Hemin-mediated DNA Strand Scission*

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Hemin (ferric protoporphyrin IX chloride) has been shown to promote differentiation and influence gene expression in a variety of cell systems (1-3). For example, in the Friend erythroleukemia cell system, hemin induces globin mRNA accumulation for globin protein synthesis (4) and is required for the differentiation of Friend erythroleukemia cells (5-7). The mechanism by which hemin mediates these effects is unknown. Recently, however, it was found that hemin, when properly coordinated to a protein, mediates a rapid and selective degradation of that protein by the localized generation of reduced oxygen species.¹ Since it has been demonstrated that hemin plays a critical role in the differentiation of Friend erythroleukemia cells (5-7) and it has been reported that DNA nicking occurs during this phenomenon (8, 9), we were prompted to test whether or not this agent could damage DNA directly. In this paper evidence is presented to show that hemin in the presence of oxygen and the reducing agent, 2-mercaptoethanol, causes nicking and subsequent degradation of supercoiled plasmid DNA. The reaction is influenced markedly by certain cations; the transition metal cations, cobalt and zinc, facilitate the reaction whereas calcium and magnesium inhibit the degradation of DNA.

These findings raise the possibility that hemin mediates some of its effects on gene expression by nicking of the DNA with resultant rearrangement or modification of the chromatin structure at specific locates. It is postulated that this action of hemin might account for its influence in the differentiation of erythroid (5-7, 10) and other cell types (1, 2).

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Fig. 1. Effect of 2-mercaptoethanol concentration on the hemin-mediated degradation of pBR322 DNA. pBR322 DNA was incubated in an atmosphere of air for 30 min at 20 °C with 50 μM hemin and the indicated concentrations of 2-mercaptoethanol. The DNA products were analyzed on 1% neutral agarose gels as described under "Experimental Procedures." The gel was stained with ethidium bromide and the DNA patterns were visualized using ultraviolet light. (Lane 1, DNA + hemin; lane 2, DNA alone; lanes 3–7, DNA + hemin + 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM 2-mercaptoethanol, respectively; lane 8, DNA + 100 mM 2-mercaptoethanol; lane 9, linear DNA standard.)

Fig. 2. Effect of hemin concentration on the degradation of pBR322 DNA. Samples of DNA (0.2 μg) were incubated in an atmosphere of air for 20 min at 20 °C with 10 mM 2-mercaptoethanol and the indicated concentrations of hemin. The amount of DNA degradation was determined by analyzing the samples on 1% neutral agarose gels. The DNA was visualized by staining with ethidium bromide and examining the gel under ultraviolet light. (Lane 1, DNA alone; lanes 2–6, DNA + 2-mercaptoethanol + 10 μM, 50 μM, 100 μM, 500 μM, and 1.0 mM hemin, respectively; lane 7, linear DNA standard.)

Fig. 3. Dependency of the hemin-mediated degradation of pBR322 DNA on oxygen. Samples of DNA were incubated with 10 μM and 10 mM 2-mercaptoethanol under an atmosphere of oxygen, air, or nitrogen for the indicated times. All reactions were carried out in septum-stoppered test tubes. Operationally the samples were purged for 15 s with air, nitrogen, or oxygen immediately after the addition of the 2-mercaptoethanol. The DNA products remaining at the end of the reaction were then analyzed on a 1% alkaline agarose gel. (Lane 1, DNA alone; lanes 2–4, complete system incubated in an atmosphere for 5, 10, or 15 min, respectively; lanes 5–7, complete system incubated in a nitrogen atmosphere for 5, 10, or 15 min, respectively; lanes 8–10, complete system incubated in an oxygen atmosphere for 5, 10, or 15 min, respectively. Control samples (i.e. lanes 11–13) were incubated in an oxygen atmosphere for a period of 15 min. Lane 11, DNA + 2-mercaptoethanol; lane 12, DNA + hemin; lane 13, linear DNA standard.)

Introduction breaks in the supercoiled plasmid DNA at concentrations as low as 10 μM (Fig. 2, lane 3). As the hemin concentration is increased, DNA is converted more rapidly to the open circle and linear forms.

Reduced oxygen species have been shown to be involved in DNA degradation (11, 12). To determine whether oxygen plays a role in the hemin-mediated nicking of DNA, the reaction was carried out under either oxygen, air, or nitrogen atmospheres. As shown in Fig. 3, an atmosphere of nitrogen essentially precluded scission of DNA (lanes 5–7). In an atmosphere of 100% oxygen, the decrease of the supercoiled DNA (lanes 8–10), and the appearance of the linear form is more rapid than in air in which case some supercoiled DNA still remains after a 15-min incubation (lane 4). Hemin alone or 2-mercaptoethanol alone, even in an atmosphere of 100% oxygen, failed to cause measurable strand scission (lanes 12 and 11, respectively). These data clearly demonstrate the dependence of the hemin-mediated scission of DNA on the presence of all three reagents: oxygen, hemin, and 2-mercaptoethanol.

The hemin-mediated degradation of DNA is time-dependent and progressive (Fig. 4). In the first 10 min the DNA damage is largely reflected in a conversion of supercoiled DNA to the open circle form (compare lanes 3 and 4). By 20 min (lane 5) this conversion appears complete and further nicking leads to the appearance of linear forms and smaller DNA fragments that migrate as a smear in the gel. By 70 min of incubation (lane 9), only small DNA fragments remain.

In search of additional insights into the mechanism of the hemin interaction with DNA, various cations were tested for their effects on the hemin-mediated DNA degradation reaction. Fig. 5 and Table 1 show the results of adding monovalent and divalent cations to the DNA, hemin, and mercaptoethanol reaction mixture. NaCl and KCl at 50 and 100 μM levels had little or no effect on the amount of DNA degraded (lanes 5–7). Magnesium and calcium added at 50 μM (lanes 10 and 12) allowed the initial nicking to occur with the production of the nicked open circle and linear forms; however, the progressive degradation of these products to smaller fragments was largely inhibited. At higher concentrations of magnesium and calcium, all of the DNA could be recovered as the nicked open circle and linear forms (data not shown). The reason for the reaction stopping at this point is not clear from available data; however, the possibility exists that calcium or magnesium precipitate or otherwise condense the DNA structure.
of the reaction was increased, allowing for a significant reduction of DNA to oligonucleotides. Although the chemical mechanism of the reaction was not elucidated, the requirement for a reducing compound and molecular oxygen suggests that a reactive reduced oxygen species, such as a hydroxyl radical, or an iron oxygen complex may be generated. Earlier findings have already demonstrated that oxygen radicals cause DNA strand scission (13, 14).

In such a process it is projected that the ferric iron in hemin is first reduced to the ferrous form which can then react with molecular oxygen to produce the reactive species that attack the DNA. While not proven in these experiments, this oxidation-reduction process may well be cyclic. In this view, the mechanism of the hemin-mediated DNA strand scission is similar to the ferrous oxidase cycle proposed for iron chelated with bleomycin. In the latter reaction, ferrous-bleomycin can bind oxygen leading to the production of reduced oxygen species (15, 16).

The contrasting effects of the different divalent cations on the degradative process is especially interesting when viewed from the aspect of their influence on DNA structure. While all of the tested divalent cations can interact with the phosphate residues in the DNA backbone, they have different affinities for the bases themselves (17). For example, Mg

\( \text{II} \) does not readily interact with the bases in double-stranded DNA whereas the transition metals do. Thus, in contrast to Mg

\( \text{II} \), whose interaction is suggested to contribute to a more compact structure of the DNA, Co

\( \text{II} \) and related transition metal ions might open up the DNA structure for a more effective interaction or intercalation of hemin with this macromolecule. It is significant to this discussion that cations can alter DNA conformation to a more condensed form (18, 19) or cause the helix to change from the B form to a left-handed Z DNA structure (20, 21). While we are not aware of

### Table 1

| Addition | [Hemin] (µM) | Relative enhancement |
|----------|--------------|---------------------|
| None     | 50.0         | 1                   |
| CoCl₂    | 0.5          | 100 ×               |
| ZnCl₂    | 1.0          | 50 ×                |
| NiCl₂    | 1.0          | 50 ×                |
| MnCl₂    | 5.0          | 10 ×                |
| CuSO₄    | 10.0         | 5 ×                 |

**FIG. 4.** The effect of incubation time on the scission of pBR322 DNA in the presence of hemin and oxygen. Samples, in an atmosphere of air, were incubated for 15 min as described in the legend to Fig. 5 with a 50 µM level of the indicated cation and hemin concentrations ranging from 0.5 to 50 µM. The DNA products were analyzed by electrophoresis in 1% neutral agarose gels. The level of hemin required to produce a destruction of DNA comparable to that obtained with 50 µM hemin and 10 mM 2-mercaptoethanol in the absence of cation additions was ascertained by visual comparison of the ethidium bromide-stained electrophoresis.

**FIG. 5.** Effect of cations on the hemin-mediated nicking of DNA. Reaction mixtures containing pBR322 DNA, 50 µM hemin, and 10 mM 2-mercaptoethanol were incubated for 15 min in an atmosphere of air at 20 °C with the indicated cation additions. Control samples (i.e., lanes 1–3) containing hemin or 2-mercaptoethanol alone were incubated for 80 min; lane 1, DNA + hemin; lane 2, DNA + 2-mercaptoethanol; lane 3, DNA alone. The complete system was incubated for 10, 20, 30, 40, 50, 60, 70, and 80 min in lanes 4–11, respectively. Lane 12, linear DNA standard.

**DISCUSSION**

This study demonstrates that hemin in the presence of 2-mercaptoethanol and oxygen can introduce nicks into DNA. The reaction is rapid and leads to an extensive degradation of DNA to oligonucleotides. While we are not aware of...
any data showing that hemin can intercalate into DNA, this phenomenon has been demonstrated for certain synthetic porphyrins (22, 23). In this connection, it is important to note that while this paper was in preparation, Fiel et al. (24) published data showing that synthetic metalloporphyrins with the capacity to intercalate into DNA can cause DNA strand scission; however, they did not report the dependency of the process on the presence of molecular oxygen.

Chelated iron complexes, such as the antitumor antibiotics bleomycin and doxorubicin have been shown previously to nick DNA $\textit{in vitro}$ (11, 12) and $\textit{in vivo}$ (25). This study with hemin, however, appears to be the first example of a physiological iron chelator that can nick DNA. Hemin possesses two charged carboxylic groups, and these could also be involved in the selective actions of the different divalent cations. However, it seems unlikely that a simple neutralization of the negatively charged carboxylic acid groups of the hemin and phosphates of the DNA underlies the cation effects since $\text{Na}^+$ and $\text{K}^+$ had no effect on the degradation process.

With respect to the possible relevance of the DNA nicking action by hemin in the differentiation of Friend erythroleukemia cells and immature red blood cells, it should be noted that heme accumulates in the nucleus as these cells differentiate (5, 6, 28). In Friend cells, nicks occur in the DNA during differentiation (8, 9) and the number of topological turns in the DNA appears to decrease (29). As the result of these correlations we are led to propose that hemin may implement the changes in gene expression associated with erythroid differentiation by the generation of localized reactive oxygen species with localized damaging effects on the chromatin structure.

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