irradiations were carried out in a Gammacell 220 ^{60}Co gamma ray source (Atomic Energy of Canada Limited, Ottawa, Ontario, Canada) at a dose rate of 46 Gy/min as measured by Fricke dosimetry [26]. The samples were irradiated in either a sealed vial, or on glass dishes placed within leakproof aluminium chambers [27].

In the first case, 1-mL samples containing 50 μM

† Corresponding author.

of the 2-nitroimidazole, 100 mM sodium formate, 50 mM phosphate buffer and 0.5 mg BSA (final pH 7.0) were sealed in 2.0-ml Reactivials with septum closures (Pierce Chemical Co., Rockford, IL, U.S.A.). The solutions were deaerated over a period of 60 min by means of nitrogen gas flowing over the rapidly stirred solutions and exiting from the vials through a needle inserted through the septa.

In the second case, 1.5-mL samples of the above solutions containing various macromolecular components (i.e. BSA, RNAase, DNA or RNA at 0.5 mg/mL) were added to glass Petri dishes. The glass dishes were placed inside leakproof aluminium chambers and the gas phase of the chamber was replaced by nitrogen (oxygen content less than 10 ppm) using a series of gas exchanges over a period of 30 min [28].

Following irradiation, the solutions were mixed with an equal volume of cold 10% trichloroacetic acid and centrifuged at 0° for 15 min 2500 rpm. The supernatant was drawn off, and the amount of unreduced 2-nitroimidazole was determined by ultraviolet spectroscopy at 320 nm using a measured molecular extinction coefficient of $\varepsilon = 7400$. The tritium content of the supernatant was measured by adding a sample to liquid scintillation fluid (Scintiverse I; Fisher Scientific Co., Edmonton, Alberta, Canada) and counting the sample in a Beckman LS7800 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, U.S.A.). The precipitated macromolecules were suspended in 1.0 mL of 5% TCA, reisolated by centrifugation for 15 min at 2500 rpm, and the supernatant discarded. This rinsing procedure was repeated to minimize background counts in those experiments where the binding rate was extremely low. With the repeated acid washes, the tritium content of the pellet reached a constant value. There was no evidence that the measured binding was acid labile under these conditions. It is possible that acid labile, binding products were formed but they were not detected by the procedure used. One of the objects of the study was to relate the test tube results reported here to those obtained previously in cellular studies in which similar TCA procedures were used to detect macromolecular binding so the possibility of acid labile products was not pursued further. The macromolecular pellet was dissolved in 300 μL of 1 N NaOH. This solution was neutralized, added to scintillation fluid, and analyzed for tritium content.

The thiol content in the irradiated solutions was determined with Ellman's reagent [29]. This could be done with untreated samples since the other components did not interfere with the absorbance changes at 412 nm. It was confirmed in control, protein precipitation experiments that the thiol groups were associated with the protein. Glyoxal or its equivalent was measured as glyoxal bisoxime ($[CH{=}NOH]_2$) according to a literature procedure [30]. For this, an equal volume of 0.25 M hydroxylamine followed by 100 μL of 0.1 N NaOH was added to the irradiated solutions. The solutions were held at room temperature for 2 hr and the absorbance at 268 nm was measured. Calibration curves were established with 40% aqueous glyoxal (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) whose glyoxal content was determined gravimetrically by means of the bis(2,4-dinitrophenylhydrazone) derivative of glyoxal.

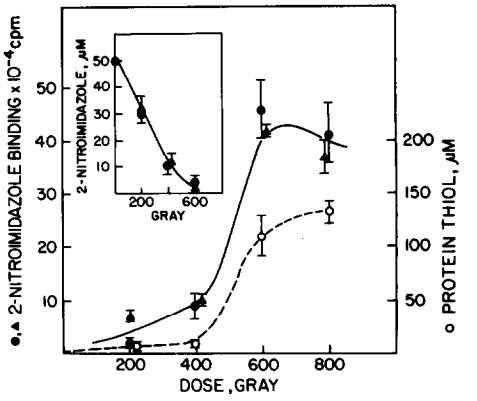


Fig. 1. Dose-dependent reduction of parent compound (inset) and formation of acid insoluble adducts to BSA for the 2-nitroimidazoles, misonidazole (▲) and CCI-103F (●). The dose dependence of thiol production is shown by the dashed line (○). Each point is the mean ± SD of three independent experiments.

RESULTS

The radiation-induced binding of the 2-nitroimidazoles to BSA in the presence of formate showed a lag response increasing sharply after approximately 80% of the 2-nitroimidazole had been reduced (Fig. 1). For radiation doses which completely reduced the 2-nitroimidazoles, approximately 1% of the total tritium in radioactivity-labeled CCI-103F or misonidazole bound to the protein. The rate of loss of the 2-nitroimidazoles as measured by ultraviolet spectroscopy was the same for misonidazole and CCI-103F (Fig. 1). Approximately 1 mmol of 2-nitroimidazole was consumed for each 6.2 mEq of electrons generated in the solution containing 0.5 mg/mL BSA assuming a G-value of 6 for reducing equivalents $e^-_{aq} + CO_2^-$ [31]. The G value is the number of molecules changed per 100 eV of energy absorbed in the solution. In the absence of protein, the efficiency of nitroheterocyclic reduction was increased so that the 2-nitroimidazoles consumed approximately 4 mEq of electrons for each mmol reduced (cf. Refs. 24 and 25).

Several possible reasons for the lag in binding were tested. One possibility was that residual oxygen in the solutions was inhibiting the binding process [32, 33]. However, a number of experimental findings led us to conclude that oxygen was not the primary cause of the lag in binding. First, in those cases where less than complete deaeration was intentional, the consumption of 2-nitroimidazole did show a lag until the residual oxygen was consumed by radiation chemical reduction. However, the passage of a stream of nitrogen over the stirred solutions in the sealed vials for 60 min invariably produced a linear dose-yield response (Fig. 1, inset) and, therefore, the lag in binding was not due to a lag in 2-nitroimidazole reduction. Second, residual oxygen

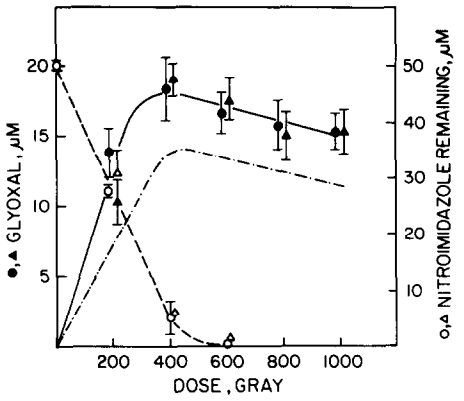


Fig. 2. Dose-dependent formation of glyoxal in solutions of 50 μM misonidazole (▲) or CCI-103F (●) and dose-dependent consumption of misonidazole (△) or CCI-103F (○) in solutions containing BSA. The yield of glyoxal bisoxime in the absence of BSA is also shown (-●-●-).

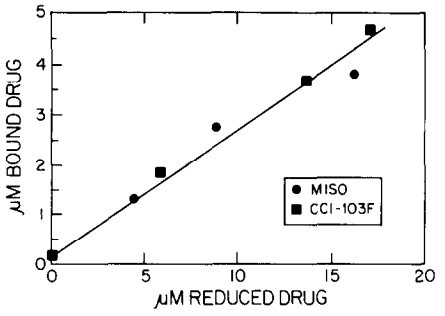


Fig. 3. Yield of acid insoluble adducts bound to prereduced BSA as a function of reduced drug for misonidazole (●) and CCI-103F (■). Initial nitroimidazole concentration was 20 μM.

might oxidize thiols in BSA. It was found, however, that protein thiols were stable for many hours in the sealed vials as measured by Ellman's reagent. In addition, the extent of 2-nitroimidazole binding to BSA, which had been prereduced by the radiation chemical procedure, was unchanged by storage of the prereduced BSA in the sealed vials prior to the binding experiment. The possibility that hydrogen peroxide or superoxide produced by trace amounts of oxygen in the deaerated solutions might affect binding was excluded by showing that binding was unaffected by the presence of 0.6 units of superoxide dismutase and 8 units of catalase either separately or in combination during the course of the radiation reduction (data not shown). Finally, results similar to those obtained in the sealed Reactivials were observed using a sealed chamber and glass dish system where the oxygen concentrations can be monitored directly (see below).

A second possibility for the lag in binding was that binding intermediates appeared only after four electrons on average had reacted with each 2-nitroimidazole molecule. This would imply that the hydroxylamine **II** was stable and that further reduction of **II** was required to produce binding intermediates. The known extreme lability of **II** at neutral pH with respect to the addition of water [7, 30, 34] made this unlikely. Nevertheless, the hydrolysis of **II** was monitored in terms of the formation of glyoxal or a closely related compound which is known to be formed by the hydrolysis of **II** [23, 35]. Using hydroxylamine (NH_2OH) as the reagent and the resulting glyoxal bisoxime as the analyte (268 nm) [30], it was found that 38% of the 2-nitroimidazole originally present could be accounted for as glyoxal or its equivalent. It was also found that the dose-yield response for glyoxal formation showed no lag but was linear with dose (Fig. 2) and mirrored the linear dose-yield response for 2-nitroimidazole consumption. Finally, if the 2-nitroimidazoles were first reduced and then added to prereduced BSA, then no binding to BSA occurred. Taken together, these results indicate that there was no build-up of the four-electron reduction product (**II**) in the solutions.

The glyoxal or closely related species was relatively stable to the reducing conditions of the irradiated solutions in contrast to commercially available aqueous solutions of glyoxal in which the glyoxal is readily consumed at the rate of one molecule for every two electrons delivered to the solution (data not shown). In keeping with the likelihood that free glyoxal was not formed in the solutions, we found that the initial rate of glyoxal bisoxime formation (268 nm) was eleven times slower than that with comparable concentrations of free glyoxal (data not shown). In addition to these results, it was found that the measured yield of glyoxal in the presence of BSA was 20% higher than that formed in the absence of BSA (dotted line, Fig. 2), perhaps indicative of an interaction between BSA and a reactive intermediate which led to an increased yield of glyoxal.

Protein thiol formation increased sharply just at the point where increased binding occurred (Fig. 1). The large G value (~6) suggests that the thiols arose from the reduction of the 17 disulfide groups in BSA [21]. The sharp increase in thiol production indicates that the disulfides (*ca.* 130 μM) compete effectively for electrons only when the 2-nitroimidazole concentration has dropped to approximately 10 μM. Whether there is concomitant unfolding of the protein which facilitates the reaction is not known. Prereduction of BSA by irradiation in hypoxia in the presence of 100 mM formate and 50 mM phosphate buffer but without 2-nitroimidazole was linear with dose (data not shown). This facile radiation chemical reduction of protein disulfides is analogous to that for the disulfides of glutathione and dithiothreitol [36]. Under the conditions of the present experiments, the reduction of approximately one-half of the available disulfides in BSA (molecular weight 66,000) [21] occurred. The protein thiols appeared to be quite stable as measured by Ellman's reagent. No precipitation of the reduced proteins was observed which might have occurred if there were extensive intermolecular cross-linking of the thiols. The use of radiation-chemically prereduced BSA removed the lag in binding and gave a very high yield of bound sensitizer (Fig. 3) with the overall rate of binding under these conditions accounting for more

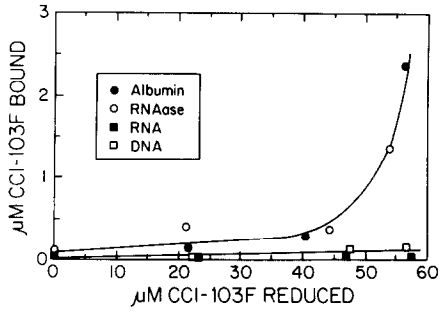


Fig. 4. Yield of acid insoluble adducts bound to various macromolecules as a function of CCI-103F reduced (initial concentration 55 μM). Key: BSA (●), RNAase (○), RNA (■) and DNA (□).

than 20% of the 2-nitroimidazole consumed. No attempt was made to measure the relatively small (4–8%) decrease in thiol content in the prereduced BSA which might be expected to accompany the binding of the reductively-activated 2-nitroimidazole.

Of the hydroxyl radical scavengers examined, only sodium formate efficiently promoted the formation of thiol groups in irradiated BSA. None of benzyl alcohol, dextrose, dioxane, isopropanol, sodium carbonate or tetrahydrofuran at 100 mM in the phosphate-buffered BSA solutions led to thiol production to an extent greater than 2% of that produced in the presence of 100 mM sodium formate (data not shown). The results with sodium formate clearly established the importance of the presence of protein thiol groups for efficient binding, and therefore binding in the presence of the other hydroxyl radical scavengers was not investigated.

Since even non-reduced albumin contains ~1 free SH per molecule, a comparison was made for binding to various macromolecules, all present at 0.5 mg/mL (Fig. 4). Binding to DNA and RNA was negligible over a wide range of reduced 2-nitroimidazole concentrations. Although the low concentrations of thiols expected in the solutions containing RNAase were not measured, the course of binding to bovine RNAase (containing 4 disulfides but no free protein thiol) [37] was similar to that for BSA in the sense that efficient binding occurred only after most of the 2-nitroimidazole had been reduced (Fig. 4).

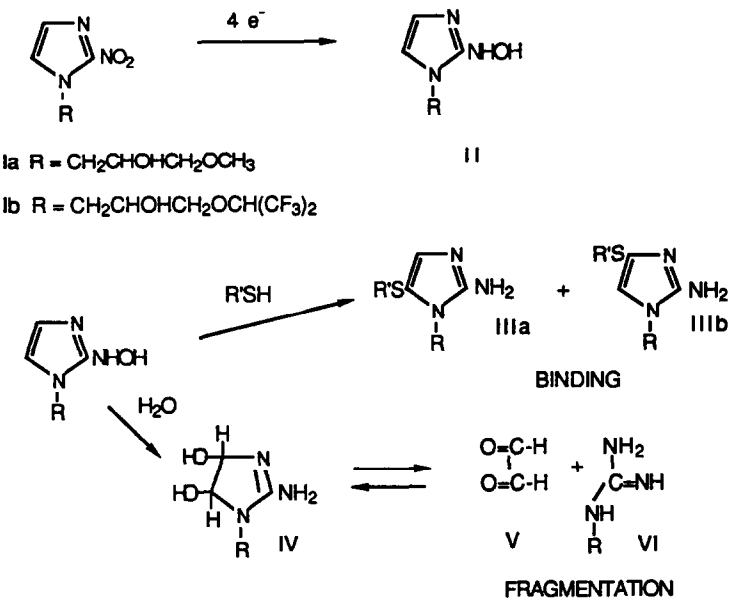
DISCUSSION

The mechanism by which reductively-activated 2-nitroimidazoles bind to macromolecules in hypoxic cells is largely unknown. The majority of the binding occurs to cellular protein [5, 18] which is similar to the macromolecular binding of nitrofurans in bacteria [1] and rat microsomes [2] and of nitrothiazoles in schistosomes [4] and is in agreement with the observation that the binding of 2-nitroimidazoles is concentrated in the cytoplasm of canine tissue cells [38]. The macromolecular binding of 2-nitroimidazoles has the temperature dependence of a metabolic reaction, is inhibited by thiols such as cysteine and glutathione [9, 18] and shows an oxygen dependence similar to that for the radiobiological oxygen effect [33, 39, 40]. The binding incorporates all of the atoms of the original 2-nitroimidazole molecule [41, 42], and antibodies raised against CCI-103F which had been reductively bound to proteins *in vitro* recognize the nitroaromatic bound to hypoxic cells *in vivo* [14, 15, 38].

No information about the macromolecule-hapten linkage in cells is available from the immunochemical studies because the antigenic determinants for the polyclonal antibodies were associated with the side-chain of the 2-nitroimidazoles [14]. However, in the case of the thiol containing tripeptide, glutathione, the linkage has been shown to occur between the thiol group of glutathione and either of the 4 or 5 positions of the imidazole ring of the reductively-activated 2-nitroimidazole (**IIa,b**) [43, 44]. The results for the reductive binding of misonidazole or CCI-103F to BSA reported here are consistent with a similar mechanism for the binding to proteins. For example, BSA has 23 arginine, 59 lysine, 17 histidine, 28 serine, 31 threonine, 41 aspartate and 59 glutamate nucleophilic moieties [21], but only when some of the 34 latent thiol groups are released by disulfide reduction does the binding of reduced 2-nitroimidazoles become efficient. Comparing baseline binding in the lag phase with that following thiol release (Fig. 1) shows that the thiol groups are at least twenty times more effective than other protein nucleophiles in binding reductively-activated 2-nitroimidazoles. The data in Fig. 4 suggest that this factor is even larger when the comparison is made with the nucleophilic groups in polynucleotides.

In addition to 2-nitroimidazole consumption in the binding process, reduced 2-nitroimidazoles are subject to hydrolysis in aqueous solution [25, 30, 34]. One indicator of the hydrolytic pathway is the appearance of glyoxal or a closely related species (Scheme 1) [25, 34, 35, 45]. Glyoxal reacts in a reversible manner with nucleophiles such as water, amines, thiols and guanidines (**VI**) [46] so that the glyoxal measured in completely equilibrated solutions can be expected to be the sum of hydrated glyoxal, compound **IV**, and a variety of reversible adducts to BSA including those to lysine and to arginine, whose structure in the latter case would be analogous to **IV** [i.e. R = $(CH_2)_3CH(NH_2)COOH$]. The kinetics of the hydroxylamine (NH_2OH) reaction with various glyoxal adducts may be different but no investigation was made of this or whether complete equilibration among the various adducts was achieved. It was observed that the rate of formation of glyoxal bisoxime in the irradiated solutions was eleven times slower than that for free glyoxal. In addition, free glyoxal was observed to be readily reduced under the conditions of the binding experiment but very little reduction of the glyoxal precursor occurred in the irradiated solutions. Both of these observations are consistent with the conclusion that glyoxal is formed as a complex such as **IV** with little, if any, free glyoxal present [30, 34, 45].

The reaction of glyoxal with protein thiol, amine or guanidine residues and with nucleic acid guanine residues might contribute to the toxicity of reduced 2-nitroimidazoles [7, 35, 45, 46]. It is less likely that



Scheme 1. Reductive activation of 2-nitroimidazoles.

these reactions contribute to the binding observed in the present study because the 4 and 5 positions in the imidazole ring from which glyoxal is derived are unlabeled. It is conceivable that an intermediate such as **IV** could serve as a binding intermediate but the limited diffusion of the binding intermediate and the long-term stability of the macromolecular binding in hypoxic cells [9, 13] argues against the involvement of reversibly bound glyoxal or closely related aldehyde precursor such as might arise in a step-wise opening of the reduced 2-nitroimidazole [25].

The reductive binding of the 2-nitroimidazoles to BSA represents more than 20% of the nitroheterocycle consumed. An additional 38% of the reductively activated 2-nitroimidazoles can be accounted for in terms of glyoxal (glyoxal bisoxime) formation. This leaves approximately 40% of the 2-nitroimidazole unaccounted for. The hydrolytic instability of the hydroxylamine derivative **II** [30, 34] could account for the incomplete material balance if hydrolytic fragmentation proceeded by routes other than that in Scheme 1. For example, attack of water molecules at position 2 of the imidazole ring has been proposed [25] to account for the formation of products of 2-nitroimidazole fragmentation other than glyoxal [25, 47]. The interaction of reductively-activated 2-nitroimidazoles with phosphate [34] or formate anions might also lead to undetected products and, hence, to an incomplete material balance. While the 6 electron reduction, 2-aminoimidazole product may be present [25], the extensive degradation of the reductively-activated 2-nitroimidazoles rules out the possibility that the 6 mEq per mmol stoichiometry for 2-nitroimidazole reduction (Fig. 1) is related simply to the formation of the 2-aminoimidazole product since it is stable to hydrolysis under the conditions of the experiment [25].

The hydroxylamine derivative **II** seems most plausible as the intermediate leading to both glyoxal-like products and protein adducts but the data do not rule out possible alternatives. For example, hydrolysis and binding schemes analogous to Scheme 1 can be drawn for the 2 electron nitroso intermediate and, even though the dose-yield response for 2-nitroimidazole consumption (Fig. 1, insert, and Fig. 2) is more compatible with the 4 electron reduction hydroxylamine intermediate, a nitroso binding intermediate cannot be rule out. Likewise, the data do not rule out a free radical intermediate.

The reduction of the disulfides via a free radical chain reaction mechanism has been shown by Elliot *et al.* [36] to occur in oxidized dithiothreitol and glutathione. It is assumed that similar reactions are possible with protein disulfides, and a simplified version of the possible reactions is listed below. Chain terminating steps include the recombination of $CO_2^{\cdot}$ or RS· radicals.

$$H_2O + \text{Radiation} \longrightarrow e_{aq}^-, OH\cdot, H\cdot, H_2O_2, H^+ \quad (1)$$

$$HCO_2^- + OH\cdot\,(H\cdot) \longrightarrow CO_2^{\cdot} + H_2O\,(H_2) \quad (2)$$

$$CO_2^{\cdot} + RSSR \longrightarrow RSSR^- + CO_2 \quad (3)$$

$$e_{eq}^- + RSSR \longrightarrow RSSR^- \quad (4)$$

$$RSSR^- + H^+ \longleftrightarrow RSH + RS\cdot \quad (5)$$

$$RS\cdot + HCO_2^- \longleftrightarrow RSH + CO_2^{\cdot} \quad (6)$$

While the involvement of non-radical intermediates in the binding process is favored, free radical mechanisms involving, for example, the addition of protein thiyl radicals to the 2-nitroimidazoles or their reduction products must also be considered. If the critical thiyl radicals are formed from the disulfide reduction (reactions 3–5), then the data of Fig. 1 cannot rule out the possibility that these thiyl radicals interact with residual 2-nitroimidazole in a binding reaction. However, the fact that prereduction of

BSA disulfide groups to thiols produces efficient binding indicates that the thiol group and not the disulfide group is the important moiety in the binding process. Thiols are reported to have high rates of reaction with hydrated electrons [48] and thiyl radicals might conceivably arise from this reaction. However, the protein thiols, once formed, appear to be stable to hydrated electron attack (Fig. 1 and additional data not shown), and the production of significant amounts of thiyl radicals by this route seems unlikely. However, these arguments are not conclusive and, ultimately, a complete understanding of the mechanism(s) of binding will require that the structure of the protein adducts be determined.

This report is the first to demonstrate a major pathway of reductive binding of 2-nitroimidazoles to macromolecules in a model system. The levels of binding were comparable to those observed for nitrofurans in rat microsomal systems [2], and the thiol pathway to protein binding might be expected to be important in the cellular milieu where protein concentrations are 20-fold higher and the protein thiol concentrations comparable to those in the BSA model system. Limited quantitative measures of the reductive binding of 2-nitroimidazoles to living tissue are available for comparison with binding in the BSA model system. However, in one study [42], the extent of binding to the acid insoluble fractions of EMT6 tumors in Balb/C mice amounted to 2% of the radioactively labeled misonidazole injected into mice. About one-half of the 2-nitroimidazole moiety is excreted unchanged [49] so approximately 4% of the metabolized 2-nitroimidazole was bound to tumor cell macromolecules—primarily to protein. Whether this somewhat lower level of *in vivo* binding results from an enhanced hydrolysis of the hydroxylamine intermediate is not known but it is perhaps significant in this regard that the hydrolysis marker, glyoxal or a closely related derivative, has been recovered from the urine of patients injected with misonidazole [50].

In principle, carbon centered radicals produced from hydroxyl radical scavengers other than formate can reduce disulfide bonds to produce disulfide radical anions, and subsequently, thiyl radicals ([51]; cf. reactions 3 and 5). However, a reaction rate constant of the order of $10^4\,M^{-1}\,sec^{-1}$ for hydrogen atom abstraction by thiyl radicals from the other hydroxyl radical scavengers examined (cf. reaction 6) [52] apparently is not sufficient to promote chain reaction reduction of protein disulfides under the conditions of the experiments performed here. In the initial studies of the binding of 2-nitroimidazoles to proteins for the preparation of immunogens [14], isopropranol was used to scavenge hydroxyl radicals and provide a reducing environment for the binding process. However, the present results have established the importance of protein thiol groups in the binding process and, since hydroxyl radical scavengers other than formate salts do not efficiently promote thiol production from protein disulfides, it would appear that radiation chemical reduction in the presence of formate salts is the method of choice for binding nitroheterocyclic compounds to BSA or other disulfide containing proteins. The importance of thiols in the binding process may also explain the low efficiency of misonidazole binding to albumin which was observed when the solutions were irradiated under hypoxia but in the absence of sodium formate [53].

In summary, greatly increased binding of reductively-activated 2-nitroimidazoles was associated with the appearance of thiol groups on BSA which indicates an important role for the thiol groups in the binding process. The nature of the linkage in proteins is unknown but possibly involves a sulfide bond to the imidazole ring similar to that reported by others for the linkage of glutathione to reduced 2-nitroimidazoles. The reductive-activation of 2-nitroimidazoles in the presence of BSA involved both binding and competing hydrolysis of the imidazole ring. Hydrolytic fragmentation predominated under the conditions described. The radiation chemical reduction of both protein disulfides and nitroheterocyclics is an efficient and convenient way of preparing immunogens for the production of antibodies against reductively-activated 2-nitroimidazoles bound to hypoxic cells.

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