A Divalent Metal Site in the Small Subunit of the Manganese-Dependent Ribonucleotide Reductase of *Corynebacterium ammoniagenes*†

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ABSTRACT: Based on its metallo-cofactor, the manganese-dependent ribonucleotide reductase (Mn-RRase) responsible for delivery of DNA precursors in the Mn-requiring Gram-positive bacterium *Corynebacterium* (formerly *Brevibacterium*) *ammoniagenes* ATCC 6872 is no longer considered as a simple analogue of the aerobic Fe−RRase of *Escherichia coli* but as the prototype of the class IV enzymes (*1*). Deliberate dissociation of the Mn-RRase holoenzyme and an improved sample preparation of the dimeric CA2 protein allowed further characterization of the inherent metallo-cofactor by Q-band electron paramagnetic resonance (EPR) spectroscopy. At 40 K, a distinct hyperfine sextet ($I = {}^5/_2$,^{55}Mn) pattern with a weak zero-field splitting was detected in the CA2 protein prepared from manganese-sufficient cells displaying high RRase activity as expected. This Q-band Mn(II) signal was absent in the apo-CA2 protein obtained from manganese-depleted cells devoid of this enzymatic activity. The presence of a mixed valence manganese cluster in the *C. ammoniagenes* RRase is excluded since no complex multiline EPR signals were detected in the CA2 protein even at very low (8 K) temperature. The observed Mn(II) spectrum indicates a protein-bound manganese which was modified in the presence of 5.7 mM *p*-methoxyphenol, but is insensitive toward 10 mM EDTA. Thus, the manganese appeared to be either strictly bound or buried within a hydrophobic pocket of the CA2 protein, inaccessible for EDTA.

The RRases (EC 1.17.4) catalyze the irreversible reduction of ribonucleotides to deoxyribonucleotides and thus provide the building blocks for DNA replication (*2*). Since no other route to DNA exists *in vivo*, this enzymatic reaction is the bottleneck for DNA synthesis and cell proliferation. Despite a uniform mechanism which involves organic radicals (*1*), nature has evolved a great diversity of enzymes which use different metallo-cofactors or metallo-proteins for catalysis (*3*, *4*). Thus, trace elements such as Co, Fe, and Mn control growth and DNA production in different organisms (*5*−*7*). The manganese-dependent ribonucleotide reductase (Mn-RRase)[1] of *Corynebacterium ammoniagenes* was detected (*8*) in a search for the primary target of manganese depletion in this industrial nucleotide producer strain (*9*).

The manganese character of the *C. ammoniagenes* RRase has been demonstrated by (I) an absolute manganese requirement for DNA formation in growing cells (*10*), (II) the loss of the ability to reduce ribonucleotides upon manganese depletion (*11*), (III) co-chromatography of the radioactive metal isotope ^{54}Mn and enzymatic activity during purification of the holoenzyme to homogeneity, starting from isotope-labeled cells, and (IV) acid denaturation of the purified holoenzyme with subsequent release of a Mn(II) EPR signal (*12*).

To confirm the manganese state of the *C. ammoniagenes* RRase with its $\alpha\beta_2$ subunit structure (*12*), a new strategy was necessary, considering the facile dissociation of the Mn-RRase holoenzyme resulting in substantial loss of activity during purification (*12*). This paper presents Q-band EPR spectroscopic data of the metal-containing small subunit (CA2 protein) of the *C. ammoniagenes* Mn-RRase using the approach of Blasczyk (*13*) for deliberate dissociation of subunits under controlled conditions.

MATERIALS AND METHODS

General Methods. All chemicals, reagents, and nucleotides were of analytical grade. 2′,5′-ADP−Sepharose 4B, Sephadex G-100, and Superose12 were obtained from Pharmacia LKB GmbH (Freiburg, Germany). An E-Pure-System (Millipore GmbH, Eschborn, Germany) served for purification of water for preparation of solutions and buffers. ^{3}H- and ^{14}C-labeled nucleotides were from Amersham-Buchler (Braunschweig, Germany). Radioactivity was determined using a Wallac 1410 (Pharmacia, LKB, Freiburg, Germany) after mixing of fractions eluted by HPLC with

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[1] Abbreviations: EPR, electron paramagnetic resonance; Mn-RRase, manganese-dependent ribonucleotide reductase; CA1 protein, large subunit of the *Corynebacterium ammoniagenes* Mn-RRase; CA2 protein, small subunit of the *Corynebacterium ammoniagenes* Mn-RRase; DTT, dithiothreitol; ICP-MS, inductively coupled plasma-mass spectrometry; TCA, trichloroacetic acid; zfs, zero-field splitting.

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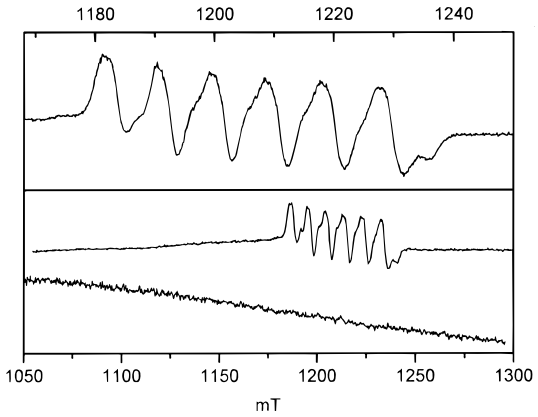

FIGURE 1: Q-band EPR spectra of the manganese(II) center of FPLC-purified native CA2 protein of *C. ammoniagenes* Mn-RRase. Conditions: 40 K, 5 mW, 33.5 GHz, modulation amplitude 0.8 mT. Top spectrum scan, 82 mT; middle scan, 250 mT; bottom, base line scan, 250 mT.

10 mL of Quicksafe A (Zinsser Analytik GmbH, Frankfurt, Germany).

Incorporation of ^{14}C-labeled nucleic acid precursors into the KOH-stable, TCA-insoluble DNA fraction was studied with growing cultures in order to determine DNA formation activity as described previously (*10*). Large-scale incubation of *C. ammoniagenes* ATCC 6872 was carried out according to Griepenburg et al. (*14*). The protocol of Willing et al. (*12*) was used for large-scale incubation in a mineral medium under conditions of manganese deficiency in order to prepare apo-RRase. Likewise, for preparation of Mn-RRase, the strain was grown under the same conditions and then reactivated in vivo by addition of 10 μM $MnCl_2$ (*10*, *11*). This incubation regime leads to biomass with enhanced enzyme activity (*7*).

Assays for Determination of Enzymatic Activity for Ribonucleotide Reduction. The standard assay for ribonucleotide reduction was done according to Willing et al. (*12*). After deliberate dissociation of the Mn-RRase holoenzyme into the larger (CA1 protein) and the smaller metal-containing subunit (CA2 protein), a biochemical complementation assay (*14*) was used to determine the activity of the latter. Protein was determined by protein−dye binding (*15*) with bovine serum albumin as a standard.

Preparation of CA2 Protein, Free of Unspecifically Bound Mn^{2+} Ions. All enzyme procedures were carried out at 0−6 °C. High concentrations of FPLC-purified native CA2 protein and apo-CA2 were obtained and enzymatically assayed using exactly the same protocol as in the flow scheme of Figure 1 presented by Griepenburg et al. (*14*). The initial conventional gel filtration on Sephadex G-100 (30 × 900 mm) with self-packed columns and ultrafiltration with an Amicon cell (PM 10 filter, Amicon GmbH, Witten, FRG) were carried out as previously described (*13*).

Analysis of Metals and Calculations. Manganese was determined by inductively coupled plasma-mass spectrometry (ICP-MS). Because its mass number, 55, does not change due to any intrinsic artifacts of this spectroscopic technique, manganese is a 100% isotope. The values obtained by ICP-MS for the elements Fe, Co, Ni, Cu, and Zn were recalculated via their isotope occurrence according to deBievre and Barnes (*16*). A VG PlasmaQuad PQ2 Turbo (VG Elemental, Winsford, U.K.) equipped with a graphite tube allowing application of very small volumes (25 μL) was used, and the temperature of the samples was increased stepwise from room temperature to 2750 °C as follows: for evaporation of water, 15 s 125 °C, 14 s constant; for pyrolysis of organic compounds, 10 s 600 °C, 10 s constant; for transition of inorganic material into gaseous compounds, 39 s 2400 °C, 4 s constant; and for cleaning of the graphite tube, 3 s 2750 °C.

The manganese content was calculated in the following four step-procedure: (I) sensitive Mn determination by ICP-MS in 100 kDa fractions containing the enriched and highly concentrated CA2 protein as the only manganoprotein (*12*); (II) subtraction of the Mn background from simultaneously enriched apo-CA2; (III) estimation of the concentration levels of native CA2 by determination of activity for ribonucleotide reduction in the biochemical complementation assay and recalculation using the published value for the specific activity of the purified holoenzyme (*12*); (IV) final calculation of the Mn/protein ratio taking into consideration the subunit structure ($\alpha\beta_2$) of the *C. ammoniagenes* Mn-RRase, inclusive molecular mass of subunits.

EPR Spectroscopy. For Q-band measurements (34 GHz, Bruker ER 220 D), the samples were filled in fused quartz tubes (2.4 mm outer diameter, Wilmad). The spectra were obtained with an ER 5106 QT cavity (Bruker) in a continuous flow cryostat CF 935 (Oxford Instruments) using a temperature range from 8 to 80 K.

RESULTS

Initial Experiments with CA2 Protein Enriched by Conventional Chromatography. The previous unequivocal autoradiographic allocation of ^{54}Mn to the smaller subunit of Mn-RRase (*12*) allowed us to focus on this part of the enzyme for metal quantification. Thus, the dimeric (2 × 50 kDa) metal-containing subunit, here referred to as CA2 protein, was selectively enriched by deliberate separation of CA2 from the holoenzyme through affinity chromatography. Retention of the allosterically regulated CA1 protein with its nucleotide effector binding sites was achieved on 2′,5′-ADP−Sepharose 4B. Initially, conventional chromatography (gel filtration, Sephadex G-100) was used for further purification. Routinely, 100 μL-solutions of a 100 kDa fraction (50 mg of protein/mL), free of unspecifically bound Mn^{2+} ions, were obtained. CA2 protein and inactive apo-CA2, simultaneously enriched from parallel cultures as described under Materials and Methods, were analyzed for Mn and other transition metals (not shown). Differential enrichment of transition metals in the smaller subunit (CA2 protein) of the Mn-RRase, depending on the presence or absence of Mn during growth, was observed. By ICP-MS, 75 ng/mg of Mn per inactive apo-Ca2 protein and 104 ng/mg of Mn per active CA2 protein were found. By subtracting the manganese background in the simultaneously enriched apo-CA2, the intrinsic manganese content of the active CA2 protein was determined as 29 ng/mg of protein. Further calculation as described under Materials and Methods yielded 2.7 Mn per CA2 dimer, a value that should be corrected to 1 Mn atom per CA2 monomer since no loss of manganese occurs during chromatographic enrichment as shown here (see below) whereas the ability to reduce ribonucleotides in the biochemical complementation assay is rapidly lost due

to the short half-life of the intrinsic stable free radical (*14*). This estimated 1:1 stoichiometry of protein and manganese favors a mononuclear metal site in the *C. ammoniagenes* Mn-RRase over a binuclear manganese cluster postulated thus far (*12*, *17*) and induced us to develop an improved sample preparation for EPR spectroscopy.

Experiments with CA2 Protein Enriched by FPLC. Using the FPLC protocol developed by Griepenburg et al. (*14*) led to an improved purification of both the native CA2 protein and apo-CA2 with regard to the metal composition since EPR spectroscopy did not detect any significant signals of metals other than Mn in the native CA2 protein whereas the apo-CA2 was devoid of any metals (see below). At first, the presence of a low-spin Mn-porphyrin, providing an analogy to the bacterial Co-corrin-dependent ribonucleotide reductases (*18*, *19*, *5*), was considered as a distinct possibility (*13*). However, no characteristic EPR signals at $g = 6$ and 2, typical for a Mn-porphyrin system (*20*), were detected in the temperature range 8−80 K.

Here we present the first successful Q-band EPR measurements of the native CA2 protein of the *C. ammoniagenes* Mn-RRase. The spectrum shown in Figure 1 (top trace) exhibits a well-resolved manganese hyperfine sextet ($I = {}^5/_2$) with splittings between 9.2 and 9.6 mT centered around $g = 2$, typical for Mn(II) in an octahedral environment. The line width amounts to 3.2 mT for the individual hyperfine lines. Additional weak features are visible at the low- and high-field flanks of the sextet lines which are indicative of a small zero-field splitting (zfs) with rhombic distortion. Similar spectra of the central $-{}^1/_2 \leftrightarrow {}^1/_2$ transition with various zfs contributions have been described for other manganese enzymes (*21*). No manganese lines were observed in the inactive apo-CA2 protein.

Apart from signal intensity, no spectral changes were observed upon variation of temperature in a range from 8 to 80 K and of microwave power between 50 mW and 15 μW. In particular, no spectral broadening or splitting nor the appearance of multiline signals was observed, which have been encountered in binuclear manganese systems with strong dipolar coupling or exchange interactions between adjacent ions (*22*−*24*). In Figure 1 (middle trace), the field scan spanning 250 mT indicates that extremely broad flanks down to 1118 mT are present. For comparison, the base line is shown in the bottom trace. Apart from this broad line, no additional fine structure transitions ($\pm{}^5/_2 \leftrightarrow \pm{}^3/_2$ etc.) at lower or higher fields could be resolved so far. According to the scheme proposed by Reed and Ray (*25*), for Mn(II) complexes with axial symmetry the complex in the CA2 protein can qualitatively be associated with a rather small zfs parameter *D*.

Sensing of the native CA2 protein with specific inhibitors of the *C. ammoniagenes* RRase appeared as the fastest strategy for additional correlation of the Mn(II) Q-band EPR signal. For this kind of experiment, *p*-alkoxyphenols, recently introduced by Pötsch et al. (*26*) as new inhibitors of the iron-type ribonucleotide reductases, were chosen. However, prior to their use, it was necessary to demonstrate that the *C. ammoniagenes* Mn-RRase is also targeted by these inhibitors. Indeed, using the biochemical complementation assay, it was shown that the CA2 protein is inactivated by *p*-alkoxyphenols (Figure 2). For EPR spectroscopy, a final concentration corresponding to the I_{50} value (inhibition 50%) was applied. Interestingly, *p*-methoxyphenol is able to modify the characteristic Q-band EPR Mn(II) spectrum of the native CA2 protein shown in Figure 1 toward a higher symmetry, noticeable from the reduced line width especially in the high field (Figure 3, trace B). This observation clearly correlates the well-resolved manganese hyperfine sextet to the metal center of the *C. ammoniagenes* RRase. On the other hand, the chelating agent EDTA did not change the EPR Mn(II) signal (Figure 3, trace A)—even not when added to give a final concentration which corresponds to the I_{50} value (10 mM, *12*).

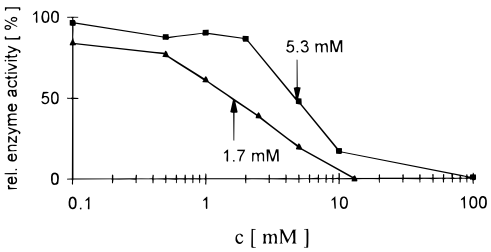

FIGURE 2: Inactivation of the native CA2 protein of the *C. ammoniagenes* RRase in the presence of increasing amounts of *p*-methoxyphenol (■) and *p*-propoxyphenol (▲). The degree of inhibition of FPLC-purifed native CA2 protein was examined by assaying for the ability to reduce ribonucleotides after biochemical complementation with the catalytic CA1 protein as described under Materials and Methods.

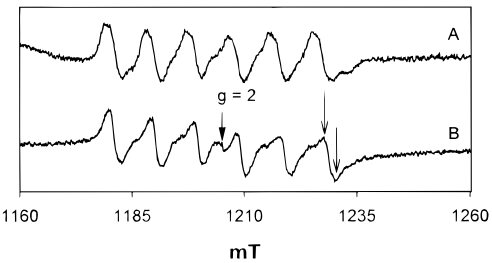

FIGURE 3: Significant spectral changes of the CA2 protein by addition of *p*-methoxyphenol (5.7 mM, final concentration). Trace A shows the rhombic distorted Q-band Mn(II) spectrum of the native CA2 protein which is identical to the spectrum obtained in the presence of 10 mM EDTA. Trace B presents the spectrum of *p*-methoxyphenol-treated CA2 protein. The line width is distinctly reduced, especially in the high field-part of the spectrum (empty arrows). Conditions: identical to Figure 1.

DISCUSSION

Thus far, a binuclear manganese cluster, analogous to the μ-oxo-bridged diferric cluster in the aerobic RRase of *E. coli* (*27*, *28*), was hypothesized for the Mn-RRase of *C. ammoniagenes* (*7*, *1*). This idea was generated on the basis of similar UV/visible spectra recorded from the Mn-RRase holoenzyme (*12*), the pseudocatalase of *Lactobacillus plantarum* (*29*), and binuclear inorganic manganese complexes (*30*, *31*, *17*). In retrospect, it is of interest that although a 1:1 stoichiometry of protein and manganese by X-band EPR of acid-denatured B2 (equivalent to CA2) was determined (*12*), the result obtained was considered as an approximation only because the idea of a mononuclear metal center was too strange at that time and more weight was put on spectral similarities. However, this spectral similarity has less significance in light of the ICP-MS data on the CA2 protein (this work) which favor a mononuclear manganese site in the *C. ammoniagenes* RRase. Nevertheless, a breakthrough

was required in order to substantiate our present view. This was provided only by the modified sample preparation using FPLC (*14*) yielding an improved signal-to-noise ratio for EPR spectroscopic characterization of the metal center in the *C. ammoniagenes* Mn-RRase.

The Q-band EPR spectra obtained have the characteristic features of isolated Mn(II) in an octahedral environment with a small zero-field splitting parameter *D*, indicating a reduced symmetry of the coordination sphere of the manganese ion. Comparable spectra were obtained from other enzymes with an isolated Mn(II) like concanavalin A (*32*) formyltetrahydrofolate synthetase with MnADP (*33*) or creatine kinase-Mn(II)-ADP with creatine and SCN^- (*34*). The relatively large line width of the sextet hyperfine lines associated with the central $-1/2 \leftrightarrow 1/2$ transitions is related to a variation of zfs parameter *D* (*21*, *24*). This variation in *D* is also the origin of the extreme broadening of the other fine structure transitions ($\pm 5/2 \leftrightarrow \pm 3/2$ and $\pm 3/2 \leftrightarrow \pm 1/2$), since they are explicitly dependent on *D* in first order (*21*). In contrast to enzymes with a binuclear Mn(II) cluster, e.g., *S*-adenosylmethionine synthetase (*35*), dimanganese concanavalin A (*23*), or the thiosulfate oxidizing system in *Thiobacillus versutus* (*36*), no additional splitting of the hyperfine lines due to exchange or strong dipolar coupling was found in the native CA2 protein, which would require distances close to 4 Å between the manganese sites. Furthermore, we exclude the presence of a mixed valence manganese cluster in the CA2 protein of the *C. ammoniagenes* RRase, since no complex multiline EPR signals were detected even at very low (8 K) temperature, a condition favorable for recording of antiferromagnetically coupled Mn(III)−Mn(IV) clusters (*37*, *22*, *38*). Likewise, we conclude from the insensitivity of the Q-band EPR Mn(II) signal toward EDTA that the manganese is more strongly bound to its ligands in the metal center than in a complex with EDTA. Another explanation for the great stability of the Mn(II) signal even at higher (10 mM) concentrations of EDTA would be that the manganese is buried in the interior of the native CA2 protein, possibly in a hydrophobic pocket, and that EDTA has no access to it.

In summary, the calculation of a 1:1 stoichiometry of protein and manganese in the CA2 monomer based on ICP-MS data together with Q-band EPR results provides strong evidence for a mononuclear, isolated Mn(II) site in the RRase of *C. ammoniagenes*. Recently, the purification of the manganese-containing ribonucleotide reductase was repeated independently in another laboratory (*39*), and the manganese content was determined as 0.5 mol/mol of Mn per R2F polypetide (R2F = CA2) by AAS, confirming our present view of a mononuclear manganese center. Interestingly, these authors were unable to detect iron in the protein. It is fascinating that the deduced sequence of the cloned gene encoding the R2F of *C. ammoniagenes* (*39*, *40*) contains all six residues that act as ligands for the μ-oxy-bridged diiron site in the *E. coli* R2 protein in equivalent positions. With regard to its metal site, the Mn-RRase of *C. ammoniagenes* clearly has evolved as an independent catalyst in the RNA to DNA transition and as such represents a distinct class of ribonucleotide reductase of its own right.

Finally, we would like to emphasize that the same sample of CA2 protein studied here was used previously for X-band EPR spectroscopic detection of an organic radical in the Mn-RRase of *C. ammoniagenes* (*14*). As this radical, showing up again in the Q-band EPR spectroscopy with identical characteristics (*41*), is not present in apo-CA2 prepared from manganese-depleted cells, one might speculate on the function of the metal center in the Mn-RRase. We conclude that manganese in the native CA2 protein is one prerequisite for the existence of the stable free radical in the enzymatically active *C. ammoniagenes* RRase.

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REFERENCES

1. Stubbe, J., and van der Donk, W. (1995) *Chem. Biol. 2*, 793−801.
2. Reichard, P. (1993) *Science 260*, 1773−1777.
3. Follmann, H. (1982) *Naturwissenschaften 69*, 75−81.
4. Lammers, M., and Follmann, H. (1983) *Struct. Bonding 54*, 27−91.
5. Hogenkamp, H. P. C., & McFarlan, S. (1984) *Pharmacol. Ther. 32*, 393−404.
6. Harder, J. (1993) *FEMS Microbiol. Rev. 12*, 273−292.
7. Auling, G., and Follmann, H. (1994) *Met. Ions Biol. Syst. 30*, 131−161.
8. Auling, G. (1980) "Habilitationsschrift", Thesis, University of Hannover, Germany.
9. Teshiba, S., and Furuya, A. (1989) *Production of nucleotides and nucleosides by fermentation. Jpn. Technol. Rev. Sect. E*, Vol. 1/1, Gordon and Breach Science Publishers, Reading.
10. Auling, G., Thaler, M., and Diekmann, H. (1980) *Arch. Microbiol. 127*, 105−114.
11. Schimpff-Weiland, G., Follmann, H., and Auling, G. (1981) *Biochem. Biophys. Res. Commun. 102*, 1276−1282.
12. Willing, A., Follmann, H., and Auling G. (1988) *Eur. J. Biochem. 170*, 603−611.
13. Blasczyk, K. (1994) Ph.D. Thesis, University of Hannover, Germany.
14. Griepenburg, U., Laβmann, G., and Auling, G. (1996) *Free Radical Res. 26*, 473−481.
15. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
16. deBievre, P., and Barnes, I. L. (1985) *Int. J. Mass Spectrom. Ion Processes 65*, 211−230.
17. Wieghardt, K. (1989) *Angew. Chem. 101*, 1179−1198.
18. Schepler, K. L., Dunham, W. R., Sands, R. H., Fee, J. A., and Abeles, R. H. (1975) *Biochim. Biophys. Acta 397*, 510−518.
19. Blakley, R. J. (1982) in B_{12} (Dolphin, D., Ed.) Vol. 2, pp 381−418, John Wiley, New York.
20. Yonetani, T., Drott, H. R., Leigh, J. S., Reed, G. H., Watesman, M. R., and Asakura, T. (1970) *J. Biol. Chem. 245*, 2998−3003.
21. Reed, G. H., and Markham, G. D. (1984) in *Biological Magnetic Resonance* (Berliner, L. J., and Reuben, J., Eds.) Vol. 6, pp 73−142, Plenum Press, New York.
22. Dismukes, G. C., and Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274−278.
23. Antanaitis, B. C., Brown, R. D., Chasteen, N. D., Freedman, J. H., Koenig, S. H., Lilienthal, H. R., Peisach, J., and Brewer, C. F. (1987) *Biochemistry 26*, 7932−7937.
24. Bogumil, R., Kappl, R., Hüttermann, J., Sudfeldt, C., and Witzel, H. (1993) *Eur. J. Biochem. 213*, 1185−1192.
25. Reed, G. H., and Ray, J. R. (1971) *Biochemistry 10*, 3190−3197.
26. Pötsch, S., Drechsler, H., Liermann, B., Gräslund, A., and Lassmann, G. (1995) *Mol. Pharmacol. 45*, 792−796.
27. Sjöberg, B.-M., Loehr, T. M., and Sanders-Loehr, J. (1982) *Biochemistry 21*, 96−102.

28. Nordlund, P., and Eklund, H. (1993) *J. Mol. Biol. 232*, 123−164.
29. Kono, Y., and Fridovich, I. (1983) *J. Biol. Chem. 258*, 6015−6019.
30. Wieghardt, K., Bossek, U., Ventur, D., and Weiss, J. (1985) *J. Chem. Soc., Chem. Commun. 1985*, 347−349.
31. Sheats, J. E., Czernuszewics, R. S., Dismukes, G. C., Rheingold, A. L., Petrouleas, V., Stubbe, J., Armstrong, W. H., Beer, R. H., and Lippard, S. J. (1987) *J. Am. Chem. Soc. 109*, 1435−1444.
32. Reed, G. H., & Cohn, M. (1970) *J. Biol. Chem. 245*, 662−664.
33. Buttlaire, D. H., Reed, G. H., and Himes, R. (1975) *J. Biol. Chem. 250*, 261−270.
34. Markham, G. D., Nageswara, R., and Reed, G. H. (1979) *J. Magn. Reson. 33*, 595−602.
35. Markham, G. D. (1981) *J. Biol. Chem. 256*, 1903−1909.
36. Cammack, R., Chapman, A., Lu, W.-P., Karagouni, A., and Kelly, D. P. (1989) *FEBS Lett. 253*, 239−243.
37. Cooper, S. R., Dismukes, G. C., Klein, M. C., and Calvin, M. (1978) *J. Am. Chem. Soc. 100*, 7248−7252.
38. Zweygart, W., Weyhermüller, T., Renger, G., Wieghardt, K., and Lubitz, W. (1992) in *Research in photosynthesis* (Murata, N., Ed.) Vol. 2, pp 289−292, Kluwer Academic, Dordrecht.
39. Fieschi, F., Torrents, E., Toulokhonova, L., Jordan, A., Hellmann, U., Barbe, J., Gibert, I., Karlson, M., and Sjöberg, B.-M. (1998) *J. Biol. Chem. 273*, 4329−4337.
40. Oehlmann, W., Griepenburg, U., and Auling, G. (1998) *Biotechnol. Lett.* (in press).
41. Griepenburg, U. (1998) Ph.D. Thesis, University of Hannover, Germany.

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