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The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin NADP$^+$ reductases

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Three soluble ferrisiderophore reductases (FsrA, FsrB and FsrC) were detected in *Escherichia coli*. FsrB was purified and identified as the haemoglobin-like protein (HMP) by size and N-terminal sequence analyses. HMP was previously isolated as a dihydropteridine reductase and is now shown to have ferrisiderophore reductase activity. Database searches revealed that the C-terminal region of HMP (FsrB) is homologous to members of a family of flavoprotein oxidoreductases which includes ferredoxin NADP$^+$ reductase (FNR). The combination of FNR-like and haemoglobin-like regions in HMP (FsrB) represents a novel pairing of functionally and structurally distinct domains. Structure–function properties of other FNR-like proteins, including LuxG and VanB, are also discussed.

Ferrisiderophore reductase; Haemoglobin; Ferredoxin NADP$^+$ reductase; Mosaic protein; LuxG; VanB

1. INTRODUCTION

Iron is a vital constituent of many key cellular components. The biological importance of iron is due, in part, to its ability to fluctuate between two oxidation states (FeII and FeIII). However, owing to the insolubility of FeIII, the amount of free iron in neutral aerated solutions is extremely low. Many bacteria scavenge iron by secreting low molecular weight chelators known as siderophores [1]. Siderophores bind FeIII with high specificity and affinity, forming soluble complexes which are taken up by cognate membrane-associated receptors.

Upon internalisation iron is released from the siderophores, but the mechanism by which this is achieved is poorly understood. It is possible that the complexed iron is reduced to FeII by a ferrisiderophore reductase (FSR). The ferrous iron would then dissociate from the siderophore due to its lower affinity. Indeed, FSRs could be responsible for controlling the absolute and relative levels of intracellular FeII and FeIII.

FSR activity has been detected in a variety of bacteria and in some cases the enzymes have been isolated [2–4]. However, these enzymes have not been characterised in detail, their role in the cell has not been established, and in most cases the corresponding genes have not been cloned and sequenced.

This paper reports the detection of three discrete and soluble FSR enzymes (FsrA, FsrB and FsrC) in *Escherichia coli*. The purification of one of these enzymes (FsrB) is described and its identity with the haemoglobin-like protein (HMP) of *E. coli* [5] is demonstrated. HMP was previously detected by virtue of its dihydropteridine reductase activity, but the present results indicate that it also has FSR activity. Homology between the C-terminal region of FsrB and members of a family of flavoprotein-oxidoreductases is also reported. The functional and structural implications of these homologies are discussed.

Abbreviations: HMP, haemoglobin-like protein; FSR, ferrisiderophore reductase; FNR, ferredoxin NADP$^+$ reductase; Fre, flavin reductase enzyme.

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2. EXPERIMENTAL

2.1. *Preparation of soluble-cell supernatants*

The following *E. coli* K-12 strains were used: JRG2157 (*Δbfr::kan, Δlac-proAB, supE, thi*, F$^-$; Timms, A.R., Andrews, S.C., Harrison, P.M. and Guest, J.R. unpublished data) and W3110. Strains were grown in 50 ml L-broth at 37°C. Cells were harvested by centrifugation and sonicated in FSR buffer (60 mM Tris-HCl, pH 8, 30 mM KCl, 30 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol) using an MSE Soniprep 150 sonicator at 10 microns for six 10 s pulses with 30 s intervals at 4°C. Soluble cell supernatants were then obtained by centrifugation at 15,000 g for 15 min at 4°C.

2.2. *Electrophoresis and activity staining*

Proteins were analysed by polyacrylamide gel electrophoresis (PAGE) [6] under either denaturing conditions (0.1% sodium dodecylsulphate (SDS) in gel and running buffer) or non-denaturing conditions (no SDS). Prior to SDS-PAGE, samples were heated at 100°C for 10 min in loading buffer (0.1 M Tris-HCl, pH 6.8, 10% v/v glycerol, 2.3% w/v SDS, 5% v/v β-mercaptoethanol). Proteins separated by electrophoresis were stained either with silver or with Coomassie blue. FSR activities were detected in native gels using an activity stain [4].

2.3. *Purification of FsrB*

E. coli K-12 strain W3110 was cultured in two-fold concentrated L-broth at 37°C in a 20 l Biolafitte fermentor for 16 h. Cells were harvested by centrifugation (10,000 × *g* for 30 min) and washed by resuspension in FSR buffer followed by centrifugation as before. Cells (66 g) were resuspended in 100 ml of FSR buffer containing 0.04% phenyl methane sulphonyl fluoride and then lysed by two passes through a French pressure cell at 20,000 psi. The whole-cell homogenate was centrifuged at 20,000 × *g* for 30 min and the resulting soluble cell supernatant was fractionated by anion-exchange chromatography using a Q–Sepharose FF column (30 × 20 cm^2) equilibrated with FSR buffer. Proteins were eluted using a 1,500 ml linear gradient of 0.03–0.53 M KCl in FSR buffer at a flow rate of 5 ml/min. Fractions containing FsrB were detected after native PAGE by activity staining, and purity was assessed by native- and SDS-PAGE. Active fractions were pooled and concentrated by ultra-filtration in a Filtron Novacell stirred cell (5 kDa nominal molecular weight limit), and then subjected to gel-permeation chromatography using Sephacryl S200-HR (90 × 5 cm^2; FSR buffer, at 30 ml/h). Fractions containing FsrB were precipitated with 50% (w/v) ammonium sulphate and dialysed against FSR buffer. The anion-exchange chromatography step was then repeated and the concentrated fractions were applied to a Phenyl–Sepharose hydrophobicity column (10 × 1.5 cm^2; equilibrated with 1 M $(NH_4)_2SO_4$ in FSR buffer). The column was washed with distilled H_2O and FsrB was eluted with a 0–100% linear gradient of ethanol at a flow rate of 30 ml/h. The FsrB-containing fractions were precipitated and dialysed and then purified by preparative-PAGE using a 10% T-native polyacrylamide gel. FsrB was located by activity staining, recovered by electroelution, precipitated and purified to homogeneity by repeating the hydrophobicity chromatography.

2.4. *Computation*

Database searches were carried out using the SEQNET computing facility at the SERC Daresbury laboratory, Daresbury, Warrington. The NEWSWEEP program was used to search the OWL protein database [7,8], FASTA was used to search the NBRF and SWISSPROT protein databases [9], and TFASTA was used to search the GenBank, EMBL and DAILY nucleic acid sequence databases [9]. The Profile Analysis suite of programs was used both to construct sequence alignments and for protein database searches [10].

2.5 Other methods

FSR activity assays were as described by Fischer et al. [2]. Superose 6 HR (10/30; Pharmacia) and Sephacryl S200-HR (90 × 5 cm^2) columns, each equilibrated in FSR buffer, were used for analytical gel-permeation chromatography. The columns were calibrated using commercial standards (M_r 12,300–700,000, Sigma) and bacterioferritin (M_r 37,000 and 452,000, [11]) as molecular weight markers. Automated solid-phase amino acid sequence determination [12] was performed using a MilliGen/Bioscarch 6600 ProSequencer according to the manufacturer's instructions.

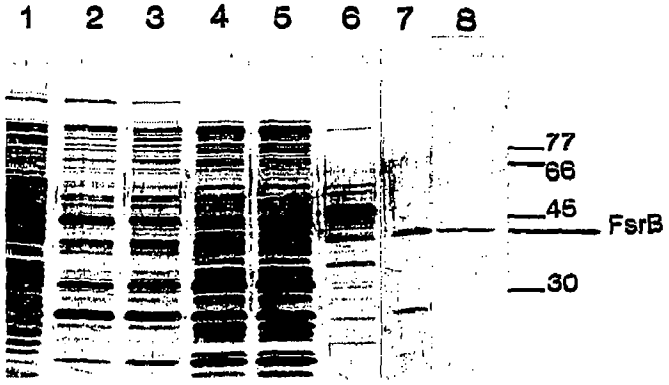

Fig. 1. Purification of FsrB. Silver stained SDS-PAGE (10% T) of samples obtained at different stages of purification (see section 2). Lane 1, soluble-cell extract; lane 2, Q-Sepharose; lane 3, ultrafiltration; lane 4, Sephacryl S200 HR; lane 5, second Q-Sepharose; lane 6, Phenyl- Sepharose; lane 7, preparative-PAGE; lane 8, second Phenyl-Sepharose. The positions and relative molecular masses (10^{-3}) of markers are indicated on the right-hand side.

3. RESULTS AND DISCUSSION

3.1. *Detection of three soluble FSR enzymes in* E. coli

The soluble proteins from two strains of *E. coli* K-12, W3110 (wild-type) and JRG2157 (*Δbfr*), were fractionated by native-PAGE and stained for FSR activity. Three active components, denoted FsrA < FsrB < FsrC in the order of their relative mobilities and staining intensities, were detected. There was no significant difference in the relative FSR-staining intensities between the two strains or between samples from exponential and stationary phase cultures, indicating that FSR activity is not influenced by the growth phase or the absence of bacterioferritin. It has previously been ob-

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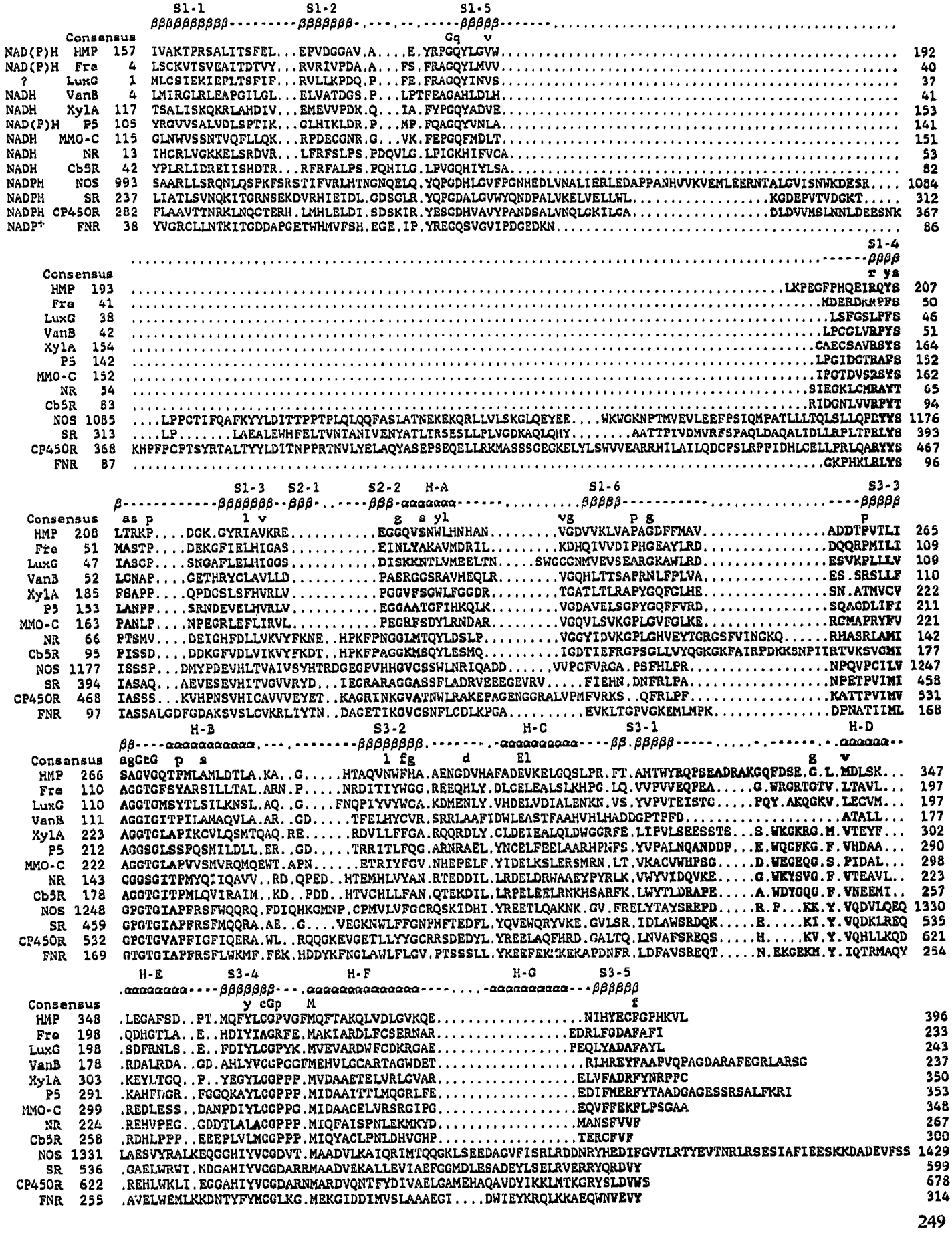
Fig. 2. Multiple sequence alignment of FNR-like domains. Residues thought to be involved in the binding of either FAD or NAD(P)(H) [14] are shown in bold. Secondary structure elements are designated according to Karplus et al. [14]: α-helix (α) and β-strand (β) regions of spinach FNR are indicated above the sequences, and regions that are neither strand nor helix in spinach FNR are indicated with hyphens. Residue numbers are shown at the left and right of each sequence line. Dots in the alignment denote padding characters added to optimise the alignment. The pyridine dinucleotide specificities are indicated. The consensus sequence indicates those regions that are absolutely or partially (<100% and >50%) conserved in upper and lower case, respectively. HMP (FsrB), haemoglobin-like protein from *E. coli* [5]; Fre (FsrC), flavin reductase enzyme from *E. coli* [15]; LuxG, product of the *luxG* gene (possibly a component of the luminescent system) from *V. harveyi* [16]; VanB, vanillate demethylase component B from *Pseudomonas* [17]; XylA, xylene monooxygenase component A from *Pseudomonas putida* [18]; P5, phenol hydroxylase component 5 from *Pseudomonas* [19]; MMO-C, methane mono-oxygenase component C from *Methylococcus capsulatus* [20]; NR, maize nitrate reductase [21,22]; Cb5R, human cytochrome b5 reductase [23]; NOS, rat nitric oxide synthase [25]; SR, sulphite synthase (a subunit) from *E. coli* [26]; CP450R, human cytochrome P_{450} reductase [27]; FNR, ferredoxin NADP$^+$ reductase from spinach [24].


S1-1
S1-2
S1-5
Consensus
NAD(P)H HMP 157 IVAKTPRSALITSFEL...EPVDGGAV.A...E.YRPGQYLGVW 192
NAD(P)H Fre 4 LSCKVTSVEAITDTVY...RVRIVPDA.A...FS.FRAGQYLMVV 40
? LuxG 1 MLCSIEKIEPLTSFIF...RVLLKPDQ.P...FE.FRAGQYINVS 37
NADH VanB 4 LMIRGLRLEAPGILGL...ELVATDGS.P...LPTFEAGAHLDLH 41
NADH XylA 117 TSALISKQKRLAHDIV...EMEVVPDK.Q...IA.FYPGQYADVE 153
NAD(P)H P5 105 YRGVVSALVDLSPTIK...GLHIKLDR.P...MP.FQAGQYVNLA 141
NADH MMO-C 115 GLNWVSSNTVQFLLQK...RPDECGNR.G...VK.FEPGQFMDLT 151
NADH NR 13 IHCRLVGKKELSRDVR...LFRFSLPS.PDQVLG.LPIGKHIFVCA 53
NADH Cb5R 42 YPLRLIDREIISHDTR...RFRFALPS.PQHILG.LPVGQHIYLSA 82
NADPH NOS 993 SAARLLSRQNLQSPKFSRSTIFVRLHTNGNQELQ.YQPGDHLGVFPGNHEDLVNALIERLEDAPPANHVVKVEMLEERNTALGVISNWKDESR 1084
NADPH SR 237 LIATLSVNQKITGRNSEKDVRHIEIDL.GDSGLR.YQPGDALGVWYQNDPALVKELVELLWL KGDEPVTVDGKT 312
NADPH CP450R 282 FLAAVTTNRKLNQGTERH.LMHLELDI.SDSKIR.YESGDHVAVYPANDSALVNQLGKILGA DLDVVMSLNNLDEESNK 367
NADP+ FNR 38 YVGRCLLNTKITGDDAPGETWHMVFSH.EGE.IP.YREGQSVGVIPDGEDKN 86
S1-4
Consensus
HMP 193 LKPEGFPHQEIRQYS 207
Fre 41 MDERDKRPFS 50
LuxG 38 LSFGSLFFS 46
VanB 42 LPCGLVRFYS 51
XylA 154 CAECSAVRSYS 164
P5 142 LPGIDGTRAFS 152
MMO-C 152 IPGTDVSRSYS 162
NR 54 SIEGKLCMRAYT 65
Cb5R 83 RIDGNLVVRFYT 94
NOS 1085 LPPCTIFQAFKYYLDITTPPTPLQLQQFASLATNEKEKQRLLVLSKGLQEYEE WKWGKNPTMVEVLEEFPSIQMPATLLLTQLSLLQPRYYS 1176
SR 313 LP LAEALEWHFELTVNTANIVENYATLTRSESLLFLVGDKAQLQHY AATTPIVDMVRFSPAQLDAQALIDLLRPLTPRLYS 393
CP450R 368 KHPFPCPTSYRTALTYYLDITNPPRTNVLYELAQYASEPSEQELLRKMASSSGEGKELYLSWVVEARRHILAILQDCPSLRPPIDHLCELLPRLQARYYS 467
FNR 87 GKPHKLRLYS 96
S1-3 S2-1 S2-2 H-A S1-6 S3-3
Consensus
HMP 208 LTRKP...DGK.GYRIAVKRE EGGQVSNWLHNHAN VGDVVKLVAPAGDFFMAV ADDTPVTLI 265
Fre 51 MASTP...DEKGFIELHIGAS EINLYAKAVMDRIL KDHQIVVDIPHGEAYLRD DQQRPMILI 109
LuxG 47 IASGP...SNGAFLELHIGGS DISKKNTLVMEELTN SWGCGNMVEVSEARGKAWLRD ESVKPLLLV 109
VanB 52 LGNAP...GETHRYCLAVLLD PASRGGSRAVHEQLR VGQHLTTSAPRNLFPLVA ES.SRSLLF 110
XylA 185 FSAPP...QPDGSLSFHVRLV PGGVFSGWLFGGDR TGATLTLRAPYGQFGLHE SN.ATMVCV 222
P5 153 LANPP...SRNDEVELHVRLV EGGAATGFIHKQLK VGDAVELSGPYGQFFVRD SQAGDLIFI 211
MMO-C 163 PANLP...NPEGRLEFLIRVL PEGRFSDYLRNDAR VGQVLSVKGPLGVFGLKE RCMAPRYFV 221
NR 66 PTSMV...DEIGHFDLLVKVYFKNE..HPKFPNGGLMTQYLDSLP VGGYIDVKGPLGHVEYTGRGSFVINGKQ RHASRLAMI 142
Cb5R 95 PISSD...DDKGFVDLVIKVYFKDT..HPKFPAGGKMSQYLESMQ IGDTIEFRGPSGLLVYQGKGKFAIRPDKKSNPIIRTVKSVGMI 177
NOS 1177 ISSSP...DMYPDEVHLTVAIVSYHTRDGEGPVHHGVCSSWLNRIQADD VVPCFVRGA PSFHLPR NPQVPCILV 1247
SR 394 IASAQ...AEVESEVHITVGVVRYD...IEGRARAGGASSFLADRVEEEGEVRV FIEHN.DNFRLPA NPETPVIMI 458
CP450R 468 IASSS...KVHPNSVHICAVVVEYET..KAGRINKGVATNWLRAKEPAGENGGRALVPMFVRKS..QFRLPF KATTPVIMV 531
FNR 97 IASSALGDFGDAKSVSLCVKRLIYTN..DAGETIKGVCSNFLCDLKPGA EVKLTGPVGKEMLMPK DPNATIIML 168
H-B S3-2 H-C S3-1 H-D
Consensus
HMP 266 SAGVGQTFMLAMLDTLA.KA..G....HTAQVNWFHA.AENGDVHAFADEVKELGQSLPR.FT.AHTWYRQPSEADRAKGQFDSE.G.L.MDLSK... 347
Fre 110 AGGTGFSYARSILLTAL.ARN.P....NRDITIYWGG.REEQHLY.DLCELEALSLKHPG.LQ.VVPVVEQPEA G.WRGRTGTV.LTAVL... 197
LuxG 110 AGGTGMSYTLSILKNSL.AQ..G....FNQPIYVYWGA.KDMENLY.VHDELVDIALENKN.VS.YVPVTEISTC PQY.AKQGKV.LECVM... 197
VanB 111 AGGIGITPILAMAQVLA.AR..GD....TFELHYCVR.SRRLAAFIDWLEASTFAAHVHLHADDGPTPFD ATALL... 177
XylA 223 AGGTGLAPIKCVLQSMTQAQ.RE....RDVLLFFGA.RQQRDLY.CLDEIEALQLDWGGRFE.LIPVLSEESSTS S.WKGKRG.M.VTEYF... 302
P5 212 AGGSGLSSPQSMILDLL.ER..GD....TRRITLFQG.ARNRAEL.YNCELFEELAARHPNFS.YVPALNQANDDP E.WQGFKG.F.VHDAA... 290
MMO-C 222 AGGTGLAPVVSMVRQMQEWT.APN....ETRIYFGV.NHEPELF.YIDELKSLERSMRN.LT.VKACVWHPSG D.WEGEQG.S.PIDAL... 298
NR 143 CGGSGITPMYQIIQAVV..RD.QPED..HTEMHLVYAN.RTEDDIL.LRDELDRWAAEYPYRLK.VWYVIDQVKE G.WKYSVG.F.VTEAVL.. 223
Cb5R 178 AGGTGITPMLQVIRAIM..KD..PDD..HTVCHLLFAN.QTEKDIL.LRPELEELRNKHSARFK.LWYTLDRAPE A.WDYGQG.F.VNEEMI.. 257
NOS 1248 GPGTGIAPFRSFWQQRQ.FDIQHKGMNP.CPMVLVFGCRQSKIDHI.YREETLQAKNK.GV.FRELYTAYSREPD R.P...KK.Y.VQDVLQEQ 1330
SR 459 GPGTGIAPFRSFMQQRA.AE..G....VEGKNWLFFGNPHFTEDFL.YQVEWQRYVKE.GVLSR.IDLAWSRDQK E....KI.Y.VQDKLREQ 535
CP450R 532 GPGTGVAPFIGFIQERA.WL..RQQGKEVGETLLYYGCRRSDEDYL.YREELAQFHRD.GALTQ.LNVAFSREQS H....KV.Y.VQHLLKQD 621
FNR 169 GTGTGIAPFRSFLWKMF.FEK.HDDYKFNGLAWLFLGV.PTSSSLL.YKEEFEKMKEKAPDNFR.LDFAVSREQT N.EKGEKM.Y.IQTRMAQY 254
H-E S3-4 H-F H-G S3-5
Consensus
HMP 348 .LEGAFSD..PT.MQFYLCGPVGFMQFTAKQLVDLGVKQE NIHYECFGPHKVL 396
Fre 198 .QDHGTLA..E..HDIYIAGRFE.MAKIARDLFCSERNAR EDRLFGDAFAFI 233
LuxG 198 .SDFRNLS..E..FDIYLCGPYK.MVEVARDWFCDKRGAE PEQLYADAFAYL 243
VanB 178 .RDALRDA..GD.AHLYVCGPGGFMEHVLGCARTAGWDET RLHREYFAAFVQPAGDARAFEGRLARSG 237
XylA 303 .KEYLTGQ..P..YEGYLCGPPP.MVDAAETELVRLGVAR ELVFADRFYNRPPC 350
P5 291 .KAHFDGR..FGGQKAYLCGPPP.MIDAAITTLMQGRLFE EDIFMERFYTAADGAGESSRSALFKRI 353
MMO-C 299 .REDLESS..DANPDIYLCGPPG.MIDAACELVRSRGIPG EQVFFEKFLPSGAA 348
NR 224 .REHVPEG..GDDTLALACGPPP.MIQFAISPNLEKMKYD MANSFVVF 267
Cb5R 258 .RDHLPPP..EEEPLVLMCGPPP.MIQYACLPNLDHVGHP TERCFVF 300
NOS 1331 LAESVYRALKEQGGHIYVCGDVT.MAADVLKAIQRIMTQQGKLSEEDAGVFISRLRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKKDADEVFSS 1429
SR 536 .GAELWRWI.NDGAHIYVCGDARRMAADVEKALLEVIAEFGGMDLESADEYLSELRVERRYQRDVY 599
CP450R 622 .REHLWKLI.EGGAHIYVCGDARNMARDVQNTFYDIVAELGAMEHAQAVDYIKKLMTKGRYSLDVWS 678
FNR 255 .AVELWEMLKKDNTYFYMCGLKG.MEKGIDDIMVSLAAAEGI....DWIEYKRQLKKAEQWNVEVY 314

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                1     .     .     .     .     .     .  63
HMP (FsrB)  MLDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPQLKEIFNMSNQRNGDQREALFNAIAAY
                      : *::* :  : : :****:*** ***:**************:********:*
Urf1            MRAILFRHTVSLNITRSLQ.AAHFYQRMFHHNPELKEIFNMSNQRNGNQREALFNTI
                1     .     .     .     .     .  56

              359     .     .     .  396
HMP (FsrB)  FYLCGPVGFMQFTAKQLVDLGVKQENIHYECFGPHKVL
              *****:**** *:**:::** : :***********:
Urf2          LCGPVAFMQFIARQLLEFGVTESQIHYECFGPHKVI
              1     .     .     .  36
```

Fig. 3. Alignments for Urf1 and Urf2 of *E. chrysanthemi* with the N- and C-terminal regions of HMP (FsrB). Identities and conservative substitutions (scoring >0.1 in the relatedness odds matrix MDM78 of Dayhoff [13]) are indicated by asterisks and colons, respectively.

served that cellular FSR activity is not dependent on Fur, the ferric uptake regulator [2]. Thus it would appear that FSR activity is not regulated by the level of cellular iron.

3.2. *Purification of FsrB and FsrC; identity of FsrB with HMP*

The three FSRs were separated into discrete fractions by anion-exchange chromatography, suggesting that they are structurally distinct components. FsrA, FsrB and FsrC represented approximately 7, 23 and 70%, respectively, of the total FSR activity. FsrB was purified to homogeneity, as judged by SDS-PAGE (Fig. 1) and native-PAGE. It exhibited a high affinity for Phenyl–Sepharose and had to be eluted with an ethanol–water gradient. Analytical gel-permeation chromatography and SDS-PAGE with FsrB gave estimated M_r's of 52,000 and 43,000, respectively, indicating that the protein is monomeric.

A 30-residue N-terminal amino acid sequence was determined for FsrB (data not shown) and found to resemble those of the *Vitreoscilla* haemoglobin-like protein ([28] 69% identity) and other haemoglobins (up to 25% identity) in the OWL database. The N-terminal sequence of FsrB was also found to be identical to that of the haemoglobin-like protein (HMP) of *E. coli*, recently deduced from the *hmp* gene [5]. The HMP of *E. coli* is a monomeric protein (M_r 44,000) containing one haem and one FAD [5]. It is therefore concluded that FsrB and HMP are identical.

The FsrC of *E. coli* was partially purified (Shipley, D. and Andrews, S.C, unpublished data) using a procedure similar to that described for FsrB. SDS-PAGE and gel-permeation chromatography revealed that FsrC is a monomer of M_r 26,000. This enzyme probably corresponds to the ferrisiderophore and FAD-reducing enzyme purified previously and designated 'NAD(P)H:flavin oxidoreductase' [29] or 'soluble ferrisiderophore reductase' [2]. The corresponding gene, *fre* (flavin reductase enzyme, Fre) has been cloned and sequenced [15].

3.3. *Similarity between HMP (FsrB) and other flavoenzyme oxidoreductases including Fre (FsrC)*

Although the N-terminal region (residues 1–140) of HMP (FsrB) is haemoglobin-like, no relationships involving the C-terminal region (residues 141–396) have been reported [5]. Database searches using the complete amino acid sequence of HMP (FsrB) as a probe have now revealed significant similarities between the C-terminal region and the NAD(P)$^+$-dependent FAD-binding oxidoreductase domains of proteins in the ferredoxin NADP$^+$ reductase (FNR) family, including Fre (FsrC). These similarities are highlighted in the multiple alignment shown in Fig. 2. The three-dimensional structure of one member of the FNR family, spinach FNR, is known [14]. It comprises two structural domains: an FAD-containing domain (residues 19–161) and an NADP$^+$-binding domain (residues 162–314). The two domains seem to form a functionally inseparable evolutionary unit, the FNR-functional domain [14]. Residue conservation in the FAD–NAD(P)-binding functional domains of members of the FNR family is particularly

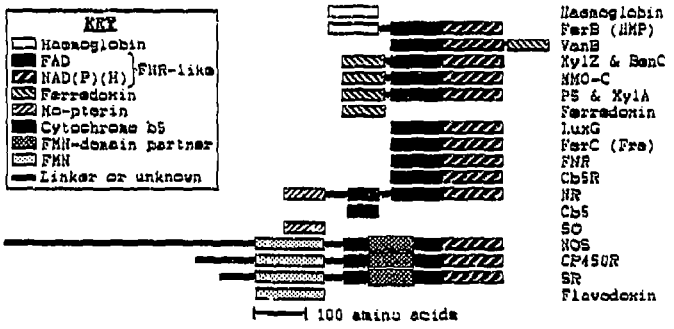

Fig. 4. Domain organisations of members of the FNR family. Schematic representations of the modular relationship between the FNR domain and other linked domains. Abbreviations are as in Fig. 2, except for: BenC, benzoate 1,2-dioxygenase component C from *Acinetobacter calcoaceticus* [31]; Cb5, mammalian cytochrome b_5 [32]; SO, chicken sulphite oxidase [33]; XylZ, toluate 1,2-dioxygenase component Z from *P. putida* [34].

strong in those regions thought to interact directly with FAD or NAD(P)$^+$ [14] (Fig. 2).

Database searches revealed that the LuxG protein [16] from the marine bacterium *Vibrio harveyi* is also a member of the FNR family (Fig. 2), and like Fre does not possess any major structural units other than the FNR domain. The corresponding gene (*luxG*) is part of the luciferase (*lux*) operon. Although the function of LuxG is unknown it may be responsible for the flavin reductase activity providing reduced flavin for the luciferase reaction in many luminous marine bacteria [30]. It is therefore possible that LuxG has a similar role to that of the *E. coli* homologue, Fre (FsrC).

3.4. *Putative* hmp *genes in* Erwinia chrysanthemi *and* Salmonella typhimurium

Similarities were detected between HMP (FsrB) and two sequences (Urf1 and Urf2) translated from adjacent but different reading frames, located upstream of the *pelA* gene of *Erwinia chrysanthemi* [37]. The sequence of residues 1–57 in Urf1 is 59% identical to the N-terminal region (residues 5–61) of HMP (FsrB) and residues 1–36 of Urf2 are 69% identical to the C-terminal segment of HMP (Fig. 3). These findings suggest that an *E. chrysanthemi* homologue of the *E. coli hmp* gene has suffered a deletion at the junction between the two open reading frames (i.e. between nucleotides 506 and 507 [37]). Interestingly, the 4,978 bp *pel* fragment already contains a deletion in the *pelD* gene at position 2,324 [37].

The database searches also detected an 87 bp segment of the *Salmonella typhimurium* chromosome encoding a sequence that is 100% identical to residues 1–29 of HMP (FsrB). This segment of DNA is presumed to be part of the *hmp* gene of *S. typhimurium*, located next to the *glyA* gene [38], as in *E. coli* [5].

3.5. *Functional and structural implications of the observed similarities*

Most members of the FNR family can be described as multi-redox centre proteins because, as illustrated in Fig. 4, they possess flavodoxin-like (FMN-containing), ferredoxin-like ([2Fe-2S]-containing), cytochrome b_5-like (haem-containing), Mo-pterin-containing or haemoglobin-like (haem-containing) domains, as well as the FNR domain. The FNR-like proteins containing flavodoxin domains also possess subdomains of approximately 130 residues in the FAD domains which are thought to mediate interaction with the FMN domains [35] (Figs. 2 and 4). The alignment shown in Fig. 2 suggests that these subdomains are in a region corresponding to an external loop between β-strands 1–5 and 1–4.

Those members of the FNR family which possess more than one functional domain are 'mosaic proteins' since they appear to have arisen by the 'shuffling' of domain-encoding regions during evolution. An interesting example of modularization in the FNR family is the VanB protein of *Pseudomonas* [17]. This protein, comprising a ferredoxin-like C-terminal domain and an FNR-like N-terminal domain, forms part of the multicomponent vanillate demethylase complex. However, the domain order is reversed in all of the other known examples of partnership between FNR- and ferredoxin-like domains (see BenC, MMO-C, P5, XylA and XylZ in Fig. 4). Nevertheless, the relative locations and orientations of the domains in three-dimensional space could be very similar for VanB and the BenC group of proteins.

The multiple alignment (Fig. 2) indicates that the α-helices and β-strands of FNR are mainly conserved, supporting the view that the proteins have similar structures. However, the region corresponding to an external loop (β-strands 2–1 and 2–2) is apparently missing in the bacterial proteins (HMP, Fre, LuxG, VanB, XylA, P5 and MMO-C; Fig. 2) suggesting that the main chain follows a relatively short path from β-strand 1–3 to helix A.

There are two approximately parallel V-shaped loops at the surface of FNR formed by helices D and E, and helices F and G [14]. The alignment further indicates that nitrate reductase, cytochrome b_5 reductase and the bacterial proteins lack helix G. This could be accommodated if helix F takes a more direct route from β-strand 3–4 to 3–5. Those proteins which seem to lack helix G also appear to lack the two residues forming the turn between helices D and E (FNR residues 253 and 254) suggesting that helices D and E may be fused to form a single α-helix. If so, the D–E helix could form a more direct link between β-strands 3–1 and 3–4.

The combination of haemoglobin-like and FNR-like domains in HMP (FsrB) represents a novel partnership which raises interesting questions regarding their structure–function relationships. The FNR-like domains are thought to function as flavoprotein reductases passing reducing equivalents between electron acceptors or donors, and pyridine dinucleotides. The FNR-like cytochrome b_5 reductase participates in methaemoglobin reduction in erythrocytes. It is possible that HMP (FsrB) functions in a similar way such that the FNR-like domain reduces the haem-iron of the haemoglobin-like domain by transferring electrons from NAD(P)H to the haem moiety via the associated FAD group. Alternatively, the haem moiety of HMP (FsrB) may be involved in oxygen-dependent regulation of FsrB activity. The ferric reductase activity of FsrB is probably mediated by flavinonucleotides, as is thought to be the case for FsrC-dependent ferric reduction [15]. HMP (FsrB) also possesses dihydropteridine reductase (DHPR) activity [5], although the HMP (FsrB) studied here is distinct from the previously purified DHPR of *E. coli* [36].

HMP (FsrB) provides an interesting example of the way in which different structural units can be brought together during evolution to generate proteins with

unique structural and functional properties. However, despite the DHPR and FSR activities of HMP (FsrB), the electron acceptor utilised in vivo, and therefore the physiological function of the protein, remains uncertain.

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