Biochimica et Biophysica Acta, 1020 (1990) 115–145
Elsevier

BBABIO 43291

Review

The structure and mechanism of iron-hydrogenases

Michael W.W. Adams

Department of Biochemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, GA (U.S.A.)

(Received 5 February 1990)
(Revised manuscript received 16 July 1990)

Key words: Hydrogenase; Iron-sulfur cluster; Nickel; ESR

Contents

Abbreviations: *Cp, Clostridium pasteurianum; Me, Megasphaera elsdenii; Dv*H, *Desulfovibrio vulgaris* strain Hildenborough; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; MCD, magnetic circular dichroism; EXAFS, extended X-ray absorption fine structure; ESEEM, electron spin echo envelope modulation.

Correspondence: M.W. Adams, Department of Biochemistry, University of Georgia, Athens, GA 30602, U.S.A.

I. Introduction

Hydrogenases are a class of enzymes that catalyze the reversible activation of the simplest of molecules, molecular H_2, according to Eqn. 1 (hydrogen : acceptor oxidoreductase, EC 1.18.99.1 and 1.12.2.1):

$$H_2 = 2H^+ + 2e^- \quad (1)$$

Representatives of most bacterial genera have the ability to either evolve H_2 or oxidize H_2, or both, during their normal growth modes, and to date hydrogenases have been purified from about twenty different species. A comparison of their properties reveals a fairly heterogeneous group of enzymes, that differ in molecular composition, specific activity in catalyzing H_2 production and H_2 oxidation, electron carrier specificity, cofactor content and sensitivity to inactivation by O_2. However, they have one common feature, all are iron-sulfur proteins, and the majority also contain nickel. Extensive research in the last few years has shown that the Ni-hydrogenases have many properties in common, and they are very distinct from the hydrogenases that lack nickel. Since the Ni-containing enzymes have been reviewed recently (see Section III), I will concentrate here on the 'Fe-only' hydrogenases, with an emphasis on the nature of their iron-sulfur clusters and the mechanism of catalysis. These enzymes contain a new type of Fe-S cluster that is thought to be intimately involved in oxidizing and producing H_2. The nature of this novel cluster is the main focus of this review. It has been the subject of intense biochemical and spectroscopic investigations over the last decade or so, and a complete understanding of its structure and properties is presently one of the most challenging problems in bioinorganic chemistry.

Hydrogenase was recognized as an enzyme capable of activating H_2 almost 60 years ago, and so it has an illustrative history, reflecting in particular the development of our understanding of iron-sulfur proteins in general. I therefore begin with a historical account of the role of iron in the structure and function of hydrogenases.

II. Historical perspective

Stephenson and Stickland [1] named the enzyme in 1931 after they and others [2–6] had demonstrated that colon bacteria evolved H_2 during growth and could use H_2, but not N_2, to reduce both artificial and physiological substrates. Shortly thereafter, Farkas et al. [7] showed that *Escherichia coli* cells catalyzed an exchange reaction between H_2 and D_2O, according to Eqn. 2, and hydrogenase was postulated to be the enzyme responsible.

$$H_2 + D_2O = HD + HDO \quad (2)$$

From the 1940's onwards, a lively controversy existed as to the nature of the enzyme and its prosthetic groups. Hoberman and Rittenberg [8] found that the deuterium exchange activity of *Proteus vulgaris* cell suspensions was inhibited by oxygen, cyanide and carbon monoxide. The CO effect was reversed by light, a characteristic of a porphyrin-containing enzyme. This was supported by Waring and Werkman [9], who showed that *Azotobacter* lacked both hydrogenase activity and known iron-containing enzymes, e.g., catalase and peroxidase, when grown in iron-deficient media. However, others found that, although the hydrogenase activity of both *E. coli* and *Azotobacter* was inhibited by CO, the inhibition was insensitive to light [10,11].

These early studies were all performed with whole cells. Joklik [12] made the first attempt to purify hydrogenase, and concluded that O_2 inactivation of partially purified *E. coli* hydrogenase arose by the oxidation of essential sulfhydryl groups. The H_2 oxidation activity was insensitive to cyanide, but inhibited by CO in a light-independent reaction. Some doubt was therefore cast on the iron-porphyrin theory. In contrast to these results, Gest [13] found that the partially purified *E. coli* enzyme was not inhibited by sulfhydryl-blocking compounds, and the O_2-inactivated enzyme was not reactivated by sulfhydryl reagents. He also made the important contribution of realizing that H_2 oxidation by bacteria probably involved a complex system of intermediate electron carriers between hydrogenase and the electron acceptor (both artificial and physiological); thus, apparent contradictions could be expected between whole cell studies and various cell-free systems, depending on the presence of the intermediate carriers. Gest concluded that further purification of the enzyme was required to elucidate the prosthetic groups involved [13].

One of the major problems in this field up to the mid-50's was that the organisms under study all contained membrane-bound hydrogenases, which made extensive purification difficult to achieve. Hydrogenase activity had been detected in a wide range of organisms, including rumen bacteria, methanogens, sulfate reducers, photosynthetic bacteria, anaerobic fermentors, and aerobic hydrogen and aerobic N_2-fixing bacteria (for review, see Ref. 14). A major breakthrough was the discovery that soluble preparations of hydrogenase could be obtained from strict anaerobes such as *Clostridium* and *Desulfovibrio* sp. (e.g., Refs. 15–17). Studies of the partially purified enzymes from these organisms revealed the essential role of iron. Iron-complexing reagents caused inhibition, activity was stimulated by the addition of ferrous ions (but not Mo, Cu or Co salts), and metal analyses showed that iron was present in all active preparations. Functional -SH groups also appeared to be required for the activity of some hydrogenases, and it was generally agreed that hydrogenase

contained an iron-containing prosthetic group, though not of the heme type [18].

It also became evident at this time that the methods used to measure hydrogenase activity were not satisfactory in many cases. Almost all preparations exhibited a lag phase in the deuterium exchange assay, considered the ideal system since additional electron acceptors were not involved, and hydrogenase-catalyzed H_2 uptake with methylene blue as the acceptor, the most widely used assay system, gave variable results depending on the purity of the enzyme preparation and the degree to which O_2 had been excluded during the purification. A major advancement was therefore made when two laboratories independently reported a procedure for the manometric determination of hydrogenase-catalyzed H_2 evolution using methyl viologen as the electron carrier and sodium dithionite as the electron donor [19,20]. The new assay eliminated any inhibitory effects of O_2, which were removed by the powerful reducing mixture of dithionite and the viologen, and it enabled more extensive and quantitative estimations of hydrogenase activity in a wider range of bacteria, e.g., Ref. 20.

The hydrogenases of various species of *Desulfovibrio* and *Clostridium* were extensively investigated in the early 60's, e.g., Refs. 21–24. Iron but not molybdenum was shown to be essential for the expression of the activity by *C. pasteurianum* cells [25], and iron was detected in hydrogenase preparations from various species. Sadana and Rittenberg [26,27] were the first to propose that the active center of hydrogenase might consist of a ferrous-sulfhydryl complex. The activity of the partially purified enzyme from *D. desulfuricans* required added Fe^{2+} and was inhibited by sulfhydryl-blocking reagents, and the addition of silver (Ag^+) ions, which bind strongly to -SH groups, caused the release of iron from the enzyme. Moreover, they demonstrated that the enzyme preparation also contained acid-labile sulfide. It was at this time that Mortenson and coworkers [28] discovered ferredoxin in *C. pasteurianum,* and Tagawa and Arnon [29] isolated a similar nonheme iron-containing protein from chloroplasts. Curiously, the presence of iron and acid-labile sulfide in both bacterial ferredoxin and hydrogenase was demonstrated in the same year [26,30,31], although the implications of this in the hydrogenase field were not realized until several years later.

The lack of a pure preparation of hydrogenase precluded any detailed examination of the properties of its iron-sulfur center(s). Indeed, the precise arrangement of the metal complex in the simple ferredoxins was still in doubt in the late 60's, e.g., Refs. 32, 33. Progress was being made though with regard to the physiological role of hydrogenase. At least three different types of hydrogenase were apparent. Tamiya et al. [34] showed that the enzyme from *D. desulfuricans* evolved H_2 from reduced cytochrome c_3 but not reduced ferredoxin, whereas Valentine et al. [35] found that ferredoxin functioned as the physiological electron carrier to *C. pasteurianum* hydrogenase. The third type was evident from the work of Bone et al. [36], who showed that the partially purified soluble hydrogenase of the aerobic hydrogen bacterium, *Pseudomonas ruhlandii,* would reduce NAD directly with H_2 in the absence of other cofactors. These different types of hydrogenase were in accord with their proposed physiological roles (for reviews, see Refs. 37, 38). Thus, H_2 production enables some organisms to dispose of excess reductant without the need for terminal electron acceptors other than protons, thereby regenerating the oxidized electron carriers. Alternatively, H_2 oxidation provides organisms with a source of reductant which can also be coupled to energy generation. Aerobes use O_2 as a terminal electron acceptor and generate ATP via electron transport phosphorylation. They also use H_2 as a source of reductant for CO_2 fixation. For example, some aerobic hydrogen bacteria were found to contain two hydrogenases, one membrane-bound, the other soluble. The latter reduces NAD directly with H_2, whereas the former oxidizes H_2 and diverts reductant to O_2 reduction. In contrast, various anaerobic bacteria use compounds such as nitrate, sulfate and CO_2 (reduced to CH_4) as the terminal electron acceptors of an electron transport phosphorylation system with H_2 as the reductant.

The 1960's saw one controversy end and another begin in the hydrogenase field. A prior bone of contention was the observation that CO inhibition of *P. vulgaris* hydrogenase could be reversed by light (which led to the porphyrin theory), but other researchers could not repeat this with other hydrogenases. Purec and Krasna [39] solved this problem by showing that the hydrogenase activity of *P. vulgaris* was in fact activated by light, even in the presence of CO. Thus, the apparent increase in activity was not due to the reversal of CO inhibition by light. Interestingly, the effect of light on CO-inhibited hydrogenases is of great relevance to the structure and mechanism of these enzymes today (subsection V-B.2). The understanding of the structure and role of hydrogenases was complicated by the observation of Ackrell et al. [40] that almost all of the twenty bacterial species they examined contained multiple forms of hydrogenase. The criterion was the mobility of hydrogenase activity after electrophoresis of cell extracts. Some organisms such as *C. pasteurianum* and *Rhodospirillum rubrum* were found to contain at least four apparently distinct hydrogenases. Although more recent work has shown that some of these results were artifacts, arising from the strong association of hydrogenase with other cellular components, e.g., Ref. 41, several organisms do contain more than one hydrogenase. For example, *C. pasteurianum* has two [42] and *D. desulfuricans* [43] and *E. coli* [44] each have three,

although a physiological role for each is not always readily apparent (subsection IV-A).

With an improvement in purification methods (and the use of strictly anaerobic techniques), the first reasonably pure preparations of hydrogenase were obtained in the early 1970's, and the presence of non-heme iron was unequivocally demonstrated. Mortenson and coworkers [41,45] purified hydrogenase from *C. pasteurianum* and showed that it contained 4Fe and 4 acid labile sulfide (S^{2-}) atoms/molecule. In addition, three laboratories isolated hydrogenase from two strains of *D. vulgaris* and found 1–8 Fe atoms/molecule of enzyme with similar amount of acid-labile sulfide [46–48]. The reduced forms of the hydrogenases from both *C. pasteurianum* [45] and *D. vulgaris* [48] exhibited electron paramagnetic resonance (EPR) signals characteristic of reduced ferredoxins, suggesting that the iron-sulfur clusters in these enzymes were probably involved in electron transfer. Both of these hydrogenases were found to be very sensitive to inactivation by O_2.

A tremendous amount of research was carried out during the 1970's on H_2-evolving organisms and on the purification and characterization of hydrogenase. This was due in part to the realization that, in a very energy-conscious world, the production of H_2 from biomass and waste organic compounds might prove a viable alternative energy source (see, for example, reviews by Calvin [49] and Hall [50,51]). For example, Krampitz [52] and Benemann et al. [53] had shown that the reducing power generated by isolated plant chloroplasts through water biophotolysis could be converted to H_2 via hydrogenase, and this stimulated an enormous amount of interest (see review by Rao and Hall [54]). About a dozen hydrogenases were purified and extensively characterized from photosynthetic bacteria, strict anaerobes including sulfate reducers, facultative anaerobes and aerobic hydrogen bacteria, and a similar number of hydrogenases were partially purified, including those from methanogens, aerobic N_2-fixing bacteria and cyanobacteria. Their properties were summarized in our 1981 review (Ref. 55, see also Refs. 56 and 57).

All of the pure hydrogenases were shown to be iron-sulfur proteins containing equimolar amounts of iron and acid-labile sulfide, with values ranging from 4 to 24 gatoms/mol. Interestingly, with one exception, the iron-contents reported were all multiples of four. Besides iron-sulfur content, the enzymes showed great variation in molecular weight (50 000–205 000), subunit composition, and electron carrier specificity, but some generalizations could be made. For example, most were relatively stable to O_2 during purification and readily catalyzed H_2 oxidation, but the rates of H_2 production were very low (using artificial electron carriers). The exceptions seemed to be some of the enzymes from strict anaerobes, e.g., *C. pasteurianum* and *Megasphaera elsdenii,* which were extremely sensitive to inactivation by O_2 and catalyzed both H_2 production and H_2 oxidation at extremely high rates. This distinction in catalytic activity between the two groups tended to correlate with their physiological roles, i.e., the 'bidirectional' hydrogenases functioned in vivo to evolve H_2 whereas the 'uptake' enzymes were present in organisms that used H_2 as a source of energy and reductant. Most of the enzymes in their reduced states gave rise to EPR signals typical of those exhibited by reduced ferredoxins, whereas the oxidized hydrogenases gave rise to either axial or rhombic type EPR signals with $g_{av} > 2$. At that time the latter signals were reminiscent of those seen from the $[4Fe\text{-}4S]^{3+}$ cluster in oxidized Hipip (although their redox potentials in the hydrogenases were much lower). We therefore concluded [55] that hydrogenases contain ferrodoxin-type 4Fe-clusters and a novel type of 4Fe-center, probably involved in H_2 catalysis, that when oxidized showed EPR absorption similar to a Hipip center.

Two developments in the metalloenzyme field occurred in the early 1980's that dramatically changed our understanding of hydrogenase. The first was the discovery of 3Fe-clusters [58–60]. Oxidized 3Fe-clusters also exhibit axial-type EPR signals, and a re-evaluation of the EPR properties of several oxidized hydrogenases showed that most had characteristics of an oxidized 3Fe-cluster [61]. The exceptions to this were the 'bidirectional' hydrogenases of the strictly anaerobic bacteria which exhibited sharp, rhombic EPR signals, unlike those of either an oxidized 3Fe-cluster or oxidized Hipip. The second development had its beginnings in the work of Lancaster [62], who in 1980 observed novel EPR signals from the membranes of methanogenic bacteria, and tentatively assigned them to a Ni(III) species. At that time it was known that nickel was required for the growth on H_2 of various bacteria, including methanogens and aerobic hydrogen bacteria [63–65]. In 1981, Thauer and co-workers [66] first demonstrated the presence of nickel in a purified hydrogenase. They found about 1 Ni atom/molecule in the enzyme from *Methanobacterium thermoautotrophicum,* and the following year reported that the enzyme exhibited the novel EPR signal that Lancaster had earlier assigned to nickel [67]. The new rhombic EPR signal, with g values of 2.3, 2.2 and 2.0, was unambiguously shown to arise from nickel by examining the hydrogenase purified from cells grown in the presence of ^{61}Ni ($I = 3/2$; thus a four-line spectrum was observed). In the same year and independently, (a) Lancaster confirmed that the novel EPR signal from *M. bryantii* did derive from nickel using ^{61}Ni substitution [68], (b) two research groups showed using metal analyses together with EPR and isotope substitution that purified hydrogenase from the sulfate-reducing bacterium, *Desulfovibrio gigas,* contained

nickel [69,70], and (c) nickel was found in both the soluble and membrane-bound hydrogenases of the aerobic hydrogen bacterium, *Alcaligenes eutrophus* [71].

It was therefore clear at the beginning of 1983 that nickel, in addition to iron-sulfur clusters, was an essential component of some, if not all hydrogenases. There was also a report of a hydrogenase containing selenium (in the form of selenocysteine) in *Methanococcus vannielii* [72], although at the time this did not receive much attention. The direction of research in this field thus switched to address the questions, do all hydrogenases contain nickel, and what are properties and role of the nickel center?

III. Ni-hydrogenases

Nickel-containing hydrogenases have now been isolated and characterized to varying degrees from well over a dozen different organisms, including photosynthetic bacteria [73–75], methanogens [76–81], sulfate-reducing bacteria [69,70,82–84], colon bacteria [85,86], aerobic hydrogen bacteria [87–90], aerobic N_2-fixing bacteria [91–94], and from an extremely thermophilic archaebacterium that grows optimally at 100 °C [95,96]. In addition, hydrogenases containing equimolar amounts of selenium and nickel have been purified from various sulfate-reducing bacteria [97–99]. The absence of nickel has been demonstrated only in the hydrogenases of a few anaerobic bacteria, as discussed below, and it is now evident that 'Fe-only' hydrogenases are the exception rather than the rule. For the sake of comparison, the properties of the Ni-hydrogenases will be briefly described. These enzymes have been extensively reviewed in the last few years [100–104], and the reader is referred to these reviews for further details of their properties.

With one exception [95], the Ni-hydrogenases are purified aerobically in an inactive state, even from strictly anaerobic bacteria. They therefore require reductive activation to give the fully active enzymes, which are then very sensitive to irreversible inactivation by O_2. Indeed, it was reported [105] that the hydrogenase from the strict anaerobe, *Methanobacterium thermoautotrophicum*, rapidly lost activity if purified under anaerobic conditions. Hydrogenases lacking iron-sulfur clusters have yet to be isolated, and until recently it was thought that the minimum metal content of a Ni-hydrogenase was a Ni center with one Ni atom that is spin-coupled to a [4Fe-4S] cluster. The hydrogenases from the photosynthetic bacteria, *Chromatium vinosum* and *Thiocapsa roseopersicina* [106,107], were originally proposed to contain just that, one Ni atom and a single 4Fe-cluster, but more recent analyses [108,109] have shown that these enzymes contain at least two [4Fe-4S] clusters per Ni atom. It therefore appears that all Ni-hydrogenases contain at least two 4Fe-clusters, which are of the ferredoxin type. Some Ni-hydrogenases, such as the enzyme from *D. gigas* [110,111], also contain stoichiometric amounts of a [3Fe-4S] cluster, but in others this cluster is a minor component probably arising from the aerobic degradation of a 4Fe-cluster [112].

The structure of the Ni center is unknown and may vary in different hydrogenases. X-ray absorption spectroscopy (EXAFS) and EPR studies have suggested that Ni is 5- to 6-coordinate and bound by at least one [100], and more likely three [113] or four [114] sulfur atoms, but not to Fe. Electron spin echo envelope modulation (ESEEM) studies of three hydrogenases [115–117] have all indicated a weak interaction between the Ni center and a ^{14}N nucleus. In one case this was assigned to one N atom of a bound flavin [115], and in the other two to the distal N of a histidine imidazole [116,117]. Extensive EPR studies using the hydrogenases of *C. vinosum*, *D. gigas* and *M. thermoautotrophicum* [111,118–124] have shown that three different EPR signals (termed A, B and C) are exhibited by the Ni center during activation of the inactive, aerobically-prepared enzymes and during catalysis. However, there is controversy as to whether the nickel cycles between four (III to 0 [118,124]), three (III to I [119,124]) or two (III, II [111,120]) redox states during these processes. Although it is generally accepted that the EPR signal exhibited by the as isolated enzymes arises from a Ni(III) site, this has now been challenged by Maroney and co-workers [125]. Based on the properties of novel Ni thiolate complexes, they proposed that the 'Ni(III)' EPR signal arises from an Ni(II) center ($S = 1$) that is spin coupled to a thiyl radical to yield an $S = 1/2$ system [125]. Moreover, a recent study of the reaction of O_2 with the same Ni complexes led to the suggestion that inactivation of Ni-hydrogenases by O_2 occurs by oxidation of a Ni-cysteinate ligand [126]. In this regard, a redox transition of a ligand to the Ni atom during the reductive activation of *M. thermoautotrophicum* hydrogenase has recently been postulated on the basis of EPR and potentiometric analyses [124].

There is general agreement, however, that the Ni center of the Ni-hydrogenases is the site of reaction with H_2 [120,122,123]. For example, an Ni(I)-hydride and an Ni(I)-CO species, both sensitive to visible light, have been proposed as respective intermediates in catalysis and the reversible inhibition by CO of *C. vinosum* hydrogenase [123]. The nature of the [4Fe-4S] clusters in the Ni-hydrogenases is not known, since, in contrast to the Ni center, they are not detectable by EPR spectroscopy in most redox states of these enzymes. A recent Mössbauer study of *D. gigas* hydrogenase showed that the two 4Fe-clusters in this enzyme have different redox potentials and have atypical magnetic properties compared to ferredoxin-type 4Fe-centers [111].

The general picture of a redox active, Ni-S center at the catalytic site of the Ni-hydrogenases is complicated

by the fact that some Ni-hydrogenases show very weak or no EPR absorption from Ni and/or have very low Ni contents (much less than 1 atom/molecule; see below and Ref. 100). This emphasizes the heterogeneity in this group of enzymes and suggests some may lose metal during purification, or that they have different mechanisms of H_2 activation. In addition, it has been demonstrated that the hydrogenase of the aerobic hydrogen bacterium, *Nocardia opaca,* contains two loosely-bound Ni atoms, which appear to aid in holding together the subunits of the enzyme, as well as two tightly-bound Ni atoms which are thought to be at the catalytic site [127,128]. Furthermore, the Ni-containing hydrogenase of the extremely thermophilic archaebacterium, *Pyrococcus furiosus,* seems to be very distinct from all the other Ni-hydrogenases [95,96]. It contains only a single Ni atom per apparent $\alpha_2\beta_2\gamma_2$ structure, does not exhibit any EPR signal characteristic of Ni (or of the Fe-only hydrogenases, see below), is insensitive to the inhibitors of Ni- (and Fe-) hydrogenases, and has the unique property of preferentially catalyzing H_2 evolution under the standard assay conditions. These data suggest that H_2 catalysis by the *P. furiosus* enzyme may not occur at the same type of Ni-S (or Fe-S) center found in other hydrogenases.

As mentioned above, some hydrogenases from methanogens and sulfate-reducing bacteria contain selenium, in addition to nickel and iron-sulfur clusters. The selenium is in the form of selenocysteine [72], and it has recently been shown using EPR and X-ray absorption spectroscopy that in *D. baculatus* hydrogenase the selenium is directly coordinated to nickel [129,130]. The effect of selenium substitution appears to be a very subtle one, since the general properties of the selenium-containing enzymes are very similar to those without it [99]. However, the NiSe-hydrogenases do exhibit distinctly different kinetics in catalyzing the H/D exchange reaction, and it was proposed [99] that selenium serves to modulate or fine-tune the catalytic properties of the enzyme. It remains to be established how this is related to the physiological roles of the NiSe- and Ni-hydrogenases. The selenium-containing hydrogenases appear to be a subset of the Ni-enzymes, since in *Desulfovibrio* sp. they share a significant degree of sequence homology at the gene level and have very similar metal contents and spectroscopic properties [131]. It should be noted that elucidating the in vivo functions of the Ni-hydrogenases is exceedingly complex in some cases. As an example, the sulfate-reducing bacterium *D. vulgaris* (strain Hildenborough) contains three different membrane-bound Ni-hydrogenases, in addition to an 'Fe-only' enzyme located in the cytoplasm [84].

In spite of the above discussion, the Ni-hydrogenases are a fairly cohesive group of enzymes, with the possible exception of that of *P. furiosus.* They are usually referred to as 'uptake' hydrogenases, a term which reflects their physiological roles, i.e., in the use of H_2 as a growth substrate, and the fact that in *in vitro* assays they preferentially catalyze H_2 oxidation. Indeed, many exhibit rates of H_2 production that are barely detectable. All Ni-hydrogenases are comprised of (at least) two dissimilar subunits ($\alpha\beta$) with molecular weights of approx. 60000 and 30000, and the majority are also membrane-bound. Those that are not, usually contain one or more additional subunits which function to interact with specific (cytoplasmic) electron carriers, such as NAD in the aerobic hydrogen bacteria or cofactor F_{420} in the methanogenic bacteria. The similarities between the different Ni-hydrogenases is shown by immunological and DNA sequence analyses, which reveal extensive structural homology between the enzymes of different genera [104,132].

IV. Fe-Hydrogenases

The dramatic changes that occurred in the hydrogenase field during the 1980's are exemplified by the fact that not until 1984 was it unequivocally demonstrated that some hydrogenases do not contain nickel. Although this would seem a rather trivial problem, purified hydrogenases (and most other proteins) contain trace amounts of various transition metals, notably iron, nickel, zinc and copper. Since some Ni-hydrogenases contain much less than stoichiometric amounts of nickel and/or do not exhibit the 'classical' $g = 2.3$ EPR signal of the O_2-inactivated enzymes, the finding of only trace amounts of nickel in a preparation of a pure hydrogenase did not necessarily mean that one had an 'Fe-only' hydrogenase. As a further complication, iron is readily measured by colorimetric methods, but accurate nickel quantitations require plasma emission or atomic absorption spectroscopy, techniques that may not be available to all researchers. In addition, it was apparent that nickel was present in a wide variety of hydrogenases, and the onus was therefore to definitively prove the absence of nickel.

In 1984, Peck, LeGall and co-workers showed that the periplasmic hydrogenase from *D. vulgaris* (strain Hildenborough) lacked nickel [133], and we demonstrated that the same was true with two hydrogenases (I and II) from *C. pasteurianum* [134]. All three enzymes appeared to contain approximately equimolar amounts of iron and acid-labile sulfide, but much less than 0.1 gatoms of Ni/mol. For example, plasma emission spectroscopy of hydrogenase II of *C. pasteurianum* revealed that only iron and copper were above the limits of detection, and the Fe:Cu ratio was 280:1 [135]. The hydrogenase from another strict anaerobe, *Megasphaera elsdenii,* was found to contain quite high concentrations of both nickel and copper (0.2–0.6 gatoms/mol) in addition to iron and sulfide (approx. 12 gatoms/mol of each), but the absence of a well-defined EPR signal

from nickel led the authors to conclude that both it and copper are adventitiously bound [136]. The similarity in the properties of the hydrogenases of *M. elsdenii, C. pasteurianum* and *D. vulgaris* confirms this [137], as discussed below.

On the other hand, Yagi and co-workers isolated a hydrogenase from the membranes of the Miyazaki F strain of *D. vulgaris* in the mid-70's [138,139], but its nickel content was very low (up to 0.2 gatoms/mol [139]) and the classical $g = 2.3$ Ni EPR signal was not apparent, so this enzyme was also considered to be an Fe-hydrogenase, e.g., Ref. 104. This was in spite of the fact that its molecular weight (89000), subunit structure ($\alpha\beta$), catalytic properties, and sensitivity to CO and O_2, more resembled those of a Ni hydrogenase [138–141]. Accordingly, it has now been shown that this hydrogenase does, in fact, give rise to a weak $g = 2.3$ EPR signal (Yagi, T., personal communication: see also, Fig. 3 in Ref. 140), and its classification as a Ni-hydrogenase has also been confirmed by analysis of its amino acid sequence (deduced from the gene sequence), which shows high homology with that of the Ni-hydrogenase of *D. gigas* (Voordouw, G., personal communication).

The only other 'Fe-hydrogenase' so far reported is that isolated by Ragsdale and Ljungdahl [142] from the acetogenic bacterium, *Acetobacterium woodii.* The final enzyme preparation they obtained lacked nickel, but it was not completely pure and its iron and sulfur content and EPR properties were not reported. That this organism contains an Fe-hydrogenase has therefore still to be substantiated. Recent analyses of *A. woodii* DNA using DNA probes to the Ni-hydrogenases of *Desulfovibrio* species have indicated that this bacterium also contains an Ni-hydrogenase (Peck, H.D., Jr., personal communication).

IV-A. Physiological roles

To date, the absence of nickel has been unequivocally demonstrated only in some hydrogenases from strictly anaerobic bacteria. This is not a general rule, however, since some strict anaerobes, such as methanogens, appear to contain only Ni-hydrogenases, while some species of the sulfate-reducing genus, *Desulfovibrio,* contain both Fe- and Ni-hydrogenases. The Fe-hydrogenase of *D. vulgaris* strain Hildenborough has been postulated to play a role in H_2 recycling [104]. It is located in the periplasm and uses cytochrome c_3 as an electron carrier [143]. Only anaerobic bacteria that grow by fermentation seem to lack Ni-hydrogenases, e.g., *C. pasteurianum* and *M. elsdenii.* These organisms obtain energy by substrate level phosphorylation, and the hydrogenase serves to dispose of the excess reductant generated during fermentation in the form of H_2. Reduced ferredoxin is the natural electron donor to the Fe-hydrogenases in these bacteria. However, not all fermentative anaerobes contain Fe-hydrogenases. The extremely thermophilic archaebacterium, *Pyrococcus furiosus,* grows by fermenting carbohydrates to organic acids, CO_2 and H_2, but this organism contains an atypical Ni-containing hydrogenase [95,144]. Like the mesophilic eubacteria though, ferredoxin is the natural electron donor for this H_2-evolving hydrogenase.

The mesophilic anaerobe, *C. pasteurianum,* is so far unique in that it contains two different hydrogenases, neither of which contain nickel. Hydrogenase I, first isolated in the early 70's by Mortenson and co-workers [41,56], functions to evolve H_2 in vivo. However, Chen and Blanchard [42] identified a second hydrogenase (II) in *C. pasteurianum,* and this enzyme preferentially catalyzes H_2 oxidation (see below). The role (or necessity) of

TABLE I

Properties of iron-hydrogenases

	Organism			
	Clostridium pasteurianum		*Megasphaera elsdenii*	*Desulfovibrio vulgaris* (Hildenborough)
	I	II		
Location:	cytoplasmic	cytoplasmic	cytoplasmic	periplasm
Purification conditions:	anaerobic	anaerobic	anaerobic	aerobic
O_2 sensitivity as isolated:	extremely	extremely	extremely	insensitive
Molecular weight:	61944 [a]	54738 [a]	58000 [b]	45820 + 10000 [c]
V_m, H_2 evolution [d]:	5500	10	7000	10400
V_m, H_2 oxidation [e]:	24000	34000	9000	50000
Fe/mol:	20.1 ± 0.7	13.8 ± 0.4	15.6 ± 2.7	9–15
S^{2-}/mol:	17.8 ± 1.2	11.4 ± 0.2	15.5 ± 2.4	≈13
Reference:	56, 134, 145, 146	42, 134, 146, 147	136, 148–150	133, 143, 151–153

[a] Based on amino acid composition.
[b] Based on sedimentation equilibrium analysis.
[c] Based on amino acid (gene) squence.
[d] Expressed as μmol of H_2 evolved/min per mg using dithionite-reduced methyl viologen as electron donor.
[e] Expressed as μmol of H_2 oxidized/min per mg using methylene blue or benzyl viologen as electron acceptor.

an enzyme with these catalytic properties in an organism that must continually dispose of excess reductant in the form of H_2 is not known, nor is the in vivo electron carrier for this unique Fe-hydrogenase.

In the following I will therefore focus on the two Fe-hydrogenases from *C. pasteurianum* and on the Fe-enzymes from *D. vulgaris* (strain Hildenborough) and *M. elsdenii.* These hydrogenases have been extensively characterized by a variety of biochemical and spectroscopic techniques.

IV-B. Molecular and catalytic properties

As shown in Table I, the three hydrogenases of the saccharolytic anaerobes, *C. pasteurianum* (*Cp*, I and II) and *M. elsdenii* (*Me*) have many properties in common. They are monomeric, cytoplasmic enzymes with molecular weights of approx. 60000. They are extremely sensitive to inactivation by O_2, with $t_{1/2}$ values of only minutes in air, and must be purified under strictly anaerobic conditions, i.e., all buffers are degassed and maintained under a positive pressure of an inert gas, and contain sodium dithionite to remove any contaminating O_2. The amino-acid or gene sequences of these hydrogenases are not known, so their molecular weights are based on analyses using techniques such as gel-filtration, SDS-gel electrophoresis and sedimentation equilibrium analysis.

In contrast, the Fe-hydrogenase from the sulfate reducing bacterium, *D. vulgaris,* strain Hildenborough (*Dv*H) is located in the periplasm and can be purified aerobically [151]. Although aerobic purification is convenient from a practical point of view, this property appears to complicate the interpretation of the spectroscopic data obtained from this enzyme (see below). *Dv*H hydrogenase can be removed from the periplasm of intact cells by washing with Tris/EDTA buffer [151], and the enzyme is stable to air if the intact cells are well aerated in the growth medium before harvesting [154]. However, like the Ni-hydrogenases, the *Dv*H enzyme in its O_2-stable state is not fully active and requires some form of *reductive* activation (not just removal of O_2), otherwise a lag phase is observed in the H_2 oxidation assay [155]. Moreover, the O_2-stable enzyme becomes sensitive to inactivation by O_2 upon reduction with H_2, and the O_2-stable state is regained only by anaerobic oxidation with the dye, 2,6-dichlorophenol indophenol (DCPIP), in the presence of Fe and EDTA [154], suggesting that some form of Fe-S cluster interconversion occurs (see below).

The *Dv*H enzyme is the only Fe-hydrogenase that has been cloned [156] and sequenced (Ref. 157: see also Ref. 158). Initial studies on purified *Dv*H hydrogenase [133,151] established that it was a monomeric protein of $M_r \approx 50000$. However, characterization of the enzyme prior to the genetic analyses showed that it contained an additional, smaller subunit of $M_r \approx 13000$. In accordance, the hydrogenase operon was found to comprise two genes coding for polypeptides of M_r 45820 and 13493 [156,157]. Subsequent amino acid sequencing of the smaller subunit by Prickril et al. [159] revealed that the mature polypeptide lacked the 34 N-terminal residues coded by the small subunit gene, and it was suggested that these represented a signal peptide involved in the transport of this periplasmic hydrogenase. The small subunit after processing therefore had a molecular weight of about 10000, rather than about 13500, and the molecular weight of the holoenzyme was about 56000 (46000 + 10000). As discussed below, an accurate molecular weight is crucial in determining the metal contents of these hydrogenases. The finding of an additional subunit in the *Dv*H enzyme prompted a reevaluation of the subunit compositions of the two clostridial Fe-hydrogenases. However, both were found to be monomeric enzymes [146].

As mentioned above, the Fe-hydrogenases of *Me*, *Cp* and *Dv*H are all extremely active enzymes, and their specific activities in the in vitro assays are one or two orders of magnitude greater than those observed with the Ni-hydrogenases, e.g., Ref. 104. However, as shown in Table I, hydrogenase II of *Cp* is unique in that it preferentially catalyzes H_2 oxidation. It is about 50% more active than *Cp* hydrogenase I in H_2 uptake activity, but about 550-fold less active in evolving H_2. A possible mechanistic reason for this is discussed in subsection V-B. The distinct nature of the *Cp* II enzyme was recently demonstrated by immunological studies [132]. Antibodies to this hydrogenase reacted with several Ni-hydrogenases, but not with the *Cp* I or *Dv*H enzymes, and antibodies to the Ni-hydrogenase of *Bradyrhizobium japonicum* reacted with the *Cp* II enzyme but not with the *Cp* I or *Dv*H hydrogenases. Interestingly, although the *Cp* I and *Dv*H hydrogenases show immunological cross-reactivity [132], DNA probes derived from the gene for the large subunit of the *Dv*H enzyme (see below) do not hybridize to total *Cp* DNA (Peck, H.D., Jr., personal communication). In addition to differences in their relative rates of catalyzing H_2 evolution and H_2 oxidation, the two *Cp* hydrogenases also differ in their ability to carry out the H/D exchange reaction (Eqn. 2: Bonam, D. and Mortenson, L.E., unpublished results). The H/D exchange activity of *Dv*H hydrogenase in comparison with Ni- (and NiSe-)hydrogenases was recently summarized [104].

Carbon monoxide has been known as a potent inhibitor of hydrogenase for many years (see Ref. 55). Only the Ni-hydrogenases of the extreme thermophile, *P. furiosus* [96], and of the aerobic hydrogen bacterium, *Alcaligenes eutrophus* [160], have been shown to be insensitive. Peck, LeGall and co-workers have shown that Fe- and Ni-hydrogenases of various *Desulfovibrio*

TABLE II

Effect of CO on the activity and EPR properties of iron-hydrogenases

Source and type of hydrogenase	Assay	[CO] for 50% effect [a] (μM)
*Dv*H (Fe)	H/D exchange	0.1
	H_2 evolution	0.4
	EPR	3.9
Cp I (Fe)	H_2 evolution	0.90
	EPR	1.1
Cp II (Fe)	H_2 evolution	2.8
	EPR	0.31
D. gigas (Ni)	H/D exchange	20
	H_2 evolution	35

[a] Indicates the CO concentration required to give 50% inhibition of enzyme activity or to give half maximal intensity of the CO-induced EPR signal. Data taken from Refs. 133, 138, 161–163. See text for details.

species can be differentiated by the sensitivity of their H/D exchange and H_2 evolution activities to inhibition by CO [104,161]. Some of the inhibition results are summarized in Table II, together with the relevant data for the *Cp* hydrogenases. It is evident that the Fe-hydrogenases are much more sensitive to inhibition by CO (by at least an order of magnitude) than the Ni-hydrogenase of *D. gigas,* which is representative of several Ni-hydrogenases that have been examined [104]. It should be noted that the inhibition of H_2 evolution by CO is an *irreversible* process with the two *Cp* hydrogenases. That is, the inhibition observed when these enzymes are assayed by H_2 evolution from dithionite-reduced methyl viologen in the presence of CO (where [CO] > K_i, e.g., 10 μM) is not reversed by removing the CO (by repeated flushing and degassing with an inert gas or with H_2), i.e., there is no increase in the observed rate of H_2 production [134]. Whether the CO inhibition of H/D exchange by *Dv*H hydrogenase is reversible has not been reported. The exchange activity of the latter enzyme is also much more sensitive to inhibition by both nitrite and NO than is the exchange activity of Ni-hydrogenases [104]. These reagents have yet to be tested with the *Cp* enzymes.

IV-C. Iron and acid labile sulfide content

The iron content of the Fe-hydrogenases, notably in the *Cp* and *Dv*H enzymes, has been and continues to be the subject of much debate. Determining the Fe content of a protein might appear to be rather simple, since very sensitive colorimetric assays based on the chelation of Fe by phenanthroline or bipyridyl derivatives have been available for many years (e.g., Ref. 31). However, in addition to suitable controls for buffers, salts, reagents, etc., a reliable Fe determination depends upon (a) a pure protein, (b) accurate quantitation of the protein, (c) an accurate molecular weight for the protein, (d) the absence of apoprotein (or protein lacking a full complement of Fe), and (e) total release of Fe from the protein. The problems of determining accurately the acid labile sulfide (S^{2-}) content of Fe-S proteins by methylene blue formation have been well documented (see Ref. 164) and will not be reiterated here, except to note that prerequisites (a)–(d) also apply.

In hindsight, the iron and acid-labile sulfide contents reported for the Fe-hydrogenases over the years appear to have lacked some or all of these considerations. For example, the first reasonably pure preparations of *Cp* hydrogenase I were obtained in the mid-70's, and these contained 11–12 gatoms/mol of both Fe and S^{2-} [56,145]. These were based on a molecular weight of 60 000, and the Lowry colorimetric method [165] was used to determine protein concentration. The enzyme preparations exhibited a specific activity in the H_2 evolution assay of about 500 units/mg (using 1 mM methyl viologen, where 1 unit = 1 μmol H_2 evolved/min). Quite remarkably, these Fe and S^{2-} values went unchallenged for over 10 years, in spite of the fact that the specific activity of the enzyme preparations had increased and the results of several spectroscopic studies (including many involving the author of this paper) were inconsistent with the Fe and S^{2-} values (see below and Ref. 163). Thus, we recently analyzed six preparations of *Cp* hydrogenase I, which had specific activities of about 900 units/mg, and they were found to contain 14.7 ± 0.4 and 13.0 ± 0.9 gatoms/mol of Fe and S^{2-}, respectively [146]. However, quantitative amino acid analysis revealed that the Lowry method of protein determination, using bovine serum albumin as the reference, overestimated the amount of protein in hydrogenase I samples by a factor of 1.37. (It should be added that amino acid and iron analyses can be performed on the same acid hydrolysates, thereby ensuring that the iron is fully released from the protein.) Correcting for this error, and using the molecular weight determined by physical methods and amino acid analysis (61 144), hydrogenase I contains 20.1 ± 0.7 and 17.8 ± 1.2 gatoms/mol of Fe and S^{2-}, respectively, values almost twice those relied upon for so many years.

The Fe content of *Cp* hydrogenase II has undergone a similar transformation. The original preparations of this enzyme had specific activities in the H_2 oxidation assay of about 3000 units/mg and they contained about 8 gatoms/mol of both Fe and S^2-based on the Lowry protein assay [134]. These values were also used for the interpretation of data from several spectroscopic studies (see below). An evaluation of three more recent preparations with specific activities of about 3400 units/mg gave values of 10.6 ± 0.5 and 8.8 ± 0.2 gatoms/mol of Fe and S^{2-}, respectively [146]. However, quantitative amino acid analysis showed that the Lowry method also

overestimates the amount of protein in preparations of this enzyme, this time by a factor of 1.30 (the calculated molecular weight was 54738 [146]). Thus, hydrogenase II contains 13.8 ± 0.4 and 11.4 ± 0.2 gatoms/mol of Fe and S^{2-}, respectively. These values, together with the revised values for hydrogenase I, are listed in Table I (the activities of the two enzymes are also based on quantitative amino-acid analysis to determine protein concentration).

*Dv*H hydrogenase was originally reported [148,151] to contain approximately 12 gatoms/mol of both Fe and S^{2-}, based on colorimetric protein measurements and a molecular weight determined by physical methods. These values have now been revised by two different groups. Hagen et al. [166] analyzed nine preparations of the enzyme using a colorimetric method (microbiuret) to determine protein concentration, and they based their results on a molecular weight of 59313 (from the complete gene sequences of the two subunits). They found that the Lowry method overestimated the protein concentration by 1.24-fold, and that the enzyme contained 15.3 ± 1.3 and 13.1 ± 1.3 gatoms/mol of Fe and S^{2-}, respectively. Based on the revised molecular weight of 55800 for this enzyme [159], one can correct these values to 14.4 ± 1.2 and 12.3 ± 1.2 gatoms/mol, respectively. More recently, Patil et al. [153] analyzed four preparations of the *Dv*H enzyme using quantitative amino acid analysis to determine protein concentration (they obtained good agreement between the determined amino acid content and the composition derived from the gene sequences). The Fe values (S^{2-} was not determined) were 10 ± 1 gatoms/mol, based on a molecular weight of 56000. However, they also found that the Lowry method overestimated the protein content of the enzyme preparations by a factor of 1.20, thus validating the method of protein determination used by Hagen et al. [166]. The current value for Fe content of *Dv*H hydrogenase is therefore between 9 and 15 gatoms/mol, with the S^{2-} content probably about 85% of the Fe value [166].

The iron content of *Me* hydrogenase has also been recently revised. This enzyme was originally reported to contain about 12 gatoms/mol of both Fe and S^{2-} [148], but Hagen, Veeger and co-workers [150] have now shown that highly purified preparations of the enzyme contain 15.6 ± 2.7 and 15.5 ± 2.4 gatoms/mol of Fe and S^{2-}, respectively. To calculate these values, protein concentrations were measured by a colorimetric method and a molecular weight of 58000 (based on sedimentation equilibrium analysis [150]) was used rather than the previous value of 50000 (based on SDS-gel electrophoresis [148]).

The prerequisites listed above for an accurate determination of Fe and S^{2-} contents have therefore not been satisfied with any of the Fe-hydrogenases. The values for the hydrogenases of *Me* and *Cp* are limited by the lack of accurate molecular weights (their genes have not been isolated) and those for the *Me* enzyme are based on a colorimetric protein assay. The values for *Dv*H hydrogenase do not suffer from these shortcomings, but very different results have been obtained by two research groups. Moreover, the *Dv*H enzyme is purified aerobically and there is evidence for oxidative degradation/interconversion of its Fe-S cluster(s) (see subsection V-A). It is therefore likely that some apoprotein is present in purified preparations, i.e., protein lacking a full complement of Fe. As discussed below, the Fe (and S^{2-}) values are critical in rationalizing the spectroscopic properties of these enzymes. Conversely, an analysis of the spectroscopic data can shed light on the validity of the current values. As an aside, it should be mentioned that ribonucleotide reductase from *E. coli*, an enzyme that has been extensively characterized over the last two decades, has recently been found to contain twice the amount of iron previously assumed (four instead of two atoms/molecule). Like the *Cp* hydrogenases, a re-evaluation of the method used to determine the protein concentration of this enzyme was prompted by an inconsistency between spectroscopic data and the apparent iron content [167].

IV-D. Electron paramagnetic resonance spectroscopy

IV-D.1. C. pasteurianum hydrogenase I

Over the years, EPR has proved to be an exceptionally useful spectroscopic probe of the number and types of Fe-S clusters in Fe-hydrogenases. EPR spectra obtained from the oxidized and reduced forms of *Cp*

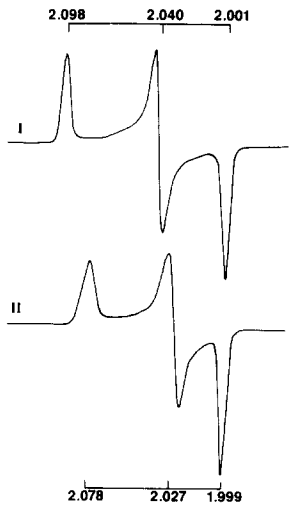

Fig. 1. EPR spectra of oxidized hydrogenase I (I) and oxidized hydrogenase II (II) of *C. pasteurianum*. Each enzyme (about 100 μM) in 50 mM Tris-HCl buffer (pH 8.0) was oxidized anaerobically with a slight excess of thionine prior to freezing in liquid N_2. The temperature and microwave power settings were: I, 20 K and 10 mW; II, 40 K and 5 mW. The instrument settings were: modulation amplitude, 0.8 mTesla; time constant, 0.128 s; scan time, 600 s; microwave frequency, 9.23 GHz.

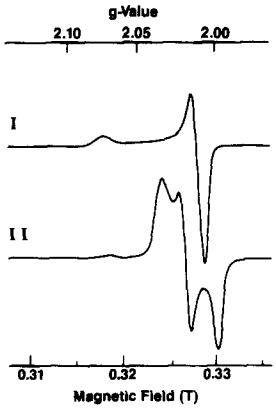

Fig. 2. EPR spectra of oxidized hydrogenase I (I) and oxidized hydrogenase II (II) of *C. pasteurianum* after treatment with CO. Samples were prepared as in Fig. 1 except that they were exposed to CO (1 atm) for 1 min prior to freezing in liquid N_2. The spectra were recorded using 1 mW microwave power at 18 K (I) and 14 K (II). The spectrometer settings were: modulation amplitude, 0.8 mTesla; time constant, 0.128 s; scan time, 600 s; microwave frequency, 9.23 GHz.

hydrogenase I were first reported in the mid-70's from the groups of Orme-Johnson [168] and Mortenson [145], and almost identical spectra have been reported since then [137,163,169–172]. The oxidized form, prepared either by the anaerobic addition of a dye, e.g., thionine ($E_{m,7} = 60$ mV), or by allowing the enzyme to 'auto-oxidize' by evolving H_2 under Ar, gives rise at 10 K to a sharp and narrow rhombic signal with g values of 2.098, 2.040 and 2.001 (henceforth referred to as the rhombic 2.10 signal: Fig. 1). Although significantly broadened, the spectrum is observed at 70 K [163]. The lineshape and temperature dependence of the spectrum is unlike that of any other Fe-S cluster. The quantitation of this EPR signal was a source of controversy for many years, since it was found to represent only about 0.6 spins/mol, rather than one [145,163]. However, these results were based on colorimetric protein determinations, and a re-evaluation based on quantitative amino acid analyses [146] gave values (0.84 ± 0.06) that were closer to, though still less than, one spin/mol. Redox titrations have shown that the species giving rise to the rhombic EPR signal had a midpoint potential at pH 8 ($E_{m,8}$) of about -400 mV, and that the signal remained unchanged up to $+200$ mV [163]. Above this potential, the signal intensity decreased with a corresponding decrease in activity, indicative of cluster degradation. A very recent examination of the thionine-oxidized enzyme using high protein concentrations and high microwave power has revealed additional low field EPR resonances at $g = 6.1$ and 5.0 [172]. The relative intensities of these resonances were strongly temperature-dependent, and they were shown to arise from the lower and upper zero-field doublets of an $S = 3/2$ ground state, interpreted by a conventional spin Hamiltonian ($E/D = 0.15$, $D = -3.4$ cm^{-1}). However, spin quantitations showed that the $S = 3/2$ species is a minor component representing < 0.05 spins/mol [172].

Oxidized *Cp* hydrogenase I therefore exhibits a rhombic 2.10 EPR signal, apparently arising from an $S = 1/2$ system, and this represents almost one spin. A key in attempts to understand the nature of the species giving rise to this signal has been the effect of the inhibitor, CO. First reported by Erbes et al. [168], the addition of CO to the oxidized enzyme quantitatively converts the rhombic 2.10 EPR signal to an axial signal, $g_{\|} = 2.074$, $g_{\perp} = 2.011$ (the 'axial 2.07', Fig. 2: [137,163,169–171]). The axial 2.07 signal saturates much more readily than the rhombic 2.10 signal but can be readily observed at 100 K [163]. Titrations have shown that the oxidized enzyme has an extremely high affinity for CO: half-maximal intensity of the axial 2.07 signal is induced by a CO concentration (in solution) of only 1.1 μM (Table II: Ref. 163). A puzzle for several years was that the axial 2.07 signal was also observed upon treatment of the enzyme with excess oxidant in the *absence* of CO [137]. This has recently been resolved [171] by the finding that the addition of low concentrations of O_2 to the oxidized enzyme induces an axial 2.07 EPR signal with the same relaxation properties (and photolytic properties, see below) as the CO-induced signal. The appearance of this signal in the absence of CO is therefore due to trace O_2 contamination, since only the rhombic 2.10 signal is observed after oxidation of the enzyme under rigorously anaerobic conditions or under the controlled conditions of a redox titration [163]. The axial 2.07 signal, induced either by CO or O_2, is photosensitive at low temperatures [171]. That is, illumination (at 8 K) led to dissociation of the bound CO or O_2 from the EPR-active center and the appearance of the rhombic 2.10 signal. Upon raising the temperature (to 200 K) and then re-analyzing the sample at 8 K, the axial 2.06 signal reappeared indicating recombination of the dissociated ligand. Curiously, the dominant product after illuminating the CO- (or O_2-) treated enzyme at 30 K was a new rhombic EPR signal, with $g \approx 2.26$, 2.12 and 1.89 (the 'rhombic 2.26 signal'). The rhombic 2.26 signal is unlike that seen from any other Fe-S cluster, and it was suggested that it arose by a unique photo-induced redox process [171]. The photolytic behavior serves to emphasize the novel properties of the center that exhibits the rhombic 2.10 EPR signal (see below).

In contrast to the novel EPR signals of oxidized *Cp* hydrogenase I with and without CO, the reduced enzyme (as isolated in the presence of excess sodium dithionite) exhibits EPR absorption indicative of multiple $[4Fe\text{-}4S]^{1+}$ clusters of the type found in 4Fe-ferredoxins (Fig. 3: [137,145,163,168–172], note that in the earlier papers the enzyme was probably not fully reduced, see Ref. 163). This spectrum was originally found to represent about 1.8 spins/mol [145]. Since the en-

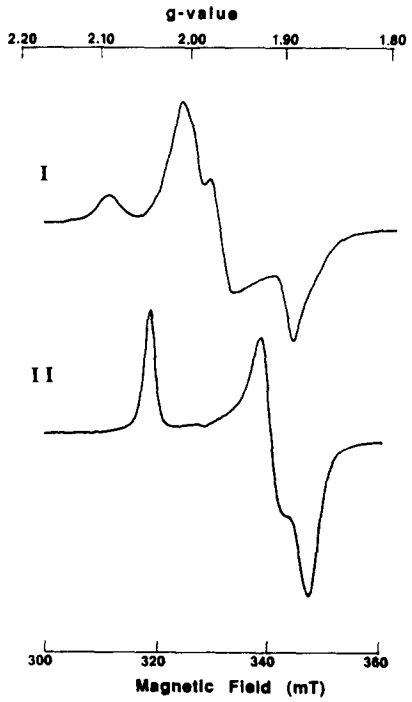

Fig. 3. EPR spectra of reduced hydrogenase I (I) and reduced hydrogenae II (II) of *C. pasteurianum*. The enzymes (about 100 μM) were used as isolated in 50 mM Tris/HCl buffer, pH 8.0, containing sodium dithionite (1 mM) and were maintained under H_2 (1 atm) prior to freezing.[1] The temperature and microwave power settings were: I, 15 K and 10 mW; II, 14 K and 1 mW. The instrument settings were: modulation amplitude, 0.8 mTesla; time constant, 0.128 s; scan time, 600 s; microwave frequency, 9.23 GHz.

zyme was thought to contain 12 Fe and 12 S^{2-} atoms, it was proposed that 8 of these are in form of two ferredoxin-type [4Fe-4S] clusters, while the remaining comprise a different type of [4Fe-4S] cluster, which is involved in H_2 catalysis and gives rise to the novel 2.10 EPR signal of the oxidized enzyme [55,145,173]. Like the Fe content, this was the working hypothesis for several years. However, more recent analyses [146,174] have shown that the spin content of the EPR signal of the reduced enzyme is about 2.6 spins/mol (based on colorimetric protein assays), suggesting that the enzyme should contain more than the two ferredoxin-type 4Fe-clusters suggested by the Fe analysis. After correcting for the overestimation of the protein content of the samples by the colorimetric assay, the reduced enzyme contains 3.58 ± 0.25 $[4Fe\text{-}4S]^{1+}$ clusters per molecule [146]. These clusters could not be distinguished by redox potentiometry, since their $E_{m,8}$ values were all -420 mV [163].

Additional EPR absorption at low field has recently been observed from reduced *Cp* hydrogenase I by Johnson and co-workers [172]. Resonances at $g = 5.80$ and 4.76 were identified, and on the basis of their temperature dependence, they were assigned to different Kramers doublets of an $S = 3/2$ spin system (where $E/D = 0.15$ and $D = -2.8 \pm 0.3$ cm^{-1}). Since variable temperature MCD (see subsection V-A.2) showed that the reduced enzyme contained $[4Fe\text{-}4S]^{1+}$ clusters of both the $S = 1/2$ and $S > 1/2$ type, the observed EPR was assigned to $S = 3/2$ $[4Fe\text{-}4S]^{1+}$ clusters. Quantitation of the low-field signals gave values in the range of 0.6–1.4 spins/mol [172].

Thus, to summarize (see Table III), *Cp* hydrogenase I contains about 20 Fe atoms/molecule. Of these, 16 are proposed to be in the form of four ferredoxin-type [4Fe-4S] clusters, or 'F' clusters. Three of the F clusters have an $S = 1/2$ ground state in the reduced enzyme and give rise to the EPR spectrum shown in Fig. 3. The remaining reduced F cluster appears to exist in a mixed spin state, with approximately equal amounts of $S = 1/2$ and $S = 3/2$ forms. This is not without precedent, since a single $[4Fe\text{-}4S]^{1+}$ cluster existing in these different spin states has been found in several other proteins, including nitrogenase Fe protein [175], glutamine phosphoribosylpyrophosphate amidotransferase of *Bacillus subtilis* [176], and in ferredoxins from *D. africanus* (Fd III: Ref. 177) and *Pyrococcus furiosus* (Conover, R.C., Park, J.-B., Adams, M.W.W. and Johnson, M.K., unpublished results). The remaining Fe in hydrogenase I is proposed to comprise a novel type of Fe-S cluster, termed the 'hydrogenase' or 'H' cluster, which exhibits the rhombic 2.10 EPR signal of the oxidized enzyme.

TABLE III

EPR properties of iron-hydrogenases

Hydrogenase	Fe (atoms/molecule)	Reduced (spins/mol)		Oxidized (spins/mol)		Oxidized + CO (spins/mol)	Fe as F clusters (atoms/molecule)	Fe as H cluster (atoms/molecule)
		$S=1/2$	$S=3/2$	$S=1/2$	$S=3/2$	$S=1/2$		
Cp I	20	3.6	0.5 (?)	0.84	< 0.03	0.81	16	4 (6?) [a]
Cp II	14	1.7	n.d.[b]	1.00	n.d.	0.95	8	6
*Dv*H	9–15	1.5	n.r.[c]	> 0.4 (?)	n.r.	0.95	8	1–7
Me	13–18	1.5–2.5	n.r.	0.85–1.1	n.r.	n.r.	8	5–10

[a] See text for discussion.
[b] n.d., none detected.
[c] n.r., not reported.

Before considering the nature and function of the H cluster, the EPR properties of the other Fe-hydrogenases will be described.

IV-D.2. C. pasteurianum hydrogenase II

Oxidized *Cp* hydrogenase II also exhibits a sharp rhombic 2.10-type EPR signal (Fig. 1: Refs. 134, 163, 172, 178). However, in contrast to the homogeneous rhombic 2.10 signal of hydrogenase I, that of hydrogenase II is a mixture of almost identical species, the relative amounts of which are pH-dependent [163]. They are differentiated by their relaxation properties: one form ($g = 2.088, 2.033, 1.999$) is clearly seen only below 20 K, while the other ($g = 2.078, 2.027, 1.999$) is apparent even at 100 K [163,172]. The complete EPR absorption of oxidized hydrogenase II (< 20 K) was originally found to represent about 0.7 spins/mol [163], but after correcting for protein concentration [146] this becomes 1.00 ± 0.06 spins/mol. In accord, no additional EPR resonances are observed from the oxidized enzyme at low field [172]. The $E_{m,8}$ value for the rhombic 2.10 signal was estimated at -400 mV, the same as hydrogenase I [163].

The addition of CO to oxidized hydrogenase II results in the loss of the rhombic 2.10-type EPR signal and the appearance of a new rhombic signal ($g = 2.032, 2.017, 1.988$: Ref. 163, see Fig. 2), the lineshape of which is independent of pH [163]. This conversion by CO is quantitative since the latter signal represents 0.95 ± 0.04 spins/mol (after correction: Ref. 146). The CO-induced signal relaxes much faster than the rhombic 2.10-type signal, in contrast to the effect of CO on oxidized hydrogenase I [163]. However, like hydrogenase I, the CO-induced signal of oxidized hydrogenase II is also induced by low concentrations of O_2, and the CO- (O_2-) induced signal is photosensitive (Kowal, A.T., Adams, M.W.W. and Johnson, M.K., unpublished data). Illumination at 8 K leads to the dissociation of the CO (O_2) and the appearance of the rhombic 2.10-type EPR signal of the oxidized, untreated enzyme. Moreover, illumination of CO-treated, oxidized hydrogenase II also generates the rhombic 2.26 EPR signal seen after photolysis of CO-treated oxidized hydrogenase I [171], although the two enzymes differ in the temperature dependence of its formation. This indicates that the photoinduced redox process proposed for hydrogenase I [171] also occurs with hydrogenase II, and demonstrates the similarity in the structure and properties of their H clusters.

Cp hydrogenase II in its reduced state (as isolated in the presence of excess sodium dithionite) exhibits an EPR spectrum at 14 K typical of an $S = 1/2$ $[4Fe\text{-}4S]^{1+}$ cluster (Fig. 3; Ref. 134). Quantitation of this resonance ($g = 2.06, 1.93, 1.90$) gave values of 1.07 ± 0.06 spins/mol (after correction [145,172]). Its $E_{m,8}$ value was estimated at -180 mV [163]. However, at lower temperatures, an additional underlying and very broad resonance becomes apparent from a more rapidly relaxing species (Fig. 4 [163,172]). The complete spectrum recorded at 4 K represents about 1.7 spins/mol (after correction) and no additional low-field resonances were detected [172], indicating that the reduced enzyme contains two paramagnetic $S = 1/2$ centers. Parallel MCD analyses [172] showed that both centers comprise $[4Fe\text{-}4S]^{1+}$ clusters (see below). Thus, like *Cp* hydrogenase I, hydrogenase II exhibits EPR absorption from reduced F clusters, and in this case these represent 8 of the 14 Fe atoms present in the enzyme. The remaining Fe is again proposed to constitute a novel H cluster, which, from the EPR, redox and photolytic properties of oxidized hydrogenase II, both with and without CO, would appear to more or less identical to the H cluster of hydrogenase I (Table III).

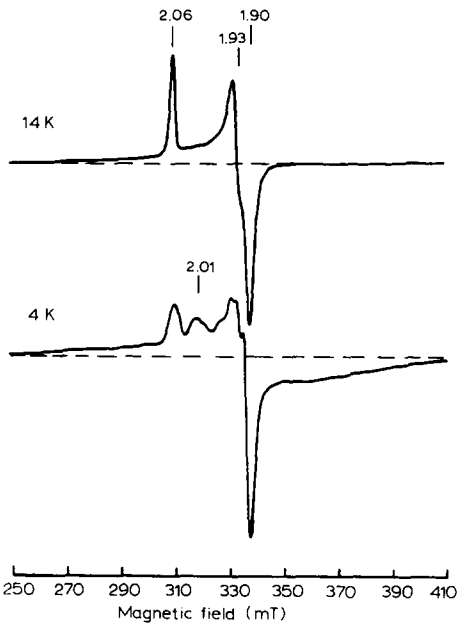

Fig. 4. EPR spectra of reduced hydrogenase II of *C. pasteurianum*. The sample was prepared as in the legend to Fig. 3 except that the buffer contained ethylene glycol (50%, v/v). The conditions of measurement were: microwave power, 1 mW; modulation amplitude, 0.63 mTesla; time constant, 0.128 s; scan time, 600 s; microwave frequency, 9.40 GHz; temperature, 4 K and 14 K as indicated. Both spectra were recorded at the same spectrometer gain (adapted from Ref. 172).

IV-D.3. M. elsdenii hydrogenase

The EPR properties of *Me* hydrogenase appear to be more or less the same as those of *Cp* hydrogenase I, except in respect to quantitation. The reduced *Me* enzyme (as isolated) exhibits EPR absorption typical of $[4Fe\text{-}4S]^{1+}$ clusters, and this was originally reported to represent about 1.5 spins/mol, while the oxidized form gives rise to a rhombic 2.10-type spectrum ($g = 2.101, 2.052, 2.005$) which accounts for about 0.6 spins/mol [136,149]. In a more recent report [150], similar spin quantitations were obtained using enzyme samples that

were estimated to be 70–80% pure. By extrapolating to 100% purity, the spin concentration for the oxidized and reduced forms of the enzyme ranged from 1.5 to 2.5 and from 0.85 to 1.1 spins/mol, respectively. From the former value it was concluded that the enzyme contains two F clusters, while the H cluster is comprised of the remaining iron (some 5–10 atoms/molecule). However, from the near identity of the EPR spectra of the *Me* enzyme and those of *Cp* hydrogenase I, it would seem likely that they have the same Fe-S cluster composition, i.e. four F clusters. This cannot be ruled out at present, since the data for *Me* hydrogenase were based on a colorimetric protein assay.

IV-D.4. D. vulgaris (strain Hildenborough) hydrogenase

The EPR properties of *Dv*H hydrogenase are much more complex than those of the *Cp* and *Me* enzymes, but common properties are beginning to emerge. A complicating factor is that the *Dv*H enzyme is purified aerobically and appears to undergo reductive activation [155]. For example, the *Cp* and *Me* enzymes, as isolated in a reduced and fully active state, exhibit EPR absorption from reduced F clusters. These signals disappear upon anaerobic oxidation to give the rhombic 2.10 signal of the oxidized H cluster (which is quantitatively converted to the axial 2.07-type signal upon addition of CO). Re-reduction with H_2 or sodium dithionite is immediate and leads to complete loss of the rhombic 2.10 signal and the reappearance of the EPR signal of the as isolated enzyme. During this redox cycling there is no change in the catalytic activity of the *Cp* hydrogenases, as measured by either H_2 evolution of H_2 oxidation [163]. In contrast, *Dv*H hydrogenase as isolated in an oxidized O_2-stable state exhibits an EPR signal typical of an oxidized 3Fe-cluster. The amount seems to vary with the enzyme preparation and represents up to 0.2 spins/mol [133,143,153,179,180]. Treatment of the enzyme with excess oxidant (potassium ferricyanide) leads to an increase in signal intensity, with a concomitant decrease in catalytic activity [143]. Since 3Fe-clusters can be generated by the oxidative degradation of 4Fe-clusters [181], the finding of less than stoichiometric amounts of a 3Fe-cluster in the *Dv*H enzyme suggests that it is a degradation artifact of the isolation procedure.

Reduction of *Dv*H hydrogenase with sodium dithionite or by prolonged incubation under H_2 (up to 48 h [133,136,179,180]) leads to the appearance of an EPR spectrum typical of reduced F clusters (1.4–1.7 spins/mol). Superimposed upon this is an axial EPR signal ($g = 2.06$, 2.00), accounting for about 0.03 spins/mol, that is very similar to the axial 2.07 signal of CO-treated, oxidized hydrogenase I of *Cp*. Huynh et al. [133] found that the EPR signal from the F clusters disappeared and the axial 2.07-type signal increased in intensity (to about 0.3 spins/mol) upon re-oxidation of the *Dv*H enzyme with DCPIP ($E_{m,7} = +217$ mV), or by the addition of CO. In contrast, Hagen et al. [180] found that the re-oxidation of this enzyme (by allowing it to autooxidize under Ar) did not increase the axial 2.07-type signal, but led to the appearance of a rhombic EPR signal ($g = 2.11$, 2.05, 2.00: about 0.1 spin/mol), identical to the rhombic 2.10 signal of oxidized hydrogenase I. Moreover, both they [180] and we [137] attributed the appearance of the axial 2.07-type signal from the *Dv*H enzyme in the absence of CO to incomplete anaerobicity during re-oxidation. As discussed in subsection V-B.2, this conclusion is supported by our data with *Cp* hydrogenase I, which show that both O_2 and CO convert the rhombic 2.10 signal to the axial 2.07 signal [171]. Subsequent work with *Dv*H hydrogenase by Patil et al. [162] showed that the axial 2.07-type signal is maximally induced to 0.94 spins/mol by very low CO concentrations. In addition, they first demonstrated (with the *Dv*H enzyme) that the axial 2.07-type EPR signal is photosensitive [162]. More recent analyses have shown that, like the two *Cp* hydrogenases, illumination of CO-treated *Dv*H hydrogenase at 30 K also generates the rhombic 2.26-type EPR signal (Kowal, A.T., LeGall, J. and Johnson, M.K., unpublished data).

*Dv*H hydrogenase therefore exhibits the EPR signals characteristic of the *Cp* (and *Me*) hydrogenases, namely, a complex signal from reduced F clusters, the rhombic 2.10-type signal from an oxidized H cluster, the photosensitive axial 2.07-type signal from the oxidized enzyme treated with CO (or inadvertently with O_2), and the rhombic 2.26-type signal as a product of photolysis. The EPR data from the *Dv*H enzyme is complicated, however, by the quantitation of these signals and also by the finding of additional EPR resonances. For example, Patil et al. [153] have shown that during reductive titration of the as isolated *Dv*H enzyme, the rhombic 2.10 signal increased in intensity and reached a maximum of 0.4 spins/mol at -300 mV. This signal abruptly disappeared at lower potentials, with the concomitant appearance of the complex signal of the reduced F clusters ($E_{m,7} \approx -305$ mV). However, a new rhombic EPR signal was observed at higher potentials ($g = 2.06$, 1.96, 1.89: the 'rhombic 2.06') which reached a maximum of 0.7 spins/mol at -110 mV before decreasing.

If one assumes that the F clusters in the *Dv*H enzyme are of the conventional type, i.e., undergo a simple $1e^-$ redox reaction, and that the oxidized H cluster in its active, catalytic state exhibits the rhombic 2.10 signal, the data of Patil et al. [153] suggest that the following sequences of events occur during the 'reductive titration'. The H cluster in the as isolated enzyme appears to be in an inactive conformation, which is EPR silent and perhaps has O_2 bound (the oxidized 'pre-H cluster'). Upon reduction, the pre-H cluster becomes EPR active and exhibits the rhombic 2.06 signal, but it remains catalytically incompetent. Some form of

cluster rearrangement then occurs in which the reduced pre-H cluster converts to the oxidized H cluster (without further reduction), perhaps involving release of O_2. The catalytically-active oxidized H cluster then gives rise to the rhombic 2.10 signal. At this point the enzyme is fully active and is equivalent to the anaerobically-oxidized forms of the *Cp* and *Me* hydrogenases. That is, upon subsequent reduction of the enzyme, the H cluster and each F cluster accept one electron to give the EPR silent reduced H cluster and EPR active reduced F clusters.

Unfortunately, the 'reductive titration' experiments of Patil et al. [153] do not discriminate between reductive activation of the as isolated enzyme (involving cluster modification and/or interconversion) and a conventional (reversible) redox titration. The analysis presented above suggests that incubation of the as isolated *Dv*H enzyme at about −300 mV would lead to a form of the enzyme identical to that of oxidized *Cp* hydrogenase I, that is, a form that exhibits only a near stoichiometric amount of the rhombic 2.10 EPR signal and shows no lag phase in H_2 evolution assay. Moreover, it predicts that the rhombic 2.06 signal would not be seen upon re-oxidation of the active enzyme under anaerobic conditions. A final point concerns the apparent midpoint potentials of these clusters. From the data of Patil et al. [153], the values for the H cluster and F clusters of *Dv*H hydrogenase are approximately the same, namely −300 mV, although curiously, they were determined using Tris buffer ($pK_a = 8.3$) at pH 7.0 (the actual pH may therefore have been lower). For *Cp* hydrogenase I, the midpoint potentials (in Tris buffer, pH 8.0) of the H and F clusters are also very similar, −400 mV and −420 mV, respectively [163]). The $E_{m,8}$ value for the H cluster of *Cp* hydrogenase II is also −400 mV [163]. Thus, the differences in midpoint potentials of the H clusters of the *Dv*H and *Cp* (I and II) enzymes is very likely a reflection of their pH dependence.

Like oxidized *Cp* hydrogenase I, (reduced and) re-oxidized *Dv*H hydrogenase has also been reported to exhibit low-field EPR absorption. In addition to the rhombic 2.10 signal, a $g = 5$ signal was observed, and this was assigned to a $\Delta m = 2$ transition between the $m_s = \pm 1$ levels of an $S = 2$ system [180]. Moreover, based on the $g = 5$ linewidth, the $S = 2$ species was estimated at 1 spin/mol. As pointed out [172], this is speculative at best, since the low-field resonance is readily rationalized in terms of an $S = 3/2$ system, analogous to the low-field resonances of oxidized *Cp* hydrogenase I [172]. In this case, it represents negligible spin intensity. The overall similarities in the EPR properties of these two hydrogenases would tend to support this, as discussed above.

In summary, the Fe-hydrogenases of *Cp*, *Me* and *Dv*H have many EPR properties in common (Table III). All exhibit EPR absorption from reduced F clusters: the *Cp* II, *Me* and *Dv*H enzymes appear to each have two F clusters, while *Cp* hydrogenase I has four. All the oxidized enzymes exhibit a rhombic 2.10-type EPR signal. This is quantitatively converted to another type of EPR signal (an axial 2.07-type signal with *Cp* I and *Dv*H and a different type of rhombic signal with *Cp* II) upon treatment with CO (or O_2). Illumination leads to dissociation of the CO (O_2) and, depending on the temperature of photolysis, the appearance of a rhombic 2.26 EPR signal. All of the EPR signals of the oxidized enzymes have been assigned to a new type of Fe-S center unique to the Fe-hydrogenases, termed the H cluster. The remainder of this review will address the question, what is the structure and function of the H cluster?

V. The novel Fe-S cluster of Fe-hydrogenases

V-A. Structure of the H cluster

V-A.1. ENDOR and Mössbauer spectroscopy

The unusual EPR signals of the oxidized Fe-hydrogenases, first observed in the mid-70's [145,168], were always assumed to arise from an Fe-containing center, but this was not actually demonstrated until 1984 by Hoffman, Münck and colleagues [169]. It was shown that the rhombic 2.10 EPR signal of *Cp* hydrogenase I, enriched with ^{57}Fe ($I = 1/2$) by purifying the enzyme from ^{57}Fe-grown cells, exhibits two well-resolved ^{57}Fe ENDOR resonances, i.e., the paramagnetic H cluster comprises two magnetically-distinct types of Fe atom. The unique nature of this center was shown by its ^{57}Fe hyperfine coupling constants ($A_1 = 17$ MHz, $A_2 = 9.5$ MHz), which are substantially smaller than those observed for conventional 2Fe-, 3Fe- or 4Fe-clusters. Mössbauer analysis of the oxidized enzyme showed that the majority ($\approx 70\%$) of the Fe in the oxidized enzyme was present as $[4Fe\text{-}4S]^{2+}$ (F) clusters. However, the small magnitude of the A values for the H cluster meant that the magnetic Mössbauer spectrum of the oxidized cluster was hidden under the strong quadrupole doublet of the oxidized F clusters. For the same reason, Huynh et al. [133] found that the nature of the H cluster in oxidized *Dv*H hydrogenase could not be determined using Mössbauer spectroscopy.

A more detailed ENDOR investigation of oxidized *Cp* hydrogenase I [170] showed that a ^{13}C resonance was associated with the axial 2.07 EPR signal generated by treating the oxidized enzyme with ^{13}C-labelled CO ($I = 1/2$). The ^{13}C coupling constant was about 21 MHz. The ^{57}Fe coupling constants associated with the rhombic 2.10 EPR signal of the oxidized enzyme were shown to change dramatically and in opposite directions upon induction of the axial 2.07 EPR signal by CO ($A_{1C} = 30$–34 MHz, $A_{2C} = 6$ MHz). These results

indicate that the oxidized H cluster remains intact upon binding a single CO molecule, and that CO binding causes a change in the spin distribution within the cluster. This is consistent with the photolysis results described above, which suggest that CO simply binds to and (photo)dissociates from the oxidized H cluster without structural modification.

An analogous ENDOR study with *Cp* hydrogenase II [178] revealed the great similarity between the oxidized H clusters in the two *Cp* enzymes. For example, the rhombic 2.10 signal of oxidized hydrogenase II also gave rise to two ^{57}Fe resonances ($A_1 = 18$ MHz, $A_2 = 7$ MHz) and these also changed upon the binding of a single CO molecule ($A_{1C} \approx 30$ MHz, $A_{2C} \approx 9.5$ MHz). The major difference between the two hydrogenases is that the A^C value for hydrogenase II was 50% larger (about 34 MHz). Unfortunately, the A^C and A^{Fe} values obtained with both enzymes did not allow the determination of the details of CO binding. All hyperfine values fall within the range observed for inorganic iron-carbonyl complexes [182], in which several types of CO binding have been proposed. These include terminal CO binding to a single Fe atom, symmetrical and asymmetrical bridging between two Fe atoms, and even bridging between three Fe atoms. Moreover, the terminal binding of CO to the Fe atom of an Fe-S cluster could occur either by addition, ligand displacement, or by insertion into an Fe-ligand bond.

The ^{1}H-ENDOR signals from the two *Cp* hydrogenases in their oxidized states, both with and without CO, provided further evidence for a lack of disruption of the H cluster upon CO binding [170,178]. For example, changes in the resonances were observed upon CO binding, but the coupling constants remained small (< 7 MHz), and were similar to those observed for other Fe-S clusters, where they can be reasonably assigned to the β-protons of coordinating cysteine residues. It had previously been suggested [183,184] that the novel EPR signals of oxidized *Dv*H and *Me* hydrogenases arose from a sulfur radical species, analogous to the cysteinyl disulfide proposed to exist in a superoxidized state of a ferredoxin [185]. This was based on the observations that all of these proteins exhibit $g_{av} > 2$ EPR spectra that are seen at high temperature and, in contrast to conventional paramagnetic Fe-S clusters, have negligible low-temperature MCD (see below). However, the low ^{1}H hyperfine coupling values from the oxidized *Cp* hydrogenases are inconsitent with a cysteinyl sulfide or disulfide radical as a source of the observed EPR signals, since these would give rise to proton couplings above 35 MHz [186].

The ENDOR studies of the *Cp* hydrogenases therefore show that in both enzymes, the H cluster is comprised of two types of Fe site, and it covalently binds a single CO molecule. This binding causes almost identical changes in the magnetic properties of the Fe subsites

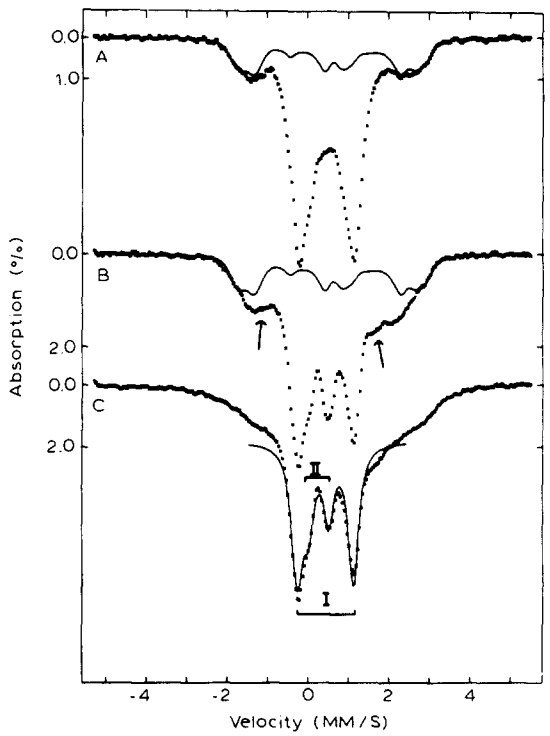

Fig. 5. Mössbauer spectra of the reduced and intermediate states of ^{57}Fe-enriched hydrogenase II of *C. pasteurianum*. (A) Spectrum of the intermediate state (the sample was poised at about −300 mV, pH 8.0) taken in a 0.05 T parallel field (the corresponding EPR spectra are given in Fig. 6, see text for details). (B) Spectrum of the reduced enzyme recorded in 0.05 T parallel field and (C) in zero field. The solid lines in (A) and (B) are theoretical spectra for the $g = 1.94$ form of a $[4Fe\text{-}4S]^{1+}$ center, normalized to 30% of the total iron. This represents a single F cluster (4Fe out of total of 14Fe). The solid line in (C) represents two quadrupole doublets (indicated I and II) with a relative intensity of 2:1 normalized to 40% of the total iron (6Fe out of a total of 14Fe). See text for details (adapted from Ref. 135).

of the two clusters. Further insight into the structure of this cluster came from a Mössbauer investigation of hydrogenase II [135]. As shown in Fig. 5, the EPR-silent H cluster of the reduced enzyme comprised 40% of the total Fe and exhibited, in zero field, a 2:1 quadrupole pattern very similar to that of reduced 3Fe-clusters. However, whereas the latter are paramagnetic ($S = 2$, evident in the Mössbauer spectrum at fields of 0.01 Tesla), the reduced H cluster was diamagnetic ($S = 0$, up to 6.0 Tesla). The remaining 60% of the Fe in hydrogenase II was assigned to clusters (F) of the $[4Fe\text{-}4S]^{1+}$ type. Redox titrations had shown [163] that the $g = 1.94$ ferredoxin-type EPR signal of reduced hydrogenase II (Fig. 3) disappeared upon oxidation with $E_{m,8} = -180$ mV, whereas the $E_{m,8}$ of the H cluster was −410 mV. Thus, as shown in Fig. 6, a sample poised at about −300 mV exhibits EPR absorption from both the oxidized H cluster and the reduced F cluster. Mössbauer analysis of such a sample (Fig. 5) showed that upon oxidation to this 'intermediate' redox state, approximately half of the paramagnetic compo-

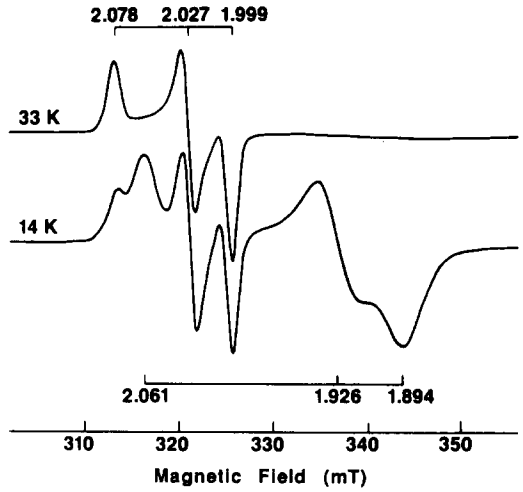

Fig. 6. EPR spectra of the intermediate redox state of *C. pasteurianum* hydrogenase II. Hydrogenase II (95 μM) was prepared in 50 mM Tris-HCl buffer (pH 8.0) containing sodium dithionite (1.0 mM) and methyl viologen (1.0 mM). The enzyme was allowed to autooxidize by H_2 evolution while evacuating and flushing with Ar until the blue solution decolorized, and the sample was then rapidly frozen. This corresponds to a redox potential of about −300 mV (see Ref. 163 for experimental details). EPR spectra were recorded at the indicated temperature using a microwave power of 10 mW. The spectrometer settings were: modulation amplitude, 0.8 mTesla; time constant, 0.128 s; scan time, 600 s; microwave frequency, 9.23 GHz.

nent was lost, indicating that in the reduced enzyme there are two distinct F clusters. The one with $E_{m,8} = -180$ mV could be simulated as a conventional [4Fe-4S] cluster (termed F) while the other, with $E_{m,8} < -300$ mV, represented a [4Fe-4S] cluster in an atypical configuration, and this was termed F′. Note that the F′ cluster is paramagnetic in the reduced enzyme, and the broad, rapidly-relaxing EPR signal (Fig. 4) is now assigned to this cluster. This signal is not apparent in the 'intermediate' state [172], as predicted by the Mössbauer analysis.

Mössbauer analysis [135] of oxidized hydrogenase II treated with CO showed that of the two types of Fe present in the H cluster (shown by the ENDOR study described above), the more strongly coupled resonance ($A_{1C} \approx 30$ MHz) arose from two distinct types of Fe site, one with a positive and one with a negative hyperfine coupling constant. Together they accounted for 26% of the total Fe. Simulations using a $S = 1/2$ spin Hamiltonian yielded hyperfine values of $A_{1C}a = +26.5$ MHz and $A_{1C}b = -30$ MHz (where a and b are the Fe subsites). Moreover, simulations showed that the more weakly coupled site identified by ENDOR ($A_{2C} \approx 9.5$ MHz) represented 13% of the total Fe. These results were therefore in excellent agreement with the data from the reduced enzyme, namely, that the H cluster contains two types of spin-coupled Fe atoms which are in a 2:1 ratio and account for 39% of the total Fe in hydrogenase II.

At the time this study [135] was carried out, hydrogenase II was thought to comprise about 8 Fe atoms/molecule. The H cluster was therefore proposed to be a novel 3Fe cluster, while the remaining Fe was thought to be arranged in a single [4Fe-4S] cluster that existed in two conformations (F and F′). By this analysis the enzyme would actually contain 7Fe/mol. Such an assignment was inconsistent with the EPR data, however, since quantitations of the signals from the oxidized H cluster, both with and without CO, and from the reduced F cluster (the $g = 1.94$-type EPR signal: see Fig. 3) yielded respective values of about 0.7 and 0.9 spins/mol, rather than 1.0 and 0.5 spins/mol as predicted by the Mössbauer analysis. However, the recent revision in the Fe content of hydrogenase II to 14 Fe/mol [146] has allowed complete rationalization of both the Mössbauer and EPR data (see Table III). Thus, 60% of the total Fe (8Fe/mol) is now assigned to two [4Fe-4S] clusters, one F and one F′. The EPR signal of the reduced F cluster represents about 1 spin, while those of F and F′ clusters combined (Fig. 4) account for nearly 2 (1.7) spins/mol. The remaining 40% of the Fe (6Fe/mol) is assigned to the H cluster. This is consistent with the Mössbauer data which show that the H cluster Fe atoms are in a 2:1 ratio and the cluster must comprise a multiple of 3Fe atoms. Since the EPR signal of the oxidized H cluster, with and without CO, now accounts for 1 spin/mol, this cluster is proposed to contain six spin-coupled Fe atoms [146].

Additional Mössbauer analysis of *Cp* hydrogenase I [135] showed the striking similarities between its H cluster and that of hydrogenase II. For example, the more strongly coupled Fe sites of the oxidized H cluster of hydrogenase I were also found to split into two components with positive and negative hyperfine values ($A_{1C}a = +33$ MHz and $A_{1C}b = -33$ MHz). Since the EPR, ENDOR and Mössbauer parameters of the oxidized H cluster, with and without CO, were more or less identical in both hydrogenases, it was concluded [135] that both contain fundamentally the same structure. The photolytic properties of the two H clusters with CO (or O_2) bound further emphasize their identity. Thus, based on a 6Fe-H cluster and four F clusters (Table III), hydrogenase I should contain 22 Fe/mol. This is consistent with the Mössbauer data [169], which indicated that about 70% of the Fe was associated with ferredoxin-type [4Fe-4S] clusters. The finding of about 20Fe/mol in hydrogenase I and slightly less than integral spin values (Table III) suggests either some apoprotein is present in preparations of hydrogenase I, or that its molecular weight is slightly underestimated [146]. However, in spite of the great similarities in the spectroscopic properties of the two *Cp* enzymes, the possibility that their H clusters have a different Fe content cannot be ruled out. The spectroscopic data obtained with hydrogenase I show only that its H cluster must comprise at least 3Fe atoms (two strongly coupled and one weakly coupled site), although 3Fe

would represent only 15% (rather than about 30%) of the total Fe.

To date, *Me* hydrogenase has not been examined by Mössbauer or ENDOR spectroscopy. However, the Fe content and the EPR properties of both the oxidized and reduced forms, including lineshape, temperature dependency and spin quantitations [136,148–150], are very similar to those of *Cp* hydrogenase I *before* the revision in the Fe and protein content of the *Cp* enzyme, e.g., Ref. 145. Based on the current values for the *Me* enzyme (Table III), between 5 and 10 Fe atoms in this enzyme are assigned to the H cluster [150].

ENDOR data are not available for *Dv*H hydrogenase and only preliminary Mössbauer results have been reported [133]. As shown in Table III, the EPR data indicate that the enzyme contains two F clusters. This is supported by the amino acid sequence of the large subunit derived from the gene sequence which shows a region that is homologous to 8Fe-ferredoxins [157], indicating the presence of two [4Fe-4S] clusters. In this regard, it is interesting to note that the genes for *Dv*H hydrogenase have been expressed in *E. coli* [187]. The resulting protein contained F clusters but did not exhibit the EPR signals of the native re-oxidized enzyme, showing that it lacked the H cluster [187]. Presumably, *E. coli*, which does not appear to contain an Fe-hydrogenase, lacks the genetic information to synthesize and/or insert the novel H cluster. The presence of two F clusters (8Fe) in the *Dv*H enzyme leaves 1–7 Fe atoms for the H cluster (Table III). The near identity in the EPR properties of the oxidized H clusters in this hydrogenase and that of *Cp* hydrogenase I again suggest a common or at least very similar structure. These properties include the rhombic 2.10 signal, the axial 2.07-type signal induced by CO and by O_2, the photosensitivity of the CO-bound H cluster, and the photogenerated rhombic 2.26-type EPR signal. Furthermore, a component of the Mössbauer data from the CO-treated *Dv*H enzyme (labelled M_A: see Ref. 133) has parameters very similar to the strongly coupled Fe sites of the oxidized CO-bound H cluster of hydrogenase I [135].

It is therefore tempting to speculate that the H clusters in all four of these hydrogenases, from *Me*, *Dv*H and the two *Cp* enzymes, are the same or have remarkably similar structures. In the case of *Cp* hydrogenase II, all of the available spectroscopic and molecular data are consistent with the cluster comprising six spin-coupled Fe atoms, and there is no evidence to suggest that this is also not the case with the other three enzymes. Taken individually and in isolation, the data on the H cluster of hydrogenase I show that it must comprise $\geqslant 3$ Fe atoms, the results on the *Dv*H enzyme show only that its H cluster contains Fe (1–7 atoms based on Fe content), and no information is available on the H cluster of the *Me* enzyme (5–10 Fe atoms based on Fe content). Coincidentally, Hagen et al. [166] first suggested that the novel Fe-S cluster in *Dv*H hydrogenase might be a 6Fe-cluster, although this was based solely on the determined Fe content (14–16 Fe/mol), after subtracting the Fe in the two [4Fe-4S] clusters that were indicated by the gene sequence.

V-A.2. Magnetic circular dichroism, resonance Raman and electron spin echo spectroscopy

Variable temperature magnetic circular dichroism (MCD) is a particularly powerful technique in discriminating between various paramagnetic Fe-S clusters and in providing information on their electronic properties. For example, a study of reduced *Cp* hydrogenase I first indicated that one of its F clusters had an $S > 1/2$ spin state, and confirmed that the other three F clusters, as well as the F clusters of hydrogenase II (F and F′), were $S = 1/2$ [172]. However, MCD investigations of the oxidized forms of the hydrogenases from *Cp* (Refs. 137, 172; see below), *Me* [183] and *Dv*H [184] showed that there are no detectable temperature-dependent transitions in the visible region that are uniquely associated with the $S = 1/2$ species giving rise to the 2.10 and 2.07-type EPR signals of these hydrogenases. These results for the *Dv*H and *Me* enzymes were originally interpreted as indicating that the 'H cluster' was not an $S = 1/2$ Fe-S cluster and prompted the proposal of a radical species as the source of the observed EPR signals [183,184]. Yet the EPR, ENDOR and Mössbauer data for *Cp* hydrogenases I and II unequivocally establish that the oxidized H cluster, the source of the EPR signals seen from the oxidized enzymes ($\pm$CO), is a novel cluster of spin-coupled Fe atoms that has an $S = 1/2$ spin state. As discussed above, the available data suggest that the same is true for the H clusters of the *Dv*H and *Me* enzymes [169,170,178].

So, why are the MCD data obtained from the oxidized hydrogenases inconsistent with the EPR, ENDOR and Mössbauer results, and why does the paramagnetic Fe-containing H cluster, in contrast to all known types of paramagnetic Fe-S cluster, exhibit negligible MCD intensity in the visible region? Before considering these questions, it is relevant to mention the results of a study of *Cp* hydrogenases I and II by resonance Raman spectroscopy [188]. With this technique, the Raman bands observed correspond to stretching vibrations of bridging and terminal Fe-S bonds within an Fe-S cluster, the frequencies and intensities of which are diagnostic of the cluster type. Hence, as shown in Fig. 7, both oxidized hydrogenases gave rise to bands from their oxidized F clusters, similar to those seen from the [4Fe-4S] clusters found in 8Fe-ferredoxins. However, hydrogenase I also exhibited strong bands near 280 and 390 cm^{-1}, which are seen from [2Fe-2S] clusters but from no other known Fe-S cluster type. Analogous bands were extremely weak, if present at all, in hydrogenase II (Fig. 7). Although these data were the first to

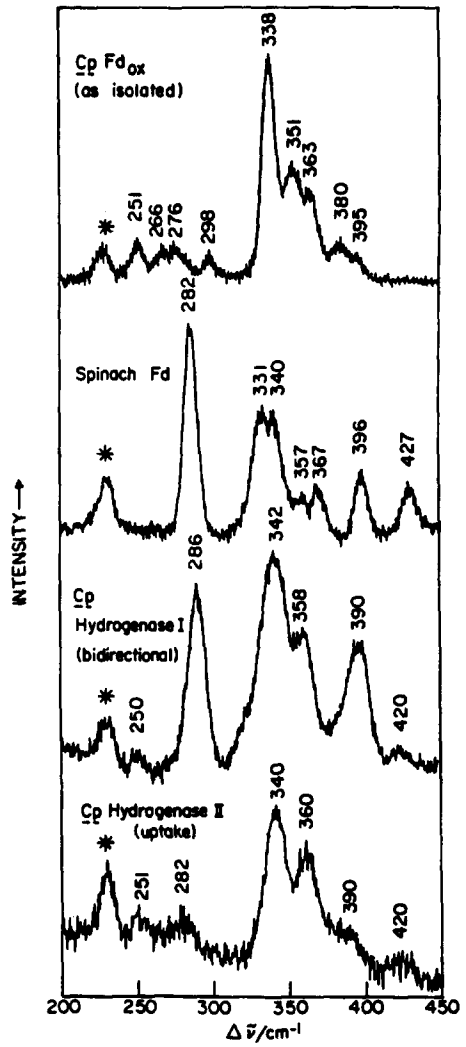

Fig. 7. Low-temperature resonance Raman spectra of oxidized hydrogenase I and oxidized hydrogenase II of *C. pasteurianum*. The hydrogenases were prepared as described in the legend to Fig. 1. Excitation was carried out at 406.7 nm and spectra were recorded at 12 K. The spectra of the $[4Fe\text{-}4S]^{2+}$ clusters of oxidized *C. pasteurianum* ferredoxin (*Cp* Fd_{ox}) and the $[2Fe\text{-}2S]^{2+}$ cluster of oxidized spinach ferredoxin, both recorded at 77 K, are given for comparison (adapted from Ref. 188).

indicate that significant differences do exist between the H clusters of the two *Cp* enzymes, they also contradict the conclusions from the EPR, ENDOR and Mössbauer studies, which show that the H cluster of hydrogenase I is comprised of at least three Fe atoms, and possibly six.

The key to rationalizing all of these spectroscopic data is that the techniques of MCD and resonance Raman are dependent on the visible absorption of the H cluster, and as such are very dependent on the number, type and geometry of the bridging and terminal ligands. That is, results inconsistent with electron and nuclear resonance data might be expected if the H cluster has a degree of non-sulfur coordination, since the absorption of Fe-O and Fe-N bonds in the visible region is greatly decreased compared to Fe-S. Thus, the presence of significant Fe-O/N ligation in the H cluster would dramatically decrease its visible absorption such that, in comparison to homogeneous 'all-sulfur' Fe-S clusters, e.g. F clusters, the H cluster would not be detected by MCD. Similarly, the observed Raman bands might reflect particular Fe-S bonding within and to the cluster [188]. That is, the data would be expected to indicate structural features that comprise the cluster rather than the cluster itself. An arrangement of Fe and S atoms like those in a [2Fe-2S] cluster is therefore a possible component of the H cluster of hydrogenase I (see subsection V-A.4).

What evidence is there for non-sulfur ligands to the H clusters of these hydrogenases? In the case of the *Cp* hydrogenases, this is suggested by their relative Fe, S^{2-} and cysteine contents. For example, hydrogenase II contains more Fe (14 atoms/mol) than S^{2-} (11 atoms/mol) or cysteine residues (12 per mol), and hydrogenase I also contains more Fe (20 atoms/mol) than S^{2-} (18 atoms/mol) but not cysteine residues (21 per mol [145]). Assuming a stoichiometry of $[4Fe\text{-}4S](cys)_4$ for the F clusters, this leaves $[6Fe\text{-}3S](cys)_4$ for the H cluster of hydrogenase II. Although accurate determinations of acid labile sulfide are difficult (see Ref. 164), less S^{2-} than Fe has also been reported in *Dv*H hydrogenase [166]. Sequence analysis shows that this enzyme contains 18 cysteine residues [157], which are all located in the large subunit [159].

More definitive evidence for non-sulfur coordination to the H cluster has recently come from electron spin echo envelope modulation (ESEEM) spectroscopy of the *Cp* hydrogenases (Ref. 189: Thomann, H., Bernardo, M. and Adams, M.W.W., unpublished results). The echo envelope waveforms from oxidized hydrogenase I (exhibiting the rhombic 2.10 EPR signal) and reduced hydrogenase I (exhibiting the $g = 1.94$ ferredoxin-type EPR signal) are shown in Fig. 8. Intense low frequency modulations characteristic of coordinating ^{14}N nuclei ($I = 1$), e.g., Ref. 190, are clearly evident in the spectrum of the oxidized form (from the oxidized H cluster) but not in that of the reduced form (from the reduced F clusters). The frequency of these modulations are revealed by cosine Fourier transformation. For example, Fig. 9 shows the ESEEM spectra of the oxidized enzyme recorded at constant g value using three different microwave frequencies. Analogous spectra have been reported for ^{14}N coordination to an $S = 1/2$ iron-sulfur center (Refs. 191–193; see also Refs. 115–117). For oxidized hydrogenase I, three lines in each of the ESEEM spectra (Fig. 9) are assigned to zero-field nuclear quadrupole transitions, ν_0, ν_- and ν_+, which arise when the local magnetic field is approximately cancelled by the applied field (for discussion, see Ref. 191 and references therein). The other broader lines in Fig. 9 arise from transitions in which the two fields are additive. Although a full analysis of the data will not be

presented here, spectral simulations have indicated a relatively small hyperfine coupling value ($A_{iso} \approx 2$ MHz) and an unusually large quadrupole coupling parameter ($e^2qQ/h \approx 5$ MHz) for the coordinated N (see, for example, Ref. 194). Interestingly, the latter value is inconsistent with direct coordination of an imidazole or peptide N to the H cluster [191,192], which suggests some novel mode of Fe(S)-N ligation, while the small hyperfine value explains why no ^{14}N ENDOR resonances were observed with oxidized hydrogenase I [170]. Preliminary ESEEM data obtained with oxidized hydrogenase II suggest that its H cluster has additional N co-ordination not observed in hydrogenase I. This would be consistent with the resonance Raman results [188], which suggest a difference in the coordination structure of the H clusters in the two *Cp* enzymes. The important point though is that some degree of non-sulfur ligation to the H cluster in these hydrogenases provides some rationalization for the anomalous MCD results described above.

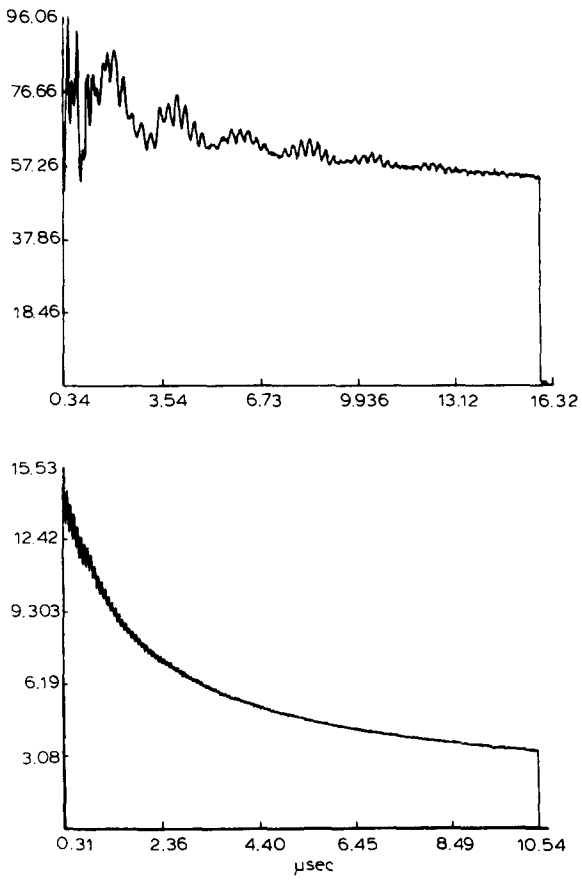

Fig. 8. ESEEM spectra of the oxidized (*upper*) and reduced (*lower*) forms of hydrogenase I of *C. pasteurianum*. The samples were prepared as described in the legends to Figs. 1 and 3. The conditions for recording the three pulse stimulated echo waveforms were: temperature, 4.3 K; microwave frequency, 9.087 MHz. For oxidized hydrogenase I, $\tau = 150$ ns (where τ the time between pulse I and II) and the magnetic field was 318 mTesla. This corresponds to $g = 2.04$ (see Fig. 1). For reduced hydrogenase I, $t = 120$ ns and the magnetic field was 331 mTesla ($g = 1.96$, see Fig. 3). See text and Ref. 190 for details.

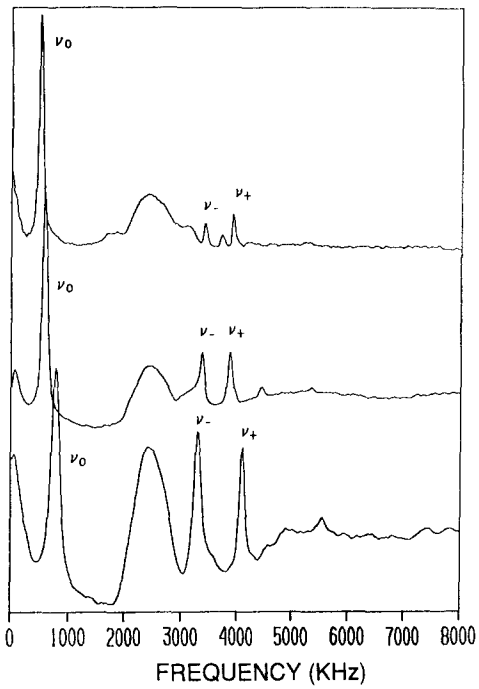

Fig. 9. Cosine Fourier transforms of the stimulated echo waveforms of oxidized hydrogenase I of *C. pasteurianum*. The ESEEM spectra were recorded at 1.7 K under the following conditions: *upper*, 7.930 GHz, 270.6 mTesla, $\tau = 290$ ns; *middle*, 9.087 GHz, 310.0 mTesla, $\tau = 180$ ns; *lower*, 10.631 GHz, 372.7 mTesla, $\tau = 180$ ns. The raw data were Fourier transformed and phase corrected after removing the stimulated echo magnetization decay using a polynominal fit. See Ref. 190 and the legend to Fig. 8 for details.

Even if one accepts that the $S = 1/2$ oxidized H cluster is not detectable by MCD because of non-sulfur ligation, it should be noted that the oxidized states of the *Cp* (I and II [172]), *Me* [183] and *Dv*H [184] hydrogenases all exhibit temperature-dependent MCD bands that arise from a paramagnetic center with a $S > 1/2$ ground state. For example, the MCD spectra of oxidized *Cp* hydrogenase I are shown in Fig. 10. The forms of the spectra are very similar for the *Me* and *Dv*H enzymes, but that of *Cp* hydrogenase II is somewhat different and is possibly a mixture of two different species, one of which resembles the spectra of hydrogenase I [172]. However, the corresponding magnetization data for oxidized hydrogenase I show that the observed transitions do not arise from an $S = 1/2$ species. Such a species would afford data in which all points fall on a smooth curve, irrespective of the temperature of measurement, and would be well fit by the theoretical curve constructed for the average observed g value. As shown in Fig. 10, this is obviously not the case. So, what is the source of the paramagnetic MCD exhibited by the oxidized hydrogenases? It must arise from an Fe-S cluster, since Fe is the only metal found in these enzymes and is the only moiety that has significant visible absorption. Whatever it may be, this center is undetectable by EPR spectroscopy, since the low-field

resonances corresponding to an $S > 1/2$ species observed from both the *Cp* I and *Dv*H enzymes respresent a very minor component (subsection IV-D), and would have to exhibit MCD intensity at least 10-times that of any known Fe-S cluster to account for the observed spectra. Moreover, oxidized *Cp* hydrogenase II does not exhibit similar low-field EPR absorption. This suggests that the MCD-detectable paramagnetic Fe-S center is an integer spin system, although a half-integer spin system with $D < 0$ could yield resonances not seen by EPR due to relaxation effects [172].

We recently suggested three possibilities for the MCD-detectable, EPR-silent ($S > 1/2$) Fe-S cluster of the oxidized hydrogenases [172]. First, it could be an oxidized F cluster with a $S > 0$ ground state, but this would be unprecedented for biological and synthetic $[4Fe\text{-}4S]^{2+}$ clusters. Second, it could represent an alternative conformation of the oxidized H cluster. However, it would have to exhibit unparalleled MCD intensity, since the $S = 1/2$ state of this cluster represents approximately one spin in both the *Cp* enzymes (Table III) and probably does so in the other two hydrogenases under the appropriate conditions (subsection IV-D). Third, it could be a new type of Fe-S cluster, unrelated to the H and F clusters, Again, this appears improbable, since all of the spectroscopic data (except MCD) readily accommodates all of the Fe (and more) as H and F clusters in the two *Cp* hydrogenases. Another possibility is that the observed MCD is somehow connected to the unique properties of the H cluster itself. That is, the same cluster exhibits EPR from an $S = 1/2$ ground state but appears to be $S > 1/2$ when examined on the basis of its visible absorption properties. Again, this would be unprecedented and irrational in terms of our current understanding of Fe-S clusters. However, substantial non-sulfur ligation in a multinuclear asymmetric cluster might confer rather unusual electronic properties. At the present time, none of these possibilities can be ruled out, and solving the puzzle of the source of the paramagnetic MCD from the oxidized hydrogenases will no doubt impart considerable insight into the structure and properties of the H cluster.

V-A.3. X-ray absorption spectroscopy

Only one of the Fe-hydrogenases has been examined by this technique so far, but again the unique properties of the H cluster were apparent [195]. The Fe K-edge absorption spectra of the reduced and oxidized forms of *Cp* hydrogenase II (Fig. 11) were similar to those of other biological Fe-S proteins, e.g., Ref. 196, indicating that the majority of the Fe in the enzyme is tetrahedrally coordinated to sulfur. The Fourier transforms of the EXAFS data are also shown in Fig. 11. The oxidized enzyme exhibited two major peaks, which from simulations were assigned to Fe-S and Fe–Fe interactions at distances of 2.27 Å and 2.76 Å, respectively, values typical of those of 2Fe-, 3Fe- and 4Fe-clusters. However, reduced hydrogenase II gave rise to three major peaks in the transform (Fig. 11). Two corresponded to the Fe-S and Fe–Fe interactions seen with the oxidized enzyme, with similar distances, while simulations showed that the additional peak not seen in the oxidized enzyme could be represented by an Fe–Fe interaction at 3.3 Å. Since the Fe–Fe distances in ferredoxin-type [4Fe-4S] clusters change very little upon oxidation and reduction (e.g., Ref. 197), the same was assumed for the

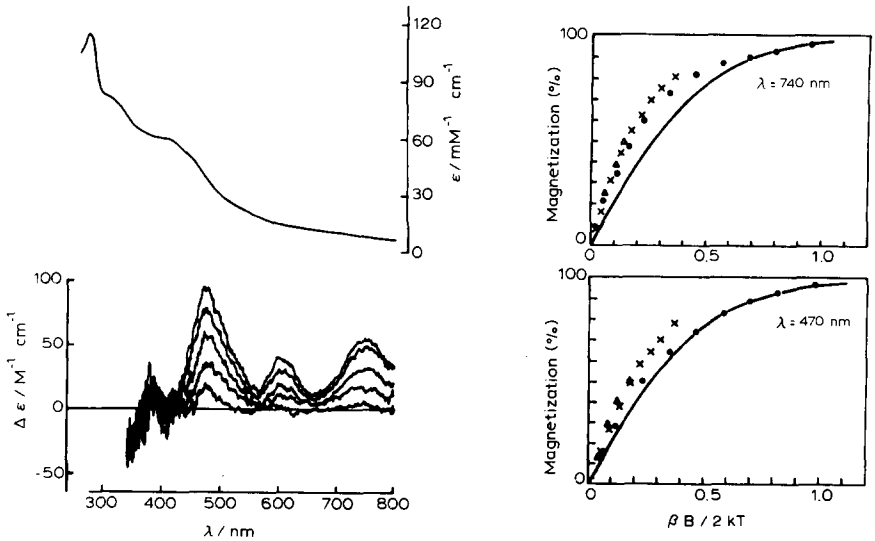

Fig. 10. Variable-temperature MCD and magnetization data for oxidized hydrogenase I of *C. pasteurianum*. The enzyme (110 μM) was prepared as described in the legend to Fig. 1 except the buffer contained ethylene glycol (50%, v/v) as a glassing agent. *Upper left*, room temperature absorption. *Lower left*, MCD spectra at 4.5 Tesla using a 0.17 cm pathlength recorded at 1.60, 4.22, 8.8, 20 and 92 K (the intensity increases with decreasing temperature). *Right*, magnetization plots using magnetic fields (B) between 0 and 4.5 Tesla. The temperatures were 1.6 K (●), 4.22 K (×) and 8.8 K (▲) with wavelengths as indicated. The solid lines are theoretical curves for an isotropic $S = 1/2$ ground state with $g = 2.04$. See text for details (adapted from Ref. 172).

hydrogenase F clusters. Thus, the 3.3 Å interaction is from the reduced H cluster. Furthermore, the amplitudes of the different Fe–Fe interactions in the reduced enzyme corresponded to those expected for two 4Fe-F clusters and one 6Fe-H cluster, where the bulk of the Fe in the H cluster is separated by a distance of 3.3 Å. It should be noted that this technique cannot readily distinguish Fe–O and Fe–N interactions in a system where there is significant Fe-S bonding, and a number of Fe-(O,N) ligands within the H cluster would not have been detected.

Therefore, the very surprising and unique conclusion is that the H cluster (of *Cp* hydrogenase II) does not simply accept an electron upon conversion of the oxidized $S = 1/2$ state to the reduced $S = 0$ state. Rather, in contrast to any known biological Fe-S cluster, there is concomitant rearrangement of the cluster to give a more expanded conformation wherein most, if not all, of the Fe atoms are about 0.5 Å further apart than they were in the oxidized cluster. The implications of this are discussed below.

V-A.4. Other biological and synthetic Fe-S clusters

The spectroscopic data presented so far unequivocally establish that the Fe-hydrogenases all contain a novel Fe-S cluster with very similar and unique properties. In the case of the *Cp* enzymes, the cluster appears to comprise 6Fe atoms and has a substantial amount of non-sulfur ligation. Although this is so far unprecedented in biological systems, several clusters with at least 6Fe atoms have been synthesized, mainly in the laboratories of Holm and Coucouvanis. These include [6Fe-6S] (two isomers), [6Fe-8S] and [6Fe-9S] clusters [198–203], and [7Fe-6S] [204] and [18Fe-30S] [205] clusters. However, all have total S coordination and contain too much S compared with likely structures that could be derived for the H cluster. Furthermore, all contain Fe–Fe distances of about 2.7 Å rather than 3.3 Å, as observed in reduced *Cp* hydrogenase II. Fe–Fe distances of this magnitude (3.00–3.57 Å) have been found in proteins containing a binuclear Fe center of the type found in hemerythrin [206], ribonucleotide reductase [207], purple acid phosphatase [208] and methane monooxygenase [209,210], and in various synthetic analogs [211]. In hemerythrin, the Fe atoms are bound by histidine residues and are bridged by two carboxylates (of aspartate and glutamate) and a μ-oxy group [206,212].

These comparisons suggest that a binuclear structure similar to that in hemerythrin might be a component of the reduced H cluster of hydrogenase II. Such a unit would have to undergo a conformational change upon oxidation to give rise to Fe–Fe interactions at 2.7 Å. It is interesting that the resonance Raman data from oxidized hydrogenase I [188] suggest a $[2Fe\text{-}2S](cys)_4$ structure as part of its H cluster (Fig. 7). The latter would have an Fe–Fe distance of about 2.7 Å, consistent with the EXAFS data from the oxidized H cluster of hydrogenase II (Fig. 11). Since the H cluster appears to contain both S and non-S ligands, it is possible that reduction of the H cluster with H_2 involves some form of ligand exchange or rearrangement whereby a $[2Fe\text{-}2S](cys)_4$ unit converts to a more expanded $[2Fe\text{-}(N/O)](cys)_4$ or $[2Fe\text{-}S,N](cys)_x(O/N)_y$ structure. Further speculation at this point is obviously not warranted, although it might prove useful in stimulating the eventual synthesis of mixed Fe-S-N/O clusters as possible analogs of the H cluster.

As previously noted [195], it is interesting that our current understanding of the structure and properties of

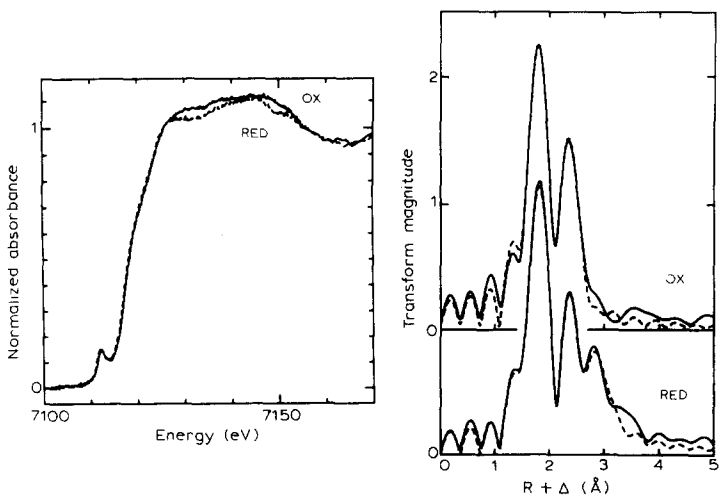

Fig. 11. Iron K-edge X-ray absorption spectra (*left*) and EXAFS Fourier transforms (*right*) of the oxidized (OX) and reduced forms (RED) of hydrogenase II of *C. pasteurianum*. The broken lines shown in the transforms are simulations based on the following interactions (per Fe atom): oxidized, 4.1 Fe-S at 2.27 Å and 3.5 Fe–Fe at 2.76 Å; reduced, 4.0 Fe-S at 2.28 Å, 2.3 Fe–Fe at 2.76 Å, and 1.1 Fe–Fe at 3.30 Å. See text and Ref. 195 for details (adapted from Ref. 195).

the H cluster bears some resemblance to those of the FeMo-cofactor, which is thought to be the site of N_2 reduction by the MoFe protein of nitrogenase, e.g., see Ref. 213. This cofactor contains $\geq$ 6Fe atoms (Fe_{6-8}-$S_{8-10}Mo$ [214]), is probably involved in catalyzing H_2 production (see Ref. 213), has some non-sulfur ligation [215], and has some Fe–Fe interactions of at least 3.0 Å [216]. Moreover, with the recent discovery of an H_2-evolving, 'Fe-only' nitrogenase in *Azotobacter vinelandii* [217,218], in addition to the alternative vanadium-containing (VFe) enzyme [219], it will be intriguing to see whether its analogous 'FeFe-cofactor' has additional properties in common with the H cluster of the Fe-hydrogenases.

V-B. The function of the H cluster

V-B.1. The pathways of electron transfer

The discovery of *Cp* hydrogenase II by Chen and co-workers [42] was an important development in attempts to understand the mechanism of H_2 production and H_2 oxidation, not only by Fe-hydrogenases, but by hydrogenases in general. This enzyme catalyzes H_2 oxidation in the standard in vitro assays at rates comparable to, or more than, those observed with the Fe-hydrogenases of *Cp* (I), *Me* and *Dv*H, yet it supports rates of H_2 production that are between two and three orders of magnitude less than those of the other enzymes (Table I). As discussed in previous sections, all of these enzymes contain at least two ferredoxin-type 4Fe clusters, or F clusters, and the novel H cluster. Thus, the central questions are, why does hydrogenase II preferentially catalyze H_2 oxidation, and what is the role of the H cluster? This novel cluster had been proposed since the mid 70's to be intimately involved in H_2 catalysis [55,145,168,173], but the key to answering these questions came from redox titrations with the two *Cp* enzymes [163]. The $E_{m,8}$ values for their H clusters were both about -400 mV in the absence of the inhibitor, CO, and about -360 mV in its presence. A similar value (-420 mV) was found for the F clusters of hydrogenase I, but the F cluster of hydrogenase II had a much higher potential, -180 mV. The E_m values for the F clusters were affected by CO. Mössbauer [135] and EPR [172] studies showed that the other F-type cluster in hydrogenase II, F′, is diamagnetic at potentials of -300 mV, so its $E_{m,8}$ must be much less than -300 mV.

The redox potential of the H cluster therefore approximates that of the H_2/H^+ electrode (-480 mV at pH 8.0) and increases to more positive values upon binding a single molecule of CO [146,163,170,178], a potent inhibitor of H_2 catalysis (but see subsection V-B.2). On the other hand, the F clusters do not appear to bind CO and have spectroscopic properties, i.e., EPR, Mössbauer, resonance Raman and EXAFS, very

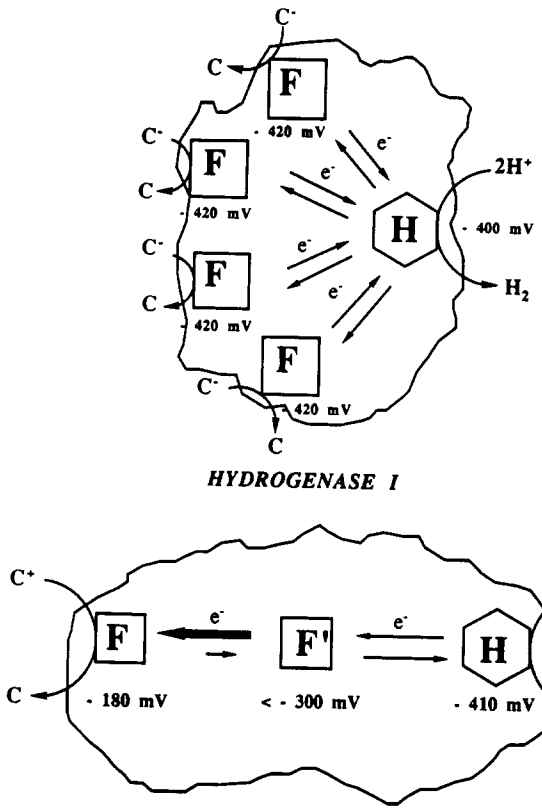

Fig. 12. Proposed pathways of electron transfer during H_2 evolution and H_2 oxidation catalysed by hydrogenase I and hydrogenase II of *C. pasteurianum*. H and F (F′) represent the H and F clusters, respectively, and C, C^- and C^+ are electron carriers for the enzymes. The heavy arrow in hydrogenase II indicates the more thermodynamically favorable direction of electron transfer. See text for details. (Reproduced with permission from Ref. 146.)

similar to the $[4Fe\text{-}4S]^{1+,2+}$ clusters of ferredoxins, in which they serve an electron transfer role. Thus, if one assumes that the H cluster is indeed the site of H_2 catalysis and the F clusters serve to shuttle electrons between the H clusters and the external electron carrier for the hydrogenases, simple models of hydrogenase I and hydrogenase II can be constructed [146] that readily explain why hydrogenase II preferentially catalyzes H_2 oxidation (shown diagrammatically in Fig. 12). Hydrogenase I is an excellent catalyst because the redox potentials of its H and F clusters overlap that of the H_2/H^+ electrode such that thermodynamically, it is a readily reversible system. Low-potential electrons (via reduced methyl viologen, $E_{m,8} = -440$ mV, or reduced ferredoxin, $E_{m,8} = -420$ mV: designated C^- in Fig. 12) reduce the F clusters, which in turn reduce the H cluster. The H cluster then reduces protons to evolve H_2. Conversely, if the enzyme is incubated under H_2 in the presence of a suitable electron acceptor, the H cluster activates H_2 and electrons readily flow in the reverse direction to reduce the acceptor via the F clusters. In hydrogenase II, the F′ cluster is proposed to

TABLE IV

Catalytic activities of hydrogenases I and II

All activities are V_m values expressed as μmol H_2 oxidized or evolved/min per mg. Taken from Refs. 134 and 220 after correcting for protein concentration [146].

Activity	Electron carrier (E_m)	H_2-ase I	H_2-ase II
H_2 evolution	methyl viologen (−440 mV)	5500	10
H_2 oxidation	methyl viologen (−440 mV)	19200	155
H_2 oxidation	ferredoxin (−420 mV)	2500	39
H_2 oxidation	methylene blue (+11 mV)	24000	34000
H_2 oxidation	thionine (+60 mV)	8400	77000

mediate electron flow between the H cluster and the F cluster. This enzyme is therefore thermodynamically 'primed' to catalyze H_2 oxidation: electrons from H_2 activation readily flow from the H to the F′ to the F cluster and the reduced F cluster reduces the electron acceptor (designated C^+ in Fig. 12). On the other hand, H_2 production catalyzed by hydrogenase II is very unfavorable thermodynamically, since it requires reduction of the low potential F′ cluster by the high potential F cluster.

From the models shown in Fig. 12 one would predict that hydrogenase II would only catalyze high rates of H_2 oxidation with electron carriers of redox potential more positive than that of its F cluster, i.e., above −180 mV. In contrast, the low potential F clusters of hydrogenase I should allow high rates of H_2 oxidation more or less independent of the redox potential of the electron acceptor. The observed V_m values for H_2 oxidation by the two enzymes using a variety of electron acceptors show that this is the case (Table IV): hydrogenase II exhibits high rates of catalysis only in the H_2 oxidation assay and only with electron acceptors of high potential. These kinetic data are therefore in accordance with only the high potential F cluster of hydrogenase II, rather than its F′ or H clusters, functioning to interact with the external electron carrier.

V-B.2. The effect of CO and O_2

Two very different effects are observed when the Fe-hydrogenases are exposed to CO. First, as described in subsection IV-B and Table II, CO is a potent inhibitor of these enzymes when they are assayed, either by H_2 evolution or H/D exchange, in the *presence* of CO. In the case of the *Cp* enzymes, this is irreversible inactivation by CO, since enzyme activity is not recovered upon removal of the CO. Second, treatment of the oxidized enzymes with CO quantitatively converts the rhombic 2.10 EPR signal to a completely different EPR signal (Table II). This arises from the binding of a single CO molecule to the oxidized H cluster (subsection V-A.1 [170,178]). However, with both the *Cp* enzymes [163] and *Dv*H hydrogenase [221], it has been shown that the CO-treated enzymes, exhibiting the CO-induced EPR signal, are *active*. That is, they show no loss of either H_2 evolution or H_2 oxidation activity when samples of the CO-treated, oxidized enzymes are assayed in the *absence* of CO.

The H clusters of the two *Cp* hydrogenases differ in the reversibility of the non-inhibitory effect of CO [163]. The oxidized H cluster of hydrogenase II binds CO reversibly, since CO was removed upon reducing the CO-treated oxidized enzyme (exhibiting the axial 2.07 EPR signal) with H_2 (subsequent oxidation in the absence of CO revealed the rhombic 2.10 EPR signal). This is not mere removal of CO, since CO binding was not reversed by simply degassing and flushing with Ar (the axial 2.07 signal remains). In accordance, the reduced H cluster of hydrogenase II does not react with CO (subsequent oxidation in the absence of CO revealed only the rhombic 2.10 EPR signal). In contrast, both the oxidized and reduced forms of the H cluster of hydrogenase I bind CO irreversibly, i.e., the CO-induced EPR signal was apparent after (a) treating the reduced enzyme with CO and subsequently oxidizing it in the absence of CO, and (b) after treating the oxidized enzyme with CO, removing the CO, reducing the enzyme with H_2 in the absence of CO, and then re-oxidizing the enzyme. The reaction of *Dv*H hydrogenase with CO is complicated by the fact that the active 'oxidized' enzyme exhibiting stoichiometric amounts of the rhombic 2.10 EPR signal has yet to be obtained. However, the CO-induced EPR signal (the axial 2.07-type) of the 'oxidized' enzyme can be generated by adding CO to the H_2-reduced enzyme without subsequent oxidation (although it is puzzling why CO should cause enzyme oxidation [162,221]). Since the axial 2.07-type EPR signal disappeared when H_2 was added and then reappeared upon the addition of CO, it was concluded that CO binding was reversible [162]. However, it seems more likely that H_2 simply reduced the CO-bound oxidized H cluster to the EPR silent state, and that the axial 2.07 signal would have subsequently appeared upon re-oxidation whether CO was added or not. The reactivity of the *Dv*H enzyme is therefore probably very similar to that of *Cp* hydrogenase I.

As discussed in subsection IV-D, low concentrations of O_2 induce the same changes as CO in the EPR properties of the oxidized *Cp* hydrogenases, i.e., conversion of the rhombic 2.10 signal to the photosensitive axial 2.07-type signal [171]. This result settled the controversy in the literature as to whether the appearance of the axial 2.07 EPR signal with the *Dv*H enzyme in the *absence* of CO was due to simple dye oxidation of the enzyme or to the presence of O_2-inactivated enzyme arising from incomplete anaerobicity (see Refs. 137, 155, 180, 221). However, there is no loss of catalytic activity when CO is added to the oxidized or reduced forms of the two *Cp* enzymes or *Dv*H hydrogenase (in

its active state), yet it has been known for many years that O_2 causes irreversible inactivation of these enzymes. So, why do CO and O_2 have the same effect on the EPR properties of the oxidized H cluster but very different effects on activity? It is now clear [171] that the induction of the axial 2.07 signal by O_2 does *not* cause enzyme inactivation: both the O_2- and CO-bound oxidized H clusters are fully active. Inactivation by O_2 is indicated by a decrease in the intensity of the EPR signal (the rhombic 2.10 as well as the axial 2.07) to much less than stoichiometric amounts, presumably as a result of cluster degradation. Thus, upon exposure of an oxidized Fe-hydrogenase to O_2, the oxidized H cluster may (1) not bind O_2, continue to exhibit the rhombic 2.10 signal and remain catalytically active, (2) bind O_2, exhibit the axial 2.07 EPR signal and remain catalytically active, and (3) be degraded by O_2 to EPR silent intermediates with loss of catalytic activity. The relative extent of these three processes will depend upon the O_2 concentration and the time of exposure, and step 2 is obviously a prerequisite for step 3. The important point is that the appearance of the axial 2.07 signal during redox (re)cycling of a hydrogenase in the absence of CO is an indicator of O_2 contamination (step 2). Although this itself does not lead to loss of activity, it indicates that irreversible inactivation is occurring (step 3). These results suggest that O_2 and CO have a common mechanism of reaction with the H cluster of the hydrogenases, although, in contrast to CO, O_2 has additional reactivity such that prolonged exposure leads to destruction of the H cluster.

The various effects of CO on the hydrogenases of *Cp* and *Dv*H can be summarized as follows. CO reacts with three different states of the H cluster (and the same is probably true with O_2). With all three hydrogenases the oxidized H cluster binds CO but only with *Cp* hydrogenase II is the binding reversed by H_2. The reduced H cluster of *Cp* hydrogenase I and the *Dv*H enzyme, but not that of hydrogenase II, also bind CO and again this is irreversible. None of these interactions leads to loss of catalytic activity. In addition, some form of the H cluster binds CO during catalysis by all three enzymes, but the reaction of this 'catalytic' state of the H cluster with CO leads to irreversible inactivation. The differences in the sensitivity to CO of these two quite distinct reactions, i.e., inactivation by CO and induction of a different EPR signal by CO, is listed in Table II for the three enzymes. The *Cp* I and *Dv*H enzymes are more sensitive to inhibition, whereas the oxidized H cluster of *Cp* hydrogenase II exhibits the CO-induced EPR signal at much lower CO concentrations than that required to inhibit the enzyme. The differences in the H cluster between the latter and the former enzymes is discussed below. Possible mechanisms for CO binding and how this relates to mechanisms for H_2 activation will now be considered.

V-B.3. The mechanism of catalysis

It is now fairly well established that activation of H_2 by hydrogenase involves the heterolytic cleavage of the molecule with the formation of a hydride and a proton (Eqn. 3; see Ref. 55 and references therein).

$$H_2 = H^- + H^+ \tag{3}$$

Assuming that the H cluster is the site of H_2 catalysis (Fig. 12), any proposed mechanism for H_2 activation

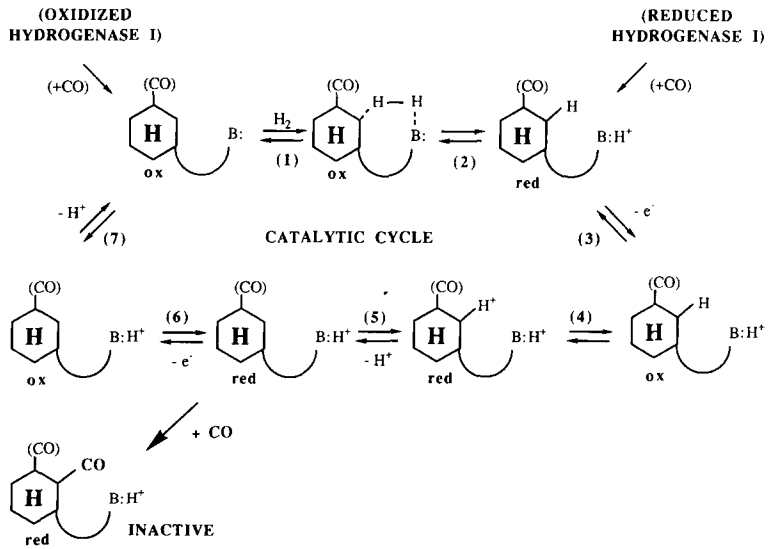

Fig. 13. The proposed states of the H cluster of hydrogenase I of *C. pasteurianum* during the catalytic cycle. The H cluster is represented by H inside a hexagon, 'B:' is an adjacent base, and ox and red indicate the oxidized ($S = 1/2$) and reduced ($S = 0$) forms of the H cluster. The effects of adding CO to the oxidized and reduced forms of the enzyme and their subsequent participation in the cycle (in the *absence* of CO) are indicated by parentheses. The production of an inactive CO-bound H cluster is indicated when catalysis takes place in the *presence* of CO. See text for details.

must accommodate the fact that the *oxidized* H cluster binds and oxidizes H_2. This is shown by the ability of the two *Cp* hydrogenases in their oxidized states, i.e., in the presence of a large excess of an oxidized dye, to catalyze H_2 oxidation and dye reduction when H_2 is added [163]. It is therefore proposed that H_2 binds to a specific Fe atom(s) of the oxidized H cluster. The polarization of the H–H bond required for bond cleavage could be achieved either within the H cluster itself, i.e., it accepts both the hydride ion and the proton, or by the cluster and an adjacent base such as a histidine residue, i.e., the cluster accepts the hydride ion with the transfer of the proton to the base. Other mechanisms include the participation of ligands either within the cluster or to the cluster, as proposed for the Ni center of Ni-hydrogenases (Section III), or the binding of H_2 to an Fe site by a metal σ-donor interaction [222]. The net result, shown in Fig. 13, is reduction of the H cluster by one electron and loss of one proton (in Fig. 13 the proton is proposed to bind to an adjacent base: reactions 1 and 2). The subsequent steps in Fig. 13 are based on the mechanisms previously suggested [134,163], whereby the H cluster transfers an electron to an F cluster (reaction 3), and the reduced F cluster is then re-oxidized by the external electron acceptor. The second proton then dissociates from the oxidized H cluster (reactions 4 and 5) and the reduced H cluster reduces an oxidized F cluster (reaction 6), which in turn is reoxidized by the external carrier. The catalytic cycle is completed by the dissociation of the first proton from the base (step 7). This mechanism is consistent with the EPR data which show that both the H and F clusters undergo only a one electron redox step.

This mechanism can be used to rationalize the effects of CO on the EPR properties and catalytic activity of the H cluster. The reaction of CO with the oxidized H cluster of hydrogenase I is shown in parentheses in Fig. 13 whereby CO binds to the cluster, but at a site remote from where H_2 binds. It is known that CO is not removed from the oxidized H cluster by reducing *Cp* hydrogenase I with H_2 in the presence of excess oxidant (section V-B.2 [163]). Since this is the same as determining enzyme activity (except that in this case the enzyme is added to excess oxidant under H_2), CO must remain bound to the H cluster throughout the catalytic cycle, and this is indicated in Fig. 13. The reduced H cluster must bind CO at the same site as the oxidized H cluster, since the CO-treated reduced enzyme upon oxidation exhibits the same EPR signal as the CO-treated oxidized enzyme. In the reduced enzyme, the reduced H cluster is in a state equivalent to the product of reaction 2 in Fig. 13: reaction 3 is blocked since it involves reduction of an oxidized F cluster by the reduced H cluster, but in the fully reduced enzyme the F clusters would be already reduced. The reduced H cluster with CO bound is also fully active in the absence of CO, i.e. reaction 3 proceeds if CO is removed from the reduced enzyme and oxidant is added to oxidize the F clusters. Irreversible inactivation of the enzyme by CO occurs when CO is allowed to react with a form of the H cluster that is generated during the catalytic cycle. Inspection of Fig. 13 shows four possibilities: the products of reactions 3 to 6. The product of reaction 6 is unlikely to bind CO irreversibly, since the H cluster is in the same state as it is in the oxidized enzyme. Since CO binding causes loss of activity, it is proposed that CO reacts with the H_2 binding site of the H cluster, which becomes exposed on the reduced cluster as the product of reaction 5, and this is indicated in Fig. 13. Note that this site is blocked in the reduced enzyme (product of reaction 2) by the bound hydride. Thus, CO is proposed to cause irreversible inactivation of the hydrogenases by binding to a reactive and transient state of the reduced H cluster in which the H_2 binding site is accessible. A consequence of this mechanism is that if oxidized *Cp* hydrogenase I is treated with CO and then assayed in the presence of CO, the inactivated H cluster will have CO bound at both sites, as shown in Fig. 13. Radiolabelling experiments with ^{14}CO should be able to test this hypothesis.

V-B.4. Cp hydrogenase I versus Cp hydrogenase II

The mechanism given in Fig. 13 describes the effect of CO on *Cp* hydrogenase I, and probably on *Dv*H hydrogenase as well. It also accounts for the irreversible inactivation of *Cp* hydrogenase II by CO, but not the effect of CO on the oxidized and reduced states of this enzyme. As discussed in subsection V-B.2, oxidized hydrogenase II binds CO reversibly, while the reduced enzyme does not react. Moreover, CO-treated oxidized hydrogenase II exhibits a unique rhombic EPR signal (which will now be referred to as the 'rhombic 2.03 signal': see Fig. 2 and subsection IV-D.2) rather than the axial 2.07-type signal of the *Cp* I and *Dv*H enzymes (Fig. 2). In addition, the rhombic 2.03 signal is induced by much lower CO concentrations than those required to induce the axial 2.07 signal (Table II), the CO-bound H cluster of oxidized hydrogenase II shows a different temperature dependence in the photoproduction of the rhombic 2.26 EPR signal compared to hydrogenase I (subsection IV-D.2), and ^{13}C ENDOR shows that the hyperfine coupling constant for the CO-bound H cluster is over 50% larger in hydrogenase II compared to hydrogenase I [178]. These data suggest that CO might not bind to the same site on the H clusters of the two enzymes. These differences are rationalized in diagrammatical terms in Fig. 14. The oxidized H cluster of hydrogenase II is proposed to bind CO at the H_2 binding site, rather than at the adjacent site suggested for the H cluster of the *Cp* I (and *Dv*H) enzyme. The latter binding gives rise to the axial 2.07 signal, while the former exhibits the rhombic 2.03 signal. Both sites with CO-bound are photosensitive. Addition of H_2 dis-

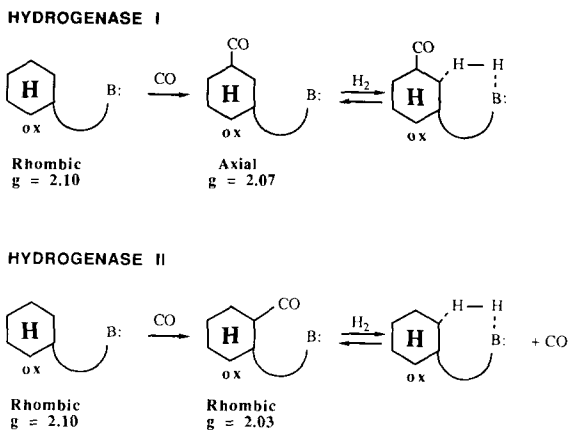

Fig. 14. The reaction of CO with the oxidized H clusters of hydrogenase I and hydrogenase II of *C. pasteurianum* and their EPR properties. An explanation of the symbols is given in the legend to Fig. 13. The type of EPR signal exhibited by the various forms of the H cluster are indicated. See text for details.

places CO from the oxidized H cluster of hydrogenase II, but this site is vacant on the oxidized H cluster of hydrogenase I and H_2 binds to the CO-bound cluster. Thus, in contrast to hydrogenase I, the H cluster of hydrogenase II does not have CO bound when it is subsequently assayed in the absence of CO. Moreover, the mechanisms shown in Figs. 13 and 14 suggest that the H cluster of hydrogenase II has only one, rather than two, CO binding sites.

The question remains as to what structural differences can account for the different CO reactivities observed with the H clusters of hydrogenase II and hydrogenase I. In spite of the fact that the two clusters have almost identical EPR, Mössbauer and ^{57}Fe ENDOR properties both with and without CO, indicating fundamentally similar structures, we cannot rule out the possibility that they contain a different number of Fe atoms (subsection V-A.1). However, it seems more likely that the difference lies in the number and/or type of non-sulfur ligands. This is suggested by the differences in their resonance Raman and electron spin echo properties (subsection V-A.2), and perhaps also in their MCD properties (accepting that the paramagnetic MCD observed from the oxidized enzymes is somehow connected to their H clusters, see subsection V-A.2). Although the models of the two enzymes given in Fig. 12 readily explain why hydrogenase II preferentially catalyzes H_2 oxidation, it is possible that the observed differences in their reactivities with CO also reflect some intrinsic ability of the H cluster of hydrogenase II to retard H_2 production. For example, the K_m for H_2 is 10-fold lower for hydrogenase II compared to hydrogenase I [134], and this could arise from structural differences in their H clusters. At this point, however, it is hard to rationalize such differences in terms of the presence or absence of non-sulfur ligands.

What is clear is that the H clusters of hydrogenase I and hydrogenase II are very similar in structure, and are unique in the field of bioinorganic chemistry. In spite of the enormous amount of spectroscopic, biochemical and kinetic information that has accumulated over the last 15 years or so, the structure of this novel Fe-S center remains a mystery, and one that is likely to be solved only by crystallographic analyses of the enzymes. In this respect, the H cluster shares another property in common with the enigmatic FeMo-cofactor of nitrogenase (subsection V-A.4). Indeed, as with the FeMo-cofactor, elucidating the structure of the H cluster will no doubt open up an as yet unexplored area of Fe-S(N/O) chemistry.

VI. Summary

Hydrogenases devoid of nickel and containing only Fe-S clusters have been found so far only in some strictly anaerobic bacteria. Four Fe-hydrogenases have been characterized: from *Megasphaera elsdenii, Desulfovibrio vulgaris* (strain Hildenborough), and two from *Clostridium pasteurianum.* All contain two or more [4Fe-4S]$^{1+,2+}$ or F clusters and a unique type of Fe-S center termed the H cluster. The H cluster appears to be remarkably similar in all the hydrogenases, and is proposed as the site of H_2 oxidation and H_2 production. The F clusters serve to transfer electrons between the H cluster and the external electron carrier. In all of the hydrogenases the H cluster is comprised of at least three Fe atoms, and possibly six. In the oxidized state it contains two types of magnetically distinct Fe atoms, has an $S = 1/2$ spin state, and exhibits a novel rhombic EPR signal. The reduced cluster is diamagnetic ($S = 0$). The oxidized H cluster appears to undergo a conformation change upon reduction with H_2 with an increase in Fe–Fe distances of about 0.5 Å. Studies using resonance Raman, magnetic circular dichroism and electron spin echo spectroscopies suggest that the H cluster has significant non-sulfur coordination. The H cluster has two binding sites for CO, at least one of which can also bind O_2. Binding to one site changes the EPR properties of the cluster and gives a photosensitive adduct, but does not affect catalytic activity. Binding to the other site, which only becomes exposed during the catalytic cycle, leads to loss of catalytic activity. Mechanisms of H_2 activation and electron transfer are proposed to explain the effects of CO binding and the ability of one of the hydrogenases to preferentially catalyze H_2 oxidation.

Acknowledgements

I thank Drs. D. Bonam and A. Juszczak for reading the manuscript, and Drs. D. Bonam, J. LeGall, M.K. Johnson, L.E. Mortenson, H.D. Peck, Jr., H. Thomann,

G. Voordouw, and T. Yagi for providing unpublished data. Results reported from the author's laboratory were supported in part by the National Science Foundation (DMB8805255), the Department of Energy (FG09-88ER13901) and the Petroleum Research Fund (ACS-PRF 21925-AC4,3) administered by the American Chemical Society.

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