SUPERCLUSTERS

A search for novel structures and functions of biological iron-sulfur clusters

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus,
Dr C.M. Karssen
in het openbaar te verdedigen op 1 oktober 1996
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

ISBN: 90 5485 629 758

ISBN 90-5485-595-9

The research described in this thesis was financially supported by the Netherlands Organization of Scientific Research (NWO) under the auspices of the Netherlands Foundation for Chemical Research (SON)

NN08201, 2146

1. Het afbeelden van de natuur als een levende, uitvoerende instantie is een vorm van wetenschappelijk animisme.

J.J.P. Fraústo da Silva & R.J.P. Williams (1991) in *The Biological Chemistry of the Elements, Oxford University Press, Oxford.*

2. Het ferredoxine uit *Desulfovibrio vulgaris* (Hildenborough) is mogelijkerwijs een ferreguline.

Dit proefschrift, hoofdstuk 5

3. De hoge redoxpotentiaal van de wolfraam in het aldehyde oxidoreductase uit *Pyrococcus furiosus* doet geen lampje opgaan over de biologische functie van dit metaal.

Dit proefschrift, hoofdstuk 6

4. Het veelvuldig citeren uit overzichtsartikelen betekent een onderwaardering van het oorspronkelijke, experimentele werk, leidt gemakkelijk tot verspreiding van onjuist geïnterpreteerde of foutief overgenomen gegevens, en dient om deze redenen vermeden te worden.

5. Het is wenselijk dat spectroscopische metingen aan enzymen zoveel mogelijk worden gedaan aan *actieve* enzympreparaten, vooral wanneer de resultaten hiervan gepubliceerd worden in *bio*chemische tijdschriften.

S. Mukund & M.W.W. Adams (1991) J. Biol. Chem. 266, 14208-14216.

6. De driedimensionale structuur van metallo-proteïnen verschaft geen inzicht in het redoxgedrag van het metaal noch in het katalytisch mechanisme.

W.R. Hagen, H. Wassink, R.R. Eady, B.E. Smith, & H. Haaker (1987) Eur. J. Biochem. 169, 457-465.

J. Kim & D.C. Rees (1992) Science 257, 1677-1682.

7. De term "zorgverzekeraar" blijkt bijzonder goed gekozen nu velen zich, als gevolg van de strenge eisen die gelden bij het afsluiten van een ziekenkostenverzekering, zich van zorg verzekerd weten.

8. Het plan om apen dezelfde rechten toe te kennen als mensen teneinde een "mens"waardig bestaan te garanderen zal, gezien de wereldwijde martelpraktijken, niet op voorhand gunstig uitpakken voor deze dieren.

Science (1996) 273, 39

9. Met aardappelzetmeel als voer voor *Pyrococcus furiosus* blijft de grootste kruidenier van het land op de kleintjes letten.

Dit proefschrift, hoofdstuk 3

10. De architectuur van veel prehistorische, megalithische bouwwerken wijst erop dat de makers van deze bouwwerken een zogenaamde "nieuwjaarsborrel-cultus" kenden.

Superclusters. A quest for novel structures and functions of biological iron-sulfur clusters.

A.F. Arendsen

1 oktober 1996

Voorwoord

De wetenschappelijke traditie wil dat een promotie-onderzoek wordt afgesloten met een proefschrift. Een dergelijk exemplaar ligt nu voor u. Omdat er één naam op de omslag prijkt zou de lezer de indruk kunnen krijgen dat dit boekje het resultaat is van de inspanningen van die ene persoon, de auteur. Dat zou een foute indruk zijn. De rol van de auteur is in werkelijkheid zeer beperkt. Sterker nog, zonder de inzet van vele anderen zou dit boekje nooit geschreven hebben kunnen worden. Een dankwoord is hier dan ook op zijn plaats.

Allereerst wil ik mijn promotor Prof. Fred Hagen bedanken. Door mij op sleeptouw te nemen naar talloze congressen, zijn aanstekelijk enthousiasme en zijn oorspronkelijke ideeën is bij mij de bio-anorganische snaar geraakt. Ik had mij geen betere leermeester kunnen wensen. Mijn promotor Prof. C. Veeger ben ik zeer erkentelijk voor het feit dat ik mijn promotie-onderzoek heb kunnen uitvoeren op de vakgroep biochemie. Het was een voorrecht om aan zijn vakgroep werkzaam te mogen zijn. Zijn opvolger, Prof. Colja Laane, is weliswaar niet mijn promotor, maar heeft mij wel als zodanig gesteund, waarvoor dank.

Yvonne Bulsink heeft met haar grote inzet niet alleen aan de wetenschappelijke productie bijgedragen, maar -minstens zo belangrijk- voor de goede sfeer op het lab gezorgd. Ik heb de samenwerking met haar als bijzonder plezierig ervaren. Veel werk is verzet door "mijn" studenten: Jos Schalk, Jeroen Kramer, Peter Veenhuizen, Mark Moorthamer, Giuliano Andreoli, Bart Keijser, Marcel de Vocht, Dora Pinho en Femke Merkx. Zonder hun inzet zou dit boekje er bepaald anders hebben uitgezien. Ik wil mijn collega-AIO's Dr. Marc Verhagen en Dr. Dirk Heering bedanken voor de prettige samenwerking en de vele nuttige tips en aanwijzingen. Dr. Julia Butt, Dr. Sophie Marritt en Marco Hulstein wil ik bedanken voor de prettige samenwerking en voor de hulp bij de afwikkeling van dit proefschrift. Met mijn nieuwe "labgenoten" Hans Wassink, Martina Duyvis en Dr. Huub Haaker heb ik een korte, doch daarom niet minder prettige samenwerking mogen beleven. Voorts wil ik bedanken Laura Ausma voor haar hulp bij allerlei administratieve rompslomp en Martin Bouwmans voor het tekenen van de figuren. Zo zou ik alle leden van de vakgroep wel kunnen opnoemen. Dat zal ik niet doen; ik dank een ieder die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit proefschrift.

De samenwerking met Ine van Kuijk en Dr. Fons Stams heeft mij doen kennismaken met het enzym fumarase. Via Dr. Siem Albracht, Trienke van der Spek, Randolph Happe en Winfried Roseboom ben ik geïntroduceerd in de infrarood-spectroscopie. Door de samenwerking met Dr. Martin Feiters ben ik in aanraking gekomen met EXAFS spectroscopie.

I thank Dr. Julia Steuber-Fritz for the very pleasant collaboration on sulfite reductase. Prof. Trautwein, Dr. Eckhard Bill and Monika Kröckel have introduced me to Mössbauer spectroscopy.

Prof. Peter Lindley, Jonathan Hadden, Graeme Card, Alan McAlpine, Slava Zaitsev and Sue Bailey are acknowledged for their support and assistance with the crystallization of the 'prismane' protein. Special thanks to Peter Lindley for his kind invitation to work at the CCLRC.

Tenslotte wil ik mijn ouders en Yvonne bedanken voor hun onvoorwaardelijk en onmisbare steun.

CONTENTS

ABBREVIATIONS

A	hyperfine constant, absorbance
Å	Ångström (10^{-10} m)
Ac	acetate
ATP	adenosine triphosphate
B	magnetic field
ß	Bohr magneton
C	cysteine
CoA	coenzyme A
D	axial zero-field splitting parameter
Da	Dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DSM	Deutsche Sammlung für Mikroorganismen
DTT	dithiothreitol
E	rhombic zero-field splitting parameter or redox potential
E_m	reduction potential
EDTA	ethylenediaminetetraacetate
EPPS	N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonate]
EPR	electron paramagnetic resonance
F	Farady constant
FAD	flavin adenine dinucleotide
FeMoco	iron-molybdenum cofactor
FTIR	Fourier transform infrared
FPLC	fast protein liquid chromatography
g	g-factor
H	Hamiltonian operator
Hepes	4-[2-hydroxyethyl]-1-piperazineethane sulphonic acid
HiPIP	high potential iron-sulfur protein
I	nuclear spin quantum number
i	current
IEF	isoelectric focussing
J	Joule
K	Kelvin
K_m	Michaelis-Menten constant
Mes	2-[N-morpholino]ethanesulphonic acid
NAD(P)H	reduced nicotinamide-adenine dinucleotide (phosphate)
NHE	normal hydrogen electrode
n	number of electrons
NMR	nuclear magnetic resonance
PEG	polyethyleneglycol
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point

R	gas constant
RNA	ribonucleic acid
S	electron spin (operator)
SCE	saturated calomel electrode
SDS	sodium dodecyl sulfate
T	absolute temperature, tesla
Tricine	N-tris[hydroxymethyl]methylglycine
U	unit of enzyme activity (μmol product/minute)
UV	ultraviolet.

GENERAL INTRODUCTION

1.1 REDOX CHEMISTRY IN BIOLOGY

In the living cell **redox reactions** play an important part in metabolic processes, e.g. photosynthesis, oxydative phosphorylation, and nitrogen fixation. Proteins involved in redox processes have to possess devices capable of 'storing' electrons, in other words these proteins have to be redox-active. Some contain an organic redox active group like flavin, pyrroloquinoline quinone, topa quinone, or tryptophan tryptophylquinone. Alternatively, redox proteins may contain **transition metals** as redox-active cofactors. Transition elements have partially filled valency orbitals, and usually exist in two or more redox states. Many transition elements are found in nature involved in redox processes; vanadium, manganese, iron, cobalt, nickel, copper, molybdenum and tungsten. Most are 3d elements; molybdenum and tungsten are 4d and 5d elements, respectively, which are also the only 4d and 5d elements found in biology, which is possibly explained by their bio-availability[1]. The ability of a protein to catalyze a redox reaction largely depends on the reduction potential of the redox-active group. As all biological processes occur in aqueous solutions, the physiological bounderies of life are determined by the oxidation and reduction of water (E_m H_2O/O_2 = 830 mV, E_m H_2/H^+ = –420 mV; at 25°C and pH 7); nevertheless, redox proteins with a reduction potential lower than -420 mV have been found.

1.2 IRON-SULFUR CLUSTERS

Although iron can exist in many redox states, it occurs mainly as Fe^{2+} and Fe^{3+}. A fixed reduction potential of the Fe^{2+}/Fe^{3+} couple in solution would be of little biological relevance, but the highly specific spacial organization of the iron in a protein matrix apparently provides an excellent means to 'tune' the redox potential of the iron atom(s). Thus, iron in proteins is mainly found in **hemes** and in **iron-sulfur clusters**. Four types of 'classical' iron-sulfur (Fe-S) clusters can be distinguished: a one-iron cluster (rubredoxin-like cluster) (Fig. 1.1), a two-iron cluster ([2Fe-2S]) (Fig. 1.2), a three-iron cluster ([3Fe-4S]) (Fig. 1.3), and a four-iron cluster ([4Fe-4S]) (Fig. 1.4). These four 'standard' Fe-S clusters are found in all organisms, but their existence was only recognized since the 60's. Since then, hundreds of Fe-S cluster containing proteins have been isolated, and this number is still increasing[2-7]. The clusters shown in Fig. 1 all share a basic building plan. They are ligated to the protein by the sulfur atoms of cysteine residues to the iron atoms[8-11]. The iron atoms in the cluster experience a distorted tetrahedral co-ordination. Because the energy splitting in a tetrahedral co-ordination is relatively small, the iron is always high-spin. The iron atoms are μ-S^{2-} bridged, except for the mononuclear cluster (rubredoxin) which, strictly spoken, is no Fe-S cluster because of the absence of acid-labile sulfide atoms.

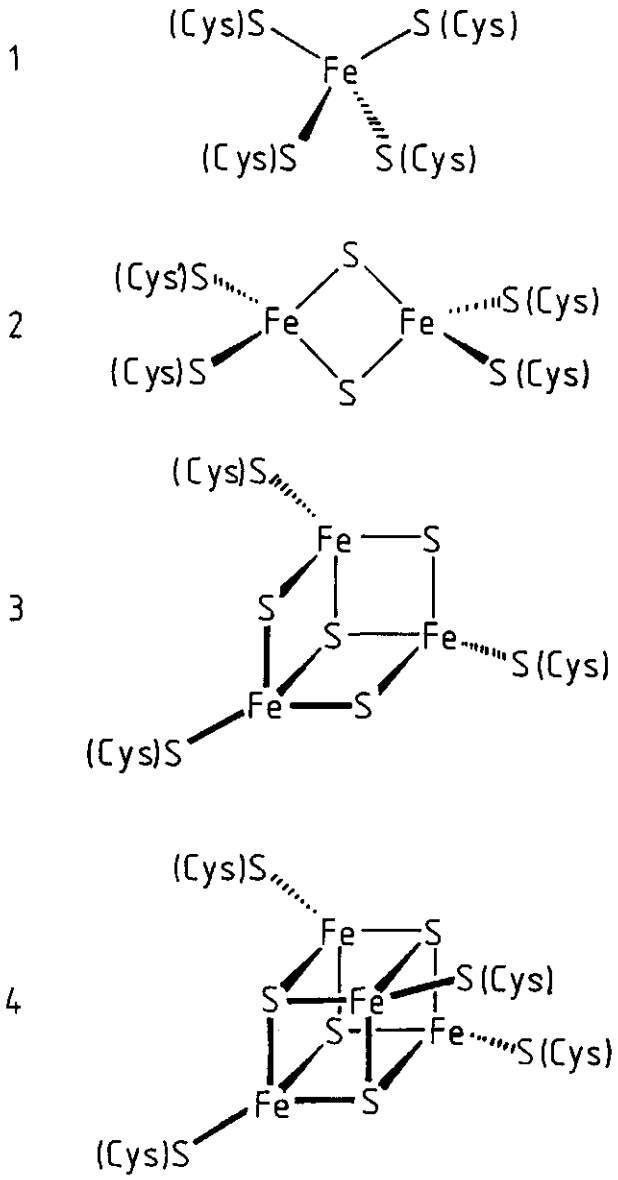

Fig. 1. Common Fe-S clusters.

Each iron-atom can formally exist in two redox states, i.e. Fe^{2+} and Fe^{3+}. Therefore, a [2Fe-2S] cluster can theoretically exist in three redox states. However, this cluster can only take up one electron under physiological conditions[12,13]. The valency of a cluster is usually expressed ignoring the charge of the ligands. Thus, a [2Fe-2S] cluster with one Fe^{2+}, one Fe^{3+}, and two μ-S^{2-} bridging atoms has a formal net charge of 1+. The one-electron (and also fully) oxidized cluster has a charge of 2+. This Fe-S cluster is denoted as $[2Fe\text{-}2S]^{(2+;1+)}$. Accordingly, four-iron clusters can be described as $[4Fe\text{-}4S]^{(2+;1+)}$, and three-iron clusters as $[3Fe\text{-}4S]^{(1+;0)}$[14]. High-spin Fe^{3+} has five unpaired electrons in its 3d orbitals, and, therefore, has an electron spin S = 5/2. Fe^{2+} has four unpaired electrons and is S = 2. Mössbauer spectra of reduced [2Fe-2S] clusters (with one Fe^{2+} and one Fe^{3+}) can be explained when one Fe^{2+} and one Fe^{3+} atom are assumed to be present[12]. If so, then this cluster would be expected to exhibit an S = 5/2 and an S = 2 system in EPR spectroscopy. Nevertheless, only a single S = 1/2 EPR spectrum is found. This effect can be explained by **exchange interaction** of the individual spins of the iron atoms in the cluster (reviewed by Hagen[15]). In the case of the $[2Fe\text{-}2S]^{(2+;1+)}$ cluster, coupling of the spins results in the lowest possible combination; i.e 5/2 - 2 = 1/2. This effect is called **super exchange**. Super exchange results in antiferromagnetic coupling. On the other hand, exchange interaction may also result in the highest possible combination; i.e. 5/2 + 2 = 9/2. This ferromagnetic coupling is a result of **double exchange**, which occurs when charge is delocalized. For example, in the case of [4Fe-4S] clusters two pairs of iron atoms couple ferromagnetically to a high-spin state, and these in turn couple to each other antiferromagnetically to a low-spin state. Thus, the reduced [4Fe-4S] cluster is S = 1/2, whereas the oxidized cluster is diamagnetic (S = 0).

1.2.1 Mononuclear iron-sites

Mononuclear iron-sites (Fig. 1.1) occur in bacterial redox proteins called **rubredoxins**. These are red colored, low molecular mass, redox active ($Fe^{(3+;2+)}$) proteins of typically 5-12 kDa molecular mass; the crystal structure was solved in 1979[16]. Their function is still unknown, although a role as electron carrier has been proposed[17]. However, redox behaviour *in vitro* does not necessarily imply a redox role *in vivo*. For example, aconitase catalyzes a non-redox reaction, although its [4Fe-4S] cluster is redox active *in vitro*[18].

Rubredoxin-type iron centers have been found in a few proteins with unknown function, like **desulforedoxin**[19-21], **desulfoferrodoxin**[22], **neelaredoxin**[23], **rubrerythrin**[24], and **nigerythrin**[25], all of which are found in sulfate-reducing bacteria. Interestingly, the latter two proteins contain in addition a dinuclear cluster (see 1.2.4).

1.2.3 Two-, three-, and four-iron clusters

Two-iron ([2Fe-2S], Fig. 1.2) and four-iron ([4Fe-4S, Fig. 1.4) clusters are very common in nature[2-7]. They are present in **ferredoxins** and in numerous redox and non-redox enzymes. In ferredoxins [2Fe-4S] and [4Fe-4S] clusters occur as single clusters, or in pairs. They are involved in almost all redox processes in life, like in glycolysis, oxidative phosphorylation, and many others. Ferredoxins are small, rigid, Fe-S containing proteins, with a typical molecular mass of 6-12 kDa. They are supposed to function as electron carriers, i.e they shuttle electrons between enzymes involved in redox catalysis. Most redox enzymes containing Fe-S clusters contain additional metals or cofactors, like nickel ([NiFe] hydrogenase), molybdenum (xanthine oxidase), FAD (fumarate reductase), heme (cytochrome reductase or bc_1 complex), or tungsten (aldehyde oxidoreductase), although at least two redox enzymes contain solely Fe-S clusters as redox cofactors, namely Fe-only hydrogenase and alternative (i.e. Fe-only) nitrogenase. The reduction potential of $[2Fe\text{-}2S]^{(2+;1+)}$ clusters ranges from -240 to -460 mV, whereas $[4Fe\text{-}4S]^{(2+;1+)}$ clusters have a very broad potential range, i.e. from 0 to -645 mV[26]. Although almost all Fe-S clusters are ligated to the protein through cysteine residues, some [2Fe-2S] clusters do not obey this rule. For example, **Rieske-type proteins** contain a [2Fe-2S] cluster with two cysteine ligands and two non-cysteine ligands, probably histidine[27]. They were first isolated from beef heart mitochondria by Rieske and co-workers[28]. These [2Fe-2S] clusters feature more positive potentials than normal [2Fe-2S] clusters, i.e. from +300 to -155 mV[29]. Although $[2Fe\text{-}2S]^{(2+;1+)}$ clusters switch from the two-ferric state to the mixed-valency ferrous/ferric state, it is possible to 'superreduce' the cluster to the two-ferrous state *in vitro*. Verhagen *et al.*[30] were able to reduce the water soluble Rieske fragment from bovine heart bc_1 complex by cyclic voltammetry, and Sykes and co-workers could reduce 'normal' [2Fe-2S] clusters to the two-ferrous state by pulse radiolysis[31]. These phenomena do probably not convey a biological meaning. Likewise, some [4Fe-4S] clusters do not follow the all-cysteine rule. The fourth ligand of the [4Fe-4S] cluster of *Pyrococcus furiosus* ferredoxin was shown to be an aspartic acid by ^1H NMR[32]. Moreover, one of the two [4Fe-4S] clusters of the [NiFe] hydrogenase from *Desulfovibrio gigas* is ligated by three cysteines and one histidine[33].

A special class of [4Fe-4S] clusters containing proteins feature high reduction potentials, ranging from +50 to +500 mV[29,34]. These proteins are called High Potential Iron Proteins (**HiPIP**'s)[35,36]. They are unique in the sense that they contain $[4Fe\text{-}4S]^{(2+;3+)}$ clusters. However, Heering *et al.*[37] were able to 'superreduce' a HiPIP $[4Fe\text{-}4S]^{(2+;1+)}$ cluster to the 1+ state, both by cyclic voltammetry and by chemical reduction, thereby yielding a $[4Fe\text{-}4S]^{(3+;2+;1+)}$ cluster.

It has long been known that oxidative damage of a [4Fe-4S] cluster can result in the loss of an iron atom, leading to the formation of a 3Fe cluster (Fig. 1.3)[5]. However, the presence of [3Fe-4S] clusters as constituant parts of proteins is now fully established, and the crystal structure of a ferredoxin containing a [3Fe-4S] cluster was solved in 1988[9,10]. A [3Fe-4S] cluster can be thought of as a cubane with one iron atom missing. $[3Fe\text{-}4S]^{(1+;0)}$ clusters have been found in 7Fe ferredoxins, e.g. from *Azotobacter vinelandii* and *Desulfovibrio africanus* III. A special feature of the

latter protein is its ability to undergo so-called cluster-interconversion, i.e. the cluster can reversibly be converted from a [3Fe-4S] to a [4Fe-4S] cluster[38]. In addition to two [4Fe-4S] clusters the NiFe hydrogenase from *Desulfovibrio gigas* contains a [3Fe-4S] cluster[33], and [3Fe-4S] clusters have been suggested to be part of several (redox) enzymes, for instance in the respiratory complex II or succinate dehydrogenase.

[4Fe-4S] clusters are not only involved in redox catalysis, but also in non-redox catalysis. In the latter case the Fe-S cluster functions as a Lewis acid. For example, aconitase catalyzes the hydroxylation of of citrate to cis-aconitate[18]. This reaction does not involve electron transfer. Binding of the substrate to one of the iron atoms changes the coordination of the iron to six coordinate[39,40]. Another example comes from the DNA repair enzyme Endonuclease III from *Escherichia coli*, which contains a [4Fe-4S] cluster[41]. The [4Fe-4S] cluster is supposed to be involved in positioning basic residues with the DNA phosphate backbone. Finally, Fe-S clusters may be involved in gene regulation[42,43].

The protein matrix determines the reduction potential of the Fe-S cluster to a large extent. Table 1 lists some Fe-S clusters with their reduction potentials[26,34,44,45]. Clearly, the potentials deviate largely from that of aqueous Fe^{2+}/Fe^{3+} under standard conditions. We can also observe that [4Fe-4S] clusters show the widest range in reduction potentials, whereas the one-iron containing rubredoxin has only a narrow potential range. Apparently, increasing the number of iron atoms in a cluster is a means to extend the redox span of biological iron.

Table 1. Reduction potentials of Fe-S clusters[26,34,44,45]

Clustertype	Source	E_m range (mV)
$[Fe]^{(3+;2+)}$	Rubredoxins	-20 to +37
$[2Fe\text{-}2S]^{(2+;1+)}$	Ferredoxins	-240 to -460
$[Fe_2S_2(RS)_2N_2]^{(0;-1)}$	Rieske proteins	-155 to +312
$[4Fe\text{-}4S]^{(2+;1+)}$	Bacterial ferredoxins	-645 to 0
$[4Fe\text{-}4S]^{(3+;2+)}$	HiPIP	+50 to +500

1.2.3 Dinuclear μ-oxo bridged clusters

A rather special class of iron structures are the **μ-O bridged dinuclear centers**[46] (Fig. 2). These centers have been found in a number of proteins with various functions, e.g. the O_2-transporting protein hemerythrin from invertebrates[47], and several redox enzymes, like ribonucleotide reductase[48,49], purple acid phosphatase[50,51], and methane monooxygenase[52-53].

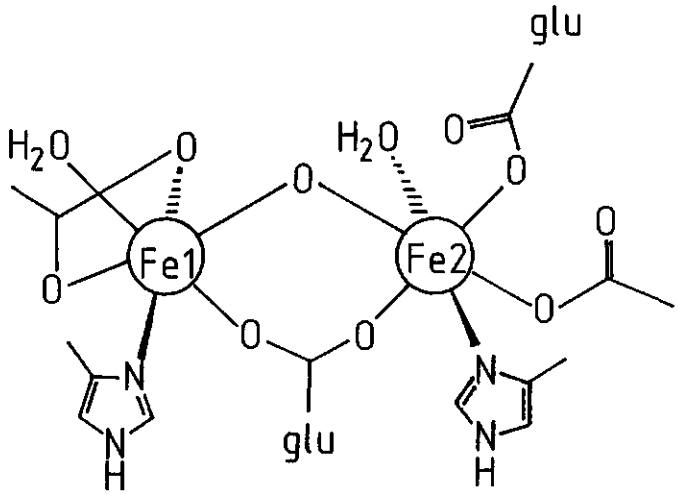

Fig. 2. Dinuclear iron center in R2 subunit of *E. coli* ribonucleotide reductase[54]

1.3 SUPERCLUSTERS

There is growing evidence that, in addition to common Fe-S clusters, there are clusters containing more than four iron atoms. Table 2 lists five enzymes supposedly containing so-called **'superclusters'**. These enzymes all catalyze multi-electron transfer reactions.

Table 2. Multi-electron transferring enzymes

Enzyme	reaction	equation
Nitrogenase	$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$	(1)
[Fe-only] hydrogenase	$2H^+ + 2e^- \rightarrow H_2$	(2)
Dissimilatory sulfite reductase	$SO_3^{2-} + 6H^+ + 6e^- \rightarrow S^{2-} + 3H_2O$	(3)
CO dehydrogenase	$CO_2 + 2H^+ + 2e^- \rightarrow CO + H_2O$	(4)
Prismane protein	?	

Reactions 1-4 are all multi electron redox reactions involving the activation of small, inorganic molecules (e.g. N_2, H_2, SO_x, NO_x). These compounds are important because they may be a threat to the environment (NO_x,SO_x), or an important source of energy (H_2) or fertilizer (H_2 plus N_2). The afore mentioned enzymes catalyze these reactions at relatively mild conditions, compared to industrial catalysis. Despite the

simplicity of these reactions on paper, the responsible enzymes require complex Fe-S clusters exhibiting peculiar spectroscopic behaviour (e.g. S ≥ 3/2 EPR). The enzymes are usually large and multimeric, and they contain high numbers of iron and acid-labile sulfide.

1.3.1 Nitrogenase

The FeMoS enzyme **nitrogenase** catalyzes the eight-electron reduction of molecular nitrogen to ammonia at the expense of 16 ATP molecules (1)[55-61]. The enzyme occurs in bacteria like *Clostridium, Azotobacter*, and *Rhodospirillum*. It consists of two proteins; component I and II. Crystal structures of both components have recently been solved[55,62-64]. The so-called MoFe protein (component 1) contains 2 molybdenum atoms and 30 iron atoms, divided over 2x2 special iron-sulfur structures. The first one is the Fe_7MoS_8 containing iron molybdenum cofactor, or **FeMoco** (Fig. 3a).

The FeMoco is supposed to be the substrate-binding site. The cofactor can exist in three redox states; the oxidized form is diamagnetic, the reduced form has an S = 3/2 ground state. Under turnover conditions FeMoco is superreduced, probably having integer S ≥ 1 electron spin[65,66].

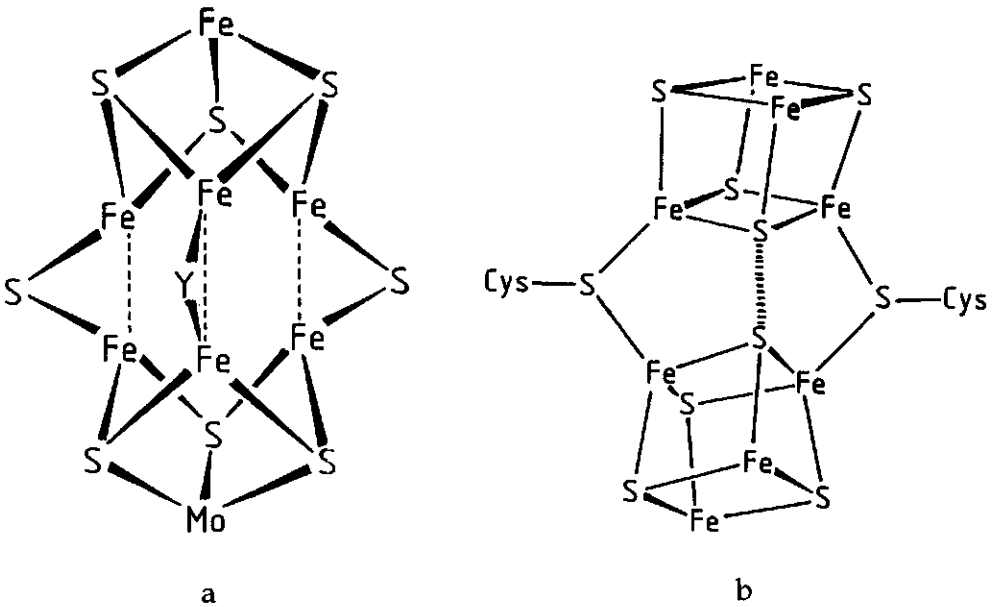

Fig. 3. Iron-molybdenum cofactor (a) and P-cluster (b) from nitrogenase[63]

The second special cluster in nitrogenase is the **P-cluster**. Hagen and co-workers proposed an eight-iron containing Fe-S cluster on the basis of EPR spectroscopy[67]. EPR spectra of the three-electron oxidized P-cluster revealed a spin mixture of S = 1/2 and S = 7/2[68]. The two-electron oxidized P-cluster has integer spin, probably S = 3, and the fully reduced cluster is diamagnetic. The eight-iron proposal proved to be correct with the elucidation of the crystal structure by Kim and Rees[62-64] (Fig. 3b). Although the cluster appears as a set of two cubanes, all irom atoms are magnetically coupled into one net spin system.

1.3.2 (Iron-only) hydrogenase

A second class of enzymes reported to contain uncommon Fe-S clusters consists of **hydrogenases**. Hydrogenase is one of the four nickel containing enzymes. In addition to nickel, some hydrogenases contain the biologically rare element selenium. However, a small number of hydrogenases is known to contain only iron (Table 3). Thus, hydrogenases can be divided into three subgroups depending on their metal content; iron-only, NiFe, and NiFeSe hydrogenases[69-71].

Already in 1986 Hagen and co-workers proposed a novel iron-sulfur cluster to be the active site of the periplasmic iron-only hydrogenase from the sulfate-reducing bacterium *Desulfovibrio vulgaris* strain Hildenborough[72]. This enzyme contains an uncommon iron-sulfur cluster as deduced from a rhombic EPR spectrum (g = 2.10) in the partially reduced enzyme. The signal was first detected in the iron-only hydrogenase from *C. pasteurianum*, in 1975[73], and it was also found in the iron-only hydrogenases from *M. elsdenii*[74], *D. vulgaris* (Hildenborough)[75], and *Trichomonas vaginalis*[76]. This uncommon cluster is supposed to be the site where hydrogen activation takes place[77], and because of this it was given the name **H-cluster**[78]. Based on the metal content of the Fe-only hydrogenase from *D. vulgaris* (Hildenborough) it was proposed that the H-cluster contains approximately six iron atoms[72]. The discovery of a high-spin EPR signal (g = 5) in the same enzyme also indicated that the cluster is of unprecedented nature[75]. Likewise, magnetic circular dichroism measurements on the oxidized Fe-hydrogenases of *Clostridium pasteurianum*[79], *Megasphaera elsdenii*[80], and *Desulfovibrio vulgaris* (Hildenborough)[81] revealed the presence of an unknown Fe-S cluster, probably with S ≥ 1/2. Furthermore, five conserved cysteine residues in the C-terminal part of the protein are supposed to ligate the H-cluster[82-87].

1.3.3 Sulfite reductase

A third class of enzymes that has been proposed to contain superclusters comprise the **dissimilatory sulfite reductases**. In biology sulfite is reduced for two purposes. Firstly, sulfur must be incorporated into sulfur containing biomolecules, like the amino acids cysteine and methionine. The enzyme responsible for this process is called assimilatory sulfite reductase. Secondly, sulfite can be used for respiration. The

Table 3. Iron-only hydrogenases

Organism	physiology	Fe/molecule	H-cluster*)	ref.
Desulfovibrio vulgaris (Hildenborough)	anaerobic, sulfate-reducing bacterium	12-15	s,e	[75,83,110]
Desulfovibrio desulfuricans ATCC 7757	anaerobic, sulfate-reducing bacterium	14	e	[111]
Desulfovibrio vulgaris (subsp. *oxamicus* Monticello)	anaerobic, sulfate-reducing bacterium	?	s	[86]
Desulfovibrio fructosovorans	anaerobic, sulfate-reducing bacterium	?	s	[85]
Clostridium pasteurianum (*Cp*I and *Cp*II)	anaerobic, fermentative N_2 fixating bacterium	20 (*Cp*I) 18 (*Cp*II)	e,s	[73,82,112]
Clostridium acetobutylicum P262	fermentative bacterium	?	s	[84]
Megasphaera elsdenii	fermentative bacterium	13-18	e	[74]
Thermotoga maritima	hyperthermophilic bacterium	20	?	[113,114]
Trichomonas vaginalis	protozoon	?	e	[76]

*) from sequence (s) and/or characteristic EPR signal (e)

enzyme catalyzing the latter reaction is called dissimilatory sulfite reductase. Both enzymes contain siroheme and iron-sulfur. For *Escherichia coli* assimilatory sulfite reductase the active site has been modeled as the siroheme bridged to a [4Fe-4S] cluster[88] (Fig. 4). This proposal has been confirmed with the elucidation of the crystal structure[89]. Although both assimilatory and dissimilatory sulfite reductases catalyze the six-electron reduction of sulfite to sulfide (3), they show considerable differences. In assimilatory sulfite reductase the siroheme is always low-spin; the enzyme can be either monomeric, homodimeric, or heteropolymeric, and contains typically five iron atoms per subunit[89-100]. Dissimilatory sulfite reductases can be divided into four classes based on their UV-visible spectra: desulfoviridin (625 nm), desulforubidin (525 nm), P-582 (582 nm), and desulfofuscidin (576 nm). Dissimilatory sulfite reductases are probably either $a_2\beta_2$ or $a_2\beta_2\gamma_2$, and they may contain up to 11 iron atoms per half molecule[101-109]. S = 9/2 EPR signals were observed in the dissimilatory sulfite reductase from *D. vulgaris* (Hildenborough)[101]. The dissimilatory sulfite reductases have been proposed to contain a supercluster.

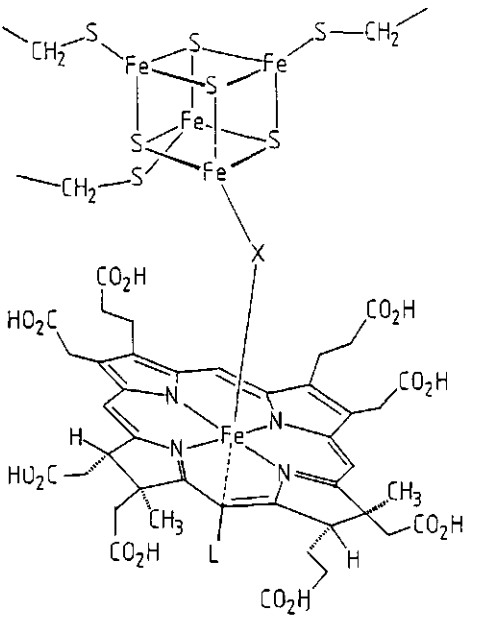

Fig. 4. Bridged siroheme-cubane model as the active site for *E. coli* assimilatory sulfite reductase[88,92]

1.3.4 CO dehydrogenase

The fourth Fe-S redox enzyme exhibiting very high-spin EPR signals is the NiFeS containing **CO dehydrogenase**. This enzyme catalyzes the reversible oxidation of CO to CO_2 (4); it occurs in methanogenic, actetogenic and sulfate-reducing

bacteria, which use the acetyl-CoA pathway, or Wood pathway, for the formation of acetyl-CoA from a methyl donor and a carbonyl moiety. A reversed Wood pathway is operative in *Methanothrix* and *Methanosarcina*, which oxidize acetate to CH_4 and CO_2. CO dehydrogenase has been suggested to play a role in both the formation of acetate and the cleavage of the carbon-carbon bond[115-119]. In partially reduced, anaerobically isolated CO dehydrogenase from *Methanothrix soehngenii* EPR signals were observed with all g-values < g_e, and it was hypothesized that these signals arose from an iron-sulfur cluster with electronic and magnetic properties similar to the [6Fe-6S] cluster[120]. The discovery of S = 9/2 EPR signals in the oxidized enzyme provided further support for the suggestion that this enzyme contains a supercluster[121].

1.3.5 Prismane protein

The EPR spectrum of the reduced six-iron containing, monomeric protein from the sulfate-reducer *Desulfovibrio vulgaris* (Hildenborough) shows an S = 1/2 spectrum almost identical to the $[Fe_6S_6(L)_6]^{3-}$ (prismane) model compound[122-124] (Fig. 5) which led Pierik *et al.*[123] to propose the name **prismane protein**. As isolated, the prismane protein exhibits a second S = 1/2 EPR spectrum with all g-values below the g-value of the free electron. It was also shown that the protein is capable of three one-electron transitions, and in the as-isolated protein they found high-spin EPR signals which they ascribed to an S = 9/2 system[125].

Unfortunately, the prismane protein has no established function yet, but sequence homology with CO dehydrogenase from *Methanothrix soehngenii* and *Clostridium thermoaceticum* may give a clue as to what the biological function of the prismane protein may be[126]. Moreover, a recent resonance Raman study indicates the presence of a Fe-O-Fe species, suggesting a novel function for the Fe-S cluster[127]. Unfortunately, no crystal structure of the prismane protein is available yet.

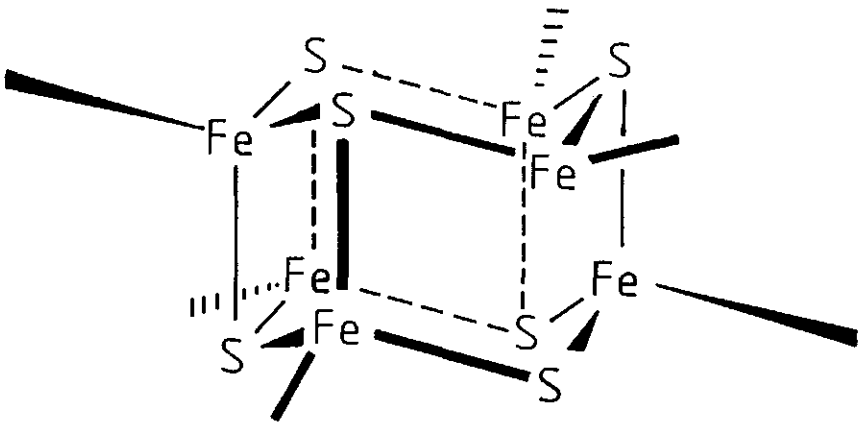

Fig. 5. $[6Fe\text{-}6S]^{3+}$ (prismane) cluster

1.4 TUNGSTEN ENZYMES

Tungsten (W) is a group 6 element from the 5d series; in the earth it occurs predominantly as tungstate ($[WO_4]^{2-}$)[128]. Tungsten has only recently become known as a bio-element. In 1974 it was discovered that formate dehydrogenase activity in crude extracts from *C. thermoaceticum*[129] and *C. formicoaceticum*[130] was stimulated by additional tungsten in the growth medium. It was found that ^{185}W corporation co-incided with carboxylic acid reductase activity from *C. thermoaceticum*[131] and formate dehydrogenase activity from *C. acidiurici* and *C. cylindrosporum*[132]. The first enzyme for which tungsten was proven to form a constituant part is formate dehydrogenase from *C. thermoaceticum*[133]. By now seven distinct tungsten enzyme, or **tungstoenzymes** activities have been reported: formate dehydrogenase (**FDH**), acetylene hydratase, carboxylic acid reductase (**CAR**), glyceraldehyde-3-phosphate oxidoreductase (**GAPOR**), aldehyde oxidoreductase (**AOR**), formaldehyde oxidoreductase (**FOR**), and formylmethanofuran dehydrogenase (**FMDH**). The corresponding activities are listed below (Table 4).

Table 4. Tungsten biochemistry

Enzyme	reaction	eq.	ref.
GAPOR	$RCOH + Fd(ox) \rightarrow RCOOH + Fd(red)$	(5)	[134]
acetylene hydratase	$C_2H_2 + H_2O \rightarrow CH_3CHO$	(6)	[135]
CAR	$RCHO + 2MV^{++} + OH^- \rightarrow RCOO^- + 2H^+ + 2MV^{+\bullet}$	(7)	[136]
FDH	$CO_2 + NADPH \rightarrow HCOOH + NADP^+$	(8)	[136]
AOR	$RCHO + Fd(ox) \rightarrow RCOOH + Fd\ (red)$	(9)	[137]
FMDH	$CO_2 + MFR + 2[H] \rightarrow$ formyl-MFR + H_2O	(10)	[138]
FOR	$HCOH + Fd\ (ox) \rightarrow HCOOH + Fd\ (red)$	(11)	[139]

Fd = ferredoxin; MV = methyl viologen; MFR = methylmethanofuran;
R = CH_2OCH_2OP

Equations 5-11 are all two-electron transfer reactions, except for the hydration reaction catalyzed by acetylene hydratase. FMDH catalyzes the reversible reduction of CO_2 with methanofuran to N-formylmethanofuran, which is the first step in methanogenesis[140,141]. Until now, about a dozen tungstoenzymes have been purified, as listed in Table 5. Tungsten is often present as a tungstopterin cofactor, comparable to molybdopterin[134-136,138,142-145].

Several tungstoenzymes are known to have a molybdenum-containing counterpart. For example, carboxylic acid reductase from *C. formicoaceticum* was shown to occur in a W and a Mo form[144,146], and it was shown that these two forms are not iso-enzymes, but catalytically distinct enzymes[147]. Tungsten also sometimes substitues for molybdenum in case of Mo-deficiency, which was recognized already in 1974 by Gottschalk and co-workers, who found that tungsten addition to molybdenum-deprived cells partly restored formate dehydrogenase activity in *C.*

thermoaceticum[130]. *Methanobacterium wolfei* contains two isoenzymes of formylmethanofuran dehydrogenase (FMDH), one containing molybdenum and the other one containing tungsten[138,148]. When the organism was grown on a medium supplemented with tungsten, but in the absence of molybdenum, the molybdenum-containing FMDH was synthesized as a tungsten-containing, catalytically active FMDH[149]. Similarly, in *M. thermoautotrophicum*, which also contains both a W- and a Mo-FMDH, molybdenum was shown to substitute for tungsten in the W-FMDH, however, no active Mo-substituted W-FMDH could be detected[150]. The latter observation is in accordance with the finding that most tungsten-substituted Mo-enzymes are inactive, e.g. nitrogenase from *Azotobacter vinelandii*[151], formate dehydrogenase and nitrate reductase from *Escherichia coli*[152], rat liver sulfite oxidase[153] and xanthine oxidase[154].

The substitution effect of tungsten for molybdenum can be understood when we compare these two metals from a chemical point of view. Tungsten resembles molybdenum, the 4d element from group 6, with respect to bio-availability, ionic radius, coordination and redox chemistry; both metals are mainly oxo or hydroxo complexed[156]. Being elements from the left half of the transition metal period, tungsten and molybdenum have a high coordination number and high oxidation states (IV,V,VI). They favor O/N ligation[1]. Molybdoenzymes function primarily in oxygen atom transfer (not to be confused with molecular oxygen transport), the single exception being nitrogenase[56]. In transferring oxygen molybdoenzymes catalyze a hydroxylation reaction, where water is used as the source of the oxygen atom to be incorporated into the product (12)[157].

$$RH + H_2O \rightarrow ROH + 2e^- + 2\,H^+ \qquad (12)$$

Clearly, this reaction is similar to the two-electron transfer catalyzed by tungstoenzymes; eqs. 5-11. The majority of tungstoenzymes purified sofar is from thermophilic or extreme thermophilic organisms. The best studied Archaeon, *Pyrococcus furiosus*, is known to contain at least three distinct redox tungstoenzymes, namely: aldehyde oxidoreductase[137], formaldehyde oxidoreductase[139], and glyceraldehyde-3-phosphate oxidoreductase[134]. All three enzymes exist solely as tungstoenzymes; no molydenum isoenzymes have been found[158]. One could argue that the 'choice' for tungsten over molybdenum is simply explained by the fact that tungsten is more abundant than molybdenum in those habitats where (hyperthermophilic) tungsten-dependent organisms are found; mainly in geothermally heated marine environments (both shallow and deep waters)[159]. On the other hand, tungsten-substituted molybdenum enzymes have also been found in mesophiles[131,133,136,143,144,149,150]. Moreover, the observation that tungsten-substituted molybdenum enzymes are catalytically distinct from the native molybdenum enzymes suggests that the choice between tungsten and molybdenum is not simply a matter of availability[147,149]. Alternatively, tungsten may stabilize the enzyme at elevate temperatures.

Table 5. Tungstoenzymes

Organism	enzyme	substrate	subunits	mass (kDa)
ARCHAEA				
Pyrococcus furiosus	AOR	RHCHO	α_2	66
Pyrococcus furiosus	GAPOR	glyceraldehyde 3-phosphate	α	63
Thermococcus litoralis	FOR	formaldehyde	α_4	69
Methanobacterium wolfei	FMDH	formyl methanofuran	$\alpha\beta\gamma$	35,51,64
Methanobacterium thermoautotrophicum	FMDH	formyl methanofuran	$\alpha\beta\gamma\delta$	65,53,31,15
Thermococcus strain ES-1	AOR	RHCHO	α_2	67
BACTERIA				
Clostridium thermoaceticum	FDH	CO_2	$\alpha_2\beta_2$	97,76
Clostridium thermoaceticum	CAR (NADPH dep.)	RHCOOH	$\alpha_3\beta_3\gamma$	64,14,64
Clostridium thermoaceticum	CAR	RHCOOH	$\alpha\beta$	64,14
Clostridium formicoaceticum	CAR	RHCOOH	α_2	67
Desulfovibrio gigas	AOR	RHCHO	α_2	73
Pelobacter acetylenicus	acetylene hydratase	acetylene	α	73

CAR, carboxylic acid reductase
AOR, aldehyde oxidoreductase
FDH, formate dehydrogenase
FOR, formaldehyde oxidoreductase
FMDH, formylmethanofuran dehydrogenase
GAPOR, glyceraldehyde-3-phosphate oxidoreductase

electron carrier	metals	W(V) EPR	Fe-S clusters	refs.
ferredoxin	5Fe $4S^{2-}$ 1W 1Mg	+	[4Fe-4S]	[137,164] [chapter 6]
ferredoxin	6Fe 1W	NR	NR	[134]
ferredoxin	16Fe $12S^{2-}$ 3W	NR	[4Fe-4S]	[139,148]
?	2Fe 0.3-0.4W	+*	NR	[138]
?	8Fe 0.4W	NR	NR	[150]
ferredoxin	4-5Fe 1Mg 1W 2P	NR	[4Fe-4S]	[166]
NADPH	36Fe $50S^{2-}$ 2Se 2W	+	[4Fe-4S] [2Fe-2S]	[131,133]
NADPH	82Fe $54S^{2-}$ 4.3W 1.7 FAD	NR	NR	[131,155]
?	29Fe $25S^{2-}$ 0.8W	NR	NR	[131,155]
?	11Fe $16S^{2-}$ 1.4W	NR	NR	[144]
?	5Fe $3S^{2-}$ 0.7W	+	[4Fe-4S]	[143]
---	5Fe $4S^{2-}$ 0.4W	NR	NR	[135]

* W-substituted Mo-MFDH
NR, not reported

Tungsten occurs in three redox states, two, one, and zero electrons in the valency (5d) orbitals, respectively. W^{6+} is always diamagnetic, W^{5+} is always paramagnetic (S = 1/2), and W^{4+} is either paramagetic (S = 1) or diamagnetic, depending on the ligand field. Of these redox states only W^{5+} is readily detected with 'normal' (perpendicular) EPR spectroscopy. Because W^{5+} is a d^1 system its EPR spectrum is expected to exhibit slow relaxation properties, which makes detection possible up to high temperatures, and the g-values should all be less than 2.00. Nevertheless, g-values of W^{5+} have been reported up to 2.10. Relatively litte is known about tungsten-EPR of proteins. Table 6 shows tungsten-EPR properties of tungstoenzymes, as well as reduction potentials.

Knowledge on redox potentials of tungstopterins is rather parsimonious. The reduction potential of the substrate/product couples (carboxylic acid/aldehyde) is very low at room temperature and pH 7.0, i.e. < -420 mV[160]. This implies that the enzyme catalyzing this reaction should also have a low potential reaction center. However, given the variation in reduction potentials of tungstopterins, it remains an open question whether the relevant redox chemistry takes place on the tungsten, or on the pterin cofactor.

Table 6. W-EPR of tungstoenzymes

Enzyme	W^{5+} g-values	^{183}W hyperfine splitting (Gauss)	(W) reduction potential mV vs NHE	refs.
P. furiosus AOR	1.982, 1.953, 1.885	A_{xyz}, 77,45,42	W^{4+}/W^{5+}; + 180 mV	[chapter 6]
D. gigas AOR	1.84-1.79	NR	$W^{4+}/W^{5+} \geq -400$	[143]
C. thermoaceticum FDH	2.10, 1.98, 1.95[1)]	NR	$W^{5+}/W^{6+} \leq -420$	[161]
C. formicoaceticum CAR	30 K: 2.035, 1.959, 1.899 15 K: 2.03, 2.01, 2.002	NR	NR	[147]
M. wolfei MFDH[2)]	2.049, 2.012, 1.964	A_{xyz}, 50,46,31	NR	[149]
Rat liver sulfite oxidase[3)]	$g_{\|\|}$= 1.982, $g_{\perp}$= 1.936	$A_{\|\|}$, 90; $A_{\perp}$, 40	NR	[162]

NR, not reported
[1)] EPR signals possibly originating from W^{5+}
[2)] W-substituted Mo-FMDH
[3)] W-substituted Mo-sulfite oxidase

AOR, aldehyde oxidoreductase
FDH, formate dehydrogenase
FMDH, formylmethanofuran dehydrogenase
CAR, carboxylic acid reductase

OUTLINE OF THE THESIS

The existence of iron-sulfur (Fe-S) proteins holding one-, two-, three-, or four-iron sulfur clusters has been known for many years. These clusters have been extensively studied, and many such Fe-S proteins have been crystallographically characterized. In contrast, despite increasing evidence for the presence of larger Fe-S clusters in multi-electron transferring enzymes, the electronic and structural properties of these clusters are still poorly understood. The aim of this thesis is to investigate physical, chemical and biological properties of multi-electron transferring enzymes, in a quest for possible novel structures and functions of biological Fe-S clusters.

The first multi-electron transferring enzyme studied in this research is the dissimilatory sulfite reductase from *Desulfosarcina variabilis* (chapter 2). This enzyme contains heme and Fe-S; it exhibited S = 9/2 EPR signals, suggesting the presence of a larger Fe-S (super) cluster. Similar high-spin EPR signals had already been detected in *Desulfovibrio vulgaris* desulfoviridin[101], but in contrast to the latter enzyme the *D. variabilis* enzyme was found to contain fully metalated sirohemes, which makes it a less complex system for spectroscopy.

In the soluble NiFe hydrogenase from the hyperthermophile *Pyrococcus furiosus* a rhombic, high-potential EPR signal was discovered. This signal titrated as a two-electron reducible species, suggesting the presence of a supercluster. In chapter 3 the results of an EPR/redox study are be described.

Novel FTIR resonances were detected in the Fe-only hydrogenases of *Megasphaera eldsenii* and *Desulfovibrio vulgaris* (Hildenborough) as well as in several NiFe hydrogenases (chapter 4). Fe-hydrogenases have been proposed to contain a six-iron cluster[72]. A systematic, comparitive FTIR study of NiFe and Fe-only hydrogenases, as well as a broad range of Fe-S proteins, was carried out, in order to investigate whether these FTIR signals arise from Fe-S clusters, or whether they are unique for hydrogenases.

From the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) a small, unusual Fe-S protein was purified. The protein was identified as a ferredoxin. The ferredoxin absorbed strongly at 260 nm, indicative of association with nucleic acid. Since Thomson has hypothesized that bacterial ferredoxins may play a role in gene regulation[163], it was decided to characterize the ferredoxin, and to investigate the nature of the bound nucleic acid (chapter 5).

The tungsten-containing aldehyde oxidoreductase (AOR) from the hyperthermophilic Archaeon *Pyrococcus furiosus*[137] has been proposed to contain a $[WFe_3S_4]$ cluster[164], which prompted a detailed spectroscopic investigation (chapter 6). The unexpectedly high redox potential of tungsten in *P. furiosus* AOR leads to the proposal that the biologically relevant redox chemistry may not be take place on the tungsten. Recently, the crystal structure has been solved[165].

The putative [6Fe-6S] proteins from *Desulfovibrio vulgaris* (Hildenborough) and *D. desulfuricans* ATCC 27774 have been extensively studied by several spectroscopic techniques, but crystallographic evidence for the presence of a [6Fe-6S] cluster in this protein, and in other proteins, is still lacking. Work was initiated to crystallize the prismane protein in collaboration with the group of prof. P.

Lindley. In chapter 7 the crystallization and preliminary crystallographic analysis of the prismane protein from *D. vulgaris* (Hildenborough) is presented.

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2. THE DISSIMILATORY SULFITE REDUCTASE FROM *DESULFOSARCINA VARIABILIS* IS A DESULFORUBIDIN CONTAINING UNCOUPLED METALATED SIROHEMES AND S = 9/2 IRON-SULFUR CLUSTERS

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Biochemistry (1993) **32**, 10323-10330

Reprinted from Biochemistry, 1993, 32.

The Dissimilatory Sulfite Reductase from *Desulfosarcina variabilis* Is a Desulforubidin Containing Uncoupled Metalated Sirohemes and $S = {}^9/_2$ Iron–Sulfur Clusters†

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Received February 19, 1993; Revised Manuscript Received June 15, 1993•

ABSTRACT: The active site of *Escherichia coli* NADPH–sulfite reductase has previously been modeled as a siroheme with its iron bridged to a nearby iron–sulfur cubane, resulting in antiferromagnetic exchange coupling between all iron atoms. The model has been suggested to hold also for other sulfite reductases and nitrite reductases. We have recently challenged the generality of the model with the finding that the EPR of Fe/S in dissimilatory sulfite reductase (desulfoviridin) from *Desulfovibrio vulgaris* indicates that an $S = {}^9/_2$ system is not subject to coupling. Siroheme in desulfoviridin is to a large extent demetalated, and therefore coupling is physically impossible. We have now studied examples from a second class of dissimilatory sulfite reductases, desulforubidins, which have their siroporphyrins fully metalated. Desulforubidin from *Desulfosarcina variabilis* is a 208-kDa $\alpha_2\beta_2\gamma_2$ hexamer. The α- and β-subunits are immunologically active with antibodies raised against the corresponding subunits from *D. vulgaris* desulfoviridin, whereas the γ-subunit is not. The desulforubidin contains two fully metalated sirohemes and a total of ≈15 Fe and ≈19 S^{2-}. Quantification of high-spin plus low-spin heme EPR signals accounts for all sirohydrochlorin. The frequency-independent (9–35 GHz) effective perpendicular g-values of the high-spin $S = {}^5/_2$ siroheme (6.33, 5.19) point to quantum mixing with an excited (≈770 cm^{-1}) $S = {}^3/_2$ multiplet. Similar anomalous g-values are observed with sulfite reductases from *Desulfovibrio baarsii* and *Desulfotomaculum acetoxidans*. The *D. variabilis* enzyme exhibits very approximately stoichiometric $S = {}^9/_2$ EPR ($g = 16$). None of the EPR signals give indication for dipolar and/or exchange coupling between siroheme and iron–sulfur clusters. $S = {}^9/_2$ EPR is not detected in concentrated samples of assimilatory sulfite reductases from *E. coli* and from *D. vulgaris*. Thus, the functional difference between dissimilatory and assimilatory sulfite reductases appears to have a structural parallel in the presence or absence, respectively, of an $S = {}^9/_2$ EPR iron–sulfur cluster.

The reduction of the bisulfite ion is catalyzed in biology by sulfite reductases for two purposes. Sulfur must be assimilated for the biosynthesis of organosulfur compounds, *e.g.*, cysteine. On the other hand, the electron acceptor sulfite may be dissimilated in respiration. The overall reaction involves the transfer of six reducing equivalents:

$$6H^+ + 6e^- + HSO_3^- \rightarrow 3H_2O + HS^- \qquad (1)$$

The relevant p*K*s are 6.91 (HSO_3^-/SO_3^{2-}) and 7.04 (HS^-/H_2S) at 18 °C. All sulfite reductases contain siroheme and iron–sulfur. *In vitro*, the assimilatory sulfite reductases supposedly produce sulfide in a single six-electron step, *i.e.*, with no intermediate sulfur compounds released (Asada, 1967; Yoshimoto et al., 1967; Lee et al., 1973). The *in vivo* released product is not indentified. By far the most thoroughly studied system is the heterododecameric $\alpha_8\beta_4$ NADPH–sulfite reductase from *Escherichia coli* (Siegel et al., 1973). Monomeric 66-kDa β-subunit isolated from this complex contains siroheme and iron-sulfur and has reduced methyl viologen–sulfite reductase activity (Siegel & Davis, 1974). The *E. coli* enzyme shows (primary) structural and also enzymological homology with spinach leaf assimilatory nitrite reductase (Krueger & Siegel, 1982; Back et al., 1988; Ostrowski et al., 1989).

Other putative assimilatory sulfite reductases consist of only a single subunit. Some are low-molecular-mass, apparently monomeric enzymes with relatively high specific activity. They differ from all other sulfite reductases in that their siroheme is low-spin in the purified protein. The sequence of the assimilatory *D. vulgaris* enzyme has two Cys-containing motifs with some homology to the sequences of the heme–protein subunits from *E. coli* and *Salmonella typhimurium* sulfite reductase and spinach leaf nitrite reductase (Back et al., 1988; Ostrowski et al., 1989; Tan et al., 1991).

There is a different group of sulfite reductases with a *dissimilatory* function. These proteins are heteropolymers with relatively high iron contents. On the basis of visible absorption spectroscopy (especially the peak position in the red for oxidized enzyme), four classes have been reported: desulfoviridin (628 nm), desulforubidin (545 nm), P-582 (582 nm), and desulfofuscidin (576 nm). After over two decades of dispute, it is still an unsettled question whether these enzymes release intermediates, *e.g.*, trithionate or thiosulfate, when assayed *in vitro* (Kobayashi et al., 1972, 1974; Akagi et al., 1974; Schedel & Trüper, 1979; Hatchikian & Zeikus, 1983) or even *in vivo* (Fitz & Cypionka, 1990). However, whatever

† This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).
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• Abstract published in *Advance ACS Abstracts*, September 1, 1993.

0006-2960/93/0432-10323$04.00/0

the precise mechanism will turn out to be, it is obvious that the reaction catalyzed by any sulfite reductase enzyme involves the transfer of one, two, or three *pairs* of reducing equivalents. By consequence, the redox active site has the capacity to take up *two* (or a multiple of two) electrons. The ferric/ferrous transition of a single siroheme does, therefore, not suffice.

For the oxidized *E. coli* enzyme, a putative multielectron-accepting site has been proposed by the groups of Siegel, Münck, and co-workers in the form of a coupled cubane–siroheme unit: a $[4Fe–4S]^{2+}$ cluster with one Fe bridged through an unidentified ligand, X, to the Fe(III) of the siroheme and with an additional "bridge" in the form of a van der Waals contact point between one of the acid-labile sulfurs and the porphyrin periphery (Siegel et al., 1973; Cristner et al., 1981). The coupled cubane–siroheme model is an inference from a remarkable pair of spectroscopic observations. The EPR spectrum of the oxidized *E. coli* protein shows only a high-spin ferric heme signal ($S = {}^5/_2$), consistent with the $[4Fe–4S]^{2+}$ cluster being diamagnetic ($S = 0$). Amazingly, the Mössbauer spectrum shows that *all* iron is paramagnetic (Christner et al., 1981).

The cubane–siroheme model attempts to reconcile these two apparently mutually inconsistent observations in the proposition of a novel type of exchange coupling, *i.e.*, that between an $S = {}^5/_2$ paramagnet and an $S = 0$ diamagnet (Christner et al., 1981; Münck, 1982). In spite of the unprecedented nature of this interaction, the model has, in the ensuing years, been the framework for the interpretation of all spectroscopy on sulfite reductase enzymes (Murphy & Siegel, 1973; Huynh et al., 1984, 1985; Moura et al., 1988). Unfortunately, although the *E. coli* β-subunit has been crystallized (McRee et al., 1986) and the primary structure is known (Ostrowski et al., 1989), a complete interpretation of the electron density map (3-Å resolution) in terms of a 3D structure encompassing the heme and the iron–sulfur has not yet been put forth.

We seek to answer two central questions regarding sulfite reductases: (1) is the Siegel–Münck model of a coupled cubane–siroheme correct and (2) is the model generally valid for all sulfite reductases? Our approach is to systematically (re)investigate sulfite reductases from different classes by EPR spectroscopy and to scan for inconsistencies between the EPR data and the cubane–siroheme model. We have previously studied the dissimilatory sulfite reductase from *D. vulgaris*, a desulfoviridin, and we have found that the iron–sulfur is *not* diamagnatic but has the unusually high system spin $S = {}^9/_2$ (Pierik & Hagen, 1991). No interaction between the iron-sulfur ($S = {}^9/_2$) and the siroheme ($S = {}^5/_2$) was detected. Thus, the coupled cubane–siroheme model does not appear to hold for this enzyme. However, matters are complicated by the fact that desulfoviridin-type reductases have their siroheme to a considerable extent (>70%) demetalated (Murphy & Siegel, 1973; Moura et al., 1987, 1988; Pierik & Hagen, 1991). Therefore, the theoretical possibility of coupling is physically limited to a minor part of the molecules.

We have now studied examples of dissimilatory sulfite reductases that have essentially fully metalated siroporphyrin. We describe one highly purified enzyme from *D. variabilis* and two partially purified enzymes from *D. baarsii* and *D. acetoxidans*. These three species all belong to the subgroup of sulfate reducers that oxidize acetate via the acetyl-CoA route; they all contain carbon monoxide dehydrogenase activity (Jansen et al., 1984, 1985; Schauder et al., 1986; Spormann & Thauer, 1988, 1989). Sulfite reductase has not hitherto been described for this subgroup. Below we describe the dissimilatory sulfite reductase to be of the desulforubidin type. The EPR data are in disagreement with the coupled cubane–siroheme model. We have also reinvestigated two assimilatory-type sulfite reductases, the complex *E. coli* enzyme and the low-molecular-mass, monomeric *D. vulgaris* enzyme.

MATERIALS AND METHODS

Strains, Growth, and Harvesting. D. variabilis, DSM 2060 (Widdel, 1980), was grown at 30 °C on 10 mM pyruvate. *Desulfotomaculum acetoxidans*, DSM 771 (Widdel & Pfennig, 1977), and *Desulfovibrio baarsii*, DSM 2075 (Widdel, 1980), were both grown at 37 °C on 10 mM butyrate plus 5 mM acetate. The growth medium contained Na_2SO_4, 21 mM; KH_2PO_4, 1.5 mM; NH_4Cl, 5.6 mM; $CaCl_2$, 1.4 mM; NaCl, 17 mM; $MgCl_2·6H_2O$, 2 mM; KCl, 6.7 mM; $NaHCO_3$, 30 mM; and Na_2S, 1 mM. For the brackish water organism *D. variabilis*, NaCl was 220 mM and $MgCl_2·6H_2O$ was 10 mM. In all cases, resazurin, 2.2 μM, was present as a redox indicator. Trace elements were H_3BO_3, 0.1 μM; $AlCl_3·6H_2O$, 40 nM; $MnCl_2·4H_2O$, 0.5 μM; $FeCl_2·4H_2O$, 10 μM; $CoCl_2·6H_2O$, 0.8 μM; $NiCl_2·6H_2O$, 0.1 μM; $CuCl_2$, 20 nM; $ZnCl_2$, 0.5 μM; $Na_2SeO_3·5H_2O$, 11 nM; Na_2MoO_4, 150 nM; $Na_2WO_4·2H_2O$, 15 nM. EDTA was also added to 1.3 μM. Vitamins used were biotin, 2 μM; folic acid, 2 μM; nicotinamide, 5 μM; thiamin HCl, 5 μM; riboflavin, 5 μM; pyridoxine HCl, 10 μM; cyanocobalamine, 5 μM; *p*-aminobenzoic acid, 5 μM; lipoic acid, 5 μM; and pantothenic acid, 5 μM.

Cells were grown in 20-L batch cultures (10% inoculum v/v) to an absorbance of approximately 0.25 at 660 nm. Growth period and wet-cell yield respectively were 10 days and 17 g for *D. variabilis* and 14 days and 8 g for both *D. acetoxidans* and *D. baarsii*. Cells were harvested by continuous centrifugation. The pellet was immediately frozen in liquid N_2 and then stored at −20 °C until use.

Purification of D. variabilis Dissimilatory Sulfite Reductase. Frozen cells of *D. variabilis* were suspended in three volumes of standard buffer (20 mM Tris-HCl, pH 8.0) plus 5 mM $MgCl_2$ and a spatula of DNase. The cells were broken in a French pressure cell by passing at 135 MPa. Cell-free extract was obtained as the supernate after a 1-h spin at 100 000*g*. The extract was dialyzed overnight against standard buffer and loaded onto a 60-mL DEAE-Sepharose Fast Flow anion-exchange column. After a 100-mL wash with standard buffer, a 1-L gradient was applied of 0–1 M NaCl in standard buffer. Fractions with desulforubidin were pooled on the basis of complete UV–visible spectra (peaks at 398 and 545 nm; see the Results section). After concentration (16×) over an Amicon YM-100 filter and overnight dialysis against 5 mM potassium phosphate, pH 7.5, the sample was applied onto a 40-mL hydroxylapatite affinity column and washed with 80 mL of potassium phosphate buffer. A 0.5-L gradient was applied of 5–200 mM potassium phosphate, pH 7.5. The pooled fractions were 10× concentrated over Amicon YM-100 and applied onto a 200-mL Sephacryl S-400 molecular sieve and eluted with standard buffer plus 500 mM KCl. The desulforubidin peak was 10× concentrated and dialyzed overnight against standard buffer. The last purification step was by FPLC (Pharmacia) at ambient temperature on a 1-mL Mono-Q anion-exchange column. The desulforubidin was eluted with a 0–1 M KCl stepped gradient as "fast" and a "slow" forms, and these fractions were concentrated (15×) separately over Amicon YM-100. Since the two fractions proved to be indistinguishable by SDS-PAGE, UV–visible spectroscopy, and siroheme EPR spectroscopy, they were pooled.

Purification of D. vulgaris Assimilatory Sulfite Reductase. D. vulgaris assimilatory sulfite reductase was purified ac-

cording to the method of Huynh et al. (1984), with a modification in the order of purification steps: the crude extract was first loaded on a DEAE anion-exchange column, and then on a G-75 molecular sieve, and finally on a hydroxylapatite affinity column.

Purification of E. coli NADPH–Sulfite Reductase. E. coli, strain C 600, was grown batchwise at 37 °C in the medium described by Siegel and Kamin (1971), with the modification glucose, 10 g/L, and the additions of L-leucine, 100 mg/L, and L-threonine, 50 mg/L. Also, *O*-acetyl-L-serine [synthesized according to the method of Sheenan et al. (1956)], a stimulant of sulfite reductase biosynthesis (Siegel & Kamin, 1971; Jones-Mortimer et al., 1968), was added to 2 mM, except in the final 200-L batch (bioreactor, Bioengineering). The purification from 700 g of cells was essentially according to the classical method of Siegel at al. (1973) for wild-type *E. coli* B (DEAE-cellulose was replaced by Q-Sepharose F.F.).

Activity Determinations, Analytical Chemistry, and Immunology. For specific activity determinations, the protein was measured by Coomassie staining (Bradford, 1976). The sulfite-dependent H_2-uptake activity was measured according to the method of Lee et al. (1973), except that *E. coli* hydrogenase was replaced by 10 μg of *D. vulgaris* (Hildenborough) periplasmatic hydrogenase and the assay was done at 30 °C, because the organism was grown at that temperature. For metal, heme, and spin stoichiometry determinations, the protein was determined using the microbiuret method (Bensadoun & Weinstein, 1976). Iron was determined colorimetrically as the ferene complex (Hennessy et al., 1984). The acid-labile sulfide content of the protein was determined according to the method of methylene blue formation [Fogo & Popowski, 1949, modified by Hennessy et al. (1984)]. Siroheme was extracted with an HCl/acetone mixture and subsequently determined as the pyridine hemochrome as described in Siegel et al. (1978). SDS polyacrylamide electrophoresis was performed with a midget system (Pharmacia) holding 8- × 5- × 0.75-cm gels, according to the method of Laemmli (1970). The composition (mass/volume) of the stacking gel was 4% acrylamide and 0.1% bisacrylamide; the running gel was 17.5% acrylamide and 0.07% bisacrylamide. Optical gel scanning was performed using the Gel Analysis Program of a Cybertech CS1 CAM 1.0. Western blotting, Immobilon blotting, and polyclonal antibodies against the α-, β-, and γ-subunits of *D. vulgaris* desulfoviridin were as previously described by Pierik et al. (1992a).

Spectroscopy. UV–visible data were obtained with a DW-2000 spectrophotometer. X-band EPR spectroscopy was done with a Bruker EPR 200 D spectrometer with peripheral equipment and data handling as described in Pierik and Hagen (1991). Q-band EPR spectroscopy was done in the laboratory of Dr. S. P. J. Albracht (The University of Amsterdam) on a Varian E-9 spectrometer with a 35-GHz cylindrical cavity. The modulation frequency was always 100 kHz.

RESULTS

D. variabilis Sulfite Reductase Is a Metalated Desulforubidin. The color of purified *D. variabilis* sulfite reductase is red to red-brown, which indicates that the protein is of the desulforubidin type. The UV–visible spectrum is presented in Figure 1. Indeed, it exhibits the peak at 545 nm that is the desulforubidin fingerprint. Other peaks are observed at 280, 398, and 582 nm (Table I). It has been shown that the siroheme in desulfoviridin from *D. gigas* and from *D. vulgaris* is to a considerable extent demetalated (Murphy & Siegel, 1973; Moura et al., 1987, 1988; Pierik & Hagen, 1991). To determine the metalation of the porphyrin in the *D. variabilis* enzyme, the heme was extracted with HCl/aceton and subsequently complexed with pyridine (Siegel et al., 1978). The resulting pyridine hemochrome spectrum is given in Figure 2. The spectrum is that of metalated siroheme (Siegel et al., 1978; Murphy et al., 1974); its intensity corresponds to approximately 0.7 heme per $\alpha\beta\gamma$ unit of 104 kDa.

Iron and acid-labile sulfur were determined colorimetrically (Table I). The numbers are typically in the range reported for other dissimilatory sulfite reductases.

The sulfite-dependent H_2-uptake activity was 41 munit per mg of protein, which is somewhat lower than values reported for dissimilatory sulfite reductases, although still in the same range (Table I).

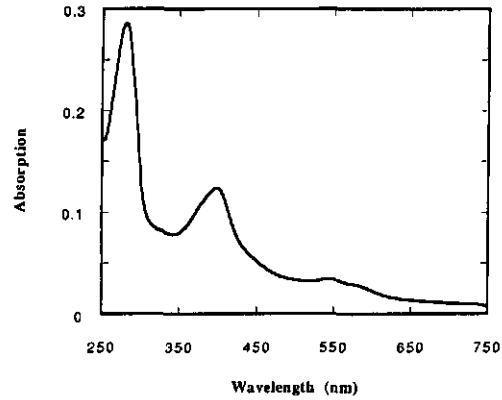

FIGURE 1: UV–visible absorption spectrum of *D. variabilis* dissimilatory sulfite reductase. The protein was 0.127 mg/mL in 20 mM Tris buffer, pH 8.

Table I: Optical and Analytical Data of *D. variabilis* Desulforubidin[a]

absorption coefficients (mM^{-1} cm^{-1})	at 280 nm	468
	at 398 nm	184
	at 545 nm	52
purity indices	398 nm/545 nm	3.65
	398 nm/280 nm	0.35
stoichiometries	siroheme	1.4
	iron	15 ± 2
	sulfide	19 ± 3
specific activity (munits/mg of protein)		41 ± 6

[a] All values refer to the $\alpha_2\beta_2\gamma_2$ holoprotein.

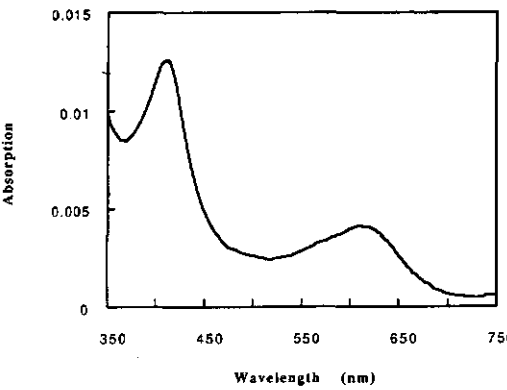

FIGURE 2: Absorption spectrum of pyridine hemochrome after heme extraction from *D. variabilis* dissimilatory sulfite reductase.

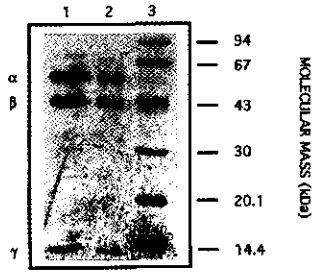

FIGURE 3: Hexameric $\alpha_2\beta_2\gamma_2$ subunit composition of *D. variabilis* desulforubidin as indicated by SDS-PAGE, followed by Immobilon blotting on a PVDF filter and subsequent staining with Coomassie Brilliant Blue. Lanes 1 and 2 contained 3 and 1.5 μg of desulforubidin, respectively; lane 3, molecular mass marker mixture.

Subunit Composition and Immunological Cross Reactivity. All dissimilatory sulfite reductases have long been taken to contain two types of subunits in the canonical $\alpha_2\beta_2$ heterotetrameric composition (Lee et al., 1973; Kobayashi et al., 1972; Schedel & Trüper, 1979; Hatchikian & Zeikus, 1983). We have recently reported multiple evidences for the occurrence of a third, small γ-subunit in $\alpha_2\beta_2\gamma_2$ hexameric desulfoviridin from *D. vulgaris* (Hildenborough) (Pierik et al., 1992a). Immunological evidence indicated that this subunit structure also applies to desulfoviridins from other species; and it was suggested that the subunit structure of other dissimilatory should be (re)examined.

We have subjected *D. variabilis* desulforubidin to analysis in SDS-PAGE. The final step in the purification over FPLC Mono-Q anion exchanger was repeated to obtain a highly purified enzyme. SDS-PAGE and subsequent Immobilon blotting, followed by Coomassie staining, revealed three bands with mobilities corresponding to molecular masses of 50, 42.5, and ≈12 kDa. Integration of densitometric scans of both the Coomassie-stained SDS-PAGE gel and the Coomassie-stained Immobilon blot resulted in a stoichiometry of 1:1.28:1.09(α:β:γ) after correction for the molecular masses (Figure 3). With a subunit composition of $\alpha_2\beta_2\gamma_2$, the molecular mass of desulforubidin from *D. variabilis* would be 208 kDa. This fits well with the molecular mass of the holoenzyme of ≈200 kDa, as determined by gel filtration.

For each of the three subunits, the immunological relation between desulforubidin from *D. variabilis* and desulfoviridin from *D. vulgaris* was explored in the cross reactivity of desulforubidin with polyclonal antibodies raised against the desulfoviridin subunits (Figure 4). Each lane of the SDS polyacrylamide gel was loaded with 650 ng of desulforubidin, corresponding to 325 ng of the α-subunit, 275 ng of the β-subunit, and 78 ng of the γ-subunit. After being blotted onto nitrocellulose membranes, the membranes were incubated with mouse antiserum raised against the individual subunits from purified desulfoviridin. The anti-α antiserum was diluted 500-fold, anti-β 2000-fold, and anti-γ 1000-fold. Goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad, Richmond, VA) was used for immunostaining. On immunoblots the antisera exhibited specific responses with the α- and the β-subunits. The response of the β-subunit was more pronounced than the responses of the α-subunit. No cross reactivity could be detected for the γ-subunit. Cross reactivity with the β-subunit was observed for both the anti-α and the anti-γ antisera (Figure 4).

EPR Spectroscopy Identifies Quantum Mixed Spin Siroheme. In Figure 5, we compare the *g*-perpendicular regions of the siroheme $S = {}^5/_2$ EPR from *D. variabilis* desulforubidin and the partially purified dissimilatory sulfite reductase from

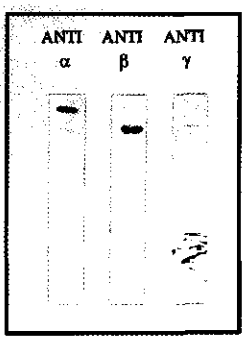

FIGURE 4: Antigenic cross reactivity of dissimilatory sulfite reductases from *D. variabilis* and *D. vulgaris*. Immunostained nitrocellulose blot; each lane was loaded with ≈650 ng of desulforubidin from *D. variabilis*, corresponding to 310 ng of α-, 260 ng of β-, and 75 ng of γ-subunit. Anti-α was 500× diluted; anti-β, 2000×; anti-γ, 1000×.

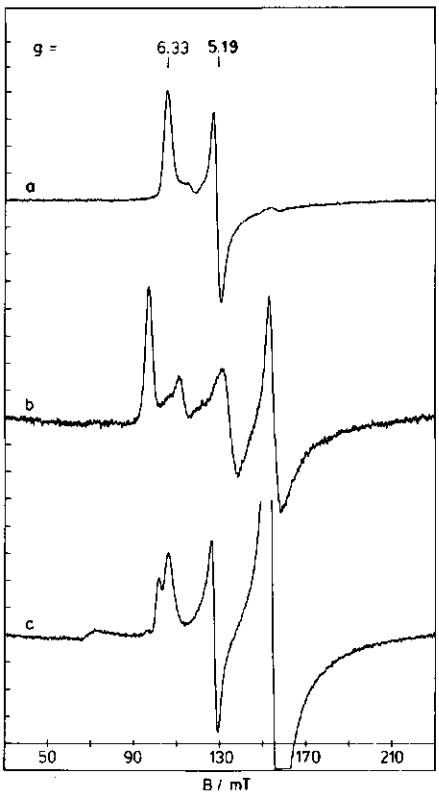

FIGURE 5: High-spin siroheme EPR spectra of desulforubidin from (a) *D. variabilis*, (b) *D. acetoxidans*, and (c) *D. baarsii*. All three spectra are indicative of quantum spin mixing. See text for details. EPR conditions: microwave frequency, 9301 MHz; microwave power, 0.2 (trace a) or 0.5 (trace b) mW; modulation amplitude, 1 mT; T, 4.2 K.

D. acetoxidans and *D. baarsii*. The spectra of the latter two also contain a rubredoxin-like signal ($S = {}^5/_2$, $E/D \approx 1/3$, $g = 4.3$ and ≈9.6); however, the high-spin siroheme signal is easily identified. All three proteins exhibit relatively simple spectra, dominated by a single major component of small rhombicity $E/D = 0.02$–0.04 [$E/D \approx \Delta g_\perp/48$ (Peisach et al., 1971)]. A similar spectrum has been observed by Moura et al. (1988) for *Desulfovibrio baculatus* desulforubidin. This simple spectrum can be contasted with the complex pattern

Table II: EPR g-Values of Siroheme and Fe/S from Desulforubidin[a]

	g_x	g_y	g_z
$S = {}^5/_2$ Siroheme			
D. variabilis	6.33(2)	5.19(2)	1.98
D. variabilis (Q-band)	6.33(8)	5.19(5)	nd[b]
D. acetoxidans	6.89	4.95	nd
D. baarsii	6.30	5.20	nd
$S = {}^1/_2$ Siroheme (*D. variabilis*)			
signal 1	1.782	2.374	2.474
signal 2	1.75	2.340	2.530
signal 3	nd	2.26	2.63
$S = {}^9/_2$ Fe/S (*D. variabilis*)			
$\|\pm{}^1/_2\rangle$ doublet	nd	16.1	nd
	(0.53)	(16.1)	(1.10)
$\|\pm{}^3/_2\rangle$ doublet	nd	≈8.6	nd
	(3.87)	(8.43)	(6.12)
$\|\pm{}^5/_2\rangle$ doublet	≈8.6	nd	nd
	(8.95)	(1.88)	(2.13)

[a] All values refer to X-band unless otherwise indicated. Values in parentheses are calculated for $S = {}^9/_2$, E/D = 0.13, $g = 1.90$. [b] Not determined.

of several rhombicities observed in desulfoviridins (Moura et al., 1988; Pierik & Hagen, 1991; Hall & Prince, 1981; Liu et al., 1979).

The spectra in Figure 5 and that of *D. baculatus* desulforubidin all have another striking property in common: the average of the two perpendicular g-values falls significantly short of the value of 6.0 predicted for a pure sextet state of Fe^{3+}. Moura et al. (1988) found $(6.43 + 5.34)/2 = 5.89$ for g_{av}; they do not comment on this unusual value. For the spectra in Figure 5, we find $g_{av} \approx 5.76, 5.92, 5.75$, respectively (*cf.* Table II).

In order to check whether these shifts from the theoretical g-value reflect intermolecular magnetic interaction, we have recorded the spectrum of *D. variabilis* high-spin heme in the presence of increasing amounts of urea (0.4, 1.2, and 3.6 M). Minor changes in the line width were observed; however, no significant change in the effective g-values was found (not shown).

Dipolar interactions between paramagnets, whether intra- or intermolecular, can usually be identified by taking EPR data at two different microwave frequencies. At the higher frequency, the relative strength of dipolar interactions *versus* the Zeeman interaction is changed in favor of the latter one. We have, therefore, recorded the spectrum of *D. variabilis* desulforubidin at Q-band frequency (Figure 6). The effective g-values observed in Q-band are identical within 0.05% to those found in X-band (*cf.* Table II). Therefore, the anomalous g-values are not caused by magnetic coupling. Deviations from $g_{av} = 6$ have been observed previously in cytochromes *c'* and in peroxidases, and they have been interpreted in terms of quantum-mechanical mixing of an $S = {}^5/_2$ and an $S = {}^3/_2$ state [reviewed by Maltempo and Moss (1976)]. The present case of desulforubidin would be the first example of a quantum mixed spin state in a protein from an anaerobic bacterium. Based on the model calculations of Maltempo and Moss (1976; Figure 5), we find the excited $S = {}^3/_2$ state at 770 cm^{-1} above the $S = {}^5/_2$ ground state.

Quantification of Siroheme EPR. The complete EPR spectrum of the high-spin siroheme in *D. variabilis* desulforubidin is given in Figure 7, trace a. The accurate quantification of these types of spectra is usually not trivial for two reasons. Depopulation of the $|\pm{}^1/_2\rangle$ ground doublet of the $S = {}^5/_2$ multiplet occurs with increasing temperature. Secondly, slight disturbances of the base line between the middle and the highest g-value result in relatively large errors in the second

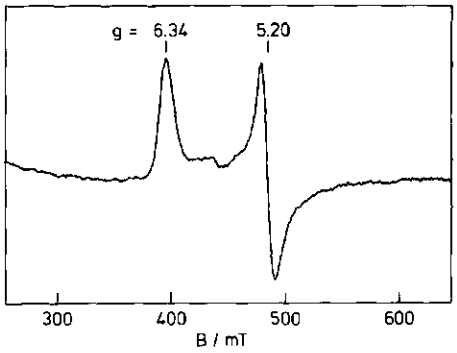

FIGURE 6: Q-band EPR spectrum of the *D. variabilis* desulforubidin high-spin siroheme. The spectrum establishes the effective g-values to be frequency independent. The sample is the same as for Figure 5, trace a. EPR conditions: microwave frequency, 35 221 MHz; microwave power, 50 mW; modulation amplitude, 1.25 mT; T, 15 K. The spectrum is an average of four scans.

integral. The depopulation problem is easily circumvented by taking data at a temperature of 4.2 K, where the ground doublet is nearly 100% populated. A possible way to avoid unreliable integrals over wide magnetic field scans is to numerically simulate the spectrum.

The angular-dependent inhomogeneous EPR line width in high-spin hemoproteins is a complicated entity encompassing contributions from several different broadening mechanisms (Hagen, 1981). However, at X-band frequencies, a major contribution is from unresolved superhyperfine interactions (Hagen, 1981; Scholes et al., 1972). This observation goes some way toward explaining why simulations of these types of effective $S = {}^1/_2$ spectra employing a Gaussian line shape with little angular dependency give satisfactory results [*cf.* Aasa and Vänngård (1975)]. We have used this approach to quantify the desulforubidin high-spin heme spectrum.

The simulation in Figure 7, trace b is the end result of minimization on a rhombic effective g-tensor and a near-isotropic Gaussian. Figure 7 also contains the experimental and simulated spectra of the external standard $[Cu(H_2O)]^{2+}$ in aqueous perchlorate. This latter simulation includes variation of the inhomogeneous line width as a function of m_I (*i.e.*, the $I = {}^3/_2$ $^{63,65}Cu$ nuclear orientation) according to the expansion

$$W(\theta,\phi) = W_o(\theta,\phi) + b(\theta,\phi)m_I + \text{higher order terms} \quad (2)$$

This pattern appears to be general for copper and low-spin cobalt complexes (Hagen, 1981; Froncisz & Hyde, 1980) and has been argued to be directly related to the phenomenon of g-strain (Hagen, 1981).

The advantage of simulating both the spectrum of the unknown and that of the standard is that the transition probability for all powder orientations is included. Therefore, quantification involves only the direct comparison of second integrals of the simulations with amplitudes normalized to those of the experimental spectra. The end result of this procedure was 0.65 high-spin siroheme per half-molecule of desulforubidin, with the concentration of the latter determined with the microbiuret method using a molecular mass of 208 kDa.

In the 4.2 K spectrum of high-spin siroheme in Figure 7, trace a, some low-spin heme is just detectable around $B \approx 280$ mT. Under the conditions employed, the low-spin heme signal is strongly saturated. Figure 8 gives the nonsaturated low-spin heme spectrum at 25 K. As we have observed before with *D. vulgaris* desulfoviridin, there are several (at least three;

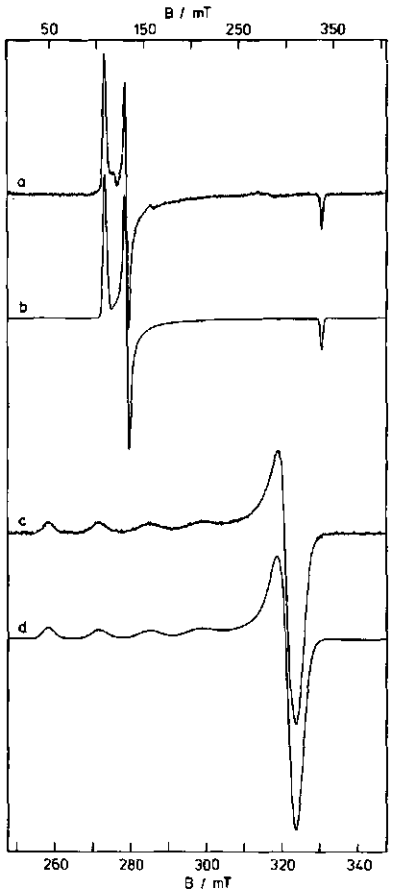

FIGURE 7: Computer simulation of the complete high-spin siroheme EPR spectrum from *D. variabilis* desulforubidin and from the external copper standard. Trace a is from desulforubidin, trace b is the simulation, trace c is the copper standard, and trace d is the simulation. EPR parameters for traces a and c: microwave frequency, 9.30 GHz; microwave power, 0.2 and 0.008 mW; modulation amplitude, 1.6 mT; T, 4.2 K. Simulation parameters for trace b: 1000 × 20 orientations; *g*-values, 6.30, 5.22, 1.98; line widths, 3.36, 2.8, 2.7 mT. Simulation parameters for trace d: 40 orientations; *g*-values, 2.071, 2.385; line widths, 3.8, 2.7 mT; asymmetry parameters (*b* in eq 2), −0.075, 0.350; copper ($I = {}^3/_2$) hyperfine splittings, 0.63, 13.2 mT.

cf. Table II) slightly different forms of low-spin heme. We have quantitated the sum of all forms by doubly integrating the spectrum of Figure 8 and correcting for the signal around $g \approx 2$ (the g_z of high-spin heme plus a minor contaminant, possibly a trace of copper). We thus find 0.20 low-spin siroheme per half desulforubidin. Added up to the high-spin heme, this gives a total of 0.85 siroheme per half desulforubidin, in reasonable agreement with the chemical heme determination. All data are summarized in Table II.

Iron–Sulfur $S = {}^9/_2$ EPR. Low-field EPR as a function of temperature is presented in Figure 9. Several weak resonances are observed with effective *g*-values up to 16. A Kramers system with effective an *g*-value > 14 implies $S \geq {}^9/_2$ (Hagen, 1992). In the weak-field limit (Pierik & Hagen, 1991; Hagen, 1992), the line at $g = 16$ and the broad feature at $g \approx 8$–9 can be fitted to the standard spin Hamiltonian

$$H_S = D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2) + g\beta \mathbf{B}\cdot\mathbf{S} \quad (3)$$

for $S = {}^9/_2$, $|E/D| = 0.13$, and $g_{real} \approx 1.9$ (*cf.* Table II). When

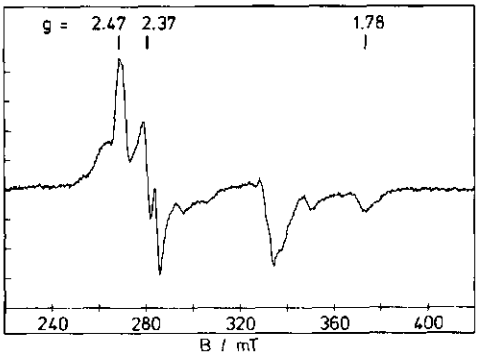

FIGURE 8: EPR of multiple low-spin siroheme components from *D. variabilis* desulforubidin. The three *g*-values of the major component are indicated. EPR conditions: microwave frequency, 9301 MHz; microwave power, 8 mW; modulation amplitude, 1.6 mT; T, 25 K.

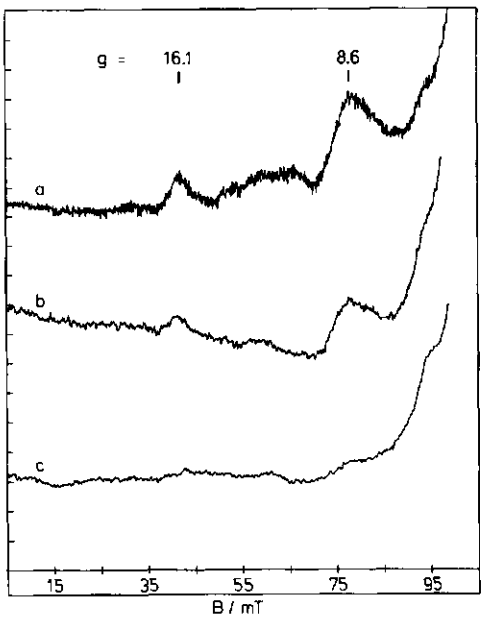

FIGURE 9: Temperature dependence of $S = {}^9/_2$ EPR from Fe/S in *D. variabilis* desulforubidin. Trace a (4.2 K), trace b (9 K), and trace c (16 K) were all recorded with the same amplification. Other settings: microwave frequency, 9.30 GHz; microwave power, 200 mW; modulation amplitude, 2 mT.

g_{real} is kept at 2.00, a fit is obtained only when allowing for a distribution in |E/D| from 0.08 to 0.12. Additional weak absorptions in the range $g_{eff} = 10$–13 also point to the occurrence of several rhombicities.

The lines are somewhat broader than those previously found for an $S = {}^9/_2$ system in *D. vulgaris* desulfoviridin (Pierik & Hagen, 1991); however, the temperature dependence is similar. After correction for depopulation, the intensities of the lines *increase* from $T = 4.2$ to 9 K, indicating a negative sign for the zero-field interaction. Lifetime broadening also sets in around 9 K. This combination of a fast spin–lattice relaxation and an inverted spin multiplet results in rather unfavorable conditions for the detection of this type of EPR, and only a very rough estimate of the spin count can be made.

From the intensity of the $g = 16$ peak at 4.2 and 9 K, we estimate $D \approx -0.3$ cm^{-1} when assuming $S = {}^9/_2$. This means

that the $|\pm^1/_2\rangle$ doublet is $2D \approx 6$ cm^{-1} above the $|\pm^9/_2\rangle$ ground state and has a population within the $S = {}^9/_2$ multiplet of some 7% at $T = 4.2$ K. We estimate the ratio of high-spin siroheme over $S = {}^9/_2$ iron–sulfur cluster using doubly integrated effective $S = {}^1/_2$ simulations (just as in Figure 7). The simulation for the $S = {}^9/_2$ system was for the $|\pm^1/_2\rangle$ doublet with the effective g-tensor given in Table II. As only one g-value ($g = 16$) is actually observed and no complete data sets for other systems available and no theory for $S = {}^9/_2$ powder line shapes are yet developed, our present quantification should be considered a rough order-of-magnitude estimate. After correction for the population at 4.2 K, we find about 0.4–2.5 $S = {}^9/_2$ per high-spin siroheme.

Upon reduction with excess dithionite, the protein becomes EPR silent.

Assimilatory Sulfite Reductases. In our previous work on *D. vulgaris* desulfoviridin, we have also reported on attempts to detect $S \geq {}^7/_2$ EPR from the Fe/S in $\alpha_4\beta_8$ *E. coli* NADPH–sulfite oxidoreductase and from the Fe/S in monomeric *D. vulgaris* assimilatory sulfite reductase (Pierik & Hagen, 1991). We have now made significantly more concentrated preparations of these proteins, namely, *E. coli* enzyme, 285 μM in total siroheme, and assimilatory *D. vulgaris* enzyme, 570 μM in low-spin siroheme, as determined by EPR integrations. With extensively signal-averaged EPR on these samples at 4.2 K, we have still not found any signal in the low-field range, where we do find $S = {}^9/_2$ signals for desulfoviridin (Pierik & Hagen, 1991) and for desulforubidin (Figure 9).

DISCUSSION

Disparity of Assimilatory versus Dissimilatory Sulfite Reductases. Sulfite reductase contains siroheme and iron–sulfur. The 3D structure of the enzyme, and therefore of the active site, is not known. The catalysis involves (multiple) electron pair transfer; therefore, a single heme is not sufficient. In the Siegel–Münck proposal, the active site is modeled as a 5Fe complex formed by a siroheme bridged to an Fe/S cubane (Siegel et al., 1973; Chistner et al., 1981). The model follows from an unorthodox interpretation (*cf.* exchange coupling between an $S = {}^5/_2$ and an $S = 0$ system) of extensive spectroscopic data on the heteropolymeric ($\alpha_8\beta_4$) assimilatory *E. coli* enzyme. By comparative spectroscopy, the model has been argued to be valid also for enzymes from other sources and with a very different subunit composition, namely, homopolymeric (α_2) spinach leaf nitrite reductase (Wilkerson et al., 1983), monomeric assimilatory sulfite reductase (low-spin) from sulfate reducers (Huynh et al., 1984, 1985), and heteropolymeric ($\alpha_2\beta_2\gamma_x$) dissimilatory sulfite reductases from sulfate reducers (Huynh et al., 1985; Moura et al., 1987, 1988).

We have previously questioned the validity of the Siegel–Münck model in general, and we have specifically provided EPR spectroscopic evidence (the detection of $S = {}^9/_2$ EPR from Fe/S) against its validity for desulfoviridin, the dissimilatory sulfite reductase from *D. vulgaris* (Pierik & Hagen, 1991). Our argumentation was complicated by the fact that the siroheme in desulfoviridin is to a considerable extent demetalated. Therefore, we have searched for $S = {}^9/_2$ EPR from Fe/S in other types of sulfite reductases. We have now found this clue also in fully metalated desulforubidin, the dissimilatory sulfite reductase from *D. variabilis*. On the other hand, we have been unable to detect any EPR whatsoever from Fe/S in oxidized assimilatory enzymes, *viz.* the monomeric *D. vulgaris* protein and the heteropolymeric *E. coli* protein. The finding of uncoupled $S = {}^9/_2$ Fe/S in dissimilatory sulfite reductases provides evidence against the validity of the Siegel–Münck model for these group of enzymes. For the class of assimilatory sulfite reductases, the coupled siroheme–[4Fe–4S] model is not contradicted by our EPR results since no high-spin Fe/S is detected.

On the basis of these observations, we propose that the *functional difference* between *assimilatory* and *dissimilatory* sulfite reductases is paralleled by structural and magnetic differences of the iron–sulfur structures. The dissimilatory enzymes have uncoupled Fe/S and siroheme prosthetic groups. In the oxidized assimilatory enzymes, the Fe/S cluster is different, as it is not detected by EPR. Its nature remains to be established.

On the basis of a Mössbauer spectroscopic study, Moura et al. have previously proposed that both desulfoviridin and desulforubidin contain a ferric siroheme exchange-coupled to a $[4Fe\text{-}4S]^{2+}$ cluster (Moura et al., 1988). An implicit ad hoc assumption in that analysis (and all previous ones) was that all iron–sulfur clusters in sulfite reductases are of the cubane type. The Mössbauer data were, by consequence, interpreted in terms of mixtures of diamagnetic and paramagnatic cubanes with the exclusion of any other interpretation. Remarkably, the authors were forced to invoke the presence of a stoichiometric extra mononuclear iron site in both proteins, although their EPR data do not show additional resonances attributable to a ferric site. It would seem to be worthwhile to readdress this question without *a priori* limitations on the modeling of the clusters, the more so since our recent Mössbauer study on a putative 6Fe cluster containing protein pointed to the hitherto not considered possibility that within a single paramagnetic cluster some of the iron ions behave as very nearly diamagnetic (Pierik et al., 1992b). We have initiated work in this direction.

Unity of Dissimilatory Sulfite Reductases. We have now determined that the dissimilatory sulfite reductase from *D. variabilis* is a metalated desulforubidin with the hexameric subunit composition $\alpha_2\beta_2\gamma_2$. The α- and β-subunits exhibit cross reactivity with antiserum raised against the corresponding subunits from *D. vulgaris* desulfoviridin. The γ-subunit, although detectable on SDS-PAGE, shows no specific response to the corresponding antibody from *D. vulgaris*. In our previous work, we have shown cross reactivity with the α-, β-, and γ-subunits of desulfoviridin from three other *Desulfovibrio* species (Pierik et al., 1992a). Especially the γ-subunit proved to be strongly cross reactive. Of all four strains tested [*D. vulgaris* (Hildenborough), *D. vulgaris oxamicus* (Monticello), *D. gigas*, and *D. desulfuricans* ATCC 27774], the anti-γ antiserum exhibited very specific responses on immunoblots. It should be noted that all four *Desulfovibrio* strains contain desulfoviridin, whereas *Desulfosarcina variabilis* contains the desulforubidin-type dissimilatory sulfite reductase.

We have found $S = {}^9/_2$ EPR in *D. variabilis* desulforubidin and in *D. vulgaris* desulfoviridin. Comparable signals are present in published spectra from *D. gigas* desulfoviridin and *D. baculatus* desulforubidin (Moura et al., 1988).

On the basis of these observations, we propose the following working hypothesis: all dissimilatory sulfite reductases have the hexameric $\alpha_2\beta_2\gamma_2$ subunit composition; they also all have Fe/S with $S \geq {}^7/_2$ and Fe ≈ 6, *i.e.*, a structure and paramagnetism analogous to that of the prismane protein (Hagen & Pierik, 1989; Hagen et al., 1991b; Pierik et al., 1992) or to that of the nitrogenase P-cluster (Hagen et al., 1987; Hagen et al., 1991a).

Presently, this hypothesis has not been tested for two other "types" (*i.e.*, based on optical spectra) of dissimilatory sulfite reductases. For P582 from *Desulfotomaculum* spp, no EPR and no subunit composition has been reported (Trudinger, 1970). For desulfofuscidin from *Thermodesulfobacterium commune*, no EPR data are available and the earlier reported

subunit composition, $\alpha_2\beta_2$, has not yet been checked for the presence of γ-subunit (Hatchikian & Zeikus, 1983).

ACKNOWLEDGMENT

We thank W. Hilbrands for help with the purification of the *E. coli* sulfite reductase. We are grateful to Dr. S. P. J. Albracht for providing supported access to the Q-band EPR facility at the Department of Biochemistry, The University of Amsterdam. We thank Professor C. Veeger for his continuous interest and support.

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3. REDOX PROPERTIES OF THE SULFHYDROGENASE FROM *PYROCOCCUS FURIOSUS*

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FEBS Lett. (1995) **368**, 117-121

Redox properties of the sulfhydrogenase from *Pyrococcus furiosus*

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Received 24 April 1995; revised version received 26 May 1995

Abstract **The sulfhydrogenase from the extreme thermophile *Pyrococcus furiosus* has been re-investigated. The $\alpha\beta\gamma\delta$ heterotetrameric enzyme of 153.3 kDa was found to contain 17 Fe, 17 S^{2-}, and 0.74 Ni. The specific activity of the purified protein was 80 U/mg. Three EPR signals were found. A rhombic $S = 1/2$ signal ($g = 2.07, 1.93, 1.89$) was observed reminiscent in its shape and temperature dependence of spectra from $[4Fe\text{–}4S]^{(2+;1+)}$ clusters. However, in reductive titrations the spectrum appeared at the unusually high potential $E_{m,7.5} = -90$ mV. Moreover, the signal dissappeared again at $E_{m,7.5} = -328$ mV. Also, two other signals appear upon reduction: a near-axial ($g = 2.02, 1.95, 1.92$) $S = 1/2$ spectrum ($E_{m,7.5} = -303$ mV) indicative for the presence of a $[2Fe\text{–}2S]^{(2+;1+)}$ cluster, and a broad spectrum of unknown origin with effective g-values 2.25, 1.89 ($E_{m,7.5} = -310$ mV). We hypothesize that the latter signal is caused by magnetic interaction of the rhombic signal and a third cluster.**

Key words: Hydrogenase; EPR; *Pyrococcus furiosus*

1. Introduction

The sulfhydrogenase of the anaerobic archaeon *Pyrococcus furiosus* was first isolated by Bryant and Adams [1]. *P. furiosus* grows near 100°C by fermentation of carbohydrates, resulting in CO_2 and H_2 as sole products. However, H_2 is only found when the organism is grown in the absence of elemental sulfur (S^0). When the culture medium contains sulfur, H_2S instead of H_2 is produced. Since H_2 inhibits growth, it was hypothesized that sulfur reduction is a means of detoxification [2]. Attempts to isolate the sulfur reductase revealed that sulfur reduction activity coincided with hydrogenase activity. Therefore, it was concluded that both H_2S and H_2 production are catalyzed by the same enzyme, and by concequence the enzyme was called sulfhydrogenase [3]. The protein is encoded by a chromosomal operon of four open-reading frames [4] for four polypeptides of 48.7, 41.8, 33.2, and 29.6 kDa [5]. Unlike other hydrogenases the *P. furiosus* enzyme catalyzed preferably H_2 production [1]. Bryant and Adams reported the enzyme to contain 31 Fe, 24 S^{2-}, and 1 Ni per 185 kDa. Despite the high metal content only two EPR signals were found. In the reduced state the enzyme exhibited resonances at $g = 2.03$, 1.93, and 1.92, which could been observed from 70 to 20 K. This signal integrated to only one spin/mol. Upon lowering the temperature a complex signal was found with fast spin-lattice relaxation rate, which accounted for another spin/mol. No Ni signals were found. The former signal was assigned to a [2Fe–2S] cluster, while the latter was proposed to arise from at least two interacting iron–sulfur clusters. In a later publication Adams reports the monitoring of a [2Fe–2S] and a [4Fe–4S] cluster in a EPR redox titration [6]. Reduction potentials of −410 mV of the [2Fe–2S] cluster and of −210 mV of the [4Fe–4S] cluster were determined. In this paper we present data on the EPR properties of *P. furiosus* sulfhydrogenase that are consistent with the presence of three, rather than two signals. We also compare differences in metal content and reduction potentials. The possibility that the rhombic signal represents a two-electron transferring cluster is discussed.

2. Materials and methods

2.1. Growth of organism

P. furiosus (DSM 3638) was grown on potato starch (5 g/l) in a stirred (150 rpm) 200 liter fermentor (lp 300, Bioengineering, Wald, Switzerland) at 90°C, in a medium described in [7]. NaCl p.a. was replaced by table salt. The medium also contained yeast extract (1 g/l) and cysteine (0.5 g/l). Trace elements were Na_2WO_4, 10 μM; $Fe(NH_4)SO_2$, 25 μM; $NiCl_2$, 2.1 μM; H_3BO_3, 0.2 μM; zinc acetate, 1 μM; $CuSO_4$, 0.04 μM; $MnCl_2 \cdot 4H_2O$, 1 μM; $CoSO_4$, 1.5 μM; $Na_2MoO_4 \cdot 2H_2O$, 0.03 μM. Vitamins used were biotin, 2 μM; folic acid, 2 μM; nicotinamide, 5 μM; thiamin HCl, 5 μM; riboflavin, 5 μM; pyridoxine HCl, 10 μM; cyanocobalamine, 5 μM; *p*-aminobenzoic acid, 5 μM; lipoic acid, 5 μM; and pantothenic acid, 5 μM. No elemental sulfur (S^0) was present. The medium was continuously flushed with nitrogen to remove evolved hydrogen gas. Growth was followed by measuring the absorbance at 660 nm. Cells were frozen and stored at −20°C until use. Potato starch and table salt were obtained from the local grocery.

2.2. Isolation procedure

Sulfhydrogenase was purified according to a modification of the procedure of Bryant and Adams [1]. Ethanol was emitted from the buffers. Because of a higher capacity a Sephacryl PG 200 molecular sieve (2.5 × 100 cm) was used instead of a Superose 6 column. As the first purification step an ammonium sulfate precipitation was performed. Ammonium sulfate was added to the extract to 55% saturation. After a 10 min spin at 5,000 × *g* hydrogenase activity was located in the pellet. The pellet was resuspended in standard buffer and dialyzed against standard buffer. As an extra purification step a 50 ml Phenyl Sepharose hydrophobic interaction column was used. Fractions containing hydrogenase activity eluting from the hydroxyapatite column were pooled and adjusted to the appropriate ionic strenght of 0.25 M by adding crystals of $(NH_4)_2SO_4$. A 1 liter linear gradient of 0.25–0 M $(NH_4)_2SO_4$ in standard buffer was applied. Hydrogenase eluted at 0 M $(NH_4)_2SO_4$.

2.3. Activity measurements, analytical chemistry

Hydrogenase activity was measured in the hydrogen production assay according to Bryant and Adams [1] using a gas chromatograph (model 3400, Varian).

Protein was measured using the microbiuret method [8]. Iron was determined colorimetrically as the ferene complex [9]. Nickel was determined by atomic absorption spectroscopy on a Hitachi 180–80 Polarized Zeeman Atomic Absorption Spectrophotometer equipped with a pyrocuvette in the laboratory of Dr. S.P.J. Albracht (The University of Amsterdam).

SDS polyacrylamide electrophoresis was performed with a midget system (Pharmacia) holding 8-× 5-× 0.75-cm gels, according to the method of Laemmli [10]. The composition (mass/volume) of the stacking gel was 4% acrylamide and 0.1% bisacrylamide; the running gel was

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SSDI 0014-5793(95)00622-2

17.5% acrylamide and 0.07% bisacrylamide. The native molecular mass of the protein was determined with a Superose 6 HR 10/30 column (Pharmacia) equilibrated with standard buffer + 0.15 M NaCl using a flow rate of 0.5 ml/min. Glucose oxidase (152 kDa), lactate dehydrogenase (109 kDa), transferrin (74 kDa), BSA (67 kDa), and ovalbumine (43 kDa) were used as the markers for the calibration. The void volume was determined with Dextran blue.

2.4. Spectroscopy

EPR measurements and mediated redox titrations were as in [11]. In titration experiments protein concentration was typically 4 μM in 100 mM HEPES pH 7.5, and the following EPR signals were monitored: the $g = 1.89$ peak of the broad signal at the wings; the $g = 1.89$ peak of the rhombic $S = 1/2$ signal; the $g = 1.92$ peak of the near-axial $S = 1/2$ signal. Titrations were done at 23°C.

3. Results

3.1. Isolation

Our first attempts to isolate the sulfhydrogenase from *Pyrococcus furiosus* using the procedure described by Bryant and Adams [1] did not result in a pure hydrogenase sample. Instead, more then four bands were observed on SDS gel. By use of a modified procedure, i.e. by using ammonium sulfate precipitation as the first step, and a Phenyl-Sepharose hydrophobic interaction column, a preparation with a higher apparent purity on SDS gel was obtained. Clearly, four subunits can be observed of 45, 43, 31, and 28 kDa (Fig. 1). These values fit reasonably with the values of the subunits deduced from the amino acid sequence, being 48.7, 41.8, 33.2, and 29.6 kDa for the α, β, γ, and δ subunit, respectively [5]. Starting with a specific activity of ≈ 1.0 U/mg, an 80-fold purification was accomplished, resulting in a specific activity of 80 U/mg (see also Table 1). This is lower than the value reported by Bryant and Adams, i.e. 360 U/mg, which accounted for a 350-fold purification [1].

3.2. Analytical data

The molecular mass of the protein was previously reported to be 185 kDa [1]. However, this value was based on an assumed hexameric subunit composition of $\alpha_2\beta_2\gamma_2$. Recently, it was shown that the the enzyme consists of four different gen products (α, β, γ, and δ) [5]. The sum of the masses of the individual subunits is 153.3 kDa. To obtain the subunit composition we performed a gel-filtration experiment. On a Superose 6 column the native protein ran with an apparent molecular mass of 135 kDa (Fig. 2). This value fits reasonably with a $\alpha\beta\gamma\delta$ subunit composition. Moreover, on a SDS gel all four subunits have similar intensities (Fig. 1). We therefore conclude that *P. furiosus* sulfhydrogenase is an $\alpha\beta\gamma\delta$ heterotetramer of 153.3 kDa. Table 1 lists the iron, sulfide, and nickel contents of three different preparations. The enzyme contained 17 ± 2 Fe, 17 ± 2 S^{2-}, and 0.74 ± 0.21 Ni atoms per 153.3 kDa.

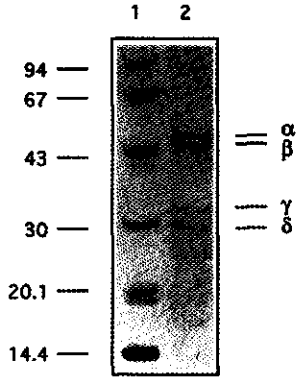

Fig. 1. SDS-polyacrylamide gel with purified *P. furiosus* sulfhydrogenase. Lane 1, molecular marker mixture; lane 2, purified sulfhydrogenase.

Table 1
Physico-chemical data on *P. furiosus* sulfhydrogenase

	Previous reports		This paper
Ni	0.98 ± 0.05	[1]	0.74 ± 0.21
Fe	31 ± 3	[1]	17 ± 2
S^{2-}	24 ± 4	[1]	17 ± 2
Spec. activity (U/mg)	360	[1]	80
E_m [2Fe–2S] (mV)	−410	[6]	−303
E_m [4Fe–4S] (mV)	nr		−90
E_m 'broad signal' (mV)	−210	[6]	−310

nr, not reported.

3.3. EPR spectroscopy

In the reduced state *P. furiosus* sulfhydrogenase exhibits two EPR signals. A near-axial ($g = 2.02, 1.95, 1.92$) $S = 1/2$ spectrum (Fig. 3C) indicates the presence a [2Fe–2S] cluster as already proposed by Bryant and Adams [1]. Moreover, a broad spectrum of unknown origin with effective g-values 2.25, 1.89 was detected (Fig. 3E). This signal showed fast relaxation properties since no saturation was observed using microwave powers up to 200 mW. Double integration of the total spectrum in the reduced state integrated to 1.8 spin/mol; the putative [2Fe–2S] cluster alone comprised 0.7 spin/mol. In addition to the two signals detected in the reduced state we discovered a third signal, which is observed when the protein is partially reduced. The shape and temperature dependence of this rhombic $S = 1/2$ signal ($g = 2.07, 1.93, 1.89$) are reminiscent of a $[4Fe–4S]^{(2+;1+)}$ cluster (Fig. 3A). Integration of this spectrum yielded ≈ 0.45 spin/mol. In the oxidized state the enzyme was EPR silent; no [3Fe–4S] signals could be found, nor did we detect any Ni resonances. No high-spin resonances were detected at low field at any stage of the reduction.

3.4. Redox titration

To investigate the redox properties of the *P. furiosus* sulfhydrogenase the enzyme was titrated in the presence of mediators. By adding sodium dithionite or ferricyanide the sample was stepwise reduced or oxidized, and EPR samples were drawn at redox equilibrium. Table 1 lists the reduction potentials of the different EPR signals. The rhombic $S = 1/2$ signal appeared upon reduction of the enzyme; a reduction potential $E_{m,7.5} = -90$ mV was determined. Further reduction causes this signal to disappear with an apparent reduction potential $E_{m,7.5} = -328$ mV; a bell-shaped curve is obtained (Fig. 4A). For the near-axial $S = 1/2$ signal a reduction potential $E_{m,7.5} = -303$ mV was determined (Fig. 4B). The broad signals at the wings of the spectrum appeared with a reduction potential $E_{m,7.5} = -310$ mV (Fig. 4C). Reversibility was tested by fully

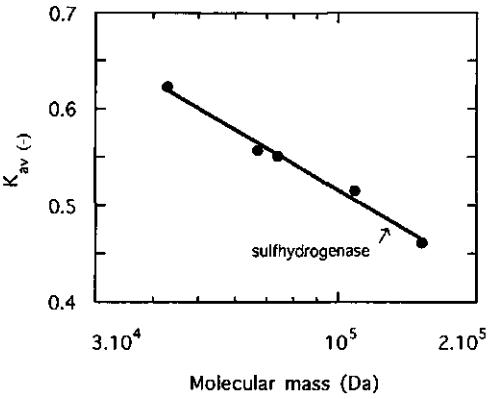

Fig. 2. Calibration of the native molecular mass of *P. furiosus* sulfhydrogenase with a Superose 6 column. Gelfiltration was performed as described in section 2. K_{av} is the ratio of the elution volume V_e minus the void volume V_0 over the total volume V_t minus the void volume V_0.

reducing the enzyme with sodium dithionite and subsequently stepwise re-oxidizing the enzyme with ferricyanide. No changes in EPR signals or reduction potentials were observed.

We have also done an aerobic isolation of *P. furiosus* sulfhydrogenase. The enzyme thus obtained was identical with respect to its activity and redox properties, as well as its EPR spectra, compared to the anaerobically isolated enzyme. Therefore, we suggest that this hydrogenase is not particularly oxygen-sensitive and there is no necessity to perform the purification under strict anaerobic conditions.

4. Discussion

4.1. Isolation and analytical data

According to the purification scheme published by Bryant and Adams it should be possible to obtain a 350-fold purification of *Pyrococcus furiosus* sulfhydrogenase in four subsequent column chromatography steps [1]. However, we were unable to reproduce their results, as SDS gel electrophoresis of our final preparation showed that the enzyme was not yet pure. Also, Mura et al. [4] found it impossible to purify the sulfhydrogenase to homogeneity using this method. By use of a modified purification scheme we were able to purify the enzyme to near homogeneity. The final preparation had a specific activity of 80 U/mg, which represents an 80-fold purification. Both the specific activity and the purification factor are lower than reported earlier [1]. Ma et al. present the isolation of a sulfur reductase of *P. furiosus* [3]. Since sulfur reductase activity and hydrogenase activity coincided during the isolation procedure, it was concluded that sulfur reductase and hydrogenase are one and the same enzyme. However, the sulfur reductase could only be purified thirty-fold instead of 350-fold for the hydrogenase. The purification factor of thirty-fold appears to be a more realistic value when compared to our results.

Enigmatic is also the difference in metal content. We find 17 Fe and 17 S^{2-}, which is almost twofold lower than reported previously, namely 31 and 24, respectively [1]. However, the numbers of Bryant and Adams [1] were based on an assumed molecular mass of 185 kDa. With the exact molecular masses of the subunits currently known [4], and with a proposed total molecular mass of 153.3 kDa, we can now correct these numbers to 27 Fe and 21 S^{2-}. These values are still higher than ours. We find 0.74 Ni, which is close to the previously published value. Unfortunately, no data on metal content have been presented for the sulfur reductase [3].

4.2. EPR spectroscopy and redox properties

We have found two EPR signals in *P. furiosus* sulfhydroge-

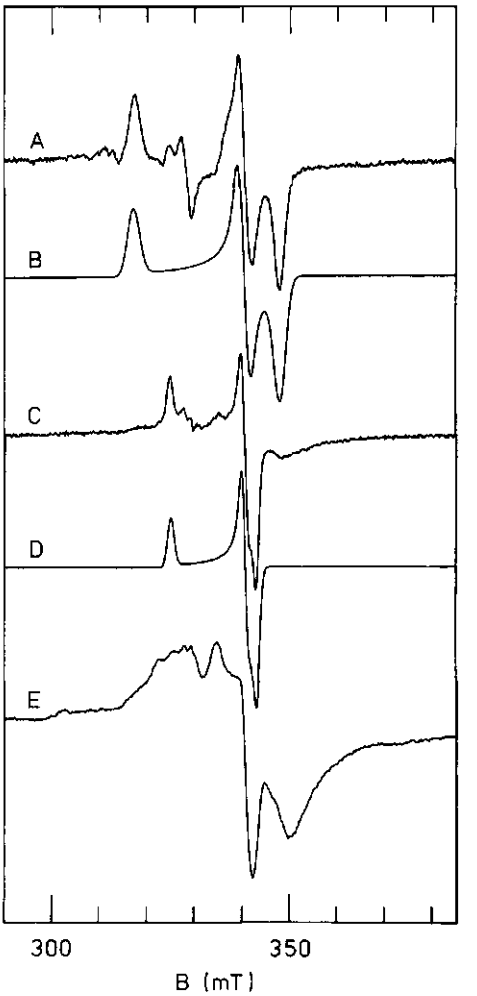

Fig. 3. EPR spectrum of purified *P. furiosus* sulfhydrogenase as a function of redox potential and microwave power. Trace A, spectrum of enzyme poised at a redox potential of −146 mV; trace B, simulation of A (see Table 2 for parameters); trace C, fully reduced enzyme; trace D, simulation of C; trace E, fully reduced enzyme at high microwave power. EPR conditions: microwave frequency, 9182 ± 1 MHz; microwave power, 5 mW or (trace E) 200 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; temperature, 20 K (trace A) or 17 K.

Table 2
Simulation parameters of EPR signals from *P. furiosus* sulfhydrogenase

	z-value	y-value	x-value
'Rhombic Signal'			
g-value:	2.068	1.927	1.886
Line width:	0.0098	0.0064	0.0080
'Axial Signal'			
g-value:	2.0185	1.926	1.913
Line width:	0.0050	0.0040	0.0045

The simulations assume $S = 1/2$ and are based on 100×50 molecular orientations. The line shape is assumed to be a symmetrical Gaussian in frequency space; the line width is described as a g-strain tensor collinear with the g-tensor [14].

nase in the reduced state. As previously proposed the near-axial signal probably represents a $[2Fe–2S]^{(2+;1+)}$ cluster [1]. A reduction potential $E_{m,7.5} = -303$ mV was determined for the putative [2Fe–2S] cluster from *P. furiosus* hydrogenase. The broad signals at the wings of the spectrum ($g = 2.25$; 1.89) appeared with a reduction potential $E_{m,7.5} = -310$ mV, which is almost identical with the observed reduction potential of the putative [2Fe–2S] cluster. We also detected a rhombic $S = 1/2$ signal in the partially reduced enzyme, which possibly arises from a $[4Fe–4S]^{(2+;1+)}$ cluster. Its reduction potential is −90 mV. Upon further reduction the signal disappeared again at $E_{m,7.5} = -328$ mV, hence a bell-shaped titration curve is obtained (Fig. 3A). Adams reported to have monitored a [2Fe–2S] cluster in a titration experiment by following the $g = 2.03$ peak. A reduction potential of −410 mV was determined [6]. Although the rhombic signal has not been observed before [1], Adams reported to have followed also a putative [4Fe–4S] cluster by monitoring the amplitude of the $g = 1.88$ signal; a reduction potential of −210 mV was determined [6]. The latter resonance is presumably identical to our 'broad signal'. There is a large disparity between the reduction potentials determined in our experiments and the potentials reported by Adams. For the [2Fe–2S] cluster we find a reduction potential which is 100 mV more positive. The [4Fe–4S] cluster reported by Adams presumably represents the broad signals for which we determined a reduction potential that is 100 mV more negative (Table 1). Our calculations were done with data from three independent titrations, we therefore propose that the data presented by Adams are not accurate.

The bell-shape of the titration curve of the rhombic signal could imply that the corresponding cluster is capable of transferring two electrons. This is unprecedented for this type of cluster. So far [4Fe–4S] clusters have only been shown to switch between either the 2+ and the 1+ state, or between the 2+ and 3+ state. Iron–sulfur clusters that can take up more than one electron are thus far limited to a few examples, e.g. the P-cluster in nitrogenase [12], and the prismane protein [13]. In the periplasmic Fe-only hydrogenase from *Desulfovibrio vulgaris (Hildenborough)* a rhombic signal ($g = 2.06$) is observed when the enzyme is partially reduced. Upon further reduction this signal disappears again, giving rise to a bell-shaped titration curve. When the enzyme is re-oxidated this signal does not re-appear. It has been hypothesized that the appearance of this signal is caused by reductive activation [11]. To exclude the possibility that the rhombic signal in *P. furiosus* sulfhydrogenase is due to reductive activation, reversibility was checked by re-oxidation after reduction. The bell shape of the curve was reproduced and is therefore not a reflection of some form of activation. However, the cluster that gives rise to the rhombic signal could be coupled to a third spin system that is not observed. In this case, reduction of this third system could lead to a transition of the rhombic signal into the broad signal, i.e. the broad signal is the resultant of a magnetic interaction between the cluster of the rhombic signal and a third cluster that has become EPR detectable upon reduction with $E_{m,7.5} = -310$ mV. Because the resulting interaction spectrum has rapid spin-lattice relaxation properties the unseen third spin system is presumably also fast relaxing. The axial signal has slow relaxation properties and the shape does not point to any interaction, therefore, it is unlikely that the coupling is caused by this spin system. The high iron and sulfide content (17 Fe, 17 S^{2-}) alllows

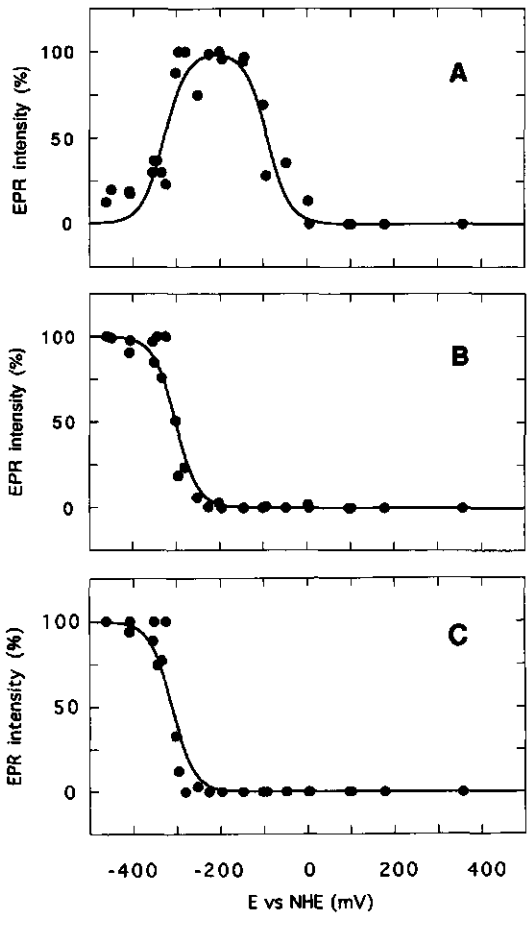

Fig. 4. Mediated redox titration of purified sulfhydrogenase from *P. furiosus*. Relative intensities of EPR signals are plotted as a function of the equilibrium redox potential in the presence of mediators (see section 2). The solid traces are least-square fits to the Nernst equation ($n = 1$) with the following E_m values: A, −328 mV, −90 mV; B, −303 mV; C, −310 mV.

for the presence of more than two clusters. Also, the four subunits have recently been shown to be homologous to the two subunits of common Ni-hydrogenases and to two of the subunits of a sulfite reductase [5] and, therefore, the $\alpha\beta\gamma\delta$ sequence carries five motifs for five putative iron–sulfur clusters. We therefore suggest that the rhombic EPR signal does not represent a two-electron transferring cluster, but that its disappearance upon reduction is due to magnetic coupling to an as yet unidentified spin system.

When we compare the results presented in this paper with previously presented data we find that there is a remarkable disparity in metal content, EPR spectra, and reduction potentials. The possibility that we have purified a different protein does not appeat to be a realistic one. The rhombic EPR signal ($g = 2.07$) has been detected only in our enzyme preparation, yet the axial signal ($g = 2.06$) and the broad signal ($g = 2.25$) have been observed in both studies; it is very unlikely that different proteins exhibit identical EPR spectra. Alternatively, the stoichiometry of the four subunits may not be constant. Because of its homology with a hydrogenase and a sulfite reductase, the *P. furiosus* sulfhydrogenase can be considered to be the sum of two functional units: a hydrogenase (α and δ subunit) and a sulfur reductase (β and γ subunit) each unit containing its own metal centers. The two units (i.e. either set of two subunits) need not necessarily be stoichiometrically expressed, for example, the expression of the subunits could be dependent on the growth conditions, and this could be reflected by the relative intensities of the different EPR signals, and, possibly, also by the metal content. However, except for some minor changes (e.g. the use of starch instead of maltose as carbon and energy source) the growth conditions used in our experiments and those of Bryant and Adams [1] are similar. Moreover, the four structural genes are organized in one transcriptional unit, and no indication was found for posttranslational processing at the amino-terminus [5]. Also, SDS gels of purified protein presented in both studies revealed the presence of four subunits. The significant discrepancies between the here presented data and the previous work remain to be resolved.

Acknowledgements: We thank W. Roseboom for help with atomic absorption spectrometry. Professor C. Veeger is acknowledged for his continuous interest and support. This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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4. UNEXPECTED SIMILARITIES IN THE ARCHITECTURE OF THE ACTIVE SITES OF NI- AND FE HYDROGENASES; AN INFRARED SPECTROSCOPY STUDY

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Eur. J. Biochem. (1996) **237**, 629-634

Eur. J. Biochem. 237, 629–634 (1996)

Similarities in the architecture of the active sites of Ni-hydrogenases and Fe-hydrogenases detected by means of infrared spectroscopy

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(Received 15 December 1995) – EJB 95 2056/4

Three groups that absorb in the 2100–1800-cm^{-1} infrared spectral region have recently been detected in Ni-hydrogenase from *Chromatium vinosum* [Bagley, K. A., Duin, E. C., Roseboom, W., Albracht, S. P. J. & Woodruff, W. H. (1995) *Biochemistry 34*, 5527–5535]. To assess the significance and generality of this observation, we have carried out an infrared-spectroscopic study of eight hydrogenases of three different types (nickel, iron and metal-free) and of 11 other iron-sulfur and/or nickel proteins. Infrared bands in the 2100–1800-cm^{-1} spectral region were found in spectra of all Ni-hydrogenases and Fe-hydrogenases and were absent from spectra of any of the other proteins, including a metal-free hydrogenase. The positions of these bands are dependent on the redox state of the hydrogenase. The three groups in Ni-hydrogenases that are detected by infrared spectroscopy are assigned to the three unidentified small non-protein ligands that coordinate iron in the dinuclear Ni/Fe active site as observed in the X-ray structure of the enzyme from *Desulfovibrio gigas* [Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E. C., Frey, M. & Fontecilla-Camps, J. C. (1995) *Nature 373*, 580–587]. It is concluded that these groups occur exclusively in metal-containing H_2-activating enzymes. It is proposed that the active sites of Ni-hydrogenases and of Fe-hydrogenases have a similar architecture, that is required for the activation of molecular hydrogen.

Keywords: hydrogenase; Fourier-transform infrared spectroscopy; hydrogen activation; nickel; iron-sulfur protein.

Activation of H_2 in a wide variety of microorganisms is performed by enzymes called hydrogenases. Two types of enzymes are known that can activate H_2 without the need for added cofactors. Ni-hydrogenases (also called Ni-Fe hydrogenases or [NiFe] hydrogenases) contain one Ni atom and at least one [4Fe-4S] cluster; more [4Fe-4S] clusters and a [3Fe-4S] cluster are often detectable. The amino acid sequences of 30 such hydrogenases are known, only a few of which have been studied in detail [reviewed by Albracht (1994)]; Fe-hydrogenases [reviewed by Adams (1990)] contain Fe as their only metal constituent and are therefore also referred to as Fe-only hydrogenases. The amino acid sequences of four Fe-hydrogenases are known [reviewed by Albracht (1994)]. Except for the amino acid sequences that encode the binding sites of at least two [4Fe-4S] clusters, there is no obvious sequence similarity to the Ni-hydrogenases. The simplest Fe-hydrogenase, in terms of prosthetic groups, is that from *Desulfovibrio vulgaris* (Van der Westen et al., 1978). It contains two classical [4Fe-4S] clusters and an Fe-S cluster, termed the H-cluster, which has been proposed (Hagen et al., 1986) to contain approximately six Fe ions and to be directly involved in the activation of H_2. Fe-hydrogenases are usually about two orders of magnitude more active than Ni-hydrogenases but have a 100-fold-higher K_m value for H_2. In addition to the metal-containing hydrogenases, an enzyme from *Methanobacterium thermoautotrophicum* has been characterized which does not contain any transition metals and yet can activate H_2 while reducing methylenetetrahydromethanopterin (Zirngibl et al., 1992). However, this enzyme cannot activate H_2 in the absence of methylenetetrahydromethanopterin.

There is significant evidence that Ni is involved in the H_2-activating site of Ni-hydrogenases [reviewed by Albracht (1994)]. Mössbauer studies of the reduced *Chromatium vinosum* hydrogenase (Surerus et al., 1994) suggested the presence of a lone low-spin Fe ion, a [3Fe-4S] cluster and two [4Fe-4S] clusters. The redox equilibrium between this enzyme and H_2 (at pH 8.0 in the absence of mediating dyes) involves an $n = 2$ redox centre at the active site, in which an $S = 0.5$ Ni species with bound H_2 participates as one of the redox partners (Coremans et al., 1992a). The Fe-S clusters are not involved in this equilibrium (Ravi, N., Roseboom, W., Duin, E. C., Albracht, S. P. J. and Münck, E., unpublished results). This $n = 2$ centre has the same midpoint potential (E_0') and pH dependence as the hydrogen electrode. It has therefore been hypothesized (Albracht, 1994) that the active site in Ni-hydrogenases involves a Ni ion and a lone Fe ion. The crystal structure of Ni-hydrogenase from *Desulfovibrio gigas* (Volbeda et al., 1995) indicates the possible presence of a lone Fe atom in close proximity to the Ni atom.

In Fe-hydrogenase from *D. vulgaris* in the presence of mediating dyes, the EPR signal proposed to represent the H-cluster

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Abbreviation. FTIR, Fourier-transform infrared.

behaves as an $S = 0.5$ Fe species with an E_0' of -307 mV ($n = 1-2$; pH 7.0; Pierik et al., 1992). In *Clostridium pasteurianum* Fe-hydrogenase this EPR signal disappears within 6 ms of mixing with H_2 (Erbes et al., 1975). The active sites in Ni-hydrogenases and Fe-hydrogenases have therefore been assumed to be very different.

Three absorption bands in the 2100–1850-cm^{-1} spectral region were described in infrared spectra of Ni-hydrogenase from *C. vinosum* (Bagley et al., 1994, 1995). The frequencies and intensity of these bands were novel for a protein. The bands were found to have a unique position in seven forms of the enzyme, which differed either in the redox state or in the state of coordination of the Ni centre. It was concluded that the bands represent groups that contain polar triple bonds and/or two adjacent double bonds very close to the Ni centre, and probably arise from ligands attached to this centre.

Recently, another enzyme has been reported to show bands at approximately 1850 cm^{-1}. In nitrile hydratase from *Rhodococcus* two bands at 1847 cm^{-1} and 1855 cm^{-1} have been ascribed to Fe-bound NO (Noguchi et al., 1995).

To examine how general the infrared-spectroscopy features of the *C. vinosum* enzyme are in Ni-hydrogenases, we have extended our infrared-spectroscopy investigations. Examination of the infrared spectra of four additional Ni-hydrogenases from widely different organisms establishes that the groups responsible for these bands are due to components of Ni-hydrogenases. For comparison, a variety of Fe-S proteins and some Ni-containing non-hydrogenase enzymes were also studied. None of the spectra from these proteins showed any bands in the 2100–1800-cm^{-1} spectral region. We investigated whether other H_2-activating enzymes showed such bands. Similar bands were also observed in two Fe-hydrogenases, but not in the H_2-forming methylenetetrahydromethanopterin dehydrogenase.

MATERIALS AND METHODS

A number of enzymes were purified by published procedures: Ni-hydrogenase from *Alcaligenes eutrophus* (soluble NAD^+-reducing enzyme; cells were obtained from G. Haverkamp and C. G. Friedrich, Dortmund, Germany; Schneider et al., 1979; Friedrich et al., 1982); Ni-hydrogenase from *C. vinosum* (strain DSM 185) (Coremans et al., 1992b); Fe-hydrogenase (Van der Westen et al., 1978) and prismane protein (Stokkermans et al., 1992) from *D. vulgaris* (Hildenborough); dissimilatory sulfite reductase from *Desulfosarcina variabilis* (Arendsen et al., 1993); ferredoxin from *Megasphaera elsdenii* (Gillard et al., 1965); and rubredoxin from *Pyrococcus furiosus* (Blake et al., 1991).

Other proteins were kind gifts from several laboratories: *Wolinella succinogenes* hydrogenase (Albracht et al., 1986) from A. Kröger (Frankfurt, Germany); *Methanococcus voltae* hydrogenase (Sorgenfrei et al., 1993) from O. Sorgenfrei and A. Klein (Marburg, Germany); *M. thermoautotrophicum* hydrogenases (Zirngibl et al., 1992) from G. Hartmann, R. Hedderich and R. K. Thauer (Marburg, Germany); Fe-hydrogenase from *M. elsdenii* (Van Dijk et al., 1980, Filipiak et al., 1989) from M. Filipiak (Wageningen, The Netherlands); the water-soluble Fe-S fragment of the Rieske protein of bovine heart bc_1 complex (Link et al., 1992) from T. A. Link (Frankfurt, Germany); *Rhodopseudomonas gelatinosa* high-potential iron protein (Bartsch, 1978) from T. E. Meyer (Tucson, AZ, USA); MoFe-protein (component I) of *Azotobacter chroococcum* nitrogenase (Yates and Planque, 1975) from R. R. Eady (Brighton, UK); *Methanothrix soehngenii* CO dehydrogenase (Jetten et al., 1991) and methyl-CoM reductase (Jetten et al., 1990) from M. S. M. Jetten (Wageningen, The Netherlands); and bovine heart Complex I (Finel et al., 1992) from R. van Belzen (Amsterdam, The Netherlands). *Spirulina platensis* [2Fe-2S] ferredoxin was from Sigma.

For the infrared-spectroscopy comparisons of the various hydrogenases and non-hydrogenase proteins, the proteins were dissolved in either 50 mM Tris/HCl, pH 8.0, or 20–120 mM potassium phosphate, pH 7–8, and concentrated to 0.12–2 mM by means of Centricon PM30 or PM10 filters. Examination of the infrared spectra of the buffers revealed no specific infrared absorption bands in the 2100–1800-cm^{-1} region. O_2-sensitive proteins were kept under Ar in the presence of 2–50 mM sodium dithionite. Samples were treated as indicated in Results and loaded into a gas-tight infrared-transmittance cell (Bagley et al., 1994) with CaF_2 windows and Teflon spacers of 50–60 μm (total sample approximately 10 μl). Aerobic samples were directly loaded into the cell and the infrared spectrometer was purged with dry air. O_2-sensitive samples were placed in a glove box, purged with Ar, and loaded into the anaerobic infrared-transmittance cell. Prior to loading the samples, the cell was filled with a mixture of glucose (80 mM) and glucose oxidase (0.4 mg/ml) to remove O_2. After 15 min the cell was extensively rinsed with Ar-saturated buffer. After loading the sample, the cell was transported to the Fourier-tansform infrared (FTIR) spectrometer in an anaerobic container and the FTIR spectrometer was purged continuously with N_2. Infrared spectra were collected at room temperature on a BioRad FTS-60A FTIR spectrometer with an MCT detector. Spectra were corrected for the water background by subtraction of the corresponding buffer spectrum. The multiple-point method of the programme WIN-IR (BioRad) was used for further correction of the base line. Spectral resolution was 2 cm^{-1}.

Studies of the pH dependence of the bands were performed with oxidized *C. vinosum* enzyme dissolved in 50 mM of each of the following buffers: Mes, pH 6.0; Mops, pH 6.75; Tris/HCl, pH 8.0; Taps, pH 9.0; glycine/NaOH, pH 9.75. Spectra were collected by means of a Perkin Elmer 1600 FTIR instrument equipped with a DTGS detector. Spectra were collected at 4-cm^{-1} resolution. For comparison of spectra measured at different pH values, the intensities of the bands were scaled with the absorbance at 280 nm for the enzyme loaded in the infrared-transmittance cell.

RESULTS

In previous infrared-spectroscopy studies, at cryogenic temperatures, of the Ni-hydrogenase from *C. vinosum* (Bagley et al., 1994, 1995) it was reported that this enzyme showed three bands in the 2100–1800-cm^{-1} spectral region. The groups responsible for these bands responded to all changes in the status of the Ni centre. We have now extended these studies by examination of the infrared absorption spectra at room temperature of several other Ni-hydrogenases to determine whether the presence of these bands is a general property of this class of enzymes.

***C. vinosum* hydrogenase.** As only limited quantities of the other enzymes were available, we first tested the minimal concentration required for reliable detection of the major infrared absorption band of the *C. vinosum* enzyme. Infrared spectra of the enzyme at different concentrations are shown in Fig. 1. A direct linear relationship was observed between the amplitude of the 1945-cm^{-1} band and the enzyme concentration. The band could still be observed at 50 μM enzyme. All subsequent samples were tested at 0.12–2 mM protein.

The bands at 2092, 2082 and 1945 cm^{-1} are typical of enzymes with trivalent Ni (Bagley et al., 1995). The bands at 2066,

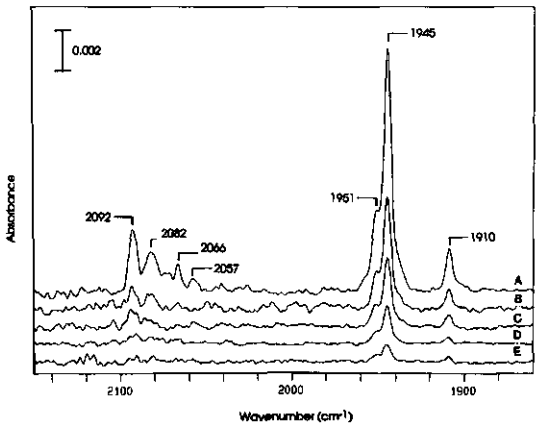

Fig. 1. Infrared spectra at room temperature of *C. vinosum* Ni-hydrogenase at several concentrations. The spectra were averages of scans at 2-cm^{-1} resolution. (A), 640 μM, 2048 scans; (B), 320 μM, 5000 scans; (C), 213 μM, 5000 scans; (D), 107 μM, 5000 scans; (E), 53 μM, 5000 scans.

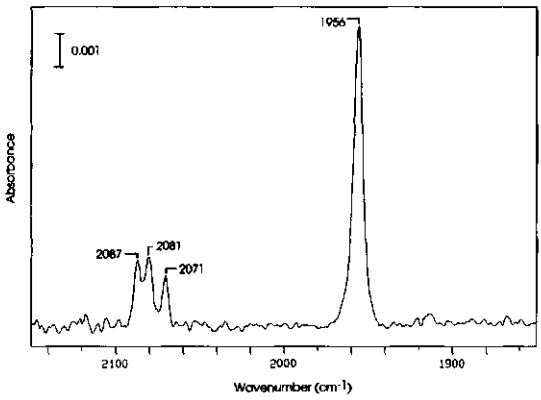

Fig. 2. Infrared spectrum at room temperature of the soluble Ni-hydrogenase from *A. eutrophus*. The spectrum was an average of 5000 scans at 2-cm^{-1} resolution.

2057 and 1910 cm^{-1} (Fig. 1) are here ascribed to an enzyme with divalent Ni. Although in an earlier study (Bagley et al., 1995) divalent Ni was ascribed to have bands at 2067, 2051 and 1910 cm^{-1} in low-temperature infrared spectra, we consistently found the second band at 2057 cm^{-1} in room-temperature spectra.

The shoulder at 1951 cm^{-1} detected in these room-temperature studies was not previously reported in spectra of *C. vinosum* at cryogenic temperatures (Bagley et al., 1994, 1995). Examination of the infrared spectra of unready enzyme (i.e., inactive enzyme which is unready to react with H_2; Fernandez et al., 1985) in its fully oxidized state suggested that this band displays significant temperature sensitivity. The band was quite large and well resolved in spectra taken at room temperature (1-cm^{-1} resolution) but decreased significantly in intensity (to nearly background level) when the sample was cooled to cryogenic temperatures (data not shown). In addition, it appeared that the 1951-cm^{-1} band was maximal in preparations where the Ni^{3+} ion was spin coupled to a system that consisted of an unknown redox group, X, and a $[3Fe\text{-}4S]^+$ cluster (X^{ox} = $[3Fe\text{-}4S]^+$) (Surerus et al., 1994; Albracht, 1994), and minimal in the oxidized enzyme where no such spin coupling could be detected (Ni^{3+} X^{red} $[3Fe\text{-}4S]^+$).

A study of the bands detected by infrared spectroscopy as a function of pH in the range 6.0–9.75 suggested that there was no significant change in frequency or intensity for any of the bands in the 2100–1800-cm^{-1} spectral region of oxidized *C. vinosum* enzyme over this pH range.

Other Ni-hydrogenases. To determine whether the bands detectable by infrared spectroscopy of the *C. vinosum* enzyme are a general property of Ni-hydrogenases, we studied several enzymes from other sources. As an example, the spectrum of an aerobic sample of the soluble hydrogenase from *A. eutrophus* is shown in Fig. 2. The major infrared band is at 1956 cm^{-1}, whereas three minor bands are present at 2087, 2081 and 2071 cm^{-1}.

Examination of the infrared spectra of three other Ni-hydrogenases in their oxidized forms shows the presence of at least one intense infrared band between 1960 cm^{-1} and 1850 cm^{-1} (Table 1). The spectrum of F_{420}-non-reducing enzyme from *M. thermoautotrophicum* showed a band at 1955 cm^{-1}, that of ^{77}Se-containing F_{420}-reducing enzyme from *M. voltae* showed bands at 1930 cm^{-1} and 1921 cm^{-1}, and that of the Ni-hydrogenase from *W. succinogenes* showed a band at 1942 cm^{-1} with a shoulder at 1953 cm^{-1}. In addition, as previously reported (Fernandez, V. M. and Hatchikian, E. C., personal communication), the enzyme from *D. gigas* shows intense bands in this region. From the observation of these bands in six Ni-hydrogenases, we conclude that the groups responsible for these bands are probably present in all Ni-hydrogenases and that they arise from very similar chemical structures.

Fe-S proteins and Ni-enzymes (non-hydrogenases). Ni-hydrogenases contain Ni and Fe-S clusters as prosthetic groups. To confirm that the presence of these types of prosthetic groups in other proteins do not give rise to the infrared absorbances described above, we examined the infrared spectra of a number of non-hydrogenase proteins that contain either Fe-S clusters, Ni ions or both. We could not detect infrared bands in the 2100–1800-cm^{-1} spectral region in any of the following proteins (Table 1): rubredoxin from *P. furiosus*; ferredoxins from *M. elsdenii* and *S. platensis*; the Rieske Fe-S protein from bovine heart; high-potential iron protein from *R. gelatinosa*; the prismane protein from *D. vulgaris* (Hildenborough); the MoFe-protein of nitrogenase from *A. chroococcum*; dissimilatory sulfite reductase from *D. variabilis*; and methyl-CoM reductase and CO-dehydrogenase from *M. soehngenii*. The published infrared spectra of CO dehydrogenase from *Clostridium thermoaceticum* (Kumar and Ragsdale, 1992) also do not display bands in this region.

The sequence similarity (Albracht, 1993) of the 49-kDa subunit and the PSST subunit, named after the first four amino-acid residues of this subunit (Arizmendi et al., 1992), of mitochondrial NADH:ubiquinone oxidoreductase (Complex I) with the large and the small subunits of Ni-hydrogenases, respectively, prompted us to study Complex I from bovine heart. No bands in the 2100–1800-cm^{-1} region could be detected in this enzyme.

Non-Ni-hydrogenases. We investigated whether other H_2-activating enzymes showed these bands. We could not find any such bands in the purified H_2-forming methylenetetrahydromethanopterin dehydrogenase. We have not tested whether such bands were induced by addition of the substrate methylenetetrahydromethanopterin. This substrate alone has no bands in

Table 1. Proteins studied by means of infrared spectroscopy. The presence (+) or absence (−) of absorption bands in the 2100−1800-cm^{-1} spectral region was investigated.

Protein	Prosthetic groups	Source	Infrared bands
Ni-hydrogenase	Ni, Fe, [3Fe-4S], [4Fe-4S]	*Chromatium vinosum*	+
Ni-hydrogenase	Ni, Fe, [3Fe-4S], [4Fe-4S]	*Alcaligenes eutrophus*	+
Ni-hydrogenase	Ni, Fe, [3Fe-4S], [4Fe-4S]	*Wolinella succinogenes*	+
Ni-hydrogenase	Ni, Fe, [4Fe-4S]	*Methanococcus voltae*	+
Ni-hydrogenase	Ni, Fe, [4Fe-4S]	*Methanobacterium thermoautotrophicum*	+
Metal-free hydrogenase	none	*Methanobacterium thermoautotrophicum*	−
Fe-hydrogenase	H-cluster, [4Fe-4S]	*Desulfovibrio vulgaris*	+
Fe-hydrogenase	H-cluster, [4Fe-4S]	*Megasphera elsdenii*	+
Rubredoxin	$Fe(Cys)_4$	*Pyrococcus furiosus*	−
Ferredoxin	[2Fe-2S]	*Spirulina platensis*	−
Rieske Fe-S protein	[2Fe-2S]	Bovine heart	−
High-potential iron protein	[4Fe-4S]	*Rhodopseudomonas gelatinosa*	−
Ferredoxin	[4Fe-4S]	*Megasphaera elsdenii*	−
Complex I	FMN, [2Fe-2S], [4Fe-4S]	Bovine heart	−
MoFe-protein	P-cluster, FeMo cofactor	*Azotobacter chroococcum*	−
Prismane protein	[6Fe-6S]	*Desulfovibrio vulgaris*	−
Dissimilatory sulfite reductase	siroheme, [xFe-4S]	*Desulfosarcina variabilis*	−
CO dehydrogenase	Ni, [xFe-yS]	*Methanothrix soehngenii*	−
Methyl-CoM reductase	Ni (F_{430})	*Methanothrix soehngenii*	−

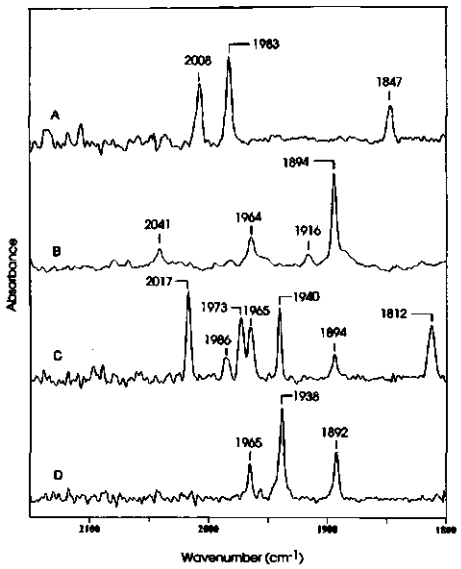

Fig. 3. Infrared spectra of Fe-hydrogenases from *D. vulgaris* and *M. elsdenii*. The spectra were averages of 5000 scans at 2-cm^{-1} resolution. (A), enzyme from *D. vulgaris* isolated in air; (B), *D. vulgaris* enzyme reduced with 50 mM dithionite for 2 h at room temperature; (C), *D. vulgaris* enzyme treated with H_2 for 15 min at room temperature then with Ar for 2 h at room temperature; (D), *M. elsdenii* enzyme prepared in the presence of 10 mM dithionite.

the 2100−1800-cm^{-1} region (G. Hartmann and A. Klein, personal communication).

The Fe-hydrogenases from *D. vulgaris* and *M. elsdenii*, however, showed bands in the 2100−1800 cm^{-1}-region (Fig. 3). The *D. vulgaris* enzyme, isolated in air (Fig. 3A), showed bands at 2008, 1983 and 1847 cm^{-1}. Upon reduction with dithionite a major band at 1894 cm^{-1} and minor bands at 2041, 1964 and 1916 cm^{-1} were observed (Fig. 3B). When the enzyme was reduced with H_2 and reoxidized with protons (i.e. by replacement of H_2 with Ar for several vacuum/Ar cycles), a number of bands appeared (2017, 1986, 1973, 1965, 1940, 1894 and 1812 cm^{-1}; Fig. 3C). The dithionite-reduced Fe-hydrogenase from *M. elsdenii* (Fig. 3D) only showed bands at 1965, 1938 and 1892 cm^{-1}. With this enzyme we found that the bands at 1965 cm^{-1} and 1938 cm^{-1} bands were maximal, and the 1892-cm^{-1} band was minimal, in the absence of dithionite, whereas the opposite occurred in the presence of excess dithionite. The major band detected by infrared spectroscopy of fully reduced Fe-hydrogenases from both bacteria was at 1892−1894 cm^{-1}. The enzyme in less-reduced states showed a variety of bands at different positions, presumably due to the presence of enzyme molecules in various redox states. These observations suggest that the Fe-hydrogenases also display the infrared features detected in the Ni-hydrogenases and that, like the Ni-hydrogenases, the bands detected by infrared spectroscopy in the Fe-hydrogenases are sensitive to the oxidation state of the active site.

DISCUSSION

***C. vinosum* hydrogenase.** The band at 1951 cm^{-1} has only been detected reproducibly in preparations which showed a large amount of spin coupling between Ni^{3+} and the $\{X^{ox} - [3Fe\text{-}4S]^+\}$ species. This coupling is not well understood. X behaves like an $n = 1$ Nernst redox component with an E'_0 of +150 mV (Coremans et al., 1992b). There are indications (Surerus et al., 1994) that X might be an Fe ion or a radical species close to the [3Fe-4S] cluster. The observation of the spin-spin interaction in EPR experiments suggests that the distance between the Ni^{3+} and the $\{X^{ox} = [3Fe\text{-}4S]^+\}$ system is much shorter than that between the Ni atom and the [3Fe-4S] cluster (approximately 2.1 nm) in the X-ray structure of the *D. gigas* enzyme (Volbeda et al., 1993, 1995). After reduction of X, the Ni^{3+} spin has no interaction with the $S = 0.5$ of the oxidized [3Fe-4S] cluster (or the $S = 2$ of the reduced cluster), which shows that they are far apart under these conditions (Albracht et al., 1984; Asso et al., 1992). It is possible that a structural change takes place when X changes oxidation state. Given the apparent correlation between the detection of the 1951-cm^{-1} band and the spin-coupled state,

we suspect that the band at 1951 cm^{-1} might represent X in its oxidized state. This possibility is currently under investigation.

Groups in metal-containing hydrogenases detectable by infrared spectroscopy. The results presented here indicate that the bands in the 2100–1800-cm^{-1} spectral region are exclusively present in metal-containing H_2-activating enzymes. They are not simply due to the presence of Fe-S clusters or Ni. This finding and the response to specific changes of the active sites in these enzymes strongly indicate that the groups involved are an integral part of the H_2-activating site. The major bands in the Ni-hydrogenases and Fe-hydrogenases are in the 2020–1810-cm^{-1} region.

As previously discussed (Bagley et al., 1994, 1995), it is unlikely that the bands in the spectrum of the *C. vinosum* enzyme are due to exchangeable CO molecules, bonds that involve an exchangeable proton, or a metal hydride. The frequency and intensity of the bands in the 2100–1800-cm^{-1} spectral region are most consistent with chemical groups that contain polar triple bonds (e.g., cyanide, CO, metal-bound N_2) or a system of adjacent double bonds (e.g., azide, thiocyanate, isothiocyanate) (Nakamoto, 1978; Colthup et al., 1990). Metal-coordinated NO has recently been proposed to be present in the Fe-containing enzyme nitrile hydratase (Noguchi et al., 1995) based on detection of two ^{15}N-sensitive bands at 1855 cm^{-1} and 1847 cm^{-1} in the infrared spectrum of this enzyme. The frequency detected in the nitrile hydratase enzyme is most consistent with a neutral or negatively charged ligand since neutral NO and negatively charged NO have ν(NO) stretching frequencies below 1880 cm^{-1}. Of the metal-containing hydrogenases studied only one of the Fe-hydrogenases showed bands which were so low in frequency (Fig. 3), which suggests that most, if not all, of the bands detected are not due to a neutral or negatively charged nitrosyl ligand. However, positively charged NO ligands (nitrosium) coordinated to metals have significantly higher frequencies and may be consistent with a number of the infrared frequencies detected in the 2100–1800-cm^{-1} spectral region of the metal-containing hydrogenases.

The X-ray structure of Ni-hydrogenase from *D. gigas* indicates that there are three non-protein groups coordinated to the Fe at the binuclear Ni/Fe active site (Volbeda et al., 1995). The three groups detectable by infrared spectroscopy in Ni-hydrogenases described here are probably the same three non-protein groups detected in the X-ray structure. Since the position of the bands detected by infrared spectroscopy is highly sensitive to the status of the Ni-centre as monitored by EPR, it was concluded (Bagley et al., 1995) that the groups were ligands to Ni. However, the X-ray data indicate that the three non-protein groups are ligands to the Fe atom. None of the EPR signals ascribed to Ni in the *C. vinosum* enzyme showed any broadening when inspected in preparations more than 90% enriched in ^{57}Fe, but all of them showed broadening or splitting in ^{61}Ni-enriched preparations (Duin, E. C. and Albracht, S. P. J., unpublished results). This finding suggests that unpaired spins in the active site are localized on Ni, rather than on Fe. If the groups detectable by infrared spectroscopy are ligands to the Fe ion, then electronic changes on the Ni ion, as monitored in EPR, are effectively transferred to the Fe ion, resulting in changes of the infrared frequency of the three unknown groups.

The finding of three minor bands in the infrared spectra of the soluble *A. eutrophus* enzyme (Fig. 2), instead of two bands in the *C. vinosum* enzyme, might be related to the finding that in the *Alcaligenes* enzyme no significant EPR signals of Ni can be evoked under any redox conditions (Cammack et al., 1986), unless artificial redox dyes are added (Happe, R., Massanz, C., Friedrich, B. and Albracht, S. P. J., unpublished results). This enzyme is not inhibited by O_2 or CO (Schneider et al., 1983). We speculate that the active site in this enzyme contains an extra group detectable by infrared spectroscopy that fixes the Ni in the divalent state and blocks the site where CO and O_2 can attack other Ni-hydrogenases.

The presence of these groups detectable by infrared spectroscopy in the active sites of metal-containing hydrogenases suggests some similarities in the structure of the H_2-activating centres of Ni-hydrogenases and Fe-hydrogenases. As one of the possibilities we speculate that the H_2-activating centre in both enzymes consists of a bi-metallic centre (Ni/Fe in Ni-hydrogenases or Fe/Fe in Fe-hydrogenases) involving a low-spin Fe ion, the groups detectable by infrared spectroscopy and thiols from Cys residues.

We thank Drs R. R. Eady, R. van Belzen, M. Filipiak, C. G. Friedrich, G. Hartmann, G. Haverkamp, R. Hedderich, M. S. M. Jetten, A. Klein, A. Kröger, T. A. Link, T. E. Meyer, O. Sorgenfrei and R. K. Thauer for kind gifts of proteins or cells. We thank Mr T. L. Snoeck and Mr A. Terpstra for useful advice during the infrared measurements and Mr C. J. Kleverlaan for stimulating discussions. The investigations were supported (in part) by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and by the European Union Human Capital and Mobility Programme via grant no. ERBCHRXCT920072 to the Masimo Network. K. A. B. acknowledges support via an award from the Research Corporation.

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5. CHARACTERIZATION OF A FERREDOXIN FROM *DESULFOVIBRIO VULGARIS* (HILDENBOROUGH) THAT INTERACTS WITH RNA

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Eur. J. Biochem. (1995) **231**, 352-357

Eur. J. Biochem. 231, 352–357 (1995)

Characterization of a ferredoxin from *Desulfovibrio vulgaris* (Hildenborough) that interacts with RNA

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(Received 9 February/5 May 1995) – EJB 95 0200/3

The purification and characterization of a ferredoxin from *Desulfovibrio vulgaris* (Hildenborough) is described. The protein can be isolated in two forms; the major form is strongly complexed to RNA, while a minor form is free from nucleic acid. Bound RNA cannot be removed by digestion with nucleases, or by heating to 70°C, and it can only be partially removed by rechromatography. The ultraviolet/visible spectrum shows typical absorption maxima at 280 nm and 400 nm for the RNA-free ferredoxin. The RNA-bound protein exhibits an additional strong peak at 260 nm. The RNA can be extracted from the protein with phenol. The ferredoxin is a dimer of subunits, each of 7.5 kDa; its pI is 3.9. The protein contains a $[4Fe\text{-}4S]^{(2+;1+)}$ cluster with an EPR spectrum (g = 1.90, 1.93 and 2.05) in the reduced state. A reduction potential of −360 mV was determined for the RNA-free ferredoxin with reversible voltammetry at glassy carbon. From the temperature dependence of the reduction potential, the unusually high standard reaction entropy was calculated as $\Delta S^{\circ} = -230\ J \cdot K^{-1} \cdot mol^{-1}$. No electrochemical response was obtained with the RNA-bound ferredoxin. Binding of RNA appears to require the presence of an intact cluster, since the absence of absorption at 400 nm runs in parallel with the absence of absorption at 260 nm. The possibility is discussed that the binding to the RNA has a regulatory function and is controlled by the state of the cluster.

Keywords. Ferredoxin; mRNA; bioelectrochemistry; EPR; *Desulfovibrio vulgaris* (Hildenborough).

Bacterial ferredoxins are small iron-sulfur (Fe-S) proteins containing one or two Fe-S clusters. The proteins are supposed to act as electron carriers, i.e. they shuttle electrons between enzymes involved in redox catalysis. Each cluster can reversibly take up one electron. In sulfate-reducing bacteria, ferredoxins have been found to contain either one [4Fe-4S] cluster, two [4Fe-4S] clusters, one [3Fe-4S] cluster, or one [4Fe-4S] cluster plus one [3Fe-4S] cluster [1, 2].

Some ferredoxins are known to undergo cluster interconversion. *Azotobacter chroococcum* contains a 7Fe ferredoxin. In the reduced state, the $[3Fe\text{-}4S]^{(0)}$ cluster has a high affinity for Fe(II), leading to uptake of a ferrous iron atom. A $[4Fe\text{-}4S]^{(2+)}$ cluster is formed, which can subsequently be reduced to a $[4Fe\text{-}4S]^{(1+)}$ cluster [3]. It was believed that uptake of iron(II) only occurs when cellular nucleic acid is bound [4]. In *Azotobacter vinelandii* strains that lacked the gene encoding for ferredoxin I, an 18-kDa acidic protein of unknown function was found to be overexpressed [5]. It was proposed that ferredoxin I acts as a repressor of this 18-kDa protein [4]. No evidence has yet been provided to support this hypothesis. In mammals,iron homeostasis is controlled by an Fe-S protein, the so-called iron-responsive-element-binding protein. Interconversion between a [3Fe-4S] and a [4Fe-4S] cluster of this protein is proposed to control protein expression at the level of mRNA [6, 7]. Based on these facts, Thomson [4] hypothesized that ferredoxins may represent a class of DNA-binding proteins that play a role in gene regulation in prokaryotes.

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Abbreviation. NHE, normal hydrogen electrode.

Ferredoxin from *D. vulgaris* strain Hildenborough was isolated by Akagi in 1967 [8]. A role in electron transfer between periplasmic hydrogenase and cytoplasmic pyruvate dehydrogenase was proposed.

In this paper, we describe the isolation and characterization of *D. vulgaris* ferredoxin which is tightly bound to nucleic acid. A possible role of this protein is discussed.

MATERIALS AND METHODS

Purification. *D. vulgaris* strain Hildenborough (NCIB 8303) was grown in our home-built 300-l fermentor in Saunders' medium [9] at 37°C under a nitrogen atmosphere. At the end of the exponential growth phase, the cell suspension was concentrated to a volume of 30 l. The cells were harvested by continuous centrifugation (Sharpless laboratory Super-centrifuge); the yield was typically 150 g wet mass. All further steps were carried out in air. The cell paste was suspended in 3 vol. 20 mM Tris/HCl, pH 8.0 (standard buffer) plus 5 mM $MgCl_2$, 2 mg DNase I and 2 mg RNase A. Cells were disrupted by passage through a chilled Manton-Gaulin press (84 MPa). A cell-free extract was obtained as the supernatant after a 1-h spin at 100000×g. All further steps were carried out at 4°C. The extract was diluted to the appropriate ionic strength (0.1 M NaCl), and subsequently applied onto a DEAE anion-exchange column (5 cm×10 cm; Pharmacia). A 2-l linear gradient of 0–0.5 M NaCl in standard buffer was applied. Proteins were routinely monitored by the absorbance at 280 nm. Ferredoxin eluted shortly after desulfoviridin at approximately 0.25 M NaCl. All fractions from 0.25 M NaCl onward were concentrated over an Amicon YM-100 filter, allowing smaller proteins to pass

through the filter whereas larger proteins (e.g. desulfoviridin) were retained. The filtrate was concentrated over an Amicon YM-3 filter to a volume of 2 ml and applied to a Sephadex-75 molecular sieve column (2.5 cm×80 cm; Pharmacia). The column was eluted with standard buffer plus 0.15 M NaCl. The last purification step was by Mono-Q chromatography (Pharmacia). A 30-ml linear gradient of 0−1 M NaCl in standard buffer was applied. Nucleic-acid-free ferredoxin eluted at 0.25 M NaCl, while the nucleic-acid-bound fraction eluted at 0.4 M NaCl. The ferredoxin was concentrated over an Amicon YM-3.

N-terminal sequencing. 100-pmol quantities of both nucleic-acid-bound and nucleic-acid-free ferredoxin were freeze dried. Gas-phase sequencing of these samples was carried out at the sequencing facility of the Netherlands Foundation for Chemical Research (SON; Dr R. Amons, University of Leiden, The Netherlands).

Cluster destruction. Destruction of iron-sulfur clusters was performed by oxidation with 1 mM ferricyanide in 20 mM Tris/HCl, pH 8.0, in the presence of oxygen for 30 min, at 30°C. After incubation, the protein was loaded onto a Mono-Q column, and eluted with a linear gradient of 0−1 M NaCl in the same buffer. The efficiency of the destruction was monitored by visible spectroscopy. After elution from the column, the protein did not absorb at 400 nm, indicating that the protein was devoid of Fe-S clusters.

Isolation of ferredoxin-bound nucleic acid. Ferredoxin-bound nucleic acid was isolated by extraction of the purified protein with phenol and with chloroform/isoamyl (24:1) [10]. The nucleic acid was precipitated overnight with 2 vol. ethanol in the presence of 0.3 M NaAc, pH 5.2, collected by centrifugation (10 min, 10000×*g*) and dissolved in 150 µl RNase-free water. For removal of 5′ terminal phosphate groups, approximately 30 µg nucleic acid was incubated for 1 h at 50°C with 5 U calf intestine alkaline phosphatase (Boehringer) in the buffer obtained from the supplier. After addition of EDTA (to 10 mM) and SDS (to 0.2%, mass/vol.), the enzyme was inactivated by incubation for 15 min at 65°C and removed by extraction with phenol (twice) and chloroform/isoamylalcohol. After overnight precipitation with ethanol, the nucleic acid was collected by centrifugation and dissolved in 20 µl sterile water.

Isolation of total DNA and RNA. Total RNA from *D. vulgaris* was isolated as described previously [11]. After removal of traces of DNA with RNase-free DNase I (in 10 mM Tris/HCl, pH 8.0, plus 5 mM $MgCl_2$), the RNA was sonicated (3×10 s, amplitude 22 microns) to a rather uniform fragment size of approximately 150 nucleotides. After extraction with phenol and precipitation with ethanol, the RNA was dephosphorylated with calf intestine phosphatase as described above.

Total DNA was isolated as described previously [12].

Labeling of the nucleic acid. Dephosphorylated nucleic acids (fragmented total RNA and nucleic acid isolated from ferredoxin) were made radioactive by transfer of the labeled phosphate of [γ-^{32}P]ATP (3000 Ci/mol, 10 µCi/ml, Amersham) with T4 polynucleotide kinase (Gibco/BRL). Highly radioactive nucleotide acid for hybridization was made in a 20-µl incubation, containing 250 ng dephosphorylated nucleic acid isolated from ferredoxin (approximately 30 pmol), 17 pmol [γ-^{32}P]ATP (50 µCi), 10 U T4 polynucleotide kinase in 50 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, for 1 h at 37°C. The reaction was stopped by addition of EDTA, followed by heat inactivation of the enzyme (10 min, 65°C). Similar conditions were used to radiolabel fragmented total *D. vulgaris* RNA.

For electrophoretic analysis, the nucleic acid was labeled with only 0.8 pmol (2.5 µCi) [γ-^{32}P]ATP and 1 U kinase. After 1 h at 37°C, the incubation mix was split into three portions. One portion received 1 µg RNase A (Sigma; made DNase-free by boiling for 10 min in 50 mM NaAc, pH 5.2), the second received 20 U DNase I (Boehringer, RNase-free); no addition was made to the third portion. After further incubation (20 min, 37°C) the reactions were stopped by addition of an equal volume of loading buffer containing 10 mM EDTA in formamide.

Size determination of the nucleic acid. The ^{32}P-labeled nucleic acid samples (untreated and digested with RNase A or DNase I) were size fractionated in a 40-cm long 15% polyacrylamide gel containing 8 M urea [10], alongside synthetic oligodeoxynucleotides of defined size (17, 22, 23, 31 and 36 residues) and a synthetic sequence ladder (10−36 residues). Oligonucleotides were also made radioactive by end labeling.

Hybridization of the nucleic acid with *D. vulgaris* DNA. *D. vulgaris* genomic DNA was digested with several restriction enzymes, size fractionated in a 0.7% (mass/vol.) agarose gel, denaturated and transferred to a nitrocellulose membrane [13]. After baking the membrane (2 h, 80°C), excess binding sites were saturated by incubation in 100 ml 1% blocking reagent (Boehringer) in 0.1 M Tris/HCl, 0.15 M NaCl, pH 7.5 (2 h, 42°C), followed by overnight incubation in 100 ml 5×0.15 M NaCl, 0.015 M sodium citrate (NaCl/Cit), 0.1% SDS, 5×Denhardt's solution and 100 µg/ml sheared, heat-denaturated herring sperm DNA. The blot was hybridized overnight with 125 ng highly radioactive end-labeled nucleic acid in 5 ml of the latter solution (but without the herring sperm DNA) in a hybridization stove at 30°C. Subsequently, the blot was washed with 2×NaCl/Cit, 0.1% SDS at 35, 43, 50 and 55°C (2×100 ml, 2×30 min at each temperature) with intermittent exposure to X-ray film.

The same blot was re-probed with radioactive total *D. vulgaris* RNA. In this case, hybridization was at 40°C, and the wash steps at 45°C and 53°C.

Analytical determinations. SDS/polyacrylamide electrophoresis was performed with a midget system (Pharmacia) holding SDS-containing polyacrylamide gels (8×5×0.75 cm) in tricine buffer [14]. The composition of the stacking gel was 4% acrylamide and 0.1% bisacrylamide; the running gel was 16.5% acrylamide and 3% bisacrylamide. Isoelectric focussing was performed on Phast System (Pharmacia) holding a PhastGel IEF 3−9 focussing gel. Both gels were stained with Coomassie blue [15].

The native molecular mass of nucleic-acid-free ferredoxin was determined with a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 8.0, plus 0.15 M NaCl, at a flow rate of 0.5 ml/min. Ovalbumin (43 kDa), carbonic anhydrase (30 kDa), cytochrome c_3 (14.1 kDa), cytochrome c_{553} (9.2 kDa) and vitamin B_{12} (1.36 kDa) were used as markers for the calibration. Dextran blue was used to determine the void volume.

Spectroscopy. Ultraviolet/visible spectra were recorded with a DW-2000 spectrometer (Aminco). EPR spectroscopy was carried out with a Bruker EPR 200 D spectrometer using peripheral equipment and data handling as described previously [16]. The modulation frequency was 100 kHz. Since only a very small amount of nucleotide-free ferredoxin could be obtained, all EPR spectra were performed with nucleotide-bound ferredoxin.

Cyclic voltammetry. Cyclic voltammograms were recorded with a BAS CV27 potentiostat (Bioanalytical systems) connected to a Philips PM 8043 x-y-t recorder (Philips). The potential axis was calibrated with a Fluka 8022-A digital multimeter. The electrochemical experiments were performed with a three-electrode microcell using the method described previously [17]. The working electrode was a nitric-acid-activated glassy carbon disc (Le Carbon Loraine); as the counter electrode, the P1312 micro platinum electrode (Radiometer) was used. The potential was measured with reference to a K-401 saturated calomel electrode (Radiometer). All reported potentials have been recal-

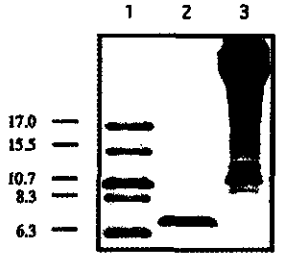

Fig. 1. Tricine-buffered SDS/polyacrylamide gel with *D. vulgaris* ferredoxin. Lane 1, molecular-marker mixture; lane 2, purified ferredoxin; lane 3, cell-free extract.

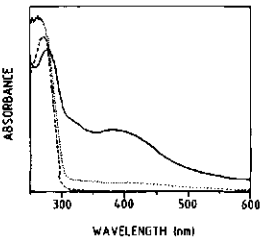

Fig. 2. Ultraviolet/visible absorption spectra of isolated *D. vulgaris* ferredoxin. Dotted line, after G-75 gel filtration; broken line, after cluster destruction; solid line, RNA-free ferredoxin.

culated with respect to the normal hydrogen electrode(NHE). During the experiments, the temperature was kept constant by immersing the electrochemical cell in a thermostated waterbath. The same setup was used for measurement of the temperature dependence. Typically, 20 µl ferredoxin ($A_{400} \approx 2$) was used, in 20 mM Hepes, pH 7.0, containing 1 mM neomycin.

RESULTS

***D. vulgaris* ferredoxin binds tightly to nucleic acid.** During isolation procedures for other cytoplasmic proteins from *D. vulgaris* (Hildenborough), it was noticed that a yellowish fraction eluted from the first anion-exchange column at high ionic strength. Moreover, this protein absorbed strongly at 260 nm. When the protein was further purified over an Amicon YM 100 filter and a Sephadex G-75 molecular sieve, an apparent homogeneous preparation was obtained. The isolated protein appeared as a single band that moved with a mobility corresponding to a molecular mass of 7.5 kDa (Fig. 1, lane 2). On a Superose 12 column, the native protein ran with an apparent molecular mass of 15.8 kDa (not shown), suggesting that the protein is a homodimer of subunits, each of 7.5 kDa. On an isoelectric focussing gel, the protein moved to a pH of 3.9 (not shown). These data are in agreement with those for ferredoxins, we therefore conclude that the isolated protein is a ferredoxin. A batch of 150 g wet cell mass yielded about 1 mg protein. It is not certain whether 1 mg corresponds to all ferredoxin present in the cell. However, SDS/PAGE of the cell-free extract (Fig. 1, lane 3) shows no protein with an apparent molecular mass of 7.5 kDa, indicating that the protein is expressed in small amounts in the cell.

Akagi [8] reports the association of absorbance at 260 nm with *D. vulgaris* ferredoxin. He used an alumina column to successfully remove this material. We were unable to reproduce this latter result. However, application of the ferredoxin to a Mono-Q column resulted in the separation of nucleic-acid-bound and the nucleic-acid-free ferredoxin. The majority of the ferredoxin eluted at 0.4 M NaCl as nucleic-acid-bound protein ($A_{400}/A_{280} \approx 0.02$), while a minor fraction eluted at ≈ 0.3 M NaCl as nucleic-acid-free protein ($A_{400}/A_{280} \approx 0.44$). In this way, typically ≈ 100 µg of nucleic-acid-free protein could be obtained, although there is a variation in the yield; some isolations yielded essentially nucleic-acid-bound ferredoxin. When the nucleic-acid-bound ferredoxin was rerun on a Mono-Q column, the nucleic acid could be partially removed. After repeated chromatography (i.e. six or seven times), the initial A_{400}/A_{280} ratio of 0.02 could be increased tenfold to 0.2. The amount of protein at this stage was hardly detectable by ultraviolet/visible spectroscopy. Apparently, part of the ferredoxin exists in the bacterial cell as nucleic-acid-free protein, which can be isolated on Mono-Q. The remaining ferredoxin is strongly associated with nucleic acid, and it can only be partly dissociated from the nucleic acid.

The nucleic-acid-bound ferredoxin eluting from the G-75 gel-filtration column (Fig. 2, dotted line) exhibits a broad peak at 400 nm, typical for Fe-S proteins, but also a strong absorption at 260 nm is observed, indicative of nucleotide binding. The A_{260}/A_{280} ratio of this sample is greater than one. For RNA and DNA, this ratio is 1.8–2.0, whereas for proteins it is less than one. The spectrum of the nucleic-acid-free ferredoxin (Fig. 2, solid line) lacks the strong absorbance at 260 nm. The A_{260}/A_{280} ratio for this preparation is smaller than one and the A_{400}/A_{280} ratio is 0.44, which is somewhat lower than the value of 0.63 reported by Akagi [8].

Several attempts were made to determine whether or not the bound nucleic acid could be removed. Treatment with RNase, DNase or Benzon Nuclease (Merck) followed by chromatography did not lead to dissociation of the nucleic acid, nor did heating of the protein to 70°C followed by digestion with nucleases and chromatography. Rechromatography on Sephadex-75 or hydroxyapatite could not remove the nucleic acid. Repeated rechromatography on Mono-Q afforded only partially nucleic-acid-free protein. Chromatography under reducing conditions, e.g. using buffers containing 2 mM dithionite, did not remove the bound nucleotides; this was checked by Mono-Q and hydroxyapatite chromatographies. Adding Fe(II) to the buffers did not lead to dissociation of the nucleic acid. Destruction of the Fe-S cluster (by ferricyanide treatment in the presence of oxygen) removed the absorption at 260 nm, indicating that an intact cluster is required for nucleotide binding (Fig. 2, broken line).

Attemps to sequence the N-terminus of the ferredoxin failed. It appeared that the protein chains of both nucleic-acid free and nucleic-acid-bound ferredoxin were blocked.

Characterization of the ferredoxin-bound nucleic acid. To study the nature of the nucleic-acid-bound to the ferredoxin, the nucleic acid was isolated with phenol extraction and labeled with [γ-^{32}P] ATP. It should be emphasized that the ferredoxin had been treated previously with DNase I and RNase A; therefore, the isolated nucleic acid represents fragments that are protected from digestion by the ferredoxin. The size of these protected, labeled nucleic acid fragments was estimated on a denaturating polyacrylamide gel. The majority had a size of 9–17 nucleotides (Fig. 3). After incubation with DNase I, the integrity of the nucleic acid fragments is maintained, however, they are completely digested with RNase A, confirming the nature of the nucleic acid as RNA (Fig. 3).

To further investigate the nature of the ferredoxin-bound RNA (for example, to establish if it originates from a specific messenger) the extracted, radiolabeled RNA was used as a probe

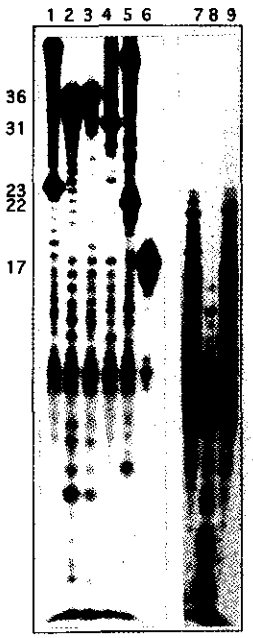

Fig. 3. Size fractionation of nucleic acid fragments extracted from ferredoxin. Lanes 1–6, oligomer markers (23, 36, 36, 31, 22, 17 nucleotides, respectively); lanes 7–9, isolated ferredoxin-bound nucleotides; lane 7, no addition; lane 8, with RNase A; lane 9, with DNase I.

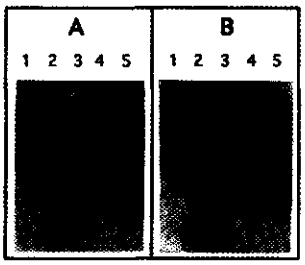

Fig. 4. Southern blot of restricted total *D. vulgaris* genomic DNA. (A) Hybridized with extracted, [γ-^{32}P]ATP labeled RNA from ferredoxin; (B) hybridized with [γ-^{32}P]ATP labeled isolated total *D. vulgaris* RNA. Lane 1, *D. vulgaris* DNA + *Eco*RI; lane 2, *D. vulgaris* + *Pst*I; lane 3, *D. vulgaris* + *Sst*II; lane 4, *D. vulgaris* + *Kpn*I; lane 5, *D. vulgaris* + *Sma*I.

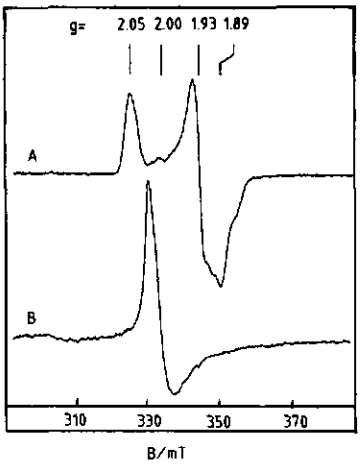

Fig. 5. EPR spectrum of *D. vulgaris* RNA-bound ferredoxin. Trace A, reduced ferredoxin; trace B, as isolated ferredoxin. EPR conditions: microwave frequency, trace A, 9.308 GHz; trace B, 9.309 GHz; microwave power, trace A, 0.32 mW; trace B, 51 mW; modulation amplitude, 2.0 mT; temperature, 17 K.

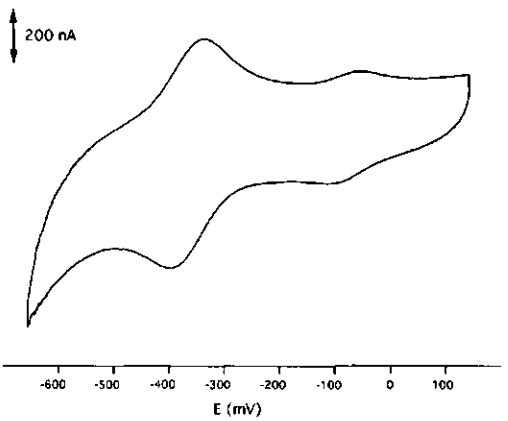

Fig. 6. Cyclic voltammetry at glassy carbon of RNA-free *D. vulgaris* ferredoxin. The protein was 0.5 mg/ml in 20 mM Hepes, pH 7.0, in the presence of 1 mM of the promotor neomycin. The potential scan rate was 5 mV/s. Both [4Fe-4S] and [3Fe-4S] transitions are seen; midpoint potentials are −360 mV and −80 mV, respectively, relative to the NHE.

for hybridization with Southern blots of digested *D. vulgaris* genomic DNA. Hybridization was under conditions of low stringency, followed by washing the blot at successively increasing stringency with intermittent exposure to X-ray film. As a control, the same DNA blot was reprobed with radiolabeled total *D. vulgaris* RNA. 5–7 DNA fragments appeared to hybridize in the different digests with the labeled ferredoxin-extracted RNA (Fig. 4). The hybridization pattern was identical when total *D. vulgaris* RNA was used as a labeled probe for hybridization. With total *D. vulgaris* RNA, the hybridization is kinetically driven by the most abundant RNA species, i.e. ribosomal RNA, which composes >90% of the total RNA. As the hybridization patterns obtained with the ferredoxin-bound RNA and total *D. vulgaris* RNA are similar, it appears that the ferredoxin-bound RNA is not part of a specific messenger; in this case, a specific hybridizing DNA fragment would be expected that is not found after hybridization with total *D. vulgaris* RNA. It, therefore, appears that ferredoxin binds either randomly to total RNA or specifically to rRNA.

EPR spectroscopy. The EPR spectrum of isolated *D. vulgaris* ferredoxin consists of a near isotropic signal around $g = 2.00$, typical for a [3Fe-4S] cluster (Fig. 5B). When the sample is reduced with dithionite, a rhombic spectrum appears with g values of 2.05, 1.93 and 1.89 (Fig. 5A). This spectrum arises most probably from a [4Fe-4S] cluster. [3Fe-4S] cluster signals are

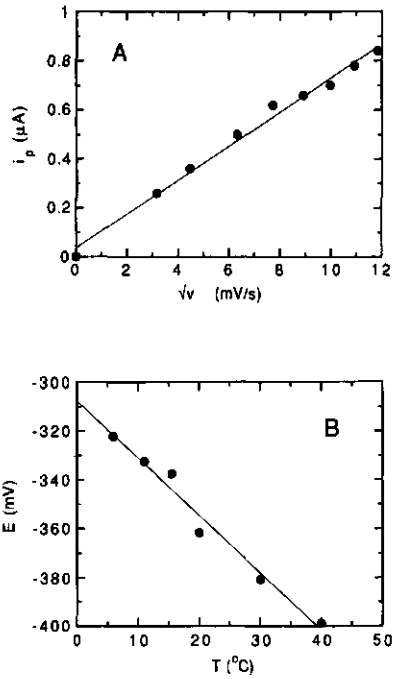

Fig. 7. Scan rate dependence of the anodic peak current (A) and (B) temperature dependence of the midpoint potential of the [4Fe-4S] cluster at glassy carbon. See text for details.

frequently seen in EPR spectra of oxidized Fe-S proteins, and although [3Fe-4S] clusters are reported to form a constituent part of certain ferredoxins (e.g. in *D. gigas* ferredoxin II [18], and in ferredoxin III from *Desulfovibrio africanus* [19]), these signals do not necessarily point to the presence of such a cluster *in vivo*. The [3Fe-4S] cluster can be a degradation product. It is known that oxidative damage of a [4Fe-4S] cluster can lead to the formation of a [3Fe-4S] cluster [20]. When we compare the concentration of the [4Fe-4S] cluster to the concentration of the [3Fe-4S] cluster of four different ferredoxin samples, we find that the ratio [4Fe-4S]/[3Fe-4S] is not constant, being 24.6, 11.3, 12.3 and 4.5, respectively. It, therefore, seems likely that the [3Fe-4S] cluster is an artifact, due to oxidative damage.

In *D. africanus* ferredoxin III and *A. chroococcum* ferredoxin, cluster interconversion is possible between [4Fe-4S] and [3Fe-4S] clusters [3, 21]. However, for the nucleic-acid-bound ferredoxin from *D. vulgaris*, the [3Fe-4S] cluster concentration did not increase upon anaerobic oxidation with ferricyanide. Therefore, *D. vulgaris* ferredoxin does not appear to undergo cluster interconversion.

Cyclic voltammetry. Cyclic voltammetry of *D. vulgaris* nucleic-acid-free ferredoxin at glassy carbon resulted in a voltammogram (Fig. 6). Two transitions are seen relative to the NHE, one at −80 mV, the other at −360 mV. It is obvious that the former transition is from a minor component. Since these potentials are typical for [3Fe-4S] and [4Fe-4S] clusters, respectively, and with the EPR spectra in mind, we can assign a midpoint potential $E_{m,7.0} = -360$ mV to the $[4Fe\text{-}4S]^{(2+;1+)}$ cluster and $E_{m,7.0} = -80$ mV to the $[3Fe\text{-}4S]^{(1+;0)}$ cluster. Interestingly, only ferredoxin free from RNA afforded a well-defined voltammogram; when nucleic acid was bound, no signal was detected.

The peak current is proportional to the square root of the potential scan rate (Fig. 7A), therefore, electron transfer is reversible and diffusion controlled. This conclusion is further borne out by the observations that the cathodic/anodic peak separation of both transitions is 60 mV at a potential scan rate of 5 mV/s and that the ratio of cathodic peak current/anodic peak current is near unity.

The slope of the curve (dE°/dT), obtained by a least squares fit, is -2.38 mV · K^{-1} (Fig. 7B). Given the standard entropy change $\Delta S^\circ = n \cdot F \cdot (dE/dT)$, and taking $n = 1$, an entropy change of -230 J · K^{-1} · mol^{-1} for the reduction is calculated. There is no kink in the slope of the plot of temperature versus potential, therefore, *D. vulgaris* ferredoxin does not appear to be subject to a temperature-induced conformational change.

DISCUSSION

Ferredoxins are small proteins containing one or two iron-sulfur clusters. They usually function in electron-transfer processes, e.g. in N_2 fixation, H_2 metabolism, respiration and photosynthesis. In this respect, they are comparable to other redox proteins such as cytochromes; these too are small and are expressed in large amounts. However, some ferredoxins may not fit this picture. Thomson [4] hypothesized that bacterial ferredoxins may be DNA-binding proteins involved in controlling gene expression.

We have now isolated a ferredoxin from the strict anaerobic, sulfate-reducing bacterium *D. vulgaris* (Hildenborough). An attempt was made to sequence both nucleic-acid-bound and nucleic-acid-free ferredoxin from *D. vulgaris* (Hildenborough). Knowledge of the N-terminal sequence could help classify this protein. Unfortunately, both protein chains were blocked. The ferredoxin was isolated in small amounts (≈ 1 mg); the absence of a 7.5-kDa band in the cell-free extract on a SDS/polyacrylamide gel indicates that this low yield is not an artifact, but that the protein is expressed in small amounts. If the protein played a role as a redox carrier, e.g. in the process of sulfate reduction, a higher abundance would be expected. There is a similarity between ferredoxin from *D. vulgaris* (Hildenborough) and ferredoxin II from *D. vulgaris* (Miyazaki). The latter ferredoxin is a dimer of subunits, each of 7.2 kDa, and it is isolated in a low yield [22]. These similarities suggest that the two proteins may have the same function. Akagi [8] proposed that *D. vulgaris* ferredoxin functions in electron transfer between periplasmic hydrogenase and cytoplasmic pyruvate dehydrogenase. The electrons released during pyruvate oxidation would be accepted by ferredoxin and subsequently transferred to cytochrome c_3. The latter would act as an electron donor for periplasmic hydrogenase [8]. However, this cannot be correct, since cytochrome c_3 is located in the periplasm [23] whereas ferredoxin is located in the cytoplasm.

The temperature dependence of the reduction potential is -2.38 mV · K^{-1}; the standard reaction entropy is -230 J · K^{-1} · mol^{-1}. These values are large when compared to those of other small redox proteins. For example, for the Rieske [2Fe-2S]-containing water-soluble fragment of bovine heart bc_1 complex, a standard reaction entropy of -155 JK^{-1} · mol^{-1} is calculated [24]. The temperature dependence of the reduction potential of the 2[4Fe-4S]-containing ferredoxin from *Methanosarcina barkeri* strain MS was -0.39 mV · K^{-1} below 18 °C, and -1.22 mV · K^{-1} above 18 °C [25]. These values correspond to standard reaction entropies of -38 J · K^{-1} · mol^{-1} and -118 J · K^{-1} · mol^{-1}, respectively. The standard reaction entropy of reduction reflects the flexibility of the protein. If the function of a protein were solely the transfer of electrons, then a rigid, redox-state-indepen-

dent structure would be beneficial. A high standard reaction entropy points to a large conformational change, which may possibly facilitate binding to other molecules, e.g. to nucleic acid. Alternatively, the *in vitro* redox transitions may have no physiological significance as, e.g. with aconitase, where the iron-sulfur cluster acts as a Lewis acid catalyst [26].

The isolated ferredoxin is strongly associated with nucleic acid. This has been suggested to be the case also for *A. chroococcum* ferredoxin, although no data have been presented to support this view [4]. Unfortunately, because of the tight binding to the nucleic acid of *D. vulgaris*, ferredoxin reconstitution experiments could not be carried out. The nucleic-acid-bound to the ferredoxin could be identified as RNA. To our knowledge, this is the first prokaryotic Fe-S protein known to interact solely with RNA. Specific binding to RNA would support the hypothesis of Thomson [4] that bacterial ferredoxins play a role in gene regulation. However, a control experiment with total RNA isolated from *D. vulgaris* indicated that the binding of the ferredoxin to RNA may not specific. Given the fact that >90% of all RNA in the cell consists of ribosomal RNA, an assumption can be made that the ferredoxin is bound to ribosomal RNA. We have made no attempts to sequence the RNA bound to the ferredoxin. Firstly, the amount of nucleic acid obtained is low. Secondly, the RNA extracted from the ferredoxin is most likely a mixture of fragments, since it hybridized with 5–7 DNA fragments.

In summary, two observations suggest that *D. vulgaris* ferredoxin may not be a redox protein; the protein is expressed in low amounts, and it has an unusually high standard reaction entropy for the iron-sulfur cluster reduction. Moreover, the fact that the protein binds to RNA is an indication that it may have a regulatory function.

We thank M. F. J. M. Verhagen for help with electrochemistry, W. A. M. van den Berg for help with the work on RNA, and M. M. Bouwmans for the drawings. We thank Professor C. Veeger for his continuous interest and support.

This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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6. REDOX CHEMISTRY OF BIOLOGICAL TUNGSTEN: AN EPR STUDY OF THE ALDEHYDE OXIDOREDUCTASE FROM PYROCOCCUS FURIOSUS

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J. Biol. Inorg. Chem., in press

Abstract

Aldehyde:ferredoxin Oxido-Reductase (AOR) from the hyperthermophilic archaeon *Pyrococcus furiosus* is a homodimeric protein. Each subunit carries one [4Fe-4S] cubane and a novel tungsten cofactor containing two pterins. A single iron atom bridges between the subunits. AOR has previously been studied with EPR spectroscopy in an inactive form known as the Red Tungsten Protein (RTP): reduced RTP exhibits complex EPR interaction signals. We have now investigated the active enzyme AOR with EPR, and we have found an S = 1/2 plus S = 3/2 spin mixture from a non-interacting $[4Fe\text{-}4S]^{1+}$ cluster in the reduced enzyme. Oxidized AOR affords EPR signals typical for W(V) with g-values of 1.982, 1.953, and 1.885. The W(V) signals disappear at a reduction potential $E_{m,7.5}$ of +180 mV. This unexpectedly high-value indicates that the active-site redox chemistry is based on the pterin part of the cofactor.

1. Introduction

In recent years tungsten-containing enzymes have been purified from an unexpectedly wide variety of anaerobic microorganisms including the acetogenic bacteria *Clostridium thermoaceticum*[1] and *Clostridium formicoaceticum*[2], the sulfate reducing bacterium *Desulfovibrio gigas*[3], the methanogenic archaea *Methanobacterium wolfei*[4] and *Methanobacterium thermoautotrophicum*[5], and the hyperthermophilic archaea *Pyrococcus furiusus*[6] and *Thermococcus litoralis*[7]. All these enzymes are two-electron transferring oxidoreductases that catalyze one of two generic reactions, namely, carbon dioxide activation or aldehyde activation[8].

The enzyme Aldehyde:ferredoxin Oxido-Reductase (AOR) from *P. furiosus* was initially purified in an inactive form on the basis of its red color in the presence of dithionite, and it was given the trivial name Red Tungsten Protein (RTP)[9]. With the partially reduced protein an S = 3/2 EPR signal was found and tentatively ascribed to an Fe-S cluster. Upon full reduction a very complex signal appeared (center 'B') and this was assigned to the dipolar coupling of the S = 3/2 system with another system of unknown origin and spin. Later, the S = 3/2 signal was assigned to a [4Fe-4S] cluster and the second system was denoted as Fe_xS_y[8,10]. However, recently the crystal structure of *P. furiosus* homodimeric AOR was determined at 0.23 nm resolution, and the proteins was shown to contain only a single Fe-S cluster per subunit. The X-ray diffraction analysis also showed that a single, mononuclear metal ion, presumably iron, was bridged between the two identical subunits in an approximately tetrahedral site formed by a His and a Glu residue from each subunit[11].

The RTP was shown to be an inactive form of the AOR enzyme. The latter was obtained by rapid, anaerobic purification in the presence of dithiothreitol and glycerol. The two forms of the protein RTP and AOR were claimed to be both monomeric and to be indistinguishable in their EPR properties[6]. In view of the apparent discrepancy between these results (i.e. two different Fe-S clusters in a monomeric protein) and the X-ray crystal analysis (i.e. two identical Fe-S cluster and

an additional mononuclear site in a homodimeric protein) we have re-investigated the protein in redox titrations monitored with EPR spectroscopy.

2. Materials and methods

2.1 Growth, purification, and enzyme activity

P. furiosus (DSM 3638) was grown on maltose (5 g/l) as described[12]. Aldehyde: ferredoxin oxidoreductase was purified anaerobically according to a modification of the isolation method as described[6]. All steps were carried out at ambient temperature, and the isolation was completed out in two days. All buffers were thoroughly degassed prior to use and contained 10% glycerol, 2 mM sodium dithionite and 1 mM dithiothreitol (DTT). Frozen *P. furiosus* cells (typically 50 g wet cell mass) were suspended in 150 ml of 20 mM Tris/HCl, pH 8.0, containing 0.1 mg/ml each of lysozyme, DNase I and RNase A, and 2 mM $MgCl_2$. The suspension was stirred for 1h. The cells were broken in a French pressure cell by passing at 135 MPa. A cell-free extract was obtained as the supernatant after a 1-h spin at 18,000g. The extract was diluted threefold in 20 mM Tris pH 8.0 and was loaded onto a 100 ml Q-Sepharose Fast Flow anion-exchange column. After a 150 ml wash, a 1.5-l gradient was applied of 0-0.5 M NaCl in the same buffer. Fractions containing active AOR (eluting at ≈ 0.30 M NaCl) were pooled and diluted twofold in 5 mM potassium phosphate buffer, pH 7.5, and were subsequently loaded onto a hydroxylapatite column (25 ml) equilibrated with the same buffer. A 0.9l gradient was applied of 5-300 mM potassium phosphate, pH 7.5. AOR eluted at 50 mM potassium phosphate. Pooled fractions were concentrated to a total volume of 2 ml in an anaerobic glovebox (Miller Howe Ltd, Watlington, UK) using an Amicon YM 30 filter. The concentrated sample was run on a Superdex G-75 column (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 8.0. Active fractions were concentrated over Amicon YM 30 and stored in liquid nitrogen until use. Enzyme activity was measured as the crotonaldehyde oxidation according to Mukund and Adams[6] except that the temperature was 65°C.

2.2 EPR spectroscopy, redox titration.

EPR measurements and redox titrations were as described by Pierik *et al.*[13]. Oxidative redox titrations were done at ambient temperature in 50 mM Hepes, pH 7.5 in the presence of 10% glycerol. Computer simulations of W(V) spectra were based on the spin Hamiltonian $\underline{H} = ßB \cdot g \cdot S + S \cdot A \cdot I$ with S = 1/2, I = 1/2 (14.4% ^{183}W). The g- and A-tensors were assumed to be colinear and the hyperfine interaction was taken as a perturbation to the Zeeman interaction to second order[14]. The intensity was calculated as described by Aasa and Vänngård[15]. Approximate simulations of the S = 3/2 $[4Fe\text{-}4S]^{1+}$ spectra were made as effective S = 1/2 spectra broadened by g-strain[16] assuming full population of the $|\pm 1/2\rangle$ doublet at low temperatures.

2.3 Analytical procedures

Protein was determined using the microbiuret method[17]. SDS-polyacrylamide electrophoresis was performed on Phast System (Pharmacia) holding a PhastGel SDS 8-25%. Analytical gel filtration was performed with a Superdex 200 HR 10/30 column (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 8.0 containing 10% glycerol, 2 mM sodium dithionite, 1 mM DTT, and 0.15 M NaCl, using a flow rate of 0.5 ml/min. Glucose oxidase (152 kDa), human transferrin (74 kDa), bovine serum albumin (67 kDA), ovalbumin (43 kDa), myoglobin (17.6 kDa) and cytochrome c_3 (13 kDa) were used as the markers for the calibration. The void volume was determined with dextran blue.

3. Results

3.1 Purification and quarternary structure

The aldehyde: ferredoxin oxidoreductase from *Pyrococcus furiosus* was purified in three successive column chromatography steps in two days, resulting in a purification factor of 10, and a specific activity of 20 U/mg (at 65°C) in the crotonaldehyde assay. These values are comparable to those previously published, i.e. a purification factor of 11 and a specific activity of 54 U/mg (at 80 °C)[6]. No activity was detected when glyceraldehyde-3-phosphate was used as a substrate. The purified enzyme appeared as a single band on a SDS gel with a mobility corresponding to 80 kDa (not shown). Recently, the crystal structure of *P. furiosus* AOR was determined at 0.23 nm resolution, and in the crystal the enzyme was shown to be a dimer of two identical subunits of 66 kDa[11]. In a gel-filtration experiment on a Superdex 200 column the native enzyme ran with an apparent molecular mass of 110 kDa (not shown). This fits reasonably well with the expected 132 kDa of the homodimer. We conclude that the purified aldehyde-oxidoreductase is a homodimer also in solution.

3.2 EPR of reduced AOR

The EPR spectrum of active, fully reduced enzyme in the presence of 2 mM dithionite is shown in Fig. 1A. Peaks at g = 4.70 and 3.45 indicate the presence of an S = 3/2 species. These values can be fit to the standard spin Hamiltonian $H = g\beta B \cdot S + D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2)$ with the parameters S = 3/2, g = 2.05, E/D = 0.103. Also, a typical S = 1/2 spectrum is observed with g-values of 2.03, 1.93, and 1.89. Both spectra point to the presence of a $[4Fe\text{-}4S]^{1+}$ cluster, existing as a mixture of an S = 1/2 and an S = 3/2 ground state. No change in the EPR spectra was observed when the enzyme was incubated with 10 mM crotonaldehyde for 10 minutes at ambient temperature. When the enzyme was oxidized with ferricyanide these signals disappeared.

Mukund and Adams reported an additional EPR signal which they labeled center 'B'[9]. We found no trace of center 'B' in the spectra of our active enzyme preparations. However, Fig. 1B shows the EPR spectrum of inactive AOR, which was obtained as a side fraction during the purification of sulfhydrogenase[12]. The enzyme was monitored on the basis of its red color, and it was found to be inactive when assayed for crotonaldehyde oxidation.

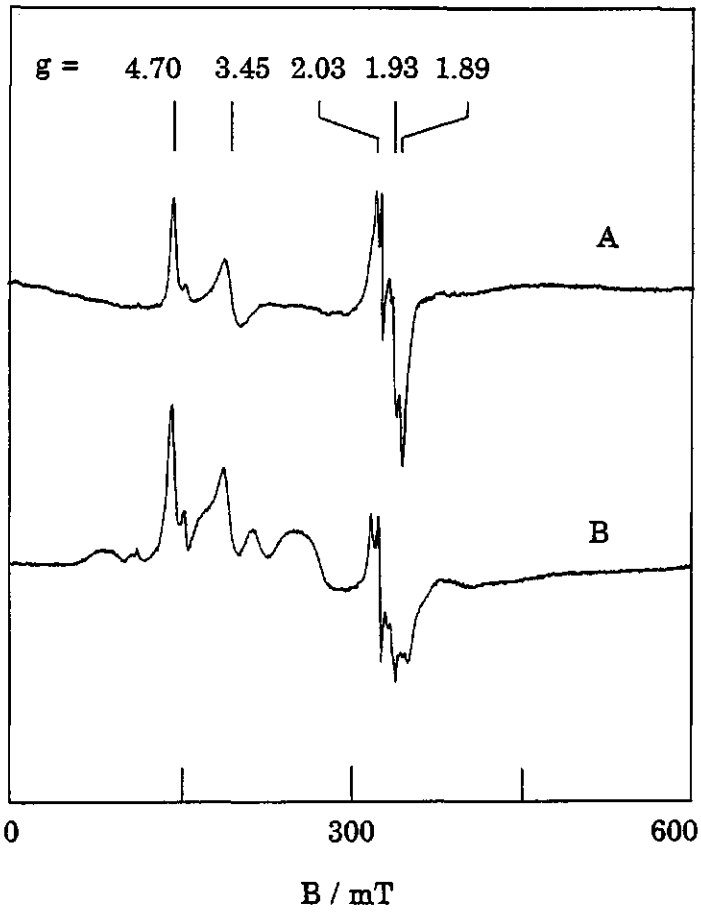

Figure 1. EPR of reduced *P. furiosus* aldehyde oxidoreductase. Trace A, active AOR (20 U/mg) in 20 mM Tris/HCl, pH 8.0 containing 2 mM dithionite, 1 mM DTT, and 10% glycerol. Trace B, inactive AOR, in 20 mM Tris/HCl pH 8.0 + 2 mM dithionite. EPR conditions: microwave frequency, 9.18 GHz; microwave power, 80 mW, modulation frequency, 100 kHz, modulation amplitude, 1.0 mT, temperature, 13 K.

3.3 EPR of oxidized AOR

AOR could be anaerobically, reversibly oxidized in the presence of redox mediators. Figure 2A shows the EPR spectrum of the enzyme poised at +328 mV. Signals are observed at g = 1.982, 1.953, and 1.885. The spectrum remained unchanged up to 50 K, and only a slight broadening was observed at 125 K, indicating slow spin-lattice relaxation. This observation, plus the fact that all g-values are below 2.002, points to a d^1 configuration. Therefore, these signals can be assigned to W(V).

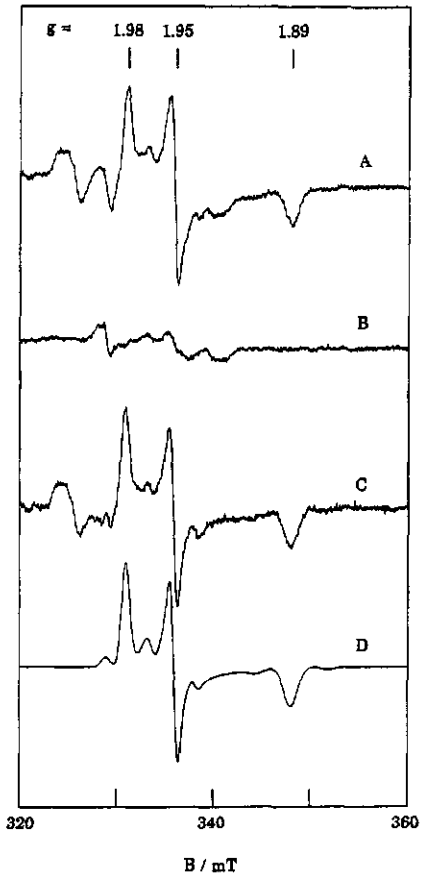

Figure 2. EPR of oxidized *P. furiosus* aldehyde oxidoreductase. Trace A, enzyme poised at +328 mV; Trace B, enzyme poised at +59 mV; Trace C, difference of A minus B; Trace D, simulation of spectrum C. The enzyme was in 50 mM Hepes, pH 7.5 containing 10% glycerol, 250 mM NaCl, and 40 µM of each mediator (see ref. [13] for details). EPR conditions: microwave frequency, 9.18 GHz; microwave power, 3.1 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; temperature, 41 K. Spectra A and B are the average of five scans. Simulation parameters for trace D: 51 x 51 orientations; g-values, 1.8855, 1.9533, 1.9820; line widths, 12, 6, 7 mT; tungsten hyperfine splittings, 7.5, 4.5, 4.2 mT; relative ^{183}W concentration, 14.4%.

The EPR spectrum in Fig. 2A exhibits an additional signalwhich is more clearly seen in Figure 2B where the enzyme is poised at +59 mV. The g = 1.982 spectrum has disappeared, and a second rhombic spectrum can be seen. This weak signal was found to be present in all preparations, and it is detected over a wide potential range, i.e. from -100 mV to at least +328 mV. When this rhombic spectrum is subtracted from the spectrum at high potential, a typical d^1-spectrum is obtained (Figure 2C). The spectrum could be best fitted using ^{183}W hyperfine splittings A_{xyz} of 7.7, 4.5, and 4.2 mT (Figure 2D). Simulation of both the spectrum of the unknown and that of the copper standard provides a simple, accurate means for double integration[18]. Thus, the intensity of the W(V) spectrum was determined to be 0.2 spin/monomer. With the EPR spectrum of the fully reduced enzyme the ratio of $[4Fe\text{-}4S]^{1+}$ (S = 3/2; 1/2) over W(V) was estimated to be 0.9. The S = 3/2 over S = 1/2 ratio of the cubane was found to be approximately 3 by simulation.

Upon addition of 10 mM crotonaldehyde (10 minutes incubation at ambient temperature) the W(V) signals completely vanished, indicating that the crotonaldehyde is able to reduce the W(V) to W(IV).

In a mediated, oxidative redox titration tungsten (V) signals became only detectable at potentials above 150 mV (Figure 3). The data points could be fitted to the Nernst equation assuming a one-electron transition. A reduction potential of +180 mV was determined. At higher potentials redox equilibrium was slow, and it usually took several minutes before the potential was sufficiently stable to draw an EPR sample. This unstability is reflected in a slight scatter of the data points.

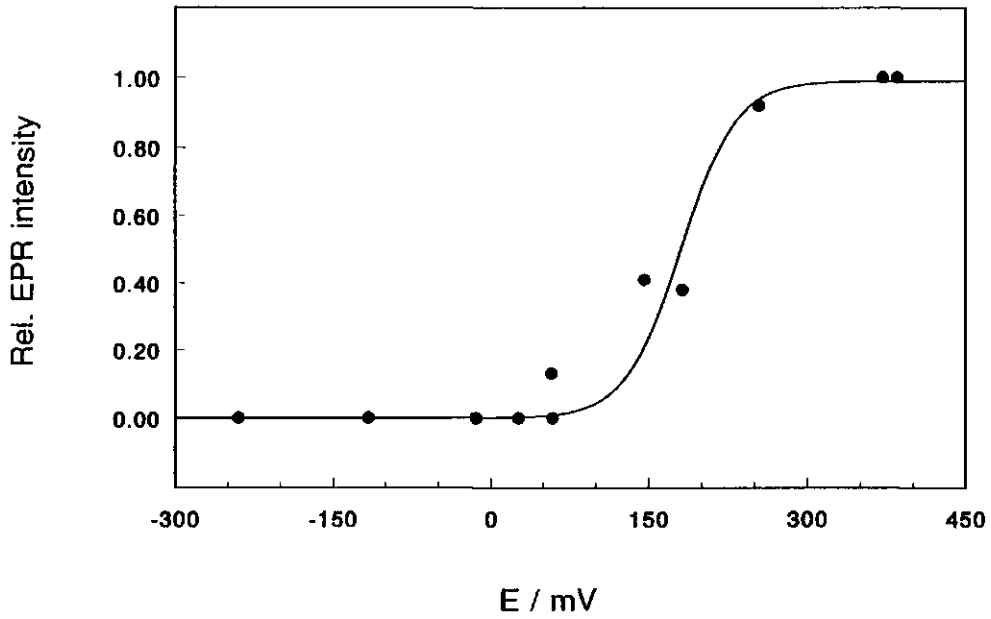

Figure 3. EPR redox titration of the tungstopterin. The relative intensities of the g = 1.953 signal are plotted versus the potential. The solid line represents a least-square fit to the Nernst equation with n=1.

4. Discussion

4.1 Reduced AOR contains a non-interacting [4Fe-4S] cluster

P. furiosus AOR was first purified in an inactive form, called RTP. When the enzyme was partially reduced an S = 3/2 spectrum was found. Upon full reduction a complex spectrum appeared (center 'B'). This was explained by dipolar coupling of the S = 3/2 system with another system of unknown origin[9]. Later, the S = 3/2 signal was assigned to a [4Fe-4S] cluster and the second system was denoted as Fe_xS_y[8,10]. *P. furiosus* RTP was found to be the inactive form of an aldehyde oxidoreductase (AOR), and the EPR spectra of the active form of the enzyme (AOR) were reported to be indistinguishable from the inactive form[6]. No EPR spectra of the active form were presented.

We have now found that the EPR spectrum of reduced, active AOR is from a single $[4Fe\text{-}4S]^{1+}$ cluster, observed as a spin mixture of two, non-interacting spectra, i.e. an S = 1/2 and an S = 3/2 spectrum. These findings are in agreement with the recently published crystal structure, which showed that the enzyme contains only one Fe-S cluster per monomer[11]. When the enzyme was isolated in an inactive form, we detected a complex EPR spectrum that was very similar to the center 'B' reported previously[9]. It is concluded that the complex interaction spectrum (center 'B') is an artifact caused by inactivation of the enzyme.

4.2 Oxidized AOR contains W(V)

P. furiosus aldehyde: ferredoxin oxidoreductase (AOR) has previously been shown to contain iron, acid labile sulfide, and a tungstopterin cofactor[9]. No tungsten EPR signals have been reported. In a mediated, oxidative titration we have now detected W(V) EPR signals. As expected for a d^1 system all g-values are less than 2.00. Previously, tungsten enzymes have been reported to exhibit g-values significantly greater than 2.00 possibly as a consequence of covalency, e.g. 2.10 for *Clostridium thermoaceticum* formate dehydrogenase[19], or 2.05 for the tungsten substituted molybdenum formylmethanofuran dehydrogenase from *Methanobacterium wolfei*[20]. *P. furiosus* AOR and the tungsten-containing aldehyde oxidoreductase from *Desulfovibrio gigas*[3] exhibit a W-EPR spectrum with g-values all less than 2.00.

In addition to the W(V) signal a weak, second rhombic signal was detected in *P. furiosus* AOR. This signal (Fig. 2B) is detected over a potential range from -100 mV to at least +328 mV. The nature of the weak signal is not clear; it may arise from another W(V) species, e.g. due to inactivation.

The W(V) EPR spectrum of *P. furiosus* AOR can be simulated using ^{183}W hyperfine splittings A_{xyz} of 7.5, 4.5, and 4.2 mT. This is the second enzyme for which ^{183}W hyperfine splittings are reported. For the tungsten-substituted formylmethanofuran dehydrogenase from *Methanobacterium wolfei* hyperfine splittings A_{xyz} of 5.0, 4.6, and 3.1 mT have been reported[20], which are in the same range as the values for the native *P. furiosus* enzyme. Similar values have been

reported for some tungsten thiolate compounds[21], suggesting that these ^{183}W hyperfine splittings may be common in thiolate-coordinated tungsten complexes or enzymes.

We have demonstrated that oxidized *P. furiosus* AOR contains W(V); its EPR spectrum appears with an apparent reduction potential $E_{m,7.5}$ of +180 mV. This implies that reduced AOR contains W(IV). However, George *et al.* concluded from EXAFS data that reduced *P. furiosus* AOR contains W(VI), and that the W(V)/W(VI) reduction potential should be less than -500 mV[22]. These apparently mutually inconsistent observations may have a bearing on studies on Mo model compounds which showed that an overall one-electron oxidation of a formally Mo(VI)=S complex can be coupled to two-electron oxidation of the sulfur ligands to give a Mo(V) S_2^{2-} complex[23]. While this type of reaction could also apply to tungsten, neither the X-ray analysis[11] nor EXAFS data[22] give any indication for the presence of either a W=S group or a tungsten disulfide. However, we cannot rule out the possibility of internal electron transfer by some other, not yet understood mechanism. Nevertheless, whether or not induced internal electron transfer takes place, the unexpectedly high oxidation potential fot the appearance of the W(V) in *P. furiosus* AOR (+180 mV) suggests that the biologically relevant redox chemistry of the enzyme in the strictly anaerobic archaeon may well take place on the pterin ring system rather than on the tungsten atom.

Acknowledgements: We thank two reviewers for pointing out the possibility of induced internal electron transfer. We thank P.Th.M. Veenhuizen for help with the initial purification of AOR. This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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7. CRYSTALLIZATION AND PRELIMINARY CRYSTALLOGRAPHIC ANALYSIS OF THE PUTATIVE [6Fe-6S] PRISMANE PROTEIN FROM *DESULFOVIBRIO VULGARIS* (HILDENBOROUGH)

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Acta Crystallografica, in press.

ABSTRACT

Crystals of the prismane protein from *Desulfovibrio vulgaris* (Hildenborough) containing a putative [6Fe-6S] cluster have been obtained and X-ray data collected to a resolution of 1.7 Å using synchrotron radiation. The unit cell is orthorhombic with $\underline{a}$ = 63.87 Å, $\underline{b}$ = 65.01 Å, $\underline{c}$ = 153.49 Å, space group $P2_12_12_1$ (No. 19). The unit cell will readily accomodate four molecules of molecular mass 60 kDa with a corresponding solvent content of approximately 48%.

INTRODUCTION

Iron-sulfur proteins are found in a wide variety of organisms and are usually involved in electron transfer processes. These proteins contain one or more iron-sulfur centers, consisting of iron, inorganic sulfur and/or sulfur atoms from cysteine residues organized in a cluster. In size they can vary from around 50 residues, rubredoxin[1], to several hundred in for example nitrogenase[2-5] and the redox potentials also show a wide variation[6,7]. Rubredoxin represents the simplest type of cluster with an iron surrounded by four cysteine sulfurs, the plant ferredoxins have two-iron clusters [2Fe-2S] with an additional two cysteine ligands, whereas the larger clusters are comprised of three or four iron atoms and four sulfur atoms arranged in the shape of a cube and there are often more than one cluster per molecule. Even more clusters are found in enzymes such as nitrogenase, the central enzyme in nitrogen fixation. A novel iron-sulfur cluster is supposed to be present in the active site of Fe-only hydrogenases, possibly a [6Fe-6S] cluster[8]. Larger clusters are proposed to be also present in dissimilatory sulfite reductase[9,10] and carbon monoxide dehydrogenase[11]. A useful summary of the field of iron-sulfur proteins is that by Johnson[12].

Hagen, Pierik and Veeger[13] reported an unusual iron-sulfur protein isolated from *Desulfovibrio vulgaris* (Hildenborough) and suggested a putative [6Fe-6S] cluster on the basis of EPR measurements and comparison with synthetic clusters of the type $[Fe_6S_6(L)_6)^{3-}$ where L = Cl^-, Br^-, I^-, RS^- and RO^-. It was noted that the spin concentration for the protein as isolated was substoichiometric and sample dependent and this, together with a later study[14], suggested that the cluster can exist in four different redox states, namely: $[6Fe\text{-}6S]^{6+}$ with all irons in the fully oxidized Fe(III) state and spin S = 0, the one electron reduced state $[6Fe\text{-}6S]^{5+}$ with a mixture of S = 1/2 (10%) and S = 9/2 (90%), the two electron reduced state $[6Fe\text{-}6S]^{4+}$ with S = 0 or integer, and the three electron reduced state $[6Fe\text{-}6S]^{3+}$ formally containing three Fe(III) and three Fe(II) ions and with spin S = 1/2. This work was followed by a comprehensive biochemical and biophysical characterization[15] and a primary structure sequence determination by Stokkermans *et al.*[16]. The latter showed that the protein contained nine cysteine residues and that four of these, located towards the N-terminus, had a sequence C-2X-C-7X-C-5X-C which could be at least part of a cluster binding domain. Additional studies using EPR[17], multi-frequency EPR and Mössbauer spectroscopy[14] and low-temperature magnetic circular dichroic spectroscopy[18] have shown further evidence for a [6Fe-6] cluster. Recent Resonance Raman studies suggest the presence of a Fe-O-Fe structure, indicating a novel

functionality for this special cluster[19]. Crystallographic studies are now in progress at Daresbury Laboratory and the preliminary work is described herein.

EXPERIMENTAL

Bacterial growth and protein purification

Desulfovibrio vulgaris (Hildenborough) NCIB 8303 holding an overproducing plasmid (pJSP104)[17] putative prismane protein was grown anaerobically as described by van den Berg *et al.*[20]. The prismane protein was purified according to the procedure by Pierik *et al.*[15]. The protein was further purified by FPLC (Pharmacia) on a Q-Sepharose HiLoad column. The protein was finally dialyzed against 5 mM Tris (pH 8.0), 50 mM NaCl and concentrated to 24 mg/ml. Protein samples were then stored at 203 K prior to use.

Crystallization

The prismane protein crystallized over a range of pH, 5.9-8.0 and PEG 8000 concentrations of 18-24 %, using the sitting drop method. However, the best quality crystals were obtained using the following procedure; (i) 1.3 - 1.5 µl of protein (24 mg/ml in 5 mM Tris, pH 8.0, 50 mM NaCl) was diluted to 4 µl with 0.1 M MES (pH 5.9), 66 mM $MgAc_2$, (ii) 4 µl samples were set-up in Crischem plates and equilibrated against 600 µl of well solution (0.1 M MES at pH 5.9, 66 mM $MgCO_3$), and (iii) plates were incubated at 277 K. Crystals usually appeared within four days and grew to a maximum size of 0.7 mm within ten days as shown in Figure 1.

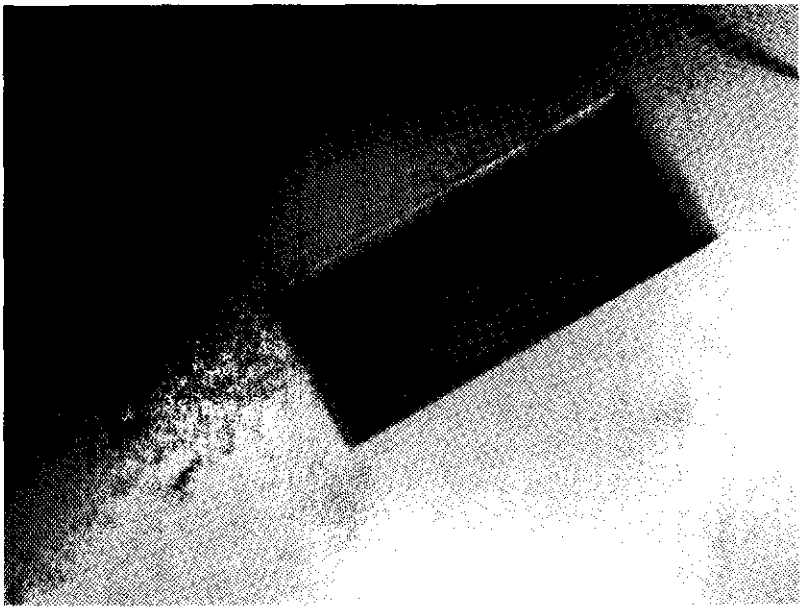


Figure 1. Crystals of the prismane protein used for X-ray data collection.

X-ray data were collected from one crystal mounted in thin-walled capillary containing a small volume of mother liquor on station 9.6 of the Synchrotron Radiation Source at CCLRC Daresbury Laboratory (2 GeV energy with average circulating current of 200 mA). This station derives its synchrotron radiation from a 3-pole wiggler magnet operating at 5 T and has a platinum coated cylindrically curved fused quartz mirror and a bent triangular Si(III) monochromator as optical elements. The wavelength selected was 0.87 Å and a distance of 265 nm was set between the sample and a 30 cm diameter Mar-Research image plate detector system. The sample was cooled to 277 K and a total of 87 one degree oscillation images were recorded with exposure times of 60 seconds per image. All the images were recorded using the MOSFLM suite of programs[21] and a final scaling and data reduction was achieved using ROTAVATA and AGROVATA from the CCP4 suite of programs (CCP4, 1994).

RESULTS AND DISCUSSION

Crystals of the prismane protein were shown to diffract to 1.5 Å or even higher, but in order to collect a complete data set at this higher resolution oscillation ranges considerably smaller than one degree would have had to be used and this was not possible in the synchrotron time available; it may well be the subject of further studies. The statistics of the data set collected to 1.7 Å are given in Table 1 and Figure 2 shows typical diffraction images. The native data set is clearly of high quality and a search for putative heavy atom derivatives indicates that *p*-hydroxymercurybenzoic acid may be one likely candidate. Further X-ray analysis is in progress.

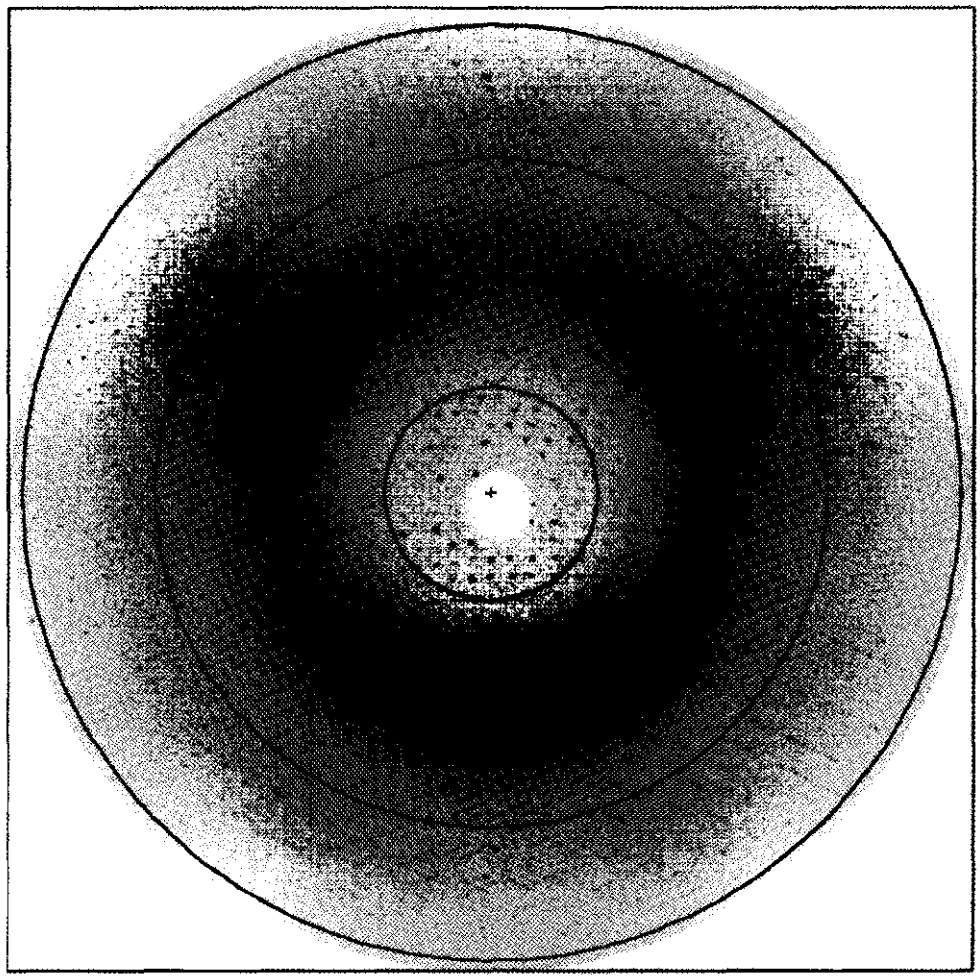
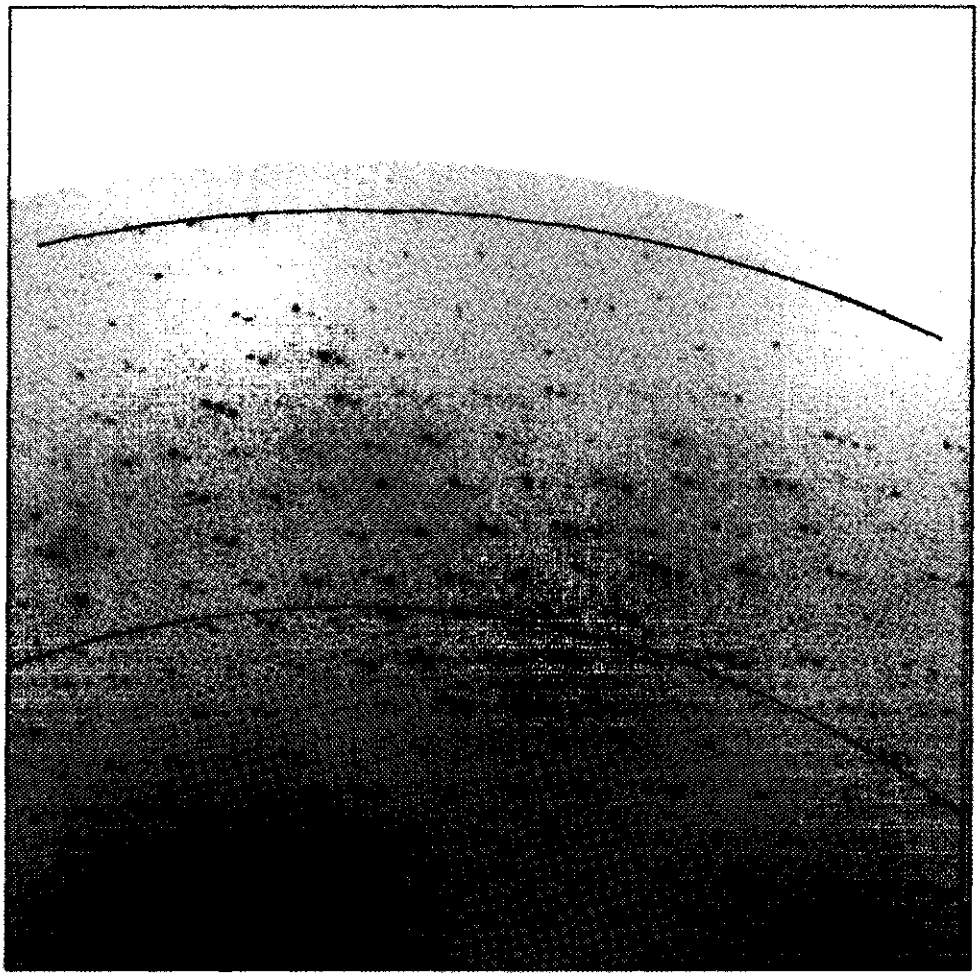


Figure 2. (a) A typical diffraction pattern of prismane recorded on station 9.6 at the SRS, Daresbury Laboratory; l = 0.87 Å, crystal to detector distance = 265 mm.



(b) An outer portion of a typical diffraction pattern showing that the resolution extends to at least 1.75 Å; diffraction data can be recorded to 1.5 Å or even higher.

Table 1. X-ray data of prismane protein at 1.7 Å resolution.

Resolution (Å)	R_{sym}	$I/\sigma(I)$	$N_{symmetry}$	N_{unique}	% possible collected	N_{total}	% with $I>3\sigma(I)$
6.61	0.031	9.0	3190	921	82.6	1111	96.5
4.68	0.025	22.9	7356	1929	95.0	2203	99.0
3.82	0.024	18.7	9432	2480	96.7	2848	99.0
3.31	0.026	18.9	11609	3023	98.9	3413	99.0
2.96	0.030	20.2	13011	3424	98.4	3826	98.2
2.70	0.034	15.7	14437	3841	98.9	4234	97.4
2.50	0.041	8.0	15869	4277	100.0	4646	97.0
2.34	0.045	8.7	16999	4587	99.4	4937	96.5
2.21	0.050	9.3	18130	4943	99.9	5264	95.5
2.09	0.057	11.2	19087	5257	100.0	5567	94.7
2.00	0.067	9.4	20046	5481	99.7	5822	92.5
1.91	0.081	8.7	20893	5725	99.8	6089	89.9
1.84	0.104	6.9	21805	5969	100.0	6350	87.0
1.77	0.126	5.7	22613	6206	99.7	6573	83.1
1.71	0.190	3.3	16550	4873	85.1	5791	70.2
	0.039		231027	62936	97.6	68674	91.5

(Average multiplicity = 3.4)

ACKNOWLEDGEMENTS

The authors would like to acknowledge UK inter-research council Biology Program Joint Steering Committee (MRC, BBSRC and EPSRC) which supports the structural biology program at the SRS, Daresbury Laboratory.

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8. SUMMARY

Iron sulfur (Fe-S) proteins are found in a variety of organisms. They usually function in electron transport, but they may also be involved in other functions like gene regulation and Lewis acid catalysis. The structure and spectroscopic properties of Fe-S clusters holding one, two, three, or four iron atoms is known to a great extent. These 'common' clusters share some basic properties. Firstly, they contain not more than four iron atoms. Secondly, despite the fact that they may contain more than one iron atom, they exist in two (physiological) redox states only. Thirdly, they are characterized by a low electron spin, i.e. they are usually S = 1/2. However, there is a strong indication that Fe-S clusters exist which do not obey these general rules. These clusters may hold more than four iron atoms, they are usually high-spin ($S \geq 3/2$), and they may exist in more than two redox states. Because of these properties these clusters are referred to as **superclusters**. When I started this research project, six potential systems were proposed to contain larger (≥4Fe) or uncommon (WFe3S4) Fe-S clusters. These enzymes are involved in (multi-electron) redox catalysis:

1. Nitrogenase
2. Fe-only hydrogenase
3. Dissimilatory sulfite reductase
4. carbonmonoxide dehydrogenase
5. Prismane protein
6. *Pyrococcus furiosus* aldehyde oxidoreductase

The aim of my thesis is to study physical, chemical, and biological properties of multi-electron transferring enzymes, in a quest for possible new structures and functions of biological Fe-S clusters.

Chapter 2 describes the purification and characterization of a dissimilatory sulfite reductase from *Desulfosarcina variabilis*. The enzyme belongs to the class of desulforubidins, as was deduced from its UV/vis absorption spectrum. It is a $a_2ß_2\gamma_2$ hexamer of ≈ 208 kDa, and it was found to contain ≈15 Fe and ≈19 S^{2-}. The oxidized enzyme exhibited S = 9/2 Fe-S EPR signals (g = 16). Similar signals have previously been found in *Desulfovibrio vulgaris* (Hildenborough) desulfoviridin by Pierik and Hagen, who suggested the presence of a larger Fe-S cluster. With the finding of similar very high spin signals also in *D. variabilis* desulforubidin, it appears that the presence of a S = 9/2 Fe-S (super) cluster is common in all dissimilatory sulfite reductases. The sirohemes in *D. variabilis* desulforubidin were found to be fully metalated, and none of the Fe-S EPR signals gave indication for dipolar and/or exchange coupling with siroheme. These observations are interpreted as supportive evidence against the previously proposed model of a bridged cubane/siroheme as the active site for dissimilatory sulfite reductases.

The extreme hyperthermophile *Pyrococcus furiosus* contains a NiFe hydrogenase which not only reversibly oxidizes hydrogen but also reduces elemental sulfur (S^0) to H_2S. The Archaeon was succesfully grown in a 200 l fermentor at 90°C

on potato starch, and the hydrogenase could be purified aerobically without loss of activity. In contrast to previous reported data the enzyme was found to contain 17 Fe, 17 S^{2-}, and 0.74 Ni. Three EPR signals were found; a near-axial (g = 2.02, 1.95, 1.92) S = 1/2 signal ($E_{m,7.5}$ = -303 mV) indicative of a $[2Fe\text{-}2S]^{(2+;1+)}$ cluster, a broad spectrum of unknown origin (g = 2.25, 1.89; $E_{m,7.5}$ = -310 mV), and a novel rhombic S = 1/2 EPR signal (g = 2.07, 1.93, 1.89) reminiscent of a $[4Fe\text{-}4S]^{(2+;1+)}$ cluster. This rhombic signal appears with a reduction potential of $E_{m,7.5}$ = -90 mV, and disappears at $E_{m,7.5}$ = -328 mV. The latter observation suggested that this cluster is capable of taking up two electrons, and, therefore, that it is a supercluster. However, it is hypothesized that the disappearance of the signals at low potential is caused by magnetic interaction of the rhombic g = 2.07 signal with a third paramagnet, resulting in a broad interaction signal. Hence, there is no indication for the presence of a supercluster in *P. furiosus* NiFe hydrogenase (chapter 3).

In the NiFe hydrogenases of several organisms as well as in the Fe-only hydrogenases of *Megasphaera eldenii* and *Desulfovibrio vulgaris* (Hildenborough) novel Fourier transform infrared (FTIR) detectable groups were found. The bands occur in the region of 2100-1800 cm^{-1}, which corresponds to stretching vibrations of polar triple bonds, metal hydrides, or asymetrically coupled vibrations of two adjacent double bonds. The position of these bands shifted upon oxidation and reduction of the enzymes. FTIR bands in this region were not detected in a large control group of Fe-S and/or nickel containing proteins including a metal-free hydrogenase. The FTIR groups in NiFe hydrogenases are assigned to the three unidentified small non-protein ligands that coordinate the Fe as observed in the X-ray structure of *Desulfovibrio gigas* NiFe hydrogenase. Thus far, the structural difference between NiFe- and Fe-only hydrogenases had been thought to reside in the absence or presence, respectively, of a novel Fe-S cluster (H-cluster) which is proposed to be the site of hydrogen activation. The finding of similar FTIR groups in both Fe-only and NiFe-hydrogenases might suggest that the hydrogen-activating site of both classes of hydrogenases encompasses of a bimetallic center involving a low spin Fe ion with FTIR-detectable groups.

During the purification of several proteins from *Desulfovibrio vulgaris* strain Hildenborough a yellowish fraction eluted from the first anion exchange column at high NaCl concentration. It was observed that this fraction absorbed strongly at 260 nm. Attempts were made to purify the protein. The protein turned out to be a ferredoxin, as concluded from its size (a homodimer of subunits, each of 7.5 kDa), its pI (3.9) and its EPR spectrum in the reduced state, indicating the presence of a $[4Fe\text{-}4S]^{(2+;1+)}$ cluster. The protein was associated with RNA having a typical size of 9-17 nucleotides. Hybridization experiments with extracted, radiolabeled RNA and digested *D. vulgaris* genomic DNA indicated that the ferredoxin binds either to total RNA or specifically to rRNA. The suggestion is made that *D. vulgaris* ferredoxin may not be a redox protein, but that it may have a regulatory function. This suggestion is supported by the unusually high standard reaction entropy of reduction of -230 $J \cdot K^{-1} \cdot mol^{-1}$. This would be the first prokaryotic Fe-S protein known to function in translation regulation.

In chapter 6 an EPR/redox study is presented on the tungsten-containing aldehyde oxidoreductase (AOR) from the hyperthermophile *Pyrococcus furiosus*. The

enzyme had previously been suggested to hold a [WFe3S4] cluster. Highly active AOR could be obtained by rapid, anaerobic purification (i.e. within two days). Only active enzyme was used for this study. The fully reduced enzyme exhibited a mixture of S = 1/2 and S = 3/2 Fe-S EPR signals. Oxidized AOR afforded signals typical for W^{5+} (g = 1.982, 1.953, 1.885). Shortly after this research project started the X-ray structure of *P. furiosus* AOR was elucidated by Chan *et al.*, who showed that the enzyme contains one [4Fe-4S] cluster and one tungsten cofactor per subunit. Our data are in agreement with the crystal structure, which excluded the possibility of a [WFe3S4] cluster. Such a cluster would have been a completely novel Fe-S cluster. The W^{5+} spectrum could be simulated using ^{183}W hyperfine splitting constants A_{xyz} of 7.7, 4.5, and 4.2 mT. A reduction potential $E_{m,7.5}$ = +180 mV was determined for the couple W^{4+}/W^{5+}. Given the low reduction potential of the substrate, it is suggested that the biologically relevant redox chemistry may not not be located on the tungsten, but rather on the pterin cofactor.

The prismane proteins of *Desulfovibrio vulgaris* (Hildenborough) and *Desulfovibrio desulfuricans* ATCC 27774 are proposed to contain a [6Fe-6S] cluster. A similar cluster has been proposed to be present in the active site of Fe-only hydrogenases. Unfortunately, crystallographic evidence for the presence of [6Fe-6S] clusters is still lacking. Several attempts were made to crystallize the *D. vulgaris* (H) protein in our lab, but these were all unsuccessful. Eventually, high quality crystals were obtained in Daresbury, U.K. in collaboration with prof. Lindley. Crystals grew within four days, and grew to a maximum size of 0.7 mm within ten days. The resolution of the diffraction pattern extends to 1.7 Å. The unit cell is orthorhombic, with spacegroup $P2_12_12_1$. The unit cell will readily hold four molecules of molecular mass of 60 kDa, with a solvent content of approximately 48%.

Generally, I looked for superclusters in the following multi-electron transferring enzymes: dissimilatory sulfite reductase, hydrogenase, and *P. furiosus* aldehyde oxidoreductase. For *Desulfosarcina variabilis* dissimilatory sulfite reductase I found indication for the presence of a supercluster, whereas for hydrogenases the finding of novel FTIR resonances suggest a unique metal structure to be part of the active site. No indication was found for the presence of a supercluster in *Pyrococcus furiosus* aldehyde oxidoreductase, but the enzyme showed to be an interesting case for the study of biological tungsten. An apparent supercluster in *Pyrococcus furiosus* NiFe hydrogenase turned out to be most likely a [4Fe-4S] cluster. An RNA-binding ferredoxin from *Desulfovibrio vulgaris* (H) may be the first example of a prokaryotic, gene regulating Fe-S protein. Finally, elucidation of the crystal structure of the prismane protein from *Desulfovibrio vulgaris* (Hildenborough) will be a major step towards the development of the concept of larger Fe-S clusters.

SAMENVATTING

Doel van het onderzoek

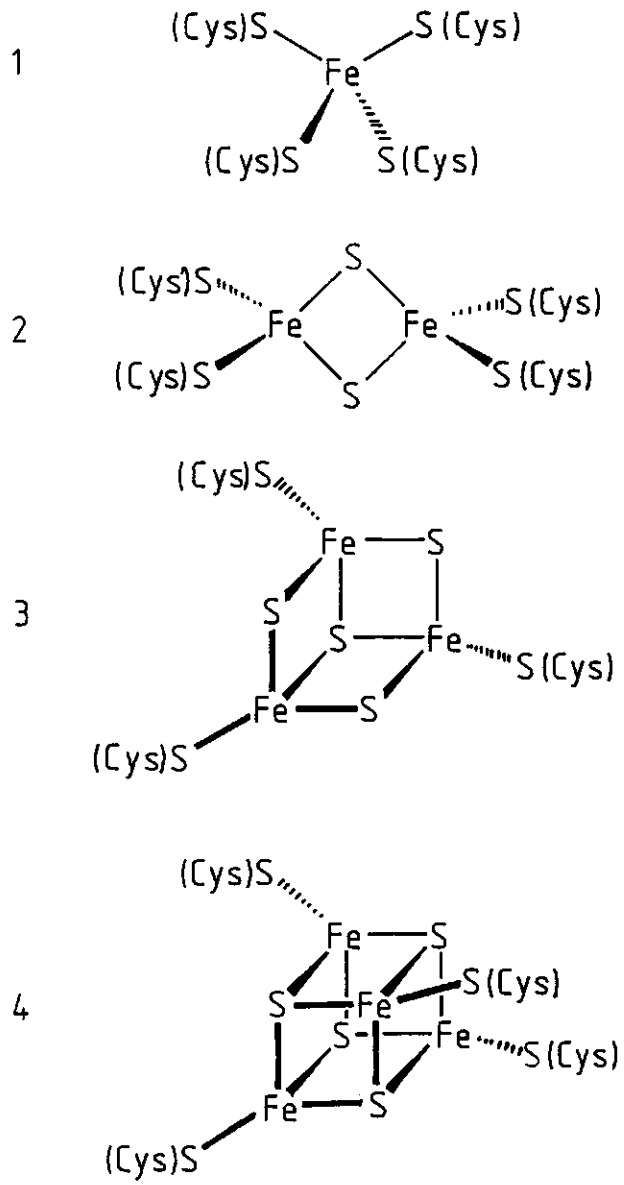
In dit proefschrift beschrijf ik een onderzoek naar de biologische, chemische en fysische eigenschappen van multi-electronoverdragende enzymen, met speciale aandacht voor nieuwe structuren en funkties van biologische ijzer-zwavelclusters.

Inleiding

In de biochemie wordt de moleculaire basis van het leven bestudeerd. Alle levende wezens (bacteriën, planten, dieren en mensen) zijn opgebouwd uit grote moleculen zoals DNA en eiwitten. **Eiwitten** zijn lange opgevouwen ketens van tientallen tot honderden aminozuren. Er zijn twintig verschillende aminozuren. Door eindeloze variatie in combinatie van aminozuren kunnen ontelbaar veel unieke eiwitten worden gevormd. Eiwitten hebben vele functies: ze zorgen ondermeer voor structuur, ze transporteren allerlei belangrijke stoffen en ze voeren reacties uit. Eiwitten die reacties katalyseren (= versnellen) worden **enzymen** genoemd. Om alle processen in het leven gaande te houden is energie nodig. We hebben energie nodig om te groeien, te bewegen, ons voort te planten en om te denken. Die energie ligt opgeslagen in electronenrijke verbindingen en kan vrijkomen wanneer de electronen aan die stof worden onttrokken en worden overgedragen op een andere verbinding; er stromen electronen. Een en ander kan worden uitgelegd aan de hand van een waterkrachtcentrale. Waterkrachtcentrales maken gebruik van de potentiaal van water. Stuwdammen houden het water tegen dat met grote kracht tegen de dam drukt. Als het water gaat stromen komt die energie vrij om er bij voorbeeld een turbine mee aan te drijven. De potentiële energie van water wordt zo omgezet in electrische energie. Electronen (kleine, geladen deeltjes) kunnen van de ene naar de andere verbinding "stromen". Dit proces is een **redox-reactie**. Redox staat voor reductie-oxidatie. Het onttrekken van electronen wordt **oxidatie** genoemd, het toevoegen van electronen **reductie**. De energierijkdom van een verbinding wordt uitgedrukt in de **redoxpotentiaal** van die verbinding. De redoxpotentiaal is een electrochemisch concept. Om de energie te kunnen gebruiken is het van belang dat redoxreacties in kleine stapjes plaatsvinden; anders gezegd: het potentiaalverschil tussen twee verbindingen mag niet te groot zijn. In de analogie van de waterkrachtcentale: als het water met enorm geraas door de dam dendert kunnen we er niets mee doen, we willen dat het netjes langs de schoepen stroomt. Om de redoxreacties in goede banen te leiden zijn er speciale eiwitten, zogenaamde **redoxenzymen**. Net als de electronenrijke verbindingen worden ook redoxenzymen gekenmerkt door een zekere redoxpotentiaal. Stel nu eens dat er een redoxreactie plaatsvindt waarbij electronen worden overgedragen van verbinding A met een potentiaal van bijvoorbeeld tien, naar verbinding B met een potentiaal van nul. Het potentiaalverschil is dus tien. Om deze reactie gecontroleerd te laten verlopen wordt zij opgedeeld in tien kleinere reacties die alle een potentiaalverschil van één hebben. De biochemicus verwacht nu dat elk van de tien subreacties wordt uitgevoerd door een apart redoxenzym met een potentiaal die overeenkomt met die van de uit te voeren reactie. Redoxenzym nummer één heeft een potentiaal van tien, redoxenzym twee een potentiaal van negen

enzovoort. Met de kennis van de potentiaal van een redoxenzym kan men een voorspelling doen over de reactie die het zal uitvoeren. Een redoxenzym draagt electronen over van de ene verbinding op de andere. Zo'n enzym moet over de mogelijkheid beschikken om electronen (tijdelijk) op te slaan. Daartoe bezitten redoxenzymen zogenaamde **"redox-actieve groepen"**. Men kan zich dit aldus voorstellen: een redoxenzym bindt aan een verbinding, haalt daar een electron af, stopt dit op de redox-actieve groep in het enzym, bindt aan een andere verbinding, haalt het electron weer van de redox-actieve groep af en plaatst het op die tweede verbinding. Veel redoxenzymen bevatten metalen, die als redoxgroep blijken op te treden. Metalen kunnen één of meer electronen afstaan. We kennen dit uit de praktijk wanneer een ijzeren voorwerp roest. Bij het roesten worden er drie electronen uit een ijzeratoom gehaald; roesten is een redoxreactie. Biologisch ijzer kent twee **redoxtoestanden**: Fe^{2+} en Fe^{3+}. Een ijzeratoom in een redoxenzym zou aldus als een redoxgroep kunnen dienen. Echter, de potentiaal van ijzer ligt vast (tenminste als de zuurgraad en de temperatuur niet veranderen). Om alle subreacties uit te kunnen voeren is ijzer dus niet geschikt. Toch vinden we in veel redoxenzymen ijzer als redoxgroep (en andere overgangsmetalen als nikkel, koper, cobalt, molybdeen, wolfraam, mangaan en vanadium). Kennelijk is er een manier om de redoxpotentiaal van ijzer (en andere metalen) te variëren. Gebleken is dat de omgeving van een ijzeratoom de potentiaal beïnvloedt. We vinden ijzer dan ook nooit zomaar "los" in het redoxenzym, maar op heel specifieke wijze gecoördineerd. De meest bekende en meest voorkomende ijzerstructuur zijn **ijzer-zwavelclusters**. Figuur 1 laat vier "gewone" ijzer-zwavelcluster zien. De cluster in figuur 1.4 bestaat uit vier ijzer- en vier zwavelatomen die een kubus vormen; er zijn echter ook clusters met één, twee en drie ijzeratomen bekend (figuur 1.2 t/m 1.4). De cluster wordt via de ijzeratomen in het redoxenzym op zijn plaats gehouden door (doorgaans vier) aminozuren. Dit op zijn plaats houden noemt men liganderen; de betrokken aminozuren zijn **liganden**. Alhoewel de ijzer-zwavelcluster in figuur 1.4 vier ijzeratomen bezit kan hij slechts één electron opnemen in plaats van vier. Dit blijkt een kenmerk te zijn van alle "gewone" ijzer-zwavelclusters. Voor ijzer-zwavelclusters geldt dat hoe meer ijzeratomen de cluster bevat, des te groter het potentiaalbereik is. Op deze wijze is het probleem van een te gering potentiaalbereik van ijzer opgelost.

Alhoewel de meeste eiwitten met ijzer-zwavelclusters (Fe/S-eiwitten) betrokken zijn bij electronentransport blijkt dit toch niet de enige functie te zijn. In zoogdieren is een eiwit gevonden dat betrokken is bij de ijzerhuishouding. Dit eiwit heeft een regulerende functie en het blijkt een ijzer-zwavelcluster te bevatten. Van sommige Fe/S-eiwitten is bekend dat ze reacties uitvoeren waarbij helemaal geen electronen worden overgedragen.



Figuur 1. "Gewone" ijzer-zwavelclusters met één (1), twee (2), drie (3) en vier ijzeratomen.

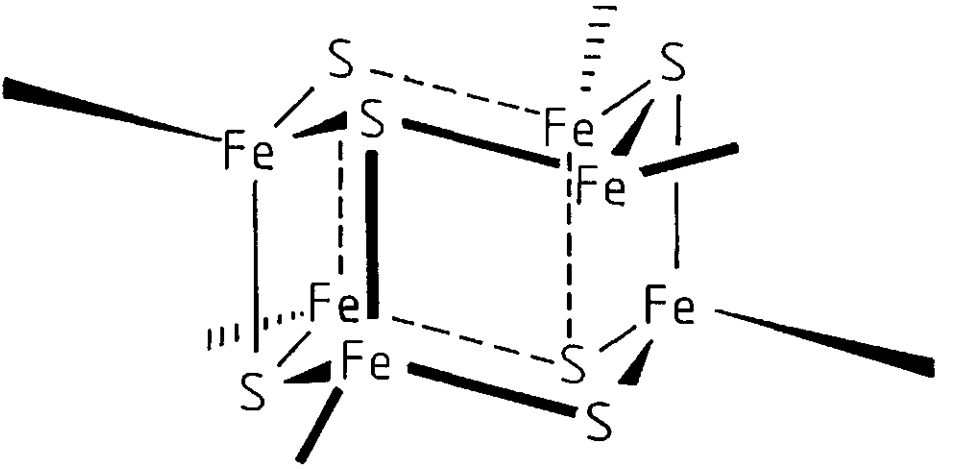
Een speciaal type ijzer-zwavelenzymen katalyseert de omzetting van kleine, anorganische moleculen. Voorbeelden van deze moleculen zijn stikstof (N_2), waterstof (H_2), koolmonoxide (CO), zwaveloxides (SO_x) en stikstofoxides (NO_x). Deze moleculen vormen een potentiële energiebron (H_2), zijn een grondstof voor kunstmest (N_2), of bedreigen het milieu (NO_x, SO_x). Industriële omzetting van dergelijke moleculen is een moeizaam en duur proces; het kan alleen onder stringente condities plaatsvinden (hoge druk en temperatuur). Sommige bacteriën daarentegen voeren deze reacties schijnbaar moeiteloos uit. Daartoe bezitten zij speciale enzymen. Deze multi-electronoverdragende enzymen bevatten ijzer-zwavelclusters. De complexiteit van deze ijzer-zwavelclusters staat in scherp contrast met de schijnbare eenvoud van kleine, anorganische moleculen die worden omgezet. Kennelijk voldoen "gewone" ijzer-zwavelclusters niet meer wanneer deze moleculen moeten worden omgezet. Onderzoek heeft uitgewezen dat deze clusters waarschijnlijk meer dan vier ijzeratomen bevatten en dat ze meer dan één electron kunnen opnemen. Bovendien bezitten deze clusters bijzondere magnetische eigenschappen. Door deze drie kenmerken onderscheiden ze zich van "gewone" ijzer-zwavelclusters. We noemen ze "**superclusters**". Een voorbeeld van een supercluster is de [6Fe-6S] cluster (figuur 2), waarvan verondersteld wordt dat hij voorkomt in het prismaaneiwit en in ijzerhydrogenases. Bij aanvang van dit onderzoek waren er zes potentieel-superclusterbevattende enzymen:

Tabel 1. Mogelijk-superclusterbevattende enzymen

Enzym	**reactie**	
Nitrogenase	$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$	(1)
IJzerhydrogenase	$2H^+ + 2e^- \rightarrow H_2$	(2)
Sulfietreductase	$SO_3^{2-} + 6H^+ + 6e^- \rightarrow S^{2-} + 3H_2O$	(3)
CO dehydrogenase	$CO_2 + 2H^+ + 2e^- \rightarrow CO + H_2O$	(4)
Prismaaneiwit	?	
Aldehyde oxidoreductase:	$RCHO + Fd(ox) \rightarrow RCOOH + Fd(red)$	(5)

(Fd = ferredoxine)

Dit proefschrift beschrijft een onderzoek naar de biologische, chemische en fysische eigenschappen van multi-electronenoverdragende enzymen, met speciale aandacht voor nieuwe structuren en funkties van biologische ijzer-zwavelclusters.



Figuur 2. [6Fe-6S] cluster

Resultaten

Zwavel is een element dat voorkomt in de aminozuren cysteïne en methionine. Mens en dier moeten deze aminozuren via het voedsel opnemen maar planten en bacteriën kunnen ze zelf maken. Hiertoe bezitten zij speciale enzymen waaronder sulfietreductase. Dit enzym reduceert sulfiet tot sulfide waarna het kan worden ingebouwd (geassimileerd), zie reactie 3. Dissimilatoir sulfietreductase voert dezelfde reactie uit maar in dit geval wordt de sulfiet gebruikt voor ademhaling; sommige bacteriën kunnen ademen met sulfaat, sulfiet is een intermediair in dit proces. Uit de sulfaatreducerende *bacterie Desulfosarcina variabilis* is een dissimilatoir sulfietreductase geïsoleerd (hoofdstuk 2). Er werden bijzondere electron paramagnetische resonantie (EPR) signalen gemeten. Dergelijke signalen waren reeds eerder gevonden in het vergelijkbare enzym uit de sulfaatreduceerder *Desulfovibrio vulgaris* (Hildenborough), hetgeen werd uitgelegd als een aanwijzing voor het voorkomen van een supercluster. Nu dergelijke EPR-signalen ook zijn aangetroffen in *D. variabilis* desulforubidine is het aannemelijk dat alle dissimilatoire sulfietreductases een S = 9/2 supercluster bevatten. *Desulfosarcina variabilis* dissimilatoir sulfietreductase bevat ook heem. Er is geen indicatie voor interactie (koppeling) tussen de ijzer-zwavelclusters en de heem, hetgeen wordt uitgelegd als bewijs tegen een model waarin een gekoppelde vier-ijzercluster/siroheem-structuur wordt voorgesteld als het actieve centrum van dissimilatoire sulfietreductases.

Het hyperthermofiele organisme *Pyrococcus furiosus* groeit bij temperaturen rond het kookpunt van water en komt o.a. voor bij vulkanische bronnen. Dit organisme bezit het enzym hydrogenase, dat niet alleen reversibel waterstof oxideert (tabel 1, reactie 2) maar ook elementair zwavel (S^0) reduceert tot zwavelwaterstof (reactie 6).

$$S^0 + 2e^- + 2H^+ \rightarrow H_2S \qquad (6)$$

Het organisme werd gekweekt in een 200-literfermentor bij 90°C, en het hydrogenase kon aeroob worden gezuiverd. In hoofdstuk 3 wordt een studie beschreven naar de redox- en spectroscopische eigenschappen van dit enzym. Een nog niet eerder gemeten EPR (electron paramagnetische resonantie) signaal werd gevonden. Dit signaal komt op bij een redoxpotentiaal van -90 mV, maar opmerkelijk genoeg verdwijnt het weer bij lagere potentiaal. Hoewel dit erop zou kunnen wijzen dat de cluster twee keer één electron kan opnemen, wordt verondersteld dat het verdwijnen van het EPR signaal wordt veroorzaakt door magnetische interactie met een andere, onzichtbare cluster of metaal, als gevolg waarvan een interactiespectrum onstaat.

In de ijzerhydrogenases van de bacteriën *Desulfovibrio vulgaris* (Hildenborough) en *Megasphaera elsdenii*, alsmede in diverse nikkel/ijzer-hydrogenases werden infrarood (FTIR) signalen gevonden (hoofdstuk 4). De positie van deze signalen veranderde wanneer de enzymen werden geoxideerd of gereduceerd. De FTIR groepen konden worden toegeschreven aan de drie niet-geïdentificeerde liganden zoals die uit de recente kristalstructuur van een nikkelhydrogenase naar voren kwamen. Het feit dat deze signalen nu ook zijn aangetroffen in ijzerhydrogenases, maar niet in een grote (controle)groep eiwitten met nikkel en/of ijzer-zwavelclusters, zou erop kunnen wijzen dat alle hydrogenases eenzelfde actieve centrum hebben: een bimetallisch centrum, bestaande uit een ijzeratoom in een lage electronspin-toestand, plus de FTIR-detecteerbare groepen.

Tijdens zuiveringen van andere eiwitten uit de bacterie *Desulfovibrio vulgaris* (Hildenborough) werd een gele fractie aangetroffen die bij hoge zoutconcentratie van de anionenwisselaarkolom eluëerde. Er waren aanwijzingen dat het eiwit sterk aan nucleïnezuren bond. De zuivering en karakteristieken van dit eiwit worden beschreven in hoofdstuk 5. Het bleek dat het eiwit niet aan DNA maar aan RNA bond. Het eiwit bevat een [4Fe-4S] cluster; het bleek een ferredoxine te zijn. Vanwege de sterke binding aan RNA (en niet aan DNA) wordt voorgesteld dat dit ferredoxine niet betrokken is bij electronentransport, maar dat het een regulerend eiwit is. Deze hypothese wordt gesteund door de van de onverwacht grote standaard-reactie-entropie van de reductie van -230 $J \cdot K^{-1} \cdot mol^{-1}$.

Er zijn slechts enkele enzymen bekend die het metaal wolfraam (W) bevatten; ze worden voornamelijk in thermofiele en hyperthermofiele organismen aangetroffen. De hyperthermofiel *Pyrococcus furiosus* bezit drie wolfraamenzymen waaronder aldehydo-oxidoreductase (AOR). Van het AOR werd verondersteld dat het een [WFe3S4] cluster bevat. Een studie van dit enzym wordt beschreven in hoofdstuk 6. Aktief AOR werd verkregen na een snelle, anaerobe zuivering. Volledig gereduceerd, aktief *P. furiosus* AOR vertoonde electron paramagnetische resonantie (EPR) signalen die wijzen op de aanwezigheid van een [4Fe-4S] cluster; er waren geen

aanwijzingen dat het enzym een [WFe3S4] cluster bevat. In het geoxideerde enzym werden EPR-signalen gemeten die konden worden toegeschreven aan wolfraam (W^{5+}). Deze resultaten zijn in overeenstemming met de inmiddels beschikbare kristalstructuur van *P. furiosus* AOR, die de aanwezigheid van een [4Fe-4S] cluster, alsmede een wolfraam-cofactor toont. De gemeten reductiepotentiaal van het W^{4+}/W^{5+} koppel is +180 mV. Deze onverwacht hoge potentiaal wijst erop dat niet wolfraam, maar de pterine-cofactor de redoxactieve groep is.

Van de prismaaneiwitten uit *Desulfovibrio vulgaris* (Hildenborough) en *D. desulfuricans* ATCC 27774 wordt verondersteld dat ze een [6Fe-6S] cluster bevatten. Tevens wordt verondersteld dat een dergelijke cluster voorkomt in het aktieve centrum van ijzerhydrogenases. Helaas is er nog geen kristalstructuur van deze cluster beschikbaar. Na enkele pogingen om het prismaaneiwit van *Desulfovibrio vulgaris* (Hildenborough) te kristalliseren in ons lab, kon het eiwit uiteindelijk worden gekristalliseerd in Daresbury, Engeland. Kwalitatief zeer goede kristallen werden verschenen binnen vier dagen; na tien dagen waren ze volgroeid tot 0.7 mm. De resolutie van het diffractiepatroon is minimaal 1.7 Ångström, hetgeen een nauwkeurige kristalstructuur mogelijk maakt. De resultaten worden beschreven in hoofdstuk 7.

Samenvatting en conclusies

Op zoek naar superclusters heb ik de volgende multi-electronoverdragende enzymen bestudeerd: dissimilatoir sulfietreductase, hydrogenase en *Pyrococcus furiosus* aldehyde oxidoreductase. Voor *Desulfosarcina variabilis* dissimilatoir sulfietreductase heb ik aanwijzing gevonden dat dit enzym een supercluster bevat. De ontdekking van infraroodsignalen in hydrogenases wijst in de richting van een unieke metaalstruktuur in het aktieve centrum van alle waterstof-oxiderende hydrogenases. Ik heb geen aanwijzing gevonden voor de aanwezigheid van een supercluster in *Pyrococcus furiosus* aldehyde oxidoreductase, maar het enzym bleek een interessante "case" om de funktie van biologische wolfraam te bestuderen. Het RNA-bindende ferredoxine uit *Desulfovibrio vulgaris* (Hildenborough) is mogelijk het eerste prokaryotische genregulerende ijzer-zwaveleiwit. Ten slotte zal de verwachte kristalstructuur van het prismaaneiwit uit *Desulfovibrio vulgaris* (Hildenborough) een belangrijke stap voorwaarts zijn in het onderzoek naar de structuur van superclusters.

CURRICULUM VITAE

Alexander Ferdinand Arendsen werd geboren op 12 maart 1966 te Doetinchem. In 1984 werd het VWO diploma behaald aan het Christelijk 'Revius-Lyceum' te Doorn. In 1987 ving hij aan met de studie biologie aan de Rijksuniversiteit te Groningen. Na de propaedeuse werd de studie voortgezet aan de Landbouwuniversiteit te Wageningen. Het doctoraalprogramma omvatte een gecombineerd afstudeervak bij de vakgroepen Microbiologie (Prof. Dr. A.J.B. Zehnder en Dr. A.J.M. Stams) en Biochemie (Prof. Dr. C. Veeger en Dr. W.R. Hagen). Een stage werd uitgevoerd in Tromsø (Noorwegen) aan het Norwegian Institute of Fishery Sciences (Dr. P. Wassmann).

Sinds 1 januari 1993 is hij werkzaam als onderzoeker-in-opleiding (OIO) aan de vakgroep Biochemie van de Landbouwuniversiteit te Wageningen. Het promotie-onderzoek wordt gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk onderzoek (NWO) en heeft geleid tot dit proefschrift.

De laatste fase van het promotie-onderzoek wordt momenteel uitgevoerd aan het CCLRC Daresbury Laboratory (GB), waarvoor een NWO-beurs uit het Stimuleringsfonds voor Internationalisering is toegekend.

In 1994 trouwde hij met Yvonne Wilms.

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