Effect of Misonidazole on Radiation-induced Reduction of DNA Bases in Deaerated Aqueous Solution

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The effect of a hypoxic-cell radiosensitizer misonidazole on the radiation-induced reduction of DNA bases (thymine, cytosine and adenine) was studied in deaerated aqueous solution containing sodium formate (100 mM) at pH 7.0. The reductive decomposition of DNA bases proceeded in proportion to the irradiation dose in the absence of misonidazole with G-values of 3.7 for thymine, 2.1 for cytosine and 0.5 for adenine. On irradiation in the presence of misonidazole, the induction period proportional to the initial concentration of misonidazole was observed prior to the initiation of base decomposition. A stoichiometrical investigation showed that one misonidazole molecule reacts stepwise with four electrons originated from reducing species such as e\(_{aq}\), CO\(_2\) and base radical anions (or their protonated forms) during the course of its reduction. The four-electron reduction products of misonidazole retarded the base decomposition occurring after the induction period. The degree of the retardation increased with increasing the concentration of the four-electron reduction products of misonidazole.

INTRODUCTION

Since Adams et al. demonstrated a correlation between the radiosensitivity of hypoxic cell and the electron affinity of the radiosensitizer used\(^1\), the radiosensitizing effects of a great number of electron-affinic compounds on the degradations of DNA and its related compounds have been investigated extensively in aqueous solutions to elucidate the sensitization mechanism\(^2\)-\(^4\).

Among the electron-affinic radiosensitizers tested so far in vivo as well as in vitro, misonidazole (1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol, Ro 07-

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0582) is recognized to be one of the most effective radiosensitizers for hypoxic cells\(^6\). However, the radiation chemical behavior of misonidazole associated with its radiosensitizing activity is still a subject of considerable investigations. Recently, we reported the radiosensitizing characteristics of misonidazole in the oxidative decomposition of thymine in N\(_2\)O-saturated aqueous solution\(^7\)\(^-\)\(^8\).

In this work, the radiolysis of the deaerated aqueous solution of DNA base such as thymine, cytosine or adenine containing sodium formate has been performed in the absence and the presence of misonidazole. The purpose of this paper is to clarify the effect of misonidazole on the base decompositions via reactions with reducing species of e\(_{aq}\) and CO\(_2^-\).

**MATERIALS AND METHODS**

**Materials**

Thymine, cytosine, adenine and dihydrothymine were obtained from Sigma. Misonidazole (1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol, Ro 07-0582) was supplied by Roche Products Ltd. Japan. All other chemicals were of the best available grades and were used without further purification. Predistilled water was purified by the successive distillations from acid dichromate, from alkaline permanganate, and finally without additives.

**\(\gamma\)-Irradiation**

Aqueous DNA-base solution (1 mM) containing mISONIDAZOLE (0—1 mM) and sodium formate (100 mM) was buffered at pH 7.0 ± 0.1 (S.E.) with sodium phosphate (2 mM). The solution was deaerated by repeated freeze-pump-thaw cycles on a vacuum line. All irradiations were carried out in a sealed glass ampoule at room temperature with a \(^{60}\)Co \(\gamma\)-ray source at a dose rate of 380 Gy h\(^{-1}\).

In order to determine the effect of the reduction products of misonidazole on the radiolytic reduction of thymine (0.5 mM), the deaerated aqueous formate solution (100 mM) of misonidazole (0—2 mM) was preirradiated at pH 7.0 ± 0.1 (S.E.) up to the complete disappearance of misonidazole. The total concentration of the reduction products was assumed to be equal to the initial concentration of misonidazole.

Irradiation of an aqueous solution of adenine (1.0 mM, pH 7.0 ± 0.1 (S.E.)) containing sodium formate (100 mM) was performed under N\(_2\)O-saturated conditions to clarify the intrinsic reactivity of adenine toward CO\(_2^-\).

**High performance liquid chromatography (HPLC)**

The irradiated solutions (0.1 cm\(^3\)) were subjected to HPLC analysis using a Toyo Soda Model HLC-803 apparatus equipped with an ODS (C\(_{18}\))-type column of Toyo Soda LS 410. The phosphate buffer solutions (pH 2.0 for thymine,
4.0 for cytosine and adenine) containing methanol (3 vol% for thymine, 1 vol% for cytosine and 10 vol% for adenine) were delivered as the mobile phase at a flow rate of 0.6 cm³ min⁻¹. The eluents were monitored by the uv absorption at 210 or above 250 nm, using a Toyo Soda Model UV-8 variable wavelength-type detector.

RESULTS AND DISCUSSION

HPLC analysis

Figure 1 shows the HPLC chromatograms of the (a) nonirradiated, (b) 1.52-kGy irradiated, and (c) 5.32-kGy irradiated thymine (1 mM)-misonidazole (1 mM) solutions when the eluents were monitored by the uv absorption at 210 nm. The area of the elution band assigned to thymine was invariant during the 1.52-kGy irradiation, while the preferential decomposition of misonidazole occurred to give a major product (designated by * in chromatogram (b)) with several by-products. On prolonged irradiation up to 5.32-kGy, misonidazole disappeared completely and the decomposition of thymine was also observed at the same time (chromatogram (c)). The formation of 5,6-dihydrothymine as a major product of thymine decomposition was confirmed along with those of 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrothymine-5-carboxylic acid and
5,6-dihydrothymine-6-carboxylic acid by the reference to the previous reports\textsuperscript{7,9).}

Similar results were obtained in the cases of cytosine and adenine, in which the DNA bases did not decompose until misonidazole was converted completely into a major product identical with that observed in Fig. 1 (b) and (c). Further attempts were not made to confirm the structures of the reaction products derived from the DNA-bases in these systems\textsuperscript{10).}

When the eluents were monitored at 250 nm, the major radiolysis product of misonidazole detected at 210 nm was not observed. The lack of the uv absorption at 250 nm and the considerably short retention time indicate that the major product may be hydroxyamino or oxime derivative of misonidazole which has been characterized recently\textsuperscript{11).} Since no product peak with a retention time longer than that of misonidazole was detected, the formation of azo and azoxy derivatives of misonidazole\textsuperscript{12) seems minor under the present experimental conditions.}

\textit{Radiation-induced reduction of DNA bases}

Figure 2 shows variations of the concentrations of DNA bases (thymine, cytosine and adenine) as a function of irradiation dose for deaerated solutions containing sodium formate irradiated in the absence and the presence of misonidazole (0.5 mM). In the absence of misonidazole the concentrations of these bases decreased linearly with irradiation dose at different rates (broken lines in Fig. 2). From these linear relationships the G-values for the base decompositions are evaluated as $G(-T)=3.7$, $G(-C)=2.1$ and $G(-A)=0.5$ for thymine, cytosine and adenine, respectively.

![Fig. 2. Plots of the concentrations of the DNA bases determined by HPLC analysis against the irradiation dose in the (-----) absence and (—) presence of misonidazole; (•) thymine, (○) cytosine and (△) adenine. Variation of the misonidazole concentration is also represented with corresponding closed symbols.](image-url)
It is well known that OH radicals ($G(\cdot \text{OH})=2.7$) and H atoms ($G(\text{H} \cdot)=0.55$) produced by radiolysis of water are converted into carbon dioxide radical anion ($\text{CO}_2^-$) in the presence of an excess amount of formate ion (reactions 2 and 3).

\[
\begin{align*}
\text{H}_2\text{O} & \rightarrow \cdot \text{OH}, \text{ H} \cdot, \text{ e}_{\text{aq}} \\
\cdot \text{OH} + \text{HCO}_2^- & \rightarrow \text{H}_2\text{O} + \text{CO}_2^- \\
\text{H} \cdot + \text{HCO}_2^- & \rightarrow \text{H}_2 + \text{CO}_2^-
\end{align*}
\] (1) (2) (3)

Therefore, $\text{e}_{\text{aq}}$ ($G(\text{e}_{\text{aq}})=2.7$) and $\text{CO}_2^-$ ($G(\text{CO}_2^-)=3.25$) are responsible for the reactions of DNA bases under the present conditions.

Hydrated electrons react with DNA bases (B) to give radical anions (reaction 4)\textsuperscript{13}.

\[
\text{e}_{\text{aq}} + \text{B} \rightarrow \text{B}^-
\] (4)

The radical anions ($\text{B}^-$) thus formed give mainly the dihydro and hydroxydihydro derivatives via protonation (reaction 5) followed by disproportionation (reactions 6 and 7); e.g. for thymine\textsuperscript{9},

\[
\begin{align*}
\text{[Diagram]} \rightarrow \text{[Diagram]} \rightarrow \text{[Diagram]} \rightarrow \text{[Diagram]} \\
\text{[Diagram]} \rightarrow \text{[Diagram]} \rightarrow \text{[Diagram]} \rightarrow \text{[Diagram]}
\end{align*}
\] (5) (6) (7)
Another reducing species $\text{CO}_2^-$ reacts with pyrimidine bases (thymine and cytosine) to give either adducts (reactions 8 and 9)\textsuperscript{9,10} or base radical anion in an analogous manner as $e_{\text{aq}}$ (reaction 10); e.g., for thymine\textsuperscript{9},

\[
\begin{align*}
\text{Thymine} + \text{CO}_2^- & \rightarrow \text{Thymine CO}_2^- \\
\text{Cytosine} + \text{CO}_2^- & \rightarrow \text{Cytosine CO}_2^- \\
\end{align*}
\]

An aqueous adenine solution (1 mM) containing sodium formate (100 mM) was also irradiated under N$_2$O-saturated conditions to characterize the intrinsic reactivity of adenine toward CO$_2^-$. Since $e_{\text{aq}}$ reacts with N$_2$O to give $\cdot$OH ($e_{\text{aq}} + \text{N}_2\text{O} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{N}_2$) which is subsequently converted to CO$_2^-$ by reaction (2), the reducing species is essentially limited to CO$_2^-$ ($G(\text{CO}_2^-) \approx 5.95$) under these conditions. The obtained $G(-\text{A})$-value=0.1 in N$_2$O-saturated formate solution ($G(\text{CO}_2^-) \approx 5.95$) is much lower than $G(-\text{A})$-value=0.5 in deaerated formate solution ($G(e_{\text{aq}}^-)=2.7$, $G(\text{CO}_2^-) \approx 3.25$), indicating much less reactivity of adenine as a purine base toward CO$_2^-$. Thus, it is likely that CO$_2^-$ makes minor contribution to the reductive decomposition of adenine relative to $e_{\text{aq}}$, while both species ($e_{\text{aq}}^-$ and CO$_2^-$) are equally responsible for the pyrimidine decompositions.

The overall efficiency of $e_{\text{aq}}^-$ for adenine decomposition ($G(-\text{A})/G(e_{\text{aq}}^-)$) can be evaluated as about 0.2 from the data, $G(-\text{A})=0.5$ and $G(e_{\text{aq}}^-)=2.7$. A comparison with the corresponding value for thymine (0.5) reported by Loman and Ebert\textsuperscript{14}, and Infante et al.\textsuperscript{15} indicates that the apparent reactivity of adenine toward $e_{\text{aq}}^-$ is lower than thymine by a factor of 0.6.
The decomposition of DNA bases began to occur after the complete disappearance of misonidazole.

Variation of thymine decomposition as a function of the initial concentration of misonidazole (0.1–1.0 mM) is shown in Fig. 3. In all cases thymine decomposition occurred after the complete disappearance of misonidazole. More explicitly, Fig. 4 shows that the dose required for the complete disappearance of misonidazole ($D_m$) is equal to that during induction period of the base decomposition ($D_i$). This result indicates that misonidazole can effectively inhibit the radiolytic decomposition of DNA bases in deaerated aqueous solution containing sodium formate.

It is likely that misonidazole (M) reacts with $e_{aq}^{-}$ (reaction 11) in competition with DNA bases (thymine, cytosine and adenine) in the DNA base-misonidazole systems.

$$e_{aq}^{-} + M \longrightarrow M^-$$ (11)

Taking into account the rate constants of the reactions with $e_{aq}^{-}$: $1.8 \times 10^{10}$ (thymine)$^{13}$, $1.3 \times 10^{10}$ (cytosine)$^{13}$, $0.9 \times 10^{10}$ (adenine)$^{13}$ and $3.0 \times 10^{10}$ M$^{-1}$s$^{-1}$ (misonidazole)$^{16}$, radical anions of the DNA bases seem to be produced in considerable yields even in the presence of misonidazole; e.g. G-values of base radical anions produced by the reaction with $e_{aq}^{-}$ are estimated as 1.5, 1.3 and 1.0 for thymine, cytosine and adenine, respectively, under the conditions of [base]=1.0 mM and [misonidazole]=0.5 mM.

Nevertheless, no decomposition of DNA bases occurred in the presence of misonidazole (Fig. 2 and Fig. 3). It follows that electron transfer from base radical anion ($B^-$) or its protonated from ($B^++H$) to misonidazole (M) (reactions 12a, b) occurs in preference to several subsequent reactions to give final products (reaction 5, 6 and 7).
This is consistent with a number of pulse radiolysis investigations that the electron transfer as in reaction 12a or 12b is a very fast process occurring at a diffusion-controlled rate\(^{16,17}\).

While few kinetic study has been accomplished on the reactions of DNA bases with CO\(_2\), the rate constant for the reaction of thymine with CO\(_2\) has been estimated to be about \(10^5\) M\(^{-1}\) s\(^{-1}\).\(^{14}\) Taking into account a fact that the rate constants for the electron transfer from CO\(_2\) to heterocyclic nitro-compounds are much larger than that for the reaction between thymine and CO\(_2\) by a factor of \(10^3\) - \(10^4\),\(^{18}\) it is likely that most of CO\(_2\) does not react with DNA bases but reduces misonidazole preferentially into its radical anion (reaction 13).

\[
\text{CO}_2^- + M \rightarrow \text{CO}_2 + M^-
\]

(13)

The G-values of the conversion of misonidazole evaluated in this work (G(M) ≈ 1.5 as summarized in Table 1) agree well with literature values 1.4\(^{11}\), 1.5\(^{12}\) and 1.0\(^{16}\) obtained in the radiolysis of aqueous misonidazole solution containing 2-propanol or sodium formate. From the ratio of the total G-value of reducing species (\(G_t=G(e_{aq})+G(\text{CO}_2)=5.95\)) to the G(M) value, \(G_t/G(M)≈4\), it is suggested that one misonidazole molecule reacts with four electrons originated from various reducing species such as \(e_{aq}\), CO\(_2\), B\(^-\) and B\(^2-\)H (see reactions 11, 12a, 12b and 13) during the course of its reduction.

In view of our recent report on the reduction of nitroimidazole derivatives including misonidazole\(^{11}\), hydroxyamino derivative of misonidazole is possibly produced via four-electron reduction in the present system but readily isomerizes to an oxime form and/or decomposes as in reaction (14).

\[
\text{R} = \text{CH}_2\text{CH(OH)CH}_2\text{OCH}_3
\]
It should be noted in reaction (14) that the possible nitroso intermediate is derived on the whole from two-electron reduction of misonidazole, whereas the hydroxyamino derivative is due to four-electron reduction. In agreement with all the results mentioned above, it is likely that the nitroso intermediate reacts preferentially with various reducing species in an analogous manner as misonidazole.

Retardation of the radiolytic reduction of thymine by reduced misonidazole

The values of G(-T) after induction period in the thymine-misonidazole system (Table 1), and G(-T)* and G(dihydrothymine)* (=G(DHT)*) in the thymine-reduced misonidazole (MISred) system (Table 2) are plotted against the ratio [MISred]/[Thymine] in Fig. 5. For the thymine-misonidazole system the [MISred] was estimated from the total dose during induction period and the initial concentration of misonidazole.

It is noteworthy in Fig. 5 that the variation of G(-T)* as a function of [MISred]/[Thymine] is in good agreement with that of G(-T). This result indicates that the four-electron reduction products, accumulated during induction period, have the abilities to retard the reductive decomposition of thymine, which is distinct from misonidazole as an inhibitor. Thus, the reactivity of MISred toward reducing species, particularly less reducing species CO_2^-, B^- and B^-H (see reactions 12a, 12b and 13), may be much lower than that of misonidazole. It seems possible to predict that further reduction of MISred (possibly hydroxyamino and/or oxime derivative of misonidazole) by eaq (and CO_2^-) occurs in competition with the reductive decomposition of thymine.

Figure 5 also shows that G(DHT)* decreases in proportion to the decrease in G(-T)*. Moreover, the selectivity of the dihydrothymine formation given by the ratio G(DHT)*/G(-T)* is almost independent of MISred and approximately

### Table 2.

<table>
<thead>
<tr>
<th>[MISred]</th>
<th>[MISred]/[Thymine]</th>
<th>G(-T)*</th>
<th>G(DHT)*</th>
<th>G(DHT)<em>/G(-T)</em></th>
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<td>2.5</td>
<td>1.0</td>
<td>0.39</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>2.4</td>
<td>0.8</td>
<td>0.34</td>
</tr>
</tbody>
</table>

1) Initial concentration of the reduction products of misonidazole [MISred] was assumed to be equal to that of misonidazole.
equal to 0.4 (Table 2). This suggests that MISred does not affect a major process (reaction 6) to give dihydrothymine. This is consistent with above prediction that MISred will be less reactive toward Br−H. The decreases in G(-C) and G(-A) observed after induction period (Table 1) may also be ascribed to a similar effect of MISred.

In conclusion, the present results suggest that misoniazole undergoes preferentially reduction to consume stepwise four electrons per molecule, thereby inhibiting the decomposition of DNA bases by reducing species. The product(s) derived from the four-electron reduction of misonidazole can also retard the reductive decomposition of DNA bases. These are in contrast to the radiosensitizing ability of misonidazole that promotes the hydroxylation of thymine to thymine glycol, even in the absence of oxygen, by one-electron oxidation of the intermediate hydroxythymyl radical7,8. Further details of the retardation effect of reduced misonidazole on the radiolytic reduction of DNA bases will be reported in a future paper.
REFERENCES


