Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 793-799

RIBONUCLEOTIDE REDUCTASE: A STRUCTURAL STUDY OF THE DIMERIC IRON SITE

Britt-Marie Sjöberg, Medical Nobel Institute, Department of Biochemistry, Karolinska Institute, S-104 01 Stockholm, Sweden.

Astrid Gräslund, Department of Biophysics, University of Stockholm, Arrhenius Laboratory, S-106 91 Stockholm, Sweden.

Joann Sanders Loehr, Department of Chemistry, Portland State University, Portland, Oregon 97207, USA.

Thomas M. Loehr, Department of Chemistry and Biochemical Sciences, Oregon Graduate Center, 19600 N.W. Walker Road, Beaverton, Oregon 97006, USA.

Received April 17,1980

SUMMARY: The iron-containing B2 subunit of ribonucleotide reductase from *Escherichia coli* has been investigated by Raman spectroscopy. Both the tyrosyl radical-containing native protein and the radical-free protein exhibit a resonance-enhanced Raman band at 500 cm^{-1}. This band is assigned to an Fe-O vibrational mode arising from an oxygen-containing ligand. The failure to observe any tyrosinate ring modes makes it unlikely that ribonucleotide reductase is an iron-tyrosinate protein and rules out tyrosinate oxygen as a ligand. It is proposed that the 500 cm^{-1} band in ribonucleotide reductase is analogous to the 510 cm^{-1} Fe-O vibrational mode of methemerythrin and arises from an oxo- or carboxylate-bridge between the antiferromagnetically-coupled Fe(III) ions.

INTRODUCTION: Ribonucleotide reductase from *Escherichia coli* catalyzes the formation of deoxyribonucleotides from the corresponding ribonucleotides (1). The enzyme consists of two non-identical subunits, proteins B1 and B2, each alone devoid of enzymatic activity (2). Protein B2 (molecular weight 78,000) consists of two identical polypeptide chains. It contains two iron atoms (3) and at least one tyrosyl free radical (4-6) necessary for enzymatic activity.

The iron center of protein B2 has previously been studied by electronic and Mössbauer spectroscopy (7) and by magnetic susceptibility measurements (6). The results have shown that the iron center in the native protein consists of an antiferromagnetically-coupled pair of high spin Fe(III), similar to the iron center of hemerythrin (8,9). Selective destruction of the free radical by treatment with hydroxyurea (HU) does not appear to alter the iron center, as judged by the Mössbauer and electronic spectra of the radical-free protein B2 (7). Furthermore, by use of a chelating agent the iron atoms can be removed from protein B2 (7). During this treatment, the radical disappears and the result is an iron-free, radical-free apoprotein B2, which can be reconstituted to a native protein B2 by the addition of Fe(II) and oxygen.

0006-291X/80/110793-07$01.00/0

Resonance Raman (RR) spectroscopy has previously been used to study the various forms of hemerythrin (10-12). In oxyhemerythrin, two Raman bands were observed at 844 and 503 cm^{-1}. They were assigned to O-O and Fe-O vibrations, respectively, of the bound dioxygen molecule (9). Methemerythrin-azide exhibited an Fe-O vibration near 510 cm^{-1} (11,12) but in this case the oxygen appears to have been derived from solvent oxygen rather than molecular oxygen (8).

We have now applied RR spectroscopy to study protein B2 in both its native and radical-free forms. No Raman bands related to the radical were observed. Instead, the spectra show a 500 cm^{-1} band which is assigned to an Fe-O vibration of an oxygen-coordinated ligand. The close similarity between hemerythrin and ribonucleotide reductase previously reported on the basis of magnetic and optical and Mössbauer spectroscopic properties (6) is further extended by the present study.

EXPERIMENTAL SECTION: Protein B2 was prepared and enzyme activity determined as described by Eriksson et al. (13). Apoprotein B2 was prepared according to Atkin et al. (7). The proteins were stored as frozen solutions (-70°C) in 50 mM Tris-HCl, pH 7.6, 20% glycerol.

Hydroxyurea was obtained from E.R. Squibb & Sons, Ltd., England. For inactivation of the radical, protein B2 at a concentration of 1-2 mM was incubated in the presence of 20 mM HU at room temperature for 20 minutes. Enzyme assays showed that the resulting enzyme activity was less than 1% of the original value.

Protein B2 (native, radical-free or apo) was precipitated in 80% saturated $(NH_4)_2SO_4$ and centrifuged (27,000 x g for 15 min). The pellet was washed with 80% saturated $(NH_4)_2SO_4$, centrifuged a second time and the supernatant was removed. The pellet was highly viscous having a protein concentration ∿1 mM (2 mM in iron). For Raman spectroscopic samples, ∿10 µl of the pellet was drawn into an open-ended glass capillary (i.d. ∿1.2 mm), then sealed with paraffin at the bottom.

Resonance Raman spectra were recorded on a computerized Jarrell-Ash spectrophotometer as previously described (14). Excitation wavelengths were provided by either a Spectra-Physics Model 164-01 Kr ion laser equipped with a high-field magnet or a Coherent Radiation Laboratory Model 52 Ar ion laser. Laser light was passed through a Pellin Broca prism monochromator (Instruments SA, Inc., Metuchen, NJ) to minimize plasma emission and/or to separate the 406.7/413.1 nm Kr lines. Scattered light was detected on a cooled ITT FW-130 (S-20) photomultiplier whose output was further processed in an ORTEC Model 9302 Amplifier/Discriminator. Capillaries containing ∿10 µl of sample were inserted into a copper rod cold finger immersed in liquid N_2 and irradiated in a quasibackscattering geometry.

RESULTS AND DISCUSSION: The electronic spectra above 320 nm of the native, radical-free, and apo forms of protein B2 are shown in Figure 1. The optical absorption above 320 nm of the radical-free protein originates from its iron center, and the difference in absorption between native and radical-free protein is due to the free radical (6). The wavelengths chosen for Raman

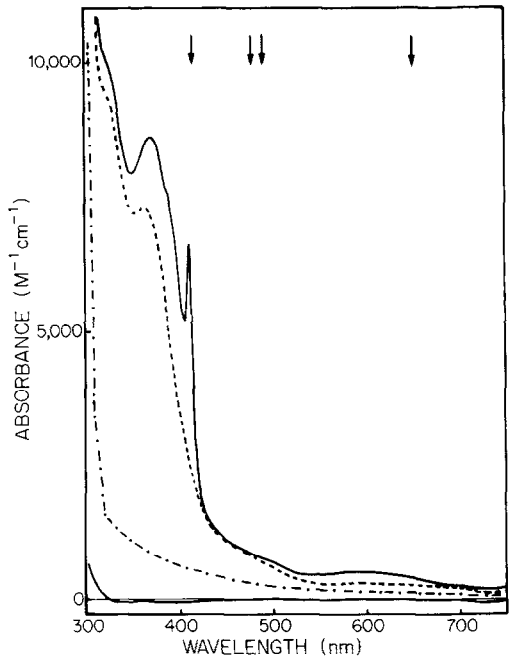


Figure 1: Electronic absorption spectra of protein B2 in native (———), radical-free (- - -) and apo (-·-·-) forms. The proteins were diluted to a concentration of ∿ 0.2 mM in 50 mM Tris-HCl, pH 7.6, 20% glycerol. Arrows indicate laser excitation wavelengths used for Raman spectroscopy.

excitation, corresponding to electronic absorption bands in native and radical-free protein B2, are indicated in Figure 1.

Laser Raman spectra of protein B2 with 413.1 nm excitation are shown in Figure 2. At this excitation wavelength both the iron center and the radical have nearly equal molar absorptivities (3000 $M^{-1}cm^{-1}$) while the apoprotein is essentially transparent. The apoprotein (Fig. 2c) shows Raman bands only from the concentrated ammonium sulfate at 462, 620 and 975 cm^{-1}. These bands are narrowed and slightly shifted from their room temperature positions ($\nu_1(SO_4^{2-})$ at 981 cm^{-1}). Although higher quality spectra were obtained with the radical-free protein, both native (Fig. 2a) and radical-free (Fig. 2b) proteins show a new resonance-enhanced Raman band at 500 cm^{-1} whose presence is dependent on the integrity of the iron center.

The 500 cm^{-1} band of the native and radical-free B2 proteins is also observed by excitation with the 406.7 nm line of the Kr laser. However, excitation at higher wavelengths, either with the 476.5 or 488.0 nm lines of the Ar laser or the 647.1 nm line of the Kr laser produced no resonance-enhanced Raman bands in the protein. Furthermore, no resonance-enhanced Raman bands were observed between 1000 and 1700 cm^{-1} in the native or radical-

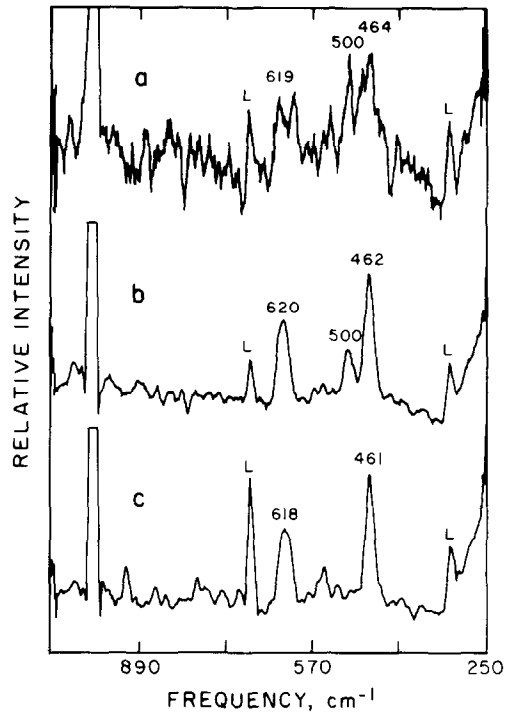


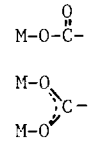
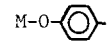
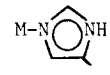
Figure 2: Raman spectra of protein B2 of ribonucleotide reductase from *E. coli* in (a) native, (b) radical-free and (c) apo forms. The samples were ammonium sulfate precipitates (∿ 1 mM in protein) at 77 K. The spectra were obtained with ∿ 5 mW of 413.1 nm excitation, and are accumulations of (a) 2, (b) 3 and (c) 11 scans; scan rate 2.0 cm^{-1}/s, slit width ∿ 10 cm^{-1}. A strong background fluorescence under the entire spectral range has been subtracted. The high-frequency noise has been reduced by a 25-point smoothing procedure (14). Laser plasma lines are denoted by L.

free protein with any of the wavelengths employed, precluding tyrosinate coordination.

The resonance-enhanced Raman band at 500 cm^{-1} was also observed in radical-free protein B2 stabilized by 20% glycerol at 0°C. However, the RR band was partially obscured by a 485 cm^{-1} spectral contribution from glycerol. Since equally stable preparations of concentrated protein B2 were obtained by ammonium sulfate precipitation which eliminated the Raman lines from glycerol, this latter method of sample preparation was preferred. The 400-500 cm^{-1} region was further sharpened by use of frozen samples to minimize the spectral interference of water.

After completion of the Raman experiment the protein precipitate was dissolved in Tris-glycerol buffer. The native enzyme was checked for activity and the radical-free enzyme was examined with respect to its electronic absorption spectrum. Exposure of the native enzyme at 77°K to 413 nm

TABLE: Typical Metal-Ligand Vibrational Frequencies for Divalent and Trivalent Metal Ions and Various Ligands.

Ligand	Type of Binding	Range of ν(M-L), cm^{-1} M(II)	M(III)	Ref.
Carboxylato		350-530 400-560		15
Phenolato		545-560		16
Aqua	$M-OH_2$	310-405	490-540	17
Hydroxo (methemoglobin)	M-OH		497	18
Hydroxo-bridged		480-560	500-580	19
μ-Oxo-bridged	M-O-M 		363 (sym) 538 (sym)	20 21
Peroxo (oxyhemerythrin)	M-O-O		503	10
Superoxo (oxyhemoglobin)	M-O-O		570	22
Amine	$M-NH_2$	300-440	510-590	17,23
Histidine (oxyhemocyanin)		265-285		24
" (azurin)		400-425		25
Cysteinato (azurin)	M-S	369		25
" (Fe-S proteins)			300-400	26

laser light (5 mW) for 15-20 minutes caused a loss of 40% in enzymatic activity. This observation raises the possibility that the 500 cm^{-1} band is due to a denatured form of the enzyme, which could be similar or identical to the radical-free protein B2. However, the rates of the decay of both activity and intensity at 500 cm^{-1} for the native protein have been observed to be greater than for the radical-free protein, indicating that radical-free protein B2 is not responsible for the 500 cm^{-1} band in the native protein B2.

Since the 500 cm^{-1} band in protein B2 is independent of the presence of the radical, we assign it to the iron center as an Fe-ligand vibration. Possible iron ligands are listed in the Table together with typical values expected for such Raman bands. Those which appear to be too low to account

for the 500 cm^{-1} vibrational mode are histidine and cysteine. Although the M-O frequencies of amino acid complexes of divalent metal ions are typically in the 200-400 cm^{-1} range (27), the corresponding vibrations in alkyl carboxylate complexes can reach 560 cm^{-1} (15).

Tyrosinate or peroxide and superoxide ligands are expected to have Fe-O vibrations near 500 cm^{-1}, but these should all be accompanied by intense bands due to tyrosine ring or O-O vibrations, respectively, none of which were observed for protein B2. Typical Fe(III)-tyrosinate RR bands have been reported at ca. 1605, 1505, ~1270, and 1175 cm^{-1} in the Fe(III)-transferrins (28) and protocatechuate 3,4-dioxygenase (29). The absence of such RR signals in protein B2 indicates that tyrosine is not an iron ligand, even though tyrosyl radical is formed during the reconstitution of the apoprotein with Fe(II) and O_2 (6,7). This conclusion is supported by the failure to observe any effect of the radical on the Mössbauer spectrum of protein B2 (7). Vibrational frequencies associated with peroxide O-O stretching have been reported at 844 cm^{-1} in oxyhemerythrin (9) and 744 cm^{-1} in oxyhemocyanin (24) and superoxide O-O stretching has been observed at 1107 cm^{-1} in oxyhemoglobin (30). The lack of an O-O stretching vibration in the RR spectrum of native or radical-free protein B2 makes a peroxide or superoxide coordinated species unlikely. This is also in agreement with the observation that catalase or superoxide dismutase did not interfere with the reconstitution reaction (6).

Likely possibilities for an Fe-L vibration at 500 cm^{-1} are a carboxylate (β or γ) from the protein or an oxygen-containing group from the solvent (H_2O, OH^-, or μ-oxo bridge). In the case of methemerythrin-azide, isotope exchange experiments with ^{18}O in place of ^{16}O and deuterium in place hydrogen have led to the proposal that the RR band at 510 cm^{-1} is due to a μ-oxo bridged structure (8). In view of the similarities of the Mössbauer and electronic spectra and magnetic properties of protein B2 and methemerythrins (6), similar isotope exchange experiments are being planned for protein B2. Furthermore, since the isotope-labeling experiments with oxy- and met-hemerythrins have not unambiguously established the existence of a μ-oxo bridge (8,31), studies with the B2 subunit of ribonucleotide reductase may help to clarify the situation.

ACKNOWLEDGMENTS: This research was supported by grants from the Swedish Medical Research Council, the Swedish Natural Science Research Council, the Wallenbergstiftelsens Jubileumsfond, the Magn. Bergvalls Stiftelse, and the U.S. Public Health Service, National Institutes of Health (GM 18865). We thank Professors Peter Reichard and Anders Ehrenberg for their encouragement and support of this work.

REFERENCES:

1. Thelander, L., and Reichard, P. (1979) Annu. Rev. Biochem. 48, 133-158.
2. Brown, N., Canellakis, Z.N., Lundin, B., Reichard, P., and Thelander, L. (1969) Eur. J. Biochem. 9, 561-573.
3. Thelander, L. (1973) J. Biol. Chem. 248, 4591-4601.
4. Ehrenberg, A., and Reichard, P. (1972) J. Biol. Chem. 247, 3485-3488.
5. Sjöberg, B.M., Reichard, P., Gräslund, A., and Ehrenberg, A. (1978) J. Biol. Chem. 253, 6863-6865.
6. Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.M., and Reichard, P., (1980) J. Biol. Chem. 255, in press.
7. Atkin, C.L., Thelander, L., Reichard, P., and Lang, G. (1973) J. Biol. Chem. 248, 7464-7472.
8. Kurtz, D.M., Jr., Shriver, D.F., and Klotz, I.M. (1977) Coord. Chem. Rev. 24, 145-178.
9. Loehr, J.S. and Loehr, T.M. (1979) Adv. Inorg. Biochem. 1, 235-252.
10. Dunn, J.B.R., Shriver, D.F., and Klotz, I.M. (1973) Proc. Nat. Acad. Sci. USA 70, 2582-2584.
11. Dunn, J.B.R., Shriver, D.F., and Klotz, I.M. (1975) Biochemistry 14, 2689-2694.
12. Dunn, J.B.R., Addison, A.W., Bruce, R.E., Loehr, J.S., and Loehr, T.M. (1977) Biochemistry 16, 1743-1749.
13. Eriksson, S., Sjöberg, B.M., Hahne, S., and Karlström, O. (1977) J. Biol. Chem. 252, 6132-6138.
14. Loehr, T.M., Keyes, W.E., and Pincus, P.A. (1979) Anal. Biochem. 96, 456-463.
15. McConnell, A.A. and Nuttall, R.H. (1977) Spectrochim. Acta 33A, 459-462.
16. Percy, G.C. and Stenton, H.S. (1976) Spectrochim. Acta 32A, 1615-1621.
17. Adams, D.M. (1968) Metal-Ligand and Related Vibrations, St. Martin's Press, New York.
18. Asher, S.A., Vickery, L.E., Schuster, T.M., and Sauer, K. (1977) Biochemistry 16, 5849-5856.
19. Ferraro, J.R. (1971) Low-Frequency Vibrations of Inorganic and Coordination Compounds, Plenum Press, New York.
20. Burke, J.M., Kincaid, J.R., and Spiro, T.G. (1978) J. Am. Chem. Soc., 100, 6077-6083.
21. Hewkin, D.J. and Griffith, W.P. (1966) J. Chem. Soc. A, 472-475.
22. Brunner, H. (1974) Naturwissenschaften 61, 129.
23. Nakamoto, K. (1970) Infrared Spectra of Inorganic and Coordination Compounds, 2nd ed., Wiley-Interscience, New York.
24. Freedman, T.B., Loehr, J.S. and Loehr, T.M. (1976) J. Am. Chem. Soc., 98, 2809-2815.
25. Thamann, T.J. (1980) Ph.D. Dissertation, Oregon Graduate Center.
26. Spiro, T.G., and Loehr, T.M. (1975) in Advances in Infrared and Raman Spectroscopy, Clark, R.J.H. and Hester, R.E., Eds., London, Heyden and Son, 98-142.
27. Percy, G.C. (1976) Spectrochim. Acta 32A, 1287-1290, and references therein.
28. Tomimatsu, Y., Kint, S., and Scherer, J.R. (1976) Biochemistry 15, 4918-4924
29. Keyes, W.E., Loehr, T.M., and Taylor, M.L. (1978) Biochem. Biophys. Res. Commun. 83, 941-945.
30. Barlow, C.H., Maxwell, J.C., Wallace, W.J., and Caughey, W.S. (1973) Biochem. Biophys. Res. Commun. 55, 91-95.
31. Duff, L.L., and Shriver, D.F., personal communication.