Fumarate reductase of *Helicobacter pylori*—an immunogenic protein

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Summary. An immunogenic protein with an apparent mol. wt of 80 kDa that was recognised by 55 % of sera from patients infected with *Helicobacter pylori* in Western blots was found in butanol extracts of *H. pylori* membranes. The N-terminal amino-acid sequence of the 80-kDa protein showed 80 % identity with the N-terminal sequence of subunit A of the fumarate reductase of *Wolinella succinogenes*, suggesting the existence of a fumarate reductase in *H. pylori*. The membrane fraction of *H. pylori* catalysed succinate oxidation with methylene blue at a specific enzyme activity of 0.06 U/mg of protein. The enzyme was purified by Triton X100 extraction followed by ion-exchange chromatography. The purified enzyme contained an 80-kDa protein which was recognised by rabbit serum raised against subunit A of fumarate reductase of *W. succinogenes*. A second protein band with a mol. wt of 31 kDa was recognised by rabbit serum raised against subunit B of fumarate reductase of *W. succinogenes*. Two-dimensional gel electrophoresis demonstrated that the 80- and 31-kDa proteins were subunits of one protein complex. These results indicate that *H. pylori* contains an enzyme that is very similar to *W. succinogenes* fumarate reductase. The 80-kDa subunit was recognised in sonicates of all 32 *H. pylori* strains tested by rabbit antibodies raised against subunit A of fumarate reductase of *W. succinogenes*, indicating that fumarate reductase is a common protein in *H. pylori*. The fumarate reductase of *H. pylori* might enable the bacterium to perform anaerobic respiration in a similar fashion to other anaerobic or facultative bacteria.

Introduction

Fumarate reductase is the key enzyme in fumarate respiration of anaerobic and facultatively anaerobic bacteria such as *Wolinella succinogenes* or *Escherichia coli*.^{1,2} In these bacteria, the enzyme is membrane bound and exhibits both fumarate reduction and succinate oxidation activities.^{3,4} The free energy yield of fumarate reduction by donors such as H₂ and NADH is sufficient for the synthesis of one molecule of ATP from ADP and inorganic phosphate.^{1,2} The fumarate reductase is composed of several subunits. In the case of *W. succinogenes*, the enzyme consists of three subunits with apparent mol. wts of 79, 31 and 25 kDa.³

The micro-aerophilic gram-negative bacterium *Helicobacter pylori* is a pathogenic organism that can inhabit the human stomach and cause type B gastritis. It contributes to the development of peptic ulcer disease^{5–7} and is probably a significant risk factor for the development of gastric carcinoma.^{8,9} Antibodies can be detected against various *H. pylori* proteins in the sera of patients infected with *H. pylori*.^{10,11} Unfortunately, the identity of most of these imm-

unogenic proteins is not well established. Immunogenic proteins that have been characterised include the 62–66- and 26.5–30-kDa subunits of the urease,^{12,13} the 53-kDa flagellin,¹⁴ the 87-kDa cytotoxin,¹⁵ the heat shock protein homologues with mol. wts of 54 and 60 kDa^{16,17} and a 120–128-kDa protein of unknown function.^{17,18}

In studies of membrane-bound and membrane-associated immunogenic proteins of *H. pylori*, an enriched immunogenic protein was found in a butanol extract of the membrane of *H. pylori* that was identified as a subunit of fumarate reductase. Enzyme activity was measured and the enzyme was partially purified; the distribution of this enzyme among various *H. pylori* strains was also investigated.

Materials and methods

Bacteria and antisera

H. pylori strains 898-1, 004, 005, 007, 008, 010, 012, 023 and 032 were clinical isolates. Sera from patients infected with *H. pylori* were obtained from Dr R.-J. Adamek (St Josef-Hospital, Bochum, Germany). Antisera against fumarate reductase subunits of *W.*

succinogenes were produced by immunisation of rabbits with subunit A and subunit B as described by Lauterbach *et al.*¹⁹ Briefly, fumarate reductase complex from *W. succinogenes* was isolated as described previously³ and subunits A and B were separated by SDS-PAGE. The protein bands were cut out and homogenised in 50 mM K_2HPO_4 (pH 7.4). Rabbits were immunised with six injections containing 0.1 mg of isolated subunit A or B in Freund's complete adjuvant. The antisera were obtained 40 days after the first injection.

Butanol extraction of bacterial membranes

H. pylori strain 898-1 was grown in brain-heart infusion (BHI) medium with horse serum 10% as described previously.²⁰ Harvested bacteria were sonicated and centrifuged at 48 500 *g* for 90 min at 4°C. The sediment was suspended in cold phosphate-buffered saline (PBS) and *n*-butanol was added slowly (5 vol of PBS to 4 vol of *n*-butanol). After stirring for 1 h at 0°C, the suspension was centrifuged (27 000 *g*, 90 min, 4°C) and the aqueous phase was dialysed against 5 mM morpholinoethane sulphonate buffer, pH 6.0.²¹ The solution was lyophilised and dissolved in water.

Polyacrylamide gel electrophoresis

Native gel electrophoresis with partially purified fumarate reductase of *H. pylori* was performed according to the method described by Schägger *et al.*²² Briefly, a native separation 9% gel contained a 30% acrylamide-bisacrylamide mixture (AB-mixture; Roth, Germany) 2.25 ml, gel buffer (1.5 M 6-aminocaproic acid and 150 mM Bistris, pH 7.0) 2.5 ml, double distilled water 2.71 ml, 10% ammonium persulphate solution (APS) 32.25 μ l and Temed 6.45 μ l. The 3.75% sample gel contained AB-mixture 625 μ l, gel buffer 1.665 ml, double distilled water 2.66 ml, APS 40 μ l and Temed 4 μ l. The samples (20 μ g protein/trace) were diluted in sample buffer (750 mM aminocaproic acid, Triton X100 0.1% and 50 mM Bistris, pH 7.0) to 10 μ l final volume and 1 μ l of 500 mM aminocaproic acid with Serva Blue G 5% was added. The cathode buffer contained 50 mM tricine, 15 mM Bistris, and Serva Blue G 0.02%, pH 7.0; 50 mM Bistris adjusted with HCl to pH 7.0 was used as anode buffer. Electrophoresis was performed in a Mini Protean II chamber (BioRad) at 120 V and 4°C for 4 h. The track containing the fumarate reductase complex was cut out and the compounds of the protein complexes were separated by SDS-PAGE by the method of Lugtenberg *et al.*²³ Protein mixtures of myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa) were used as standards for mol. wt determination.

Western blotting

Western blots were done by the method of Towbin *et al.*²⁴ The proteins that had been separated by SDS-PAGE were blotted on nitrocellulose paper sheets at 200 mA for 2 h (blotting buffer: 285 mM glycine, 60 mM Tris and methanol 13.3%). The nitrocellulose paper sheets were incubated for 1 h in powdered skimmed milk 5% in PBS to saturate non-specific binding sites. The strips were overlaid with 2 ml of antiserum—diluted as follows: human 1 in 200, rabbit 1 in 2000 (α -fumarate reductase subunit A), and 1 in 800 (α -fumarate reductase subunit B)—incubated for 2 h at room temperature and washed three times with 5 ml of PBS containing Tween 20 0.05%. The sheets were treated with peroxidase-labelled goat anti-human or goat anti-rabbit immunoglobulin (Nordic Immunology, Netherlands; dilution 1 in 1500) for 1 h, washed again, and finally incubated with 5 ml of substrate containing 4-chloro-1-naphthol 15 mg in methanol 5 ml, Tris-buffered saline 25 ml, pH 8.2, and H_2O_2 10 μ l.

Purification of the 80-kDa protein

Butanol-extracted proteins were separated by SDS-PAGE (8% gel). The 80-kDa protein band was cut out. Protein bands of four gels were combined and a second, preparative SDS-PAGE (8% gel) was performed. The 80-kDa protein band was electroblotted of an Immobilon-P membrane (Millipore, Germany) according to the manufacturer's instructions. The amido black-stained 80-kDa protein band was cut out and an amino-acid sequence analysis was performed.

Amino-acid sequence analysis

The amino-acid sequence analysis was performed with a pulse liquid sequencer (model 476A protein sequencer, Applied Biosystems) in pmol size according to the principles first described by Edman and Begg²⁵ and the methods described by Tarr.²⁶

Preparation of Triton extract

Harvested bacteria were suspended and stirred for 15 min at 0°C in degassed and N_2 -flushed buffer containing 10 mM EDTA, 2 mM malonate, 0.25 mM phenylmethylsulphonylfluoride (PMSF) and 50 mM Tris-HCl, pH 7.8. Lysozyme 1.25 g/L (Serva) and, after 90 min, 15 mM $MgCl_2$ and DNAase I 1 mg/L were added. The suspension (cell homogenate) was stirred for a further 30 min and centrifuged for 25 min at 27 000 *g*. The sediment (membrane fraction) was suspended in degassed and N_2 -flushed buffer containing Triton X100 1%, 2 mM malonate, 1 mM dithiothreitol (DTT), 0.25 mM PMSF and 50 mM Tris-HCl, pH 7.8, and stirred at 0°C for 90 min (Triton homogenate). The Triton extract (supernate) was

Fig. 1. Protein profiles of *H. pylori* sonicate and butanol-extracted proteins obtained by SDS-PAGE (11.7% gel). Lane 1, standard proteins; 2, butanol-extracted membrane proteins; 3, sonicate of bacteria. (Stained with Coomassie Blue).

obtained after centrifugation for 30 min at 40000 *g* and 4°C.

Chromatography on DEAE-sepharose CL-6B

The Triton extract was passed through a DEAE-sepharose CL-6B column (volume 15 ml) equilibrated with a buffer containing Triton X100 0.05%, 2 mM malonate, 1 mM DTT, 0.25 mM PMSF and 50 mM Tris-HCl, pH 7.8. After rinsing with 20 ml of the same

buffer, the enzyme was eluted with 80 ml of a linear NaCl gradient (0–0.3 M NaCl) in the equilibration buffer at a flow rate of 20 ml/h. The enzyme eluted at 65–100 mM NaCl. The fractions containing the enzyme at sufficiently high specific activity were pooled and concentrated four-fold in Centricon tubes with membranes of 10 kDa exclusion pore size.

Enzyme activity

Fumarate reductase activity was measured as succinate oxidation by methylene blue at 37°C in a cuvette of 0.5-cm path length that contained 1 ml of 0.2 mM methylene blue (Serva), 10 mM succinate and 10 mM Tris-HCl, pH 7.9.²⁷ The solution was bubbled with N₂ for 2 min before the enzyme was added, and kept under an atmosphere of N₂. Measurement was done at a wave-length of 578 nm ($\Delta\epsilon = 17.1/\text{mM} \times \text{cm}$).

Results

Butanol-extracted proteins

Sonicate preparations of *H. pylori* and butanol-extracted proteins showed different protein profiles in SDS-PAGE (fig. 1). Proteins with a mol. wt > 80 kDa were not found in the SDS-PAGE of butanol extract whereas other proteins were enriched. The butanol-extracted proteins were tested for their immunogenicity with several sera from patients infected with *H. pylori*. As well as recognition of protein bands with mol. wts of 64 and 32 kDa (probably the urease subunits), 54 kDa and, at a lower frequency, some other protein bands (data not shown); 55% of the 49 sera recognised an enriched 80-kDa protein (fig. 2).

N-terminal amino-acid sequence of the 80-kDa protein

The amino terminal amino-acid sequence of the 80-kDa protein showed 80% identity with the amino terminal amino-acid sequence of subunit A of the fumarate reductase of *W. succinogenes* (fig. 3).

Fig. 2. Western blot with purified, 80-kDa protein. A, sera from patients infected with *H. pylori*; B, amido black stain of electroblotted 80-kDa protein.

80 kDa protein	1	5	10	15	20
<i>H. pylori</i>	MK I T Y C D A L I I G G G L A G L R A				
	:	:	:	:	:
Fumarate reductase	MK V Q Y C D S L V I G G G L A G L R A				
<i>W. succinogenes</i>	1	5	10	15	20

Fig. 3. N-terminal amino-acid sequence analysis. Comparison of amino-acid sequence of the 80-kDa protein of *H. pylori* with the 79-kDa subunit A of the fumarate reductase of *W. succinogenes*.

Table. Purification of the fumarate reductase of *H. pylori*

Source	Enzymic activity*	
	Total (U)	Specific (U/g of protein)
Cell homogenate	11.2	19.4
Triton homogenate	20.0	61.8
Triton extract	10.4	98.9
Pooled fractions from ion-exchange chromatography	5.4	769.0

* Fumarate reductase activity was measured as succinate oxidation by methylene blue.

Purification of the fumarate reductase of *H. pylori*

The cell homogenate obtained after lysis of *H. pylori* catalysed the oxidation of succinate by methylene blue (table). This enzyme activity is typical of either succinate dehydrogenases or fumarate reductases.²⁸ The addition of Triton X100 to the membrane fraction caused a two-fold increase in the total activity, while the specific activity increased three-fold. After centrifugation of the Triton homogenate the supernate (Triton extract) was subjected to ion-exchange chro-

matography. The fractions containing the highest specific activities were pooled and concentrated by pressure dialysis. The specific activity of this preparation was 13-fold that of the Triton homogenate.

Fumarate reductase activity could not only be shown by measuring the succinate oxidation to fumarate by methylene blue but also as fumarate reduction to succinate by reduced benzyl viologen (data not shown).

Identification of subunits

In comparison to the Triton extract, bands of apparent mol. wts 116, 80, 31, 25, 23.5 and 22 kDa were enriched in the partially purified *H. pylori* fumarate reductase preparation (fig. 4A) after SDS-PAGE. In this preparation, rabbit antiserum raised against subunit A reacted specifically with the 80-kDa polypeptide (fig. 4B) and antiserum raised against subunit B of *W. succinogenes* fumarate reductase reacted with a 31-kDa polypeptide (fig. 4C) as demonstrated by Western blotting. The size of the 31-kDa polypeptide is consistent with that of subunit B (31 kDa) of *W. succinogenes* fumarate reductase.³

Fig. 4. SDS-PAGE (12.8% gel) of the fumarate reductase of *H. pylori*. Lane 1, standard proteins; 2, Triton extract; 3, pooled fractions after ion-exchange chromatography. A, Coomassie Blue stain; B, Western blot developed with rabbit antiserum recognising subunit A (79 kDa) of *W. succinogenes* fumarate reductase; C, Western blot developed with rabbit serum recognising subunit B (31 kDa) of *W. succinogenes* fumarate reductase.

Fig. 5. SDS-PAGE (12.8% gel) after native gel electrophoresis. Protein bands forming a common lane belong to one protein complex. The arrows mark protein bands (A, 80 kDa; B, 31 kDa) that are immunoreactive with rabbit sera against (A) subunit A and (B) subunit B of *W. succinogenes* fumarate reductase.

Native gel electrophoresis and SDS-PAGE of *H. pylori* fumarate reductase indicated that the two polypeptides (80 and 31 kDa) belong to the same protein complex (fig. 5). In addition, polypeptides with apparent mol. wts 25, 23.5 and 22 kDa were present in the same lane. The protein bands between the 80- and 31-kDa proteins seemed to be degradation products of

the 80-kDa protein because they also were recognised by the antiserum against subunit A of *W. succinogenes* fumarate reductase.

Frequency of the 80-kDa protein in different H. pylori strains

Western blots were developed with rabbit antiserum against subunit A of fumarate reductase complex of *W. succinogenes* (fig. 6). The 80-kDa protein was detected in all 32 sonicates of the *H. pylori* strains tested.

Discussion

This report describes a membrane-associated 80-kDa protein of *H. pylori* that was frequently recognised by antibodies in the sera of patients infected with *H. pylori*. The protein was similar to *W. succinogenes* fumarate reductase subunit A not only in N-terminal amino-acid sequence but also in mol. wt (79 kDa for *W. succinogenes* fumarate reductase subunit A^{3,29}) and cross-reaction with antibodies. This similarity was supported by the presence of a second subunit with a mol. wt of 31 kDa in the purified preparation of *H. pylori* fumarate reductase. This subunit cross-reacted with antibodies against subunit B of *W. succinogenes* fumarate reductase, that has the same apparent mol. wt in SDS-PAGE.^{3,29} The 31-kDa subunit of *H. pylori* could not be shown in the bacterial sonicate but was demonstrated in the Triton extract and was strongly enriched after ion-exchange chromatography. The 80-

Fig. 6. Western blot showing the recognition of the 80-kDa protein band in sonicates of different *H. pylori* strains by rabbit serum against subunit A of *W. succinogenes* fumarate reductase. Lane 1, strain 032; 2, strain 023; 3, strain 012; 4, strain 010; 5, strain 008; 6, strain 007; 7, strain 005; 8, strain 004.

and 31-kDa proteins of *H. pylori* are present in one protein complex, as are *W. succinogenes* subunit A and B. The native protein complex appears to contain three more protein bands, one of which has the same mol. wt as subunit C of *W. succinogenes* fumarate reductase (25 kDa) which has been identified as cytochrome b.³⁰ The involvement of cytochrome in the protein complex is possible, because the active fractions obtained from ion-exchange chromatography showed a reddish-brown colour that is characteristic of cytochromes. It is still unclear whether the other proteins belong to the enzyme complex or whether they co-purified with the fumarate reductase complex. Enzyme activity could be found only in the membrane preparation but not in the cytoplasmic fraction of the bacterium, suggesting that the fumarate reductase of *H. pylori* is membrane-bound like the fumarate reductase of *W. succinogenes*. Recently, fumarate catabolism in *H. pylori* was investigated by ¹H and ¹³C nuclear magnetic resonance spectroscopy and fumarate reductase activity was also demonstrated.³¹

H. pylori and *W. succinogenes* not only show

homology in one protein, the fumarate reductase. In previous studies a close phylogenetic relationship between *W. succinogenes* and *H. pylori* (formerly *Campylobacter pylori*) was demonstrated by comparison of partial 16S rRNA sequences of these bacteria with the bacteria of the genus *Campylobacter* and some other gram-negative bacteria.^{32, 33}

Fumarate reductase seems to be a common protein of *H. pylori*, as demonstrated by the presence of the 80-kDa protein of the fumarate reductase in all strains tested.

Fumarate can be formed easily from widely abundant compounds including proteins.^{1, 2} This may account for the fact that fumarate respiration is the most widespread type of anaerobic respiration. Therefore, it is possible that the presence of fumarate reductase enables the micro-aerophilic bacterium *H. pylori* to carry out anaerobic respiration.

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