Nitric Oxide-forming Reaction between the Iron-N-Methyl-p-glucamine Dithiocarbamate Complex and Nitrite*

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The objective of this study was to elucidate the origin of the nitric oxide-forming reactions from nitrite in the presence of the iron-N-methyl-p-glucamine dithiocarbamate complex ([MGD]Fe²⁺). The ([MGD]Fe²⁺) complex is commonly used in electron paramagnetic resonance (EPR) spectroscopic detection of NO both in vivo and in vitro. Although it is widely believed that only NO can react with ([MGD]Fe²⁺) complex to form the ([MGD]Fe²⁺-NO) complex, a recent article reported that the ([MGD]Fe²⁺) complex can react not only with NO, but also with nitrite to produce the characteristic triplet EPR signal of ([MGD]Fe²⁺-NO) (Hiramoto, K., Tomyama, S., and Kikugawa, K. (1997) Free Radical Res. 27, 505–509). However, no detailed reaction mechanisms were given. Alternatively, nitrite is considered to be a spontaneous NO donor, especially at acidic pH values (Samouilov, A., et al (1994) Arch Biochem. Biophys. 357, 1–7). However, its production of nitric oxide at physiological pH is unclear. In this report, we demonstrate that the ([MGD]Fe²⁺) complex and nitrite reacted to form NO as follows: 1) ([MGD]Fe²⁺-NO) complex was produced at pH 7.4; 2) concomitantly, the ([MGD]Fe²⁺) complex, which is the oxidized form of ([MGD]Fe²⁺), was formed; 3) the rate of formation of the ([MGD]Fe²⁺-NO) complex was a function of the concentration of [Fe²⁺]²⁺, [MGD], [H⁺] and [nitrite].

Nitric oxide (NO)¹ has many important physiological roles which include that of a cytotoxic mediator of the immune system, regulation of vasomotor tone in the cardiovascular system, and as a neurotransmitter in the central nervous system (1, 2). NO is thought to be identical to the endothelium-derived relaxing factor (1), and its insufficiency is believed to contribute to the pathogenesis of vascular disease such as atherosclerosis, hypertension, and myocardial ischemia. As a result, much attention has been focused on the potential therapeutic ability of nitrovasodilators (e.g. nitroglycerin and nitroprusside) (3) and the anti-cancer drug hydroxyurea (4) to release NO.

In order to understand the mechanisms by which NO, a diffusible free radical with a short lifetime, mediates various biological processes, accurate methods for its measurement are required. Several methods for the quantitation of NO such as chemiluminescence (1), methemoglobin formation (5), and electron paramagnetic resonance (EPR) spectroscopy of nitrosyl-metal complexes (6) have been developed (7). Production of NO can also be indirectly assessed by measuring the nitric oxide oxidation product, nitrite, with the Griess reaction.

EPR spectroscopy is the only specific general technique available for the detection and measurement of radical production, but has severe quantum mechanical limitations for diatomic molecules. EPR methods have been developed which stabilize NO as a polyatomic adduct using endogenous and exogenous spin traps (8–14). Conventional nitro- and nitroso-based spin traps are not capable of trapping NO as stable radical adducts, and nitromethane is an effective spin trap only at very alkaline pH values (15). The diethylthiocarbamate (DETC) ferrous complex is a commonly used spin trap for NO (16), and the resultant (DETC)Fe²⁺-NO complex has a characteristic triplet EPR signal. Although the (DETC)Fe²⁺ complex has been widely used to trap NO from cells and tissues (12, 13), quantitation of NO using (DETC)Fe²⁺ requires complicated procedures to overcome its low solubility in water. Recently, N-methyl-d-glucamine dithiocarbamate (MGD) has been used to overcome the poor solubility of (DETC)Fe²⁺ (14). The (MGD)Fe²⁺ complex (Fig. 1) is water-soluble (17) and forms a characteristic triplet EPR spectrum after trapping NO. NO is produced by the nitric oxide synthase-catalyzed oxidation of L-arginine. Ultimately, NO is oxidized to nitrite and nitrate. Although the nitrite is generally believed to be a fairly stable product of NO oxidation, Samouilov et al. (18) demonstrated an enzyme-independent pathway of NO generation from nitrite at acidic conditions (pH ≤ 7) by EPR and chemiluminescence techniques. They also indicated that the conversion rate of nitrite to NO was too slow to be directly measured at physiological pH (18). However, Hiramoto et al. (19) reported that the coexistence of Fe²⁺-dithiocarbamate complexes and nitrite produced a triplet EPR spectrum which corresponded to that of the dithiocarbamate-Fe²⁺-NO complex even at physiological pH.

Although it is widely believed that Fe²⁺-dithiocarbamate complexes specifically react with NO to form dithiocarbamate-Fe²⁺-NO complex, its production from nitrite by (MGD)Fe²⁺ may lead to misinterpretation regarding the actual presence of NO. Nitrite in biological systems originates as an oxidation product of endogenous NO, as a food component (20), and as the reduction product of nitrate by facultative anaerobic bacteria (21–23). Therefore, it is important to investigate any reaction between nitrite and the Fe²⁺-dithiocarbamate complex at physiological pH. In this study, EPR spectroscopy was utilized to investigate whether (MGD)Fe²⁺ complex produces (MGD)Fe²⁺-Fe²⁺-NO in the presence of nitrite under anaerobic conditions.

EXPERIMENTAL PROCEDURES

Materials—MGD was synthesized, according to a previous report (17), from N-methyl-D-(-)glucamine (Sigma) and carbon disulfide (Sigma). The purity and molecular weight of synthesized MGD were verified with commercially available MGD (OMRF, Oklahoma, OK) by high
**NO-forming Reaction from (MGD)₂Fe²⁺ and NO₂**

**FIG. 1.** Chemical structure of water soluble dithiocarbamate-iron complexes and their NO radical adducts. a, MGD. b, N-(dithiocarboxy)sarcosine. c, 2-hydroxyethyl dithiocarbamate. d, proline dithiocarbamate.

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Performance liquid chromatography and mass spectrometry, respectively. Sodium nitrite and FeCl₃·6H₂O were purchased from Sigma. High purity (99.999%) FeSO₄·7H₂O was obtained from Alfa (Ward Hill, MA). Gaseous NO was commercially obtained (National Welders Supply Co., Inc., Raleigh, NC). NO was purified from higher oxides such as NO₂ and N₂O₃ by passing NO through a trap containing 1 M KOH solution. NO-saturated aqueous solution was prepared by bubbling NO gas for 10 min through water which had been previously deoxygenated by bubbling with purified argon for 30 min (24). All other chemicals were analytical grade.

**Preparation of the Iron-MGD Complex—**Stock solutions of FeSO₄·7H₂O (0.1 M), FeCl₃·6H₂O (0.1 M), MGD (0.5 M), and NaNO₂ (0.1 M) were prepared immediately before measurement and were used within a few hours. All solutions were prepared in argon-purged distilled water (24). The iron-MGD complex was prepared by adding the appropriate amount of iron and MGD from stock solution into HEPES solution (1.9 mM NO) deoxygenated by bubbling with purified argon for 30 min.

**EPR Measurements—**All EPR measurements were carried out at room temperature (25 °C) with a 17-mm quartz flat cell. For anaerobic measurements, samples were directly transferred to the argon-purged flat cell which was, in turn, placed in the cavity of the EPR spectrometer. All solutions were mixed prior to EPR measurement to provide the final concentrations indicated in the figure legends, and the reactions were initiated by the addition of iron. A Bruker 106 ESR spectrometer (Bruker, Co., Billerica, MA) with a TM₁₁₀ cavity was employed to collect all EPR spectra. The typical instrumental conditions were: microwave, 20 mW; modulation amplitude, 2.0 G; modulation frequency, 50 kHz; sweep time, 168 s; scan range, 100 Gauss; and 100 gain. Spectra were stored on an IBM/PC computer for analysis. Quantitation of NO was carried out by double integration of the EPR spectrum. The standard for quantitation of NO was prepared utilizing a NO-saturated aqueous solution (1.9 mM NO). A 20-gauss calibration chart was included with each EPR spectrum, and all EPR spectra were calibrated with a NO-saturated aqueous solution (1.9 mM NO).

**VIS Spectrophotometry—**Nitrite-induced changes of absorption spectra of iron-MGD complexes were monitored by measuring the absorbance between 450 and 700 nm with an SLM-AMINCO DW-2000 spectrophotometer at room temperature.

**NO Electrode Studies—**NO was measured by a commercially available NO electrode (ISO-NO, World Precision Instruments, Inc., Sarasota, FL) with an associated nitric oxide probe (ISO-NOP). Calibration of the electrode was performed with saturated aqueous NO solution just prior to the experiment. The undiluted, saturated aqueous NO solution (1.9 mM) was prepared by bubbling the purified NO gas into the deoxygenated distilled water at room temperature.

**RESULTS**

**NO Electrode Studies of Spontaneous NO Formation from Nitrite—**Fig. 2 shows the production of NO from nitrite under anaerobic conditions. The production of NO was observed when nitrite was introduced into acetic acid buffer (0.1 M, pH 4.0–5.5) as reported (25), but was strongly dependent on the pH of the solution. At pH 4.0, 0.8 μM NO was produced from 1 mM nitrite within 3 min (Fig. 2a), while barely detectable NO was found at pH 5.5 (Fig. 2f). The initial rate of the NO production was calculated to be 1.3 × 10⁻⁷ m/s at pH 4.0, but no NO was detected utilizing an NO electrode at pH 7.4 (data not shown).

**EPR Measurement of NO Formation from Nitrite—**When...
nitrite was added to a solution containing (MGD)$_2$Fe$^{2+}$-NO complex in 0.5 M HEPES buffer (pH 7.4), a triplet signal characteristic of the (MGD)$_2$Fe$^{2+}$-NO complex with a nitrogen hyperfine coupling constant of 12.5 Gauss (14) was detected (Fig. 3). The intensity of the (MGD)$_2$Fe$^{2+}$-NO radical adduct steadily increased with incubation time under anaerobic conditions. The rate for the production of (MGD)$_2$Fe$^{2+}$-NO from 1 mM nitrite under these conditions (Fig. 3) was calculated to be 4.1 × 10$^{-8}$ m/s. With lower concentrations of nitrite and (MGD)$_2$Fe$^{2+}$ complex, the production of (MGD)$_2$Fe$^{2+}$-NO complex was still detectable with a rate of 1.0 × 10$^{-10}$ m/s (Fig. 3B). To elucidate whether these apparent differences of NO production as determined with the NO-electrode and EPR were dependent on the (MGD)$_2$Fe$^{2+}$ complex or not, we studied in detail the concentration dependence of the Fe$^{2+}$/MGD, H$^+$, and nitrite for the production of NO as measured by EPR.

As shown in Figs. 4, 5, and 6, the production of (MGD)$_2$Fe$^{2+}$-NO complex from nitrite increased with incubation time (Figs. 4A, 5A, and 6A). The rate of NO production was not simply linear with the iron concentration, but increased with the square of the iron concentration (Fig. 7B), i.e. second order. The third-order rate constant for the formation of (MGD)$_2$Fe$^{2+}$-NO from nitrite was calculated from Fig. 4 as 1.46 ± 0.08 m$^{-1}$ s$^{-1}$ (Table I). When authentic NO (0.1 mM) was introduced into a solution containing (MGD)$_2$Fe$^{2+}$ complex under the same experimental conditions as in Figs. 5–7, no significant change in the concentration of the (MGD)$_2$Fe$^{2+}$-NO complex was observed as a function of MGD, Fe$^{3+}$, or hydrogen ion concentration (data not shown).

Absorption Spectrum of Iron-MGD Complex after Addition of Nitrite—If nitrite is being reduced to (MGD)$_2$Fe$^{2+}$-NO at physiological pH, then (MGD)$_2$Fe$^{2+}$ will be converted to (MGD)$_2$Fe$^{3+}$-NO and (MGD)$_2$Fe$^{3+}$ in a 1:1 ratio (see below). Fig. 8A shows the time course of the absorption spectra of the (MGD)$_2$Fe$^{2+}$ complex in the presence or absence of nitrite in 20 mM HEPES buffer (pH 7.4) under anaerobic conditions. The optical absorption increased with time in the presence of nitrite (Fig. 8A, d-i, every 10 min), whereas (MGD)$_2$Fe$^{3+}$ was stable in the absence of nitrite (Fig. 8A, b, 10 min; and c, 60 min of incubation). The absorption maxima at 515 nm is characteristic of the six-coordinate Fe$^{3+}$-dithiocarbamate complex of MGD (26, 27). Fig. 8B, f and h, are the absorption spectra of 0.1 mM (MGD)$_2$Fe$^{2+}$-NO and 0.1 mM (MGD)$_2$Fe$^{3+}$, respectively. If the (MGD)$_2$Fe$^{2+}$ complex has completely been changed to (MGD)$_2$Fe$^{3+}$-NO and (MGD)$_2$Fe$^{3+}$ in a 1:1 ratio in the presence...
of nitrite, the absorption spectrum will be the average of these spectra, as shown in Fig. 8B (i). This absorption spectrum agreed with that of (MGD)_2Fe^{2+} complex in the presence of nitrite (Fig. 8A, i). These data indicate that the (MGD)_2Fe^{2+} complex was converted to the (MGD)_2Fe^{2+}-NO and (MGD)_2Fe^{3+} complex by nitrite at pH 7.4. The third-order rate of this reaction was calculated to be 4.84 ± 0.13 m s\(^{-1}\) (Table I).

**DISCUSSION**

Iron dithiocarbamate complexes such as DETC (12), MGD (14), proline dithiocarbamate (28), and N-(dithiocarboxy)sarcosine (29) have been used as spin-trapping agents for NO. Co-existence of these iron complexes and NO leads to an intense three-line EPR signal at room temperature. The (DETC)_2Fe^{2+}-NO complex is very hydrophobic, which is a limitation in its use in vivo (13, 30–54). Recently, the application of hydrophilic iron dithiocarbamate complexes such as MGD-Fe, N-(dithiocarboxy)sarcosine-Fe, and proline dithiocarbamate-Fe for NO detection in vivo has been investigated by several researchers (14, 29, 45, 48–50, 55–66).

Nitrite in biological systems is known to be derived mainly from diet and as the oxidation product of NO, which is synthesized by nitric oxide synthases (NOS) from L-arginine. In 1997, Hiramoto et al. (19) reported the appearance of the EPR spectrum of the dithiocarbamate-Fe^{2+}-NO radical adduct in the presence of nitrite after prolonged incubation. If this were true, it would require a careful interpretation of biological data because the formation of the dithiocarbamate-Fe^{2+}-NO complex would not always indicate the systemic production of NO, but could also detect systemic nitrite.

However, the mechanisms for the formation of dithiocarbamate-iron-NO complex from dithiocarbamate-iron complex and nitrite have not been thoroughly investigated. In the present investigation, we explore the reaction mechanisms for the formation of the (MGD)_2Fe^{2+}-NO complex from nitrite and the water soluble dithiocarbamate-iron complex, (MGD)_2Fe^{3+}. As determined with the NO-electrode (Fig. 2), nitrite can produce NO at acidic pH because acidified nitrite can undergo disproportionation to produce NO (67),

**TABLE I**

| Fe^{2+} complex | Reactant  | Rate constants | Ref. |
|-----------------|-----------|----------------|------|
| (MGD)_2Fe^{2+}  | NO\(_5\)  | 4.84 ± 0.13 (m s\(^{-1}\)) | This work\(^a\) |
| (MGD)_2Fe^{2+}  | NO\(_6\)  | 1.46 ± 0.08 (m s\(^{-1}\)) | This work\(^b\) |
| (PDTC)\(_2\)Fe^{2+} | O\(_2\)  | 5 × 10\(^{-5}\) (M\(^{-1}\) s\(^{-1}\)) | 27 |
| (MGD)\(_2\)Fe^{3+} | NO\(_8\)  | 1.21 ± 0.53 (M\(^{-1}\) s\(^{-1}\)) | 64 |
| (DTCS)\(_2\)Fe^{3+} | NO\(_9\)  | 1.71 ± 0.30 (M\(^{-1}\) s\(^{-1}\)) | 64 |
| (PDTC)\(_2\)Fe^{3+} | NO\(_{10}\) | 1.1 ± 0.3 (M\(^{-1}\) s\(^{-1}\)) | 28 |

\(^a\) (MGD) = 2.50 × 10\(^{-3}\) m and pH 7.4 from formation of (MGD)_2Fe^{2+}-NO and (MGD)_2Fe^{3+} measured optically.

\(^b\) MGD = 2.50 × 10\(^{-3}\) m and pH 7.4 from formation of (MGD)_2Fe^{2+}-NO measured by EPR.
NO-forming Reaction from (MGD)$_2$Fe$^{2+}$ and NO$_2^-$

3NO$_2^-$ + 2H$^+$ $\rightarrow$ 2NO + NO$_3^-$ + H$_2$O (Eq. 1)

The rate of NO production from 1 mM nitrite at pH 4.0 was calculated to be $1.3 \times 10^{-7}$ m/s, which was in good agreement with the literature value ($1.7 \times 10^{-7}$ m/s)/(18). Although no NO was detected with an NO-electrode at neutral pH, the triplet EPR spectrum of the (MGD)$_2$Fe$^{2+}$-NO complex was generated with time (Fig. 3). The rate for the production of NO from nitrite at pH 7.4 was calculated to be $4.1 \times 10^{-8}$ m/s utilizing EPR quantitation of the rate of formation of (MGD)$_2$Fe$^{2+}$-NO as a measure of the rate of NO formation. However, if all NO were generated by spontaneous decomposition of nitrite via Equation 1, the rate of NO production at pH 7.4 would be $6.9 \times 10^{-11}$ m/s (18). This value was 1000 times less than the observed rate of NO formation. This difference clearly indicated that another NO generation pathway must exist at neutral pH in the presence of the (MGD)$_2$Fe$^{2+}$ complex and nitrite.

Next, we investigated the individual components of the (MGD)$_2$Fe$^{2+}$ complex for their effect on the generation of NO from nitrite. As shown in Figs. 4, 5, and 6, the rate of NO production was first-order in the concentration of nitrite, MGD, and hydrogen ion, respectively. However, it was second-order in iron concentration (Fig. 7).

When Fe$^{2+}$ is added to MGD, the (MGD)$_2$Fe$^{2+}$ complex is formed under anaerobic conditions.

$$\text{Fe}^{2+} + 2\text{MGD} \rightarrow (\text{MGD})_2\text{Fe}^{2+} \quad (\text{Eq. 2})$$

When nitrite is introduced into the solution containing the (MGD)$_2$Fe$^{2+}$ complex at neutral pH, (MGD)$_2$Fe$^{3+}$, which is an oxidized form of (MGD)$_2$Fe$^{2+}$, appears (Fig. 8). We propose that (MGD)$_2$Fe$^{2+}$ reacts with nitrite, possibly as a transient Fe$^{3+}$-nitric oxide complex (68), and then oxidizes (MGD)$_2$Fe$^{2+}$ to (MGD)$_2$Fe$^{3+}$.

$$(\text{MGD})_2\text{Fe}^{3+} + \text{NO}_2^- \rightarrow (\text{MGD})_2\text{Fe}^{3+}\cdot\text{NO} + \text{OH}^- \quad (\text{Eq. 3})$$

The ferric-NO complex, upon reduction by excess (MGD)$_2$Fe$^{2+}$, will form the (MGD)$_2$Fe$^{2+}$-NO complex,

$$(\text{MGD})_2\text{Fe}^{2+}\cdot\text{NO} + (\text{MGD})_2\text{Fe}^{2+} \rightarrow (\text{MGD})_2\text{Fe}^{2+}\cdot\text{NO} + (\text{MGD})_2\text{Fe}^{3+} \quad (\text{Eq. 4})$$

If the reduction of (MGD)$_2$Fe$^{3+}$ is the rate-limiting step, then the net reaction between nitrite and (MGD)$_2$Fe$^{2+}$ complex can be expressed by adding Equations 3 and 4,

$$2(\text{MGD})_2\text{Fe}^{3+} + \text{NO}_2^- + \text{MGD} + \text{H}^+ \rightarrow (\text{MGD})_2\text{Fe}^{3+} + (\text{MGD})_2\text{Fe}^{2+}\cdot\text{NO} + \text{OH}^- \quad (\text{Eq. 5})$$

Then, the rate of (MGD)$_2$Fe$^{2+}$-NO complex formation ($v$) is expressed as,

$$v = k \cdot [(\text{MGD})_2\text{Fe}^{3+}]\cdot[\text{NO}_2^-]\cdot[\text{MGD}]\cdot[\text{H}^+] \quad (\text{Eq. 6})$$

The (MGD)$_2$Fe$^{2+}$ complex concentration is proportional to iron (Equation 2) because MGD is present in excess (more than 5 times the [Fe$^{2+}$]). Accordingly, the rate law of Equation 6 accounts for the results of Figs. 4–7.

These results demonstrate that: 1) the reaction of nitrite and (MGD)$_2$Fe$^{2+}$ complex can produce (MGD)$_2$Fe$^{2+}$-NO complex via the reduction of nitrite by the (MGD)$_2$Fe$^{2+}$ complex; and 2) the rate of formation of (MGD)$_2$Fe$^{2+}$-NO complex is a function of [NO$_2^-$], [MGD], [H$^+$], and the square of [Fe$^{2+}$]. On the basis of these results, we propose a mechanism for (MGD)$_2$Fe$^{2+}$-NO complex production from nitrite under anaerobic conditions (Scheme 1).

The plasma nitrite concentration was reported to be as high...
as 100 μm by the Griess method after lipopolysaccharide administration in rats (69). The concentration of (MGD)Fe²⁺ complex in the blood of an animal during the quantitation of NO is initially estimated to be 0.01–5.71 μM (14, 49, 50, 55, 61–63). As shown in Fig. 3B, the (MGD)Fe²⁺-NO EPR spectra increased with time in the presence of 100 μm nitrite and 0.5 mM (MGD)Fe²⁺ complex. This data clearly demonstrates that the observed (MGD)Fe²⁺-NO EPR spectrum does not necessarily indicate genuine NO production, but can also detect nitrite when the (MGD)Fe²⁺ complex is introduced into an animal treated with the endotoxin depending on the local nitrite and Fe²⁺ (MGD) concentrations.

We have previously demonstrated that under aerobic conditions, (MGD)Fe²⁺ rapidly air oxidizes to form reactive oxygen species capable of oxidizing some nitrogen-containing compounds to nitric oxide (Table I) (27). We have now demonstrated that under anaerobic conditions, (MGD)Fe²⁺ reduces nitrite to form (MGD)Fe²⁺-NO at physiological pH values. In vitro, these two reactions will compete with each other. Both of these reactions will also compete with the trapping of authentic NO by the various reaction rates of (MGD)Fe²⁺. Which of these reactions will dominate in vivo will depend, in large measure, on the relative concentrations of molecular oxygen, nitrite, and NO ([O₂] ≳ [NO₂⁻] ≳ [NO]) as well as the various reaction rates of (MGD)Fe²⁺ (Table I).

In summary, the development of the (MGD)Fe²⁺-NO complex from biological samples containing nitrite and NO is not necessarily the presence of genuine NO. Special attention to this fact is needed to correctly interpret results obtained by the use of the (MGD)Fe²⁺ complex for the detection of NO from nitrite-containing samples. If all nitrite originates from nitric oxide, then the biological interpretations of nitrite-dependent (MGD)Fe²⁺-NO complex formation will not change except for the time course, which may be artificially extended. On the other hand, if diet is the source of nitrite, then even more serious misinterpretations of biological significance may result. In any case, the formation of the (MGD)Fe²⁺-NO complex cannot be critically taken as evidence for the presence of NO in biological systems.

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