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The hydrogenases and formate dehydrogenases of *Escherichia coli*

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Abstract

Escherichia coli has the capacity to synthesise three distinct formate dehydrogenase isoenzymes and three hydrogenase isoenzymes. All six are multisubunit, membrane-associated proteins that are functional in the anaerobic metabolism of the organism. One of the formate dehydrogenase isoenzymes is also synthesised in aerobic cells. Two of the formate dehydrogenase enzymes and two hydrogenases have a respiratory function while the formate dehydrogenase and hydrogenase associated with the formate hydrogenlyase pathway are not involved in energy conservation. The three formate dehydrogenases are molybdo-selenoproteins while the three hydrogenases are nickel enzymes; all six enzymes have an abundance of iron-sulfur clusters. These metal requirements alone invoke the necessity for a profusion of ancillary enzymes which are involved in the preparation and incorporation of these cofactors. The characterisation of a large number of pleiotropic mutants unable to synthesise either functionally active formate dehydrogenases or hydrogenases has led to the identification of a number of these enzymes. However, it is apparent that there are many more accessory proteins involved in the biosynthesis of these isoenzymes than originally anticipated. The biochemical function of the vast majority of these enzymes is not understood. Nevertheless, through the construction and study of defined mutants, together with sequence comparisons with homologous proteins from other organisms, it has been possible at least to categorise them with regard to a general requirement for the biosynthesis of all three isoenzymes or whether they have a specific function in the assembly of a particular enzyme. The identification of the structural genes encoding the formate dehydrogenase and hydrogenase isoenzymes has enabled a detailed dissection of how their expression is coordinated to the metabolic requirement for their products. Slowly, a picture is emerging of the extremely complex and involved path of events leading to the regulated synthesis, processing and assembly of catalytically active formate dehydrogenase and hydrogenase isoenzymes. This article aims to review the current state of knowledge regarding the biochemistry, genetics, molecular biology and physiology of these enzymes.

Introduction

Formic acid and dihydrogen are both valuable sources of reductant for anaerobically growing organisms. *Escherichia coli* is no exception and the presence of enzymes capable of utilising both compounds was recognised in early studies (Stephenson & Stickland 1931, 1932; Ordal & Halvorson 1939). The name formate dehydrogenase (FDH) was given to the enzyme catalysing the oxidation of formate and hydrogenase (Hyd) given to that catalysing the reversible oxidation of hydrogen. It was also recognised that both enzymes could fulfill two roles: molecular hydrogen is not only produced by *E. coli* by the formate hydrogenlyase (FHL) reaction (Stephenson & Stickland 1932) but it also can be used as a source of energy by coupling its oxidation to the reduction of fumarate (Macy et al. 1976); the oxidation of formate serves as a source of electrons for proton reduction by the FHL complex or for the reduction of nitrate to nitrite by the formate-nitrate reductase respiratory pathway (Wimpenny & Cole 1967). Nevertheless, it was considered for a long time that in each instance a single enzyme species was responsible for the catalysis of both reactions. Current-

ly, three FDH isoenzymes and three Hyd isoenzymes have been identified and each has been characterised to a different degree; all are genetically distinct. The FDH isoenzymes are named according to the growth condition in which their induction is optimal. Thus, FDH-O is synthesised in the presence of oxygen or nitrate, FDH-N synthesis is induced by anaerobiosis and nitrate while FDH-H synthesis is optimal when *E. coli* grows fermentatively (Sawers et al. 1991). The Hyd isoenzymes are named according to the chronological order of their discovery and are termed Hyd-1, Hyd-2 and Hyd-3. Hyd-1 and Hyd-2 are involved in the energy conserving oxidation of dihydrogen, while Hyd-3 together with FDH-H forms part of the FHL complex.

All six isoenzymes have iron-sulfur clusters but they also contain redox-active metals; molybdenum and selenium in the case of the FDHs and nickel in the three Hyd isoenzymes. They also have a complex subunit structure and are all membrane-associated which hampered, to a certain extent, their characterisation. However, our understanding of the enzymes has flourished with the advent of molecular biology. The availability of a multitude of mutants accumulated from several laboratories has made these advances possible. The contribution made by these many different laboratories to our current knowledge of the biology of the FDH and Hyd isoenzymes is reviewed here. Many excellent reviews, particularly in the field of hydrogenase, have been published recently and these will be referred to where appropriate as sources of further detailed information.

Multiple formate dehydrogenase isoenzymes in *E. coli*

The study of FDH function in *E. coli* has been influenced strongly by two seminal pieces of work in the 1950s. First, Pinsent (1954) demonstrated the absolute requirement of selenium and molybdenum as growth medium supplements for the production of FDH activity in both aerobically and anaerobically cultured cells. Second, Peck & Gest (Gest & Peck 1955; Peck & Gest 1957) provided evidence suggesting that *E. coli* synthesises more than one distinct FDH isoenzyme species. It is now clear that there are three distinct FDH isoenzymes in *E. coli* (Giordano et al. 1983; Sawers et al. 1991). Although the results from early studies attested to this (Pinsent 1954; Peck & Gest 1957) it was finally a genetic approach that provided irrefutable evidence for the existence of multiple enzyme species (Zinoni et al. 1986; Berg & Stewart 1990; Sawers et al. 1991).

Formate, the substrate of the FDH isoenzymes, is produced in large amounts by anaerobically growing *E. coli* cells and results from the CoA-dependent cleavage of pyruvate by the pyruvate formate-lyase (PFL) enzyme (Knappe & Sawers 1990). If nitrate is available to anaerobically growing cells, a formate-nitrate respiratory chain is synthesised that comprises a formate dehydrogenase (FDH-N, for nitrate), a quinone and a terminal nitrate reductase (Ruiz-Herrera et al. 1969; Enoch & Lester 1974, 1975). This enzyme complex is exclusively membrane-associated (Itagaki et al. 1961; Enoch & Lester 1975) and catalyses the oxidation of formate to carbon dioxide with the concomitant transfer of the reducing equivalents to nitrate. As would be anticipated for a respiratory chain, the pathway is energy-conserving (Garland et al. 1975; Jones 1980a; Jones et al. 1980).

Fermentative growth of *E. coli* results in the formation of a distinct enzyme complex that decomposes formate to carbon dioxide and dihydrogen. This pathway was coined formate-hydrogenlyase (FHL) by Stephenson & Stickland (1931, 1932). Gest & Peck (1955), by mixing extracts derived from different gas-negative 'coli-aerogenes' variants, managed to reconstitute FHL activity and concluded that the pathway was constituted by a 'soluble' formate dehydrogenase (FDH-H), two electron carriers and a hydrogenase (now termed Hyd-3, see below) (Gest & Peck 1955; Peck & Gest 1957). The tentative conclusions drawn regarding the structure of the complex at the time from these cogent and elegant studies have proved to be correct as determined by recent genetic and physiological studies (Zinoni et al. 1986; Böhm et al. 1990; Sauter et al. 1992).

The function of the third FDH isoenzyme is less clear. It is active in catalysing the oxidation of formate in aerobic cells using oxygen as terminal electron acceptor (Pinsent 1954; Sawers et al. 1991). Due to this activity it has been termed formate oxidase (Pinsent 1954) and hence is abbreviated as FDH-O (Sawers et al. 1991). It was shown by selenium labelling experiments to be a selenoprotein (Leinfelder et al. 1988a; Sawers et al. 1991) and in analogy with the FDH-N enzyme it has been proposed that FDH-O oxidises formate and transfers the electrons to the quinone pool (Sawers et al. 1991). Recently, it has been shown that FDH-O also transfers electrons to nitrate and nitrite (Pommier et al. 1992; Darwin et al. 1993a) and FDH-O is indeed synthesised when *E. coli* respires with nitrate

which would be congruent with it having a respiratory function. It appears not to be synthesised to significant levels during fermentation (Sawers et al. 1991).

A recent study has indicated that a newly discovered enzyme encoded by the *purU* gene provides a source of formate for purine biosynthesis in aerobically growing cells (Nagy et al. 1993). This may also be the source of substrate for the FDH-O isoenzyme. The *purU* gene product has no similarity with PFL but does exhibit significant homology with 5′-phosphoribosylglycinamide transformylase (Nagy et al. 1993); its substrate *in vivo* is unknown.

Properties of the FDH isoenzymes

Measurement of FDH isoenzyme activity

Three FDH enzyme activities are distinguishable depending on the growth condition the cell is subjected to: 1. The formate-dependent reduction of oxygen to water (Pinsent 1954; Sawers et al. 1991); 2. The formate-dependent reduction of two-electron-accepting redox dyes such as methylene blue (MB) or 2,6-dichlorophenolindophenol (DCPIP) using catalytic amounts of phenazine methosulphate (PMS) catalysed by FDH-N after anaerobic growth with nitrate (Peck & Gest 1957; Ruiz-Herrera et al. 1969; Enoch & Lester 1975); 3. The formate-dependent reduction of one-electron dyes of low redox potential, for example benzyl viologen (BV), induced by fermentative growth (Peck & Gest 1957; Cox et al. 1981; Axley et al. 1990). Although controversy raged in the literature as to whether these different activities were catalysed by a single formate-activating enzyme modified under the different conditions (Chippaux et al. 1977) or whether they were catalysed by distinct isoenzymes (Ruiz-Herrera & DeMoss 1969; Ruiz-Herrera & Alvarez 1972; Giordano et al. 1983), it has now been clearly established that oxygen-, PMS(DCPIP)- and BV-reduction is diagnostic of the isoenzymes FDH-O (Sawers et al. 1991), FDH-N (Enoch & Lester 1975) and FDH-H (Axley et al. 1990), respectively.

Subunit structure of the FDH isoenzymes and their cofactor content

The first FDH enzyme to be purified and characterised in detail was FDH-N. The enzyme was purified to near homogeneity from deoxycholate-solubilised membranes and shown to be composed of three subunits of molecular weights 110,000, 32,000 and 20,000 (Enoch & Lester 1975). Sedimentation analysis indicated an α_4 β_4 γ_4 structure for the native enzyme, but the content of the γ subunit was variable from preparation to preparation. Earlier studies using partially purified enzyme preparations of FDH-N and nitrate reductase had indicated that each had a cytochrome *b* component as part of the intact enzyme species (Itagaki et al. 1961, 1962; Ruiz-Herrera & DeMoss 1969). Enoch & Lester (1975) confirmed that FDH-N indeed contains cytochrome *b* and this was proposed to be associated with the 20 kD subunit because the variable haem content of the purified enzyme correlated strongly with the stoichiometry of the γ subunit (see also Scott & DeMoss 1976). Analysis of the data derived from DNA sequencing together with spectral studies of a specific mutant provided strong corroborative evidence to indicate that the cytochrome component is located on the γ subunit (Berg et al. 1991b).

FDH-N also proved to contain 1 mole of molybdenum, 1 mole of selenium and approximately 14 moles each of non-haem iron and acid-labile sulfide per mole of enzyme (Enoch & Lester 1975). This confirmed evidence provided in former studies indicating the requirement for molybdate, selenite and iron for formation of FDH activity (Pinsent 1954; Fukuyama & Ordal 1965; Lester & DeMoss 1971; Shum & Murphy 1972). Selenium could be shown definitively to be tightly associated with the 110 kD subunit (Enoch & Lester 1975; Cox et al. 1981) and is present in the form of selenocysteine located at amino acid 196 in the polypeptide chain (Berg et al. 1991a,b).

A strong correlation between FDH-H enzyme activity and a specific 80 kD selenopolypeptide was demonstrated by Cox et al. (1981). Again, cloning of the gene (Pecher et al. 1985) and determination of the DNA sequence (Zinoni et al. 1986) identified that the 80 kD selenopolypeptide was the major component of the FDH-H isoenzyme. Subsequent mutagenesis and amino acid sequence analysis proved conclusively that selenocysteine was located at amino acid position 140 (Zinoni et al. 1987; Stadtman et al. 1991). FDH-H was purified by Axley and coworkers (Axley et al. 1991) from an over-producing strain and was shown to contain 3.3 g atoms of iron and 1 g atom of molybdenum, suggesting that the enzyme may contain one 4Fe-4S cluster. It was also reported that molybdenum could be isolated as molybdenum cofactor (molybdopterin guanine dinucleotide), having a similar structure to that identified in the dimethylsulfoxide (DMS) reduc-

Table 1. Properties of the formate dehydrogenase isoenzymes.

	FDH-H	FDH-N	FDH-O
Subunit composition	α (β?) FHL complex	$\alpha_4\beta_4\gamma_4$	$\alpha_4\beta_4\gamma_4$[a]
Metals & cofactors	FeS, Mo, Se	FeS, Mo, Se, cytochrome *b*	FeS, Mo, Se, cytochrome *b*[a]
Associated with the reduction of:	H^+	NO_3^-	O_2, NO_3^-
Artificial electron acceptor specificity	benzyl viologen	phenazine methosulphate	phenazine methosulphate
Km for formate (mM)	26	0.12	unknown
Inhibition by iodoacetamide	enhanced in the presence of formate	enhanced in the absence of formate	unknown

[a] Assumed through analogy with FDH-N.

tase from *Rhodobacter sphaeroides* (Johnson et al. 1990).

In a recent comparison of the gene sequences of four molybdenum cofactor-dependent FDHs, Heider & Böck (1993) identified four conserved cysteine residues, three of which form a CysxxCysxxxCys motif, that they propose may be ligands for the 4Fe-4S cluster.

Unfortunately, very little is known regarding the composition of the FDH-O isoenzyme from biochemical studies, other than it has a 110 kD selenopolypeptide subunit (Sawers et al. 1991). A *selC* mutant, which encodes the selenocysteine-specific tRNA, lacks both formate oxidase activity and the aerobic 110 kD selenopolypeptide. This would be commensurate with FDH-O also possessing a selenocysteinyl residue in its polypeptide chain. The fact that FDH-O has a respiratory function, that it has a 110 kD selenopolypeptide and that a FDH:PMS oxidoreductase activity has been purported to be present in aerobic cells (Schlindwein et al. 1990; Pommier et al. 1992) is very suggestive that the FDH-O isoenzyme has a very similar structure to that of FDH-N. Indeed, recent sequencing of a portion of the *E. coli* chromosome has identified three genes whose products are almost identical with those of the *fdnGHI* operon (coding for FDH-N) gene products (Plunkett et al. 1993; see below). These genes have been assigned the nomenclature *fdoGHI* and probably encode the FDH-O isoenzyme.

Catalytic activity of FDH-N and FDH-h

Detailed kinetic analyses have been performed with purified forms of both FDH-H and FDH-N (Axley & Grahame 1991; Enoch & Lester 1975), the results of which are summarised in Table 1. The FDH-N isoenzyme is characterised by having a Km for formate that is two orders of magnitude lower compared with that of the FDH-H enzyme. This may have relevance for the physiological roles of both isoenzymes (see below).

The studies of Axley & Grahame (1991) demonstrated that the Vmax of FDH-H remained essentially unchanged when deuteroformate was used as a substrate; however, the Km was increased 3-fold compared with that for proteoformate. This indicated that the formate oxidation step is not rate-limiting. Subsequent electron transfer is then proposed to occur in successive one-electron steps with concomitant reduction of two molecules of BV. A careful study comparing the kinetics of the (Se)FDH-H with a (S)FDH-H form, derived by specifically replacing the selenocysteine with cysteine, showed that although the sulfur analogue reduced the Km for formate, the reaction rate was diminished approximately 300-fold (Axley et al. 1991). Therefore, simply through the exchange of a single atom the result was that the sulfur enzyme is 20 times less active than its selenium counterpart. This was further supported by examining the pH-dependent alkylation profiles of both enzymes which indicated that the selenol and thiol groups have very different ionisation states at neutral pH (Axley et al. 1991). Furthermore, these studies prove that the selenol group is directly involved in the oxidation of formate.

Clues as to the physiological electron acceptor of the FDH-H isoenzyme were provided by the recent analysis of the deduced amino acid sequence of the other FHL components. Two ferredoxin-like proteins have been identified based on sequence comparisons (Böhm et al. 1990; Sauter et al. 1992). One of these, HycB, shows convincing homology with the β subunit of formate dehydrogenase from *Wolinella succinogenes* (Bokranz et al. 1991), nitrate reductase from *E.*

coli (Blasco et al. 1989) and DMSO reductase, also from *E. coli* (Bilous et al. 1988). HycB may therefore represent the second subunit and hence the electron transfer partner of FDH-H. This would also be consistent with a one-electron transfer mechanism.

In contrast, the reaction catalysed by FDH-N appears to involve two-electron carriers. Enoch & Lester (1975) could demonstrate strong formate-dependent reduction of the cytochrome *b* component of FDH-N and coupling to quinone reduction. Moreover, the different sensitivities of the selenocysteinyl group in FDH-N and FDH-H to alkylation would be entirely consistent with different modes of electron transfer; formate protects the FDH-N enzyme from iodoacetamide-dependent inhibition while it enhances the sensitivity of FDH-H to alkylation (Enoch & Lester 1975; Axley et al. 1990; Axley & Grahame 1991). Recently, Heider & Böck (1993) proposed a mechanism for the transfer of electrons from formate via the molybdenum cofactor to the iron-sulfur clusters and speculated, based on the findings described above, that both enzymes employ different electron-channelling mechanisms.

Although essentially nothing is known regarding the kinetics of FDH-O, Azoulay et al. (1978) presented evidence to suggest that ubiquinone and not menaquinone serves as electron acceptor for the formate oxidase activity in aerobically grown cells (Pinsent 1954).

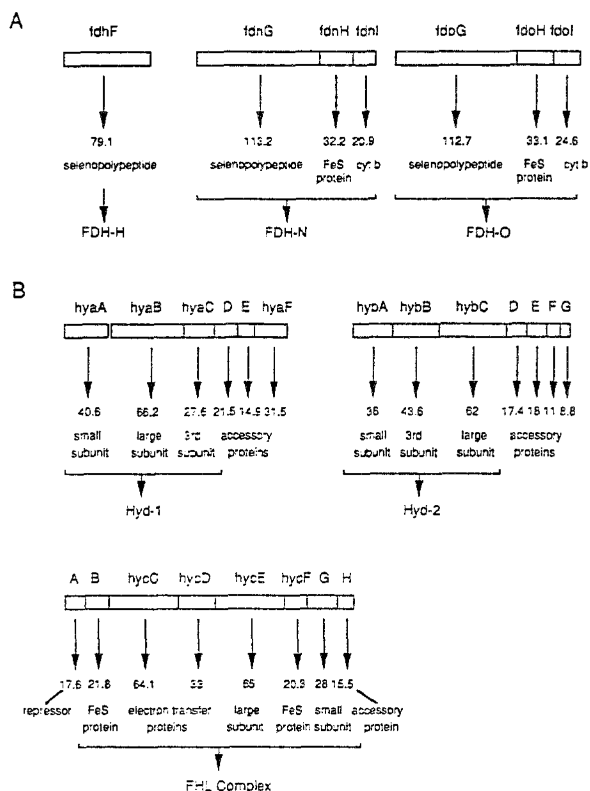

Fig. 1. The structure of the operons encoding the formate dehydrogenase and hydrogenase isoenzymes of *Escherichia coli*. The genes of each operon are presented as rectangles. The molecular masses of the respective gene products, as deduced from the nucleotide sequences, are given below the arrows in kilo daltons. The proposed function of each polypeptide is also displayed; however it should be noted that those functions assigned to the FdoH, FdoI, HyaC and HybB proteins are purely hypothetical and must be confirmed experimentally.

Genetics of FDH isoenzyme formation

The structural genes encoding the FDH isoenzymes

A major breakthrough in FHL research was achieved by the isolation of two mutants employing a simple screening procedure based on the physiology of FHL synthesis (Pecher et al. 1983). This involved mutagenesis with Mu d1 and screening for colonies that synthesised β-galactosidase only anaerobically. One of these mutants (M9s) proved to have the *lacZ* gene fused with the structural gene encoding FDH-H while the other mutant (M17s) carried a lesion in one of the structural genes of an operon encoding Hyd-3 (see below). The Mu d1 insertion in M9s resulted in the synthesis of a truncated 60 kD selenopolypeptide (Pecher et al. 1985). The synthesis of this 60 kD selenopolypeptide was regulated in a manner identical to that of the FDH-H isoenzyme and the FHL complex, i.e. induced by formate but repressed by oxygen and nitrate. Cloning and sequencing of the wild type gene, termed *fdhF*, indicated that it had the potential to code for a protein of molecular weight 79,087 (Zinoni et al. 1986). Surprisingly, however, the coding sequence carried an opal (UGA) nonsense codon at a position corresponding to amino acid 140 of the FDH-H polypeptide. Construction of various hybrid *fdhF-lacZ* gene fusions demonstrated that the presence of the UGA codon required that selenium be supplied in the culture medium to generate a full-length translated product; hybrid derivatives lacking the UGA codon did not exhibit this selenium requirement (Zinoni et al. 1986). These findings provided the first indication that selenium is incorporated into proteins by a co-translational mechanism. Corroboration of this presumption was

obtained by mutagenising the UGA codon to serine or to cysteine codons which abrogated the dependence on selenium for translation of the *fdhF* mRNA (Zinoni et al. 1987).

The structural genes encoding the FDH-N isoenzyme (Fig. 1A) were isolated by complementing a Mu d1 insertion mutant (WL24) that produced a truncated 110 kD selenopolypeptide (Leinfelder et al. 1988a; Berg & Stewart 1990). The paucity of *fdn* (nomenclature for the FDH-N structural genes) mutants described in the literature may reflect the fact that FDH-O can complement partially the deficiencies in nitrate metabolism caused by the absence of the FDH-N isoenzyme.

Unlike the *fdhF* gene which is monocistronic, the three genes encoding the α, β and γ subunits of the FDH-N enzyme are colinear and form an operon (Berg et al. 1991b). The deduced amino acid sequence of the *fdnGHI* genes indicates that they code for polypeptides of molecular mass 113 kD, 32 kD and 21 kD, respectively, which correlates well with biochemical data (Fig. 1A; Enoch & Lester 1975). Like the *fdhF* gene, the *fdnG* gene contains an opal nonsense codon in its coding sequence and evidence has been provided to indicate that this codon specifies selenocysteine (Berg et al. 1991a).

Comparison of the deduced amino acid sequences of the α polypeptides of the FDH-H and the FDH-N isoenzymes of *E. coli* with the α subunit of the FDH enzyme from *Methanobacterium formicicum* (Shuber et al. 1986) and the large subunit of FDH from *W. succinogenes* (Bokranz et al. 1991) indicates there is a considerable degree of homology between the proteins (Berg et al. 1991b; Heider & Böck 1993). The latter two polypeptides have a cysteinyl residue at the position corresponding to the selenocysteine in FDH-H and FDH-N. The *fdnH* gene product has four cysteine clusters characteristic of electron transfer components of several multisubunit reductases and dehydrogenases (Sauter et al. 1992).

The genes encoding the FDH-O isoenzyme have been identified recently (Plunkett et al. 1993). An opal nonsense codon is also present in the coding sequence of the *fdoG* gene. The *fdoGHI* operon is located between two genes, *fdhD* and *fdhE*, previously identified through a mutagenesis study as being required for the biosynthesis of fully functional FDH isoenzymes (Mandrand-Berthelot et al. 1988; Schlindwein et al. 1990). The *fdhE* gene is contiguous with the *fdoGHI* gene and all four genes may form an operon. The *fdhD* gene is located next to the *fdoG* gene but is transcribed divergently.

The genes required for selenocysteine biosynthesis and incorporation

Four *sel* genes have been identified whose products are required for the biosynthesis and insertion of selenocysteine into selenoproteins (Leinfelder et al. 1988a). However, only one of these gene products is required for the selenylation of tRNA species (Stadtman et al. 1989; Leinfelder et al. 1988b). The features of these four genes and their products are listed in Table 2. Mutants carrying lesions in the *sel* genes were originally isolated in both *E. coli* and *Salmonella typhimurium* using quite different screening procedures. The *E. coli* mutants were isolated after overlaying anaerobically cultivated bacterial colonies with a mixture of formate and BV (Mandrand-Berthelot et al. 1978; Haddock & Mandrand-Berthelot 1982). This screening system is based on the fact that the wild type colonies become dark violet in colour due to the rapid FDH-dependent reduction of the normally colourless BV; FDH^- mutants remain colourless. Three classes of mutants, originally termed *fdhA*, *fdhB* and *fdhC* were characterised and their location on the chromosome determined. It was only after (^{75}Se)-selenium labelling experiments were performed that it became clear that they had defects in selenoprotein and seleno-tRNA biosynthesis (Leinfelder et al. 1988a). The *fdhA* locus could be shown by complementation analysis to comprise two genes, *selA* and *selB*. The *fdhB* and *fdhC* loci were renamed *selD* and *selC*, respectively (Leinfelder et al. 1988a).

By employing a screening system initially designed to isolate $FDH\text{-}N^-$ mutants, mutations in the same genes could be isolated in *S. typhimurium* (Barrett et al. 1979; Kramer & Ames 1988). The screening system used was based on a modified MacConkey's medium which included nitrate, glycerol and a small amount of glucose and formate. $FDH\text{-}N^-$ mutants accumulate formate which causes a change in the colour of the pH indicator dye phenol red; wild type colonies are normally white while *fdn* mutants are a deep red colour. Although only proven definitively for the *selA1* gene (*selD* gene in the *E. coli* nomenclature; Kramer & Ames 1988), it can be assumed from the FDH isoenzyme phenotypes of the mutants and the chromosomal locations of the respective genes that their products have a function equivalent to their *E. coli* counterparts.

Table 2. Nomenclature and function of the accessory gene products in formate dehydrogenase biosynthesis.

Gene	Former nomenclature	Proposed function of gene product
selA	*fdhA*	selenocysteine synthase; conversion of seryl-tRNA^{sec} to selenocysteinyl-tRNA^{sec}
selB	*fdhA*	selenocysteinyl-tRNA^{sec}-specific elongation factor
selC	*fdhC*	tRNA^{sec}
selD	*fdhB*	enzyme catalysing ATP-dependent conversion of selenite to phosphoselenoate
moaABCDE	*chlA*	synthesis of molybdopterin
mob	*chlB*	addition of GMP to molybdopterin
modABCD	*chlD*	molybdenum transport
moeAB	*chlE*	synthesis of molybdopterin
mog	*chlG*	molybdate 'activation'
fdhD	*fdhD*	protease, FDH maturation
fdhE	*fdhE*	chaperone (?), membrane integration

Oxygen does not affect the expression of the *sel* genes, commensurate with the requirement for their gene products both in aerobic as well as in anaerobic metabolism (Sawers et al. 1991). The cloning and the determination of the nucleotide sequences of the *sel* genes greatly facilitated the characterisation of the functions of their respective gene products. Briefly, the first step in the biosynthesis of selenocysteine is the conversion of selenide by the SELD enzyme to a phosphoselenoate derivative (Veres et al. 1992; Ehrenreich et al. 1992) which is one of the substrates for the enzyme selenocysteine synthase, the *selA* gene product (Forchhammer et al. 1991a,b). The other substrate is seryl-tRNA^{sec} derived from the aminoacylation of the *selC* gene product by the seryl-tRNA synthetase with serine (Leinfelder et al. 1988b). Selenocysteine synthetase converts seryl-tRNA^{sec} to selenocysteyl-tRNA^{sec} (Leinfelder et al. 1990; Forchhammer et al. 1991a,b) which is then bound by a unique elongation factor, SELB (Forchhammer et al. 1989). SELB delivers the selenocysteyl-tRNA^{sec} to the translating ribosome where this special tRNA molecule decodes the UGA codon effecting the cotranslational insertion of selenocysteine into the polypeptide chain. The details of the reaction mechanisms of the *sel* gene products and the peculiarities specific to the tRNA and the codon context in the vicinity of the UGA codon have been considered in several recent reviews (Stadtman 1990; Böck et al. 1991a,b; Heider & Böck 1993).

The only aspect of selenoprotein biosynthesis not yet analysed in detail concerns the mechanisms of transport of selenite into the cell. Selenocysteine incorporation into FDH-H has been shown to be saturated at 100 nM (Zinoni et al. 1987) which is strongly suggestive of a specific transport system. Furthermore, selenite transport is not affected by cysteine (Brown & Shrift 1982) indicating that it enters the cell by a pathway distinct from the sulphate transport system.

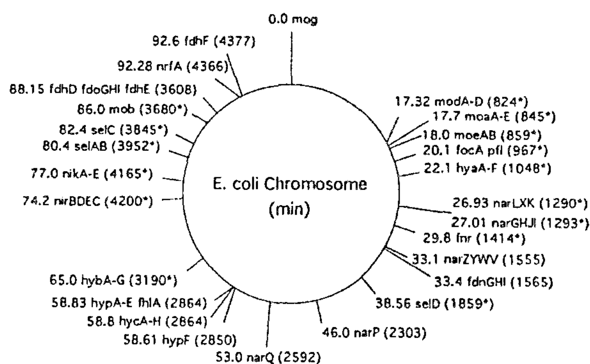

Fig. 2. The location of the genes and operons on the chromosome whose products have a function relevant to formate and hydrogen metabolism in *E. coli*. The values in brackets signify the exact, or approximate (*), locations of the respective genes on the physical genome map of *E. coli* (Kohara et al. 1987).

Genes required for molybdenum incorporation

Current data indicate that there are minimally 13 genes, distributed over 5 distinct chromosomal loci, whose products are required for the transport of molybdenum and subsequent synthesis, processing and insertion of the molybdenum cofactor. Mutations in these genes were identified by isolating mutants that were

resistant to chlorate. A comprehensive review of the characterisation of these mutants and the genetics of molybdenum cofactor biosynthesis has been published (Stewart 1988). Recently, a new nomenclature has been proposed for the *chl* genes to signify their involvement in molybdenum cofactor biosynthesis (Shanmugam et al. 1992). The gene symbol *mo-* is employed together with the existing fourth letter of the former *chl* nomenclature, for example the *chlD* genes are now referred to as *mod*. The genes and the proposed function of their gene products are listed in Table 2. The *mo-* genes, as well as those encoding other functions in FDH and Hyd isoenzyme biosynthesis, can also be located on the chromosomal map of *E. coli* in Fig. 2.

Structural studies have shown that the dimethylsulfoxide reductase from *Rhodobacter capsulatus* has its molybdenum contained within a pterin cofactor to which 5′-GMP is linked via a pyrophosphate bridge (Johnson et al. 1990). The basic scaffold is the molybdopterin (MBT) complexed with molybdenum and this has been found in all molybdoenzymes analysed so far, with the exception of dinitrogenase. Variations occur with respect to the ribonucleotide moiety attached to the basic cofactor; the function of this moiety is still unclear (for a review see Rajagopalan & Johnson 1992). However, it has been reported that molybdopterin guanine dinucleotide (MGD) is the structure of the cofactor in both nitrate reductase and formate dehydrogenase (Rajagopalan & Johnson 1992).

The roles played by the *mo-* gene products in the various steps of molybdenum cofactor assembly are gradually being revealed by combined genetic and biochemical analyses. The four gene products of the *mod* operon likely constitute a specific molybdenum-binding-protein-dependent transport system (Johann & Hinton 1987; Shanmugam et al. 1992; Corcuera et al. 1993). The products of the *moa* and *moe* operons are probably involved in MBT synthesis and recent sequence analysis (Rivers et al. 1993) of the *moa* operon indicates that the gene products show stretches of amino acids exhibiting striking similarity with folate-binding proteins (MoaB and C), tryptophan biosynthetic enzymes (MoaD) and a methyltransferase (MoaE). MoaA shows 43% identity with NifB which catalyses the initial step in the synthesis of the dinitrogenase iron-molybdenum cofactor of *Klebsiella pneumoniae* (Rivers et al. 1993).

The products of the *mog* and *mob* loci are involved in the final stages of MGD biosynthesis, since mutants accumulate MBT but cannot convert it to MGD (Miller & Amy 1983; Johnson et al. 1991; Santini et al. 1992). The *mog* gene product(s) has been proposed to function by activating the transported molybdate so that it can be introduced into molybdenum-free MBT, while the *mob* product(s) catalyses the addition of GMP to molybdenum-complexed MBT (Rajagopalan & Johnson 1992). Santini et al. (1992) have provided evidence to suggest that this latter step can occur after the MBT-Mo complex has been attached to the apoenzyme of nitrate reductase. The reader is referred to the review by Rajagopalan & Johnson (1992) for a more detailed account of recent advances in molybdopterin cofactor biosynthesis.

Genes encoding other accessory proteins

Two genes have been identified in both *E. coli* (Mandrand-Berthelot et al. 1988) and *S. typhimurium* (Barrett & Riggs 1982; Paveglio et al. 1988), mutations in which result in a FDH-N$^-$ phenotype. The genes have been termed *fdhD* and *fdhE* in *E. coli* and *fdnC* and *fdnB* in *S. typhimurium*. The *fdhD* and *fdnC* gene products are functionally homologous, as are the *fdhE*- and *fdnB*-encoded proteins (Stewart et al. 1991). These two proteins are interesting because they are neither structural components of an FDH isoenzyme nor have they a function in selenocysteine or molybdenum cofactor biosynthesis. Furthermore, they are not involved in the transcriptional regulation of the *fdnGHI* operon (Stewart et al. 1991). The FdhD and FdhE proteins have molecular weights of 30,500 and 32,000, respectively, and the nucleotide sequence of both genes has been established but gives no clue as to the possible function of the proteins (Schlindwein et al. 1990; Schlindwein & Mandrand 1991; Plunkett et al. 1993).

Schlindwein et al. (1990) reported that the expression of the *fdhE* gene was reduced in a *selA* mutant. In the light of the recent sequence information (Plunkett et al. 1993) it appears that the *fdhE* and *fdoGHI* genes are cotranscribed. Therefore, the apparent dependence of *fdhE* transcription on *sel* gene products probably is due to a polarity effect caused by abberant translation termination at the UGA codon within the *fdoG* coding sequence.

Stewart and colleagues could show clearly that neither *fdhD* nor *fdhE* lesions affected *fdn* operon expression (Stewart et al. 1991). However, Mandrand-Berthelot et al. (1988) could demonstrate that no immunologically detectable FDH-N enzyme was present in extracts of *fdhE* mutants. This anomaly can be explained by an enhanced turnover of the FDH-

N polypeptides, suggesting that the FdhE protein may have a function in the processing or assembly of the FDH-N isoenzyme, and perhaps also the FDH-O isoenzyme, in the cytoplasmic membrane. Pulse-labelling experiments with concomitant subcellular localisation studies may resolve this question.

Paveglio et al. (1988) described an unusual phenotype of their *fdhD* mutants in *S. typhimurium* in which extracts from these strains regained FDH-N and FDH-H enzyme activity after 2 to 3 hours incubation. However, this recovery of activity could be prevented by inclusion of protease inhibitors in the extracts. Moreover, the conditional nature of the FDH-H activity could be abolished by reducing the glucose content of the growth medium (Paveglio et al. 1988). These features would be compatible with a 'leaky' phenotype and FdhD functioning as a protease required for maturation of the three FDH isoenzymes. Reducing the glucose concentration in the medium would lower the cellular energy status and consequently the rate of protein synthesis, thus perhaps allowing the low levels of FdhD protein (or the reduced activity of the protein if the mutation is in the structural gene) to maintain pace with enzyme synthesis. Naturally these suggestions are hypothetical but would provide an explanation for the strange phenotypes of the *fdhD* and *fdhE* mutants. Interestingly, a *fdhD* homologue is also present in the operon encoding the FDH of *W. succinogenes* (Bokranz et al. 1991; H. Heider, personal communication).

Multiple hydrogenase isoenzymes in *E. coli*

Numerous studies have been undertaken to characterise the molecular reactions catalysed by hydrogenase. Early work concentrated on *E. coli* and it was recognised that anaerobically grown intact cells were capable of supporting hydrogen production from formate (Ordal & Halvorson 1939). These authors proposed that this FHL activity was constituted by a complex comprising a FDH, a hydrogenase and electron carriers. FHL activity was observed principally in fermenting cells and was strongly inhibited by the presence of electron acceptors such as oxygen or nitrate and activity was induced by inclusion of formate in the growth medium (Pinsky & Stokes 1952; Peck & Gest 1957; Fukuyama & Ordal 1965; Ruiz-Herrera & Alvarez 1972; Sawers et al. 1985).

Hydrogen can also act as a source of reductant and *b*-type cytochromes and menaquinone could be shown to be involved in this reaction (Macy et al. 1976; Haddock & Jones 1977). Fumarate or malate can function as non-fermentable carbon sources with reductant being supplied by dihydrogen or formate (Macy et al. 1976). Bernhard and Gottschalk (1978b) then demonstrated that up to 1 mole of ATP could be generated per mole of fumarate reduced by fumarate reductase with dihydrogen as energy source, thus defining a new respiratory pathway in anaerobically growing *E. coli*. Further work demonstrated that nitrate and trimethylamine N-oxide (TMANO), as well as fumarate, can act as acceptors (Yamamoto & Ishimoto 1978). The energy-conserving role of hydrogenase in this respiratory pathway could be underscored by the demonstration that it was proton-translocating (Jones 1980b).

The evolution of dihydrogen from formate is not energy-conserving. This fact, together with the observation that multiple stained bands in non-denaturing polyacrylamide gels could be ascribed to hydrogenase activity (Ackrell et al. 1966; Yamamoto & Ishimoto 1978; Ballantine & Boxer 1985) suggested the presence of more than one hydrogenase enzyme species in *E. coli*. Ballantine & Boxer (1985) identified two immunologically distinct, membrane-associated hydrogenase isoenzymes. These authors also demonstrated that both isoenzymes contained nickel. Hydrogenase isoenzyme 2 (Hyd-2) is less abundant than Hyd-1 and could be released from the membrane as a soluble active fragment by treatment with trypsin. A further criterion supporting their distinction is that Hyd-2 can be irreversibly inactivated by high pH (Ballantine & Boxer 1985, 1986).

In an attempt to assign either H_2-oxidation or proton-reducing functions to these isoenzymes Sawers et al. (1985) demonstrated a strong correlation between Hyd-2 and dihydrogen-uptake activity in cells grown with hydrogen and fumarate or glycerol and fumarate. Although Hyd-1 activity could be shown to correlate with FHL activity in the wild type, this correlation broke down in a mutant unable to synthesise the transcriptional regulator FNR. In a *fnr* mutant FHL activity could be shown to attain almost wild type levels when formate was included in the growth medium while both Hyd-1 and Hyd-2 were essentially absent (Sawers et al. 1985). This result had two implications: first, Hyd-1 was not part of the FHL pathway and thus has a distinct function in anaerobic metabolism, probably dihydrogen oxidation; second, if neither Hyd-1 nor Hyd-2 could be correlated with FHL activity then this indicated that a third hydrogenase activity must exist. Indeed, by employing immunoprecipitation analyses with Hyd-1- and Hyd-2-specific polyclonal antisera

these authors could show categorically that a third immunologically distinct activity existed that correlated exactly with FHL activity. The activity of this Hyd-3 isoenzyme was shown to be unstable (Sawers et al. 1985).

This assignment of isoenzyme function was challenged by Stoker et al. (1988, 1989a) who isolated a mutant no longer able to synthesise what they termed the 'labile' hydrogenase activity. This mutant, however, still synthesised an active FHL complex but could not oxidise dihydrogen with fumarate as electron acceptor. The mutation was localised to 64.8 min on the *E. coli* chromosome and most probably is in one of the structural genes encoding Hyd-2 (Lee et al. 1985; Przybyla et al. 1991, 1992). Stoker et al. (1988) based their assignment on activity-staining of polyacrylamide gels and enzyme activities after analysing cells scraped from agar plates. Consequently, the hydrogenase specific activities measured were very low and since Hyd-3 activity is so unstable it was likely to be absent in their preparations leaving Hyd-2 as the principle hydrogenase activity.

As with the FDH isoenzymes, the unequivocal allocation of function to the respective Hyd isoenzymes was obtained by the cloning, sequencing and specific deletion analysis of their structural genes. Hyd-1 and Hyd-2 are uptake hydrogenases (Menon et al. 1990, 1991; Przybyla et al. 1992) while Hyd-3 is the hydrogenase associated with the FHL complex (Pecher et al. 1983; Zinoni et al. 1984; Böhm et al. 1990; Sauter et al. 1992).

Properties of the hydrogenase isoenzymes

Subunit structure of the hydrogenase isoenzymes

The natural electron acceptors or donors of the three Hyd isoenzymes have not been identified, although from our current knowledge it can be inferred what they may be (see Section 6). The activity of all three isoenzymes is determined in an identical manner by taking advantage of the reversibility of the hydrogenase reaction and measuring the reduction of BV with dihydrogen as electron donor. Methyl viologen (MV) and methylene blue (MB) are also functional but yield much lower activities (Adams & Hall 1979; Ballantine & Boxer 1986; Sawers & Boxer 1986). Naturally, because only one assay is available for all three enzymes this escalated some of the initial problems encountered in trying to define the number of isoenzymes; it also made purification somewhat problematic.

Hydrogenase was partially purified from the membrane fraction of *E. coli* grown in H_2 and fumarate medium (Bernhard & Gottschalk 1978a). Solubilisation was effected by treatment with sodium deoxycholate and trypsin, suggesting that it was Hyd-2 that these authors characterised (Ballantine & Boxer 1985, 1986). Later a further hydrogenase preparation was reported (Adams & Hall 1979) from apparently aerobically grown cells. These cells must have become anaerobic, however, because hydrogenase is not synthesised aerobically (Yamamoto & Ishimoto 1978; Sawers et al. 1985). Again, the enzyme was purified after protease treatment of membrane fractions (Adams & Hall 1979). The native molecular weight of the enzyme was 113,000 and proved to be a homodimer of 56 kD subunits. Later studies also demonstrated that this enzyme was Hyd-2 that, apparently, had lost the small subunit during purification (Ballantine & Boxer 1986; Hallahan et al. 1987).

Hyd-2 was eventually purified to homogeneity as an active tryptic fragment from anaerobic glucose-grown cells (Ballantine & Boxer 1986). Sedimentation equilibrium ultracentrifugation identified a native molecular weight of 180,000 for the enzyme and it was composed of equimolar amounts of 61 kD and 30 kD subunits, suggesting an $\alpha_2\ \beta_2$ structure. Immunological analysis of membrane-associated Hyd-2 indicated that an approximately 5 kD fragment was removed from the small subunit by the trypsin cleavage (Ballantine & Boxer 1986). It is not known whether this cleavage occurs at the N- or C-terminus. The nucleotide sequence-derived molecular weights have been reported as being 62,000 and 36,000 (including signal-peptide sequence) for the large and small subunits, respectively (Przybyla et al. 1992).

The first report of Hyd-1 characterisation was based on an immunological approach whereby precipitin arcs exhibiting Hyd activity were excised and used to prepare hydrogenase-specific antiserum (Graham et al. 1980). Using this antiserum the authors precipitated a 58 kD ^{35}S-methionine-labelled polypeptide. Further immunological studies combined with analyses employing radioactive membrane-impermeant probes demonstrated that this hydrogenase spanned the cytoplasmic membrane (Graham 1981). The subunit molecular mass was also revised to 63 kD.

Hyd-1 could eventually be purified to near homogeneity from anaerobic glucose-grown cells (Sawers & Boxer 1986). The pure enzyme has a native molec-

ular weight of 200,000 and is composed of polypeptides of apparent Mr 64,000, 31,000 and 29,000. The apparent stoichiometry of the respective polypeptides was 2:1:1 and immunological studies demonstrated that the 31,000 and 29,000 polypeptides were proteolytic degradation products of a 35,000 subunit. Thus, the isolated enzyme has an $\alpha_2\ \beta_2$ structure (Sawers & Boxer 1986) and the estimated size of the subunits is in good agreement with those deduced for the large and small subunits from the DNA sequence of the respective genes (Menon et al. 1990).

Hyd-1 has also been purified from a *mod* mutant and it has been reported that the enzyme lacks the small subunit when purified from cells grown to the stationary phase (Francis et al. 1990). That the small subunit of Hyd-1 can be readily dissociated from the large subunit without an apparent loss of the ability to catalyse the hydrogen-dependent reduction of BV had been reported earlier (Graham et al. 1980; Sawers & Boxer 1986).

The characterisation of Hyd-3 has posed a great many problems. Although the activity of the isoenzyme could be measured after dispersion of membrane vesicles with detergent (Sawers et al. 1985), it could not be visualised by staining for enzyme activity subsequent to non-denaturing polyacrylamide gel electrophoresis nor could it be purified in a catalytically active form (Sawers 1985). It should be stressed that no anaerobic precautions were taken during these procedures. These studies indicated that Hyd-3 enzyme activity was extremely labile. The HycE polypeptide, which constitutes the large subunit of Hyd-3, could be isolated in a native form only by monitoring the purification immunologically, after first having identified the *hycE* gene (Böhm et al. 1990; see below). An intensive mutagenesis study led Sauter et al. (1992) to conclude that both the HycE and FDH-H polypeptides form a loose association with the other components of the FHL enzyme complex, which is probably located on the inner face of the cytoplasmic membrane. Taken together, these findings provide an explanation for the difficulties encountered in earlier studies of FDH-H and Hyd-3 enzyme activites (Sawers et al. 1985) and accounts for the inherent instability of the FHL complex.

It is likely that Hyd-3 also has a small subunit that functions in electron-transfer within the complex. The deduced amino acid sequences of six putative structural components of the FHL complex encoded by the *hyc* operon suggest that the HycG protein is a candidate for the small subunit of Hyd-3 (Böhm et al. 1990; Sauter et al. 1992).

Cofactor content of the hydrogenase isoenzymes

All three hydrogenase isoenzymes in *E. coli* are nickel, iron-sulfur proteins. More than 10 years ago it was shown that nickel present in the hydrogenase of *Methanobacterium thermoautotrophicum* is redox-sensitive, suggesting that it is the site of interaction with H_2 in the enzyme (Graf & Thauer 1981; Albracht et al. 1982). Evidence has been presented that nickel in hydrogenase does undergo a direct interaction with dihydrogen (Van der Zwaan et al. 1985) and the various aspects of nickel chemistry and function in the catalysis of hydrogenase have been reviewed recently (Przybyla et al. 1992).

That both Hyd-1 and Hyd-2 are nickel enzymes was demonstrated by Ballantine & Boxer (1985). Subsequently, the presence of nickel in the purified enzymes could be confirmed (Ballantine & Boxer 1986; Sawers & Boxer 1986). Only more recently could it be shown conclusively that Hyd-3 also contains nickel (Rossmann et al. 1994) although this had been speculated upon for a number of years, based on genetic evidence (Waugh & Boxer 1986; Wu & Mandrand-Berthelot 1986b; Wu et al. 1989; Navarro et al. 1993).

Nickel has been clearly shown to be associated with the large subunits of isoenzymes 1 and 3 (Ballantine & Boxer 1985; Sawers & Boxer 1986; Rossmann et al. 1994), consistent with it also being liganded to this subunit in other hydrogenases (Eidsness et al. 1989; He et al. 1989). One mole of nickel is associated with 1 mole of enzyme (Przybyla et al. 1992). Ballantine & Boxer (1986) estimated that Hyd-2 contained approximately 1.5 mole nickel / mole enzyme while Sawers & Boxer (1986) only found 0.3 mole nickel per mole of Hyd-1 enzyme. The latter finding may indicate that Hyd-1 loses nickel more readily than Hyd-2. Purified HycE contained roughly 1 mole nickel per mole of enzyme as judged by atomic absorption spectroscopy (Rossmann et al. 1994).

Both Hyd-1 and Hyd-2 could be shown to have approximately 6 moles of non-haem iron and 6 moles of acid-labile sulfide per mole of enzyme (Ballantine & Boxer 1986; Sawers & Boxer 1986; Francis et al. 1990). The structure and location of the Fe-S clusters in the Hyd isoenzymes of *E. coli* have yet to be determined biophysically but the general opinion at the moment is that the small subunits of hydrogenases bear the majority of the iron-sulfur clusters (Pryzbyla

et al. 1992; Voordouw 1992). The number of cysteinyl residues in the HyaA (Menon et al. 1990), HybA (Przybyla et al. 1992) and HycG (Böhm et al. 1990) proteins are consistent with this hypothesis.

Catalytic activity of the hydrogenase isoenzymes

Hydrogenase isoenzyme 3 has yet to be purified in a catalytically active form, thus there is no kinetic data available for this enzyme. The other two isoenzymes have been characterised only partially. The apparent Km values for H_2 of both is very low (2–4 μM) which is appropriate for a dihydrogen-oxidising function (Ballantine & Boxer 1986; Sawers & Boxer 1986). Isoenzyme 2 could be shown to have a greater capacity for H_2-oxidation than Hyd-1 (Ballantine & Boxer 1986). Hyd-2 has an apparent Km for BV of 4.5 mM and a Vmax with this substrate of 630 μmole H_2 oxidised per min · mg protein. The Vmax with MV as electron acceptor is reduced by a factor of 30 compared with the value attained with BV (Ballantine & Boxer 1986). The Hyd-2 preparation of Adams & Hall (1979) had a 6-fold better Vmax for MV than the enzyme preparation of Ballantine & Boxer (1986) and a much higher affinity for BV. The reason for these quite dramatic differences is not immediately clear but it may be a reflection of differences in the purification of the enzyme. The physiological electron acceptor for Hyd-2 is not known and the purified enzyme could not reduce FAD, FMN, NAD^+ or $NADP^+$ (Adams & Hall 1979). Nor, indeed, could the enzyme interact with ubiquinone, menadione or duraquinone (Ballantine & Boxer 1986).

Both Hyd-1 and Hyd-2 are susceptible to the same spectrum of inhibitors; these include carbon monoxide, heavy metal salts, and N-bromosuccinimide (Adams & Hall 1979; Ballantine & Boxer 1986; Sawers & Boxer 1986). However, these two isoenzymes do have different pH stability profiles. While Hyd-1 is stable over a wide pH range from 4 to 10, Hyd-2 is rapidly and irreversibly inactivated at pH values above 9.0. The properties of the three hydrogenase isoenzymes are summarised in Table 3.

Genetics of hydrogenase formation

Isolation of hyd mutants

At least ten research groups have isolated and characterised '*hyd*' mutants in *E. coli*. Two major problems hampered early studies aimed at resolving the genetics of hydrogenase. First, although it had been known for a long time that both H_2 oxidation and H_2 evolution were catalysed by anaerobic *E. coli* cells, it was not until 1985 that it became clear that more than one isoenzyme was present in *E. coli* (Ballantine & Boxer 1985; Sawers et al. 1985). Second, the three isoenzymes cannot be distinguished based on their electron acceptor specificities. Because of this, almost all mutants isolated using BV or MV-based screening systems are pleiotropic, i.e. they lack all hydrogenase functions (Pascal et al. 1975; Graham et al. 1980; Lee et al. 1985; Waugh & Boxer 1986). These mutants have defects in genes whose products are involved in nickel metabolism or in the processing and assembly of catalytically active hydrogenase (see below). Mapping studies localised the mutations in these strains to the 58–59 min region of the *E. coli* chromosome.

Either by using modifications of this screening procedure or by using a different approach other mutants with more specific defects in hydrogen metabolism could be isolated. Thus, Shanmugam and colleagues made use of the superoxide radical-generating capacity of reduced BV to 'select' for mutants that could not grow in the presence of dihydrogen (Lee et al. 1985). Along with mutants having lesions at 58 min they also isolated mutants which could not oxidise dihydrogen with fumarate as electron acceptor; however, these mutants retained other hydrogenase functions. The mutation mapped at 65 min on the chromosome. Similar mutants were isolated using penicillin-enrichment on H_2-fumarate medium (Krasna 1984) followed by screening for lack of dihydrogen-dependent reduction of MV (Stoker et al. 1989a).

Wu & Mandrand-Berthelot (1986b) used a MacConkey-based medium, which included fumarate and formate, to attempt to isolate mutants that were defective specifically in the FHL pathway. These mutants proved to be pleiotropic but the mutations were all located at 77 min on the *E. coli* chromosome. The Hyd^+ phenotype could be restored to one class of these mutants by growth in the presence of high concentrations of nickel salts, suggesting a defect in transport.

Finally, novel mutants were isolated that were defective specifically in FHL pathway synthesis by researchers in two laboratories. These mutants were isolated subsequent to Mu d1 insertion mutagenesis but were obtained by slightly different screening programmes; Pecher et al. (1983) screened for mutants that no longer showed increased β-galactosidase synthesis anaerobically; Yerkes et al. (1984), in contrast,

Table 3. Properties of the hydrogenase isoenzymes.

	Hyd-1	Hyd-2	Hyd-3
Subunit composition	$\alpha_2\beta_2(\gamma_2)^a$	$\alpha_2\beta_2(\gamma_2)^a$	$\alpha\beta$ associated with FHL complex
Metals & cofactors	FeS, Ni	FeS, Ni	FeS, Ni
Function	H_2 oxidation	H_2 oxidation	proton reduction
Artificial electron acceptor specificity	benzyl viologen	benzyl viologen	benzyl viologen
Apparent Km for H_2 with BV	2–4 μM	2–4 μM	unknown
pH stability	stable over broad pH range	irreversibly inactivated above pH 9	unknown
Inhibitors	CO, heavy metals	CO, heavy metals	unknown

[a]The existence of the γ subunit is hypothetical and is based on the homologies exhibited by the HyaC and HybB proteins with the third subunit of the hydrogenase of *Wolinella succinogenes* (see Dross et al. 1992).

Table 4. Nomenclature of the '*hyd*' genes of *E. coli.*

New nomenclature	Former nomenclature	References
hypB	*hydB*	Waugh & Boxer 1986
	hydE	Sankar et al. 1985
	hydE	Chaudhuri & Krasna 1987
hypD	*hydB*	Sankar & Shanmugam 1988a
hypE	*hydF*	Sankar & Shanmugam 1988b
hypF	*hydA*	Karube et al. 1984; Lee et al. 1985; Sankar et al. 1985
	850	Pascal et al. 1975
fhlA	*fhl*	Sankar et al. 1985
hycA	*ant-4*	Yerkes et al. 1984
hycB	M17s	Pecher et al. 1983
hycC	*ant-2*	Yerkes et al. 1984
hycD	*ant-3, ant-5*	Yerkes et al. 1984
hycE	*ant-1*	Yerkes et al. 1984
hyb operon	*hup*	Lee et al. 1985
	hydL	Stoker et al. 1989a
	hydF	Stoker et al. 1989a
nik operon	*hydC*	Wu & Mandrand-Berthelot 1986b

screened for mutants incapable of reducing BV with formate.

Essentially, the same mutants have been isolated recurrently in different laboratories. Naturally, the same alleles often have received different names and this has led to considerable confusion in the literature. Fortunately, the large amount of information acquired recently through DNA sequencing has enabled us to assign functions to a large number of gene products. Moreover, sequencing has revealed that there are many more genes whose products are involved in hydrogenase synthesis than was previously supposed; there are minimally 34 gene products with a direct involvement in hydrogenase isoenzyme biosynthesis.

The introduction of a uniform nomenclature (Menon et al. 1990; Böhm et al. 1990) has helped clarify the earlier confusion. Thus, the structural genes of hydrogenases 1, 2 and 3 have the three-letter code *hya*, *hyb* and *hyc*, respectively. Those genes whose products are required for the synthesis of minimally two hydrogenase isoenzymes have the gene symbol *hyp* (*p*leiotropic) and those genes specifically involved in nickel transport are called *nik* (Wu et al. 1991). A

compilation of the old and new nomenclature is presented in Table 4.

The structural genes encoding the hydrogenase isoenzymes

Of the currently known genes involved in hydrogen metabolism in *E. coli* only those encoding Hyd-1 have never been identified by random mutagenesis and screening programmes. Instead, the genes of the *hya* operon were identified by hybridisation analysis using DNA probes from conserved regions of the hydrogenase structural genes of *Desulfovibrio vulgaris* (Menon et al. 1990). Two distinct sets of hydrogenase genes were identified; the second set were those encoding Hyd-2 (Przybyla et al. 1992). The *hyb* genes are located at 65 min on the *E. coli* chromosome (Fig. 2) and are probably those affected in the mutants isolated by Krasna (1984), Lee et al. (1985) and Stoker et al. (1989).

The first major surprise was that the structural genes of both isoenzymes are part of polycistronic operons. The *hya* operon comprises six genes and the *hyb* operon seven genes (Fig. 1B). The unifying feature of NiFe-hydrogenases is a large subunit of 50–60 kD. Comparison of the N-terminal amino acid sequence of the large subunit of Hyd-1 with the deduced amino acid sequences of the open reading frames (ORFs) of the *hya* operon identfied *hyaB* as that encoding the large subunit. This subunit shows over 60% similarity to the large subunit of other NiFe-hydrogenases, including *hybC*, which is the gene specifying the large subunit of Hyd-2. Several recent, detailed reviews cover various aspects of the homologies between hydrogenase polypeptides from different microorganisms and this will not be dealt with in this review (Przybyla et al. 1992; Voordouw 1992; Wu & Mandrand 1993; Friedrich & Schwarz 1993; Vignais & Toussaint 1994). Suffice it to say that all large subunits of NiFe- and NiFeSe-hydrogenases share two highly conserved peptide sequences: a more N-terminal oriented RxCGxCxxxH motif and a C-terminal DPCxxCxxH motif. These sequences are purported to include amino acid residues that are crucial for the liganding of nickel. Indeed, mutagenesis studies that altered some of the amino acid residues in these motifs of HyaB indicate that the Arg and Cys residues in the N-terminal motif and the Asp, Pro, one of the His and two of the Cys residues of the C-terminal motif are essential for hydrogenase activity (Przybyla et al. 1992). None of the large subunits have a signal peptide.

The small subunits of many sequenced hydrogenases show significant similarity, having cysteinyl residues with an organisation characteristic of ferredoxin-like iron-sulfur clusters. Fifteen of the sequenced small subunits from various organisms have a very highly conserved signal peptide which is unusually long and contains a conserved RRxFxK motif (Wu & Mandrand 1993). The *hyaA* gene product falls into this category and direct amino acid sequencing of the N-terminus of the purified small subunit demonstrated that the signal peptide is 45 amino acids long (Menon et al. 1990).

The small subunit of Hyd-2 has been reported to be encoded by the *hybA* gene (Przybyla et al. 1992). Apart from the signal peptide it shows no other features typical of hydrogenase small subunits other than that it is ferredoxin-like. That Hyd-2 has a radically different configuration from Hyd-1 in the cytoplasmic membrane was indicated by their differential sensitivities to trypsin (Ballantine & Boxer 1985, 1986). Therefore, these findings are not inconsistent with the small subunit of Hyd-2 being atypical.

The mutant M17s isolated by Pecher et al. (1983) and the *ant* (*an*aerobic electron *t*ransport) mutants isolated by Yerkes et al. (1984) proved to have lesions in various genes of the *hyc* operon (Böhm et al. 1990). The operon encodes eight polypeptides (Fig. 1B) and it is probable that six of these encode components of the FHL complex (Sauter et al. 1992). Only the FDH-H component is encoded by a gene located elsewhere on the chromosome. However, the synthesis of FDH-H is coordinated precisely with that of the other FHL components encoded by the *hyc* operon (Birkmann et al. 1987b; Lutz et al. 1990; Rossmann et al. 1991). The large subunit of Hyd-3 is encoded by the fifth gene of the operon, *hycE* (Böhm et al. 1990). The polypeptide is 65 kD and other than the fact that it contains the two highly conserved structural motifs characteristic of large subunits, it has no significant homology with the large subunits of other hydrogenases (Böhm et al. 1990; Wu & Mandrand 1993).

The second and sixth (*hycB* and *hycF*) genes encode ferredoxin-like electron transport proteins and HycB shows similarity to the small subunits of FDHs and probably represents the docking site for FDH-H on the FHL complex (Sauter et al. 1992). The only product of the *hyc* operon that shows significant similarity to small subunits of hydrogenase is the *hycG* gene product (Böhm et al. 1990; Sauter et al. 1992). HycG does not have a signal peptide and interestingly it shows a high degree of similarity to protein G, a component of the NADH-ubiquinol oxidoreductase (NUO) complex

Table 5. Putative functions of the accessory proteins in hydrogenase biosynthesis.

Protein	Specificity	Observations
HypA	Hyd-3	antagonises Hyd-2 synthesis or activity
HypB	Hyd-1, Hyd-2 & Hyd-3	GTPase activity; nickel insertion
HypC	Hyd-1 & Hyd-3	general processing; homology with HybG
HypD	Hyd-1, Hyd-2 & Hyd-3	general processing
HypE	Hyd-1, Hyd-2 & Hyd-3	general processing
HypE	Hyd-1, Hyd-2 & Hyd-3	general processing
HypF	Hyd-1, Hyd-2 & Hyd-3	general processing
NikA, B, C, D, E	Hyd-1, Hyd-2 & Hyd-3	nickel transport
HyaD	Hyd-1	processing and assembly; homology with HybD
HyaE	Hyd-1	processing and assembly
HyaF	Hyd-1	nickel insertion?
HybD	Hyd-2	processing and assembly; homology with HyaD
HybE	Hyd-2	processing and assembly
HybF	Hyd-2	processing and assembly
HybG	Hyd-2 (Hyd-1?)	processing and assembly; homology with HypC

from chloroplasts and mitochondria (Böhm et al. 1990; Weiss et al. 1991).

Putative functions of the other polypeptides encoded by the hya, hyb and hyc operons

A total of 21 polypeptides are encoded by the three hydrogenase structural gene operons. Six of these can be accounted for as constituting the large and small subunits and the HycB polypeptide probably represents the small subunit of FDH-H. Clues to the physiological roles played by the remaining polypeptides have been obtained by conducting homology searches in the protein data bases and by constructing in-frame deletions in each gene. The latter method ensures that minimal polarity effects on the expression of the downstream-lying genes occur and at the same time permits a detailed examination of the effects caused by preventing the synthesis of one particular polypeptide. Such a detailed analysis has been performed with the *hya* and *hyc* operon genes (Menon et al. 1991; Sauter et al. 1992). Unfortunately, the DNA sequence of the *hyb* operon has not been published but information regarding the putative functions of some of the gene products can be gleaned from two different reports (Przybyla et al. 1991, 1992). The homologies exhibited by, and/or putative functions of, the various gene products are listed in Table 5. For reasons that will become apparent below I shall discuss Hyd-3 in terms of the FHL complex as a whole and the effects of the various mutations in the *hyc* operon will be considered with regard to their influence on FHL complex formation.

All of the *hya* gene products are essential for a fully functional Hyd-1 isoenzyme (Menon et al. 1991). Similarly, all of the *hyb* gene products are required for the synthesis of a fully functional Hyd-2 isoenzyme (Przybyla et al. 1991). Only in the *hyc* operon is there a single polypeptide encoded by *hycA* that is not necessary for the formation of an active FHL complex (Sauter et al. 1992). On the contrary, HycA, when overproduced prevents the synthesis of FHL; it functions as a repressor of *fdhF* gene and *hyc* and *hyp* operon expression. The HycB, HycE, HycF and HycG proteins have been discussed; however, it should be added that immunological studies have shown that the latter two proteins are tightly membrane associated (Sauter et al. 1992). Computer analyses carried out on HycC and HycD indicate that both contain putative membrane-spanning helices and portions of the amino acid sequence of both proteins exhibit significant homology to electron-transfer components of mitochondrial and plastid NUO complexes. Similarly, portions of HycE and almost the complete HycG protein also exhibit similarity to components of this complex. Thus, the current data are not inconsistent with HycB, C, D, E, F, G and the *fdhF* gene product constituting a membrane-associated FHL complex (Sauter et al. 1992).

The HycH polypeptide does not form part of this complex but is required for the synthesis of an active FHL enzyme complex. It was noted by Sauter et

al. (1992) that in a *hycH* deletion mutant the HycE polypeptide migrated slightly more slowly in a SDS-polyacrylamide gel. However, by mixing an extract containing HycH with one lacking it, there resulted a time-dependent conversion of the HycE polypeptide from the slowly migrating form to that normally observed in wild type extracts. This could be shown recently to be due to a specific endoproteolytic cleavage event resulting in the removal of the 32 C-terminal amino acids of the HycE polypeptide (Rossmann et al. 1994). Moreover, there is a strong correlation between C-terminal processing and nickel incorporation into the nascent HycE polypeptide. HycH, however, does not catalyse this cleavage but may be involved in activating or regulating a specific protease activity in the cell (R. Rossmann & A. Böck, unpublished data).

Deletion of the complete *hyc* operon has no influence on Hyd-1 or Hyd-2 enzyme synthesis and activity. Equally, *hya* and *hyb* deletions have been reported to have no measurable effect on FHL synthesis or activity (Sauter et al. 1992). Furthermore, none of the polypeptides encoded by the *hyc* operon, except for HycE and HycG, have similarity with polypeptides encoded by the *hya* and *hyb* operons.

Three of the *hya* gene products, HyaD, HyaE and HyaF are required for nickel incorporation, C-terminal processing and membrane insertion of the native Hyd-1 isoenzyme (Menon et al. 1991). The order in which these events occurs has not been elucidated but processing does not appear to be essential for membrane association, since a *hyaD* in-frame deletion resulted in a non-processed, inactive enzyme which was located in the membrane fraction. Overproduction of that HyaA and HyaB proteins in a mutant which has the *hya* operon deleted led to synthesis of a soluble apoenzyme where neither the small subunit nor the large subunit was processed. This, plus other data indicate that processing of the large and small subunits is concurrent (Menon et al. 1991). These authors have proposed that there is a specific 'processing complex' for Hyd-1, including HyaD, E and F where HyaF appears to have a catalytic function in restoring activity to the complex in the presence of nickel.

The HyaC protein is hydrophobic and possesses several putative membrane-spanning helices (Menon et al. 1990, 1991). Furthermore, it has a homologue encoded by the *hyb* operon (*hybB*; Przybyla et al. 1991) but what is more important, both are homologous to the third subunit of the *W. succinogenes* hydrogenase (Dross et al. 1992). This third subunit has been suggested to be a cytochrome *b* component required to mediate electron transfer to the quinone pool (Dross et al. 1992). Menon et al. (1991) also have provided some evidence to suggest that HyaC is required for the integrity of Hyd-1 in the membrane. These data would suggest that the reason why no physiological electron acceptor has been found previously for Hyd-1 and Hyd-2 isoenzymes (Ballantine & Boxer 1986; Sawers & Boxer 1986) may have been due to loss of a third subunit either at the membrane-solubilisation stage or during the purification itself.

It has been suggested that there may be some interaction between the constituent polypeptides encoded by the *hya* and *hyb* operons (Przybyla et al. 1991, 1992). Studies using *hyb* deletion mutants demonstrate that they have a very low Hyd-1 activity and that unprocessed HyaB could be detected in these mutants. This suggests that *hyb* operon components are required for effective Hyd-1 processing. Moreover, the HyaD and HybD proteins are homologous, suggesting that both isoenzymes may undergo analogous processing events. Finally, the HybG protein has been reported to be 70% identical with the HypC polypeptide (see below) which is essential for maturation as measured by incorporation of nickel into, and processing of, the HycE polypeptide (Jacobi et al. 1992).

Other loci specifying proteins involved in hydrogenase isoenzyme synthesis

The mutants most frequently isolated carry lesions in the *hyp operon* (Table 4) and as soon as it was realised that several hydrogenase isoenzymes exist in *E. coli* it became clear that the polypeptides encoded by these mutated genes normally must perform a function that is central to all three isoenzymes. The universal feature, of course, is nickel and the *hyp* gene products have been shown to have a crucial role in the insertion of nickel into the nascent large subunits of the three isoenzymes. The universality of the *hyp* operon is further exemplified by its evolutionary conservation in all organisms that synthesise Ni-containing hydrogenases (Jacobi et al. 1992; Friedrich & Schwarz 1993; Vignais & Toussaint 1994).

The *hyp* operon of *E. coli* encodes six polypeptides (Lutz et al. 1991), the promoter distal of which encodes the transcriptional regulator FHLA (see below). Early studies pointed to a function of the *hyp* gene products in nickel metabolism, since hydrogenase functions in *hypB* null mutants could be phenotypically restored to wild type simply by supplying high concentrations of nickel to the growth medium (Waugh & Boxer 1986;

Chaudhuri & Krasna 1987; Stoker et al. 1989a; Lutz et al. 1990; Jacobi et al. 1992; Maier et al. 1993). This phenomenon is restricted to *hypB* mutants, since mutations in any of the other four *hyp* genes cannot be suppressed by high nickel concentrations (Jacobi et al. 1992). Recent studies have demonstrated that HypB is a GTP-binding protein (Maier et al. 1993) and it has been postulated that HypB-GTP catalyses the insertion of nickel into the large subunits of the enzymes. GTP hydrolysis is then required for the dissociation of HypB from the macromolecular complex with the hydrogenase isoenzyme which would be in accord with what occurs in similar guanine nucleotide-binding protein-dependent reactions (Bourne et al. 1991). The fact that very high concentrations of nickel ions obviates the requirement for HypB protein would be in accord with it catalysing a reaction just prior to the final processing step late in the assembly pathway.

Construction of specific deletions in the *hyp* operon showed that HypB, HypD and HypE are essential for the synthesis of all three isoenzymes and mutations in these genes prevent the processing of the large subunit of all three isoenzymes and the small subunit of Hyd-1 and Hyd-2 (Lutz et al. 1991; Jacobi et al. 1992).

Mutations in *hypC* abolish Hyd-3 processing and FHL activity (Jacobi et al. 1992). A *hypC* mutant also is reduced in its content of Hyd-1, but not in its content of active Hyd-2. As mentioned above, HypC and HybG are 70% identical which suggests that either protein may be capable of fulfilling the role of processing nascent Hyd-1.

HypA is necessary for the processing of Hyd-3 but does not appear to be required for Hyd-1 processing (Jacobi et al. 1992). However, it has been reported that *hypA* mutants have enhanced Hyd-2 activity; in contrast, the *hypA* gene in multicopy reduces Hyd-2 activity. These effects are post-translational and suggest that HypA may interact with and antagonise the activity of a component of the Hyd-2 processing pathway (Jacobi et al. 1992).

The gene nomenclature *hypF* has been recently assigned to the product of the *hydA* gene (Pascal et al. 1975; Lee et al. 1985; Sankar et al. 1985; Tomiyama et al. 1991) due to the homology exhibited by its gene product with the HypF polypeptides of *Rhodobacter capsulatus* (Colbeau et al. 1993) and *Rhizobium leguminosarum* (Rey et al. 1993). Mutations in the *hypF* gene of *E. coli* strongly reduce hydrogenase-associated cellular functions (Karube et al. 1984; Lee et al. 1985). The *hypF* gene is not located in the *hyp* operon but between 4 and 5 kilobase pairs downstream of the *hyc* operon (T. Maier & A. Böck, unpublished data). It encodes a large protein of 80 kD containing putative Cys-rich clusters (Yamamoto et al. 1990; Tomiyama et al. 1991) but its role in hydrogenase processing is currently unknown. A gene termed *hydN* is located just upstream of *hypF* and both genes may be co-transcribed; it is not known whether its product has any function in hydrogenase isoenzyme biosynthesis.

Genes involved in nickel transport

Mandrand and colleagues isolated three mutants by Mu d1 insertion mutagenesis the mutations in which all mapped to a locus at 77 min on the *E. coli* chromosome (Wu & Mandrand-Berthelot 1986b). These mutants could be separated into two classes based on the effect the lesions exhibited on hydrogen metabolism. Two *nik* mutants (previously called *hydC*) had reduced FHL and H_2-uptake activities while the *hydD* mutant was devoid of both activities. A further differentiation criterion was that, like *hypB* mutants, the effects of the *nik* lesion could be overcome by adding nickel to the growth medium. The defects in hydrogen metabolism in the *hydD* mutant could not be ameliorated by nickel (Wu & Mandrand-Berthelot 1986b). Unfortunately, no subsequent reports concerning the further characterisation of the *hydD* mutant have been published so the function of HydD in hydrogen metabolism is unclear. Furthermore, although a further paper describing the isolation of *nik* mutants has appeared (Stoker et al. 1989a) no other laboratory has reported isolating a mutant with the characteristics of a *hydD* strain.

Several lines of evidence indicate that the *nik* locus may encode a specific nickel transport system: 1. *nik* mutations caused only a reduction in FHL and H_2-uptake activities, they did not abolish them (Wu & Mandrand-Berthelot 1986b); 2. *nik-lacZ* expression studies showed that addition of as little as 5 μM nickel chloride to the culture medium strongly repressed β-galactosidase synthesis; 3. *nik* mutants have intracellular nickel concentrations that are 2 orders of magnitude lower than the wild type (Wu et al. 1989); 4. The phenotype caused by the *nik* mutations could be fully suppressed by growing cells in a medium containing low magnesium ion concentrations. Nickel also can be transported by the magnesium transport system and presumably sufficient nickel can be scavenged from the medium when the synthesis of the magnesium transport system is derepressed (Jasper & Silver 1977).

Recently the *nik* locus was cloned and the genes sequenced (Wu et al. 1991; Navarro et al. 1993).

The *nik* operon encodes 5 proteins exhibiting homology with the large family of transport systems termed ABC-transporters (Higgins et al. 1990). These have a characteristic periplasmic binding protein (NikA), two integral membrane components (NikB and NikC) and either one or two ATP-binding proteins (NikD and NikE) which function as energy coupling sites. It appears therefore that the mode of nickel transport is similar to that for molybdenum (Johann & Hinton 1987). Interestingly, this is quite different to the situation in *Alcaligenes eutrophus* where the HoxN protein is an integral membrane protein involved in high-affinity nickel transport (Eitinger & Friedrich 1991).

Other hyd *loci in* E. coli

Apart from the *hydD* mutant already described the only other *hyd* mutant that has not been characterised further is mutant H61 isolated by Glick et al. (1980). This mutant was identified as being unable to reduce MV with dihydrogen and exhibited low membrane-bound hydrogenase activity which could be 'activated' by solubilisation of the membrane with detergent. Furthermore, the mutant grew poorly anaerobically in the absence of electron acceptors which is a phenotype that is not characteristic for hydrogenase mutants. The gene could be cloned and maxicell experiments showed that it coded for a 63 kD protein that was 'processed' to a smaller 60 kD product (Glick et al. 1981; See & Glick 1982). Unfortunately, the location of the mutation in strain H61 was never mapped. It has been proposed that the lesion in the H61 mutant is in a membrane protein which is required for fermentative growth and that the effect on hydrogen metabolism may be only indirect (Glick et al. 1980; Lee et al. 1985).

Regulation of FDH and hydrogenase isoenzyme biosynthesis

General information

The transcriptional regulation of the genes involved in FHL complex biosynthesis and that of the genes and operons specifying FDH-N have been well-characterised (Rossmann et al. 1991; Li & Stewart 1992), while much less is known about the details of Hyd-1, Hyd-2 and FDH-O gene expression. The regulatory features of genes and operons which have been characterised in detail are summarised in Fig. 3.

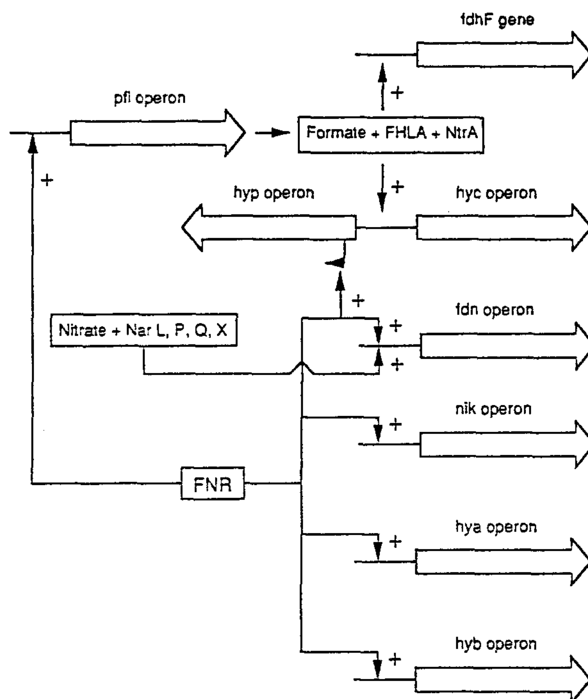

Fig. 3. Scheme showing some of the regulatory circuits involved in controlling the expression of the formate dehydrogenase and hydrogenase structural gene operons. The + indicates a requirement for transcription. The FNR-dependent regulation of the *hya* and *hyb* operons is speculative (see Sawers et al. 1985 and Wu et al. 1989).

What appears superficially to be a complicated network of signals and responses is, when analysed in detail, remarkably simple. An important point to make is that the expression of all these apparently disparately regulated genes and operons is infact elegantly integrated and should be considered in these terms. Thus, although the *fdnGHI* operon is regulated by two transcription factors that respond to quite different signals, i.e. anaerobiosis and nitrate, compared to the transcriptional regulator of the FHL genes which responds to formate, the expression of both gene systems is in essence intimately linked through the cell's metabolic requirements. This in turn is determined by the nutrients in the cell's immediate environment. Control of gene expression is therefore hierarchical (Iuchi & Lin 1991, 1993) and is poised to secure the optimal benefit for the cell, which is efficient energy generation. Hence, the synthesis of respiratory pathways will always take precedence over fermentative pathways, as long as the source of respiratory substrates is available. When this is no longer the case, changes in gene expression are required to repress synthesis

of enzymes no longer of use and to induce synthesis of other enzyme systems needed to metabolise the new substrates. Accumulation or depletion of particular metabolites is sensed and these signals are transduced to transcription factors which alter gene expression according to the cellular requirements.

To optimise control of gene expression many systems are under dual control. This means that a global regulator is required to switch on gene expression, e.g. the FNR protein in response to anaerobiosis, while a second, specific system responding to nitrate, for example, optimises expression for a particular substrate. Global and specific regulation of gene expression by oxygen and nitrate have been reviewed extensively (Spiro & Guest 1990; Iuchi & Lin 1991, 1993; Guest 1992; Gunsalus 1992; Stewart 1993). The next few sections aim to summarise the current status of knowledge on expression of genes required to synthesise functional FDH and Hyd isoenzymes.

Transcriptional regulation of the genes encoding respiratory FDH isoenzymes

It was known from biochemical studies that FDH-N is only synthesised in the absence of molecular oxygen and that the presence of nitrate is required for optimal enzyme levels (Wimpenny & Cole 1967). Further, FDH-N enzyme synthesis is coordinated to the synthesis of nitrate reductase. The isolation of the *fdnGHI* structural genes permitted a detailed study of their transcriptional regulation which confirmed that anaerobiosis and nitrate are necessary for optimal expression of the operon (Berg & Stewart 1990). It must be emphasised that anaerobiosis is essential for expression; the presence of nitrate in the culture medium of aerobically grown cells does not lead to gene activation. The FNR protein mediates the anaerobic regulation of gene expression and the NarL protein is responsible, in part, for nitrate-mediated induction of gene expression (Berg & Stewart 1990). Both of these proteins are DNA-binding proteins (Green et al. 1991; Stewart 1993).

It was noted that in a *narL* mutant residual nitrate induction of *fdn* operon expression still occurred (Berg & Stewart 1990; Li & Stewart 1992). This residual nitrate regulation could be shown to be mediated by the NarP protein, which is a structural and functional homologue of NarL (Rabin & Stewart 1993). Nitrate is sensed by one of two redundant membrane-bound sensor proteins NarX and NarQ (Chiang et al. 1992; Rabin & Stewart 1992). Only when both *narQ* and *narX* genes are inactivated is nitrate-dependent activation of gene expression completely impaired (Rabin & Stewart 1992). Both proteins share homology with the histidine kinase components of two-component sensor-regulator couples (Parkinson & Kofoid 1992). It has been proposed that upon sensing nitrate they transfer a phosphate group to NarL or NarP which consequently effects a conformational change in these proteins, converting them to their transcriptionally active forms (Stewart 1993).

A deletion analysis of the *fdn* operon non-translated regulatory region identified two specific DNA sequences that are essential for regulation by the NarL and FNR proteins (Li & Stewart 1992). The proposed FNR-binding sequence is located near the promoter while the NarL recognition sequence is more than 60 base pairs upstream on the DNA. It is likely that NarL bound to the DNA makes a specific contact with the FNR protein or RNA polymerase to activate expression of the operon. In the case of the regulation of the *narGHJI* operon, which parallels that of the *fdn* operon, a further protein called integration host factor (IHF) appears to assist in promoting a productive interaction between the FNR and NarL proteins (Rabin et al. 1992; Schröder et al. 1992); IHF does not appear to influence *fdn* operon expression.

It has been clearly shown that neither the FdhD, FdhE nor the SEL proteins are functional in regulating *fdn* operon expression (Stewart et al. 1991; Sawers et al. 1991). Whether molybdate has any direct influence on the expression of the *fdn* genes is unclear.

Nothing is known about the regulation of the *fdo* operon genes. However, if the *fdhE* gene proves to be part of the operon then the data of Schlindwein et al. (1990) would suggest that the expression is similar in aerobically and fermentatively cultured cells and is induced only marginally by nitrate. This would more or less be in agreement with the results obtained by examining polypeptide levels under these growth conditions (Sawers et al. 1991).

Transcriptional control of FHL complex formation

The isolation of mutants in which the *lacZ* gene was fused to control sequences of the *fdhF* and *hycB* genes paved the way for the elucidation of the transcriptional control mechanisms governing FHL complex synthesis (Pecher et al. 1983; Yerkes et al. 1984; Wu & Mandrand-Berthelot 1986a, 1987). Analysis of these gene fusions confirmed the results obtained by physiological studies that the expression of the *fdhF* and

hycB genes was severely repressed by oxygen and by nitrate and was induced during fermentative growth conditions by formate. Induction also required low pH conditions. Transcription of the *hycB* gene was shown to be independent of nickel by analysing expression of *hycB-lacZ* expression in cells cultured in nickel-depleted medium (Zinoni et al. 1984). These authors, however, did not determine the levels of residual nickel in the cells after growth and it could be argued that sufficient nickel was present to permit normal gene expression.

That formate is the obligate inducer of the FHL complex genes quickly became apparent through several key experiments. A strain unable to synthesise pyruvate formate-lyase (PFL) showed absolutely no expression of *fdhF* or *hycB* unless formate was supplied exogenously (Birkmann et al. 1987b). Early experiments had suggested that FHL synthesis was dependent on the FNR protein (Newman & Cole 1978); however, subsequent physiological studies indicated that this apparent FNR-dependence could be overcome by exogenously supplied formate (Sawers et al. 1985). It was suggested and later demonstrated that FNR-dependence was indirect and that reduced PFL synthesis in an *fnr* mutant lowered the intracellular formate pool below the threshold necessary to activate *fdhF* and *hycB* expression (Sawers et al. 1985; Birkmann et al. 1987b; Sawers & Böck 1988). The repressive effect of nitrate could be partially relieved either by increasing the concentration of exogenously supplied formate or by introducing a mutation in the *narGHJI* operon, so that cells no longer could metabolise nitrate (Zinoni et al. 1984; Birkmann et al. 1987b; Rossmann et al. 1991). This latter result suggested that metabolism of nitrate was essential for nitrate-mediated repression. This sharply contrasts the nitrate-dependent repression of the fumarate reductase operon which does not necessitate nitrate metabolism and which is mediated by the NarL protein (Iuchi & Lin 1987; Kalman & Gunsalus 1988; Stewart & Berg 1988). Stewart & Berg (1988) confirmed that nitrate repression of *fdhF* and *hycB* gene expression was NarL-independent and later studies showed that it was completely indirect and resulted from the sequestering of formate by the FDH-N-nitrate reductase respiratory pathway (Rossmann et al. 1991).

Since the PFL enzyme provides the major source of formate in the *E. coli* cell but is active only anaerobically, the oxygen-dependent repression of FHL synthesis could be readily explained simply through the lack of formate in this growth condition. Indeed, by overproducing the transcriptional regulator of the FHL pathway, FHLA, and supplying formate exogenously, high levels of expression of the *fdhF* and *hycB* genes in the presence of oxygen can be achieved (Schlensog & Böck 1990; Rossmann et al. 1991).

Studies on the 5′ non-coding region of the *fdhF* gene and the *hycB* operon identified two features common to formate-regulated genes. First, transcription from the promoters is dependent on the alternative sigma factor NtrA (Birkmann et al. 1987a; Lutz et al. 1990). Second, a short DNA sequence of about 25 base pairs was defined that could mediate all the regulatory effects on these promoters, i.e. the requirement for a low pH and formate for induction, and nitrate- and oxygen-dependent repression (Birkmann et al. 1987b, 1989; Birkmann & Böck 1989). This sequence was located approximately 110 base pairs upstream of the transcription start site of the *fdhF* gene. A sequence exhibiting striking similarity to this was located between the divergently oriented promoters of the *hyc* and *hyp* operons and another was found in the intergenic region between the *hycA* and *hycB* genes (Lutz et al. 1990). These studies suggested that this sequence was recognised by a specific, formate-responsive transcription factor. Such a factor was identified in two independent laboratories by isolating mutants unable to produce the protein. Shanmugam and colleagues identified a gene at the 58 min locus whose product was required for FHL and FDH-H synthesis (Lee et al. 1985; Sankar et al. 1985). The gene was termed *fhlA* and was located next to the *hyp* operon and directed the synthesis of a 78 kD protein in maxicell experiments. Using a novel approach in which the gene encoding chloramphenicol acetyltransferase was placed under the control of the *fdhF* gene regulatory region, Schlensog et al. (1989) isolated five classes of mutants which were sensitive to chloramphenicol in the presence of formate. One of these classes had the characterisitics of regulatory gene mutants. The mutations in four representatives of this class were located in the *hypB*, *hypC*, *hypD* and *fhlA* genes (Schlensog et al. 1989; Lutz et al. 1990). The phenotype of the three *hyp* gene mutants resulted from attenuation of *fhlA* gene expression.

Sequence analysis of the *fhlA* gene identified its deduced product as being a putative DNA-binding protein showing similarity with regulatory proteins of the two-component sensor-regulator class (Schlensog & Böck 1990; Maupin & Shanmugam 1990). The FHLA protein has been purified and shown to bind specifically to the sequence common to the *fdhF* and *hyc/hyp*

operon regulatory regions (Schlensog et al. 1994). Furthermore, FHLA directs specific formate-dependent transcription from the *fdhF*, *hyc* and *hyp* regulatory regions *in vitro* (Hopper et al. 1994).

It is still unclear whether FHLA binds formate directly or whether a 'formate-sensor' protein exists that is responsible for the activation of FHLA. Mutants have been sought that exhibit a phenotype similar to a *fhlA* mutant. Sankar et al. (1988) identified a gene which they termed *fdv* that was adjacent to *fhlA* on the chromsome. A *fdv* mutant had an unstable phenotype and it was shown that *fdv* in fact is the *mutS* gene, indicating that the original mutant probably carried a further lesion elsewhere on the chromosome (Schlensog & Böck 1991). A defective *mutS* gene results in an increased rate of spontaneous mutation (Radman et al. 1980).

An exhaustive mutant screening programme carried out by Schlensog et al. (1989) failed to identify a further *fdhF* gene regulator; however, this study did demonstrate that molybdate is required for maximal *fdhF* expression. Molybdate could be replaced by tungstate suggesting that it is not a functional molybdenum cofactor that effects regulation (Schlensog et al. 1989).

The HycA protein has been reported to be a negative regulator of *fdhF*, *hyc* and *hyp* operon expression (Sauter et al. 1992). Mutants lacking the protein show elevated gene expression whereas multiple copies of the *hycA* gene introduced on a plasmid, severely reduce expression. Thus, HycA appears to antagonise FHLA action. HycA does not exhibit similarity to any other currently known proteins nor does it possess a typical helix-turn-helix DNA-binding motif (Böhm et al. 1990). It could be envisaged that HycA interacts with FHLA thus preventing it from binding to the DNA or it could abrogate FHLA function by blocking formate-dependent activation of the protein.

In vitro studies have identified IHF as being a specific regulator of *hyc* and *hyp* operon transcription; IHF is not required for *fdhF* gene expression (Hopper et al. 1994). It has been proposed by Hopper and colleagues that IHF functions by binding between the divergently oriented *hyp* and *hyc* promoters and facilitates nucleoprotein complex formation by bending the adjacent DNA (for a review see Freundlich et al. 1992).

Two further mutants have been identified and partially characterised that are purported to influence hydrogenase synthesis. Mutations in a gene called *fhlB* were isolated that led to a FHL^- phenotype (Maupin & Shanmugam 1990). The authors proposed that the FHLA protein is required for the synthesis of FHLB, and it is this latter protein that activates FHL gene expression. In the light of our current understanding of FHL complex formation this cannot be correct. It may be that *fhlB* represents a further gene in the formate regulon (Rossmann et al. 1991). Moreover, the *fhlB* mutant failed to grow anaerobically in glucose-minimal medium, a phenotype discordant with the facultative nature of the FHL complex. The effects of the *fhlB* mutation on FHL and H_2-uptake activity are therefore likely to be secondary conseqences of the mutation.

A gene termed *hydG* was isolated from an *E. coli* genomic library that, when supplied in multicopy, could partially restore the labile hydrogenase activity to a *hydL* (*hyb*?) mutant (Stoker et al. 1989b). This DNA fragment also restored hydrogenase activity to a *nik* mutant. The hydrogenase enzyme activities measured in these studies were between 20 and 100-fold lower than in the majority of reported studies (Sawers et al. 1985; Sauter et al. 1992) and therefore makes the interpretation of these results difficult. The HydG protein shows strong similarity to regulators of two-component systems (Stoker et al. 1989b); however, the construction of defined chromosomal mutants has not been reported so the function of HydG in the regulation of hydrogenase gene expression remains to be elucidated.

Regulation of hyd-1 and hyd-2 synthesis

Physiological studies have indicated that Hyd-1 is synthesised anaerobically and that formate causes partial induction of enzyme synthesis (Sawers et al. 1985). Hyd-2 enzyme synthesis was also induced anaerobically and it was strongly enhanced by growth on dihydrogen and fumarate or glycerol and fumarate, suggesting that Hyd-2 synthesis may be catabolite repressed. Evidence in support of this speculation was presented for the *S. typhimurium* Hyd-2 isoenzyme where the cAMP-receptor protein (CRP)-cAMP complex was shown to be required for maximal enzyme synthesis (Jamieson et al. 1986). A more recent report has indicated that this control is exerted at the level of gene expression (Wendt et al. 1991).

Nitrate did not significantly reduce Hyd-2 isoenzyme content based on immunological analysis but Hyd-1 activity could no longer be detected under these conditions (Sawers et al. 1985). Neither Hyd-1 or Hyd-2 activity nor antigen could be detected at significant levels in *fnr* mutants suggesting that the FNR pro-

tein mediates anaerobic induction (Sawers et al. 1985). However, more recent data has indicated that the FNR dependence of Hyd-1 and Hyd-2 synthesis may be indirect (Wu et al. 1989). These authors provided evidence that in an *fnr* mutant the intracellular nickel content is depleted due to a strong FNR-dependence of *nik* operon transcription. By supplying the *nik* operon in multicopy the effect of the *fnr* mutation could be relieved (Wu et al. 1989). Moreover, Menon et al. (1990) failed to find a DNA sequence showing similarity to the FNR consensus sequence in the non-translated regulatory region of the *hya* operon which would be in accord with the findings of Wu et al. (1989). On the other hand, Wendt et al. (1991) have reported that anaerobic *hyb* operon expression does require the FNR protein. Clearly a more thorough analysis of *hya* and *hyb* operon transcription is required before any conclusions can be drawn regarding the control of Hyd-1 and Hyd-2 isoenzyme synthesis.

Regulation of genes encoding accessory proteins

The products of the *hyp* operon are required for the synthesis of all three hydrogenase isoenzymes. However, it could be shown that *hyp* operon transcription is directed by a promoter that is recognised by the alternative sigma factor NtrA (Lutz et al. 1990) and expression is formate- and FHLA-dependent (Lutz et al. 1991; Schlensog et al. 1994). In marked contrast to this regulation, the synthesis of the Hyd-1 and Hyd-2 enzymes is NtrA-independent (Birkmann et al. 1987a). This apparent contradiction was resolved by the discovery of a second promoter, located within the *hypA* gene, that is driven by the common sigma factor σ^{70} and whose expression is anaerobically inducible and FNR-dependent (Lutz et al. 1991). Therefore, the HypB, C, D, E and FHLA proteins are synthesised at low levels in the anaerobic cell. The requirement of enhanced synthesis of these proteins, e.g. during Hyd-3 and FHL complex biosynthesis, can be achieved by activating the FHLA-dependent promoter (see Fig. 3). Two important conclusions can be drawn from this novel regulatory mechanism: first, the HypA protein clearly is required only under conditions where Hyd-3 is synthesised; second, because *fhlA* is the promoter-distal gene of the *hyp* operon, FHLA synthesis is positively autoregulated (Rossmann et al. 1991). Presumably the function of the HycA protein is to act as a circuit-breaker to down-regulate FHLA synthesis.

Little is known concerning the regulation of the *hypF* gene other than a brief report which suggests that its expression is anaerobically inducible (Tomiyama et al. 1991). Further, expression has been reported to be FNR-, NtrA- and formate-dependent which is analogous to that of *hypBCDEfhlA* expression. Substantiation of these findings would provoke the interesting question as to why the *hypF* gene is not part of the *hyp* operon as appears to be the case in other organisms (Rey et al. 1993; Colbeau et al. 1993).

As mentioned above transcription of the *nik* operon is induced by anaerobiosis and this is strongly dependent on the FNR protein (Wu & Mandrand-Berthelot 1986b). Neither formate nor nitrate appears to influence *nik* expression. Nickel however, supplied at low concentration strongly represses *nik* operon transcription (Wu & Mandrand-Berthelot 1986b; Wu et al. 1989). This regulation is physiologically relevant because nickel in high concentration is toxic (Navarro et al. 1993). Moreover, this result implies that a nickel-responsive transcriptional regulator must exist in *E. coli*.

A similar argument for the existence of a molybdate-sensitive regulator can be brought forward, since transcription of the *mod* operon is strongly repressed by molybdate (Boone-Miller et al. 1987). Tungstate was an effective substitute for molybdate. Perhaps the MolR protein is involved in this regulation (Lee et al. 1990). Expression from *mod-lacZ* fusions was induced by nitrate and this was NarL-dependent; however, mutations in the *moa*, *moe* and *mog* genes did not affect *mod* expression.

The *moa* genes are subject to 20- to 25-fold anaerobic induction that does not appear to be dependent on the FNR protein (Baker & Boxer 1991). Molybdenum cofactor is a negative regulator of the *moa* operon. The low-level expression of the *moa* genes in aerobically cultured cells should be sufficient for molybdenum cofactor biosynthesis necessary to synthesise a functionally active FDH-O enzyme (Baker & Boxer 1991). Neither molybdate nor nitrate affects *moa* expression. The analysis of *mob*, *moe* and *mog* expression has not been reported.

Physiology of formate and hydrogen metabolism

Formate as reductant for nitrate and nitrite respiration

A prerequisite for an involvement in energy generation is an association with the cytoplasmic mem-

brane. Both FDH-N and nitrate reductase are energy-conserving, probably translocating two protons for every formate oxidised and a further two protons in the reduction of nitrate (Garland et al. 1975; Jones & Garland 1977; Jones 1980a; Jones et al. 1980). Chemical and immunological studies demonstrated that both enzymes are transmembranous (Graham & Boxer 1981; Stewart 1988). Although early studies, based on the use of artificial electron acceptors, suggested that the active site of nitrate reductase was oriented toward the periplasm, and that of FDH-N toward the cytoplasm (Garland et al. 1975; Jones & Garland 1977) their data are not inconsistent with both enzymes displaying the opposite topology (reviewed in Stewart 1988). The current view therefore is that nitrate reductase's active site is located in the cytoplasm with the NarI polypeptide contacting the bulk phase (Morpeth & Boxer 1985; Stewart 1988). Both the analysis of the deduced amino acid sequence of FdnG (Berg et al. 1991b) and the analogy with the periplasmic orientation of FDH from *W. succinogenes* (Bokranz et al. 1991) strongly suggest that the active site of FDH-N is toward the bulk phase. Such a topology would be in agreement with the proton-translocating capacities of both enzymes and is coherent with our current understanding of nitrate metabolism. It has been proposed that the NarK protein functions as a nitrate-nitrite antiporter, which would provide an elegant means of both supplying the substrate for, and removing the product of, the nitrate reductase reaction (Stewart & MacGregor 1982; Noji et al. 1989; DeMoss & Hsu 1991). It should be noted that although this function of the NarK protein is very appealing, it has not been proved definitively.

Nitrite not exported from the cytoplasm is reduced to ammonia by the soluble, non-energy-conserving NADH-dependent nitrite reductase (Page et al. 1990). However, the export of nitrite enables reduction of nitrite to be coupled to formate oxidation by the energy-conserving, formate-inducible Nrf (*n*itrite *r*eduction by *f*ormate) pathway (Cole & Wimpenny 1966; Abou-Jaoude et al. 1979a,b; Motteram et al. 1981). Recent data indicate that either of the three FDH isoenzymes can couple formate oxidation to the reduction of nitrite via the tetrahaem NrfA protein (cytochrome c_{552}). FDH-N and FDH-H contribute, more or less, equally to electron transfer (Darwin et al. 1993a,b). A periplasmic localisation of the NrfA protein is supported by the nascent polypeptide possessing a characteristic signal sequence (Darwin et al. 1993a).

These exciting new data naturally provoke the question of whether the FHL complex, or part of it, conserves energy by diverting electrons, possibly through the integral membrane proteins HycC and HycD, to NrfA. It should be re-emphasised that HycC and HycD show similarity to electron transfer components of the NUO complex (Sauter et al. 1992). Two pieces of evidence support this contention: 1. Early studies by Fujita & Sato (1967) showed that addition of nitrite to growing cultures reduced dihydrogen production while simultaneously enhancing carbon dioxide and ammonia production; 2. Mutants unable to synthesise ubiquinone or menaquinone retained 60% Nrf activity. Darwin et al. (1993a,b) have suggested that further uncharacterised Nrf components may be involved in this putative electron transfer process from the FHL complex to NrfA.

Energy conservation from hydrogen oxidation – the role of the Hyd-1 and Hyd-2 isoenzymes

It is clear that Hyd-2 is the major hydrogenase component when cells respire with dihydrogen and fumarate. Hyd-2 synthesis correlates with H_2-uptake activity (Sawers et al. 1985, 1986); *hyb* mutants lack this activity (Lee et al. 1985) and they fail to grow with fumarate and dihydrogen (M. Sauter & A. Böck, unpublished data). Hyd-2 is probably the proton-translocating activity analysed by Jones (1980b), because cells for these experiments were grown in conditions that optimise Hyd-2 but not Hyd-1 synthesis. On the other hand, Hyd-1 was shown to span the cytoplasmic membrane (Graham 1981). The question still to be answered concerns the exact physiological function of Hyd-1. It is more abundant than the Hyd-2 isoenzyme and although, with BV as electron acceptor, the Km for H_2 of both enzymes is similar (Sawers & Boxer 1986; Ballantine & Boxer 1986), Hyd-1 may be able to scavenge hydrogen more readily than Hyd-2 during particular growth conditions.

The topology of Hyd-1 and Hyd-2 also remains to be resolved definitively. A periplasmic orientation of the active site for both enzymes would be congruent with their H_2-oxidation functions. Both enzymes have homology with the Ni-hydrogenase of *D. vulgaris* which has been found to be exposed toward the periplasm (Rohde et al. 1989) and this orientation is also assumed for the hydrogenase of *W. succinogenes* (Dross et al. 1992). If such a topology proves to be correct then this poses intriguing problems in resolving

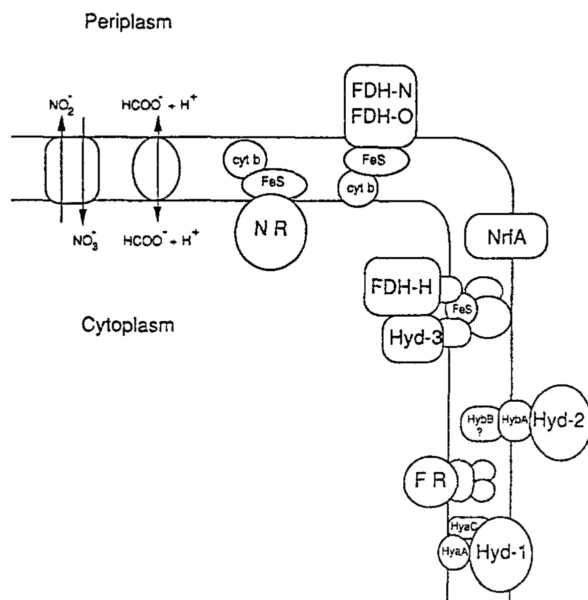

Fig. 4. Summary of the topology of the formate dehydrogenase and hydrogenase isoenzymes in the cytoplasmic membrane of *E. coli*. A simplified interpretation of transport mechanisms for formate, nitrite and nitrate is presented. It is likely that there is more than one system in the cell involved in the transport of formate (see Suppmann & Sawers 1994) and it may be the case that both nitrite and nitrate also can enter and exit the cell by more than one transport system. Abbreviations: FR, fumarate reductase; NR, nitrate reductase.

how almost the complete Hyd-2 isoenzyme is translocated across the membrane.

The current model for the FHL complex localises it to the inner side of the cytoplasmic membrane (Sauter et al. 1992). This is based mainly on the inability to recover any of the FHL components in the periplasmic fraction, despite the fact that FDH-H and HycE are peripheral membrane proteins. An early study using membrane-impermeant redox indicators localised the FHL complex and the Nrf activity to the periplasmic side of the membrane (Jones & Garland 1977). Although it is highly likely that the complex is indeed oriented toward the cytoplasm further studies are required to qualify this proposal (Sauter et al. 1992). The topology of the FDH and Hyd isoenzymes in the cytoplasmic membrane is summarised in Fig. 4.

Coordination of formate and hydrogen metabolism with gene expression

There is no absolute requirement for either the FDH or hydrogenase isoenzymes when oxygen is available. Consequently, with the exception of the FDH-O isoenzyme, neither they nor their associated terminal reductases are synthesised. The FDH-O isoenzyme is present in very low amounts in aerobically growing cells but by possessing an extremely high catalytic activity (Sawers et al. 1991) it can presumably withdraw the energy available in formate which may accrue, for example, as a by-product of purine metabolism (Nagy et al. 1993). It is probable that the electrons generated by the oxidation of formate enter the quinone pool and are transferred to one of the terminal cytochrome oxidases and ultimately to oxygen with consequent energy conservation. It is also plausible that FDH-O prevents formate accumulation and thus the wasteful activation of the FHL complex synthesis during aerobiosis.

Formate is a crucial signal molecule in anaerobic gene expression and is the major determinant of anaerobic metabolism in *E. coli*. Due to the FDH-O enzyme the formate concentration can be considered negligible during aerobic growth. In sharp contrast, however, anaerobic cells lacking all the FDH isoenzymes accumulate formate concentrations in excess of 20 mM in the culture medium (B. Suppmann & G. Sawers, unpublished data). The ability of enterobacteria to produce this molecule in such quantities distinguishes them from all other bacteria and as such confers upon them an astounding metabolic flexibility. The source of formate is PFL, an enzyme whose activity is under very strict post-translational control and whose synthesis is also strongly regulated (Knappe & Sawers 1990). *E. coli* mutants defective in the synthesis of PFL grow poorly anaerobically and in essence perform a lactic acid fermentation. However, this phenotype can be essentially completely suppressed by providing the mutant with exogenous formate, acetate and nitrate (G. Sawers, unpublished data).

Probably the most important feature of PFL is that it is catalytically active only in cells that are deprived of oxygen (Knappe et al. 1984; Wagner et al. 1992; Kessler et al. 1992). This ensures that formate is produced when it is energetically favourable. The synthesis of PFL is also enhanced during anaerobiosis and this transcriptional control is exerted by the FNR protein (Sawers & Böck 1989; Sawers & Suppmann 1992; Sawers 1993). Thus, while formate is perhaps the key

metabolite in anaerobic *E. coli*, FNR represents its molecular counterpart.

FNR is a redox-sensitive, iron-binding protein that activates the transcription of a large number of genes, and it does so only anaerobically (Spiro & Guest 1990; Guest 1992). Mutants of *E. coli* that are either unable to synthesise FNR or produce a functionally defective FNR protein lose the capacity to grow with nitrate or fumarate as terminal electron acceptor anaerobically; however, their aerobic metabolism is unaffected (Lambden & Guest 1976; Spiro & Guest 1990). Defective fumarate and nitrate respiration results from an inability to synthesise fumarate reductase and the FDH-N-nitrate reductase pathway enzymes, respectively.

FNR, therefore, can be classified as a global regulatory determinant of anaerobic metabolism. It functions coordinately with specific regulatory determinants that direct synthesis of particular respiratory pathways when *E. coli* is confronted by the oxidant used by that pathway. Currently, the best characterised of these specific regulatory determinants is the NarL protein (Stewart 1993). Through the NarL, NarP, NarQ and NarX proteins, nitrate directs the synthesis of the nitrate respiratory pathway as well as that of the two nitrite reductases. Nitrate is the preferred anaerobic oxidant and as such represses synthesis of other terminal reductases, e.g. fumarate, TMAO and DMSO reductases (Stewart 1993).

Nitrate also 'represses' the synthesis of the fermentative FHL pathway; however, this is achieved not by NarL-dependent repression rather by removing the inducer, formate (Rossmann et al. 1991). At neutral pH formate is excreted from the cell (Belaich & Belaich 1976; Suppmann & Sawers 1994). This effectively compartmentalises the formate on the periplasmic side of the cytoplasmic membrane where it is efficiently oxidised by the FDH-N and FDH-O isoenzymes. Because of this, and when nitrate is plentiful, formate never accumulates to a concentration sufficient to activate the FHLA protein. Nitrate, therefore, maintains a low intracellular formate pool and prevents FHL complex synthesis. When nitrate becomes limiting, the extracellular formate concentration increases with a concomitant decrease in the pH of the culture medium. This shifts the equilibrium such that formate now accumulates in the cytoplasm. Eventually the threshold concentration is attained and FHLA becomes activated. The FHL complex offsets the pH drop by catalysing the disproportionation of formate to carbon dioxide and dihydrogen. The dihydrogen either diffuses away from the cell rapidly or is oxidised by Hyd-1 or Hyd-2 with the electrons being channelled into the quinone pool to be eventually used to reduce fumarate to succinate with concomitant energy conservation. Removal of nitrate derepresses fumarate reductase synthesis (Iuchi & Lin 1987; Gunsalus 1992).

Under fermentative growth conditions all the hydrogenase isoenzymes are present in the cell with Hyd-1 and Hyd-3 being synthesised at optimal levels (Sawers et al. 1985). It has been proposed, but not yet unequivocally demonstrated that Hyd-1 could be involved in hydrogen-recycling (Sawers et al. 1986). It is surprising that there is still so little understood about the physiological function of the Hyd-1 isoenzyme. It is a relatively abundant protein (Sawers & Boxer 1986) which contrasts rather with Hyd-2 (Ballantine & Boxer 1986). Furthermore, Hyd-1 is catalytically less active than Hyd-2. This situation is reminiscent of the two cytochrome oxidases in *E. coli* where it has been proposed that cytochrome *o* oxidase is mainly functional when oxygen is abundant and cytochrome *d* oxidase is active at low oxygen partial pressures (Anraku & Gennis 1987; Cotter & Gunsalus 1992). The cytochrome *o* oxidase has a low affinity for oxygen, while the cytochrome *d* oxidase has a high affinity. Perhaps an analogous situation occurs with the Hyd-1 and Hyd-2 isoenzymes, where Hyd-1 has a scavenging role during certain growth conditions. It is clear, however, that Hyd-2 is the principle uptake hydrogenase during growth on dihydrogen with fumarate. The regulation of synthesis of Hyd-2 also correlates with this function (Sawers et al. 1985; Wendt et al. 1991). Perhaps more detailed physiological analyses with specific mutants and regulatory studies with the structural genes will shed more light on the physiology of these two enzymes.

Finally, it is essential that the synthesis and incorporation of metal cofactors in the FDH and Hyd isoenzymes are coordinated with the expression of the structural genes. The *sel* genes are all expressed constitutively, since selenocysteine is required for the translation of the mRNA encoding the selenopolypeptide subunits of three FDHs (Sawers et al. 1991). Although the expression of all the molybdenum cofactor biosynthetic genes has not been elucidated in detail, the *moa* operon has been characterised and it appears that the polypeptides are synthesised at a low background level in aerobic cells to accommodate molybdenum cofactor biosynthesis for the FDH-O enzyme (Baker & Boxer 1991). Anaerobically, there is a greater than 20-fold increase in *moa* gene expression reflecting the

need to satisfy the molybdenum cofactor requirement of the other two FDH isoenzymes, nitrate reductase and under particular circumstances that of DMSO and TMAO reductase (Baker & Boxer 1991; Rivers et al. 1993).

Nickel incorporation occurs only anaerobically. The synthesis of the nickel transport system and that of the *hypB-E* genes is regulated by FNR accordingly. The expression of the *nik* operon is down-regulated by free intracellular nickel (Wu & Mandrand-Berthelot 1986b; Wu et al. 1989). Onset of fermentation increases the demand for nickel incorporation and the expression of the *hyp* operon is amplified through the formate-dependent promoter to satisfy this demand (Lutz et al. 1990; Rossmann et al. 1991). It is anticipated that the *hypF* gene is regulated in a similar manner to the *hyp* operon (Tomiyama et al. 1991).

Perspectives

The recent rapid advances in our knowledge of the formate dehydrogenases and hydrogenases of *E. coli* have not simply been restricted to furthering our understanding of the roles these enzymes take in metabolism. Far more, they have led to new insights into how anaerobic metabolism is regulated and how this regulation is elegantly coordinated with gene expression; they have led to the discovery of a new amino acid, selenocysteine, and the elucidation of a new biochemical pathway involved in its biosynthesis and co-translational incorporation into proteins; they have brought to light new important information on the biosynthesis and incorporation of metal centers in redox-active enzymes; and they opened up a new avenue of research which will resolve the pathway leading to the processing, maturation and membrane association of multisubunit membrane proteins. It is also anticipated that the rapidity with which these advances is occurring will not decline.

Naturally with these advances there has been an attendant increase in the number of new problems to be solved. Is the FHL complex merely a means of maintaining pH homeostasis or can it be coupled to energy conservation, e.g. with nitrite? What are the physiological functions of the FDH-O and Hyd-1 isoenzymes? Can the proposed roles of nickel, selenium and molybdenum in the redox processes of the FDH and Hyd isoenzyme complexes be substantiated? How is selenite transported into the cell? Perhaps the most fascinating problem of all, what is the exact train of events leading to the biosynthesis of a functionally competent, membrane-associated formate dehydrogenase or hydrogenase isoenzyme? These are just some of the intriguing questions that are being posed and answered in many laboratories around the world. The answers to these problems will be forthcoming.

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