N-terminal truncations in the FhlA protein result in formate- and MoeA-independent expression of the *hyc* (formate hydrogenlyase) operon of *Escherichia coli*

William T. Self,[†] Adnan Hasona and K. T. Shanmugam

Author for correspondence: K. T. Shanmugam. Tel: +1 352 392 2490. Fax: +1 352 392 5922.
e-mail: shan@ufl.edu

Department of Microbiology
and Cell Science, Box
110700, University of
Florida, Gainesville,
FL 32611, USA

The formate hydrogenlyase complex of *Escherichia coli* catalyses the cleavage of formate to CO₂ and H₂ and consists of a molybdoenzyme formate dehydrogenase-H, hydrogenase 3 and intermediate electron carriers. The structural genes of this enzyme complex are activated by the FhlA protein in the presence of both formate and molybdate; ModE-Mo serves as a secondary activator. Mutational analysis of the FhlA protein established that the unique N-terminal region of this protein was responsible for formate- and molybdenum-dependent transcriptional control of the *hyc* operon. Analysis of the N-terminal sequence of the FhlA protein revealed a unique motif (amino acids 7–37), which is also found in ATPases associated with several members of the ABC-type transporter family. A deletion derivative of FhlA lacking these amino acids (FhlA9-2) failed to activate the *hyc* operon *in vivo*, although the FhlA9-2 did bind to *hyc* promoter DNA *in vitro*. The ATPase activity of the FhlA9-2–DNA–formate complex was at least three times higher than that of the native protein–DNA–formate complex, and this degree of activity was achieved at a lower formate level. Extending the deletion to amino acid 117 (FhlA167) not only reversed the FhlA[−] phenotype of FhlA9-2, but also led to both molybdenum- and formate-independence. Deleting the entire N-terminal domain (between amino acids 5 and 374 of the 692 amino acid protein) also led to an effector-independent transcriptional activator (FhlA165), which had a twofold higher level of *hyc* operon expression than the native protein. Both FhlA165 and FhlA167 still required ModE-Mo as a secondary activator for an optimal level of *hyc*–*lac* expression. The FhlA165 protein also had a twofold higher affinity to *hyc* promoter DNA than the native FhlA protein, while the FhlA167 protein had a significantly lower affinity for *hyc* promoter DNA *in vitro*. Although the ATPase activity of the native protein was increased by formate, the ATPase activity of neither FhlA165 or FhlA167 responded to formate. Removal of the first 117 amino acids of the FhlA protein appears to result in a constitutive, effector-independent activation of transcription of the genes encoding the components of the formate hydrogenlyase complex. The sequence similarity to ABC-ATPases, combined with the properties of the FhlA deletion proteins, led to the proposal that the N-terminal region of the native FhlA protein interacts with formate transport proteins, both as a formate transport facilitator and as a cytoplasmic acceptor.

Keywords: FhlA mutations, molybdenum, operon regulation

[†] Present address: NHLBI, NIH, Bethesda, MD, USA.

Abbreviations: FDH-H, formate dehydrogenase isoenzyme; FHL, formate hydrogenlyase enzyme complex; P_{hyc}, *hyc* operon promoter.

INTRODUCTION

Escherichia coli catalyses the cleavage of intracellular formate to H_2 and CO_2 via the formate hydrogenlyase enzyme complex (FHL) (reviewed by Böck & Sawers, 1996). This complex includes a formate dehydrogenase isoenzyme (FDH-H; encoded by the *fdhF* operon), a hydrogenase isoenzyme (HYD3; encoded by the *hyc* operon), and intermediate electron carriers (also encoded by the *hyc* operon). FHL is only produced under anaerobic conditions and in the absence of alternative terminal electron acceptors, such as nitrate. Reduced levels of expression of the *hyc* operon under these conditions have been attributed to a reduced pool of intracellular formate (Rossmann *et al.*, 1991). This lack of available formate affects the transcriptional activator FhlA, as it requires formate as an effector molecule (Maupin & Shanmugam, 1990; Schlensog *et al.*, 1994).

Molybdenum is required for FHL activity and is present in the FDH-H protein as molybdopterin guanine dinucleotide (Boyington *et al.*, 1997). Molybdenum is transported in the form of molybdate by a high-affinity transporter encoded by the *modABC* operon (reviewed by Grunden & Shanmugam, 1997). Previous studies have shown that the expression of both the *hyc* and *fdhF* operons also requires molybdenum for optimal transcription (Rosentel *et al.*, 1995; Hasona *et al.*, 1998b). MoDE protein, originally characterized as a molybdate-dependent repressor of the *modABC* operon (Grunden *et al.*, 1996), was also identified as a secondary transcriptional activator of the *hyc* operon (Self *et al.*, 1999). The FhlA protein has been proposed to serve as a molybdenum sensor (Hasona *et al.*, 1998b), possibly interacting with the product of molybdate activation by the MoeA protein (Hasona *et al.*, 1998a).

The FhlA protein has three distinguishable domains based, in part, on sequence similarity with other transcriptional activators, such as NtrC from *E. coli* (Nixon *et al.*, 1986) and NifA from *Klebsiella pneumoniae* (Drummond *et al.*, 1986). The C-terminal region contains a helix–turn–helix motif which constitutes a DNA-binding domain; this was confirmed by the DNA-binding activity of a C-terminal fragment of FhlA containing the amino acids 379–692 (Self, 1998; Leonhartsberger *et al.*, 2000). The central region of FhlA is conserved among all members of the NtrC/NifA family of activators and contains a nucleotide-binding motif (Weiss *et al.*, 1991): this region is probably responsible for formate- and DNA-dependent ATP hydrolysis and interaction with the σ^{54} form of RNA polymerase in the initiation of transcription (Hopper & Böck, 1995; Korsa & Böck, 1997). The N-terminal segment of the FhlA protein appears to contain a region which modulates the transcriptional activity of the C-terminal segment. In many proteins of the NtrC family, the unique N-terminal region (of varying length) contains a receiver domain which must be phosphorylated by a cognate sensor-protein for activity (Keener & Kustu, 1988). Some members of this family of activators

are not known to be phosphorylated, and are presumed to interact with appropriate effector molecules directly.

The N-terminal region of FhlA is one of the largest (380 aa), when compared to other response regulators of the NtrC family. The unique N-terminal region of the FhlA protein displays no significant sequence similarity to any known protein, and is not believed to be phosphorylated (Böck & Sawers, 1996). Point mutations in the N-terminal region of FhlA lead to partial independence from formate (Korsa & Böck, 1997) and molybdenum (Self & Shanmugam, 2000) in the activation of the *hyc* operon. Based on these studies, it has been proposed that the N-terminal region of FhlA binds the effector molecule formate and may also interact with the product of the MoeA protein (activated molybdenum).

Removal of the first 378 amino acids of the 692 aa FhlA protein led to a formate-independent transcriptional activator (Self, 1998; Leonhartsberger *et al.*, 2000). The N-terminal half of the protein was also shown to inhibit the ATPase activity of the C-terminal half of the protein and to inhibit the formate-dependent ATPase activity of the native protein. Since the FhlA protein has been proposed to interact with the MoeA product for optimum transcription of the *hyc* operon, and mutations in the unique N-terminal domain of FhlA lead to molybdenum-independence, it is possible that removing the N-terminal domain would also impart molybdenum-independence to the truncated FhlA protein. It is also not known which part of the nearly 400 aa N-terminal segment of the protein is essential for formate- and molybdenum-dependence for *hyc* activation. Therefore, several deletion derivatives extending to varying lengths of the N-terminal domain of FhlA were constructed to identify the critical region(s) responsible for effector-independence, and the results of this study are reported here.

METHODS

Bacterial strains. The bacterial strains, phages and plasmids used in this study are detailed in Table 1. All strains are derivatives of *Escherichia coli* K-12.

Media and growth conditions. Media used for bacterial growth were as described by Rosentel *et al.* (1995). L-broth (LB; Miller, 1972) was supplemented with glucose (0.3%), sodium formate (30 mM), sodium molybdate (1 mM) or sodium nitrate (30 mM). Antibiotics were added to the media, as required, at the following concentrations: 100 µg ampicillin ml⁻¹; 30 µg tetracycline ml⁻¹; 50 µg chloramphenicol ml⁻¹ (plates), 10 µg chloramphenicol ml⁻¹ (broth); 50 µg kanamycin ml⁻¹.

Transduction with phages P1 and λ was performed as described by Grunden *et al.* (1996). Genetic and molecular experiments were performed essentially as previously described (Self *et al.*, 1999). Plasmids pWS2, pWS164 and pWS165 are derivatives of the low-copy vector pACYC184. Plasmid pWS167 is a derivative of pT7-7. For experiments involving *pcnB* mutant (Lopilato *et al.*, 1986), the appropriate *fhlA*⁺ and *fhlA165* DNAs were transferred to plasmid vector pBR322 in the construction of pWS3 and pWS165-2, respectively. Plasmid pWS165-2 also carries the gene for spectinomycin resistance from pSJS1240 (Kim *et al.*, 1998).

Table 1. Bacterial strains, phages and plasmids used in this study

Strain, phage or plasmid	Relevant genotype*	Source/reference†
<i>E. coli</i>		
BL21(DE3)	<i>ompT gal dcm lon hsdSB λDE3</i>	Laboratory collection
GS035	$\Delta oxyS2::Cm$	G. Storz
MRi93	<i>pcnB80 zad-2084::Tn10</i>	CGSC #7066
SE1174	<i>fhla::Tn10</i>	Laboratory collection
SE1265	<i>pfl-1 zba::Tn10</i>	Laboratory collection
SE1497	$\Delta srl-fhla$	Laboratory collection
SE1910	$\Delta(modE-Km)2$	Laboratory collection
SE2007	MC4100, $\Phi(fhla-lacZ)$	Self & Shanmugam (2000)
SE2147	$\Delta moeA113 zbi::Tn10$	Laboratory collection
VJS720	<i>modB247::Tn10</i>	V. Stewart
WS113	SE1497, $\Delta lac \Delta mod \lambda WS1$	Self & Shanmugam (2000)
WS118	WS113, <i>pfl-1 zba::Tn10</i>	Self & Shanmugam (2000)
WS127	WS113, $\Delta gal mod^+$	Self & Shanmugam (2000)
WS152	SE2007, <i>pfl-1 zba::Tn10</i>	Laboratory collection
WS179	WS127, <i>moeA113</i>	This study
WS182	WS113, $\Delta(modE-Km)2$	This study
WS198	WS113, $\Delta(modE-Km)2 moeA113 (modABC)^+$	Self & Shanmugam (2000)
WS244	WS127, <i>pcnB80 zad-2084::Tn10</i>	This study
WS245	WS252, $\Delta(modE-Km)2 pcnB80$	This study
WS246	WS252, <i>moeA113 pcnB80</i>	This study
WS252	WS127, <i>modB</i>	This study
WS254	WS244, $\Delta(modE-Km)2 moeA113$	This study
Phages		
P1	Tn9 Cm^R <i>clr-100</i>	Laboratory collection
$\lambda WS1$	$\lambda bla^+ P_{hyc} lacZ$	Hasona <i>et al.</i> (1998b)
Plasmids		
pFGH100	pUC19, <i>hypA hycA hycB'</i>	Laboratory collection
pSE133	pBR322, <i>fhla</i> ⁺	Sankar <i>et al.</i> (1988)
pWS2	pACYC184, <i>fhla</i> ⁺ Cm^R	Self & Shanmugam (2000)
pWS3	pBR322, <i>fhla</i> ⁺	This study
pWS9	pT7-7, <i>fhla</i> ⁺ Ap^R	This study
pWS9-2	pT7-7, <i>fhla9-2</i> Cm^R	This study
pWS16	pT7-7, <i>fhla165</i> Ap^R	This study
pWS40	pUC19, <i>hyc-hyp</i> intergenic region	This study
pWS41	pT7-7, <i>fhla167</i>	This study
pWS164	pACYC184, <i>fhla164</i> Cm^R	This study
pWS165	pACYC184, <i>fhla165</i> Cm^R	This study
pWS165-2	pBR322, <i>fhla165</i> Sp^R	This study
pWS166	pACYC184, <i>fhla166</i> Cm^R	This study
pWS167	pT7-7, <i>fhla167</i> Ap^R	This study

* Cm , chloramphenicol; Km , kanamycin; Ap , ampicillin; Sp , spectinomycin.

† G. Storz, National Institutes of Health, Bethesda, MD, USA; CGSC, Coli Genetic Stock Center, Yale University, New Haven, CT, USA; V. Stewart, University of California, Davis, CA, USA.

Isolation of N-terminal deletion *fhla* alleles. Internal deletion mutations within the *fhla* gene were isolated by Bal31 nuclease treatment of pWS2 (Self & Shanmugam, 2000) after *HpaI* restriction endonuclease hydrolysis. *HpaI* hydrolyses pWS2 approximately in the centre of the DNA encoding the unique N-terminal region of the Fhla protein. The reactions were terminated by the addition of ethanol (70% final concn) after 5, 10 and 15 min of nuclease treatment. The DNA was precipitated and subsequently ligated with T4 DNA ligase.

The ligation mixture was transformed into strain SE1174 (*fhla::Tn10*), and colonies expressing FDH-H activity were selected by the dye-overlay procedure as described by Lee *et al.* (1985). Plasmids from selected clones were isolated and the size of the deletion was determined using restriction endonucleases. Three plasmids with a deletion of ≥ 1 kb were selected for further analysis. The extent of the deletion in each mutant *fhla* allele was determined by sequencing the DNA using the Sanger dideoxy sequencing method (Sanger *et al.*,

1977; Sequenase version 2.0 DNA sequencing kit). This procedure yielded *fhlA* alleles *fhlA164*, *fhlA165* and *fhlA166* (plasmids pWS164, pWS165 and pWS166, respectively; Fig. 1). *fhlA167* was isolated after removing the *fhlA* DNA as a *PflMI*–*SmaI* fragment and cloning it into a *SmaI*-digested vector, pT7-7. This construct, pWS41, carries a deletion of the first 117 aa of the N-terminus, and an extra 7 aa in the N-terminus derived from the vector DNA sequence (MARIRAL) which also provided the translation start site. Plasmid pWS167 was constructed by inserting a chloramphenicol-resistance gene cartridge into the *ScaI* site of pWS41. The *fhlA167* allele DNA was sequenced to confirm the deletion. DNA sequences were analysed using GENEPRO software (Riverside Scientific).

For the construction of pWS9-2, plasmid pWS9 was modified by introducing a chloramphenicol-resistance gene cartridge from plasmid pBR325 into the *SspI*–*DraI* sites, to generate pWS9-1, followed by PCR-mediated deletion of the *fhlA* DNA corresponding to amino acids 7–37 of the FhlA protein. The *fhlA* gene on both sides of the deletion was amplified using two primer pairs (pair one, 5'-AAATGTCATATACACCG-ATGGTAAAGCGTTCTGCGCTCGCCG-3' and 5'-GACT-TCGCTGACCAGTTCGTCCATA-3'; pair two, 5'-CGAG-CGCAGAACGCTTTACCATCGGTGTATATGACATT-TTGTTT-3' and 5'-TGTGGATAACCGTATTACCGCCT-3'). The PCR products were ligated together to create the deletion derivative (plasmid pWS9-2), and the PCR-amplified region was sequenced to confirm that the *fhlA* gene in plasmid pWS9-2 did not carry any additional mutations, besides the intended deletion.

Purification of the FhlA165 protein. Purification of the FhlA165 protein was essentially as described for the native FhlA protein by Schlensog *et al.* (1994). Briefly, pWS16, which carries the *fhlA165* allele under the control of phage T7 promoter, was constructed by ligating a 1.4 kb *BstBI*–*Clal* fragment from pWS165 into the *Clal* site in the expression vector pT7-7 (Tabor & Richardson, 1985). Fresh transformants of BL21(DE3) with pWS16 were grown in 1 litre of LB with ampicillin in a 2.8 litre Fernbach flask at 37 °C with shaking (225 r.p.m.). When the culture reached OD₄₂₀ 0.7 (Spectronic 710 spectrophotometer), IPTG was added (0.34 mM final concn) and the culture was incubated at 37 °C with shaking (225 r.p.m.) for an additional 3 h. Cells were harvested and washed once with Tris buffer (50 mM Tris, pH 8.0; 0.5 mM EDTA; 1 mM benzamidine; 1 mM DTT). The cells were then resuspended in 12 ml Tris buffer and broken by passage through a French pressure cell [20000 p.s.i. (138 MPa)]. All subsequent steps were carried out at 4 °C. The crude extract was centrifuged at 30000 g for 30 min, and the supernatant (14 ml) was further clarified by centrifugation for 2 h at 120000 g. Ammonium sulfate (37% saturation) was added to the supernatant, and the proteins were precipitated for 30 min. The sample was then centrifuged for 30 min at 30000 g, and the pellet was dissolved in 1.2 ml Tris buffer. This sample was dialysed overnight in 1 litre Tris buffer. Unlike the wild-type FhlA protein, which precipitated out of solution at this step (Schlensog *et al.*, 1994), the FhlA165 remained soluble. This dialysed sample was analysed by chromatography, using a 40 ml heparin-agarose affinity column (Pharmacia Biotech). The column matrix was washed with Tris buffer and the proteins were eluted with a linear gradient of KCl (0–0.5 M). FhlA165-containing fractions, as determined by SDS-PAGE analysis, were pooled and dialysed overnight in Tris buffer. Further purification of FhlA165 was achieved using a 12 ml Q-Sepharose column (Pharmacia) and a linear KCl gradient (0–0.4 M). The FhlA165 protein eluted at approximately 0.3 M KCl, and was determined to be pure

based on SDS-PAGE analysis. Fractions containing the FhlA165 protein were pooled and dialysed overnight in Tris buffer (25 mM, pH 7.5) with 1 mM EDTA and 50 % glycerol (v/v). The FhlA165 protein was stored at –20 °C.

FhlA167 and FhlA9-2 proteins were purified essentially as described for FhlA165. However, the two proteins obtained after the Q-Sepharose step were subjected to further chromatography on a Q-Sepharose column with sodium citrate as the eluent. Both proteins eluted at a citrate concentration of 0.15 M. The pure proteins were dialysed against Tris buffer (50 mM, pH 7.5) and stored on ice. Freshly prepared proteins were used immediately.

Purification of native FhlA protein. The purification procedure similar to the one used for FhlA165 protein led to the characteristic precipitation of the native protein during dialysis after the ammonium sulfate precipitation step (Schlensog *et al.*, 1994). The FhlA protein required high salt (0.2 M KCl) to remain in solution; thus, it may not have been present in the native form. In addition, most of the FhlA protein produced upon expression from a T7 promoter was present in inclusion bodies. Therefore, the conditions used for expression of *fhlA*⁺ and purification of the native FhlA were modified, and the method is described below.

The *fhlA*⁺ gene was cloned into the expression vector pT7-7, by removing a 2.4 kb *SpeI*–*Clal* fragment from plasmid pSE133 (Sankar *et al.*, 1988) and ligating this DNA to a 2.4 kb *XbaI*–*Clal* fragment from pT7-7 (Tabor & Richardson, 1985). The resultant plasmid, pWS9, carried the *fhlA* gene under the control of phage T7 gene 10 promoter. Fresh BL21(DE3) transformants of pWS9 were inoculated into 1 litre LB containing ampicillin, in a 2.8 litre Fernbach flask. The culture was grown with shaking (225 r.p.m.) at 37 °C until an OD₄₂₀ of 0.7 was reached. IPTG was added (0.5 mM final concn) to the culture, and incubation was continued at 23 °C with shaking (225 r.p.m.) for an additional 6 h. Incubating the culture at this temperature allowed a larger fraction of the newly synthesized FhlA to stay in solution. Cells were harvested at 4 °C and washed once with Tris buffer (50 mM Tris, pH 8.0; 0.5 mM EDTA; 1 mM benzamidine; 1 mM DTT). All subsequent operations were carried out at 4 °C. Cells were resuspended in 25 ml Tris buffer and broken by passage through a French pressure cell at 20000 p.s.i. (138 MPa). The crude extract was centrifuged at 30000 g for 2 h and the supernatant fraction was loaded onto Q-Sepharose column (30 cm × 2.5 cm; Pharmacia). The column was then washed with Tris buffer and the proteins were eluted with a linear gradient of NaCl (0–0.4 M): the FhlA protein eluted at approximately 0.37 M NaCl. FhlA-containing fractions, as determined by SDS-PAGE analysis, were pooled and dialysed overnight in Tris buffer. This protein mixture was loaded onto a Hi-trap heparin column (Pharmacia) and eluted using a linear NaCl gradient (0–0.4 M): the FhlA protein eluted at approximately 0.3 M NaCl. FhlA-containing fractions were pooled and dialysed overnight in HEPES buffer (30 mM HEPES, pH 7.0 with 0.5 mM EDTA, 1 mM benzamidine and 1 mM DTT). The FhlA protein was further purified using a 1 ml High S column (cation exchange, Bio-Rad) equilibrated with HEPES buffer and eluted with a linear gradient of 0–0.5 M NaCl. The FhlA protein eluted at approximately 0.25 M NaCl. Fractions containing FhlA protein were pooled and dialysed overnight in HEPES buffer. The final purification step required chromatography on a 1 ml High Q column (anion exchange, Bio-Rad) equilibrated with HEPES buffer and elution with a linear gradient of sodium citrate (0–0.3 M). The pure FhlA protein, as judged by SDS-PAGE analysis, was dialysed in storage buffer (25 mM HEPES, pH 7.0; 0.8 mM

DTT; 10% glycerol). FhlA protein was quickly frozen in liquid nitrogen and stored at -70°C , in aliquots.

Enzyme assay. FHL activities [expressed as nmol (mg cell protein) $^{-1}$ min $^{-1}$] of late-exponential phase cultures were determined as described by Lee *et al.* (1985). All experiments were carried out with whole cells to minimize O_2 inactivation of the enzyme. β -Galactosidase activities [expressed as nmol (mg cell protein) $^{-1}$ min $^{-1}$] were determined in late-exponential- to early-stationary-phase cultures as described by Rosentel *et al.* (1995).

ATPase activity. ATPase activity associated with the FhlA protein was determined as described by Korsá & Böck (1997), and expressed as pmol ATP hydrolysed (pmol FhlA) $^{-1}$.

DNA electrophoretic mobility-shift experiments. DNA mobility-shift experiments were performed essentially as described by Grunden *et al.* (1996). Briefly, DNA containing the *hyc-hyp* intergenic region was PCR-amplified from plasmid pFGH100, using two primers: 5'-CCGGATCCGT-TATTTCGAGCATATC-3' and 5'-CCCTGCAGTTTAAG-CTAAAGATGAA-3'. This amplified DNA fragment, after digestion with *Pst*I and *Bam*HI, was cloned into plasmid pUC19, also digested with *Pst*I and *Bam*HI. The resulting plasmid, pWS40, was the source of a 154 bp *Pst*I–*Bam*HI fragment used in the electrophoretic mobility-shift experiments. The 154 bp DNA fragment, after isolation from a 10–30% sucrose gradient, was labelled with ^{32}P -dNTPs by filling in the recessed 3' end of the *Bam*HI site with the DNA polymerase Klenow fragment. FhlA or FhlA165 protein was pre-incubated in binding buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 1 mM DTT; 1 mM ATP) for 5 min at 4°C before the labelled DNA was added. Binding reaction mixtures (total volume 20 μl) were incubated for 20 min at 37°C . Polyacrylamide gels (5%) were pre-run (100 V, 60 min, room temperature), with circulation of TBE buffer (Tris, 89 mM, pH 8.3; borate, 89 mM; EDTA, 2.5 mM). Samples were loaded and the gels were run for a further 60 min (100 V), in TBE buffer. Buffer circulation re-started after the samples had been loaded. The gels were then dried and exposed to pre-flashed X-ray film. The band intensity was determined using a phosphorimager (Molecular Dynamics).

Materials. Biochemicals were purchased from Sigma. Other organic and inorganic chemicals were from Fisher Scientific and were of analytical grade. Restriction endonucleases and DNA-modifying enzymes were purchased from Promega and New England Biolabs.

RESULTS AND DISCUSSION

Isolation of deletions within the N-terminal domain of FhlA

A number of deletion derivatives of *fhlA* which allowed the production of dihydrogen by an *fhlA* mutant strain, *E. coli* SE1174 (*fhlA*::Tn10) were isolated. After analysis of the size of the *fhlA* gene in these plasmids and screening for the ability to support FHL production, four *fhlA* mutations with large deletions within the unique N-terminal domain of FhlA were selected for further analysis. Based on DNA sequence data, all four alleles (*fhlA*164, $\Delta 26$ –370, L371V; *fhlA*165, $\Delta 5$ –374; *fhlA*166, $\Delta 36$ –314; *fhlA*167, $\Delta 1$ –117) carried large deletions in the unique N-terminal domain of FhlA. Although *fhlA*164 and *fhlA*166 supported production of FHL activity in a *fhlA* mutant, the level of *hyc-lac* expression in the presence of these two alleles was only

Fig. 1. Extent of the N-terminal deletions within the FhlA protein. The solid lines represent the amino acid sequence and the dashed lines correspond to the deletions in the FhlA protein sequence. The β -galactosidase activity [nmol (mg cell protein) $^{-1}$ min $^{-1}$] represents the expression of *hyc-lac* in the presence of the various FhlA alleles in a Δ (*srl-fhlA*) strain carrying λ WS1, grown in L-broth containing glucose (0.3%) and sodium formate (30 mM) under anaerobic conditions. The solid boxes represent the motif for the ATP-binding domain; the hatched boxes represent the helix-turn-helix motif (DNA binding domain).

Table 2. Transcription of $P_{hyc}lacZ$ by FhlA165 and FhlA167 is formate- and molybdate-independent

Cultures were grown in the following media: LBG, L-broth with glucose (0.3%); LBGM, L-broth with glucose (0.3%) and sodium molybdate (1 mM); LBGF, L-broth with glucose (0.3%) and sodium formate (30 mM); LBGMF, L-broth with glucose (0.3%), sodium formate (30 mM) and sodium molybdate (1 mM). β -Galactosidase activity is expressed as nmol (mg cell protein) $^{-1}$ min $^{-1}$.

Strain	Relevant genotype*	β -Galactosidase activity			
		LBG	LBGM	LBGF	LBGMF
WS127(pWS2)	<i>mod</i> ⁺	1200	1300	1800	1700
WS113(pWS2)	Δ <i>mod</i>	320	1800	720	1900
WS118(pWS2)	Δ <i>mod pfl</i>	140	470	840	1700
WS127(pWS165)	<i>mod</i> ⁺	3500	3300	4000	3500
WS113(pWS165)	Δ <i>mod</i>	2500	2700	2100	2900
WS118(pWS165)	Δ <i>mod pfl</i>	3600	3600	3100	3300
WS127(pWS167)	<i>mod</i> ⁺	2200	1800	2000	1900
WS113(pWS167)	Δ <i>mod</i>	1550	2000	1500	1850
WS118(pWS167)	Δ <i>mod pfl</i>	1500	1650	1400	2000

* All strains carry $P_{hyc}lacZ$ via λ WS1 and a deletion of the *srl-fhlA* region.

about 25% of the values obtained with native *fhlA* [approximately 350 units β -galactosidase activity, compared to approximately 1800 units activity for the strain with native FhlA]. The alleles *fhlA*165 and *fhlA*167, which supported the highest level of β -galactosidase production (Fig. 1), were studied in detail.

FhlA165 and FhlA167 are formate and molybdate independent

In the presence of native FhlA protein (pWS2), the optimum level of *hyc-lac* expression required both

molybdate and formate (Table 2). Most notably, in the *mod pfl* double mutant, strain WS118(pWS2), *hyc-lac* expression was very low (140 units β -galactosidase activity) in the absence of molybdate and formate. The addition of formate to the growth medium increased the level of *hyc-lac* expression to approximately 50 % of the maximal level (840 units); supplementing the medium with molybdate alone increased the level of expression by approximately threefold (470 units). A level of *hyc-lac* expression equivalent to that of wild-type *E. coli* was attained only when both formate and molybdate were present in the growth medium of the *mod pfl* double mutant.

In contrast, activation of *hyc-lac* expression by FhlA165 and FhlA167 was no longer dependent on formate and molybdate: this was demonstrated by the high level of β -galactosidase activity present in all three strains carrying pWS165 or pWS167 (Table 2). Although both deletion derivatives led to molybdenum- and formate-independence, only FhlA165 supported a higher level of *hyc-lac* expression than the wild-type (4000 and 2000 units β -galactosidase activity, respectively; Table 2). The higher than expected level of expression in the presence of FhlA165 suggests that the N-terminal half of the FhlA protein negatively regulates the activation of the *hyc* operon by the native FhlA protein, even in the presence of the effectors formate and molybdate. Removal of the N-terminal half of FhlA165 allowed the C-terminal DNA-binding segment to activate the *hyc* operon maximally; this was in agreement with a previous report by Leonhartsberger *et al.* (2000).

The FhlA167 mutant carried a deletion of amino acids 1–117, while the deletion in FhlA165 was of amino acids 5–374. These results show that removal of the first 117 aa is sufficient to produce an effector-independent transcriptional activator, but that this is not sufficient to overcome the inhibitory effect of the N-terminal domain of FhlA on the transcription of the *hyc* operon by the C-terminal domain. The critical structure responsible for modulating the activity of the C-terminal DNA-binding domain appears to reside between amino acids 118 and 374.

The product of the first gene of the *hyc* operon, *hycA*, appears to act by negatively modulating the level of *hyc* operon expression (Sauter *et al.*, 1992). The nature of this apparent repression is yet to be determined. Even in the presence of FhlA165 protein as activator, *hyc-lac* expression was reduced by HycA by the same level as seen with the native FhlA protein (data not shown). These results suggest that the effect of HycA on *hyc* operon expression is independent of formate and molybdate.

FhlA165 and FhlA167 activate transcription of $P_{hyc}lacZ$ in a *modE moeA* double mutant

Both ModE and MoeA proteins are required for the molybdate-dependent transcription of the *hyc* operon (Hasona *et al.*, 1998b). ModE was shown to be a

Table 3. Expression of $P_{hyc}lacZ$ by FhlA165 and FhlA167 requires ModE-Mo

Cultures were grown in L-broth containing glucose (0.3 %). β -Galactosidase activity is expressed as nmol (mg cell protein)⁻¹ min⁻¹.

Strain	Relevant genotype*	β -Galactosidase activity
WS127(pWS2)	<i>mod</i> ⁺	1200
WS182(pWS2)	Δ <i>modE</i> (<i>fhlA</i> ⁺)	800
WS179(pWS2)	<i>moeA113</i> (<i>fhlA</i> ⁺)	1400
WS198(pWS2)	Δ <i>modE moeA113</i> (<i>fhlA</i> ⁺)	180
WS127(pWS165)	<i>mod</i> ⁺	3500
WS182(pWS165)	Δ <i>modE</i> (<i>fhlA165</i>)	2000
WS179(pWS165)	<i>moeA113</i> (<i>fhlA165</i>)	3500
WS198(pWS165)	Δ <i>modE moeA113</i> (<i>fhlA165</i>)	2600
WS127(pWS167)	<i>mod</i> ⁺	2000
WS182(pWS167)	Δ <i>modE</i> (<i>fhlA167</i>)	1250
WS179(pWS167)	<i>moeA113</i> (<i>fhlA167</i>)	2500
WS198(pWS167)	Δ <i>modE moeA113</i> (<i>fhlA167</i>)	1600

* All strains carry $P_{hyc}lacZ$ via λ WS1 and a deletion of the *srl-fhlA* region.

secondary transcriptional activator and directly interacts with *hyc* promoter (P_{hyc}) DNA (Self *et al.*, 1999). The role of MoeA in this regulation is not known but it has been proposed that the FhlA protein binds the catalytic product of MoeA (activated molybdenum; Hasona *et al.*, 1998a) and modulates its activity (Self & Shanmugam, 2000). Since FhlA165 and FhlA167 activated transcription of *hyc-lacZ* even in the absence of molybdate, the need for ModE and MoeA proteins for the expression of *hyc-lac* with these deletion derivatives was determined.

A *modE moeA* double mutant containing the native FhlA protein, strain WS198(pWS2), produced a measurable, but low, level of β -galactosidase activity (approximately 180 units) when cultured in L-broth containing glucose (Table 3); the addition of molybdate to the medium had no effect on *hyc-lac* expression. In contrast, the same double mutant containing the FhlA165 protein, *E. coli* strain WS198(pWS165), produced an approximately 15-fold more β -galactosidase activity (2600 units): this level of activity is also approximately 2.2-fold higher than the activity of a *mod*⁺ strain containing the native FhlA, strain WS127(pWS2). The FhlA167 protein also overcame the requirement of ModE and MoeA proteins for *hyc* operon expression, and the level of expression of *hyc-lac* was similar to that of the *mod*⁺ strain with native FhlA (approximately 1500 units). As expected, addition of molybdate had no significant effect on the expression of the *hyc* operon when FhlA165 or FhlA167 was used as the activator. Although *hyc-lac* expression in the *modE moeA* mutant was higher in the presence of FhlA165 or FhlA167, the level of β -galactosidase activity produced was only approximately

65–75% of the value obtained with the *mod*⁺ strain with these mutated forms of the FhlA proteins [WS127(pWS165); WS127(pWS167)]. This lower than expected value was apparently a consequence of the absence of ModE protein, a required secondary activator, since a mutation in *modE* alone accounted for this difference. In the presence of either FhlA165 or FhlA167 the *moeA* mutation had no effect on *hyc-lac* expression. These results show that the deletions in FhlA165 and FhlA167 overcame the need for the MoeA-catalysed product for *hyc* operon expression, but not that for ModE-Mo, a required secondary activator of the *hyc* operon.

In a separate study, expression of *hyc-lac* was found to be limited by the concentration of FhlA in the cell when the protein was produced from a chromosomal copy of *fhlA* (J. A. Maupin & K. T. Shanmugam, unpublished data). Under the present experimental conditions, the amount of β -galactosidase activity produced by a culture carrying λ WS1 (*hyc-lac*) and a chromosomal copy of *fhlA*⁺ was approximately 600 units. This value did not change significantly when the *fhlA*⁺ gene was introduced from a plasmid (pWS3; vector pBR322) in a *pcnB* mutant background which limits the copy-number of the plasmid (Lopilato *et al.*, 1986). In a *pcnB*⁺ strain, the level of β -galactosidase activity produced by a culture carrying plasmid pWS2 (vector pACYC184), which contains *fhlA*⁺, was approximately 1300 units, and in the presence of plasmid pWS3 (vector pBR322) the level of β -galactosidase activity produced from *hyc-lac* increased further to approximately 3000 units. This observed copy-number effect is not related to the proposed antagonistic effect of HycA on *hyc-lac* expression (Sauter *et al.*, 1992), as the strain used in these experiments carries a deletion of the entire *srl-fhlA* region. In the experiments described above (Tables 2 and 3), the *fhlA* gene was present in a pBR322-based plasmid and it is possible that the observed independence from MoeA could be a result of overexpression of the *fhlA* mutant allele. However, it should be noted that in a *modE* background, the *moeA* mutation severely limited *hyc-lac* expression even in the presence of multiple copies of the *fhlA* gene. To rule out the effect of multiple copies of *fhlA*, *hyc-lac* expression was investigated in a *pcnB* mutant background with the *fhlA*165 allele.

When the overall level of *hyc-lac* expression was reduced to approximately 600–700 units β -galactosidase activity (in a *pcnB* mutant, strain WS244) in the presence of native FhlA, the requirement for ModE-Mo and MoeA for *hyc-lac* expression could be readily detected. In the presence of FhlA165, the *modE* mutation reduced the level of *hyc-lac* expression by about 33%. Even in a *modE moeA* double mutant, the level of *hyc-lac* expression catalysed by FhlA165 was only reduced by about 30% compared to the corresponding isogenic *moeA* mutant, strain WS246. However, with FhlA165, a *moeA* mutation alone had a positive effect on *hyc-lac* expression (1.4-fold higher than with the native FhlA protein) in this *pcnB* mutant, in contrast to a negative

effect of the *moeA* mutation (27% lower) in the presence of FhlA⁺. This positive effect of MoeA in the presence of the FhlA165 protein could be a result of a large pool of ModE-Mo in the cell, since molybdate is not processed in the *moeA* mutant combined with the MoeA-independence of the FhlA165 protein. The large pool of ModE-Mo could increase the level of expression of *hyc-lac* in the presence of FhlA165, but could not overcome the MoeA requirement for optimal activation by native FhlA. These results further demonstrate that transcription of the *hyc* operon by the N-terminal deletion derivative of FhlA is independent of the MoeA protein.

Activation of P_{*hyc*}*lacZ* by FhlA165 and FhlA167 is O₂- and nitrate-independent

Böck *et al.* previously proposed that the lack of *hyc* operon expression during conditions of aerobic (O₂) or anaerobic respiration (nitrate) was due to a lack of sufficient levels of formate to interact with FhlA (Rossmann *et al.*, 1991). The formate-independence of *hyc-lacZ* expression in the presence of FhlA165 and FhlA167 should lead to the production of significant β -galactosidase activity even under aerobic growth conditions.

Strain WS127(pWS2) with wild-type *fhlA*⁺ did not produce β -galactosidase activity when grown under aerobic growth conditions (Table 4). Addition of formate to the medium increased the level of β -galactosidase activity only to approximately 200 units and this represents 21% of the anaerobic level (Table 4). Exogenously added formate appears to be unable to support *hyc* expression to an optimum level when the cells are cultured under aerobic conditions. Even in the presence of O₂, P_{*hyc*}*lacZ* was activated by FhlA165 protein to a level higher than that of the wild-type strain grown anaerobically in L-broth containing glucose (1600 and 1200 units β -galactosidase activity, respectively). However, the level of β -galactosidase activity produced by WS127(pWS165) was still only about 50% of the same strain grown under anaerobic conditions. Including formate in the aerobic growth medium did not increase the level of P_{*hyc*}*lacZ* expression in the presence of FhlA165. Since FhlA165 is formate-independent for the transcription of the *hyc* operon, this O₂ effect could be due to a reduction in the expression of the *fhlA* gene. Previous work from our laboratory showed that *fhlA-lacZ* expression under aerobic growth conditions is only approximately 15% of the values obtained with anaerobic cultures (J. A. Maupin & K. T. Shanmugam, unpublished data). Recently, the *oxyS* small RNA species was shown to play a role in the regulation of *fhlA*, and this could account for O₂-dependent control of the *fhlA* and *fhlA*165 genes (Altuvia *et al.*, 1998) which indirectly controls the level of FHL in the cell. However, transducing an *oxyS* deletion mutation into strain WS127 did not affect the level of expression of *hyc-lac* or *fhlA-lac* in the presence of O₂ (data not shown). There appears to be an as yet unidentified O₂-

Table 4. Effect of O₂ and nitrate on the FhlA165- and FhlA167-mediated expression of P_{hyc}lacZ

Cultures were grown in the following media: LB, L-broth; LBF, L-broth with sodium formate (30 mM); LBG, L-broth with glucose (0.3%); LBGN, L-broth with glucose (0.3%) and nitrate (30 mM); LBGFN, L-broth with glucose (0.3%), sodium formate (30 mM) and nitrate (30 mM). Unless indicated (+O₂), cultures were grown under anaerobic conditions. β -Galactosidase activity is expressed as nmol (mg cell protein)⁻¹ min⁻¹.

Strain	Relevant genotype*	β -Galactosidase activity					
		LB	LB (+O ₂)	LBF (+O ₂)	LBG	LBGN	LBGFN
WS127(pWS2)	p <i>fhlA</i> ⁺	1100	< 50	230	1200	80	1900
WS127(pWS165)	p <i>fhlA165</i>	3000	1600	1500	3500	3900	ND
WS127(pWS167)	p <i>fhlA167</i>	1400	230	325	1900	1000	1200

ND, not determined.

* All strains carry P_{hyc}lacZ and a deletion of the *srl-fhlA* region.

FhlA	SDLGQGGGLFDITRTLLQQEDLASLCEAALSQ	7-37
PotA	LSGGQQRVALARAVVNKPRLLLDDESLSAL	148-178
ModF	LSWGQQRVALIVRAIVKHETLLILDEPLQGL	402-432
MalK	LSGGQQRVALGRITVAEPSEVLLDEPLSNL	134-164
XylG	LSLGQQQQLVEIAKAINKQVRLLILDEPTASL	142-173
LivG	LAYGDRRLEIARCMVTOFEILMLDEPAAGL	154-184
HisP	LSGGQQRVSIARALAMBEVLLFDEPTASL	153-183
ModC	LSGGQQRVALGRALLTAPELLLDDEPTASL	129-159
PotG	LSGGQQRVALARSLAKREKLLLDDEPMGAL	177-207
Consensus	LSGGQQRVALARAVVNKPRLLLDDEPTASL	
	ABC	
	Signature	

Fig. 2. Alignment of the N-terminal region of the FhlA protein with ATPases from various *E. coli* ABC transport systems. Amino acids 7–37 of the FhlA N-terminal region are shown on the top line. Below are proteins which all act as energizer proteins (cytoplasmic ATPases) in ABC-type transport systems within *E. coli*. The bottom line represents a consensus sequence derived from analysis of all nine proteins. References for each of the proteins are as follows: FhlA (Maupin & Shanmugam, 1990; Schlenso & Böck, 1990); PotA (Kashiwagi *et al.*, 1993); ModF (Grunden *et al.*, 1996); MalK (Shuman & Silhavy, 1981); XylG (Rosenfeld *et al.*, 1984); LivG (Adams *et al.*, 1990); HisP (Ames & Lever, 1970); ModC (Maupin-Furlow *et al.*, 1995); PotG (Pistocchi *et al.*, 1993).

dependent control of the *hyc* operon in *E. coli*. Although both the native FhlA and FhlA165 are produced to the same level in the cell, it is possible that there is a difference in the affinity of the two proteins for the P_{hyc} which may account for the difference in the level of expression of *hyc-lac* in the aerobic cell.

As formate is also an electron donor for nitrate reduction, the presence of nitrate *in vivo* is expected to decrease the formate pool and consequently reduce the expression of the *hyc* operon. As expected, *hyc-lacZ* was expressed at a very low level in the presence of nitrate when native FhlA served as the activator (Table

4). Supplementing the medium with formate restored *hyc-lacZ* expression. However, with FhlA165 as the activator, nitrate had no detectable effect on the expression of the *hyc* operon, supporting the model that formate is not a required effector for FhlA165.

It is interesting to note that although FhlA167 supported effector-independent expression of *hyc-lac* when the cells were grown anaerobically, in an aerobic cell this allele supported the production of only approximately 200 units β -galactosidase activity (approximately 15% of the anaerobic level). The addition of formate to the medium had a minimal effect on FhlA167-mediated *hyc* expression in the aerobic cell: this is in agreement with its formate independence. Addition of nitrate to the anaerobic culture also reduced the *hyc-lac* expression in the presence of FhlA167, but the nitrate effect was minimal compared to that of O₂. This difference in the response of FhlA165 and FhlA167 to terminal electron acceptors suggests that the amino acids between 117 and 374 play a significant role in sensing the redox properties of the environment. Alternatively, the affinity of the two proteins for the P_{hyc} may be different.

A sequence in the N-terminal region of FhlA is similar to a conserved region in ATPases of ABC transporters

As the first 117 amino acids of FhlA had been shown to be responsible for effector-mediated control of the *hyc* operon, this region of the FhlA sequence was compared with the sequences of other known *E. coli* proteins. This comparison revealed significant similarity between the N-terminal amino acid sequence of FhlA and sequences found in the energizer proteins of ABC transporters (Fig. 2). The C-terminal end of the presented ABC protein sequences corresponds to the 'Walker' B site, and the N-terminal end represents the unique ABC signature sequence (Linton & Higgins, 1998). Upstream of this

ATPase-like sequence resides a helical domain which interacts with the membrane component of the ABC transport system (Hunke *et al.*, 2000). Although the critical aspartate and proline amino acids, present in all 'Walker' B sites of the ABC proteins and the unique ABC signature sequence, were not present in the FhlA protein 18 of the 31 amino acids within this sequence (between amino acids 7 and 37) were similar or identical to a derived consensus sequence of the ABC proteins (Fig. 2). The observed sequence similarity between the FhlA and ABC-ATPases raised interesting questions about the role of this N-terminal region of the FhlA protein. Does this region of FhlA along with the central domain of the protein contribute to formate-dependent ATP hydrolysis? However, it should be noted that the N-terminal domain of FhlA by itself has no detectable ATPase activity (Leonhartsberger *et al.*, 2000).

To evaluate the significance of the N-terminal region of the FhlA protein, the DNA sequence corresponding to the amino acids 7–37 was deleted from the *fhlA* gene, and the resulting *fhlA9-2* allele was used in *hyc-lac* expression studies (Fig. 1). The *fhlA9-2* allele failed to support *hyc* expression or production of FHL activity, even when the medium was supplemented with formate at levels as high as 30 mM and/or 1 mM molybdate. These results show that this segment of the FhlA protein is critical for *in vivo* activity of the FhlA protein. It is possible that the FhlA9-2 protein is still formate-dependent for its activity and the inability to produce a FhlA9-2–formate complex *in vivo* apparently negated its activity. Removal of an additional 80 amino acids (to amino acid 117) completely reversed this defect, as FhlA167 is formate-independent (Fig. 1, Table 2).

Production of active FHL is independent of formate in the presence of FhlA165

Since transcription of the *hyc* operon was at its highest in the presence of FhlA165, the need for formate in the conversion of apo-FDH-H to active FDH-H was determined. Strain SE2007, which carries a mutation in the chromosomal *fhlA* gene, produced approximately 125 units FHL activity with plasmid pWS165. This value was slightly higher than the 110 units FHL activity produced by strain SE2007(pWS2) with wild-type *fhlA*. However in a *fhlA/pfl* double mutant, only the strain carrying *fhlA165* produced FHL activity (approximately 90 units), again confirming the formate-independence for *hyc* and *fdhF* expression in the presence of FhlA165. These results further demonstrate that formate is only required for transcriptional activation of *fdhF* and *hyc*, and not for maturation and activation of the apoproteins to activate the FHL.

FhlA165 has a higher affinity for *hyc* DNA

The higher level of *hyc-lac* expression when activated by FhlA165 (Table 2) prompted investigation into the affinity of FhlA165 protein for the target DNA. Wild-type FhlA (250 nM) shifted the electrophoretic mobility

Fig. 3. DNA electrophoretic mobility-shift experiments with purified FhlA and mutated FhlA proteins with P_{hyc} DNA. All reactions contained 50 fmol of 154 bp *hyc* upstream DNA, and (A) FhlA, (B) FhlA165, (C) FhlA9-2 and (D) FhlA167. The concentration of each of the FhlA proteins added is given in μ M below each image. Arrows indicate the DNA–protein complex.

of the P_{hyc} DNA and completely shifted the DNA at a concentration of 1 μ M FhlA (Fig. 3). In contrast, the FhlA165 protein shifted the mobility of the DNA at a concentration of 100 nM and completely shifted the DNA at 500 nM FhlA165, suggesting that FhlA165 had a higher affinity for the target DNA sequence. Based on phosphorimager analysis of the DNA electrophoretic mobility shift presented in Fig. 3, the apparent K_d for the binding of FhlA165 to DNA was calculated to be 125 nM, while the value for the native FhlA protein was 270 nM. In similar mobility shift experiments, including formate in the binding reaction had no effect on the apparent K_d of the interaction between DNA and protein (data not shown). The higher affinity of FhlA165 for the P_{hyc} DNA could explain the higher level of *hyc* operon expression seen in the presence of the FhlA165 protein (Tables 2 and 4), compared to the level of expression when activated by native FhlA. However, it could not be ruled out that FhlA165 had an increased ability to activate transcription.

FhlA167 had a very low affinity for P_{hyc} DNA (Fig. 3D), although this allele activated *hyc-lac* to the same level as the native protein, *in vivo*. The addition of formate to the binding reaction or to the electrophoresis conditions had no effect on the FhlA167– P_{hyc} DNA interaction. In contrast, the FhlA9-2 allele, which did not support *hyc* operon expression, did bind to the P_{hyc} DNA *in vitro* and shifted the electrophoretic mobility of the P_{hyc} DNA (Fig. 3C). This change in the electrophoretic mobility of the P_{hyc} DNA saturated at a protein concentration of approximately 100 nM FhlA9-2, and at this concentration only a small fraction of the total

Fig. 4. ATPase activity of native FhlA protein and mutant forms of the FhlA protein in the presence of formate and P_{hyc} DNA. ATPase activities are given in relative units [pmol ATP hydrolysed (pmol FhlA)⁻¹]. DNA (plasmid pFGH100) was added to give a final concentration of 0.1 μ M. (a) Native FhlA protein and FhlA165. ●, FhlA (final concn 1 μ M) and DNA; ○, FhlA only (final concn 1 μ M); ■, FhlA165 (final concn 2 μ M) and DNA; □, FhlA165 only (final concn 2 μ M). (b) FhlA9-2 and FhlA167. ●, FhlA9-2 (final concn 0.13 μ M) and DNA; ○, FhlA9-2 only (final concn 0.13 μ M); ■, FhlA167 (final concn 0.13 μ M) and DNA; □, FhlA167 only (final concn 0.13 μ M).

DNA was protein-bound. At a protein concentration of 150 nM, most of the DNA was found in larger DNA–protein complexes.

ATPase activity of FhlA mutant derivatives

Members of the NtrC/NifA family of proteins including FhlA have a promoter DNA-dependent ATPase activity (Weiss *et al.*, 1991; Hopper & Böck, 1995). This activity is associated with the formation of the DNA-open complex, a prelude to the initiation of transcription (Wedel & Kustu, 1995). Since the FhlA protein alleles had differing *in vivo* activities and promoter DNA-binding characteristics, the P_{hyc} -dependent ATPase activity of the various FhlA alleles was determined.

Purified native FhlA protein had a basal ATPase activity of 250 units (Fig. 4). This activity increased to 320 units in the presence of formate, and the optimum concentration of formate was between 10 and 25 mM. At 50 mM formate, the ATPase activity of FhlA protein decreased. In the presence of P_{hyc} DNA the ATPase activity of the FhlA protein increased by over 200 pmol ATP hydrolysed (pmol FhlA)⁻¹ (approximately 25 mM formate present) (Fig. 4a). This characteristic formate- and DNA-dependent increase in ATPase activity of the FhlA protein is similar to that reported by Hopper & Böck (1995).

The basal ATPase activity of the FhlA165 protein was only 60 pmol ATP hydrolysed (pmol FhlA)⁻¹ (Fig. 4), and as expected this activity was not increased by formate. In the presence of P_{hyc} DNA, the level of ATPase activity also increased by approximately 200 pmol ATP hydrolysed (pmol FhlA)⁻¹. In contrast to the native protein, which responded positively to lower concentrations of formate, the FhlA165 reacted nega-

tively to formate in the ATPase assay. The negative response to formate seen *in vitro* could also account for the formate-dependent decrease in *hyc*–*lacZ* activation in the *mod* mutant, strain WS113(pWS165) (Table 2). The similar response of ATPase activity of the native FhlA and FhlA165 proteins to DNA suggested that these proteins, once bound to the *hyc* upstream DNA, activated transcription at the same level, and the observed difference in the *hyc* operon expression could be attributed to the differences in the affinity of the proteins to the P_{hyc} DNA.

In these experiments the native FhlA protein had a higher than expected basal ATPase activity. This is in contrast to the ATPase activity of FhlA protein reported by Hopper & Böck (1995). This difference could be due to variations in the protein purification methods employed in these two studies. The absence of formate-dependent ATPase activity in FhlA165 suggested that the deletion of the unique N-terminal domain of FhlA removed a previously unknown ATPase activity, which may be specific for formate binding.

The FhlA167 protein had an ATPase activity of approximately 300 units which was not altered either by formate or by P_{hyc} DNA. This is in agreement with its lower affinity to P_{hyc} DNA (Fig. 4b). The FhlA9-2 protein also produced approximately 300 pmol ATP hydrolysed (pmol FhlA)⁻¹ ATPase activity which was not influenced by the presence of formate. However, the ATPase activity of this protein increased by more than threefold in the presence of P_{hyc} DNA. Addition of both P_{hyc} DNA and formate to the reaction further increased the ATPase activity of the FhlA9-2 protein by twofold. In the presence of 10 mM formate and P_{hyc} DNA the ATPase activity was over 1500 pmol ATP hydrolysed (pmol FhlA)⁻¹, the highest among the four FhlA alleles tested.

Increasing the formate concentration beyond 10 mM inhibited the DNA-dependent ATPase activity. The ability of FhlA9-2 to bind to P_{hyc} DNA *in vitro* and to hydrolyse ATP in a DNA-dependent manner shows that the FhlA9-2 protein is active *in vitro*. The formate-dependent increase in ATPase activity of this allele is similar to that of the native FhlA protein and is in contrast to that of FhlA165 and FhlA167. These results suggest that the FhlA9-2 protein requires formate for activity and the FhlA9-2-formate complex is not formed *in vivo* because of the deletion of the amino acids 7–37.

The inability of the FhlA9-2 protein to activate the expression of the *hyc* operon *in vivo* combined with the higher than expected activity *in vitro* raises the possibility that the *fhlA9-2* is not transcribed *in vivo*. To test this, the upstream region of the *fhlA* gene in both pWS9 and pWS9-2 was sequenced. The two sequences were identical for approximately 500 bases upstream of the translation start site. In a separate experiment, total RNA was isolated from strains carrying either pWS9-2 or its parent pWS9 grown under anaerobic conditions in L-broth containing glucose (0.3%). A cDNA corresponding to the *fhlA* gene was synthesized from both samples using Moloney Murine Leukaemia virus reverse transcriptase (Superscript II; Life Technologies). The cDNA was quantitated by real-time PCR using SYBR green (iCycler; Bio-Rad). The results of these experiments showed that the level of cDNA produced from the total RNA from strains with plasmid pWS9 or pWS9-2 was comparable, indicating that the *fhlA9-2* allele was also transcribed in *E. coli*. The inability to activate the *hyc* operon is apparently due to the deletion of amino acids in the protein.

CONCLUSION

Results from this study show that removal of the first 117 amino acids from the FhlA protein (FhlA167) leads to effector-independence for expression of the *hyc* operon. Extending the deletion to amino acid 374 (FhlA165) increases the ability of the protein to bind P_{hyc} DNA *in vitro*, enhances the level of *hyc* operon expression *in vivo*, and also overcomes the redox-dependent control of *hyc* operon expression. The amino acids between 117 and 374 could play a significant role in modulating the level of *hyc* operon transcription via the central ATP-binding domain and the C-terminal domain (amino acids 375–692 of the FhlA protein). Although the need for MoeA for expression of the *hyc* operon is overcome by either of the two deletion derivatives FhlA165 and FhlA167, ModE-Mo is still required as a secondary activator for the optimum expression of the *hyc* operon. These results further support the proposal that the MoeA-catalytic product interacts with the native FhlA protein for optimum expression of the *hyc* operon. Removing the sequence of amino acids with similarity to the sequences found in the ATPase components of ABC-type transport systems eliminates the *in vivo* activity, but not the formate and P_{hyc} DNA dependent *in vitro* ATPase activity. This suggests that *in vivo* the FhlA9-2 protein is unable to

produce a formate-bound complex. The FhlA protein could accept formate directly from the transporter, and the formate-dependent ATPase activity may signify this interaction. Direct transfer of formate to the FhlA protein would also minimize formate accumulation in the cytoplasm. The amino acids 7–37 may play a role in transferring formate from the transporter to the appropriate location in the FhlA protein. The sequence similarity with the ABC-ATPases, combined with the absence of formate-dependent ATPase activity in FhlA165 and FhlA167, suggests that the native FhlA protein contains two ATPase activities: one associated with DNA-binding and transcription activation and the other related to the interaction with formate.

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