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Export of the periplasmic NADP-containing glucose-fructose oxidoreductase of *Zymomonas mobilis*

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Abstract Glucose-fructose oxidoreductase (GFOR) of the gram-negative bacterium *Zymomonas mobilis* is a periplasmic enzyme with the tightly bound cofactor NADP. The preprotein carries an unusually long N-terminal signal sequence of 52 amino acid residues. A sorbitol-negative mutant strain (ACM3963) was found to be deficient in GFOR activity and was used for the expression of plasmid-borne copies of the wild-type *gfo* gene or of alleles encoding alterations in the signal sequence of the pre-GFOR protein. *Z. mobilis* cells with the wild-type *gfo* allele translocated pre-GFOR, at least partially, via the Sec pathway since CCCP (carboxylcyanide-*m*-chlorophenylhydrazone; uncoupler of proton motive force) or sodium azide (inhibitor of SecA) abolished the processing of GFOR. A *gfo* allele with the hydrophobic region of the signal sequence removed (residues 32–46; Δ32–46) led to a protein that was no longer processed, but showed full enzymatic activity (180 U/mg) and had the cofactor NADP firmly bound. A deletion in the n-region of the signal sequence (residues 2–20; Δ2–20) or exchange of the entire GFOR signal sequence with the signal sequence of gluconolactonase of *Z. mobilis* led to active and processed GFOR. Strain ACM3963 could not grow in the presence of high sugar concentrations (1 M sucrose) unless sorbitol was added. The presence of the plasmid-borne *gfo* wild-type allele or of the Δ2–20 deletion led to the restoration of growth on media with 1 M sucrose, whereas the presence of the Δ32–46 deletion led to a growth behavior similar to that of strain ACM3963, with no sorbitol formation from sucrose.

Key words Glucose-fructose oxidoreductase · *Zymomonas mobilis* · *Gfo*-deficient mutant · Sorbitol · Protein export · Signal sequence · Periplasmic NADP

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Introduction

When grown on high concentrations of sucrose or on mixtures of glucose and fructose, the gram-negative, strictly fermentative bacterium *Zymomonas mobilis* forms sorbitol and gluconic acid in addition to ethanol and carbon dioxide (Barrow et al. 1984; Leigh et al. 1984; Viikari 1984). The periplasmic enzyme glucose-fructose oxidoreductase (GFOR; EC 1.1.99.-) catalyzes both the oxidation of glucose to gluconolactone and the reduction of fructose to sorbitol (Leigh et al. 1984; Zachariou and Scopes 1986; Hardman and Scopes 1988) and contains non-dissociable $NADP^+$ as redox carrier (Zachariou and Scopes 1986; Hardman and Scopes 1988). Sorbitol is not catabolized further, but is accumulated as a compatible solute to counteract the detrimental effects of high osmolar sugar solutions (Loos et al. 1994a). A comparison of the DNA sequence of the *gfo* gene with the N-terminus of the purified enzyme has revealed an unusually long signal sequence of 52 amino acid residues (Kanagasundaram and Scopes 1992a). As mature GFOR is located in the periplasm of *Z. mobilis* (Loos et al. 1991; Aldrich et al. 1992), this signal sequence should undergo processing during its translocation (export) across the cytoplasmic membrane. In Western blots with GFOR-specific antibodies, two prominent bands have become evident in crude extracts of GFOR-overproducing *Z. mobilis* strains (Loos et al. 1991; Loos et al. 1993), pointing to the accumulation of a so-called "pre-GFOR" in the cytosol. This larger pre-GFOR has been purified from overproducing strains and has been shown to be enzymatically active (Loos et al. 1993). The N-terminal amino acid sequence of the purified protein matches that deduced from the DNA sequence (Kanagasundaram and Scopes 1992a; Loos et al. 1993).

A defined *gfo* mutant strain has not been available. Recently a mutant strain of *Z. mobilis* has been described that does not produce sorbitol (ACM3963; Kirk and Doelle 1993). In this paper, we present evidence that this mutant is deficient for GFOR and is a suitable host for the characterization of both the wild-type and the mutant alle-

les of the *gfo* gene, thus allowing the elucidation of steps in the pathway of translocation and maturation of pre-GFOR to GFOR across the inner membrane of *Z. mobilis*. To determine whether GFOR export occurs via the general bacterial secretory pathway (for a recent review, see Pugsley 1993), we established a pulse-chase regime with ^{35}S-methionine to follow the kinetics of pre-GFOR processing. Furthermore, we studied the influence of sodium azide, a known inhibitor of SecA protein from *Escherichia coli* or *Bacillus subtilis* (Fortin et al. 1990; Oliver et al. 1990; Klein et al. 1994), and of the uncoupler CCCP, which abolishes the proton motive force; proton motive force, together with ATP, is essential as an energy source for the efficient export of proteins (Schiebel et al. 1991). To examine the essence of the unusually long signal sequence, we assayed whether deletions in the specific regions of the signal sequence affected the processing and maturation of pre-GFOR and whether it could be exchanged for another signal sequence.

Table 1 Bacterial strains and plasmids

Strain or plasmid		Relevant genotype or phenotype	Reference/origin
Bacterial strains			
Escherichia coli K-12			
DH5α		*hsdR recA thi* cloning strain	Hanahan (1983)
JM109		*hsdR recA thi (lac-proAB)* Δ/F′ *proAB*$^+$ *lac*ZΔM15 *lacI*^{q}	Yanisch-Perron et al. (1985)
BW313		*dut ung* strain for phage M13 mp 18-based, oligonucleotide-directed, site-specific mutagenesis	Kunkel (1985)
S17-1		*thi hsdR pro* mobilization strain, RP4-2 integrated into the chromosome	Simon et al. (1983b)
Zymomonas mobilis			
ZM6 (ATCC 29191)		*gfo*$^+$, wild-type	Swings and DeLey (1977)
CP4 (ATCC 31821)		*gfo*$^+$, wild-type	Goncalves de Lima et al. (1970)
ACM3963		spontaneous sorbitol-non producer from ATCC 39676, *gfo*	Kirk and Doelle (1993) and this study
	Size (kb)	Features/mode of construction	Reference/origin
Plasmids			
pZVK2	9.3	*gfo*$^+$ including promoters P1 and P2 of *gfo*	Kanagasundaram and Scopes (1992a) and personal communication
pUV18	2.7	*bla, lacZ*α, multiple cloning site, high-copy-number	Vieira and Messing (1982)
pTW18	2.7	as pUC18, *Ssp*I site altered to *Not*I site, multiple cloning site altered to *Eco*RI- *Sac*I- *Ssp*I- *Eco*RV- *Sal*I	this study
pSUP104	9.5	*cat-*, *tet*-resistance genes, broad-host-range vector	Simon et al. (1983a)
pZY414	10.6	pSUP104 containing *gfo* with promoter P2	this study
pZY414Δ2–20	10.5	pZY414 with deletion of codons for amino acids 2–20	this study
pZY414Δ32–46	10.5	pZY414 with deletion of codons for amino acids 32–46	this study
pZY431	3.2	pUC19 containing *Ssp*I + *Pst*I fragment containing promoter P2 plus N-terminal region of *gfo* gene from pZVK2	this study
pZY470	4.3	pTW18 cleaved with *Ssp*I plus *Eco*RV, blunt-end ligated with 1.6-kb *Ssp*I fragment containing *gfo* from pZVK2	this study
pZY471	4.3	as pZY470, *Eco*47III restriction site engineered at bp 407 of *gfo* sequence by mutagenesis	this study
pZY474	4.1	pZY471 *Eco*RI/*Eco*47III plus 129-bp *Eco*RI/*Nae*I PCR fragment containing *gnl* signal sequence	this study
pZY507	10.1	pSUP104, *lacI*^{q}/P*tac*, multiple cloning site replacing *tet* gene	S. Schilz, H. Sahm, G. A. Sprenger (unpublished results)
pZY570	11.8	pZY507 *Sac*I plus *Sal*I restricted, ligated with 1.6-kb *Sac*I/*Sal*I fragment from pZY470 containing *gfo*	this study
pZY570Δ2–20	11.7	as pZY570, codons 2–20 removed by loop-out mutagenesis	this study
pZY570Δ32–46	11.7	as pZY570, codons 32–46 removed by loop-out mutagenesis	this study
pZY574	11.6	pZY507 *Sac*I/*Sal*I plus 1.5-kb *Sac*I/*Sal*I from pZY474, *gfo* with *gnl* signal sequence	this study

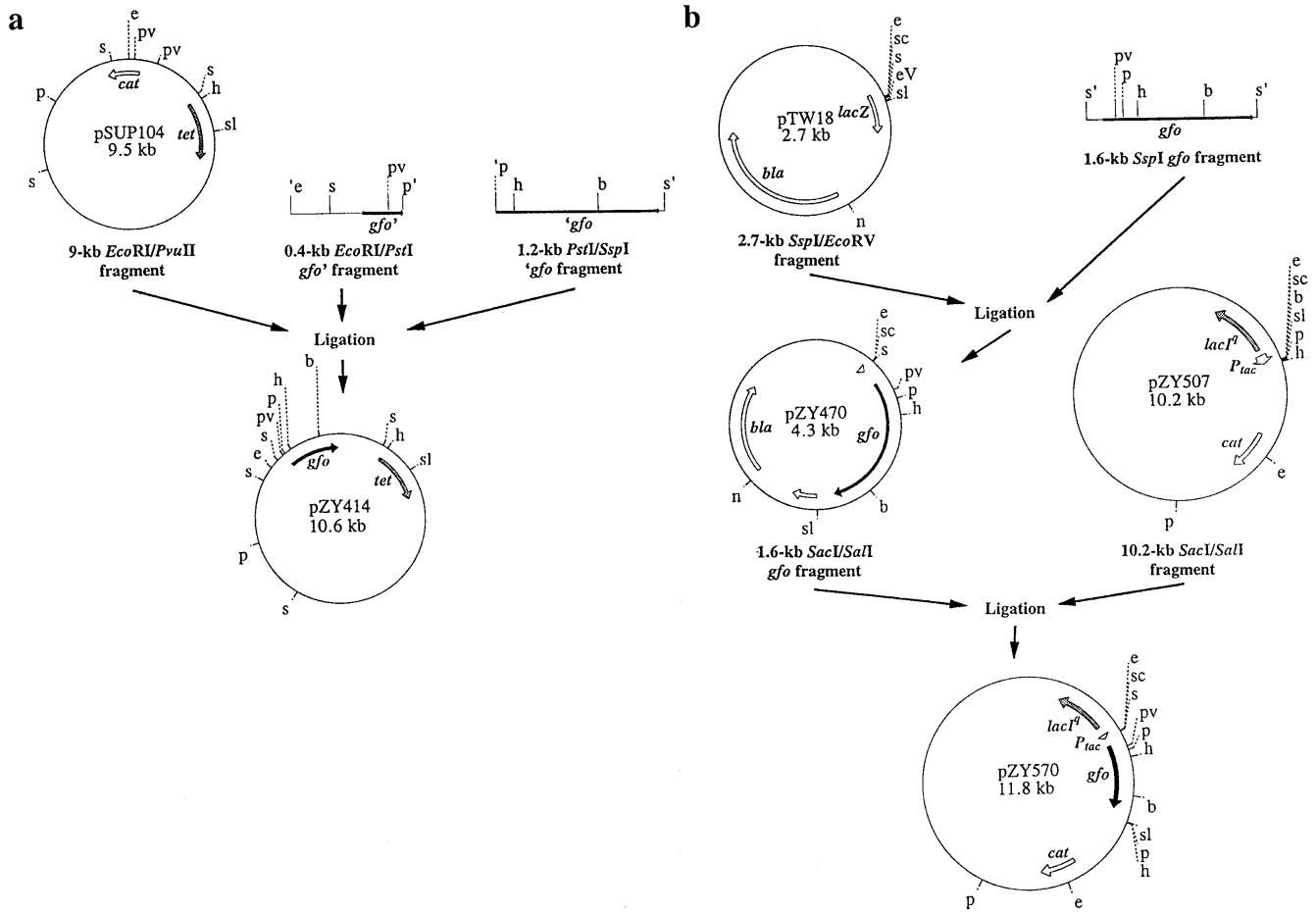


Fig. 1 a, b Construction of plasmids pZY414 and pZY570. Construction of **a** plasmid pZY414, **b** plasmid pZY570. See Table 1 for additional information. *bla* Ampicillin resistance, *cat* chloramphenicol resistance, *gfo* glucose-fructose oxidoreductase, *lacI*^{q} lactose repressor, *lacZ*α gene encoding α-peptide of β-galactosidase, *Ptac* synthetic *tac* promoter, *tet* tetracycline resistance. *b Bam*HI, *e Eco*RI, *eV Eco*RV, *h Hin*dIII, *n Not*I, *p Pst*I, *pv Pvu*II, *s Ssp*I, *sc Sac*I, *s Sal*I. For clarity, some restriction sites are omitted

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are given in Table 1. *Zymomonas mobilis* ssp. *mobilis* strains ZM6 (ATCC 29191), CP4 (ATCC 31821), ACM3963 (derivative of ATCC 39676), and its derivatives were maintained and cultivated anaerobically as described previously (Bringer-Meyer et al. 1985). For the purification of mutant protein GFORΔ32–46, cells of strain ACM3963/pZY570Δ32–46 were grown in flasks (8-l culture vol.) with stirring. To obtain high amounts of GFOR protein, isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was added to the culture. Sorbitol determination was performed with a spectrophotometrical assay as described earlier (Beutler and Becker 1987; Loos et al. 1994a).

Plasmid constructions and mutagenesis of *gfo* gene

Standard techniques for cloning (Sambrook et al. 1989), transformation (Hanahan 1983), PCR amplification of DNA (Mullis and Faloona 1987), and site-specific mutagenesis (Kunkel 1985) were applied. Construction of shuttle plasmids pZY414 and pZY570 is depicted in Fig. 1, and plasmids are listed in Table 1. For a convenient site-specific mutagenesis, a 0.5-kb *Eco*RI/*Pst*I fragment from plasmid pZY431 (Table 1) containing the 5′ end of the *gfo* gene including the P2 promoter of *gfo* (Kanagasundaram and Scopes 1992a) was cloned into the *Eco*RI/*Pst*I restriction sites of M13mp18 (Yanisch-Perron et al. 1985). Deletions in the signal sequence coding region were carried out according to the method of Kunkel (1985) with loop-out primers 5′-TGGCGTACGG GAAGCGTTCATAATCCTTGTTTCTTTCTT-3′ (Δ2–20) and 5′-TGCCTGAAGACCACTGGCGCGACGGGTCAGATTTGG-3′ (Δ32–46) and checked by DNA sequencing (Sanger et al. 1977). The mutagenized *gfo* 5′-regions were restored to complete genes using the respective *Eco*RI/*Pst*I fragment ligated to a *Pst*I/*Ssp*I fragment in the case of pZY414 (Fig. 1a), and as an *Ssp*I/*Pst*I fragment ligated to pZY470 (restricted with *Ssp*I/*Pst*I) to finally yield mutagenized alleles of pZY570 (Fig. 1b). All plasmids were checked for deletions by restriction analysis and, in the case of pZY470, by DNA sequencing. To exchange the GFOR signal sequence for the gluconolactonase (GNL) signal sequence, the *gnl* gene (Kanagasundaram and Scopes 1992b) was amplified by polymerase chain reaction with primers 5′-AGAATTCAAGCTT TTTTTCAAACTTATTTT-3′ and 5′-TACGAATTGTCGACTG-GTGCGAACGGCACA-3′ using chromosomal DNA from *Z. mobilis* CP4 and was cloned as a *Hin*dIII/*Sal*I fragment into vector pBR322. With the resulting vector as a template, the *gnl* signal sequence coding region was amplified by PCR using primers 5′-TAGAATTCGAGCTCGCGGTTAGTTGCAGGAGT-3′ and 5′-TAGAATTCGCCGGCCTGAGCTGATCCTGTAAT-3′. The product was cloned as an *Eco*RI/*Nae*I fragment into pZY471 restricted with *Eco*RI/*Eco*47III. Plasmid pZY471 was derived from pZY470 by introducing a unique *Eco*47III restriction site directly at the re-

gion coding for the processing site using M13 site-specific mutagenesis with primer 5′-ACCAGCAGGAAGCGTAGCGCTCT-GAAGACCACTGGC-3′. Note that in contrast to the published *gnl* sequence (Kanagasundaram and Scopes 1992b) at position 226, an exchange G to C (complementary base underlined in the second primer for *gnl* PCR) yielding a Gln residue instead of a Glu residue was detected. From the resulting plasmid (pZY474), a fusion (containing the coding regions for the GNL signal sequence in front of the mature GFOR) was cloned as a *Sac*I/*Sal*I fragment into pZY507 to finally yield pZY574.

After transformation into strain S17-1 (Simon et al. 1983b), the vectors were transferred to *Z. mobilis* ACM3963 by conjugation; selection was based on chloramphenicol or tetracycline resistance. To counterselect the *E. coli* donors, nalidixic acid was used (Uhlenbusch et al. 1991). After purification, the integrity of the plasmids was checked by restriction analyses and retransformation into suitable *E. coli* recipients. GFOR enzyme assays and Western blot analyses were done to check for the presence of both protein and enzyme from the *gfo* alleles.

Enzyme assay and purification of mutant enzyme of glucose-fructose oxidoreductase

Glucose-fructose oxidoreductase assays were performed at 30° C as described previously (Zacchariou and Scopes 1986). The auxiliary enzyme gluconolactonase was prepared from *Rhodotorula rubra* according to a published method (Loos et al. 1993) and was added in excess. Protein contents were determined using a dye-binding method (Bradford 1976). In SDS-polyacrylamide gels, the Combithek (Boehringer Mannheim, Germany) protein size standards were used. Mutant enzyme of GFOR was purified from 47 g of cells (wet weight), all steps being performed at 4° C. The cells were suspended in K-Hepes (20 mM, pH 7.5) and disrupted by vortexing with glass beads (0.25–0.5 mm in diameter). The cell debris was centrifuged and the supernatant clarified by ultracentrifugation. The cell-free extract was loaded onto a 500-ml DEAE-Sephacel column (negative adsorbent) coupled to a prepacked Fractogel EMD-SO_3 (Merck) column (positive adsorbent). The columns were equilibrated with K-Hepes (20 mM, pH 7.5). GFOR was eluted from the EMD-SO_3 cation exchanger with an increasing NaCl gradient. Fractions containing GFOR activity were combined and subjected to a second cation exchange chromatography on EMD-SO_3, equilibrated with K-Hepes (20 mM, pH 7.9). Fractions containing GFOR activity were combined, dialyzed, concentrated, and stored as described elsewhere (Loos et al. 1994b).

Pulse-chase experiments and Western blots

Antibodies against purified GFOR were raised from rabbits as described previously (Loos et al. 1991). SDS-PAGE and Western blotting followed standard methods (Laemmli 1970; Towbin et al. 1979). Pulse-chase experiments were carried out according to published methods (Ito et al. 1981; Gebert et al. 1988) with some modifications: 3 ml of an exponentially growing culture of *Z. mobilis* at 30° C was labeled with 70 µCi ^{35}S-L-methionine. After 5 min, 100 µl chase solution (20 mg/ml L-methionine, 40 mg/ml chloramphenicol) was added. When appropriate, sodium azide or CCCP was added immediately before addition of the chase medium. After chase times of 1, 5, and 20 min, samples (1 ml) were withdrawn and transferred to 40% trichloroacetic acid (300 µl, 4° C). After centrifugation, the precipitates were washed with 1 ml acetone (–20° C) and dried. The precipitated cells were then neutralized with 10 µl Na_2CO_3 (100 mM) mixed with 30 µl of lysis buffer 1 (2% SDS; 1 mM EDTA; 50 mM Tris-HCl, pH 8.0) and disrupted by incubation at 95° C for 5 min. After addition of 960 µl of lysis buffer 2 (2% Triton X-100; 0.15 M NaCl; 50 mM Tris-HCl, pH 8.0), the samples were centrifuged and the supernatant was subjected to immunoprecipitation; SDS-PAGE and fluorography were performed as described elsewhere (Gebert et al. 1988).

Results

Zymomonas mobilis strain ACM3963 is deficient in GFOR activity

Strain ACM3963 had been isolated as a spontaneous mutant that had overgrown the continuously subcultured strain ATCC 39676. Strain ACM3963 had been described as sorbitol-negative, but the underlying enzyme deficiency had not been reported before (Kirk and Doelle 1993). The strain was checked for its GFOR activity and/or cross-reacting immunological material. As shown in Fig. 3, compared to a wild-type strain of *Z. mobilis* (ZM6), strain ACM3963 showed no detectable GFOR activity. In a Western blot assay (Fig. 3), only a faint band was detectable in extracts from strain ACM3963 at the position of mature GFOR. Thus, it may be inferred that strain ACM3963 is a *gfo*-negative mutant. Strain ACM3963 proved to be a stable deficient mutant and, therefore, it was used as host strain for the expression of both the wild-type *gfo* gene and of several derivatives with alterations in the signal sequence (Figs. 1, 2).

In order to study the essence of the unusually long signal sequence and the mode of pre-GFOR export, we subcloned the *gfo* gene (Kanagasundaram and Scopes 1992a) as described in Fig. 1 and Table 1. Alleles of *gfo*, when on the low-copy-number vector pSUP104 (under the control of the proximal promoter P2 of the *gfo* gene), restored GFOR enzyme activity in ACM3963 to about wild-type levels (Fig. 3). In Western blot assays using crude extracts of the different strains, corresponding amounts of antibody-reactive material could be observed (Fig. 3). The low-copy-number vector was, therefore, suitable for growth experiments in comparison with the chromosomally encoded GFOR of wild-type strains. Plasmid pZY570 and its derivatives allowed an IPTG-inducible overexpression of GFOR and was used for pulse-chase experiments and purification of recombinant GFOR. Expression of the *gfo* gene from the tandem promoters, *tac* and P2 of *gfo*, led to basal GFOR activities between 8 and 11 U/mg in the absence of inducer. In the presence of IPTG (1 mM), overproduction was observed (up to 25 U/mg), with accumulation of pre-GFOR becoming visible in Western blot assays (data not shown).

ACM3963 or ACM3963/pSUP104 strains could not grow in complex medium with 1 M of sucrose in the absence of additional sorbitol (Fig. 4). When strain ACM3963 was transformed with plasmid pZY414 or other *gfo*-containing vectors, it became GFOR-positive. Despite their similarities with respect to enzyme activities, the different *gfo* alleles on vector pSUP104 led to different growth behavior when expressed in strain ACM3963. Strain ACM3963 with the wild-type allele (pZY414) could – similar to the wild-type strain ZM6/pSUP104 – grow on 1 M sucrose. However, less growth and less sorbitol formation in the culture medium was observed (Fig. 4). The reason for this is unknown, but it may reflect additional defects in the sucrose metabolism of

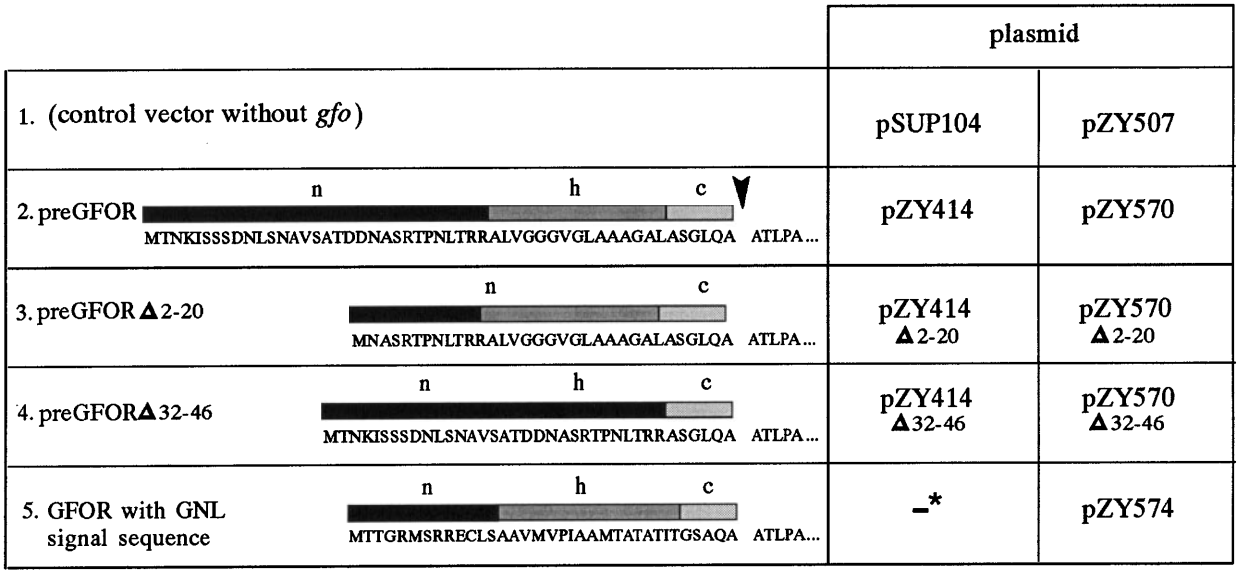


Fig. 2 Comparison of wild-type and engineered alleles in the *gfo* signal sequence. The signal sequence of *gfo* wild-type allele present in vectors pZY414 (from vector pSUP104) and pZY570 (from vector pZY507) is shown in *panel 2*. Putative n- *(black)*, h- *(grey)* and c-*(dotted)* regions are depicted. The cleavage site between amino acid residues 52 and 53 (Ala-Ala) is marked by an *arrow*. Deletions in the n-region (Δ2–20) or of the h-region (Δ32–46) are depicted in *panels 3* and *4*. Exchange of GFOR signal sequence with the GNL sequence is shown in *panel 5* for plasmid pZY574 only. *Asterisk* (*) not done

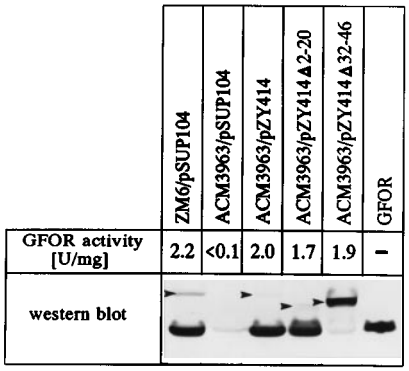


Fig. 3 Western blot assay with crude extracts of ZM6, ACM3963, and pZY414 derivatives. Crude extracts were prepared and subjected to SDS-PAGE (for details see Materials and methods). Anti-GFOR antibodies raised from rabbits were used to detect GFOR protein afterwards. Strain ZM6/pSUP104 was used as a control, and purified GFOR was used as a standard

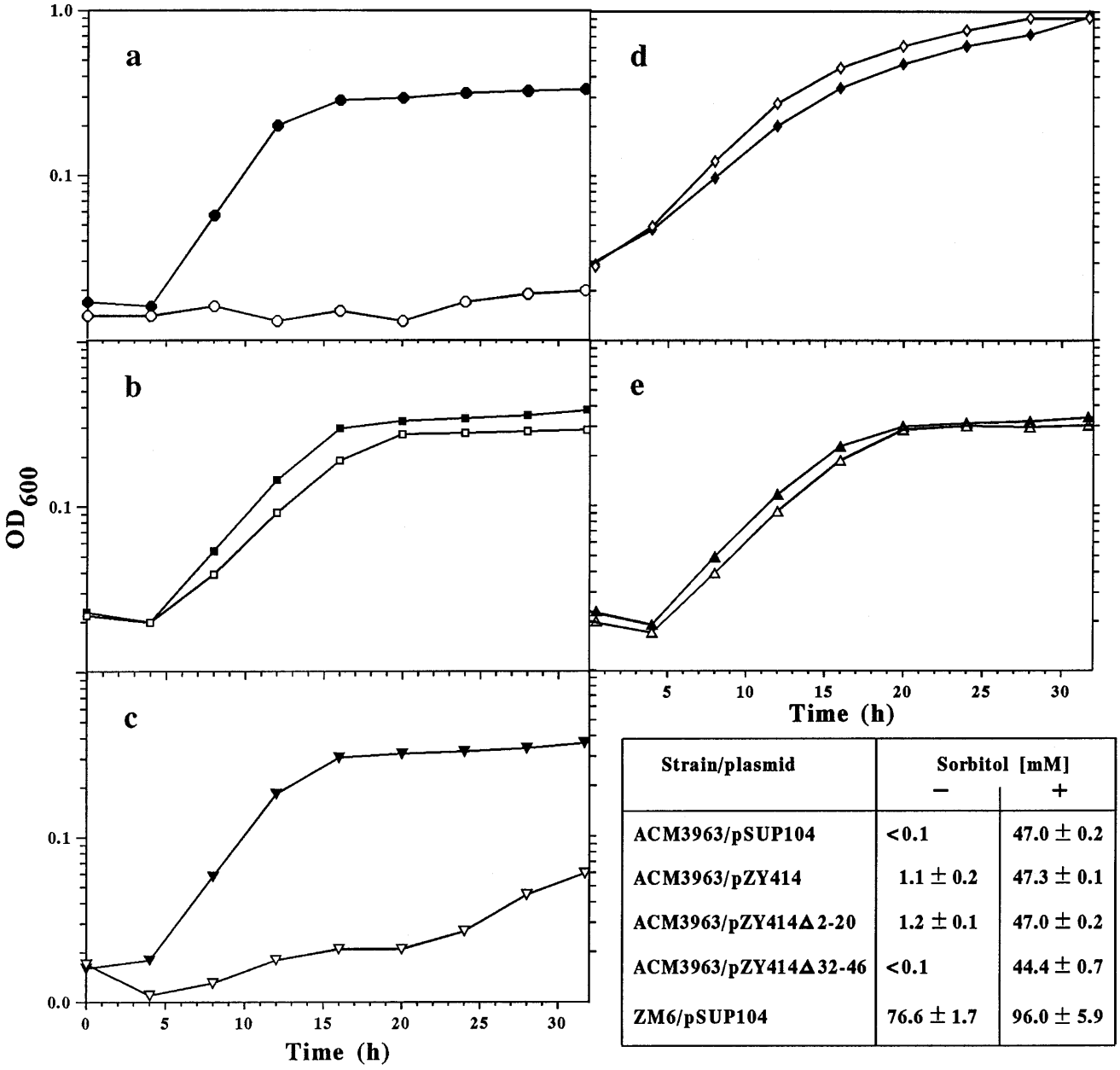
strain ACM3963 (L. Kirk, personal communication). The allele with the deletion of amino acid residues 2–20 ("n-region"; pZY414Δ2–20), gave similar results in strain ACM3963 both for growth at high sucrose and for sorbitol formation. The deletion of the hydrophobic residues 32–46 ("h-region"; pZY414Δ32–46) leads to a cytosolic, unprocessed form of GFOR (see below). Strain ACM3963/pZY414Δ32–46 formed no sorbitol and grew only in the late phase of incubation with 1 M sucrose (Fig. 4). This we take for evidence that the periplasmic localization of GFOR is essential for its proper functioning as a provider of sorbitol for osmoprotection.

Processing of the pre-GFOR depends on proton motive force and on SecA

To elucidate the process of pre-GFOR translocation across the cytoplasmic membrane, a pulse-chase technique for *Z. mobilis* strains was established. As described in the legend to Fig. 5, cells of strain ACM3963 with the cloned *gfo*-gene (pZY414) were labeled with ^{35}S-methionine for 5 min ("pulse"); thereafter an excess of unlabeled methionine ("chase") and chloramphenicol was added to stop further incorporation of the label, and the kinetics of pre-GFOR processing were followed. As can be seen from Fig. 5, processing of pre-GFOR to a smaller protein band (equivalent to the mature GFOR) could thus be followed by the pulse-chase technique. Processing of pre-GFOR was totally inhibited if sodium azide (NaN_3, 3 mM), which at the given concentration is a known selective inhibitor of the SecA protein (a component of the bacterial secretory pathway; Fortin et al. 1990; Oliver et al. 1990), was added together with the chase medium. Processing was also halted by addition of CCCP, an uncoupler of the proton motive force of *Z. mobilis* (Ruhrmann and Krämer 1992; Loos et al. 1994a). Proton motive force is essential for protein translocation (Schiebel et al. 1991). From these data we conclude that processing of pre-GFOR to the mature GFOR is, at least partially, dependent on the general secretory pathway (GSP) of bacteria.

Influence of deletions in the signal sequence on GFOR processing

The rules for bacterial signal peptides hold that, in addition to N-terminal positive charges (lysine or arginine residues, "n-region"; Inouye et al. 1982; Von Heijne 1990) and to a "c-region" preceding the cleavage site, a



Strain/plasmid	Sorbitol [mM]	
	–	+
ACM3963/pSUP104	<0.1	47.0 ± 0.2
ACM3963/pZY414	1.1 ± 0.2	47.3 ± 0.1
ACM3963/pZY414Δ2-20	1.2 ± 0.1	47.0 ± 0.2
ACM3963/pZY414Δ32-46	<0.1	44.4 ± 0.7
ZM6/pSUP104	76.6 ± 1.7	96.0 ± 5.9

Fig. 4 a–e Growth in the presence of 1 M sucrose in rich medium. Cells grown overnight in rich medium with 10% glucose (Bringer et al. 1985) were washed and inoculated to an optical density (OD_{600}) of about 0.02 into prewarmed, rich medium containing 1 M (36% w/v) sucrose. Growth was monitored over a period of 32 hours. *Filled symbols* mark cultures with addition of 50 mM sorbitol to the medium, *open symbols* are without sorbitol addition. **a** Strain ACM3963 with the control vector pSUP104 was used; **b** strain ACM3963 (pZY414); **c** strain ACM3963 (pZY414Δ32–46), (cytosolic GFOR); **d** wild-type ZM6 carrying pSUP104; **e** strain ACM3963 (pZY414Δ2–20). In the *table*, sorbitol concentrations determined at the end of the incubations are given in mM. (–) without addition of sorbitol (50 mM) from the start, (+) addition of 50 mM sorbitol from the start

hydrophobic core of 7 to 15 amino acid residues ("h-region") is required to initiate translocation across the cytoplasmic membrane (Von Heijne 1990). From the published sequences for GFOR (Kanagasundaram and Scopes 1992a; Loos et al. 1993), it became evident that the unusually long GFOR signal sequence displays a reasonable, albeit very long n-region, a c-region, and a consensus cleavage site (Leu^{-3} -X-Ala^{-1}-Ala). However, in the context of a putative h-region, an arginine residue (Kanagasundaram and Scopes 1992a) that would be incompatible with this rule (Fig. 2) is apparent. During the subcloning and mutagenesis of the *gfo* gene, we sequenced this region several times and found several errors in the published sequence. Several minor corrections were made in the nucleotide sequence; one important alteration (exchange of a GC for a CG pair) leads to the exchange of an Arg residue in the h-region at position 43 for an Ala residue, resulting in an orthodox h-region for the GFOR signal peptide. Our DNA sequencing data predicted that the nucleotide exchange GC for CG would create a rare

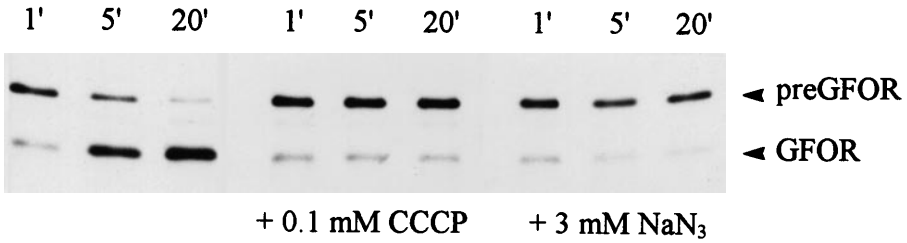


Fig. 5 Processing kinetics of Pre-GFOR to the mature form in a pulse-chase assay. Processing of Pre-GFOR over a period from 1 to 20 min was followed with strain ACM3963 (pZY570) after labeling with ^{35}S-L-methionine for 5 min ("pulse") followed by addition of an excess of unlabeled methionine ("chase") and chloramphenicol to stop further protein biosynthesis. For details, see Materials and methods. The *three lanes at the left* show processing kinetics, the *middle lanes* show kinetics in the presence of the uncoupler CCCP (0.1 mM), and the *right lanes* show processing in the presence of sodium azide (NaN_3, 3 mM final concentration), an inhibitor of SecA protein

	GFOR activity [U/mg] -IPTG	+IPTG
preGFOR	11	25
preGFORΔ2-20	10	20
preGFORΔ32-46	8	25
GFOR with GNL signal sequence	0.4	2.4

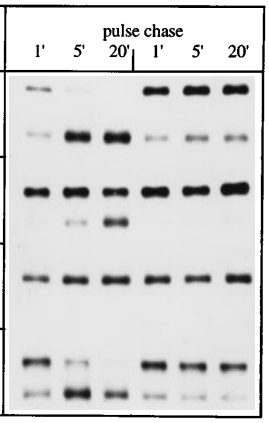


Fig. 6 Processing of wild-type Pre-GFOR and of signal sequence mutants in strain ACM3963. Pulse-chase experiments with strain ACM3963 carrying plasmid pZY414 or its signal sequence derivatives (from top to bottom) are shown in Fig. 2. Pulse-chase assays were performed as described in the legend to Fig. 5. The *left three lanes* show the kinetics in the absence, the *right three lanes* in the presence of sodium azide

restriction site for the enzyme *Pvu*II; this site, indeed, could be shown by restriction analysis to occur in the respective region (Fig. 1).

To see whether export of pre-GFOR to the periplasm is essential for NADP incorporation, for enzymatic activity, and for the physiological function of GFOR, we deleted the presumed h-region (Δ32–46) of the signal sequence, which is necessary for Sec-dependent translocation. We also shortened the extraordinarily long n-region of the GFOR signal sequence for 19 amino acids (Δ2–20) to see whether this extended region plays a specific role in GFOR export, e.g., a possible chaperone-like function. The deletions were found to have no immediate adverse effect on enzyme activity (see Fig. 3). In Western blots, extracts from the Δ2–20 derivative of strain ACM3963 showed two reactive bands, one at the position of mature GFOR and a larger, faint band that corresponded well with a (shortened) pre-GFOR (Fig. 3). Δ32–46 gave only one major band in a Western blot, at a position which corresponded to the shortened pre-GFOR. Thus, it could be deduced that Δ32–46 is no longer processed and represents a cytoplasmic form of GFOR. In pulse-chase experiments (Fig. 6), Δ2–20 performed a seemingly normal but slower processing, whereas Δ32–46 was not processed at all. However, processing was not essential to gaining full enzymatic activity. When the long *gfo* signal sequence was exchanged for the (shorter) signal sequence of the gluconolactonase gene from *Z. mobilis* (Kanagasundaram and Scopes 1992b), processing of this fusion protein occurred and an active GFOR enzyme was formed (specific activity of 2.4 U/mg), ruling out a specific need for the length or specific sequence of the GFOR signal sequence (Fig. 6).

Purification and characterization of GFOR mutant protein Δ32–46

Since the Δ32–46 allele of *gfo* had resulted in normal enzymatic activity in strain ACM3963, we assayed whether the purified protein showed differences to the wild-type GFOR. Therefore, strain ACM3963/pZY570Δ32–46 was grown in an 8-l batch with IPTG induction. GFOR was

Table 2 Purification scheme of GFOR Δ32–46 mutant protein

Sample	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Total yield (U)	%
Supernatant after ultracentrifugation	110	19.8	2,180	36.9	80,300	100
DEAE Sephacel[a] + Fractogel EMD-SO_3	34	5	170	146.2	24,800[a]	62
Fractogel EMD- SO_3	4.2	18.1	76	182	13,900	34.6[b]

[a] Only 55 ml of supernatant after ultracentrifugation was applied onto DEAE-Sephacel
[b] Fractions containing minor decomposition products only were pooled

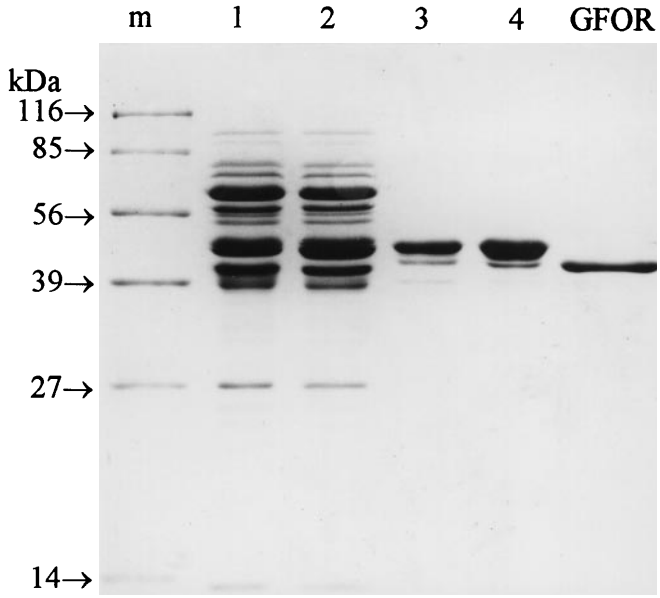


Fig. 7 SDS-PAGE of mutant protein GFORΔ32–46 purification. Cell-free extracts of strain ACM3963 (pZY570Δ32–46) were prepared by milling with glass beads, by ultracentrifugation, and by two rounds of ion exchange chromatography. *Far left lane* a protein size standard is given with molecular masses in kDa; *far right lane* purified GFOR from strain ZM6 is shown. *Lanes 1* cell-free extract, *2* after ultracentrifugation, *3* GFOR-active fractions after coupled anion/cation exchange chromatography, *4* after a second cation exchange chromatography

purified from the cell-free extract as outlined in Table 2. A protein with a subunit size of about 44,000 was obtained, which corresponded well with the expected enlargement by 37 amino acid residues (Fig. 7). A faint extra band that could be due to proteolysis was detected at 40,000. As can be seen from Table 2, Δ32–46 protein was obtained with a specific activity of 180 U/mg for the purified enzyme, which was in the range of purified GFOR (Zachariou and Scopes 1986; Loos et al. 1994b). Throughout the purification procedure, no NADP cofactor was added. The mutant protein showed the same fluorescence spectrum as the wild-type protein, and NADP could be liberated from the protein through denaturation using guanidinium hydrochloride (data not shown). Therefore, it can be assumed that Δ32–46 has NADP bound in a non-dissociable manner similar to that of wild-type GFOR.

Discussion

Protein export to the periplasmic space of gram-negative bacteria has up to now been studied mainly in *Escherichia coli* (for a recent review, see Pugsley 1993). There the involvement of several proteins of the general secretory pathway has been established. Typically, exported proteins carry an N-terminal signal sequence of 20–25 amino acid residues with distinct n-, h-, and c-regions (Von Heijne 1990), although a periplasmic enzyme from *Methylobacterium extorquens* with a signal sequence of 57 amino acid residues has been reported (Chistoserdov and Lidstrom 1991). Glucose-fructose oxidoreductase of *Zymomonas mobilis* is one of only two presently known periplasmic proteins of this organism (the other is gluconolactonase; Kanagasundaram and Scopes 1992b). Pre-GFOR carries a signal sequence of 52 amino acids, the last 20 of which are hydrophobic (Kanagasundaram and Scopes 1992a; Fig. 2). Pre-GFOR synthesized with an in vitro reticulocyte translation system has been shown earlier to be at least partially processed by membranes of *Z. mobilis* (Kanagasundaram and Scopes 1992a). To find out about the possible role of the unusually long signal sequence, we constructed deletions in both the n- and the h-regions. The removal of 19 residues from the n-region (Δ2–20) still led to complete maturation of GFOR as seen in Western blots; the kinetics of the respective pulse-chase assay, however, indicated a slower rate of processing. The removal of 15 residues of the h-region led to an unprocessed, albeit enzymatically fully active GFOR. This confirms that the h-region is essential for the export of pre-GFOR to the periplasm. Exchange of the genuine GFOR signal sequence with the other *Z. mobilis* signal sequence (from the gluconolactonase) allowed processing and maturation of GFOR. This we take for evidence that no specific signal sequence is needed to export pre-GFOR to the periplasm or to incorporate the cofactor NADP to obtain a functional GFOR enzyme. The inhibitory function of both CCCP and sodium azide on the processing of pre-GFOR is further evidence that export of pre-GFOR to the periplasm of *Z. mobilis* is dependent, at least partially, on the presence of a proton motive force and on SecA protein, respectively.

Previously it has been shown that glucose-fructose oxidoreductase acts in the provision of sorbitol as compatible solute for *Z. mobilis* wild-type cells (Loos et al. 1994a). In this paper we show that the sorbitol-negative mutant ACM3963 lacks GFOR activity and displays only faint cross-reacting immunological material. Using this stable deficient mutant, a series of *gfo* alleles could be expressed and assayed for their physiological function. Δ32–46 protein, although enzymatically fully active, was not processed and, therefore, stays in the cytoplasm. *Z. mobilis* strain ACM3963/pZY414Δ32–46 formed no sorbitol in a medium with 1 M sucrose. Without sorbitol addition, this strain showed a growth behavior similar to that of the *gfo*-deficient strain and drastically different from the pZY414 and pZY414Δ2–20 alleles. The essence of the periplasmic location of GFOR may be the simultaneous availability at saturating concentrations of both reactants, glucose and fructose (from sucrose), for sorbitol production. As shown recently, the sugar transport system (GLF) of *Z. mobilis* accepts both sugars and transports them at high rates. However, glucose is preferred over fructose and acts as an effective inhibitor (K_i of about 2 mM) of fructose uptake (Weisser et al. 1995). This might prevent saturating intracellular concentrations of fructose (K_m of GFOR for fructose is about 400 mM; Zachariou and Scopes 1986) in the presence of glucose. This would result in only residual sorbitol formation in the cytoplasm below the threshold that is necessary to act as osmopro-

tective compatible solute. The periplasmic location of GFOR also raises the problem of NADP export to this cell compartment, which contains phosphatases (Pond et al. 1989) that would destroy freely soluble NADP. One reason for the firm binding of NADP to GFOR might, therefore, be to protect the valuable cofactor from degradation. NADP can bind already to pre-GFOR, as shown earlier with purified pre-GFOR from crude extracts (Loos et al. 1993) and in this paper with the purified Δ32–46 GFOR mutant protein. Whether the cofactor is exported together with the pre-GFOR to the periplasm or is exported separately to the periplasm needs to be shown in further experiments. From *Myxococcus xanthus* the NAD(P)-containing extracellular protein CsgA is known, but the mode of NAD(P) export from the cytosol has not yet been resolved (Lee et al. 1995). Our results indicate that the signal sequence of GFOR cannot be an essential factor in NADP export as it can be deleted in the n-region or even exchanged for a different signal sequence.

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