SOLVENT-INDUCED EPR ANISOTROPY CHANGE OF THE NON-HEME CHROMOPHORE OF SPINACH FERREDOXIN

R. E. Coffman and Bruce W. Stavens*
Chemistry Department, University of Iowa, Iowa City, Iowa 52240

Received August 31, 1970

Summary

Reversible changes in the EPR magnetic anisotropy of spinach ferredoxin due to small amounts of CH_3OH and n-propanol in pH 7.4 tris buffer suggest a weak perturbation of the native conformation of the 2Fe-2S group and the protein to which it is bonded. The optical absorption, circular dichroism and average g-value of the 2Fe-2S chromophore are conserved in this transformation, while the g-value anisotropy changes are relatively large. These solvent induced variations in the g-values are shown to be consistent with the relative variations of g-values between other non-heme iron proteins, which are of the 2Fe-2S type.

The magnetic properties of the plant ferredoxin and related molecules are interesting because of the unusual paramagnetic resonance characteristics of the g=1.94 transitions and because of the importance of understanding the electronic properties of the 2Fe-2S group[1] which is common to a number of non-heme iron containing proteins.[2] We report here our results of a study of the EPR g-value anisotropy in frozen buffered solutions of spinach ferredoxin at 77°K, as a function of relatively small concentrations of CH_3OH and n-propanol as a solution component. Further studies are being carried out in an effort to make our conclusions more quantitative.

Experimental

Materials: Spinach ferredoxin, prepared by the method of Tagawa and Arnon,[3] was obtained from the Sigma Chemical Co., St. Louis Mo. Purity was checked by the optical A_{420}/A_{276} ratio and by gel electrophoresis. We found A_{420}/A_{276}=0.43. Gaussian curve fitting of the electrophoretic profile indicated 85% of the protein moved as one band with 15% in a second band. The EPR, CD and ORD data were in good agreement with published curves. Significantly, there were no EPR resonances detectable in the oxidized ferredoxin

*Present address: Department of Physiology and Biophysics, University of Iowa Iowa City, Iowa. 52240

(hereafter:Fd) samples at 77°K, and the dithionite reduced samples only exhibited the characteristic g=1.94 resonances below about 150°K. Tris buffer solutions of Fd were dialyzed in a 1 cm^3 dialysis cell against the same volume of buffer containing methanol, or other small MW solute, using a Diaflo membrane of 10,000 MW exclusion size. All dialyses were carried out in a cold room maintained at 4°C. Samples for EPR studies were reduced with a small amount of $Na_2S_2O_4$, capped and immediately frozen in liquid nitrogen.

Methods: EPR data were taken on a standard Varian X-band V-4502 EPR spectrometer at 77°K using a small quartz dewar and in the range 95°K to 150°K using a Varian V-4557 variable temperature accessroy. g-Values were measured using a dual sample cavity, Hewlett-Packard frequency meter and DPPH (diphenyl picryl hydrazyl) as a g-marker. The field scan calibration was checked against the hyperfine splittings of $(KSO_3)_2NO$[4] and Mn^{++}:SrO[5]. Optical absorption spectra were taken on a Cary model 14 spectrophotometer, and CD and ORD on a Cary model 60. Equilibrium sedimentation studies were done on a modified Spinco model E analytical ultracentrifuge.

Results

The effects of addition of methanol, n-propanol and urea to the buffered Fd solutions at 4°C were studied, allowing at least 4 hours for equilibrium to be attained. Methanol concentrations spanned the range 1 to 50 v/v%, n-propanol 3 to 40% and urea 0.5 to 4 molar. All solvent solutions were titrated to pH 7.4 with HCl before dialysis into the Fd solutions. We observed no line shape effects for these urea concentrations after 4 hours at 4°C. The linewidth effects observed in the alcohol solutions are summarized in Figure 1. The largest effects were caused by CH_3OH in the buffered solvent. These effects were shown to be reversible by an experiment, see Figure 2, which first dialyzed the CH_3OH into the buffered ferredoxin solution, and then dialyzed it back out again.**

** Later measurements, in which g-Values were determined, produced spectra with about a 5 fold increase in signal/noise ratio.

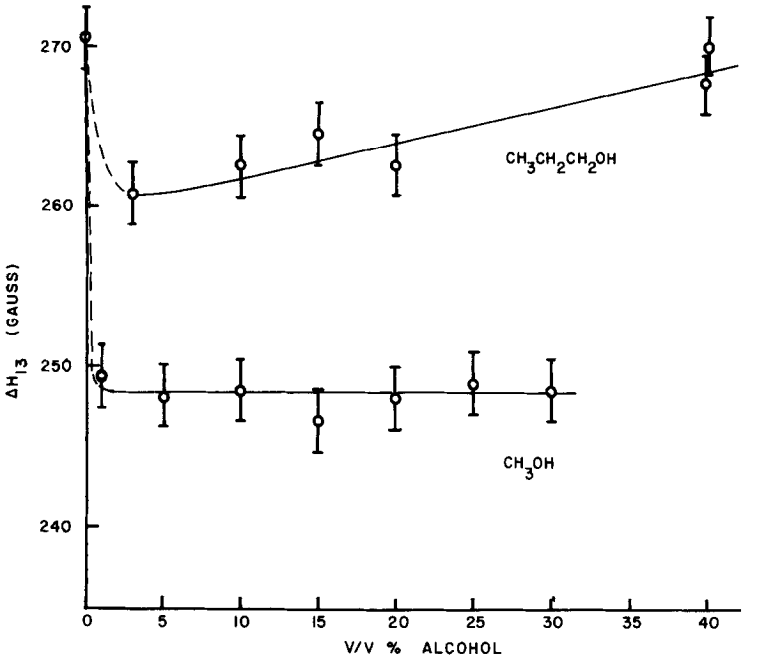

Figure 1. Line width of reduced spinach ferredoxin, measured between upper and lower field first derivative extrema (i.e. g_1 and g_3), versus per cent alcohol. Conditions: 77°K, 100 mw, 3 Gauss p-p modulation amplitude at 100 kHz and about 9.2 gHz microwave frequency. Ferredoxin concentration 2.0 mg/ml.

Discussion

Alcohols are known to cause denaturation of proteins in particular cases,[6] and the subject has been recently reviewed.[7] An equilibrium ultracentrifuge molecular weight study was done in order to see if there were any anomolies in MW in the presence of CH_3OH. None were found. The effect of n-propanol in buffered aqueous solution was also studied in order to observe the influence of alkyl group length, since for some types of denaturation the effects increase with increasing alkyl group chain length.[8] We observe the opposite here, although the upturning in the n-propanol curve of Figure 1 may be due to these effects. Close examination of the solvent induced line shape changes of Figure 2 suggests a change of g-values towards less rhombic (more axial type) symmetry. In order to explore this g-shift effect further, we define a rhombic magnetic assymmetry

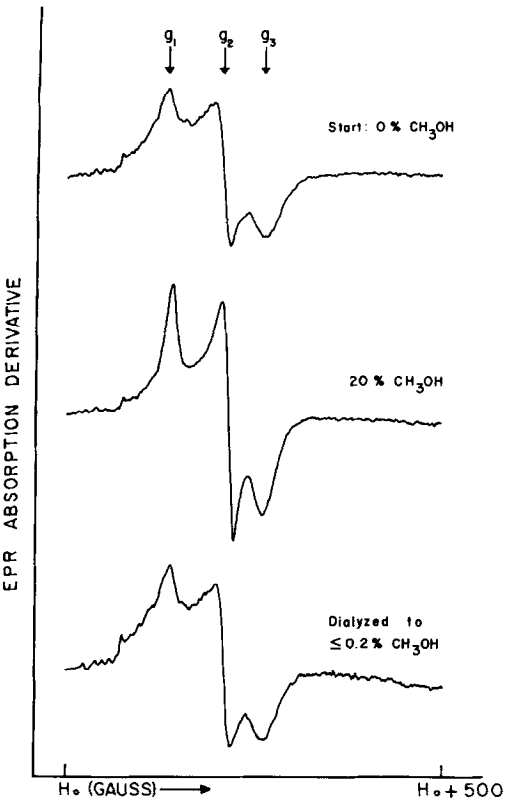

Figure 2. Demonstration of reversibility of the effect of CH_3OH on ferredoxin line shape. Conditions: 100 mw, 77°K, gain levels the same for a,b, and c, but the field modulation amplitude of b is about .8 of a and c.

parameter $x=g_2-g_3$. In Figure 3 the available literature data for the g_i are plotted versus x for putidaredoxin,[9] several adrenodoxins[9,10] xanthine oxidase,[11] spinach ferredoxin[12,13] and the ferredoxin "type-II" protein from Azotobacter Vinelandii.[14] These proteins are chosen because of the presence of independent 2Fe-2S groups, similar EPR average g-values and relaxation properties, and similar CD spectra. A uniform spreading apart of g_2 and g_3 versus x is inherent in the plot, but the slopes $\partial g_i/\partial x$ are not determined a priori. A least mean squares fitting of the data of Figure 3 gives

(1) $$\frac{\partial g_1}{\partial x} = +.34, \qquad \frac{\partial g_2}{\partial x} = +.38, \qquad \frac{\partial g_3}{\partial x} = -.62$$

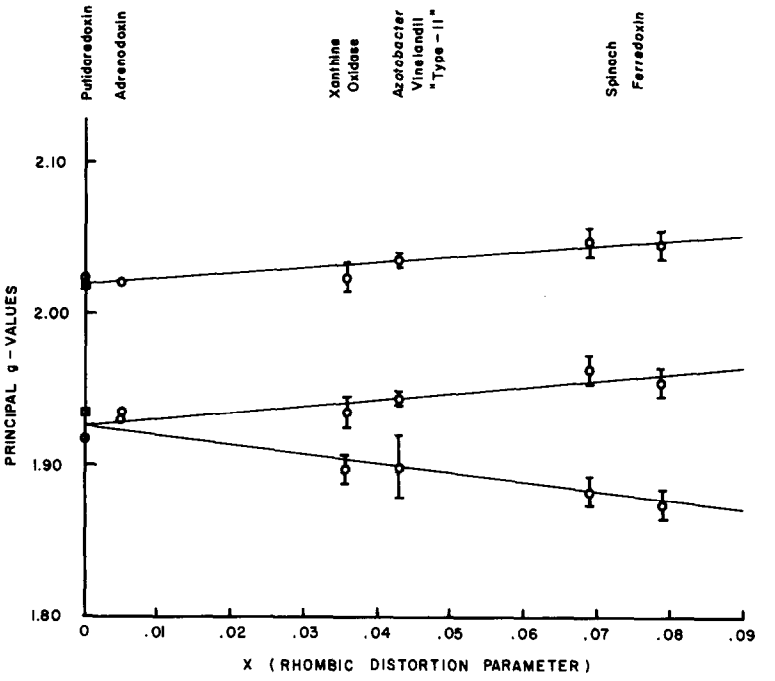

Figure 3. Published principal g values for various ferredoxin type proteins versus rhombic distortion parameter, defined in text. The straight lines are least mean squares best fits to the data.

As an approximation to the values of these derivatives representing the effect of the CH_3OH on spinach ferredoxin we carefully measured the g_i (e.g., see Figure 2) over the concentration range 0 to 20% CH_3OH and found the slopes by a least mean squares routine fitting g_i versus x. The results, which are in error due to line width changes, were: $\Delta g_i/\Delta x$ = .52, .50, and -.50 for i = 1,2,3. We estimate the true derivatives in the following manner: let

$$\frac{\partial g_1}{\partial x} + \frac{\partial g_2}{\partial x} + \frac{\partial g_3}{\partial x} = d \qquad (2)$$

where $d \simeq 0.1$ (See Equation 1). Furthermore, since $g_2 - g_3 = x$:

$$\frac{\partial g_2}{\partial x} - \frac{\partial g_3}{\partial x} = 1 \qquad (3)$$

Now, define the linewidth correction factors α, α' and β by

$$\frac{\partial g_1}{\partial x} = \alpha \frac{\Delta g_1}{\Delta x}, \qquad \frac{\partial g_2}{\partial x} = \alpha' \frac{\Delta g_2}{\Delta x}, \qquad \frac{\partial g_3}{\partial x} = \beta \frac{\Delta g_3}{\Delta x} \qquad (4)$$

We may solve Equations 2,3,4 for the experimental values of $\partial g_i/\partial x$ if we

assume $\alpha \gtrsim \alpha'$ and d = 0.1. The results, representing the experimental values of the $\partial g_i/\partial x$ due to CH_3OH solvent perturbation, are α = 1.38, β = .784 and:

(5) $$\left(\frac{\partial g_1}{\partial x}\right)_{exp} = +.38, \quad \left(\frac{\partial g_2}{\partial x}\right)_{exp} = +.36, \quad \left(\frac{\partial g_3}{\partial x}\right)_{exp} = -.64$$

These numbers compare favorably with Equations 1, obtained by comparison of different ferredoxin type proteins. Direct determination of these numbers, by curve fitting, is underway.

The unexpected similarity of the two sets of derivatives suggests a common relation between the effects causing g-value differences in the 2Fe-2S groups in different molecules and the effect of CH_3OH causing changes in the g-values of spinach ferredoxin. The different amino acid compositions and sequences of the different proteins, then, would certainly lead to protein conformation differences which must be reflected in the 2Fe-2S group itself. Thus, a conformation induced interpretation is consistent with the information available, although not the only possible interpretation. It was of great interest to see if this CH_3OH induced reversible change in the Fd molecule was similar to the reversible "I→II" protein structure rearrangement in Fd reported by Parmanabhan and Kimura.[15] The latter rearrangement, brought about 4.3 M urea, is noteable because of the large change in the CD spectrum. We could not, however, detect any significant changes in our samples in the CD or optical spectra over the 0 to 20 v/v% CH_3OH range, so these two conformation effects appear not to be related.

The general shape of the linewidth versus CH_3OH concentration curve of Figure 1 resembles an equilibrium between substrate and reactive sites where the fraction θ of combined sites approaches $\theta = 1$ for CH_3OH concentration $\gtrsim 1$ v/v%. Thus there may be a statistically small distribution of types of sites interacting with the CH_3OH molecules, at these concentrations which cause the EPR effects. That CH_3OH is more effective than n-propanol suggests that CH_3OH penetrates the protein molecule more readily due to its smaller size. A number of different interpretations are possible for the details of this process,[16,17] but none can be positively identified until

the structure of the iron, sulfur active site and its manner of bonding through the cysteine sulfurs is determined.

In conclusion, we have shown that the magnetic anisotropy of a simple 2Fe-2S non-heme iron protein changes in a way which suggests an effect due to a conformation related strain variable in the paramagnetic group of atoms. This imposes an additional constraint on the properties of possible quantum chemical models for the 2Fe-2S group, in addition to the well known property that two g-values are less than g_e=2.0023 and one greater than g_e, namely that the g-values must change with some conformation coordinate in the manner of Figure 3.

Acknowledgement: The funds for this research were granted by the Office of the Vice President for Research, University of Iowa, under Biomedical Sciences Support Grant FR-07035 from the General Research Support Branch, Division of Research Resources, Bureau of Health Professions Education and Manpower Training, National Institutes of Health. Partial support of NSF grant GP-9559 is also gratefully acknowledged.

References

1. Tsibris, J. C. M. and R. W. Woody, "Structural Studies of Iron-Sulfur Proteins", Coordination Chemistry Reviews, In press.
2. Beinert, H. and G. Palmer, Advan. Enzymol. 27, (1965).
3. Tagawa, K. and D. I. Arnon, Nature, 195, 537 (1962).
4. J. J. Windle and A. K. Wiersma, J. Chem. Phys. 39, 1139 (1963).
5. Rosenthal, J. and L. Yarmus, Rev. Sci. Instruments 36, 385 (1965).
6. Tanford, Charles and Paritosh K. De, J. Biol. Chem., 236, 1711 (1961)
7. Tanford C., "Protein Denaturation", in Advances in Protein Chemistry, 23, 122 (1968).
8. Herskovits, Theodore T., Barbara Gadegbeku and Helen Jaillet, J. Biol. Chem. 245, 2588 (1969).
9. Orme Johnson, W. H., Raymond E. Hansen, Helmut Beinert, J. C. M. Tsibris, R. C. Bartholomaus, and I. C. Gunsalus, Proc. Nat. Acad. Sci., U.S. 60, 368 (1968).
10. Orme-Johnson, W. H., Raymond E. Hansen and Helmut Beinert, in "Magnetic Resonance in Biological Systems", edited by A. Ehrenberg, B. G. Malmström and T. Vänngård, Pergamon (1967).
11. Gibson, J. F. and R. C. Bray, Biochim. Biophys. Acta 153, 721 (1968).
12. Palmer, Graham and R. H. Sands, J. Biol. Chem. 241, 253 (1966).
13. Palmer, Graham, Biochem. Biophys. Res. Communications 27, 315 (1967).
14. Dervartanian, D. V., Y. I. Sethna and H. Beinert, Biochim. Biophys. Acta 194, 548 (1969).
15. Padmanabhan, R. and Tokuja Kimura, J. Biol. Chem., 245, 2469 (1970)
16. Hollocher, Thomas C., J. Biol. Chem., 241, 1958 (1966).
17. Gersonde, K. and H. Netter, J. Mol. Biol. 10, 475 (1964).