BACTERIAL CHARACTERISATION

Cloning, sequencing, characterisation and implications for vaccine design of the novel dihydrolipoyl acetyltransferase of Neisseria meningitidis

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A λZap-II expression library of Neisseria meningitidis was screened with a rabbit polyclonal antiserum (R-70) raised against c. 70-kDa proteins purified from outer membrane vesicles by elution from preparative SDS-polyacrylamide gels. Selected clones were isolated, further purified, and their recombinant pBluescript SKI1 plasmids were excised. The cloned DNA insert was sequenced from positive clones and analysed. Four open reading frames (ORFs) were identified, three of which showed a high degree of homology with the pyruvate dehydrogenase (E1p), dihydrolipoyl acetyltransferase (E2p) and dihydrolipoyl dehydrogenase (E3) components of the pyruvate dehydrogenase complex (PDHC) of a number of prokaryotic and eukaryotic species. Sequence analysis indicated that the meningococcal E2p (Men-E2p) contains two N-terminal lipoyl domains, an E1/E3 binding domain and a catalytic domain. The domains are separated by hinge regions rich in alanine, proline and charged residues. Another lipoyl domain with sequence similarity to the Men-E2p lipoyl domain was found at the N-terminal of the E3 component. A further ORF, coding for a 16.5-kDa protein, was found between the ORFs encoding the E2p and E3 components. The identity and functional characteristics of the expressed and purified heterologous Men-E2p were confirmed as dihydrolipoyl acetyltransferase by immunological and biochemical assays. N-terminal amino-acid analysis confirmed the sequence of the DNA-derived mature protein. Purified Men-E2p reacted with monospecific antisera raised against the whole E2p molecule and against the lipoyl domain of the Azotobacter vinelandii E2p. Conversely, rabbit antiserum raised against Men-E2p reacted with protein extracts of A. vinelandii, Escherichia coli and N. gonorrhoeae and with the lipoyl and catalytic domains of E2p obtained by limited proteolysis. In contrast, the original R-70 antiserum reacted almost exclusively with the lipoyl domain, indicating the strong immunogenicity of this domain. Antibodies to Men-E2p were detected in patients and animals (rabbits and mice) infected with homologous or heterologous meningococci or other neisserial species. These results have important implications for the understanding of PDHC and the design of future outer membrane vesicle-based vaccines.

Introduction (outer-membrane proteins, OMPs). Many of these proteins are expressed constitutively, but others are environmentally regulated. Recently, there has been a major focus on OMPs for vaccine production, particularly against group B meningococci, and a number of relatively crude OMP mixtures were tested in clinical trials with various degrees of reported efficacy [1]. Following large scale placebo-controlled, randomised double-blind trials, only the vaccines produced in

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Norway and Cuba showed significant protective efficacy. The Norwegian vaccine consisted of OMPs from the Norwegian epidemic strain of Neisseria meningitidis, B:15:P1.7.16 and was given to children aged 14–16 years. It produced a point estimate of protective efficacy of 57% after a 30-month follow-up and was, therefore, considered insufficiently effective for general use [2]. The Cuban vaccine consisted of group C capsular polysaccharide mixed with OMPs from a Cuban epidemic strain, B:4:P1.15. When given to children aged 10–16 years, it offered an estimated point efficacy of 83% after 16 months of follow-up [3] and, as a result, the vaccine is now incorporated into the routine childhood vaccination programme in Cuba. Although the Cuban trial did not directly address efficacy in children <10 years old, follow-up studies of the mass vaccination have suggested that the overall protective efficacy based on vaccine coverage and incidence of disease in children under 6 years old is c. 93% [3]. However, when the Cuban vaccine was tested in a case-control study in Brazil, protective efficacy was reported to vary with age. The vaccine was effective in children aged 4 years and older, but not in younger children [4].

Although these OMP vaccine preparations are enriched for major class antigens such as serotype (class 2/3) and serosubtype (class 1) OMPs, they also contain significant amounts of other uncharacterised proteins. This makes the search for component(s) relevant to protection in man, and standardisation of preparations for future use, extremely complicated. Moreover, most of the OMP vaccines used consist of outer membrane vesicle (OMV) preparations [2–4]. Such OMVs are likely to include transmembrane, inner membrane or even cytoplasmic components. It is not known which of these components contribute most to antibody-mediated or cell-mediated immune responses and long-term protection from disease. It is possible that these OMVs contain a mixture of antigens, with varying regulating effects on the immune system. Furthermore, there are numerous cell components which are structurally, functionally or immunologically conserved across prokaryotic and eukaryotic cells and which may have been included, unwittingly, in the OMV vaccine preparations. Inclusion of such components in vaccines will carry the risk of breaking immune tolerance with concomitant risk of inducing autoimmune disease. Therefore, more information is desirable on the individual meningococcal OMV antigens in terms of structure and function to facilitate development of an ideal meningococcal vaccine.

OMVs consisting of a wide range of outer-membrane and other proteins were prepared in an earlier study. Purified proteins of c. 70-kDa were prepared from these OMVs by elution from preparative SDS-polyacrylamide gels and the antigens were used to raise rabbit polyclonal antiserum (R-70) [5]. Immuno blot experiments showed that this antiserum contained antibodies against several co-migrating meningococcal proteins, including the iron-regulated FrpB and non iron-regulated proteins [5,6]. The exact identity of these different co-migrating proteins has been unclear.

We, and others, have previously characterised FrpB and demonstrated that it is a partially surface-exposed OMP [5–7]. More recently, Pettersson et al. [8] cloned and sequenced FrpB and showed that it is related to the family of TonB-dependent proteins. However, its functional attributes remain largely unclear. With regard to the non-iron-regulated 70-kDa protein(s), very little is known. Aoun et al. [9] showed that murine sera raised against live meningococci were bactericidal to gonococci and reacted preferentially (in immunoblots) with a highly conserved and constitutively expressed 70-kDa protein. It is not clear whether this immunoreactive 70-kDa protein and the one(s) previously purified in our laboratories [6,10] are the same.

Therefore, in this study the R-70 antiserum was used to screen a DNA expression library in an attempt to clone and further identify those OMV components that were recognised by this polyclonal rabbit antiserum. The identification, sequence analysis and functional determination of one of these reactive components, the dihydriopoly acetyltransferase component of the pyruvate dehydrogenase complex (PDHC) is reported here. The detection of a gene cluster which consists of all the necessary components of the PDHC as well as a unique reading frame which has not been detected in pathogenic organisms is also reported.

Materials and methods

Bacterial strains, vectors and total protein extraction

N. meningitidis strains SD (B:15:P1.16), AS (A:4:P1.7) and EB (29E) were grown in Mueller Hinton broth (MHB) overnight at 37°C in air with CO2 5%. Iron-restriction, where required, was achieved by the addition of 25 μM desferrioxamine (Ciba-Giegy, Horsham). Escherichia coli strain XL1 Blue was obtained from Stratagene (Cambridge). E. coli strain TG2, a recA− version of TG1, i.e., thi−1, supE44, [Res− Mod− (k)], F (traD36 pro A+B+), laqIqZαAM 1, was used for cloning and expression of plasmid-encoded proteins [11]. Total protein extracts from N. meningitidis strain SD and E. coli XL1-Blue carrying plasmid, before or after isopropyl β-D thiogalactopyranoside (IPTG)-induction, were prepared as described previously [12].

Sera, SDS-PAGE and immunoblots

The rabbit antiserum against purified c. 70-kDa OMV proteins of meningococci (R-70) was raised and
characterised previously [10]. Before use, the R-70 antiserum was adsorbed once with immobilised E. coli lysate (Pierce, Pierce and Warriner, Chester) as described by the manufacturers. Rabbit antisera to whole cells (live organisms) of meningococcal strains SD, AS, EB and the commensal N. lactamica strain were raised in New Zealand White rabbits as described previously [5]. Rabbit antiserum to meningococcal E2p (Men-E2p, see below) was raised by inoculating a New Zealand White rabbit, by subcutaneous injection, with five doses of 100 µg of purified protein (eluted from SDS-polyacrylamide gels) suspended in 500 µl of phosphate-buffered saline (pH 7.2; PBS, Oxoid) and mixed with equal volumes of Freund's complete (first injection) and incomplete (the subsequent fortnightly booster injections) adjuvants. Pre-immune normal rabbit serum was also obtained. Murine polyclonal antisera to live cells of meningococcal strains SD and B16B6 (B:2a:P1.2) and murine monoclonal anti-FrpB (Mab-M70) were raised and characterised previously [6,13]. Pre-immune normal mouse serum was also obtained. Human convalescent sera were taken from patients 4 weeks after recovery from meningococcal disease (meningitis or septicaemia, or both) caused by different meningococcal strains, including strain SD, JB (B:NT) and GLD (C:2a:P1.10). A human convalescent serum was also obtained from a patient who had recovered from gonococcal pelvic inflammatory disease. Normal human serum was obtained from an individual with no prior history of infection with N. meningitidis or N. gonorrhoeae.

SDS-PAGE and immunoblots of the rabbit, murine and human antisera against total protein extracts and purified Men-E2p (below) were performed as described previously [14].

**Gene library screening, cloning and sequencing**

The R-70 antiserum was used to screen a previously constructed gene library of N. meningitidis (strain SD) DNA in λZap II [12]. Approximately 50 000 plaques, seeded on E. coli strain XL1 Blue, were lifted in duplicates onto nitrocellulose membranes (Schleicher and Schuell, supplied by Anderman, Kingston-upon-Thames) pre-impregnated with 0.5 mM IPTG. As the rabbit R-70 antiserum was raised against denatured meningococcal OMV proteins, one copy of the plaque-life membrane was denatured by heating at 56°C for 30 min in protein-denaturation buffer (containing SDS 1% and 2-mercaptoethanol 0.2%) and the other copy was left untreated. Both membranes were blocked in blocking buffer (bovine serum albumin 3%, Tween 20 0.1% in PBS) for 1 h, and then incubated with the R-70 antiserum (diluted 1 in 1000) for 3 h at room temperature followed by peroxidase conjugated goat anti-rabbit serum as previously described [5,10]. Positive plaques were isolated and purified and recombinant pBluescript SKII plasmids were excised with the ExAssist helper phage (Stratagene). Two representative recombinant plasmids (pSK18a and pSK34a) were chosen and both strands of their cloned DNA were sequenced with an Applied Biosystems 373A DNA Sequencer. Oligonucleotide primers were synthesised by an Applied Biosystems 381A DNA Synthesiser and used for the sequencing. These primers were: M13 Forward, TTG TAA AAC GAC GGC CAG 373A DNA Sequencer. Oligonucleotide primers were synthesised by an Applied Biosystems 381A DNA Synthesiser and used for the sequencing. These primers were: M13 Forward, TTG TAA AAC GAC GGC CAG

**Enzyme assays and Men-E2p characterisation**

Plasmid pSK34a was transformed into competent TG2 E. coli cells, and ampicillin-resistant colonies were isolated, grown, harvested, sonicated and examined for the presence of dihydrolipoamide transacetylase activity by previous described methods [15]. At maximum expression level, the endogenous transacetylase activity was 1–2% of total activity measured in cell extracts obtained from empty vector transformed cells [15]. This was assayed spectrophotometrically at 240 nm and 25°C in a coupled assay with phosphotransacetylase (Sigma), measuring the formation of S-acetyldihydrolipoamide as described by Schwartz and Reed [16]. Dihydrolipoamide was prepared from dl-lipoamide as described by Reed et al. [17]. Protein concentrations were estimated with the microbiuret method [18] after precipitation with deoxycholic acid and trichloroacetic acid [19]. Bovine serum albumin was used as the standard.

**Isolation of expressed Men-E2p from E. coli**

A single colony of E. coli TG2 (pSK34a) was picked from a yeast-trypette (YT) agar plate containing

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**Fig. 1.** Immunoblots of rabbit antiserum against meningococcal protein extracts. a, R-70 antiserum (absorbed with E. coli lysate) against total protein extracts of E. coli XL1 Blue; b, as (a) but after transformation with plasmid pSK34a; c, anti-Men-E2p antiserum against OMV extracts of iron-sufficient N. meningitidis strain SD; d, purified Men-E2p probed with antiserum against whole A. vinelandii E2p; e, purified Men-E2p probed with antiserum against the lipoyl domain of A. vinelandii E2p. *c. 70 kDa.
Fig. 2. The sequence data for cloned meningococcal DNA of pSK34a and the deduced amino-acid sequence of the ORFs. A putative ribosome-binding site for ORF2 is underlined. Stop = translational stop codon.
Q S Q E L T E L S K K A R E G K
1556 CA A ATG AGC CA A GAA TGG ACC GAA TGG TCC AAA AAA GCC CTT GAA GGC AAC
L K P Q E M Q G A C F T I S S L G
1607 CTC AAA CCG CA A GAT CAA GGC GCG TGG TTT ACC ATG TCC AGC TTA GGC
G I G C T G F T P I V N A P E V A
1658 GCC ATC GCC GGC AGA AGC TTC AGC CCA ATT GTG AAC GCT CCC GAA GTC GCC
I L G V C K S Q I K P V W N G K E
1709 ATC TGG GCC TGT AAC TCC CAA ATC AAA CCT GTT TGG AAC GGC AAA GAG
F A P R L M C P L S L S F D H R V
1760 GCT GCC CCG CCG CTG ATG TCC AGC CTG TCC TGC GAC CAG CTG GTC
I G A R A V I N Q T V F L A K L L
1811 ATC GAC GGT CGC CGG GTT ATG CGC TCC ACC GTA TCC TTG GCG AAG CTG TTG
K D F R R I T L Stop
1861 AAA GAC TCC CGC CGG ATT ACC TTA TAA AAT AAA ACA TCC TCT TCA AGC AGT

>--- ORF3 --->
L M G N F L Y R G I S C Q D E Q I
1980 CTT ATG GAA AAT TTC TTA TAT AGA GGC ATT AGT TGC CAA CAA GAT GAG CAA
N N Q L K P K G N K A E V A I R
2031 AAT AAT GGA CAG TTA AAA CCT AAA GGT AAT AAA GCT GAA GCT GAA CTA GCT
Y D G K F K Y D G K A T H G P S V
2082 TAT GAT GGT AAG TTT AAA TAT GAT GGT AAA GCT ACA CAT GCT CAG AGT
K N A R A V I N Q I E T G L Y D G C
2133 AAG AAT GCA GTT TAC GCC CAT CAA ATT GAA ACA ACA GTC GTC CTA TAT GAC GGA
I S T T D T T I E K A A K F A T S
2184 TAT ATA TCT ACG ACA ACA GAC AAG GAA ATT GCC AAG AAA TTT GTA CCA AGT
S G I E N G Y I V L N R D L F G
2235 TCC GCC ATC GAA AAT GCC TAT ATA TAT GTC TTA AAT AGG GAT TTT GGT
Q Y S I F E Y E V E H P E N P N E
2286 CAA TAT TCT ATT TTT GAA TAT GAG GTC GAA CAT CCA AAC CCA AAT GAG
K E V T I R A E D C G C I P E E V
2337 AAG GAA GTA ACA ATC AGA GCT GAA GTT GGT ACC GTT GTC AAT CCT GAA GAT
I I A K E L I E I N Stop
2388 ATT ATT GCT AAA GAG TTG ATA GAA ATT AAC TAA GTC GA

>--- ORF4 --->
K V N I M V E L V K P D I G G
2426 AAG GTC AAT ATA ATG GCT TTA GTT GAA TGG AAA GTG CCC GAC ATT GCC GAA
H E N V D I I A V E V N V G D T I
2477 CAC GAA AAT GTA GAT ATT ATC CGG GTT GAA GTA AAG GTG GCC GAC ACT ATT
A V D T L L I T L E T D K A T M D
2528 GCT GTG GAC GAT ACC CGT ATT ACT TTT GAA ACC GAT AAA GCG ACT ATG GAC
V P A E V A G V V K E V K V K V G
2579 GTA CCT GCT GAA GGT GTA GCA GCC GTC GTA AAA GAA GTC AAA GGG GTC GGC
D K I S E G G L I V V V A E G T
2630 GAC AAA ATC TCT GAA GGT GTT TTG ATT GTC GTT GTT GAA GCT GAA GCC AGC
A A P K A E A E A A A A A P A Q E A P
2681 GCA GCC GCT CCT AAA GGC GAA GCG GCT GCC GCC CGG CAG GAA GCC CCT
K A A P A P Q A A Q F G G S A D
2732 AAA GCT GCC GCT CCT GCT CGG CAA GCC GCC GAA TCC GCC GGT TCT GCC GAT
A E Y D V V V L G G P G G Y S A
2783 GCC GAC TAC GAC GTG GTT GCA GCC GCC GTT GCC GCC GCC GCT TAC TCC GCT
A F A A A D E G L K V A I V E Y
2834 QTA TTT GCC GCT GAT GAA GGC TTC AAA GTC GCC ATC GTC GAA GTC TAC
K T L G V C L N V G C I P S K A
2885 AAA ACT GTG GCC GCC GTT TGC GTC AAC GTC GGC GTT ATT ATC CCT TCC AAA GCC
L L H N A A V I D E V R H L A A N
2936 TTG TTG CAC AAT GCC GCC GTT ATC GAC GAA GTC GCG CAC TAC GTT GCC GAC
G I K Y P E P E L D I D M L R A Y
2987 GGT GTC AAA TAC CCC GAG CGG GAA CTC GAC ATC GAT ATG GCC GCC TAC
K D G V V S R L T G G L A G M A K
3038 AAA GAC GCA GGT GTA CTT GCC TCC TCC ACG GGC GTT TGG GTA GATG ATC GGG
S R K V D V I Q G D G Q F L
3093 AAG CGT AAA GTG GAC GTT ATC CAA GCC GAC GGG CAA TTC TTA GA

Fig. 2. (continued).
ampicillin 50 mg/L and grown overnight at 37°C in 10 ml of YT broth containing ampicillin 75 mg/L. A 100-μl sample of this culture was used to inoculate 10 ml of the same medium which was then incubated at 37°C for 8 h. Each of six 2-L flasks with 500 ml of YT containing ampicillin 75 mg/L and IPTG 20 mg/L were inoculated with 0.5 ml of this starter culture and incubated for 16 h at 37°C in a controlled environment shaker at 300 rpm. Cells (5 g) were harvested and used for isolation of Men-E2p. The cells were suspended in 27 ml of 50 mM potassium phosphate buffer containing 2 mM EDTA, 0.1 mM phenylmethane sulphonyl fluoride (PMSF) (Merck) and 1 mM benzamidine, pH 7.0, and disrupted with a French press applying two cycles at 18,000 psi. After centrifugation for 1 h at 14,000 g, the supernate was adjusted to a protamine sulphate final concentration of 0.27% w/v, in which Men-E2p remained soluble. The turbid solution was centrifuged for 45 min at 14,000 g and the clear supernate was concentrated to 5 ml by ultrafiltration (Amicon YM100, Grace Amicon, Essebaan, The Netherlands). The concentrate was then applied to a Sephacryl S-400HR column (2.4 × 100 cm) (Pharmacia), equilibrated with 50 mM potassium phosphate buffer containing 0.5 mM EDTA, 0.1 mM PMSF and 1 mM benzamidine, pH 7.0. Enzymically active fractions were analysed on SDS-PAGE and those with pure Men-E2p contents were pooled. After concentration to 3 ml by ultrafiltration (Amicon YM100) the protein was stored in liquid nitrogen.

For immunisation, 1 mg of purified Men-E2p was electrophoresed on a preparative SDS-polyacrylamide gel. The gel was then stained with Coomassie Brilliant Blue R250, and the protein band of interest was excised and recovered from the gel slice by electroelution in 2.5 mM Tris-HCl, 20 mM glycine, 0.35 mM SDS.

**Limited proteolysis and N-terminal sequence analysis**

Limited proteolysis was performed on Men-E2p in 50 ml of potassium phosphate buffer containing 0.5 mM EDTA, pH 7, with trypsin (Sigma) 0.1% w/w at 4°C for 60 min. The reaction was stopped by the addition of 1 mM PMSF and samples were subjected to SDS-PAGE.

Purified Men-E2p (400 μg) was blotted from SDS-polyacrylamide gels on to Immobilon (Millipore, Watford) and used for N-terminal amino-acid analysis, with an Applied Biosystems 475 Protein Sequencer.

**Results**

**Cloning and sequence analysis**

Screening of c. 50,000 plaques for specific protein expression identified 100 positive plaques. Most plaques were recognised by the R-70 antiserum under both native and denatured conditions. Twenty-five of the most strongly positive plaques were rescued and analysed by immunoblotting for protein expression before and after IPTG-induction. Of the examined plasmids, pSK34a (3.1-kb insert) showed the highest level of expression of cloned protein of c. 70 kDa (Fig. 1, lane b). This expression was not affected by IPTG stimulation. Plasmid pSK18a (1.5-kb insert) also showed strong expression of a protein reactive with R-70, but with lower molecular mass (c. 65 kDa, not shown).

Both plasmids were restriction-mapped and both of their strands were fully sequenced (Fig. 2), EMBL accession number X82637. The main features of the analysed DNA sequence are shown in Fig. 3. Four open reading frames (ORFs) were predicted from the sequence analysis. The deduced amino-acid sequence of ORF1, ORF2 and ORF4 showed a high degree of homology with the amino-acid sequences of *E. coli* pyruvate dehydrogenase (E1p), dihydrolipoyl acyltransferase (E2p) and dihydrolipoyl dehydrogenase (E3), respectively. These are components of the pyruvate dehydrogenase complex (PDHC). The deduced sequence of ORF2 showed amino-acid identity with the E2 component of PDHC, and the 2-oxoglutarate dehydrogenase complexes of a number of prokaryotic and eukaryotic cells, including *E. coli*, *Azotobacter vinelandii*, *Alcaligenes eutrophus* [20–22], yeasts, rats and man. Fig. 4 shows sequence alignment of meningococcal E2p (Men-E2p) with the E2p of PDHC of the other three gram-negative species.

ORF1 and ORF4 were incomplete genes, being part of larger coding regions. Indeed, subsequent sequence data (not published) confirmed that ORF1 is part of an 887 amino-acid long reading frame (included within X82637). The deduced sequence of ORF4 was almost identical (>99% homology) to the N-terminal sequence of the previously sequenced meningococcal OMP64 (Accession No. X77920) [23]. However, ORF3 failed to show significant homology with any described DNA or protein sequence. The most closely related protein was yeast mitochondrial precursor proteins import receptor and heat shock protein homologue (with c. 90 amino acid overlap, 22% homology). The DNA sequence of ORF3 showed homology with a number of unrelated genes, such as a region of the replication origin of *Bacillus subtilis* and eukaryotic ATP synthase gamma-subunit. An ORF of this size and position (between E2p and E3 components of PDHC) is thus far only found in the gene cluster encoding PDHC of *Al. eutrophus* [22]. Unlike the latter, the base composition of ORF3 deviates considerably from that of the other ORFs.

Finally, the deduced sequence of none of the ORFs within pSK34a showed significant homology with the
recently published sequences of meningococcal or gonococcal FrpB protein [8, 24].

Functional characterisation of meningococcal E2p (Men-E2p)

Plasmid pSK34a, which contained the complete ORF2, was chosen for expression of the ORF2 product (Men-E2p) in E. coli TG2. Men-E2p was expressed and purified and its properties were examined.

The E2p activity of the Men-E2p-expressing E. coli TG2 was low in the exponential growth phase (at 8 h growth); however, it increased steadily towards the end-log and stationary phase and was optimal after 16 h. Therefore, cells were harvested at this stage, when the E2p activity was 4 U/mg (Table 1). This is similar to that of A. vinelandii E2p expressed in the same E. coli strain (4.4 U/mg) [25], except that the cell yield was only 50% for Men-E2p.

When examined by SDS-PAGE, the Men-E2p-expressing E. coli TG2 showed a strongly stained protein band at c. 70 kDa (Fig. 5), as expected for an E2p possessing two lipoyl domains. The corresponding

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Protein content (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (U)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>27</td>
<td>483</td>
<td>4.0</td>
<td>1932</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulphate</td>
<td>29</td>
<td>268</td>
<td>6.8</td>
<td>1816</td>
<td>94</td>
</tr>
<tr>
<td>S400HR eluate</td>
<td>3</td>
<td>21</td>
<td>58.2</td>
<td>1243</td>
<td>64</td>
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Fig. 4. Structure-based sequence alignment of E2p from *A. vinelandii* (A.vin), *E. coli* (E.col), *Al. eutrophus* (A.eut) and *N. meningitidis* (N.men). The structural comparison of the first lipoyl domain (A) is based on the NMR data obtained for the *A. vinelandii* N-terminal lipoyl domain [30]. For the E1/E3 binding domain (B), the NMR data of the E3 binding domain from *E. coli* E20 [29] have been used. The structural comparison of the catalytic domain (C) is based on the co-ordinates of the catalytic domain from *A. vinelandii* E2p [28]. Secondary structure is indicated by the letters h (helix) and e (sheet) for each of these domains. †The catalytically important residues. *The residues involved in intertrimer interaction of the catalytic domain.
host proteins, E2o containing a single lipoil domain and E2p containing three lipoil domains, show bands in SDS gels at c. 56 kDa and c. 83 kDa, respectively [26].

The steps used in the purification of biologically active Men-E2p were similar to those used for the isolation of E2p from *A. vinelandii* [25]. The isolated Men-E2p did not contain *E. coli* E3, indicating that the E3 component of *E. coli* PDHC did not interact with Men-E2p. This made the purification procedure for Men-E2p much simpler than that for *A. vinelandii* E2p. The purified Men-E2p had a specific E2p activity of 58 U/mg (Table I), which is similar to that of
Fig. 5. Purification of meningococcal E2p expressed in E. coli TG2 (pSK34a). Samples from different stages of the isolation procedure were subjected to SDS-PAGE. Lane M, low mol.wt markers (Sigma) (kDa); 1, cell-free extract; 2, supernate after protamine sulphate precipitation; 3, concentrated supernate after protamine sulphate precipitation; 4, pooled from Sephacryl S-400HR.

The predicted amino-acid sequence of Men-E2p shows the presence of several lysine residues in all linker regions and, therefore, it was predicted that limited proteolysis with trypsin would yield a single lipoyl domain (c. 16 kDa), a catalytic domain (c. 30 kDa), a lipoyl-lipoyl di-domain (c. 36 kDa) and a lipoyl-E1/E3 binding-catalytic tri-domain (c. 54 kDa). SDS-PAGE analysis (Fig. 6) showed that all the predicted fragments were present (lane 2), with the lipoyl-E1/E3 binding-catalytic tri-domain, the lipoyl-lipoyl di-domain and the catalytic domain being the main fragments, and the single lipoyl domain being a minor product. These fragments are also present in the undigested E2p (lane 1) albeit in minor amounts. They probably originate from proteolytic activity in the TG2 cells. Rabbit anti-Men-E2p serum (Fig. 6, lanes 5 and 6) reacted strongly with all the fragments (i.e., all the domains), whereas R-70 antiserum (lanes 3 and 4) showed a stronger reaction against fragments which contained the lipoyl domain and a much weaker reaction against the catalytic domain.

Purified Men-E2p also reacted with antisera previously raised against the native dihydrolipoyl acetyltransfer-
ase of *A. vinelandii* (Fig. 1, lane d) and that raised to its lipoyl domain (Fig. 1, lane e). This further indicated that E2p is highly conserved across species in terms of immunoreactivity. Also, rabbit anti-Men-E2p antiserum cross-reacted with total proteins of *A. vinelandii*, *E. coli* and *N. gonorrhoeae* (not shown). Men-E2p failed to react with a murine monoclonal anti-FrpB antibody, MAb-M70 (not shown), indicating that this protein is different from the c. 70-kDa iron-regulated FrpB. This was further confirmed when rabbit anti-Men-E2p serum reacted equally well with the OMVs obtained from meningococci (strain SD) grown under iron-sufficient (Fig. 1, lane c) and iron-restricted conditions (not shown).

It was of interest to determine whether or not Men-E2p is immunogenic following meningococcal infection. Rabbit antisera, raised against live meningococcal strains SD (B:15:P1.16) (the strain used for Men-E2p cloning), AS (A:4:P1.7) and EB (29E), and the commensal strain, *N. lactamica*, reacted strongly with purified Men-E2p in immunoblots (Fig. 7, lanes a–d). Murine antisera raised against live meningococcal strains SD and B16B6 (B:2a:P1.2) also reacted with Men-E2p (Fig. 7, lanes e and f). No reaction was detected when rabbit or murine pre-immune sera were used (not shown).

Antibodies to purified Men-E2p were also detected in human convalescent sera obtained from three patients who had recovered from meningococcal diseases caused by three different strains, including strain SD, JB (C:NT) and GLD (C:2a:P1.10), and one patient who recovered from gonococcal pelvic inflammatory disease (Fig. 6, lanes g–j). It is important to note that normal human serum, obtained from a healthy individual with no history of meningococcal or gonococcal infection, also showed a very weak reaction with Men-E2p (not shown because the band was lost on photoreproduction).

**Discussion**

The pyruvate dehydrogenase complex of many gram-negative bacteria contains three enzymic components, pyruvate dehydrogenase (E1p), dihydrolipoyl acetyltransferase (E2p) and dihydrolipoyl dehydrogenase (E3). These are encoded by three genes (*aceE*, *aceF* and *lpd*) [27]. Thus far, three dihydrolipoyl acetyltransferases from gram-negative organisms have been sequenced and characterised [20–22]. The E2p from *N. meningitidis* (Men-E2p) characterised in this study presents the fourth example. Men-E2p has a domain structure that compares very well with that of other E2ps. From the sequence analysis it was clear that it contains two N-terminal lipoyl domains, an E1/E3 binding domain and a catalytic domain. The domains are separated by hinge regions rich in alanine, proline and charged residues. Men-E2p shares a high degree of homology with the E2p of the other three bacteria, with 50–55% sequence identity in each of the domains. Unlike *E. coli* and *A. vinelandii* enzymes, Men-E2p lacks a third lipoyl domain. Instead, and similar to the PDHC of *Al. eutrophus* [22], meningococci seem to express an additional lipoyl domain on the N-terminus of the E3 component. The function of this lipoyl domain in the overall catalysis by the complex remains an interesting aspect for future research. Another interesting feature is the presence of an additional reading frame, ORF3, between the coding regions of E2p and E3. Expression of a protein that corresponds to ORF3 (16.5 kDa) has not been observed. A similar ORF has been identified only in *Al. eutrophus*. However, unlike the latter, the codon usage of the meningococcal ORF3 is very different from the other
coding regions so it is doubtful that it codes for an additional protein.

The sequence of four highly similar E2p proteins from gram-negative organisms, together with the availability of structures of each of the domains [28–30], allows a detailed structure-based comparison (Fig. 4). The sequence around the lipoyl-lysine residues, Lys$^{39}$ and Lys$^{156}$ (A. vinelandii numbering throughout), is strongly conserved. These residues are located in a type I turn connecting beta strands Ad and Ae. The extent of lipoylation has not been analysed. Strong conservation is also observed in the E1/E3 binding domain, especially around helix BH1. This helix is thought to be involved in E3 binding [31]. Despite strong sequence conservation, the affinity of E. coli E3 for Men-E2p must be weak as this enzyme was not associated with Men-E2p during purification. In contrast, it was found that E3 from A. vinelandii bound strongly to Men-E2p (not shown) and weakly to E. coli E2p [32]. The reason for these differences in affinity is not obvious from a sequence comparison of the binding domain alone. In the catalytic domain, the important catalytic site residues Ser$^{458}$ and His$^{610}$ are, as predicted, conserved. A. vinelandii E2p is the only known E2p that has an Asn$^{614}$, this being an Asp in the other sequences. On the basis of mutagenesis experiments, it has been suggested recently that charge neutralisation of Asp$^{614}$ is required for an optimal catalytic function of the nearby His$^{610}$ [33]. Modelling experiments indicated that in E. coli E2p charge neutralisation might result from salt bridge formation between Arg$^{483}$ and Asp$^{614}$. In the sequences from Al. eutrophus and N. meningitidis, Arg$^{483}$ is replaced by Asp and Glu, respectively, which indicates that the formation of a salt bridge, as in E. coli, is unlikely.

Another interesting observation from the sequence comparison concerns the stability of the E2p core. In A. vinelandii, this core dissociates into trimers upon binding of the peripheral components, while the other E2p cores remain cubic. The intertrimer interaction is of hydrophobic character and the interface is formed by the C-terminal helix CH6, the N-terminal part of helix CH4 and one face of helix CH2 (residues indicated by asterisk in Fig. 4). In the Men-E2p sequence Ala$^{634}$ of helix CH6 is replaced by Arg and Val$^{436}$ by Glu. Modelling experiments show that these residues can form a salt bridge, thus causing additional stabilisation of the cubic core. Mutagenesis experiments are underway to verify this hypothesis.

The predicted sequence of the lipoyl domain from N. meningitidis E3 (deduced from the ORF4 amino-acid sequence) is almost identical to that of the N-terminal lipoyl domain of Men-E2p. We have recently shown that R-70 and rabbit anti Men-E2p antisera cross-react with meningococcal E3 (unpublished observations). E3 seems most likely to be the same protein as the meningococcal OMP64 characterised by Silva et al. [23], which migrates as a c. 70-kDa protein in SDS-polyacrylamide gels. Therefore, it is likely that this protein co-migrates, and hence co-purifies, with FrpB and Men-E2p. The observation that R-70 antibodies react more strongly with the lipoyl domain than with other epitopes from Men-E2p, adds to the possibility that the R-70 antibodies might react primarily with the E3 component and Men-E2p through cross-reactivity of the N-terminal lipoyl domain.

Silva et al. [23] suggested that OMP64 is an immunogenic OMP, likely to be surface exposed and, therefore, suitable for vaccine preparation. It is difficult to see a physiological role for E3 or the complex in the outer membrane. E3 might be expressed in excess to the amount required for binding to the multi-enzyme complex, for example, under regulation by ORF3. Free or complex-bound dihydrolipoyl dehydrogenase has been described as associated with the plasma membrane in some organisms [34–36], but unless an outer membrane localisation is verified, for example, by immunogold labelling of whole cells or cell sections, a contamination of the OMV preparation by other cell components cannot be excluded. The expression of meningococcal E3 in E. coli and its purification is being undertaken for further analysis of this question.

E2p is highly conserved across prokaryotic and eukaryotic species and it is interesting to note that Men-E2p is immunogenic in animals when used as purified protein or as a component of live organisms. More interestingly, patients who recovered from natural meningococcal or gonococcal disease also responded to Men-E2p, with cross-reactive antibodies. It is not known whether these antibodies are against conserved parts of Men-E2p which might be shared with man. If so, this will pose the risk of breaking immune tolerance and causing autoimmune disease. Indeed, patients with primary biliary cirrhosis produce antibodies directed against the lipoyl-lysine region of the lipoyl domains of all mammalian acyltransferases [37].

Silva et al. [23] found that antisera from vaccinees immunised with a Cuban vaccine (Va-mengoc-BC) [3] contained antibodies to OMP64 (i.e., probably E3). This indirect evidence indicates that the vaccine contains E3 protein. The presence of Men-E2p in this vaccine is also possible, considering that, at least during OMV preparation under non-denaturing conditions, E2p and E3 molecules would be expected to remain interactive and, therefore, co-purify. Similarly, the possible presence of Men-E2p or E3 in the Norwegian OMV-based vaccine preparation cannot be excluded, as it is known that this vaccine, like the Cuban preparation, contained the iron-regulated FrpB [38, 39]. It is not known whether patients with a history of neisserial infection, or vaccinees, are more likely to develop autoimmune disease than the normal
population. However, normal individuals are exposed to common respiratory neisseriae. This may explain either low quantity or affinity. Clearly, much better understanding of the immunochemistry of, and the human immune response to, meningococcal OMV components is required before a safe and effective vaccine can be developed.

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