Magnetic Susceptibility Studies of Native and Thionine-oxidized Molybdenum-Iron Protein from *Azotobacter vinelandii* Nitrogenase

(Received for publication, September 1, 1981)

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The difference between the magnetic susceptibilities of native and thionine-oxidized molybdenum-iron protein from *Azotobacter vinelandii* nitrogenase was measured by the nuclear magnetic resonance method. Reversible oxidation of the MoFe protein by 4 to 8 electron eq of thionine/mol made the protein more paramagnetic than it was in the native state. The NMR susceptibility results were analyzed in terms of a model for the spin states of the iron centers in the MoFe protein based on low temperature electron paramagnetic resonance and Mössbauer spectral studies. The model proposes that the native protein contains 2 "M" centers (S = 3/2) and 4 "P" centers (S = 0) mol/mol and that the oxidized protein has diamagnetic M centers and paramagnetic P centers with S ≥ ½. Assuming that this model holds at 280 K, the NMR susceptibility results show that the effective magnetic moment of the oxidized P centers is larger than that of the native M centers. Based on an analysis in terms of spin only magnetic moments, the susceptibility results suggest that the P centers in the oxidized protein are S = ½ systems.

Nitrogenase is composed of two distinct iron-sulfur proteins. The larger of the two proteins, which is known as the molybdenum-iron protein, contains approximately 2 molybdenum and 30 iron atoms/243,000-dalton molecular mass (1, 2). The smaller protein contains 4 iron atoms/60,000-dalton molecular mass and is known as the iron protein (2). Both of these proteins are required for the catalysis of any of the reactions. The environment of the S center iron atoms, as characterized by the Mössbauer spectra, undergoes no apparent change during thionine oxidation of the protein.

Measurement of the magnetic susceptibility can provide insight into the spin state of the iron-sulfur centers in proteins. In this paper, we report on our efforts to understand the changes due to thionine oxidation in the spin state of the P centers through a measurement of the change in the magnetic susceptibility of the MoFe protein caused by thionine oxidation. A preliminary report of our results has been published elsewhere (10).

**MATERIALS AND METHODS**

**Susceptibility Measurements**—Magnetic susceptibilities were measured by the NMR method (11). NMR spectra were obtained using a Bruker WH-270 spectrometer using 5-mm coaxial NMR tubes (Wilms Glass Co., Buena, NJ, Model 517). For anaerobic work, the inner tubes of the coaxial cells were fitted to 5 mm standard taper outer joints. This allowed the NMR tubes to be attached to the double septum seal devices that have been described previously for use in low temperature EPR studies (12). After the addition of MoFe protein and the other reagents to the inner tubes (see below) the tubes were permanently sealed with a gas-oxygen torch flame.

**Enzyme Samples**—The MoFe protein from *Azotobacter vinelandii* was prepared by the method of Shah and Brill (13). For Fourier transform NMR experiments, the solvent proton content of the samples was reduced by the following modification of the protein crystallization procedure. After the initial centrifugation of the crystals of the MoFe protein, the pellet was resuspended in 0.04 M NaCl, 0.025 M potassium phosphate, D2O (99.7 atom % D) buffer with pH 7.35. After incubation at 37 °C for 1 h, the crystals were collected by centrifugation and dissolved in 0.25 M KCl, 0.025 M potassium...
Magnetic Susceptibility of Nitrogenase

**Table 1**

| Degree of oxidation | Protein concentration (mg/ml) | Frequency shift (Hz) |
|---------------------|-------------------------------|----------------------|
| Native              | 55.8                          | 4.81 (43)^a          |
| Native              | 54.4                          | 4.99 (36)            |
| Native              | 57.0                          | 4.20 (10)            |
| Oxidized by 4 e^-   | 49.8                          | 8.73 (62)            |
| Oxidized by 5 e^-   | 49.3                          | 14.44 (55)           |
| Oxidized by 6 e^-   | 48.1                          | 12.64 (89)           |
| Oxidized by 6 e^-   | 47.0                          | 15.32 (62)           |
| Oxidized by 7 e^-   | 46.6                          | 11.66 (19)           |
| Oxidized by 7 e^-   | 44.7                          | 13.19 (70)           |
| Oxidized by 8 e^-   | 45.5                          | 13.80 (19)           |
| Oxidized by 6 e^- then reduced | 44.7 | 5.15 (50) |
| Oxidized by 7 e^- then reduced | 43.6 | 5.66 (16) |

*a The numbers in parentheses are the standard deviations of the last two significant figures of the individual measurements of \( \Delta f \).

The values of \( \Delta f \) for the MoFe protein were oxidized by 4 e^- then reduced.

The magnetic field experienced by a nuclear spin in a solution sample depends on the bulk magnetic susceptibility of the solute. Consequently, the NMR frequency of a probe nucleus in a sample solution will shift by \( \Delta f \) (in Hz) upon the addition of mg/ml of a solute with mass susceptibility \( \chi \). The susceptibility of the solute may be determined from \( \Delta f \) using the relation

\[
\Delta f = \frac{3\chi d}{4\pi fm} - x_0 \left( \frac{d - d_0}{m} - 1 \right)
\]

In Equation 1, \( f \) is the resonance frequency in Hertz, \( x_0 \) and \( d_0 \) are, respectively, the mass susceptibility and density of the reference solution, and \( d \) is the density of the sample solution containing the solute of interest. Equation 1 is written for the case where the applied magnetic field is along the cylindrical axis of the sample tube.

The values of \( x_0 \), \( d_0 \), and \( d \) must be known in order to oxidize the MoFe protein oxidized by 4 e^- then reduced with sodium dithionite. Thus, changes in the susceptibility can be interpreted as arising from a reversible oxidation rather than from an irreversible destruction of the protein.

A change in the chemical shift of the probe species due to a specific interaction with the solute is a potential source of error in susceptibility measurements by the NMR technique.

The chemical shifts of DSS and acetate ion were measured as a function of concentration in the presence of 0.1 mm MoFe protein and an internal chemical shift reference of 7 mm dioxane. The ratio of concentrations of DSS or acetate to the concentration of the MoFe protein was varied from 0.4 to 12.0.1. Over this range, the change in the chemical shifts of acetate and DSS relative to that of dioxane was less than 0.4 Hz. This suggests that at least for the native protein there is no specific interaction between DSS or acetate and the protein that leads to a change in the resonance frequencies of DSS or acetate.

There is a significant amount of scatter in the \( \Delta f \) values for oxidized MoFe protein samples with nominally equal degrees of oxidation. The scatter might be due to differences in the amount of sodium dithionite remaining after incubation with hydrogenase. Preparations of the MoFe protein contain a 5-fold molar excess of sodium dithionite over protein. A 10% variation in the concentration of dithionite remaining after treatment with hydrogenase could easily change the effective degree of oxidation by as much as 1 electron eq/mol of protein. There is considerably less scatter in the values of \( \Delta f \) for the native protein samples.

The values of the frequency shift between the solution containing the MoFe protein and the reference solution were measured for 12 separate samples of MoFe protein at 280 K (Table II). These data were taken from samples prepared and NMR measurements made on three separate occasions. We report the mean value of \( \Delta f \) and the standard deviation of the mean value determined from four to six measurements of \( \Delta f \) on the same sample. The actual protein concentrations of the samples varied due to the addition of different volumes of reagents. The concentrations reported in Table I were calculated by assuming simple dilution of the original sample by the additions. As indicated by the \( \Delta f \) values, the changes in the susceptibility of the MoFe protein resulting from thionine oxidation can be largely reversed by reduction with sodium dithionite.

RESULTS

The values of the frequency shift between the solution containing the MoFe protein and the reference solution were measured for 12 separate samples of MoFe protein at 280 K (Table II). These data were taken from samples prepared and NMR measurements made on three separate occasions. We report the mean value of \( \Delta f \) and the standard deviation of the mean value determined from four to six measurements of \( \Delta f \) on the same sample. The actual protein concentrations of the samples varied due to the addition of different volumes of reagents. The concentrations reported in Table I were calculated by assuming simple dilution of the original sample by the additions. As indicated by the \( \Delta f \) values, the changes in the susceptibility of the MoFe protein resulting from thionine oxidation can be largely reversed by reduction with sodium dithionite.
obtain a value of \( \chi \) from \( \Delta \varepsilon \). These quantities can be calculated from published susceptibility, density, and partial specific volume data (18-21). Although the limited precision of the available density data permit only a rough estimate of \( d_p - d_o \), a simple calculation shows that for the samples used in this work, the changes observed in the first term of Equation 1 are much larger than the changes in the second term. For the samples used in this work, the second term in Equation 1 has an approximately constant value of \(-52.6 \times 10^{-6}\) c.g.s., whereas the value of the first term changes from \(7.63 \times 10^{-8}\) c.g.s. for a typical sample of native MoFe protein to an average value of \(25.6 \times 10^{-8}\) c.g.s. for the thionine-oxidized protein. Consequently, the uncertainty in \( d_p - d_o \) will not have a major impact on the accuracy of a measurement of the change in solute susceptibility due to thionine oxidation.

Magnetic susceptibility measurements by the NMR method provide a value for the sum of the paramagnetic (\( \chi_p \)) and the diamagnetic (\( \chi_d \)) susceptibilities of the solute. We wish to measure the change in \( \chi \) caused by thionine oxidation in order to study changes in the spin states of the iron-sulfur centers of the MoFe protein. The change in \( \chi \) may be obtained directly from the measured change in \( \chi \) provided that thionine oxidation does not change \( \chi_d \). This appears to be a reasonable assumption to make in light of the finding (22) that the diamagnetic susceptibility of carbon monoxymoglobin is equal to the weighted sum of the diamagnetic susceptibilities of its constituent amino acids and porphyrin prosthetic groups. This shows that even gross changes in protein conformation have no major effect on protein diamagnetism. Consequently, we will assume that thionine oxidation causes no significant changes in \( \chi_d \) and that changes in the measured value of \( \chi \) directly reflect changes in \( \chi_p \).

The molar magnetic susceptibility \( \chi_m \) is equal to the mass susceptibility times the molecular weight. The molar paramagnetic susceptibility \( \chi_m \) may be interpreted in terms of an effective magnetic moment \( \mu_{\text{eff}} \) according to the Langevin-Curie equation

\[
\chi_m = \frac{\beta \mu^2 N}{3kT}
\]

where \( \beta \) is the Bohr magneton, \( N \) is Avogadro’s number, \( k \) is Boltzmann’s constant, and \( T \) is the absolute temperature. The effective magnetic moment can be related to a spin quantum number (\( S \)) for the paramagnetic center by

\[
\mu_{\text{eff}} = \gamma[S(S + 1)]^{1/2}
\]

where \( \gamma \) is the electronic g factor, provided that there are no significant contributions to the magnetic moment from incompletely quenched orbital angular momentum, or from excited electronic states (23).

According to the previously proposed model (3), there are four P centers and two M centers in the MoFe protein molecule. Mössbauer studies (1, 3) show that in the native protein, the M centers are paramagnetic and the P centers are diamagnetic whereas the converse is true in the fully oxidized protein. We define an effective magnetic moment \( \mu_p \) for each of the M centers in the native protein and \( \mu_P \) for each of the P centers in the fully oxidized protein. Provided that thionine oxidation leaves \( \chi_d \) unchanged, the difference between the molar susceptibilities of the oxidized and native proteins is given by

\[
\chi_{m,\text{ox}} - \chi_{m,\text{nat}} = (4\mu_p^2 - 2\mu_P^2)\beta N/3kT
\]

From the data in Table I, the average molar susceptibility of the native protein was found to be \(-0.110 \pm 0.002\). The average molar susceptibilities of samples oxidized (nominally) by 5, 6, 7, and 8 electron eq of thionine/mole of protein was \(-0.065 \pm 0.006\). We found, using Equation 4, that the difference in effective magnetic moments (\(4\mu_p^2 - 2\mu_P^2\)) was 101 ± 13.

The metal contents of our preparations were somewhat lower than the 2 Mo - 30 Fe/molecule discussed in the introduction. Our values were, however, well within the range of values reported in the recent literature (3) and the iron-molybdenum ratio of 14.3 was close to the canonical value of 15. If we assume that the low metal content of our preparations indicates that our samples are mixtures of holoprotein and iron-free apoprotein (3), the experimentally determined value of \(4\mu_p^2 - 2\mu_P^2\) should be scaled up to 126 ± 16.

The spectroscopic data on the native protein support the hypothesis that the M centers are \( S = \frac{3}{2} \) spin systems. Using this information and Equation 3, we calculated values of \(4\mu_p^2 - 2\mu_P^2\) for several half-integer spin quantum numbers for the oxidized P centers (Table II). There is good agreement between the experimentally determined value and the calculated value for \( S = \frac{3}{2} \) centers. Furthermore, the changes in the calculated value of \(4\mu_p^2 - 2\mu_P^2\) due to integer changes in P center spin quantum number are much larger than the uncertainty in the experimentally determined value. This demonstrates that the NMR method is sufficiently sensitive to detect integer spin state changes in the P centers.

We now summarize explicitly the assumptions made in our analysis of the susceptibility data. We know that in the native protein at low temperature the M centers are paramagnetic and the P centers are diamagnetic. The reverse situation holds for the fully oxidized protein. Furthermore, the M centers in the native protein behave as \( S = \frac{3}{2} \) spin systems. We assumed that raising the temperature from 4 to 280 K does not alter this situation. We assumed that the effective magnetic moment of the M centers may be calculated from the spin quantum number using Equation 3, implying that (a) the M centers are still in an electronic ground state at 280 K and (b) there are no contributions from unquenched orbital angular momentum to the magnetic moment, so that a meaningful “spin only” magnetic moment may be calculated. We have also assumed that thionine oxidation does not alter the contribution of the S iron atoms to the susceptibility and that thionine oxidation does not alter the diamagnetic susceptibility of the MoFe protein.

We have presented an elementary analysis of the susceptibility of the MoFe protein in an effort to understand the spin state changes which accompany thionine oxidation. In view of the complex magnetic behavior of the metal centers in iron-sulfur proteins, and the limited data available, a more sophisticated treatment of our results is unwarranted. Susceptibility data on both the native and oxidized proteins over a broad temperature range are clearly needed if the electronic structures of the metal centers in the MoFe protein are to be understood. The NMR technique is limited to temperatures between 270 K (the approximate freezing point of the samples) and 305 K (the temperature at which the MoFe protein comes

### Table II

**Calculated values for \(4\mu_p^2 - 2\mu_P^2\)**

| Spin quantum number for Oxidized P centers | \(4\mu_p^2 - 2\mu_P^2\) |
|------------------------------------------|--------------------------|
| S = 1/2                                 | -18                      |
| S = 3/2                                 | 29                       |
| S = 5/2                                 | 110                      |
| S = 7/2                                 | 222                      |
| S = 9/2                                 | 366                      |
Magnetic Susceptibility of Nitrogenase

5. Smith, B. E., and Lang, G. (1974) Biochem. J. 137, 169–180
6. Rawlings, J., Shah, V. K., Chinell, J. R., Brill, W. J., Zimmer-
mann, R., Münck, E., and Orme-Johnson, W. H. (1978) J. Biol.
Chem. 253, 1001–1004
7. Shah, V. K., and Brill, W. J. (1977) Proc. Natl. Acad. Sci. U. S.
A. 74, 3249–3253
8. Kurtz, D. M., McMillan, R. S., Burgess, B. K., Mortenson, L. E.,
and Holm, R. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4986–4989
9. Orme-Johnson, W. H., and Münck, E. (1980) in Molybdenum and
Molybdenum-containing Enzymes (Coughlan, M. P., ed) pp.
427–438, Pergamon, Oxford
10. Orme-Johnson, W. H., Lindahl, P., Meade, J., Warren, W., Nelson,
M., Groh, S., Orme-Johnson, N. R., Münck, E., Huynh, B. H.,
Emptage, M., Rawlings, J., Smith, J., Roberts, J., Hoffmann,
B., and Mina, W. B. (1981) in Current Perspectives in Nitrogen
Fixation (Gibson, A. H., and Newton, W. E., eds) pp. 79–84,
Elsevier, North-Holland, New York
11. Phillips, W. D., and Poe, M. (1972) Methods Enzymol. 24, 304–
317
12. Beinert, H., Orme-Johnson, W. H., and Palmer, G. (1978) Methods
Enzymol. 54, 111–132
13. Shah, V. K., and Brill, W. J. (1973) Biochim. Biophys. Acta 305,
445–454
14. Shah, V. K., Davis, L. C., and Brill, W. J. (1972) Biochim. Biophys.
Acta 256, 498–511
15. Cornall, A. G., Bardawill, C. J., and David, M. M. (1949) J. Biol.
Chem. 177, 751–766
16. Clark, J. L., and Axley, J. H. (1955) Anal. Chem. 27, 2000–2003
17. Van De Bogart, M., and Beinert, H. (1967) Anal. Biochem. 20,
325–334
18. Mulay, L. N. (1967) in Treatise on Analytical Chemistry (Koltoff,
I. M., Elving, P. J., and Sandell, E. B., eds) Part I, Vol. 4, pp.
1751–1876, Interscience Publishers, New York
19. Swisher, R. H., Landt, M. L., and Reithel, F. J. (1977) Biochem.
J. 163, 427–432
20. Conway, B. E., and LaLiberte, L. H. (1970) Trans. Faraday Soc.
66, 3023–3047
21. Kirshenbaum, I. (1951) Physical Properties and Analysis of
Heavy Water (Urey, H. C., and Murphy, G. M., eds) pp. 1–20,
National Nuclear Energy Series McGraw-Hill Co., New York
22. Havemann, R., Haberditzl, W., and Habe, G. (1961) Hoppe-Sey-
ler’s Z. Phys. Chem. 217, 417–425
23. Cotton, F. A., and Wilkinson, G. (1972) Advanced Inorganic
Chemistry, 3rd Ed, pp. 537–538, Interscience Publishers, New
York
Magnetic susceptibility studies of native and thionine-oxidized molybdenum-iron protein from Azotobacter vinelandii nitrogenase.
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J. Biol. Chem. 1982, 257:2310-2313.

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