Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9138–9141, August 1996
Medical Sciences

Iron chelates bind nitric oxide and decrease mortality in an experimental model of septic shock

WIESLAW M. KAZMIERSKI*†‡, GERALD WOLBERG§, JOAN G. WILSON§, SHEILA R. SMITH¶, DARRYL S. WILLIAMS¶, H. HOLDEN THORP†¶, AND LUIS MOLINA†‖

Divisions of *Organic Chemistry and §Experimental Therapy, Burroughs Wellcome, Research Triangle Park, NC 27709; ¶Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599-3290; and ‖Molichem Medicines, 2135 North Lake Shore Drive, Chapel Hill, NC 27514

Communicated by Thomas J. Meyer, University of North Carolina, Chapel Hill, NC, April 19, 1996 (received for review December 13, 1995)

ABSTRACT **The hydroxamic acid siderophore ferrioxamine B [$Fe^{III}(HDFB)^+$] and the iron complex of diethylenetriaminepentaacetic acid [$Fe^{III}(DTPA)^{2-}$] protected mice against death by septic shock induced by *Corynebacterium parvum* + lipopolysaccharide. Although $Fe^{III}(DTPA)^{2-}$ was somewhat more effective than $Fe^{III}(HDFB)^+$, the iron-free ligand H_4DFB^+ was significantly more effective than DTPA. The hydroxamic acid chelator has a much higher iron affinity than the amine carboxylate, allowing for more efficient formation of the $Fe^{III}(HDFB)^+$ complex upon administration of the iron-free ligand. Electrochemical studies show that $Fe^{III}(DTPA)^{2-}$ binds NO stoichiometrically upon reduction to iron(II) at biologically relevant potentials to form a stable NO adduct. In contrast, $Fe^{III}(HDFB)^+$ is a stable and efficient electrocatalyst for the reduction of NO to N_2O at biologically relevant potentials. These results suggest that the mechanism of protection against death by septic shock involves NO scavenging and that particularly effective drugs that operate a low dosages may be designed based on the principle of redox catalysis. These complexes constitute a new family of drugs that rely on the special ability of transition metals to activate small molecules. In addition, the wealth of information available on siderophore chemistry and biology provides an intellectual platform for further development.**

Nitric oxide (NO), a short-lived potent vasodilator, was first described as the endothelium-derived relaxation factor (EDRF) (1–3). The formation of NO from the guanidino nitrogen group of L-arginine is catalyzed by a group of enzymes termed constitutive (cNOs) and inducible (iNOs) NO synthases (3, 4). The inducible form is not present constitutively in mammalian cells but is induced by proinflammatory stimuli such as bacterial lipopolysaccharide (LPS), *Corynebacterium parvum*, and the cytokines tumor necrosis factor-α, interleukin-1, or interferon-γ, individually or in combination (5). Excess production of NO is reported to be associated with the development of hypotension associated with endotoxemia and sepsis (6–8).

Inhibition of iNOs is a potential therapeutic approach to the treatment of septic shock (6–9). However, mechanisms to protect against the deleterious effects of NO overproduction constitute an alternative approach. For instance, free hemoglobin can scavenge NO and inhibit its biological effects (10). In LPS-treated mice, formation of a paramagnetic mononitrosyl iron complex with diethyldithiocarbamate (NO·Fe·DETC) was detected and characterized by electron paramagnetic resonance (11). The NO formed in this animal model was trapped with endogenous Fe^{2+} complexed with a considerable amount (500 mg/kg) of DETC that was administered to the animals during the course of the experiment (11). Mortality was not addressed in this study. On the other hand, administration of vitamin B12A to mice prevented and reversed endotoxin-mediated hypotension and decreased mortality, possibly through binding of the vitamin with excess NO (12). Inhibition of several actions of NO, including protection of rats against endotoxin, was demonstrated with the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazolineoxyl-1-oxyl 3-oxide (13). We and others have characterized the *in vivo* models of induction of iNOs in mice and rats injected with *C. parvum* or *C. parvum* + LPS (14–18). We postulated that iron complexes could act as general NO scavengers and provide protection against septic shock. We screened a library of metal chelators and chelates and found that [Fe(III)(H_2DTPA)] and $[Fe(III)(HDFB)]^+$ (Fig. 1, **4** and **2**, respectively) offered the highest mortality decrease in an experimental model of septic shock (19). We determined that the Fe(II) form of both complexes can bind NO, which we believe is related to their biological function.

MATERIALS AND METHODS

Materials. The ferrioxamine B complex **2** was prepared by reacting deferoxamine mesylate (Sigma) and ammonium iron(III) sulfate dodecahydrate (Aldrich) in 1:10 molar ratio in water. The resulting complex **2** was desalted on C_{18} reversed phase high performance liquid chromatography (Vydac 218TP1022) using 0–20% B gradient versus A (B: 0.1% TFA in acetonitrile; A: 0.1% TFA in water) over 30 min. The fraction was collected and lyophilized. Correct MS (FAB) and C, H, N, Fe, F data were obtained for **2** containing two molecules of TFA. Iron diethylenetriamminepentaacetic acid (DTPA), disodium salt dihydrate, **3** was used as packaged by Aldrich.

***C. parvum* + LPS Shock.** Male CD-1 mice, 18–22 g (Charles River Breeding Laboratories), are injected i.v. with 100 mg of killed *C. parvum* (Coparvax, Burroughs Wellcome). Ten days later, the mice are injected i.v. with 20 mg of *Escherichia coli* 026:B6 LPS (Difco) in the presence of the analgesic butorphanol (150 mg per mouse). Compounds are dissolved in saline for i.v. dosing 2 h before and at the same time as or 30 min after the LPS. Control (non-drug-treated) animals go into shock within the first 90 min following LPS administration, and approximately 90% die within 5 h. Mice are monitored for the first 7 h and at 24 and 48 h for survival. All animal use procedures were approved by an Institutional Animal Care and Use Committee and were designed to conform to the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EDRF, endothelium-derived relaxation factor; cNOs, constitutive NO synthase; iNOs, inducible NO synthase; LPS, lipopolysaccharide; DETC, diethyldithiocarbamate; DTPA, diethylenetriaminepentaacetic acid; DTPA iron complex, [Fe(III)(H_2DTPA)] (**4**); free DTPA ligand, H_2DTPA^{3-} (**3**); ferrioxamine B, $[Fe(III)(HDFB)]^+$ (**2**); desferal, H_4DFB^+ (**1**), TFA, trifluoroacetic acid.

†W.M.K., H.H.T., and L.M. contributed equally to this work.
‡To whom reprint requests should be addressed at: Glaxo Wellcome, Inc., Department of Medicinal Chemistry, Five Moore Drive, Research Triangle Park, NC 27709.

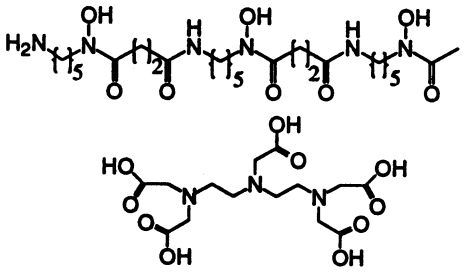

FIG. 1. Structures of desferal **1**, DTPA **3**, and their iron(III) complexes **2** and **4**, respectively.

Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86-23, 1985).

Electrochemistry. Phosphate buffers were prepared at 0.5 M concentrations using Na_2HPO_4 (Fisher) and KH_2PO_4 (EM Science) dissolved in Milli-Q water. Solutions were degassed using purified N_2 or argon gas. NO (Matheson, 99.0% pure) and $NaNO_2$ (EM Science) were used without further purification. Electrochemistry was performed using a Princeton Applied Research model 273A potentiostat and Princeton Applied Research model 270/250 analysis software. Cyclic voltammetry was conducted using a conventional three-compartment cell with ports for degassing solutions/addition of substrate in which a Bioanalytic Systems glassy carbon electrode was used as the working electrode. The auxiliary compartment contained a platinum wire auxiliary electrode separated from the main compartment by a medium glass frit. Cell potentials were measured using a sodium chloride-saturated calomel electrode as a reference. Controled-potential coulometry was performed using a vitreous carbon working electrode with a Pt gauze auxiliary. Gas analysis for NO and N_2O was performed using a Hewlett Packard model 5890A gas chromatograph with a thermal conductivity detector on a Hewlett–Packard 6-ft 5-Å 60/80 mesh molecular sieve column with helium carrier gas.

RESULTS AND DISCUSSION

Protective Effects of Iron Chelators in Experimental Septic Shock. Iron complexes are capable of forming relatively stable NO adducts (20–23). In a search for alternative approaches to septic shock therapy, we postulated that metal complexes, and in particular iron chelators, could act as "molecular sponges," mopping up the excess NO produced during septic shock. To identify lead compounds that would protect against septic shock, we screened a library of metal chelators in the *C. parvum* + LPS murine model of septic shock (19). Injection of mice with killed *C. parvum* followed 10 days later by LPS resulted in 100% mortality within 48 h (Table 1). Approximately 50% of mice were alive 2.5–3 h after LPS injection (a reflection of how acute the response to LPS is) and 90–95% mortality was reached within 5 h (Table 1). The compounds were evaluated at 0.1, 1.0, 10, and 40 mg/kg. We found that maximum protection against septic shock was offered by both **2** and **4** (Fig. 1) when administered at 10 mg/kg (19), Table 1.

Survival was greatly enhanced by the administration of **4** or **2** either 2 h before and at the time of or 30 min after LPS. In contrast, the Fe^{3+}-free ligands of these compounds, **3** and **1**, were less protective when administered before and at the time of LPS and virtually ineffective when administered after LPS. The clear advantage of **4** over **2** when administered after LPS was observed over a large number of experiments [76% survival with **4** (n = 102 mice) and 38% survival with **2** (n = 64 mice)].

The free ligand **1** was more protective than **3**. This difference probably results from the greater facility with which **1** sequesters iron (24–27). As a result, the difference in protective properties of **1** relative to **2** at 30 min were much less dramatic than those for **3** and **4**, respectively. Compound **3** has a lower affinity and selectivity for iron than **1**; when administered at 30 min, **3** lacked any protective properties. On the other hand its administration at −2.0 and 0 h provided enough time for **3** to sequester iron and to be effective (Table 1).

Table 1. Treatment of *C. parvum* + LPS-induced septic shock in mice

Compound*	Dose,† mg/kg	Dose schedule, h	% alive‡ 5 h	% alive‡ 48 h
1	10	−2.0 and 0	80	40
		+0.5	60	15
2	10	−2.0 and 0	100	65
		+0.5	60	50
3	10	−2.0 and 0	95	45
		+0.5	5	0
4	10	−2.0 and 0	95	70
		+0.5	85	75
Saline		−2.0 and 0	10	0
		+0.5	5	0

*Compounds identified in Fig. 1
†Compounds dosed iv in saline 2 h before and at the time of LPS or 0.5 h after LPS.
‡n = 20.

Alternatively, differences in biological half-lives, tissue distribution, and/or cell permeability may explain the different protective actions of **4** and **2** in septic mice. For instance, **1** penetrates into hepatocytes and comes out as **2** (28, 29). Compound **4**, on the other hand, has little tendency to cross the cell membrane (30). Also, **2** has a half-life of several hours (31) while the half-life of **4** is 1 h (unpublished data). Our results showed that **4**, which has a short half-life and low cell permeability provided the best protection against the deleterious effects of septic shock in mice.

It should be noted that iron chelators can interact with free radicals produced *in vivo* (*e.g.* hydroxyl, superoxide). Thus, oxygen-derived free radicals were implicated in the pathogenesis of endotoxemia, with the latter reduced in presence of **1** (32). Reactions of hydroxyl radical *in vitro* with **2**, **1**, **4** and the Fe(II) complex of **3** have been described (33, 34), and catalytic superoxide dismutase activity of **4** and DTPA-Fe(II) has been shown (35). Several laboratories have recently reported hydroxamic acid-based metalloproteinase inhibitors of tumor necrosis factor-α precursor processing to tumor necrosis factor-α, which is implied in many inflammatory conditions. However, the *in vivo* properties of these inhibitors, including their ability to protect against septic shock, were not demonstrated (36, 37). In addition, EDTA (at millimolar concentrations), but not its zinc complex, was able to inhibit the tumor necrosis factor-α-processing enzyme (38). This contrasted with our results, wherein both iron complexes **2** and **4** exhibited significantly better *in vivo* protective properties against septic shock than their free-ligand forms **1** and **3**, respectively.

Herein, we present *in vitro* evidence that iron chelators can sequester and (as for **2**) catalyze conversion of NO to benign products. Demonstration of mechanistic aspects of septic shock protection *in vivo*, including interaction with other free radicals, may be hampered by the detection limits of current analytical techniques. To detect the NO·Fe·DETC complex formation in livers of LPS-treated mice by the electron paramagnetic resonance, it was necessary to apply a 500 mg/kg dose of DETC (11). Our chelators demonstrated optimum protection at 10 mg/kg, and at this low concentration it was not possible to detect the electron paramagnetic resonance signal of NO-iron-chelator in circulation or in the liver of *C. parvum* + LPS-treated mice. Nonetheless, our preliminary experiments showed a decrease of the total NO–hemoglobin complex formation in septic mice treated with **2** and septic baboons

treated with **4**. This observation is consistent with the *in vivo* scavenging of NO by **2**.

NO Binding by Iron Chelates: Electrochemical Studies. In an attempt to determine the mechanism by which the iron chelates scavenge NO, we investigated the chemistry of the complexes with gaseous NO in aqueous solution using electrochemical methods. We did not observe a reaction between NO and either of the Fe(III) complexes, although both complexes are known to undergo quasireversible reduction to Fe(II) in solution, which is apparent in cyclic voltammograms (39, 40). There are numerous reports of binding and reduction of NO by Fe(II) generated chemically or electrochemically (20–23, 41–43), which suggested to us that reduction of the metal complexes to Fe(II) would provide *in vivo* NO scavengers similar to those observed with other iron complexes (44, 45). The cyclic voltammogram of **4** (Fig. 2, solid line) shows a quasireversible wave corresponding to the Fe(III/II) couple (E = −0.22 V versus sodium chloride-saturated calomel electrode). The reoxidation of Fe(II) back to Fe(III) was apparent in buffer; however, once NO was introduced, the reoxidation of Fe(II) disappeared (Fig. 2, dashed line), suggesting that the Fe(II) complex forms an adduct with NO:

$$[Fe(III)(H_2DTPA)] + e^- \rightarrow [Fe(II)(H_2DTPA)] \quad [1]$$

$$[Fe(II)(H_2DTPA)]^- + NO \rightarrow (Fe(II)(H_2DTPA)(NO)]^- \quad [2]$$

Removal of NO by bubbling with nitrogen produced the original voltammogram (Fig. 2, solid line) with the reoxidation wave. The Fe(II)–NO adduct could be generated either by reductive electrolysis of the Fe(III) complex in the presence of NO or by the direct reaction of $[Fe(II)(H_2DTPA)]^-$ with NO. The IR spectrum of the adduct showed an NO stretch at 1778 cm^{-1}, which agrees well with recent measurements on NO adducts generated *in situ* (46). As expected for Fe(II) (32), this frequency is consistent with formation of an adduct of neutral NO radical rather than of NO^+ or NO^-. The potential of the Fe(III/II) couple for **4** is easily accessed by common biological reductants (39, 47).

The electrochemistry of **2** (Fig. 3, solid line) is similar to that of **4**, except that the Fe(III/II) couple occurs at a much more negative potential (E = −0.71 V, Fig. 3) (36, 37). As with the DTPA complex **4**, reduction to Fe(II) led to a reaction with NO; however, this reaction was catalytic, producing the current enhancement shown in Fig. 3 (short dashed line). This behavior is indicative of NO binding by Fe(II) followed by further reduction to produce a reduced form of NO and the regenerated **2** complex. As shown in Fig. 3, significant reductive current for NO in the absence of the metal complex (long dashed line) was not observed until a potential of about −0.65 V was reached; both this potential and that of the $[Fe(III)(HDFB)]^+$ complex **2** are probably beyond the range of common biological reductants (39, 47). However, in the presence of the catalyst, such as $[Fe(III)(HDFB)]_2^+$, significant NO reduction current (Fig. 3, short dashed line) was observed at potentials as low as −0.3 V, which is easily accessible *in vivo*. Thus, in the absence of NO, **2** probably remains in the Fe(III) state *in vivo*: however, in the presence of NO, reduction of both the metal complex and NO are feasible.

The $[Fe(III)(HDFB)]^+$ complex **2** is a remarkably stable and efficient electrocatalyst. Both NO and NO_2^- were excellent substrates, and controlled potential electrolysis at $E_{app} < -0.5$ V in the presence of either of these species resulted in an initial increase in current to a steady-state level that remained unchanged over many turnovers (Fig. 3 inset). In one experiment, 16 equivalents of NO_2^- were consumed with essentially no change in current until the NO_2^- was depleted. In a separate experiment with excess NO_2^-, 330 equivalents of charge were passed through the solution and the steady-state current remained at or above 70% of its maximum value. The overall characteristics of the reaction were the same if electrolysis was performed at −0.6 V, which corresponds to the potential of the biological reductant NADPH (39, 47). Analysis of the gas in the electrolysis cell showed that N_2O was a reduction product

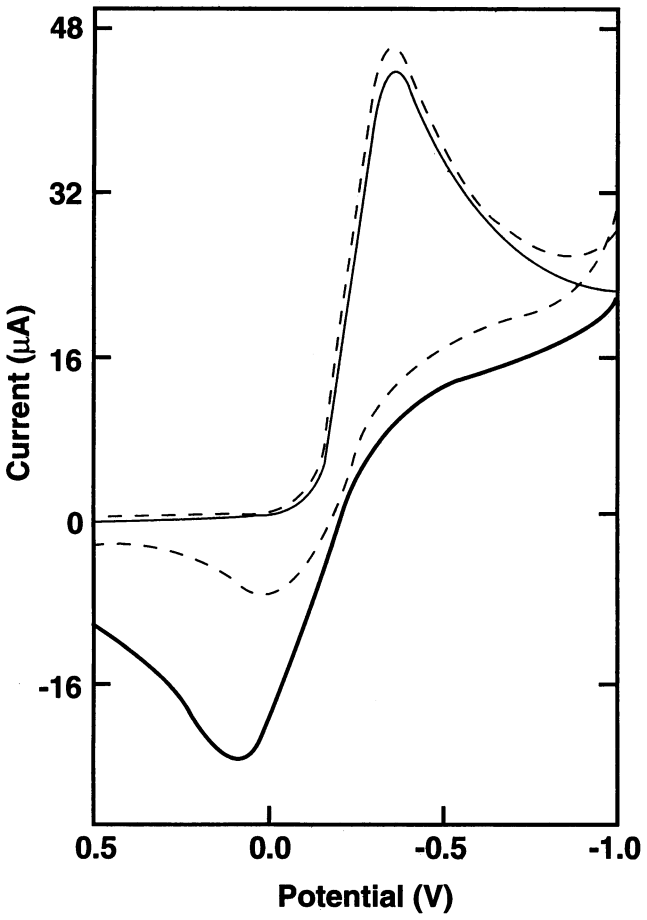

FIG. 2. Cyclic voltammogram of $[Fe(III)(H_2DTPA)]$ **4** in 0.5 M phosphate buffer at pH 7 with (dashed) and without (solid) NO. Working electrode, glassy carbon; counter electrode, Pt wire; reference electrode, sodium chloride-saturated calomel electrode; scan rate, 50 mV/s.

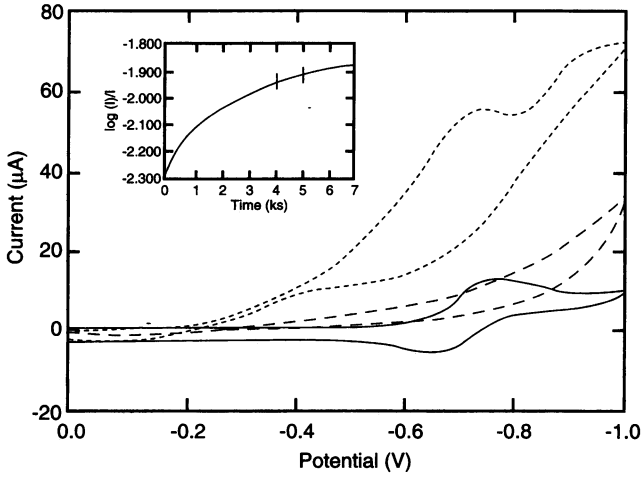

FIG. 3. Cyclic voltammogram of $[Fe(III)(HDFB)]^+$ **2** in 0.5 M boric acid buffer at pH 8 with (short dashed) and without (solid) NO. Also shown is the voltammogram of NO in the absence of metal complex (long dashed). (*Inset*) Current–time profile observed during the reduction of 2 at −0.9 V in the presence of NO.

and that NO was generated as an intermediate when NO_2^- was used as the substrate.

Possible Mechanism of Septic Shock Protection by Iron Complexes. The chemical studies suggested that **2** is a very efficient NO scavenger that operates by catalyzing NO reduction at biologically relevant potentials. These results provide a compelling stimulus for further design, synthesis, and evaluation of compounds that bind and reduce nitric oxide via transition-metal redox catalysis. Application of this newly postulated mechanism to eradicate excessive levels of NO could lead to novel treatments for diseases in which overproduction of NO is a causative factor. Because **4** protected mice against sepsis more effectively than **2**, even though **2** operates via a catalytic mechanism, the potential at which the iron center is reduced may be a more important consideration than the mechanism of NO scavenging. The DTPA complex **4** was reduced at potentials well below those of many biological reductants, while the DFB complex **2** was reduced at these potentials only as a result of the catalytic mechanism (Fig. 3). Nevertheless, these results suggest that a siderophore that decomposes NO catalytically at a lower potential than DFB might be a particularly effective NO scavenger and a prophylactic for septic shock.

1. Furchgott, R. F. & Zawadski, J. V. (1980) *Nature (London)* **288,** 373–376.
2. Palmer, R. J. M., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327,** 524–526.
3. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
4. Feldman, P. L., Griffith, O. W. & Stuehr, D. J. (1993) *Chem. Eng. News* **1,** 26–38.
5. Nussler, A. K. & Billiar, T. R. (1993) *J. Leukocyte Biol.* **54,** 171–178.
6. Kilbourn, R. G., Gross, S. S., Jubran, A., Adams, J., Griffith, O. W., Levi, R. & Lodato, R. F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3629–3632.
7. Thiemermann, C., Szabo, C., Mitchell, J. A. & Vane, J. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 267–271.
8. Klabunde, R. E. & Ritger, R. C. (1991) *Biochem. Biophys. Res. Commun.* **178,** 1135–1140.
9. Petros, A., Bennett, D. & Vallance, P. (1991) *Lancet* **338,** 1557–1558.
10. Kilbourn, R. G., Joly, G., Cashon, B., DeAngelo, J. & Bonaventura, J. (1994) *Biochim. Biophys. Res. Commun.* **199,** 155–162.
11. Kubrina, L. N., Caldwell, W. S., Mordvintcev, P. I., Malenkova, I. V. & Vanin, A. (1992) *Biochim. Biophys. Acta* **1099,** 233–237.
12. Zatarain, J., Xie, J., Miller, M. J. S. & Greenberg, S. (1993) *Clin. Res.* **41,** 783A (abstr.).
13. Maeda, H., Akaike, T., Masaki, Y. & Suga, M. (1994) *J. Leukocyte Biol.* **56,** 588–592.
14. Molina y Vedia, L., McDonald, B., Reep, B., Brune, B., Di Silvio, M., Billiar, T. R. & Lapetina, E. G. (1992) *J. Biol. Chem.* **267,** 24929–24932.
15. Chamulitrat, W., Jordan, S. J., Mason, R. P., Litton, A. L., Wilson, J. G., Wood, E. R., Wolberg, G. & Molina y Vedia, L. (1995) *Arch. Biochem. Biophys.* **316,** 30–35.
16. Mizogouchi, Y., Tsutsui, H., Miyajima, K., Sakagami, Y., Seki, S., Kobayashi, K., Yamamoto, S. & Morisawa, S. (1987) *Hepatology* **7,** 1184–1188.
17. Biliar, T. R., Curran, R. D., Stuehr, D. J., Stadler, J., Simmons, R. L. & Murray, S. A. (1990) *Biochem. Biophys. Res. Commun.* **168,** 1034–1040.
18. Harbrecht, B. G., Biliar, T. R., Stadler, J., Demetris, A. J., Ochoa, J., Curran, R. D. & Simmons, R. L. (1992) *J. Leukocyte Biol.* **52,** 390–394.
19. Kazmierski, W. & Molina, L. (1993) Appl. No PCT/GB94/00967.
20. Cheng, S.-H., & Su, Y. O. (1994) *Inorg. Chem.* **33,** 5847–5854
21. Chen, S.-M., & Su, Y. O. (1990) *J. Electroanal. Chem.* **280,** 189–194.
22. Murphy, W. R., Jr., Takeuchi, K., Barley, M. H. & Meyer, T. J. (1986) *Inorg. Chem.* **25,** 1041–1053.
23. Rhodes, M. R., Barley, M. H. & Meyer, T. J. (1991) *Inorg. Chem.* **30,** 629–635.
24. Zhang, J., Lever, A. B. P. & Pietro, W. J. (1994) *Inorg. Chem.* **33,** 1392–1398.
25. Matzanke, B. F., Mhuller-Matzanke, G., Raymond, K. N. (1989) in *Iron Carriers and Iron Proteins*, ed. Loehr, T. M. (VCH Publishers, New York), pp. 1–121.
26. Theil, E. C. & Raymond, K. N. (1994) in *Bioinorganic Chemistry*, eds. Bertini, I., Gray, H. B., Lippard, S. J. & Valentine, J. S. (University Science Books, Mill Valley, CA), pp. 1–35.
27. Carrano, C. J. & Raymond, K. N. (1979) *J. Am. Chem. Soc.* **101,** 5401–5404.
28. Laub, R., Schneider, Y. Y., Octave, J. N., Chrichton, R. R. & Tronet, A. (1983) in *Structure and Function of Iron Storage and Transport Proteins*. Proc. of the 6th Int'l Conf., Sapporo, Japan (Elsevier, Amsterdam), p. 469.
29. Kim, B. K., Huebers, H., Pippard, M. J. & Finch, C. A. (1985) *J. Lab. Clin. Med.* **105,** 440–448.
30. Peters, G., Keberle, H., Schmid, K. & Brunner, H. (1966) *Biochem. Pharmacol.* **15,** 93–109.
31. Schaich, K. M. & Borg, D. C. (1988) *Lipids* **23,** 570–579.
32. De Garavilla, L., Chermak, T., Valentine, H. L. & Hanson, R. C. (1992) *Drug. Dev. Res.* **25,** 139–148.
33. Hoe, S., Rowley, D. A. & Halliwell, B. (1982) *Chem. Biol. Interact.* **41,** 75–81.
34. Rahhal, S., & Richter, H. W. (1989) *Free Radical Res. Comm.* **6,** 369–377.
35. Pasternack, R. F. & Halliwell, B. (1979) *J. Am. Chem. Soc.* **101,** 1026–1031.
36. McGeehan, G. M. *et al.* (1994) *Nature (London)* **370,** 558–561
37. Gearing, A. J. H. *et al.* (1994) *Nature (London)* **370,** 555–557.
38. Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J. & Black, R. A. (1994) *Nature (London)* **370,** 218–220.
39. Cooper, S. R., McArdle, J. V. & Raymond, K. N. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3551–3554.
40. Crumbliss, A. L. (1990) *Coord. Chem. Rev.* **105,** 155–179.
41. Yu, A. E., Hu, S., Spiro, T. G. & Burstyn, J. N. (1994) *J. Am. Chem. Soc.* **116,** 4117–4118.
42. Mahapatra, S., Halfen, J. A. & Tolman, W. B. (1994) *J. Chem. Soc. Chem. Commun.* 1625–1626.
43. Pham, E. K. & Chang, S.-G. (1994) *Nature (London)* **369,** 139–141.
44. Komarov, A., Mattson, D., Jones, M. M., Singh, P. K. & Lai, C.-S. (1993) *Biochem. Biophys. Res. Comm.* **195,** 1191–1198
45. Lai, C.-S. & Komarov, A. M. (1994) *FEBS Lett.* **345,** 120–124.
46. Yuanjian, D., Shuqin, W. & Yahui, Z. (1987) *Sci. Sin. B* **30,** 1021.
47. Lippard, S. J. & Berg, J. M. (1994) *Principles of Bioinorganic Chemistry* (University Science Books, Mill Valley, CA), p. 11.