Monovalent nickel in hydrogenase from *Chromatium vinosum*

Light sensitivity and evidence for direct interaction with hydrogen

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Redox titrations with hydrogenase from *Chromatium vinosum* show that its nickel ion can exist in 3, possibly 4, different redox states: the 3+, 2+, 1+ and possibly a zero valent state. The 1+ state is unstable: oxidation to Ni(II) occurs unless H_2 gas is present. The Ni(I) coordination, but not that of Ni(III), is highly light sensitive. A photoreaction occurs on illumination. It is irreversible below 77 K, but reversible at 200 K. The rate of this photodissociation reaction in ^2H_2O is nearly 6-times slower than in H_2O, indicating the breakage of a nickel-hydrogen bond. This forms the first evidence for an H atom in the direct coordination sphere of Ni in hydrogenase and for the involvement of this metal in the reaction with hydrogen.

Hydrogenase *Nickel(I)* *Light sensitivity* *Active site*

1. INTRODUCTION

In the last decade, hydrogenases have been purified from a variety of microorganisms. Although the physical and catalytic properties of these purified enzymes vary widely, they all contain one or several iron-sulphur clusters [1–3]. In many of these hydrogenases the presence of nickel has been established [4–15]. In some cases, however, Ni has proved to be absent [16,17]. In the nickel enzymes both nickel and at least one Fe-S cluster seem to be required for activity [8–14], except possibly in the enzyme from *Methanobacterium thermoautotrophicum*, strain Marburg [4,5]. There is, however, no demonstration yet for direct interaction of hydrogen with either prosthetic group. Hydrogenase from *Chromatium vinosum* contains 4 Fe atoms and 4 acid-labile sulphide ions per molecule [18] with a molecular mass of 62 kDa [19]. In the oxidized state intact enzyme shows EPR signals of a $[4Fe\text{-}4S]^{3+}$ cluster in spin-spin interaction with Ni(III) [20,21].

During redox titrations we observed that after reduction of the enzyme to an EPR-silent state a new signal, that we ascribe to Ni(I), appeared upon further reduction. We discovered that in this redox state the nickel is extremely light-sensitive. In addition we have evidence that this form of nickel is in direct interaction with hydrogen.

2. MATERIALS AND METHODS

C. vinosum, strain DSM 185, was grown in a 700-l batch culture [20]. The enzyme was purified exactly as described in [19] and dissolved in 50 mM Tris–HCl buffer (pH 7.4). Redox titrations with electron-mediating chemicals in 100 mM Hepes (pH 7.3 or 7.8) were carried out in a suitable 10 ml plexiglass vessel under oxygen-free argon. The mediator mixture consisted of benzyl viologen (440 μM), methyl viologen (550 μM), menadione (30 μM), duroquinone (30 μM), pyocyanine (20 μM), indigotrisulphonate (45 μM), phenazine methosulphate (20 μM) and neutral red (30 μM).

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The potential was adjusted by addition of aliquots of anaerobic solutions of 100 mM Na_2SO_4 or 100 mM $K_3Fe(CN)_6$ and measured with a Pt-electrode, using a calomel-electrode as reference. The potential was calibrated against a quinhydrone standard. Samples were anaerobically transferred into EPR tubes, by applying overpressure to the titration vessel, and then rapidly frozen by immersing the tube in cold (140 K) isopentane. Samples were stored in liquid nitrogen until use. EPR measurements were performed on a Varian E-9 EPR spectrometer as before [21]. Illumination of samples was carried out in the helium cryostat by shining white light (Osram Halogen Bellaphot, 150 W), via a light guide, through the irradiation grid of a Varian E-231 cavity. Light intensities were varied by using a calibrated set of neutral density filters (Oriel, Stamford, USA). Freeze-quench experiments in a nitrogen atmosphere were carried out as in [22].

3. RESULTS AND DISCUSSION

3.1. *EPR spectra of oxidized hydrogenase*

An oxidized enzyme preparation shows a complex set of EPR signals. We have reported [21] that defect, inactive enzyme molecules in the preparation give rise to two EPR signals due to the independent $S = 1/2$ systems of Ni(III) and a [3Fe-xS] cluster (called signal 3 and signal 1, respectively; fig.1B). Intact enzyme molecules also show two EPR signals, in this case caused by a spin-coupled pair of Ni(III) and a $[4Fe\text{-}4S]^{3+}$ cluster (called signal 4 and signal 2, respectively). The latter signals can only be observed below 20 K (fig.1A). The nickel signals, arising from both defect and intact molecules, are each due to two forms of Ni(III), called Ni-a and Ni-b. The ratio of these forms is the same in defect and intact molecules but varies from preparation to preparation. Trace A of fig.1 is a superposition of all these EPR signals.

3.2. *Intermediate reduction stages*

During a reductive titration of the enzyme we could distinguish several stages of reduction. First a stage, from positive potentials down to about −19 mV, in which the signals of the enzyme as isolated were stable (fig.1A). A second stage appeared on lowering the potential from −19 to −38 mV. All the lines belonging to the interacting spin-pair of Ni(III) (signal 4) and the $[4Fe\text{-}4S]^{3+}$ cluster (signal 2), disappeared simultaneously in this narrow potential span. At the same time the intensity of the non-interacting Ni(III) (signal 3) and the oxidized [3Fe-xS] cluster (signal 1) increased. We ascribe this observation to a transition of the $[4Fe\text{-}4S]^{3+}$ into a $[3Fe\text{-}x\text{S}]_{ox}$ cluster whereby the spin-coupling with the Ni(III) is broken. Similar observations have been made in the $g = 2$ region with the membrane-bound hydrogenase of *Alcaligenes eutrophus* [23], but in this case the spin-coupling between the two paramagnets has not been established. On further lowering of the redox potential (stage 3) a decrease of the signal of

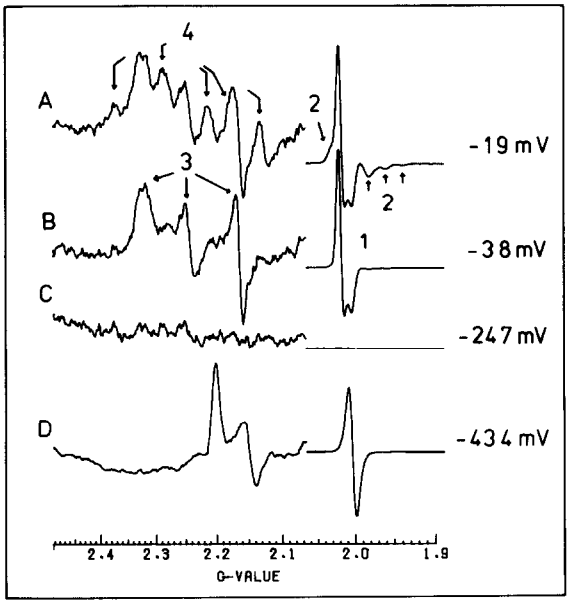

Fig.1. EPR spectra of *C. vinosum* hydrogenase during a redox titration at pH 7.3. Samples were withdrawn at different potentials. A, −19 mV; B, −38 mV; C, −247 mV; D, −434 mV. The lines of the 4 different EPR signals are indicated as 1–4. The left-hand part of the figure displays the nickel region and the right-hand part the iron-sulphur region. EPR conditions for the nickel region: microwave frequency, 9264 MHz; incident microwave power, 5 mW; modulation amplitude, 1.25 mT; temperature, 16 K. The gain for the traces A–C was 2.9 times that of trace D. EPR conditions for the Fe-S region: the same as above, except for the microwave power (2 mW) and temperature (13 K). The relative gains were 2 (A), 1.25 (B and C), and 1 (D).

the [3Fe-xS] cluster occurred ($E_{m,7.3}$ about -165 mV). Thereafter the signal in the nickel region diminished in intensity ($E_{m,7.3}$ about -175 mV). At -247 mV all signals in the Fe-S and nickel region had completely disappeared (fig.1C).

After this silent state a new signal, with two of its g values lying at $g = 2.20$ and 2.15, appeared at potentials lower than -300 mV. A similar signal has been reported recently for the F_{420}-reducing hydrogenase from *M. thermoautotrophicum*, strain ΔH [8] and for the enzyme from *Desulfovibrio gigas* [10]. In the case of *D. gigas* [10] it has been proven, with the help of ^{61}Ni, that the signal is derived from nickel. The signal at $g = 2$ in fig.1, trace D is due to radicals from the mediating dyes.

The simplest interpretation of our titration data (fig.1) is that nickel undergoes two successive redox changes: from the paramagnetic trivalent state in the oxidized enzyme, it is reduced via the diamagnetic divalent state to the paramagnetic monovalent state. When the Ni(I) signal appeared during the titrations, it was not possible to establish a stable potential in the titration vessel. This was not due to poor mediation as stable potentials could be obtained in the absence of enzyme. We interpret the observed drift of potential to higher values as a leak of reducing equivalents via the H_2-production activity of the enzyme. After replacement of Ar with H_2 gas a stable potential (about -480 mV at pH 7.8) was always obtained within a few minutes. Subsequent substitution of H_2 by Ar caused an immediate drift of the potential to higher values. A curious observation was that during this drift the intensity of the Ni(I) signal strongly increased and subsequently decreased to zero, whereby the enzyme returned to the EPR-silent state. We have earlier observed (H. van Heerikhuizen and S.P.J. Albracht, unpublished) that *C. vinosum* hydrogenase shows no hydrogen production activity with reduced methyl viologen under an H_2 atmosphere.

The Ni(I) signal, which was still detectable at 100 K, could also be evoked by incubating the enzyme with $Na_2S_2O_4$ in the absence or presence of methyl viologen, or with the natural substrate H_2. After incubation of the enzyme with H_2 (1 bar) at room temperature for 2 h, the signal (fig.2A) ascribed to Ni(I) accounted for only 3% of the

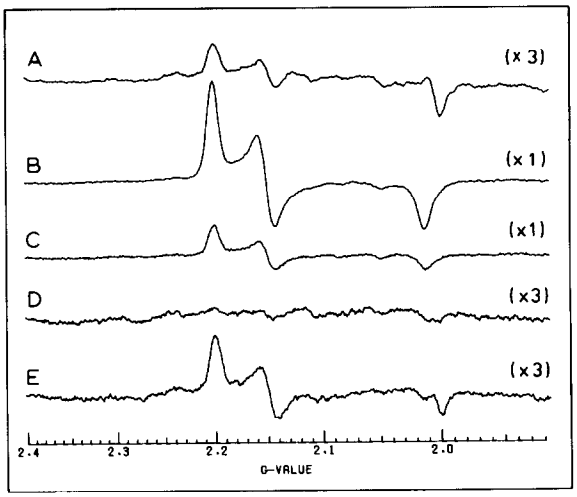

Fig.2. Behaviour of the Ni(I) signal during several treatments of the enzyme. A, enzyme treated with H_2 in a Thunberg cuvette for 2 h at 20°C. B, after removal of H_2, flushing with Ar and subsequent standing for 2 h at 20°C. C, after repeated evacuation and flushing with Ar and another period of 1.5 h at 20°C. D, as C but after standing for 20 h at 8°C. E, the cuvette was finally evacuated and flushed with H_2 once and then rapidly frozen in liquid nitrogen. This took less than 1 min. EPR conditions: microwave frequency, 9263 MHz; microwave power, 0.8 mW; modulation amplitude, 1.25 mT; temperature, 21 K. Traces A, D and E were recorded at a 3-fold increased gain.

amount of nickel maximally detectable in the trivalent state. Prolonged incubation with H_2 for 20 h did not influence this amount. Removal of H_2 by evacuation, with or without subsequent flushing with Ar or He, followed by incubation for several hours, increased the signal up to 13-fold (fig.2B). Longer incubation (fig.2C,D) and repeated evacuation and flushing with Ar finally caused a state where no signals at all could be detected, just as during the redox titration. In this EPR-silent state the enzyme is probably still in the active conformation, because after readmission of H_2, the Ni(I) signal reappeared in less than 1 min (fig.2E). Normally, with the enzyme as isolated, the appearance of the Ni(I) signal takes about 10 min incubation under H_2 (20°C).

We conclude that the transient appearance of the Ni(I) signal during the redox titrations, after replacement of H_2 by Ar, and after the same exchange of gases in a Thunberg cuvette, is due to the

same phenomenon: the enzyme in aqueous solution can be forced to low potentials only under an H_2 atmosphere. Elimination of H_2 from the gas phase results in an oxidation of the enzyme to a stable, EPR-silent state, demonstrating that the enzyme is in real redox equilibrium with H_2. Only at intermediate potentials can the Ni(I) signal be well observed. From the low intensity of this signal under H_2 we conclude that either a further reduction to Ni(O) has taken place or that the Ni(I) has become EPR-silent at this temperature by the influence of a paramagnetic component only reducible at very low potentials. An increase of this nickel signal upon removal of H_2 and replacement with Ar was also reported for the F_{420}-reducing hydrogenase from *M. thermoautotrophicum* [8]. In this case the conclusion was drawn that the nickel signal of reduced hydrogenase was an Ni(III) species, as the authors thought it unlikely that H_2-removal from reduced enzyme would lead to Ni(I). It was not reported, however, whether prolonged incubation with Ar resulted in an EPR-silent state.

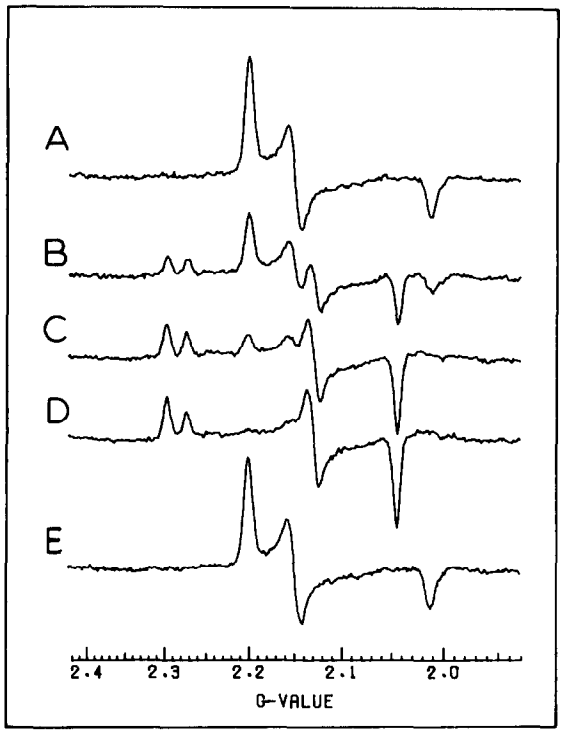

Fig.3. Light-induced transition of the Ni(I) signal of reduced hydrogenase. A, signal before illumination. B, C and D, signal after illumination for 1, 3 and 6 min, respectively. E, after thawing and freezing of the sample in D in the dark. EPR conditions, microwave frequency, 9265 MHz; microwave power, 0.8 mW; modulation amplitude, 0.63 mT; temperature, 22 K. All spectra were recorded with the same gain.

3.3. *Light-sensitivity of nickel(I)*

During this study, we repeatedly observed that after storage of H_2-reduced enzyme in liquid nitrogen, the EPR spectrum had been considerably changed. A thorough investigation revealed that the Ni(I) in reduced hydrogenase is extremely light-sensitive and that storage itself had no effect. In contrast, we could not find any light-sensitivity for Ni(III) (or the Fe-S cluster) in oxidized enzyme. Illumination of reduced enzyme at 22 K in the cavity of the EPR spectrometer caused the disappearance of the Ni(I) signal and the simultaneous appearance of a new signal (fig.3). Although irreversible below 77 K, the photolytic process was completely reversible at higher temperatures: after warming the sample in the dark to 200 K for 10 min, the original 'dark' nickel signal reappeared with the same intensity. Thawing and freezing in the dark had the same effect (fig.3D,E). The 'light-induced' signal is a summation of two overlapping signals with different g_z values and has the same double integrated intensity as the 'dark' signal. It was also still detectable at 100 K. The ratio of the two overlapping signals in trace D of fig.3 had no relation to the ratio of Ni-a and Ni-b observed in the oxidized enzyme. We interpret the light-induced changes in the EPR spectrum to be caused by breakage, as a result of the illumination, of a bonding between Ni(I) and one of its ligands, whereby the redox state of Ni does not change. Reassociation takes place only at elevated temperatures. Photosensitivity of coordination compounds of Ni has been described occasionally [24]. Light had no effect on the activities of the enzyme under the standard assay conditions.

3.4. *Influence of deuterium on the light-sensitivity of Ni(I)*

Two samples of hydrogenase were diluted 3-fold with ^2H_2O or H_2O and subsequently lyophilized. The samples were then redissolved in ^2H_2O (99.7%) and H_2O, respectively. No differences in line widths were observed for any of the signals

displayed by the oxidized enzyme. The same was true for the 'light-induced' signal of the H_2-reduced hydrogenase. For the 'dark' signal, however, we consistently detected with different preparations a narrowing of about 0.5 mT for the g_y line of the 'dark' signal with ^2H_2O as solvent (fig.4). This indicates that interaction of Ni(I) with exchangeable H atoms contributes to the width of this line. Replacement of H_2O by ^2H_2O had a drastic effect on the light sensitivity of the Ni(I) signal. From fig.5 it can be seen that the rates of transition from the 'dark' to the 'light-induced' signal in both cases were proportional to the light intensity I: $k = \epsilon\phi I$, where ϵ is the absorbance coefficient and ϕ the quantum yield. However, the slope observed for the enzyme in ^2H_2O is only 18% of that in H_2O. Because a difference in absorption coefficient of the enzyme in H_2O and ^2H_2O is not likely and the enzyme concentrations were the same, the difference in transition rates must be caused by a difference in quantum yield. The quantum yield (i.e., the ratio of the number of dissociated to excited molecules) is dependent on the bonding strength of the ligand that is removed on illumination. In view of the magnitude of the

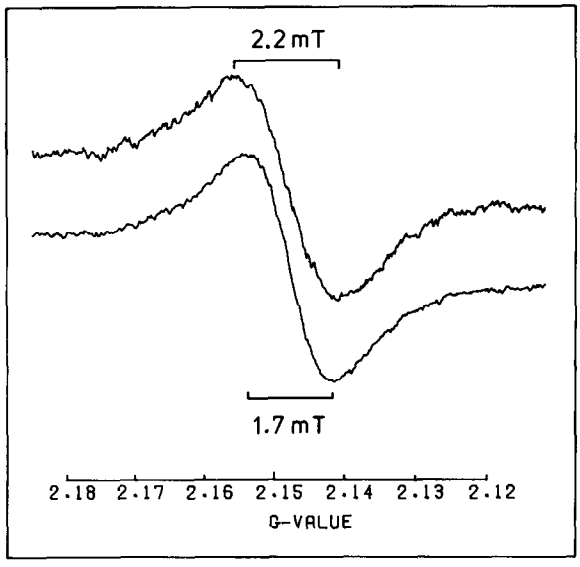

Fig.4. Effect of the substitution of H_2O by ^2H_2O on the g_y line of the 'dark' signal. Upper trace, the g_y line in H_2O. Lower trace, the g_y line in ^2H_2O. EPR conditions were as in fig.3.

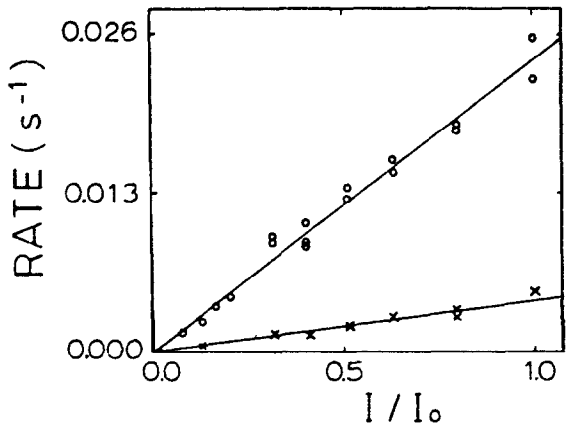

Fig.5. Effect of ^2H_2O on the light sensitivity of the Ni(I) signal in H_2-reduced hydrogenase. The initial rates of disappearance of the $g = 2.20$ line upon illumination at 22 K with different light intensities were measured with H_2O (○—○) or ^2H_2O (×—×) as solvent. EPR conditions: microwave frequency, 9264 MHz; microwave power, 2 mW; modulation amplitude, 1.25 mT; temperature, 22 K.

effect of ^2H_2O we propose that the light-sensitive bond is a direct one between Ni(I) and an exchangeable H atom. It is appropriate to mention at this point that under the conditions used, the enzyme catalyses a rapid $H/^2H$ exchange reaction between H_2 gas and ^2H_2O [25]. Thus the dramatic change in light-sensitivity of Ni(I) in the reduced enzyme in ^2H_2O provides strong evidence that hydrogen is in the direct coordination sphere of Ni(I), implying that Ni is the site of interaction of H_2 with the enzyme.

It may be added here that preliminary, anaerobically performed rapid-mixing rapid-freezing experiments have shown that the Ni(I) signals of the enzyme in H_2-saturated buffer (as the experiment was carried out in daylight, a mixture of 'light' and 'dark' signals was obtained in a control with H_2-saturated buffer) completely disappeared within 15 ms after mixing with excess of benzyl viologen (dissolved in H_2-saturated buffer). No intermediate signals were observed and the enzyme ended up in the EPR-silent state.

Light-sensitivity of Ni(I) is not restricted to the hydrogenase from *C. vinosum*. Identical results were obtained with the enzyme [15] from *Vibrio succinogenes* when treated in the same way (Z.W. van der Zwaan et al., unpublished). Since the

signal, that we call the 'dark' signal of Ni(I), has been earlier observed in enzymes from two other sources [8,10,11] we expect that the light-sensitivity of Ni(I) is a general property of Ni-hydrogenases.

4. CONCLUDING REMARKS

Our enzyme preparations contain variable amounts (15–50%) of molecules that have a [3Fe-*x*S] cluster. These molecules show no obvious activity in the normal assay [14,20] with artificial electron donors or acceptors. Both Ni and the [3Fe-*x*S] cluster are reduced, however, by H_2 along with Ni and the [4Fe-4S] cluster in intact enzyme. Since the signals in figs 2,3 amount to maximally 40% of the enzyme concentration, we cannot yet say whether they are from nickel in molecules with a 3Fe or a 4Fe cluster. Nevertheless, the results presented here demonstrate that the EPR detectable nickel is in redox equilibrium with hydrogen and in this sense we are dealing with catalytically active nickel.

Since the g_z value (2.011) of Ni(III) in the enzyme lies very close to 2 it follows that the unpaired electron resides in an orbital mainly with a d_{z^2} character [26] and that the $d_{x^2-y^2}$ orbital is highest in energy. A plausible coordination is a square-pyramidal one where at least 5 ligands are provided by the protein. On reduction to the monovalent state the free electron again appears in the d_{z^2} orbital, since once more the g_z value (2.012) is close to 2. With macrocyclic ligands for Ni(I) the unpaired spin is often found in another orbital, e.g., the $d_{x^2-y^2}$ and then none of the g values is close to 2 and $g_z > g_{y,x}$ [24,27,28]. The g_z value is defined here as g_1 in a signal where $g_1 - g_2 > g_2 - g_3$, taking $g_1 > g_2 > g_3$, or as g_3 when $g_1 - g_2 < g_2 - g_3$. To explain the observations described above we adopt the following simple model. A hydrogen atom, possibly H^-, is added in axial position to the coordination of nickel during reduction to Ni(I). In response the d_{z^2} orbital becomes highest in energy. Dissociation of this extra ligand by illumination results in a decrease of the energy of the d_{z^2} orbital to a value lower than that of the $d_{x^2-y^2}$ one. The unpaired electron is then in the latter orbital. In connection to the proposed locations of the unpaired electron under different conditions we would like to mention that no hyperfine splitting due to N ligands has been observed in any of our Ni(III) or Ni(I) spectra. This is in accordance with the recent report [29] on X-ray absorption experiments with the F_{420}-reducing enzyme of *M. thermoautotrophicum*, which indicated the presence of 3 S atoms in the direct coordination sphere of Ni(III).

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REFERENCES

[1] Weaver, P.F., Lien, S. and Seibert, M. (1980) Solar Energy 24, 3–45.
[2] Adams, M.W.W., Mortenson, L.E. and Chen, J.-S. (1980) Biochim. Biophys. Acta 594, 105–176.
[3] Mayhew, S.G. and O'Connor, M.E. (1982) Trends Biochem. Sci. 1, 18–21.
[4] Graf, E.-G. and Thauer, R.K. (1981) FEBS Lett. 136, 165–169.
[5] Albracht, S.P.J., Graf, E.-G. and Thauer, R.K. (1982) FEBS Lett. 140, 311–313.
[6] Friedrich, C.G., Schneider, K. and Friedrich, B. (1982) J. Bacteriol. 152, 42–48.
[7] Jacobson, F.S., Daniels, L., Fox, J.A., Walsh, C.T. and Orme-Johnson, W.H. (1982) J. Biol. Chem. 257, 3385–3388.
[8] Kojima, N., Fox, J.A., Hausinger, R.P., Daniels, L., Orme-Johnson, W.H. and Walsh, C.T. (1983) Proc. Natl. Acad. Sci. USA 80, 378–382.
[9] Cammack, R., Patil, D., Aguirre, R. and Hatchikian, E.C. (1982) FEBS Lett. 142, 289–292.
[10] Moura, J.J.G., Moura, I., Huynh, B.H., Krüger, H.-J., Teixeira, M., DuVarney, R.G., DerVartanian, D.V., Ljungdahl, P., Xavier, A.V., Peck, H.D. jr and LeGall, J. (1982) Biochem. Biophys. Res. Commun. 108, 1388–1393.
[11] Teixeira, M., Moura, I., Xavier, A.V., DerVartanian, D.V., LeGall, J., Peck, H.D. jr, Huynh, B.H. and Moura, J.J.G. (1983) Eur. J. Biochem. 130, 481–484.

[12] Krüger, H.-J., Huynh, B.H., Ljungdahl, P.O., Xavier, A.V., DerVartanian, D.V., Moura, I., Peck, H.D. jr, Teixeira, M., Moura, J.J.G. and LeGall, J. (1982) J. Biol. Chem. 257, 14620–14623.
[13] Lalla-Maharajh, W.V., Hall, D.O., Cammack, R., Rao, K.K. and LeGall, J. (1983) Biochem. J. 209, 445–454.
[14] Albracht, S.P.J., Albrecht-Ellmer, K.J., Schmedding, D.J.M. and Slater, E.C. (1982) Biochim. Biophys. Acta 681, 330–334.
[15] Unden, G., Böcher, R., Knecht, J. and Kröger, A. (1982) FEBS Lett. 145, 230–234.
[16] Huynh, B.H., Czechowski, M.H., Krüger, H.J., DerVartanian, D.V., Peck, H.D. jr and LeGall, J. (1984) Proc. Natl. Acad. Sci. USA 81, 3728–3732.
[17] Adams, M.W.W. and Mortenson, L.E. (1984) J. Biol. Chem. 259, 7045–7055.
[18] Gitlitz, P.H. and Krasna, A.J. (1975) Biochemistry 14, 2561–2568.
[19] Van Heerikhuizen, H., Albracht, S.P.J., Slater, E.C. and Van Rheenen, P.S. (1981) Biochim. Biophys. Acta 657, 26–39.
[20] Albracht, S.P.J., Kalkman, M.L. and Slater, E.C. (1983) Biochim. Biophys. Acta 724, 309–316.
[21] Albracht, S.P.J., Van der Zwaan, J.W. and Fontijn, R.D. (1984) Biochim. Biophys. Acta 766, 245–258.
[22] De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1982) Biochim. Biophys. Acta 681, 41–53.
[23] Schneider, K., Patil, D.S. and Cammack, R. (1983) Biochim. Biophys. Acta 748, 353–361.
[24] Haines, R.I. and McAnley, A. (1981) Coord. Chem. Rev. 39, 77–119.
[25] Krasna, A.I. (1978) Methods Enzymol. 53, 296–314.
[26] Wertz, J.E. and Bolton, J.R. (1972) Electron Spin Resonance. Elementary Theory and Practical Applications, McGraw-Hill, New York.
[27] Lewis, J. and Schröder, M. (1982) J. Chem. Soc. Dalton Trans., 1085–1089.
[28] Ansell, C.W.G., Lewis, J., Raithby, P.R., Ramsden, J.N. and Schröder, M. (1982) J. Chem. Soc. Chem. Commun., 546–547.
[29] Lindahl, P.A., Kojima, N., Hausinger, R.P., Fox, J.A., Teo, B.K., Walsh, C.T. and Orme-Johnson, W.H. (1984) J. Am. Chem. Soc. 106, 3062–3064.