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Construction of an x-band electron nuclear double resonance spectrometer and partial characterization of an iron nitrosyl complex of transferrin

Pamela Marie Proulx-Curry  
University of New Hampshire, Durham

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CONSTRUCTION OF AN X-BAND ELECTRON NUCLEAR DOUBLE RESONANCE SPECTROMETER AND PARTIAL CHARACTERIZATION OF AN IRON NITROSYL COMPLEX OF TRANSFERRIN

BY

Pamela M. Proulx-Curry
BS, University of Maine, 1990

A DISSERTATION

Submitted to the University of New Hampshire
In Partial Fulfillment of
the Requirements for the Degree of

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in
Chemistry

September, 1997
This dissertation has been examined and approved.

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Edward H. Wong, Professor of Chemistry

6 August, 1997

Date
DEDICATION

To Bob, Garrett, and Eva
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ABSTRACT

CONSTRUCTION OF AN X-BAND ELECTRON NUCLEAR DOUBLE RESONANCE SPECTROMETER AND PARTIAL CHARACTERIZATION OF AN IRON NITROSYL COMPLEX OF TRANSFERRIN

by

Pamela M. Proulx-Curry
University of New Hampshire, September, 1997

An X-band Electron Nuclear Double Resonance (ENDOR) spectrometer was constructed, tested at both room and liquid helium temperatures, and characterized at liquid helium temperature using a vanadyl-imidazole standard.

An iron nitrosyl complex of transferrin was studied. Three methods of generating nitric oxide gas were tested and resulted in no differences in product formation. The g-factors for the rhombic complex—prepared with and without carbonate addition to the buffer—were determined. For the complex prepared with carbonate \( g_x = 2.052, g_y = 2.028, \) and \( g_z = 2.013 \). For the complex prepared without addition of carbonate \( g_x = 2.051, g_y = 2.028, \) and \( g_z = 2.011 \). Complex concentration was little affected by the addition of extra Fe\(^{2+} \), but does decrease over time after reaching a maximum two to three minutes after the start of the reaction. The complex was observed to form preferentially at the C-terminal lobe of the protein. The histidine residue, which normally serves as a ligand for Fe\(^{3+} \), does not appear to be necessary for binding of the iron nitrosyl complex. An ENDOR spectrum of the complex obtained at 3.6 K yielded a signal attributed to the effect of matrix protons.
INTRODUCTION

Nitric Oxide

Much attention has been given in the last few years to the free-radical nitric oxide (NO). This small, diatomic gas molecule is synthesized by mammals [1] and is involved in such diverse biological functions as smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, and penile erection [2]. The first gas known to act as a messenger molecule in mammals, NO is manufactured in cells by the reaction of the enzyme nitric oxide synthase (NOS) with the amino acid L-arginine.

Cells may contain two kinds of the NOS enzyme—constitutive and inducible—which appear to produce NO for different purposes. Constitutive enzymes are always present in the cell and are capable of quickly producing small, short-lived amounts of NO for tasks such as neurotransmission. Inducible enzymes, on the other hand, are activated more slowly by other messenger molecules, but are able to produce 1000 times more NO, the gas so produced being used for cellular defense [3].

Once formed, NO gas is a stable paramagnetic species which does not dismutate or dimerize, and which has a solubility in water around 2 mM under 1 atm at 20°C [4]. Despite its paramagnetism, the molecule alone is EPR silent in both room temperature and frozen solutions [4]. (An EPR signal is of the NO molecule alone may be observed,
however, when the molecule is in the gaseous state.) The molecule has three interrelated redox forms: 1) the nitrosonium cation (NO⁺); 2) nitric oxide (NO); 3) and the nitroxyl anion (NO⁻) [2].

Nitric oxide (NO) has a single electron in its 2pπ* orbital [2]. The molecule can act as either an oxidant or a reductant. It reacts readily with molecular oxygen to produce NO₂, which dimerizes into N₂O₄. These in turn can produce the anhydrides N₂O₃ and N₂O₅, the acids HNO₂ and HNO₃, the anions NO₂⁻ and NO₃⁻, and other oxides such as ONOO⁻ and HN₂O₃⁻. Unlike reactions in the gas phase, in which the reaction is 2nd order with respect to NO and first order with respect to O₂, the complex reaction of NO and O₂ dissolved in water is zero order in NO. In vitro, the half-life of 10 μM NO in the presence of 50 μM O₂ is about four minutes as opposed to 3 - 30 s observed in vivo [4].

A chemical property which helps make NO so biologically important is its ability to form adducts with nucleophiles (such as amines, sulfite, and thiols) which are able to slowly and spontaneously release NO thereby making it available for cellular reactions. NO also coordinates to transition metals such as Mn, Cu, and Fe. Some well known complexes include diamagnetic nitroprusside [Fe³⁺(CN)₅NO]²⁺, and paramagnetic [Fe⁷⁺(CN)₅NO]³⁺, [Fe⁷⁺(CN)₄NO]³⁺, and [Fe⁷⁺(H₂O)₅NO]²⁺. Many ternary complexes of the form [X₋₋Fe-(NO)ₙ₋₋] (where X is a thiol residue or an amino acid) have been shown to spontaneously release NO through ligand exchange as well [4].

As stated earlier, the action of NO in a particular cell or with a particular cell part depends on the source of the gas, but its oxidation state and the particular derivative
present are also important in determining its function. Of particular interest here are the iron-nitrosyl complexes of non-heme proteins. The paramagnetic nature of many of these compounds makes them particularly amenable to study by EPR and ENDOR spectroscopy [5]. In fact, it was precisely because ENDOR can be used to identify particular ligand nuclei in metal complexes—and in some cases bond distances and angles as well—that construction of an ENDOR spectrometer was undertaken.

EPR studies of iron-nitrosyl complexes of the iron storage protein ferritin [6-8] and the iron transport protein transferrin [7,9] have been reported, but the composition and structure of these complexes is not known. The g-values obtained for these complexes are very near 2, suggesting a net spin state of $S = 1/2$.

Many dinitrosyl iron complexes have been studied [10-16]. These complexes have been shown to have tetrahedral geometry. Two nitric oxides and two Lewis base ligands are bound to the central iron which is in a high spin, $d^7$, electronic state and has a formal charge of +1 and an electron spin $S = 3/2$ [15]. The unpaired electron from NO ($S = 1/2$) antiferromagnetically couples with an electron from the central iron. The bonding of two NO$^\prime$ radicals to the iron thus results in a net spin for the complex of $1/2$ ($S_{net} = 3/2 - 1/2 - 1/2 = 1/2$) and a g value in the range of 2.02 - 2.04 at room temperature [15].

**Transferrin**

Iron, the fourth most abundant element in the Earth's crust, is essential to all life. The redox potential range of Fe(II/III) is wide (+300 to -500 mV) depending upon its
coordination. In living organisms iron is involved in such diverse processes as oxygen transport, nitrogen fixation, electron transfer reactions, and photosynthesis [17]. Indeed, life as we know it could not have evolved without this essential and ubiquitous transition metal.

Under physiological conditions the +3 oxidation state of iron is favored. Iron in this oxidation state, however, tends to precipitate out of solution due to hydrolysis at concentrations greater than $10^{-17}$ M. As a result, a family of proteins known as transferrins has evolved which help to solubilize, sequester, and transport Fe(III)[18].

Transferrins are present in the physiological fluids of all vertebrates [19] and have also been observed in spiders, crabs, and many insects [18]. The family of proteins referred to as transferrins includes: serum transferrin, found in blood; ovotransferrin, found in egg white; lactotransferrin, found in the milk and other secretory fluids of most mammals; and melanotransferrin, a membrane-bound protein found at low levels on the surface of normal cells and at high levels on the surface of melanoma cells [18,19].

Serum transferrin, the subject of the studies described herein, is a monomeric glycoprotein with a molecular weight of $\sim$80,000. Its primary biological function is the transport of iron from sites of absorption to sites of storage and utilization [18,19]. The sequence of the 678 amino acids which comprise the polypeptide chain was first determined in 1982 by Ross et al.[20].

In 1988, Bailey and coworkers presented an X-ray crystallographic structure of diferric rabbit serum transferrin determined at a resolution of 3.3 Å [21]. The polypeptide
chain is folded into two lobes, an N-terminal lobe and a C-terminal lobe. Within each lobe is a cleft which separates the lobe into two domains. A binding site for a single Fe$^{3+}$ cation and its synergistic carbonate anion is found within each cleft (Fig. 1.1).

A high degree of homology exists between the amino acid sequences of the two lobes (~40%) [20], and folding for each is essentially the same [21]. Thus the composition and structure of the metal binding sites are essentially the same in each lobe.

The orientation of the two lobes within the molecule is not the same among the transferrins, however, and the orientation of the two domains within each lobe differs slightly as well. The two lobes are joined by a short polypeptide chain consisting of 14 residues [18] (Fig. 1.2). The folding pattern of the lobes defines the metal binding site and is important in determining how it functions. Each domain consists of a mixed $\beta$-sheet overlaid with $\alpha$-helices which pack against the face of the sheet. The first ~70 residues make a coherent unit of three parallel $\beta$-strands (a,b,c) and three $\alpha$-helices (1,2,3) which form half of the first domain. A two-part (d and e) parallel strand runs behind the binding site connecting the first domain to the second, crossing over at about residue 90. The second domain is comprised of the following ~160 residues which form a number of $\beta$-strands and $\alpha$-helices. A second, long two-part strand (i and j) crosses back behind the binding site to complete the folding of the first domain. The chain then crosses a third time, terminating in a helix packed against the second domain [18,22,23] (Fig. 1.3).
Figure L.1 — Domain organization of transferrins. Each lobe is divided into two domains. The two lobes are related by a screw axis, a rotation of $-180^\circ$, and a translation of $-25 \text{ Å}$. The two iron binding sites are identified by closed circles. The connecting peptide that joins the two lobes is helical in lactoferrin (solid line) and less regular in transferrin (dashed line) [18].
Figure L2 – Ribbon diagram of human diferric lactoferrin, showing the organization of the molecule, with the N-lobe above and the C-lobe below. The four domains (N1, N2, C1, C2), the interlobe connecting peptide (H), and the C-terminal helix (C), are indicated. The glycosylation sites in various transferrin are shown by triangles and numbered (1, human transferrin; 2, rabbit transferrin; 3, human lactoferrin; 4, bovine lactoferrin; and 5, chicken ovotransferrin). The interdomain “backbone” strands in each lobe can be seen behind the iron atoms [18].

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Figure I.3 – Polypeptide folding pattern found in each lobe of human lactoferrin. Helices (cylinders) are numbered 1 to 12 and β strands (arrows) are labeled “a” to “k”. The interdomain backbone strands are shaded and the position of the hinge is indicated [18].
X-ray crystallographic structural analyses of transferrins from a number of sources have confirmed the composition and structure of the iron binding sites and shown them to be similar in both lactoferrin and serum transferrin in a variety of species.

The iron coordination in particular remains unchanged between these proteins and between the lobes of these proteins [18]. The iron is bound to two tyrosine residues, one histidine residue, and one aspartate residue. The bidentate binding to a single carbonate anion, which is in turn attached via hydrogen bonds to an arginine residue and the N-terminus of an \( \alpha \)-helix, completes the distorted octahedral coordination of the iron (Fig. I.4).

While a high degree of homology—both in composition and structure—has been observed between the N- and C-terminal lobes of transferrin, some differences do exist. Iron is bound more tightly and less reversibly at the C-lobe [24,25]. The C-terminal lobe also retains its iron at lower pH (down to pH 6.0) than the N-terminal lobe [19]. These differences are attributed to a decreased flexibility of the C-terminal lobe relative to the N-terminal lobe, probably due to the presence of an extra disulfide bridge in the C-lobe which may restrict the degree to which this lobe may "open" and "close" in order to bind and release iron [26].

Although transferrin has the highest affinity for \( \text{Fe}^{3+} \) bound synergistically with carbonate in an octahedral configuration, other metals [18] with different synergistic anions [27-29] and different coordinations [30-35] have been observed as well.
Figure 1.4 – Schematic diagram of the characteristic transferrin metal and anion binding site. Numbering is as for the N-lobe of human lactoferrin, but the same arrangement of ligands is found in the C-lobe and in the N- and C-lobes of almost all transferrins. For reference, the residue numbers for human lactoferrin and human transferrin are shown in the inset [18].
As mentioned earlier, iron-nytrosyl complexes of transferrin have recently been observed, and it is toward an understanding of the structure and composition of these compounds that the following studies are directed.
CHAPTER 1

ENDOR Theory

Transition Energies and the ENDOR Experiment

Electron Paramagnetic Resonance (EPR) and Electron Nuclear Double Resonance (ENDOR) spectroscopy are magnetic resonance techniques used to study the environment and structure of paramagnetic centers in molecules. Because these techniques are sensitive only to the paramagnetic portion of a molecule and experience little or no interference from the non-paramagnetic portions, they are often used in the study of complex biological systems [36-42]. In particular, they have proven quite useful in the study of metalloproteins in which the metal ions are often associated with protein function [36].

The first EPR experiment was performed by Zavoisky in 1945 [43] and the technique has been widely used for metalloproteins since 1960 [36]. In 1956 Feher conducted the first ENDOR experiment [44], and while the technique was applied to metalloproteins as early as 1967 by Eisenberger and Pershan [45], it was not often used for that purpose until commercial instruments became readily available after 1970 [36].

In the EPR experiment the degenerate spin states of an unpaired electron are lifted by the application of a dc magnetic field, B. Transitions between spin states are then
induced by electromagnetic radiation typically of microwave frequency [37]. For a simple spin system with one unpaired electron, S = 1/2, coupled to a nucleus with nuclear spin I = 1/2 (e.g. $^1$H, $^{15}$N, $^{31}$P), the energies (E) can be calculated from the solution to the Schrödinger equation:

$$H \Psi = E \Psi$$

(1.1)

where $\Psi$, the total spin wave function for the system, consists of electron and nuclear spin functions. $H$, the spin Hamiltonian operator [46], is given by:

$$H = \beta S \cdot g \cdot B - g_n \beta_n I \cdot B + hS \cdot A \cdot I$$

(1.2)

where $\beta$ and $\beta_n$ are the electronic and nuclear magnetons; $g$ is the electronic g-tensor which describes the electronic Zeeman interaction between the magnetic field, $B$, and the electron spin; $g_n$ is the nuclear g factor and depends on the nucleus present; and $A$ is the hyperfine tensor which describes the interaction between the electronic and nuclear spins [36,37]. The first term in equation 1.2 describes the electronic Zeeman interaction; the second the nuclear Zeeman interaction; and the third the hyperfine interaction. An energy level diagram of this simple spin system is presented in Fig. 1.1. When a paramagnetic substance is placed in a magnetic field, the degeneracy of the spin states is lifted. In the...
Figure 1.1 - Energy level diagram for an \( S = \frac{1}{2}, I = \frac{1}{2} \) spin system. The bold lines show the allowed EPR transitions, the solid lines show the NMR (ENDOR) transitions, and the dashed lines show the EPR "semiforbidden" transitions [47].
absence of any magnetic nuclei, the energy difference between electronic spin states, the
resonance condition for EPR, is given by

$$\Delta E = h \nu_e = g \beta B$$  \hspace{1cm} (1.3)

The energies given by Equation 1.3 are for EPR transitions. For an unpaired electron (S =
1/2 and g = 2.0023) an excitation frequency of 9.5 GHz will produce an absorption at a
magnetic field of about 340 mT (3400 G) [36].

The energy difference between two nuclear spin states is given by

$$\Delta E = h \nu_n = g_n \beta_n B$$  \hspace{1cm} (1.4)

The energies given by Equation 1.4 are for NMR transitions. For a proton with nuclear
spin I = 1/2 in a magnetic field of 340 mT the resonant frequency will be about 14.5 MHz
[36]. In the presence of magnetic nuclei, the electronic spin states are further split by
coupling between the electron and the nucleus. When such hyperfine interactions occur,
the third term of Equation (1.2) must be considered as the energies of the spin states will
be shifted by ± A/4 due to this interaction. The energy difference due to the hyperfine
interaction, then, is given by
\[ \Delta E = h \frac{A}{2} \] (1.5)

In the ENDOR experiment, the magnetic field is set on a particular line in the EPR spectrum (e.g. the one corresponding to the EPR transition from level 2 to 4)(See Fig. 1.1). The EPR transition is saturated (or partially saturated) with microwave power, resulting in an equalization of the electron spin populations of the two states, 2 and 4. Consequently, the EPR absorption is no longer observed. While maintaining this saturation, the radio frequency (rf) power is swept. When the rf energy matches the transition energy between levels 3 and 4, nuclear spin flips will occur, the populations of levels 2 and 4 will no longer be equal, and the EPR absorption will be restored [47].

ENDOR offers the following advantages over EPR spectroscopy: (1) it makes possible the deconvolution of inhomogenously broadened EPR lines; (2) it allows for the unambiguous identification of nuclei by their Larmor frequencies; (3) lower spectral density is afforded because the effect of nuclear interaction on an ENDOR spectrum is additive as opposed to EPR where the effect is multiplicative; (4) hyperfine interactions can be measured more accurately, and in some cases "single crystal" quality data can be obtained from powder spectra because ENDOR measurements are made at different parts of an anisotropic EPR spectrum, thus more detail regarding the environment of the paramagnetic system, up to a distance of 0.8 nm, can be obtained. The sensitivity of ENDOR is considerably less than that of EPR, however, and samples must generally be on the order of ten times more concentrated than for EPR. Also, because ENDOR lineshapes

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and amplitudes are affected by complicated relaxation mechanisms which are generally
difficult to account for, they cannot be used to quantify the number of equivalent nuclei
giving rise to a particular signal [36].

Design Considerations for the ENDOR Cavity and Coil

A resonant cavity is one of the major parts of any ENDOR spectrometer. It is
similar to an rf-tuned circuit. At microwave frequencies, however, the skin effect leads to
high resistance in wire conductors, and the dimensions of the capacitors and inductors are
of the same order as the wavelength causing energy loss by radiation. The use of cavity
resonators can significantly reduce energy loss due to such factors. A cavity resonator is a
chamber built of a highly conductive metal and fitted with devices for admitting or
extracting microwave energy [48,49]. At resonance, the cavity is able to sustain
oscillations of the electromagnetic field by reflection of the microwaves from the walls.
An interference pattern or standing wave configuration is thus formed. Various standing
wave configurations, or modes, can be obtained depending on the shape and dimensions of
the cavity [49]. The efficiency with which the cavity can maintain these oscillations is
expressed in terms of the cavity Quality factor or Q, where Q is given by the following:

\[
Q = \frac{2\pi (\text{energy stored})}{\text{energy dissipated per cycle}} \tag{2.6}
\]
Resonant cavities are most often either rectangular or cylindrical in shape. Either shape is capable of supporting both Transverse Electric, TE\textsubscript{mnp}, or Transverse Magnetic, TM\textsubscript{mnp}, modes. In TE modes the electric field lines are perpendicular to the direction of propagation of the electromagnetic wave. In the TM modes the magnetic field lines are perpendicular to the direction of propagation. The subscripts m, n, and p are used to denote the number of half-wave patterns in the various dimensions of the cavity. In a cylindrical cavity the m denotes the number of whole wavelengths around the circumference of the cavity; the n denotes the number of half wavelengths across the diameter of the cavity; and the p denotes the number of half wavelengths across the length of the cavity [48]. A diagram of a TM\textsubscript{110} mode for a cylindrical cavity is provided in Fig. 1.2.

The resonant frequency of a cylindrical cavity depends on the cavity radius, a, and length, d, [49] according to the following:

\[
(2af)^2 = \left(\frac{c(kca)}{\pi}\right)^2 + \left(\frac{cp}{2}\right)^2 \left(\frac{2a}{d}\right)^2
\]  \hspace{1cm} (2.7)

where c is the speed of light and (kca)\textsubscript{mn} is a Bessel function root as cylindrical cavity modes have Besseloid radial variations. In the case of the TM\textsubscript{110} cavity, the resonant frequency of the cavity has no dependence on the cavity length. The cavity Q, however, does depend on the cavity length [49] as follows:
Figure 1.2 - Electric (solid lines) and magnetic (dotted lines or dots) field distributions for $TM_{11}$ mode in a cylindrical waveguide [49].
\[
Q_u \frac{\delta}{\lambda} = \frac{[(k_0)^2 + (p \pi a/d)^2]^{1/2}}{2\pi(1 + a/d)}
\] (2.8)

where \( \delta \) is the skin depth of the current (i.e. the depth within the conductor at which the current decays to \( 1/e = 0.369 \) of its value at the surface), and \( \lambda \) is the wavelength.

Microwave radiation is usually coupled to the cavity by one of three methods: 1) probe (or capacitative) coupling; 2) loop (or inductive) coupling; or 3) aperture (capacitive or inductive) coupling. In probe coupling, a probe is inserted into the cavity at a point of maximum E-field. The E-field radiates out from the probe. In loop coupling, a loop is placed at a point of maximum magnetic field and introduces the magnetic field into the cavity. In aperture coupling, either the magnetic field or the electric field may be introduced into the cavity depending on the position and orientation of the aperture or slot [48]. Illustrations of these modes of coupling are provided in Fig. 1.3.

Radio frequency energy is coupled into the cavity via the ENDOR coil. Many configurations have been suggested for this coil [40,50-52]. For a cylindrical cavity at X-band frequencies the coil configuration described by Hurst and coworkers [50] is often used. This coil is a helix which is positioned around a quartz tube. The quartz tube is fashioned to permit the extension of a cryostat system into the cavity for low temperature experiments. The free standing coil is robust and can be repositioned fairly easily to permit proper tuning of the ENDOR cavity. Because the impedance of the ENDOR coil

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Figure 1.3 – Three methods of coupling energy into or out of a cavity: a) probe, or capacitive, coupling; b) loop, or inductive, coupling; c) aperture, or slot, coupling [48].
is frequency dependent, as is the impedance of all inductors, each coil is useful only over a finite range of frequencies. When this range of frequencies is exceeded the efficiency of power transfer to the coil is impaired, thus limiting the sensitivity of the spectrometer [40].

The useable frequency range is determined by the number of turns of the coil, with a 16-20 turn coil, of length up to 4 cm, being optimal for operation in the 1-30 MHz frequency range [40,51].

General requirements for a useful ENDOR cavity then are based on those aspects of construction and design which will enhance the establishment of an oscillating wave pattern within the cavity and minimize energy losses. In particular, the cavity must be constructed of a highly electrically conductive material to minimize resistive losses. The interior walls of the cavity must be highly polished to minimize losses due to interfering eddy currents in the walls. The range of acceptable radii for the cavity (and hence its resonant frequency) is limited by the bandwidth of the spectrometer's klystron. The length of the cavity is chosen to both maximize cavity Q and suppress unwanted modes. Attention to cavity dimensions is critical in order to assure that no undesired, or interfering modes, are established in the cavity. Also, the various parts of the cavity must be tightly joined in order to prevent the escape (by radiation) of microwaves at the joints. And, finally both the microwave and rf coupling systems must provide an efficient and stable transfer of energy into the cavity.
Construction of the ENDOR Spectrometer

Spectrometer Components

A schematic diagram of the complete ENDOR spectrometer is shown in Fig. 1.4. The sample is placed in a homemade TM$_{110}$ cavity which lies between the poles of an electromagnet. The voltage for generating the dc magnetic field is provided by the magnet power supply (not shown) and controlled by a field controller circuit in the Varian E-9 EPR console. The klystron in the Varian E-101 microwave bridge generates microwave energy of constant frequency in the range of 8.8 to 9.6 GHz. The frequency output of the klystron is monitored with an HP 5350A microwave frequency counter (Hewlett Packard, Santa Clara, CA). The microwave power is directed to the sample cavity via a four port circulator and waveguide. The cavity is simultaneously irradiated with a modulated rf signal, the carrier of which is generated by a computer controlled PTS 160 frequency synthesizer (Programmed Test Sources, Inc., Littleton, MA). The carrier frequency, along with a low frequency (10 kHz) modulation signal obtained from the low frequency field modulation unit of the Varian E-9 console, is fed into a surface acoustic wave oscillator-based modulator/mixer (Illinois EPR Research Center, University of Illinois, Urbana, IL)[53]. The output of this mixer is then amplified by a ENI 3200L rf amplifier (Electronic Navigation Industries, Inc., Rochester, NY) and inserted into a homemade TM$_{110}$ sample cavity via a homemade ENDOR coil and terminated in a 50 ohm dummy load (500 W, 50 ohm, Termaline Coax Resistor, Bird...
Figure 1.4 – Schematic diagram of ENDOR Spectrometer
Electronic Corp., Cleveland, OH). A swept, FM modulated magnetic field, perpendicular to the dc magnetic field, is thus induced at the coil.

The reflected microwave energy is directed to the microwave diode detector in the Varian E-101 microwave bridge. The EPR signal from the detector is passed to the receiver section of the field modulation unit in the Varian E-9 console where it is processed and converted to a dc voltage for transmission either to the recorder Y-axis or to the computer for storage. First derivative spectra, such as the one shown in Fig. 1.5, are obtained due to the phase sensitive detection.

Connections Between Modules

All connections between the various free-standing modules of the spectrometer were made using RG-58 coaxial cable (American Radio, Rochester, NH, or Electronic Surplus Supply, Manchester, NH) with BNC connectors (American Radio, Rochester, NH, or Electronic Surplus Supply, Manchester, NH) with four exceptions. The exceptions are: 1) cable plugs which mate with the output jacks of the low frequency modulation unit are Amphenol # MS 3106A 10SL-3S, 3-pin female connectors with Amphenol #AN 3057-4 cable clamps (Gerber Electronics, Norwood, MA); 2) 0.141" o.d. semi-rigid coax with a non-magnetic (copper) center conductor (Haverhill Cable & Mfg. Corp., Haverhill, MA) was used to couple the waveguide to the sample cavity; 3) a 50-conductor, shielded Telco cable with drain wire (Telecommunications Center, UNH) with two 50-pin, female Telco connectors with 90° hoods (Telecommunications Center,
Figure 1.5 - ENDOR spectrum of gamma irradiated sucrose. Experimental Conditions: field set, 3270 G; modulation amplitude, 2.5 G; time constant, 0.3 s; receiver gain, 10,000; phase, $180^\circ$; microwave power, 10 mW; microwave frequency, 9.19 GHz; rf frequency center, 14 MHz; rf scan width, 19.5 MHz; scan rate, 0.2 MHz/s; PTS output, 0.4V.

File name: 21DEC94K
UNH) was used to connect the frequency synthesizer to the computer’s A/D converter; and 4) type N connectors were used to mate with the input and output jacks of the rf power amplifier. Pinouts for the modulation cables to both the EPR modulation coils and the ENDOR coil are listed in Table 1.1 (See also Appendix 1).

Microwave Coupling and Cavity Tuning

The wave-guide carrying the microwave signal from the four port circulator is terminated by a slide-screw tuner fitted with a wave-guide to N-connector adapter. A short length of 0.141" o.d. semi-rigid coax with a non-magnetic (copper) center conductor (Haverhill Cable & Mfg. Corp., Haverhill, MA) fitted with an N-connector (Gerber Electronics, Norwood, MA) at one end and formed into a coupling loop at the other end is used to couple the microwave from the wave-guide into the cavity (see Fig. 1.6). A single loop of 1" radius is placed in the coax in order to give some flexibility to the length for tuning and adjusting purposes.

The coupling loop was inserted into the cavity and its position adjusted while observing the cavity mode on the oscilloscope screen. When a maximum in the depth of the mode dip and a minimum in the width of the mode was obtained, the loop was locked in place and has not required further adjustment since. Tuning for each sample is accomplished using both the slide screw tuner and the frequency and phase adjustments on the E-101 microwave bridge (see Appendices 2 - 4 for tuning and operating instructions).
Table 1.1 – Pinout for cable from modulation unit to mixer.

<table>
<thead>
<tr>
<th>Modulation Unit Jack – J407</th>
<th>Amphenol Plug – P407</th>
<th>RG 58/U Coaxial Cable</th>
<th>BNC – Mixer Connection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A High</td>
<td>A High</td>
<td>Center conductor</td>
<td>Center pin</td>
</tr>
<tr>
<td>B Shield</td>
<td>B Shield</td>
<td>Shield</td>
<td>Connector shell</td>
</tr>
<tr>
<td>C Low</td>
<td>No connection</td>
<td>No connection</td>
<td>No connection</td>
</tr>
</tbody>
</table>
Figure 1.6 – Detail of loop coupling of coax to ENDOR cavity.
ENDOR Cavity Design and Construction

A diagram of the fully assembled cylindrical TM_{110} cavity and engineering drawings of the cavity are shown in Fig. 1.7 - 1.13. The ENDOR cavity was constructed of aluminum alloy 6063-T5 (Central Steel and Wire Co., Chicago, IL). All cavity parts were machined at the UNH Space Science machine shop and the inside of the cavity body was polished there as well. The inside of the cavity top was polished on a polishing wheel using 180, 240, 400, 1200, and 3 micron grit paper successively with water as a lubricant. The final polish was obtained on a wheel fitted with a buffing cloth and 0.05 \( \mu \)m alumina polishing paste. A knife sharp edge was obtained on the top of the cavity body by polishing it on a polishing wheel with 180 and 240 grit paper only as finer grit paper tended to round the edges.

The cavity body (Fig. 1.10) consists of a cube of aluminum from which a cylindrical section has been cut. A smaller, circular hole is cut in the center of the base of the cavity body to permit insertion of a quartz dewar into the cavity for low temperature experiments. The cavity, with a diameter of 1.414", resonates, when fully loaded with quartz dewar, ENDOR coil, and sample, at a frequency of between 9.1 and 9.24 GHz. The frequency depends upon the particular ENDOR coil that is used and the particular sample that is inserted. The cavity body is also fitted with a hose connector (Fig. 1.12) so that nitrogen or helium gas may be blown into the cavity to cool the ENDOR coil when rf power is being applied to it.
Figure 1.7 – Diagram of total cavity assembly.
Figure 1.8 - Drawing of cavity top.

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Figure 1.9 — Drawing of spacer between cavity top and cavity body.
Figure 1.12 – Drawing of hose connector attachment.
Figure 1.13 — Drawing of pump attachment assembly (Cryo Industries).
The bottom (Fig. 1.11) can be used with cavity bodies of different dimensions to permit experimentation at a variety of frequencies in the 8.8 to 9.5 GHz range. Both the top (Fig. 1.8) and bottom consist of a flat plate with an attached, threaded stack. The threads on the cavity bottom allow it to be attached to a cryostat for low temperature experiments. The top piece is threaded to permit attachment of a pumping port (Fig. 1.13) so that cryogenic liquids and gasses may be pulled through the cryostat/cavity system in order to achieve temperatures as low as 2 K. Threaded holes on the sides of the stack permit insertion of chasis mount BNC connectors (Electronic Surplus Supply, Manchester, NH) which are thus press-fitted against the ENDOR coil. A short piece of the outer cladding of the 0.141" o.d. semi-rigid coax was fitted over the solder cup end of each BNC connector and filled with solder to give a solid connection with a relatively large surface area. A 0.500" spacer (Fig. 1.9) was added between the cavity top and the cavity body in order to provide a rigid support for the microwave coupling loop.

Construction of the ENDOR Coil

The ENDOR coil was constructed by adapting the methods described by Hurst et al. [50] and Bender [51,54,55] (Fig. 1.14). Briefly, two brass cylinders are fitted onto a teflon rod (Fig. 1.14) whose rigidity is ensured by insertion of a thin brass rod through its center. Copper wire is wrapped around the teflon rod for the desired number of turns. Each end of the wire is then soldered to a brass cylinder. The spacing between the brass cylinders is determined by the length of the cavity. They must be placed far enough apart
Figure 1.14 – Fully assembled ENDOR coil (top); reinforced teflon rod for winding ENDOR coil (center); and delrin insulators for ENDOR coil (bottom).
so that the brass will not enter into the cavity when the coil is inserted into it. The copper wire must be wound very tightly against the teflon rod and the ends of the coil must be oriented 180° from each other on the brass cylinders (see Fig. 1.14). With the brass cylinders placed the desired distance apart, and the copper wire fitted tightly around the teflon rod, the coils can then be repositioned as needed to insure uniform spacing between them.

A piece of teflon shrink wrap (Small Parts Inc., Miami Lakes, FL) is placed over the coil so that it completely covers the wire and partially covers the ends of the brass cylinders. This entire assembly is then heated in an oven to 186 °C for about 20 minutes to shrink the teflon shrink wrap tightly onto the wire. The coil and teflon rod are then placed in a freezer for about 30 minutes where the teflon rod, with its higher coefficient of thermal expansion, shrinks due to the cold more than the brass and copper. The shrink wrap coated coil can then be easily removed from the teflon rod without damage.

Once fabricated, the ENDOR coil is then inserted into the cavity via the center hole in the top stack. Delrin insulators (Fig. 1.14), fabricated by the Space Science Machine Shop (Morse Hall, UNH) line the inner walls of both the top and bottom stacks in order to electrically insulate the coil from the cavity and to hold the coil centered within the cavity. The chassis mount BNC connectors described above screw into the side of the stacks, make electrical contact with the ENDOR coil, and hold it in position.
Characterization of the Instrument

The Q of the ENDOR cavity was measured using the method described by Eaton [56]. Three samples were used to make the measurements: coal, gamma irradiated sucrose, and bis-diphenyl-phenyallyl (BDPA) polystyrene. The coal was obtained from Dr. V. K. Mathur, UNH Dept. of Chemical Engineering, and mixed with KCl. The sucrose sample was prepared by exposing a few grams of sucrose to the Cesium-137 source located in Parsons Hall for 77.5 hours. The BDPA-polystyrene sample was obtained from Dr. Gary Gerfen, M.I.T. Magnet Laboratory. Measurements were taken with the quartz dewar and ENDOR coil in place. The cavity Q was determined to be 1,088.

Initial testing of the instrument was performed at room temperature. Coal (from Dr. Mathur, UNH), DPPH, strong pitch (coal sample from Varian), gamma irradiated sucrose, and BDPA-polystyrene were all used as samples. First, EPR spectra were acquired to make sure that the ENDOR cavity was tuned and resonating properly. Once this was ascertained, the ENDOR function of the spectrometer was tested at room temperature using the gamma irradiated sucrose and the BDPA-polystyrene samples.

A vanadyl-imidazole sample \((0.02 \text{ M} \text{ VO(Im)}_{4})^{2+}\) was prepared for use as a low temperature standard (Fig. 1.15 and 1.16) by adding aqueous \(\text{VO}^{2+}\) at pH 2.0 to 2 M imidazole in a 2:1 water-glycerol solvent at pH 7.2. The sample was then purged with argon gas for 30 minutes.
Figure 1.15 — EPR spectrum of VO(Im)$_4$ ENDOR Standard. Experimental Conditions: Field center, 3200 G; scan width, 2000 G; receiver gain, 500; time constant, 0.3 s; scan time, 8 min; temperature, 10 K; modulation frequency, 1 kHz; modulation amplitude, 32 G; microwave power, 0.1 mW; microwave frequency, 9.137 GHz; [VO(Im)$_4$]$^{2+}$ = 0.02 M. Filename: 27Jul95a.fls
Figure 1.16 – ENDOR spectrum of VO(Im)$_4^{2+}$ ENDOR standard. Experimental Conditions: field center, 3200 G; time constant, 0.03 s; modulation frequency, 10 kHz; microwave power, 10 mW; microwave frequency, 9.137 GHz; temperature, 6.2 K; RF power, 0.4 V; phase, 0°; modulation amplitude, 2.00; scan rate, 0.5 MHz/s; RF field center, 10 MHz; scan width, 19.25 MHz; [VO(Im)$_4^{2+}$] = 0.02 M. Filename: 27ju95ap.fls (EW ENDOR).

*This system was previously studied by Mulks and coworkers. The set of lines around 14 MHz were interpreted as follows: 1) the large peak centered about the free proton frequency is attributed to matrix protons; 2) water exchangeable protons were said to give rise to the pair of lines with a splitting of 3.14 MHz due to axially coordinated H$_2$O; and 3) the pair of lines with splitting of 3.9 MHz were been attributed to VO$^{2+}$-bound impurities introduced with the imidazole solution. The major features between 2.5 and 6.2 MHz are attributed to a single set of equivalent nitrogens. The following parameters were calculated: $A_x = 7.00$, $Q_x = 0.32$, $A_y = 5.90$, $Q_y = 1.12$ MHz [68].

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The signal amplitude and signal-to-noise ratio were measured at a variety of microwave power settings (Fig. 1.17), RF power settings (Fig. 1.18), and temperatures (Fig. 1.19). In general, for this particular sample, the signal amplitude and S/N appeared to peak at a microwave power setting of 5 - 10 mW and decline thereafter. No saturation effects were observed, however, as the RF power was increased to a maximum of 1.0 V on the PTS frequency synthesizer. Signal amplitude increased with decreasing temperature. The maximum signal to noise ratio observed, 388, was obtained at the following settings: microwave power, 5 mW; RF power, 1.0 V; temperature, 6.0 - 6.8 K.

Signal amplitude and S/N measurements were also obtained at a variety of time constant and scan rate settings. For all scan rates tested, little improvement in S/N is observed at time constant settings above 0.1 s (Fig. 1.20). Signal amplitude decreases as the scan rate is increased from .1 MHz/s to 8 MHz/s (Fig. 1.21). S/N, however, increases with the same change in scan rate (Fig. 1.22). Little change in S/N is observed as the scan rate is increased beyond 1 MHz/s, though signal amplitude does diminish beyond that point, especially at higher time constant settings. Optimum scan rate and time constant settings appear to be about 0.1 s time constant and 1 MHz/s scan rate.
Figure 1.17 – Effect of change in microwave power on a) amplitude of ENDOR signal, and b) S/N of ENDOR spectra of VO(Im)$_4^{2+}$ ENDOR standard.
Figure 1.18 – Effect of change in RF power on a) amplitude of ENDOR signal, and b) S/N of ENDOR spectra of VO(Im)$_4^{2+}$ ENDOR standard.

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Figure 1.19 – Effect of temperature on a) amplitude of ENDOR signal, and b) S/N of ENDOR spectra of VO(Im)$_4^{2+}$ ENDOR standard.
Figure 1.20 – Effect of changes in time constant on S/N of ENDOR spectrum of VO(Im)$_2^{2-}$ ENDOR standard. S/N value normalized to time based on 0.1 MHz/s scan rate.
Figure 1.21 – Effect of scan rate on amplitude of VO(Im)\(_{4}^{2+}\) ENDOR signal.
Figure 1.22 – Effect of scan rate on S/N of ENDOR spectrum of VO(Im)$_2$$^{2+}$ ENDOR standard. S/N normalized to time based on 0.1 MHz/s scan rate.
CHAPTER 2

STUDY OF AN IRON NITROSYL COMPLEX OF TRANSFERRIN

Introduction

The transferrins are a family of proteins found in vertebrates. Their main function is the storage and transport of iron. The various transferrins are very similar in both their amino acid sequences and in their three-dimensional structure. Each protein is capable of binding two Fe$^{3+}$ cations and two carbonate anions. One cation/anion set is synergistically bound in each of two globular portions of the molecule usually referred to as the N- and C-lobes (see Introduction for a more detailed description of the protein and its binding sites) [18,19].

Although transferrin's highest affinity is for Fe$^{3+}$ and carbonate, many metal cations besides iron and many anions besides carbonate have been shown to bind to the protein [18]. Recently, studies of some iron-nitrosyl complexes of the protein have been reported [7,9]. The high external field EPR spectra of these complexes bear a strong resemblance to that of iron-nitrosyl complexes bound to imidazole and histidine [11,15], to an iron-nitrosyl complex of bacterioferritin [8], and to an iron-nitrosyl complex of human H-ferritin reported by Lee et al. [6]. In the latter case, the signal has been attributed to binding of the iron and nitric oxide to a histidine residue near one of the three-fold channels of that protein.
The actual composition and structure of the iron-nitrosyl complexes of the nonheme iron binding proteins, ferritin and transferrin, are not known. Work with iron-nitrosyl complexes and low molecular weight model compounds [11,14,15] suggests that these compounds consist of two nitric oxide ligands and two protein ligands bound to a central iron in a tetrahedral configuration. A +1 oxidation state and $d^7$ electronic configuration is postulated for the iron [10], giving it an electron spin of $S = 3/2$. Combined with the electron spin of $S = 1/2$ for each of the NO molecules, the complex exhibits a net electronic spin of $S = 1/2$ and a $g$ value around 2 [57].

Despite the electron spin echo envelope modulation (ESEEM) and ENDOR studies of dinitrosyl iron complexes with small molecules [14,15] and the ESEEM studies of some protein-dinitrosyl iron complexes [14], much uncertainty remains regarding the protein ligands and the structure of the complex in transferrin and ferritin. It was in hopes of gaining a further understanding of these complexes that this study was undertaken. In particular, the study of iron-nitrosyl complexes of serum transferrin was undertaken because the iron binding sites of the protein have been extensively studied and are very well characterized. In addition, site-directed mutants of the protein are also readily available.

**Materials and Methods**

Iron-free human serum transferrin of 100% stated purity was obtained from Calbiochem-Behring. Nonspecifically bound anions such as citrate were removed by dialysis against one liter of 0.1 M NaClO$_4$ (Aldrich) at pH 8.0 (three times). NaClO$_4$ was
removed from the protein solution by dialyzing four times against one liter of either Chelex treated 0.1 M 3-(N-morpholino) propane sulfonic acid hemi-sodium salt (MOPS buffer, Research Organics) at pH 7.5 or Chelex treated 0.1 M MOPS buffer and 25 mM Na₂CO₃ (Baker). The protein was then concentrated by ultrafiltration on an Amicon PM30 filter and the concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 8.89 x 10⁴ M⁻¹ cm⁻¹[58].

Fe(NTA) was prepared by dissolving 0.1422 g of nitrilotriacetic acid (NTA) in 50 mL of DI water and adjusting to pH 2. The NTA was then gradually added to 14 mL (1 mg/mL) of FeCl₃ AA standard (Fischer Scientific) while keeping the pH between 1.8 and 2.2 by adding 1M NaOH as needed. The final pH, prior to dilution to 50 mL, was 2.3. Following dilution, 1 mL aliquots of the Fe(NTA) were pipetted into plastic, microcentrifuge tubes and frozen in the dry ice freezer. The resulting solution was 12.10 mM in NTA and 5.014 mM in Fe³⁺.

Monoferric C-terminal transferrin (Fe₆Tf) was then prepared by adding enough iron as Fe(NTA) to a solution of apo-transferrin (Apo-Tf) to yield a final iron to protein ratio of 1:1 and allowing the mixture to incubate at room temperature for 20 minutes. The NTA was then removed by passing the protein solution through a Sephadex 25 column using 0.1 M NaClO₄, adjusted to pH 8, as the solvent. The sodium perchlorate was removed by dialyzing the protein solution four times against 0.1 M MOPS buffer and 25 mM sodium carbonate at pH 7.5.
The protein was then concentrated by ultrafiltration. Protein concentration was determined spectrophotometrically at 280 nm and at a pH of 2.0. The percent iron saturation was determined spectrophotometrically at 465 nm using an molar extinction coefficient of 2500 M\(^{-1}\)cm\(^{-1}\) [59]. The purity of the Fe\(_2\)Tf was determined by urea polyacrylamide gel electrophoresis [60]. The concentrated protein was stored at 77 K and thawed just before use.

Monoferric N-terminal transferrin (Fe\(_1\)Tf) was prepared by adding enough iron as Fe(NTA) to a solution of Apo-Tf to yield a final iron to protein ratio of 2.25:1 and allowing the mixture to incubate at room temperature overnight. Iron was then removed from the C-terminal binding site by diluting the protein-iron with a buffer containing MOPS, NaClO\(_4\), and pyrophosphate (Sigma) at pH 7.5 such that final concentrations in the solution were 50 µM protein, 0.1M MOPS, 0.5M NaClO\(_4\), and 1 mM pyrophosphate. Enough deferrioxamine (CIBA-GEIGY Ltd) was then added to complex 50% of the iron. This solution was allowed to stand for 3.5 hours at room temperature. The solution was then dialyzed against Chelex treated 0.1M MOPS and 25 mM Na\(_2\)CO\(_3\) at pH 7.5. The protein was then concentrated. The concentration, percent of iron saturation, and purity were determined as for Fe\(_2\)Tf. The concentrated protein was stored at 77 K and thawed just before use.

Diferric transferrin (Fe\(_2\)Tf) was obtained by incubating a solution of apo-Tf overnight at room temperature with enough Fe(NTA) to yield a solution with iron to protein ratio of 2.25:1. NTA was removed from the solution by dialysis against NaClO\(_4\).
at pH 8 three times. Sodium perchlorate was removed by dialysis against 0.1M MOPS and 25mM Na₂CO₃ at pH 7.5 four times. The protein was then concentrated and the protein concentration and purity of the product were determined as for the Fe₄Tf. Percent of iron saturation was determined spectrophotometrically using a coefficient of molar absorptivity of 5000 M⁻¹cm⁻¹ [59]. 50% iron saturated transferrin was obtained by adding enough ferrous sulfate to a solution of Apo-Tf to give a final iron to protein ratio of 1:1 and allowing the solution to incubate at room temperature overnight.

Recombinant N-terminal half-molecule of human serum transferrin (hTf/2N) [61] and two site-directed variants, H249Q and H249E [61], which had been used previously [62] and stored at 77 K, were reused. Iron was removed from the binding site using the dialysis method described by Harris [63] with the exception that in the final step the protein was dialyzed against 0.1 M MOPS buffer and 25 mM Na₂CO₃ at pH 7.5 (four times) instead of against water. No concentration step was necessary following dialysis.

NO gas was delivered to samples by either: 1) flushing with NO gas from a cylinder (after scrubbing the gas with a 0.1M MOPS buffer solution); 2) flushing with NO gas generated in a sidearm flask by reacting 1 M ferrous sulfate (J.T. Baker) with 1 M sodium nitrite (Mallinkrodt) in 2 M sulfuric acid (J.T. Baker); or 3) generating the NO gas in situ by adding sodium nitrite (Aldrich) and L-ascorbic acid (Aldrich) to the sample to yield final concentrations of 20 mM for both the nitrite and the ascorbate.

EPR spectra were obtained at 77 K using: a Varian E-4 spectrometer with a TE₁₀₂ single rectangular cavity and a quartz liquid nitrogen Dewar insert; or a laboratory
constructed spectrometer employing a Bruker microwave bridge with a TE_{104} dual rectangular cavity and a quartz liquid nitrogen Dewar insert. All EPR measurements were performed using calibrated quartz EPR tubes of approximately 4 mm o.d. and 3 mm i.d. Data manipulation was conducted on a 486 computer using EPRWare software (Scientific Software Services).

The spin concentration of the EPR active iron-nitrosyl-transferrin complexes was determined by comparing the double integrals of the first derivative EPR spectra of the complexes with that of a Cu^{2+} standard. The copper standard was prepared by adding enough EDTA, ascorbate, and NaClO₄ to Cu^{2+} AA standard and diluting with 3:1 water/glycerol solvent. This yielded a solution that was 0.25 mM in Cu^{2+}, 1 mM in EDTA, 0.01 M in ascorbate, and 3 M in NaClO₄. The appropriate g-factor correction was made to the EPR intensities [64].

Statistical analysis of data was performed using SAS software (SAS Institute, Inc.). Because the data in this nested experimental design was unbalanced, an ANOVA using type III sum of squares was employed [46].

Protein samples were usually about 0.1 mM in transferrin for EPR studies and about 2.5 mM in transferrin for ENDOR studies.

**Results**

**Effects of Sample Preparation Methods on Complex**

A variety of sample preparation methods were tested: 1) three methods of
generating/delivering NO gas to the sample were examined; 2) the order of addition of Fe$^{3+}$ and NO to the sample was varied; and 3) MOPS buffer solutions were prepared with and without added carbonate. EPR spectra of the resulting samples were then obtained. The double integrals of the signals around \( g = 2 \) which arose from the iron-nitrosyl complex were then calculated (e.g. Fig. 2.1). The integrals were corrected for variations in sample tube size, instrumental conditions, and transferrin concentration. The lineshapes of the spectra and their corrected double integrals were then compared. Table 2.1 lists the corrected integral values for a variety of sample preparation conditions. A standard was not run during these crude preliminary studies, so whether or not an interaction exists between the time the spectra were collected and any of the other variables cannot be ascertained. However, an ANOVA performed on the data shows no statistical difference, at the 95% confidence level, between the results obtained on different days \( (F = 0.36, df = 2, P = 0.7041, \text{Table } 2.2) \).

NO gas was delivered to the sample in one of three ways: 1) by passing NO gas from an NO gas cylinder through a buffer solution and then flushing the sample vessel with the buffered gas; 2) by generating NO gas in a sidearm flask via the reaction of Fe$^{3+}$ and sodium nitrite (Equation 2.1); or 3) by generating NO gas \textit{in situ} via the reaction of sodium nitrite or sodium nitrate with ascorbic acid.

\[
2 \text{NaNO}_2 + 2 \text{FeSO}_4 + 3 \text{H}_2\text{SO}_4 \rightarrow \text{Fe}_2(\text{SO}_4)_3 + 2 \text{NaHSO}_4 + 2 \text{H}_2\text{O} + 2 \text{NO} \quad (2.1)
\]
Figure 2.1 EPR Spectrum of Fe-NO-Transferrin prepared in (a) MOPS buffer and (b) MOPS/Na2CO3 buffer. Experimental Conditions: Field set, 3200 G; scan range, 1000 G; time constant 0.3 s; scan time, 8 min; modulation amplitude, 10 G; modulation frequency 100 kHz; receiver gain, (a) 800, (b) 100; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.15 GHz. File names: (a) 2apr95c.fls; (b) 1apr95c.fls
Table 2.1 - Effects of buffer, order of addition of reactants, and gas source on double integral of g–2 signal.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Order of Addition of Reactants</th>
<th>NO Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tf-NO-Fe</td>
<td>Tf-Fe-NO</td>
</tr>
<tr>
<td>MOPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.70 \times 10^6^a$</td>
<td>$1.77 \times 10^6^a$</td>
</tr>
<tr>
<td></td>
<td>$2.03 \times 10^6^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$1.25 \times 10^6^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$2.43 \times 10^6^b$</td>
<td>$1.45 \times 10^6^c$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.04 \times 10^6^b$</td>
</tr>
<tr>
<td>MOPS/Na$_2$CO$_3$</td>
<td>$1.02 \times 10^6^c$</td>
<td>$1.71 \times 10^6^c$</td>
</tr>
<tr>
<td></td>
<td>$2.71 \times 10^6^c$</td>
<td>$1.12 \times 10^6^c$</td>
</tr>
<tr>
<td></td>
<td>$1.82 \times 10^6^b$</td>
<td></td>
</tr>
</tbody>
</table>

Experimental Conditions: [MOPS] = 0.1 M; [Na$_2$CO$_3$] = 0.25 mM; [Transferrin] ~ 0.1 mM; [Fe$^{2+}$] ~ 0.2 mM

*a* Spectra obtained on 2 April 1995  
*b* Spectra obtained on 26 March 1995  
*c* Spectra obtained on 22 March 1995
Table 2.2 – Calculated quantities and critical values used in ANOVA of spectral data.

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>2</td>
<td>$3.04 \times 10^{11}$</td>
<td>$1.52 \times 10^{11}$</td>
<td>0.36</td>
<td>0.7041</td>
</tr>
<tr>
<td>Buffer</td>
<td>1</td>
<td>$1.11 \times 10^{11}$</td>
<td>$1.11 \times 10^{11}$</td>
<td>0.25</td>
<td>0.6368</td>
</tr>
<tr>
<td>Order</td>
<td>1</td>
<td>$8.52 \times 10^{11}$</td>
<td>$8.52 \times 10^{11}$</td>
<td>1.89</td>
<td>0.2179</td>
</tr>
<tr>
<td>Buffer*Order</td>
<td>1</td>
<td>$6.26 \times 10^{10}$</td>
<td>$6.25 \times 10^{10}$</td>
<td>0.14</td>
<td>0.7220</td>
</tr>
<tr>
<td>Source(Buffer*Order)</td>
<td>2</td>
<td>$3.28 \times 10^{11}$</td>
<td>$1.64 \times 10^{11}$</td>
<td>0.36</td>
<td>0.7089</td>
</tr>
</tbody>
</table>

* Degrees of freedom
b Probability of failure to reject the null hypothesis
c Date spectra were collected
d Buffer systems used
e Order of addition of reactants
f Interaction between buffer system and order of addition of reactants
g Source of NO gas nested within the group Buffer*Order

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The results from two sources of NO gas (from a commercially obtained cylinder and from generation in a sidearm flask) were analyzed. ANOVA of the data shows no statistical difference, at the 95% confidence level, due to source (F = 0.36, df = 2, P = 0.7089, Table 2.2). Further, spectral lineshape was unaffected by any of the three methods of NO gas generation/delivery.

The *in situ* generation method was selected for all subsequent preparations because: 1) of the potential danger of NO gas diffusing out of the hood into the lab when the cylinder was used; 2) the method of generating NO gas in a sidearm flask was particularly prone to oxygen contamination; and 3) no statistical difference in amount of product formed could be observed from day to day.

The iron-nitrosyl complex of transferrin was generated by addition of Fe$^{2+}$ as FeSO$_4$·7H$_2$O to an aqueous solution of the protein in the presence of NO gas under anaerobic conditions. Because it was not known whether complex formation was thermodynamically or kinetically controlled, the order of addition of the iron and nitric oxide was varied. An effect on the product due to the order of addition of the reagents would suggest that formation was kinetically controlled (i.e. dependent on the concentration of at least one of the reagents). The absence of an effect on product formation, on the other hand, would suggest that product was a thermodynamically favored species. The values of the double integrals of EPR signals obtained from samples prepared in two different sequences are listed in Table 2.1. The results are highly
variable; however, ANOVA of the data reveals no statistical difference, at the 95% confidence level, due to the order of addition of the reactants (F = 1.89, df = 1, P = 0.2179, Table 2.2).

Two buffer systems were tested, 0.1 M MOPS at pH 7.5, and 0.1 M MOPS with 25 mM Na$_2$CO$_3$ also at pH 7.5. Again, ANOVA of the data shows no statistical difference, at the 95% confidence level, on signal intensity due to buffer type (F = 0.25, df = 1, P = 0.6368, Table 2.2).

ANOVA further reveals that, at the 95% confidence level, there are no statistical differences due to interactions between the crossed factors of buffer type and order of addition of reactants (F = 0.14, df = 1, P = 0.7220, Table 2.2).

Qualitative information regarding lineshape may also be gleaned from these spectra. A comparison of spectra obtained using these different buffering systems shows that the presence of the carbonate anion has a definite effect on the lineshape of the spectra (see Fig. 2.1). The splitting on the peak maxima is more symmetrical in the MOPS buffer and more asymmetrical in the MOPS/Na$_2$CO$_3$ system. The position of the shoulder—which appears in the line between the positive peak maxima and the negative peak minima—shifts from well below the base line in the MOPS buffer, to very near the base line in the MOPS/Na$_2$CO$_3$ buffer.

The g = 2 signals are observed only in the presence of both iron and nitric oxide and their lineshapes in the presence of NO and iron—under anaerobic conditions—are affected by the buffer solution used in the experiment. Because the buffer system with
carbonate more closely approximates the natural conditions in which transferrin reactions normally occur, most of the preliminary studies were conducted in this medium.

**Presence of \( g = 4.3 \) Signal**

It should be noted that a small \( g = 4.3 \) signal was observed in virtually all of the samples studied (Fig. 2.2) with the exception of those samples to which no \( \text{Fe}^{2+} \) was added. This signal is due to \( \text{Fe}^{3+} \) bound to the protein at the specific iron binding sites. Because iron was added to the samples under anaerobic conditions as \( \text{Fe}^{2+} \), this result was surprising. A series of experiments was conducted to determine whether or not oxygen was contaminating the system and to attempt to eliminate the undesired high-spin \( \text{Fe}^{3+} \) signal. Samples, reagents, and syringes were thoroughly flushed with Argon gas and a positive pressure was maintained in the sample and reagent vessels to minimize the opportunity for oxygen contamination. The order of addition of the reagents was varied and samples were prepared in a variety of vessels, including the sample EPR tubes. When careful attention to these details failed to eliminate the undesired signal, the following attempts were made to chemically remove oxygen from the system by adding to the system: 1) \( \text{Fe}^{3+} \) as \( \text{Fe(NTA)} \); 2) glucose, glucose oxidase, and catalase; 3) dithionite; or 4) thioglycolic acid (TGA). Addition of iron as \( \text{Fe(NTA)} \) resulted in the loss of the \( g = 2.0 \) signal and altered the shape, but did not eliminate the \( g = 4.3 \) signal (see Fig. 2.2). Addition of glucose, glucose oxidase, and catalase diminished the size of the \( g = 2.0 \) signal and altered the signal shape (see Fig. 2.3). Addition of dithionite altered the shape of both
Figure 2.2 - EPR Spectrum of Fe-NO-Transferrin complex prepared with NTA.
Experimental Conditions: field set, 2000 G; scan range, 4000 G; time constant, 0.3 s; scan time, 8 min; modulation amplitude, 10 G; modulation frequency, 100 kHz; receiver gain, 800; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.15 GHz. File name: 4may95a.fls
Figure 2.3 - EPR Spectrum of Fe-NO-Transferrin complex prepared with glucose, glucose oxidase, and catalase. Inset: Close up of g ~ 2 signal. Experimental Conditions: Field set, 2100 G; scan range, 4000 G; time constant, 0.3 s; scan time, 500 s; modulation amplitude, 28 G; modulation frequency, 100 kHz; receiver gain, 0.1 V; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.31 GHz. File name: lau95e.fls (EWB).
the $g = 4.3$ and the $g = 2.0$ signal (see Fig. 2.4). Addition of TGA resulted in altered signal shapes for both signals (see Fig. 2.5). None of the chemicals tested were successful in eliminating the high-spin Fe$^{3+}$ signal. The $g = 2.0$ signal was still observable and measurable in regularly prepared samples despite the presence of the $g = 4.3$ signal, however, and so no further attempts were made to eliminate the $g = 4.3$ signal.

Although changes in the lineshape of the EPR signal at $g = 4.3$ of lactoferrin due to the presence of NO under aerobic conditions have been reported [9], such effects were not observed in the regularly prepared anaerobic samples of this study. Such changes occurred only when additional reagents were added to the sample in an attempt to remove oxygen. The persistence of the $g = 4.3$ signal may be due to oxygen contamination of the Teflon tip of the syringe plungers (these were exposed to the atmosphere prior to use). Alternatively, the iron may be oxidized as the nitric oxide is formed, or may be oxidized by the nitric oxide itself, or by the protein. Preparation of the samples both in a glove box under a nitrogen or argon environment, and in a completely aerobic environment, would help to determine the source of the Fe$^{3+}$ signal. This study focused primarily on the signals observed around $g = 2$ under anaerobic conditions, and so no detailed examination of the $g = 4.3$ signal was conducted.

**Measurement of the g-factor**

The g-factors for the rhombic iron-nitrosyl-transferrin complex were calculated using the spectrum of a 3.94 mM transferrin sample prepared in 0.1 M MOPS buffer
Figure 2.4 - EPR Spectrum of Fe-NO-Transferrin complex prepared with dithionite. Experimental Conditions: field set, 2100 G; scan range, 4000 G; time constant, 0.3 s; scan time, 500 s; modulation amplitude, 28 G; modulation frequency, 100 kHz; receiver gain, 0.3 V; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.31 GHz. File name: 26jul95m (EWB).
Figure 2.5 - EPR Spectrum of Fe-NO-Transferrin complex prepared with TGA. Inset: Close up of $g \sim 2$ signal.

Experimental Conditions: Field set, 2100 G; scan range, 4000 G; time constant, 0.3 s; scan time, 500 s; modulation frequency, 100 kHz; modulation amplitude, 28 G; receiver gain, 0.1 V; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.31 GHz. Filename: b9au95b.fls (EWB)
which had been treated anaerobically with 2 equivalents of Fe$^{2+}$ (added as a 0.01050 M solution of FeSO$_4$$\cdot$7H$_2$O) and NO gas (generated in situ with 1 M L-ascorbic acid and 1 M NaNO$_3$). A DPPH standard ($g = 2.0037$) was used to determine the magnetic field strength. The frequency was determined using a Hewlett Packard Model #5350A frequency counter. The calculated g-factor from this spectrum was then used to correct the magnetic field in calculating the g-factors of a spectrum obtained from a sample prepared in MOPS/Na$_2$CO$_3$ buffer. The spectra and g-factors are shown in Fig. 2.1. The calculated g-factors are tabulated in Table 2.3.

The g-factors for this rhombic iron-nitrosyl complex of transferrin have been reported by Drapier and co-workers [7]. The values of 2.055 and 2.015 reported by Drapier for $g_x$ and $g_z$, respectively, differ only in the third decimal place from those obtained in this study. Drapier's reported $g_y$ value of 2.035, however, was not properly calculated. A value of $g_y = 2.028$ was obtained in this study for spectra of samples prepared both with and without carbonate in the buffer.

Dependence of the Complex on Iron Concentration

The effect of iron concentration on complex formation was tested by preparing apotransferrin samples to which varying amounts of iron as FeSO$_4$$\cdot$7H$_2$O was added anaerobically. NO gas was then generated in situ and the sample was allowed to incubate for 15 minutes under anaerobic conditions. Samples were then quickly frozen in a dry-ice/acetone slurry and transferred to liquid nitrogen for storage. EPR spectra were

69
Table 2.3 — Calculated g-factors for iron-nitrosyl complex of Transferrin.

<table>
<thead>
<tr>
<th></th>
<th>Sample Prepared with Carbonate</th>
<th>Sample Prepared without Carbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_x$</td>
<td>2.052</td>
<td>2.051</td>
</tr>
<tr>
<td>$g_y$</td>
<td>2.028</td>
<td>2.028</td>
</tr>
<tr>
<td>$g_z$</td>
<td>2.013</td>
<td>2.011</td>
</tr>
</tbody>
</table>

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collected and the spin concentration of both the high spin iron-transferrin complex and the low spin iron-nitrosyl complex were calculated (see Table 2.4).

There was only a slight increase in the concentration of the iron-nitrosyl complex as up to 10 protein equivalents of iron is added (Fig. 2.6). Overall, there was about a 10% increase in the number of occupied iron binding sites as up to 10 protein equivalents of iron were added (Table 2.4). Interestingly, the increase in the iron nitrosyl signal is accompanied by a slight decrease in the high spin iron signal. Taken together, these observations suggest that the iron nitrosyl complex forms only in very small quantities, perhaps at the expense of the Fe$^{3+}$ transferrin complex.

**Time Dependence of the Complex**

The growth and persistence of the iron-nitrosyl-transferrin complex was tested in two ways. First a large sample was prepared anaerobically and NO gas was generated *in situ*. Aliquots were removed at specified times as the reaction progressed. These aliquots were then placed in purged EPR tubes, frozen immediately in dry-ice/acetone, and transferred to liquid nitrogen. EPR spectra of the frozen aliquots were obtained and the integrals of the signal corrected for differences in tube size and instrument conditions. The corrected intensity was then plotted against reaction time. Fig. 2.7 indicates that a maximum for both the $g = 4.3$ and $g = 2.0$ signal was obtained at 2.75 minutes. Interestingly, the ratio of the signal intensities (low spin:high spin) reached a maximum at 2.75 minutes as well (Table 2.5.). The low spin signal decreased over time to 23% of its
Table 2.4 – Effect of added iron on percent of transferrin iron-binding sites occupied.

<table>
<thead>
<tr>
<th>Fe^{2+} Added (# equivalents)</th>
<th>Conc. of Added Iron (mM)</th>
<th>Concentration of Transferrin Conc. (mM)</th>
<th>g = 4.3 Spin % Binding Sites Occupied by g = 4.3 Signal</th>
<th>g ~ 2 Spin % Binding Sites Occupied by g ~ 2 Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.1971</td>
<td>0.1008</td>
<td>0.1163</td>
<td>57.69</td>
</tr>
<tr>
<td>4</td>
<td>0.3869</td>
<td>0.1008</td>
<td>0.1149</td>
<td>56.99</td>
</tr>
<tr>
<td>7</td>
<td>0.7463</td>
<td>0.1008</td>
<td>0.0983</td>
<td>48.76</td>
</tr>
<tr>
<td>9</td>
<td>0.9166</td>
<td>0.1008</td>
<td>0.1143</td>
<td>56.70</td>
</tr>
</tbody>
</table>
Figure 2.6 – Effect of added iron on concentration of transferrin complexes.
Figure 2.7 – Effect of elapsed time on signal intensity of transferrin complexes.
Table 2.5 – Effect of elapsed time on ratio of signal intensities for transferrin complexes.

<table>
<thead>
<tr>
<th>Elapsed Time (Min.)</th>
<th>% of Maximum Signal (g ~ 2)</th>
<th>% of Maximum Signal (g = 4.3)</th>
<th>g ~ 2 Signal as % Of g = 4.3 Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.58</td>
<td>62</td>
<td>92</td>
<td>7.2</td>
</tr>
<tr>
<td>1.75</td>
<td>48</td>
<td>95</td>
<td>6.0</td>
</tr>
<tr>
<td>2.75</td>
<td>100</td>
<td>100</td>
<td>11.3</td>
</tr>
<tr>
<td>5.58</td>
<td>64</td>
<td>73</td>
<td>8.0</td>
</tr>
<tr>
<td>11.17</td>
<td>37</td>
<td>70</td>
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<td>18.58</td>
<td>35</td>
<td>92</td>
<td>4.4</td>
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<tr>
<td>45.58</td>
<td>31</td>
<td>97</td>
<td>3.6</td>
</tr>
<tr>
<td>80.00</td>
<td>23</td>
<td>89</td>
<td>3.0</td>
</tr>
</tbody>
</table>
maximum. The high spin signal, on the other hand, decreased to only 70% of its maximum signal. After having determined the optimal incubation time for sample preparation, the persistence of the signal when subjected to freezing and thawing was tested. The EPR spectrum of a sample which had been allowed to incubate for 1 min 45 seconds was obtained. The sample was thawed for one minute, refrozen, and a new spectrum obtained. The same sample was subjected to 12 freeze/thaw cycles with thaw times of varying length. The changes in peak height with each freeze/thaw cycle are shown graphically in Fig. 2.8. The decrease in signal amplitude with increasing incubation times observed in the previously described time study experiment suggested that a decrease in signal could be expected with increased thawing time. Indeed, this initially appeared to be the case. When thawing time was increased to five minutes, however, giving a total thaw time of 43 minutes, the signal actually increased until, at a total thawing time of 73 minutes, it had reached 140% of the original peak height.

Site Specificity of the Complex

During the sample preparation studies it was determined that no low spin iron signal was observed in the absence of either the protein or the nitric oxide. Experiments were then conducted in an attempt to determine whether the complex was forming at the iron binding sites or whether the iron-nitrosyl complex was binding non-specifically elsewhere on the protein. Recombinant N-terminal half-molecules of human serum transferrin (hTf2N) and two site-directed variants—H249Q and H249E—were prepared
Figure 2.8 – Effect of thawing time on signal amplitude of transferrin complexes.
and treated with NO gas generated in situ, followed by the addition of Fe$^{2+}$ anaerobically. The samples were then placed in purged EPR tubes, frozen in a dry ice/acetone slurry, and placed in liquid nitrogen. EPR spectra of the samples were obtained (Figs. 2.9 - 2.11) and the concentration of the protein-bound iron-nitrosyl complex determined. The calculated spin concentrations for the iron-nitrosyl-transferrin complex so formed, and the percent of binding sites filled by the nitrosylated iron, are listed in Table 2.6. The lineshape of the $g = 4.3$ signal was altered showing the change in the protein-ligand environment at the binding site. The $g = 2$ signal lineshape was unchanged though the signal amplitude was significantly larger indicating that a greater amount of the nitrosylated iron was bound to the protein variants than to the native protein. In view of that fact, it seems unlikely that the His residue, which usually acts as a ligand for Fe$^{3+}$, is necessary for the binding of nitrosylated iron.

Experiments were also conducted to determine if the complex formed preferentially at the N-terminal or C-terminal binding sites. Samples of human serum transferrin were treated to obtain protein selectively loaded with iron in either the N-terminal binding site (Fe$_N$Tf) or the C-terminal binding site (Fe$_C$Tf). Protein samples fully loaded with iron in both binding sites (Fe$_T$Tf) were also prepared. These samples were then treated with nitric oxide gas, generated in situ, followed by the addition of Fe$^{2+}$ under anaerobic conditions. The samples were then placed in purged EPR tubes, frozen in a dry ice/acetone slurry, and transferred to liquid nitrogen. EPR spectra of the samples were obtained (Fig. 2.12 - 2.14) and the concentration of the iron-nitrosyl complex was
Figure 2.9 – EPR spectrum of Fe-NO-Transferin complex prepared with native N-terminal half molecule. Inset: Close up of $g \sim 2$ signal. Experimental Conditions: field set, 2000 G; scan range, 4000 G; time constant, 0.3 s; scan time, 8 min; modulation amplitude, 10 G; modulation frequency, 100 kHz; receiver gain, 800; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.15 GHz. File name: 22aug95a.fls
Figure 2.10 - EPR Spectrum of Fe-NO-Transferrin Complex prepared with H249E variant. Inset: close up of $g \sim 2$ signal. Experimental Conditions: field set, 2000 G; scan range, 4000 G; time constant, 0.3 s; scan time, 8 min; modulation amplitude, 10 G; modulation frequency, 100 kHz; receiver gain, 800; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.15 GHz. File name: 22aug95b.fls
Figure 2.11 - EPR Spectrum of Fe-NO-Transferrin complex prepared with H249Q variant. Inset: close up of $g \sim 2$ signal. Experimental Conditions: field set, 2000 G; scan range, 4000 G; time constant, 0.3 s; scan time, 8 min; modulation amplitude 10 G; modulation frequency, 100 kHz; receiver gain, 150; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.15 GHz. File name: 22aug95d.fls
Table 2.6 - Changes in signal intensity of Fe-NO- transferrin complex and percent of binding sites occupied by the complex as a function of protein variant.

<table>
<thead>
<tr>
<th>Protein Variant</th>
<th>Protein Conc. (mM)</th>
<th>Spin Conc. of Iron-NO Complex (mM)</th>
<th>% Binding Sites Filled W/Iron-NO Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.1586</td>
<td>0.0246</td>
<td>15.5</td>
</tr>
<tr>
<td>H249E</td>
<td>0.1305</td>
<td>0.0823</td>
<td>63.0</td>
</tr>
<tr>
<td>H249Q</td>
<td>0.1431</td>
<td>0.1196</td>
<td>83.2</td>
</tr>
</tbody>
</table>

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Figure 2.12 - EPR Spectrum of Fe-NO-Transferrin complex prepared with Fe$_n$Tf.
Insert: close up of $g \sim 2$ signal. Experimental Conditions: field set, 2100 G; scan range, 4000 G; time constant, 0.3 s; scan time, 500 s; modulation amplitude 28 G; modulation frequency, 100 kHz; receiver gain, 0.03 V; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.31 GHz. File name: bl5au95c.fls (EWB)
Figure 2.13 - EPR Spectrum of Fe-NO-Transferrin complex prepared with Fe₃Tf. Insert: close up of $g \sim 2$ signal. Experimental Conditions: field set, 2100 G; scan range, 4000 G; time constant, 0.3 s; scan time, 500 s; modulation amplitude 28 G; modulation frequency, 100 kHz; receiver gain, 0.03 V; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.31 GHz. File name: b14au95c.fls (EWB)
Figure 2.14 - EPR Spectrum of Fe-NO-Transferrin complex prepared with Fe$_2$Tf.  
Insert: close up of g $\sim$ 2 signal. Experimental conditions: field set, 2100 G; scan range, 4000 G; time constant, 0.3 s; scan time, 500 s; modulation amplitude 28 G; modulation frequency, 100 kHz; receiver gain, 0.03 V; temperature, 77 K; microwave power, 10 mW; microwave frequency, 9.31 GHz. File name: bl4au95b.fls (EWB)
calculated. The spin concentrations calculated for each sample type, and the percent of binding sites occupied represented by these concentrations, are listed in Table 2.7. Clearly the complex is seen to form preferentially in the Fe₅Tf samples, that is in those samples with filled N-terminal binding sites and empty C-terminal binding sites, implying that the NO complex is formed at the C-terminal site.

**ENDOR Study of the Complex**

In an effort to determine the structure of the iron-nitrosyl complex under study, a concentrated sample (~3.9 mM transferrin with two equivalents of iron under ~1 atm of NO) was prepared for analysis by Electron Nuclear Double Resonance (ENDOR) spectroscopy. As the temperature was lowered to 3.5 K an ENDOR signal began to emerge. A difference spectrum was obtained by plotting the difference between the ENDOR signals obtained with the B₁ field set at EPR resonance, and that obtained off EPR resonance. The trace of this difference spectrum following a 25 point software smoothing appears in Fig. 2.15. At temperatures above 4 K only a possible hint of the matrix proton signal near 14 MHz could be seen. When the temperature is lowered to 3.6 K the proton matrix line became more pronounced with a suggestion of fine structure to it. The proton matrix line was centered at 13.747 MHz, with features observable at 12.157 MHz, 13.038 MHz, 14.329 MHz, and 15.483 MHz.
Table 2.7 – Effect of initial iron loading on spin concentration of Iron-NO complex and on percent of binding sites occupied by the complex.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein Conc. (mM)</th>
<th>Spin Conc. of Iron-NO Complex (mM)</th>
<th>% Binding Sites Filled w/Iron-NO Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe₅Tf</td>
<td>0.0945</td>
<td>0.0119</td>
<td>6.3</td>
</tr>
<tr>
<td>Fe₇Tf</td>
<td>0.0959</td>
<td>0.0792</td>
<td>41.3</td>
</tr>
<tr>
<td>Fe₂Tf</td>
<td>0.0909</td>
<td>0.0123</td>
<td>6.8</td>
</tr>
</tbody>
</table>
Figure 2.15 - ENDOR Spectrum of Fe-NO-Transferrin complex. Experimental conditions: field set, 3220 G; microwave power, 5 mW; microwave frequency, 9.15 GHz; time constant, .03 s; receiver gain, 10000; modulation frequency, 10 kHz; modulation amplitude, 1.50 V; RF field center, 10 MHz; RF scan range, 19.5 MHz; scan rate, .5 MHz/s; RF power (PTS), 0.4 V; temperature, 3.46 K. Filename: (3au95b.fls)-(3au95c.fls)
Discussion

The concentration of the iron-nitrosyl-transferrin complex changes very little as the starting concentration of Fe^{3+} is increased from two protein equivalents to 10. Likewise, product concentration is unaffected by the order of addition of reactants, which suggests that product formation is not a kinetically controlled process. The fact that only about 20% of the potential binding sites on the protein are filled with the nitrosylated iron, coupled with the observation that product concentration decreases to 23% of its maximum over the course of an hour, suggests that it is a weakly bonded complex whose equilibrium lies towards the reactant side.

The conclusion that the complex is forming at the iron binding sites on the protein is supported by the results of the studies conducted with the mono- and diferric transferrin molecules and with the protein variants. The complex is seen to form preferentially on the Fe_{n}Tf monoferric protein. That the complex is forming at the C-terminal site, rather than NO simply adding to the iron in the N-terminal site, is indicated by the results of studies done with the diferric protein in which very little product is formed. Apparently an empty binding site, preferably the C-terminal binding site in the full protein, is required for product formation.

The N- and C-terminal binding sites are not equivalent; the C-terminal site binds Fe^{3+} more strongly and releases it less readily than the N-terminal site [5]. If the iron-nitrosyl complex is in fact a weakly bound one (as suggested by the time studies described above), then it is possible that it persists somewhat longer at the C-terminal site due to the
site's stronger binding ability. Further studies involving the titration of Fe\textsubscript{NTf} and Fe\textsubscript{Tf} with Fe\textsuperscript{2+} in the presence of NO under anaerobic conditions might give more quantitative insight into the site specificity of the complex.

The small change in lineshape of the g = 2 signal with addition of carbonate to the buffer further suggests that the complex is binding at the iron binding sites and that carbonate affects, but is not required, for the binding. No rigorous attempts were made to prevent carbonate from forming in the samples to which it was not added, so it is unlikely that carbonate was completely absent from those samples. However, the small change in lineshape of the spectrum when carbonate is added to the solution suggests that it alters the binding site in some fashion, perhaps by binding nearby. Other non-synergistically bound anions have been shown to perturb the EPR spectra of transferrins [65,66].

In the absence of nitric oxide, a carbonate anion binds synergistically at the iron binding site and coordinates to Fe\textsuperscript{3+} through two of its oxygen atoms. Four amino acids, one Asp, one His, and two Tyr, complete the coordination sphere giving a distorted octahedral structure [18]. Previous studies of iron-nitrosyl complexes, however, suggest a dinitrosyl-iron complex with tetrahedral geometry [2,15]. In particular, studies conducted by Wang of a dinitrosyl-iron-imidazole complex [15] give an EPR spectrum which is similar to the nitrosyl-iron-ferritin spectrum observed by Lee [6], the nitrosyl-iron-transferrin spectrum observed by Drapier [7], and to the nitrosyl-iron-transferrin spectrum observed in these studies in the absence of added carbonate. The dinitrosyl iron complex is described as having a central iron atom with a +1 valence and a d\textsuperscript{7} electron configuration.
The iron is bound to two Lewis base ligands and the unpaired electron in each of the NO molecules $2p\pi^*$ orbital is antiferromagnetically coupled with an electron from iron, producing a complex with a total electron spin of 1/2 and a g value around 2 [2].

How, exactly, the carbonate anion is involved is difficult to say. If the complex is a dinitrosyl, tetrahedral one, then two of the coordination sites on the Fe are occupied by the NO radicals. It is likely that one of the nitric oxide radicals is substituting for the synergistically bound carbonate anion. A cyanide adduct of human serum transferrin has been reported [34,35,67] with an EPR spectrum exhibiting $g_x = 2.3$, $g_y = 2.15$, and $g_z = 1.92$. This adduct also forms preferentially in the C-terminal lobe of the protein. They suggest that the synergistic carbonate anion initially facilitates the binding of the iron-cyanide complex, but is then displaced from the first-coordination sphere by the cyanide [67]. Perhaps a similar mechanism is operating here.

The EPR signal intensity increases with the H249E variant (a change from histidine to glutamate) and the even greater increase is observed with the H249Q variant (a change from histidine to glutamine). These observations, together with the lack of a change in lineshape of the spectrum with the mutations, indicates that His 249 is not a ligand in this case.

Possible ligands for this site include: 1) one, or both, of the two tyrosines usually bound to the iron; 2) the aspartate usually bound to the iron cation; 3) one, or both, of the oxygens from a synergistically bound carbonate anion; or 4) another amino acid in the binding pocket that does not usually bind the iron cation. Further studies with different
variants which target the tyrosine and aspartate ligands would be helpful in more clearly defining the two protein ligands to the iron. Such variants have now become available.

An ENDOR study of this complex could also prove very useful in determining its exact structure and composition. Spectra of dinitrosyl-iron complexes bound to small organic molecules have been observed [11,14] including some studies performed on the ENDOR spectrometer described in Chapter 1 [15]. These latter studies were performed on samples with concentrations typically in the 20-30 mM range. Unfortunately it is difficult to concentrate transferrin much beyond about 3 mM. The emergence of a matrix proton line at temperatures below 3.6 K is, however, encouraging. With modifications to the liquid helium pumping system, much lower temperatures should be achievable. This, in turn, might permit the observation of the nitrogen coupling signals which could provide the necessary clues for definitively solving the mystery of nitrosylated iron binding to non-heme proteins.
LIST OF REFERENCES
REFERENCES


54 C. Bender, EPR Newslett, 5(3) (1934) 6.


APPENDIX 1

CABLE CONNECTIONS FOR THE ENDOR SPECTROMETER

1. A 50 conductor, shielded Telco cable with drain wire and 50-pin Telco connectors at each end runs from the 50 pin jack on the back of the A/D converter box attached to the computer to the 50-pin connector on the back of the PTS frequency synthesizer.

2. An RG58 coaxial cable with BNC connectors runs from the output BNC jack on the front of the PTS to the frequency synthesizer input jack on the front of the FM modulator/mixer.

3. An RG58 coaxial cable with an Amphenol connector on one end and a BNC connector on the other end runs from jack J407 (Amphenol) on the back of the low frequency modulation unit of the E-9 console to the modulation input jack (BNC) on the front of the FM modulator/mixer.

4. An RG58 coaxial cable with a BNC connector on one end and an N-type connector on the other end runs from the FM modulated RF output jack (BNC) on the front of the FM modulator/mixer to the RF power amplifier input jack (N-type).

5. An RG58 coaxial cable with a BNC connector on one end and an N-type connector on the other end runs from the output jack (N-type) of the RF power amplifier to the top of the ENDOR coil (BNC screw in connector on top stack of cavity).

6. An RG58 coaxial cable with a BNC connector on one end and an N-type connector on the other end runs from the ENDOR coil (BNC screw in connector on the bottom stack of the cavity) to the 50 ohm dummy load (N-type connector).
APPENDIX 2

TUNING THE ENDOR SPECTROMETER

1. Make sure the transformer and the circuit breaker for the E-9 power line are on.

2. Set the microwave power to 0.01 mW.

3. Turn the power leveler switch on the microwave bridge to on.

4. Set the bridge to tune.

5. Increase the microwave power to between 2 and 5 mW so that you can see the dip clearly.

6. Adjust the frequency so that the dip is centered on the oscilloscope screen.

7. Adjust the sample position so that no shoulder or indentation is visible on the dip curve.

8. Adjust the slide screw tuner to maximize the depth of the dip and minimize its width. In general, the shape of the curve should be rounded on both sides at the top and the dip should reach down to the baseline and be as narrow as possible. The dip is not symmetric. One side is usually narrower and shorter than the other.

9. Turn the reference arm on. Adjust the phase to get as rounded and symmetric a dip curve as possible. NOTE: the bottom of the dip will not reach to the baseline when the reference arm is on. It will, however, return to baseline when the reference arm is switched off again.

10. Turn the reference arm off and lower the power to 0.05 mW.

11. Set the bridge to operate.

12. Adjust the frequency to zero the AFC output meter.

13. Increase the power to 0.05 mW.
14. Turn the reference arm on.

15. Adjust the frequency to zero the AFC output meter.

16. Increase the microwave power to the desired setting while adjusting the screw knob on the slide screw tuner and/or the phase control on the bridge to keep the detector current below 250 microamps. The frequency may also need to be adjusted slightly.
COLLECTING EPR SPECTRA WITH THE ENDOR CAVITY

1. Tune the spectrometer according to tuning instructions in appendix 1.

2. Dial in the appropriate magnetic field setting and scan width for the sample being measured.

3. On the System Function Selector module: a) turn the cavity modulation switch to low for cavity 1; b) turn the oscilloscope monitor and recorder input switches to low frequency. Make sure the switch box on the right hand side of the console is set to low frequency as well.

4. Set the time constant at 0.30 s for a 4 or 8 minute scan.

5. Set the modulation amplitude to 32 G.

6. Set the modulation frequency to 1 kHz.

7. Adjust the phase knob as needed (usually around 0 or 180 degrees).

8. Set the receiver gain to the appropriate setting for the sample being measured – keep a close eye on the receiver level. The receiver gain should be set low enough to keep the receiver level needle below 5. **NOTE:** Usually the receiver gain cannot be set much higher than 2,000 in EPR mode.
1. Before doing anything else, make sure the modulation amplitude on the E-9 console is NOT SET ANY HIGHER THAN 3.2 G.

2. Set the time constant to a level compatible with the ENDOR scan rate you intend to use (0.1 s and 1 MHz/s works well).

3. Set the modulation amplitude on the E-9 console to 3.2 G.

4. Set the modulation frequency to 10 kHz.

5. Set the cavity modulation to Cavity II – Low.

6. Set the oscilloscope monitor and receiver input on Low.

7. Adjust the phase as needed to maximize the ENDOR signal (usually around 0 or 180 degrees).

8. Set the receiver gain to an appropriate setting.

9. Adjust the output on the PTS to obtain needed RF power (keep an eye on the meter on the RF amplifier).

10. Adjust the voltage on the modulator/mixer to achieve the desired modulation depth. The modulator mixer has a bandwidth of approximately 0.5 MHz. A setting of 5 V should produce a deviation of 531 kHz. (See table below for modulation depth under DC conditions.)
Table A4.1 – Voltage settings for modulator/mixer and resulting modulation depth.

<table>
<thead>
<tr>
<th>DC Voltage (V)</th>
<th>Modulation Depth (MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99.68</td>
</tr>
<tr>
<td>1</td>
<td>99.80</td>
</tr>
<tr>
<td>2</td>
<td>99.87</td>
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<td>6</td>
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<tr>
<td>7</td>
<td>100.47</td>
</tr>
<tr>
<td>8</td>
<td>100.53</td>
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