Split invertase polypeptides form functional complexes in the yeast periplasm in vivo

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ABSTRACT The assembly of functional proteins from fragments in vivo has been recently described for several proteins, including the secreted maltose binding protein in Escherichia coli. Here we demonstrate for the first time that split gene products can function within the eukaryotic secretory system. Saccharomyces cerevisiae strains able to use sucrose produce the enzyme invertase, which is targeted by a signal peptide to the central secretory pathway and the periplasmic space. Using this enzyme as a model we find the following: (i) Polypeptide fragments of invertase, each containing a signal peptide, are independently translocated into the endoplasmic reticulum (ER) are modified by glycosylation, and travel the entire secretory pathway reaching the yeast periplasm. (ii) Simultaneous expression of independently translated and translocated overlapping fragments of invertase leads to the formation of an enzymatically active complex, whereas individually expressed fragments exhibit no activity. (iii) An active invertase complex is assembled in the ER, is targeted to the yeast periplasm, and is biologically functional, as judged by its ability to facilitate growth on sucrose as a single carbon source. These observations are discussed in relation to protein folding and assembly in the ER and to the trafficking of proteins through the secretory pathway.

Upon entry into the endoplasmic reticulum (ER), newly synthesized proteins undergo rapid folding and assembly to acquire their functional tertiary and quaternary structure. The ER provides the appropriate environment and components that are needed to facilitate the functional assembly of translocated proteins. From the ER, proteins are distributed to various destinations in the cell via the central secretory pathway. Nascent secretory proteins are delivered to the lumen of the ER, pass through the Golgi complex, and then accumulate in specialized secretory vesicles, which fuse with the cell surface, allowing exocytosis. Movement of the proteins between these compartments occurs by budding and fusion of transporting vesicles (1, 2), and the current view is that secreted proteins will follow this pathway unless retained or diverted by specific signals in their sequence or structure. Since proteins must fold and assemble correctly to leave the ER (3), secretion out of the cell is an indication of its ability to fold in the ER.

Our current understanding of protein folding and assembly has been enhanced by the classical approach of protein fragment assembly in vitro, which allows the examination of intermediates in the folding process. For example, in vitro complementation of various combinations of overlapping fragments of staphylococcal nuclease and cytochrome c are the well-known models of this approach (4, 5). Many experiments of this sort, involving functional complementation in vitro, have been performed either with fragments produced by limited proteolysis, by chemical cleavage, or by using incomplete polypeptide chains expressed via genetic manipulation. The assembly of functional proteins from fragments in vivo has been recently demonstrated for several proteins. In fact, Bibi and Kaback (6) initiated a series of studies to exploit this possibility. When the lactose permease of Escherichia coli was expressed as two approximately equal-size fragments, an association between the two polypeptides led to a stable, catalytically active complex. To date, examples of other functional split genes, mainly of membrane, but also a few soluble cytoplasmic proteins, have been described (for example, refs. 7–9). More recently, independently exported protein fragments of the maltose binding protein have been shown to assemble in vivo into an active complex in the periplasm of E. coli (10). These studies have improved our understanding of protein folding and structure in vivo. Nevertheless, a study of split gene product assembly in the ER, which is the folding compartment of exported proteins, has not been undertaken.

Here we study a model system in which independently expressed fragments of the enzyme invertase are secreted into the ER, glycosylated, and assembled into an enzymatically active and biologically functional complex reaching the yeast periplasm, the endmost subcellular target of the secretory pathway.

MATERIALS AND METHODS

Strains, Media, and Growth. The Saccharomyces cerevisiae strains used were DGY505 (MATa, his4, ura3, trp1, ade2, sec2-?); kindly provided by D. Granot, The Volcani Center, Israel), DMM1-15A (MATa, leu2, his3, ura3, ade2; ref. 11), JTY-5186 (sec18, leu2, ura3; ref. 11), BJ1216 (pnp4, prc1-407, prb-1122, leu2, ura3, trp1; provided by Y. Ben Neriah, The Hebrew University, Jerusalem), 8979-3A (kar2-1, leu2, ura3, his4, ade2, CAN4; ref. 11), SEY5018 (sec1, leu2, ura3, ?sec2; provided by R. Schekman), and RSY586 (kar2-159, leu2, ura3, ade2; provided by R. Schekman). The following were obtained by the indicated cross: OSH4 (kar2-2159, leu2, ura3, trp1, sec2-?), RSY586 × DGY505; OSH5 (sec1, leu2, ura3, trp1, sec2-?); SEY5018 × DGY505; OSH7 (sec1, leu2, ura3, trp1, sec2-?), JTY5186 × DGY505; and OSH9 (kar2-1, leu2, ura3, trp1, ade2, his3, sec2-?), 8979-3A × DGY505. The growth medium (SD) used contained 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco) and 0.5–2.0% (wt/vol) glucose, galactose, or sucrose.

Preparation of Cell Extracts and Cell Fractionation. Extracts were prepared by growing 10 ml of yeast culture to 3.0 optical density units (at 600 nm) on SD medium at 25°C, harvesting the cells by centrifugation, and suspending them in 800 µl of TE buffer (10 mM Tris–HCl buffer, pH 8.0/1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were broken by vigorous mixing with glass beads for 1.5 min followed by centrifugation at 4°C for 4 min. The supernatant fraction obtained was used for all assays and analyses.

Abbreviations: ER, endoplasmic reticulum; endo H, endoglycosidase H.

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Subcellular fractionation using Zymolyase-20T (Siekagaku Kogyo, Tokyo) was performed as described (11). Protease inhibitors were added at the following final concentrations: α-macroglobulin, 2 units/ml; antipain, 5 μg/ml; pepstatin, 1 μg/ml; aprotinin, 5 μg/ml; leupeptin, 2 μg/ml; PMSF, 10 mM; and EDTA, 5 mg/ml. Membrane and soluble fractions of the spheroplasts were prepared by centrifugation at 120,000 × g for 90 min at 4°C in a Beckman L5-50 ultracentrifuge. The soluble fraction was removed, and the membrane pellet was resuspended in 0.5 ml of the same buffer.

Enzyme Activities and Assays. Invertase activity was based on the determination of reducing sugars (12), and protein was determined by the method of Bradford (13). For activity in whole cells, half of a cell suspension was used to make an extract as described above, and the remainder (whole cells) was treated with 0.01% NaN₃. Invertase activity of both extract and whole cells was determined by incubation in citrate buffer (pH 5) containing 1% sucrose for 2 h at 30°C and removal of cells and debris by centrifugation, followed by determination of reducing sugars. To assay invertase activity at different temperatures, cultures were grown at 22°C, and samples of their extracts were incubated in citrate buffer (pH 5) containing 8% sucrose for 2 h at 22°C, 30°C, 37°C, 50°C, or 60°C. Invertase activity was determined by the Sommer method.

To detect invertase activity on gels, nondenatured extracts were separated by PAGE (7% polyacrylamide, 0.1% sarcosyl) for 4–5 h at 4°C. The gel was incubated in 0.1 M sodium acetate (pH 4.6) containing 0.1 M sucrose at 30°C for 30 min and then stained with 0.1% 2,3,5-triphenyl tetrazolium chloride in 0.5 M NaOH at 100°C (14).

DNA Manipulations. Plasmid pTInv was created by destroying the EcoRI site of plasmid ptT7-5 (15) and cloning of the Sall–DraI fragment of plasmid pSEY304 (16) into the Sall–SmaI sites of the ptT7-5 derivative. pTInv was used for creating the following plasmids. (i) pT-EH(C135): pTInv was cleaved with EcoRI and HpaI, treated with Klenow and ligated. (ii) pT-ES(C211): pTInv was cleaved with EcoRI and Styl, treated with Klenow, and ligated. The resulting plasmid was cleaved again with EcoRI, treated with Klenow, and ligated. (iii) pT-EX(C256): pTInv was cleaved with EcoRI and XbaI, treated with Klenow, and ligated. The resulting plasmid was cleaved again with EcoRI, treated with Klenow, and ligated. (iv) pT-AH(N378): pTInv was cleaved with HpaI and AgeI, treated with Klenow and ligated. (v) pT-X(N258): pTInv was cleaved with XbaI, treated with Klenow, and ligated.

Plasmid pEF1 contains the PGK promoter on an EcoRI–BglII fragment (of plasmid pMA91; ref. 17) cloned into the EcoRI and BamHI sites of plasmid pSEY304 (16). Plasmids pRS-PGK424 and pRS-PGK426 were created in two steps. The first was the cloning of the EcoRI–XbaI small fragment of pEF1 containing the PGK promoter and part of the SUC2 into the EcoRI and SpeI sites of pRS424 and pRS426 (18). The second step was destroying the extra Sall site of the resulting plasmids by cleavage with XhoI and EcoRI, treatment with Klenow, and ligatation.

Plasmids pRS-PGK-Inv, pRS-PGK-EH(C135), pRS-PGK-ES(C211), pRS-PGK-EX(C256), pRS-PGK-AH(N378), and pRS-PGK-X(N258) were constructed by cloning of the Sall–SacI fragments of the respective pTInv derivatives into either pRS-PGK-424 and/or pRS-PGK-426. pRS-PGK-InvASP and pRS-PGK-ES(C211)ASP were created by cleaving pRS-PGK-Inv and pRS-PGK-ES(C211) with Sall and EcoRI, treating with Klenow, and ligatation. Plasmid YEg-GAL-ES(C211) was constructed by cloning the Sall–SacI fragment of pRS-PGK-ES(C211) into pRH3 (19).

Total Protein Extraction and Immunoblot Analysis. For Western blot analysis, cell extracts were boiled in endoglycosidase H (endo H) denaturing buffer (0.5% SDS/1% 2-mercaptoethanol) and split, and half the sample was subjected to endo H treatment for 2 h at 37°C. The samples were electrophoresed on SDS/10% polyacrylamide gels, transferred to nitrocellulose, and probed with rabbit anti-invertase antisera (20).

Electroelution. Triplicate samples of nondegenerated extract were fractionated as above for invertase activity detection, Coomassie blue staining, and electroelution of protein. The activity-stained portion of the gel was excised for electroelution, which was carried out in a dialysis bag in gel running buffer (without detergent) at 100 V for 5–6 h at 4°C. Electroeluted samples were concentrated [15-ml Vivaspin containers (Vivascience, Lincoln, U.K.) for 20 min at 1960 × g], denatured, and treated with endo H for 3 h at 30°C.

RESULTS

Construction of Split Invertase Genes. We have demonstrated that the E. coli lipoprotein signal sequence is functional in secretion of proteins in S. cerevisiae (11, 19, 21–23). Processing of the lipoprotein signal peptide in yeast occurs at a unique site (between Cys-21 and Ser-22), which is one residue from the signal peptidase II processing site in E. coli (11). To allow a similar targeting of N- and C-terminal fragments of invertase in yeast, we have used the lipoprotein signal peptide. The vectors that we have created, termed the PRS-PGK-lpp series, harbor either the URA3 or TRP1 genes, allowing the selection of strains harboring combinations of vectors each encoding a different polypeptide. These vectors each contain the phosphoglycerate kinase (PGK) promoter and, downstream to it, encode the lipoprotein signal peptide, followed by an EcoRI site and the entire mature invertase-encoding sequence. When the lipoprotein signal peptide is used for expression and secretion, 13 aa of mature lipoprotein and linker sequences are added to the processed form of the expressed protein. The invertase N- or C-terminal coding sequences were cloned in-frame with the signal peptide coding sequence between the EcoRI and SacI sites in this vector.

Schematically presented in Fig. 1 are two N-terminal fragments [AH(N378) and X(N258)] containing 378 and 258 aa from the invertase N terminus, respectively, and three C-terminal fragments [EH(C135), ES(C211), and EX(C256)] containing 135, 211, and 256 aa from the C terminus, respectively. These fragments are expressed as hybrids with the lipoprotein signal peptide and under the PGK promoter as described above. In addition, for control experiments, plasmids were created encoding the mature invertase protein sequence or the ES(C211) fragment both lacking signal peptides [InvASP and ES(C211)ASP, respectively]. S. cerevisiae (strain DGY505) harboring a chromosomal deletion of SUC2 were transformed with the various invertase plasmid constructions.

![Fig. 1](image-url)
Expression, Glycosylation, and Secretion of Invertase Fragments. Two complementing constructs, which represent C- and N-terminal truncated forms of invertase [AH(N378) and ES(C211), respectively], were chosen for studying the fate of independently expressed invertase fragments. Expression of invertase fragments was examined by SDS/PAGE and immunoblotting of whole cellular extracts with anti-invertase antibodies. As shown in Fig. 2, the AH(N378) deglycosylated fragment appeared as a major 45-kDa band with minor lower bands, which probably represent degradation or truncated translation products (lane 4). These forms also appear in strains defective for vacuolar proteases such as PEP4 (data not shown). The deglycosylated ES(C211) fragment appeared as a single 21-kDa band (lane 5). Both fragments were clearly detected in extracts expressing the combination (lane 2), and each was significantly smaller than Inv, the unfragmented mature invertase (compare lanes 3 and 4 to lane 5). Essentially, no invertase immunoreactive material was detected in extracts of the chromosomally deleted suc2-A9 strain, DGY505, which was used as the host strain for all our experiments (lane 1).

Core glycosylated forms of secretory proteins, such as invertase, are assembled in the ER and are further modified by addition of outer chain carbohydrates during transit through the Golgi body. Thus, glycosylation serves as an indication of secretion (24, 25). Our invertase antisera recognizes several nonspecific glycosylated proteins in yeast cell extracts (Fig. 2, lane 12), yet specific invertase bands are clearly detected on that background (compare lanes 7–11 with lane 12). Both the AH(N378) and ES(C211) polypeptides are glycosylated as concluded from the extensive changes in their electrophoretic mobility following deglycosylation with endo H (Fig. 2; for polypeptide ES(C211) compare band d, lanes 2 and 3 to band d', lanes 10 and 11; for polypeptide AH(N378) compare band c, lanes 2 and 4 to c', lanes 9 and 11). In contrast, invertase lacking a signal peptide (InvASP) is not targeted to the ER and not modified, and therefore it exhibits no change in its electrophoretic mobility upon treatment with endo H (band b, lane 6, and band b', lane 7). The fact that AH(N378) and ES(C211) are glycosylated provides evidence for their translocation into the ER.

To examine if invertase fragments exit the ER and follow the general secretory pathway, subcellular fractionation experiments were conducted. Zymolase was used to digest the cell wall in fractionation experiments (19) in which we found that the split gene products are extremely sensitive to proteolysis (Fig. 3; and data not shown). The major invertase immunoreactive bands found in whole cell extracts are those of AH(N378) and ES(C211) (Fig. 3, lane 6, bands a and b, respectively). Periplasmic fractions prepared in the presence of various protease inhibitors contain only small amounts of the full-length AH(N378) polypeptide and barely detectable amounts of the ES(C211) polypeptide, whereas these preparations contain significant amounts of invertase degradation products (lanes 3–5). This degradation is probably the result of known proteolytic activity in Zymolase enzyme preparations used for the fractionation procedure (26). In fact, the spheroplast fraction, in contrast to the periplasmic fraction, is protected by the membrane from this proteolytic activity (compare lane 2 with lanes 3–5). Under the same conditions, the unfragmented invertase (Inv) is targeted to the periplasm and is very stable, whereas the signal peptide lacking invertase (InvASP) is localized to the cytosol (data not shown). From these results, we conclude that a significant amount of the split invertase gene products reach the yeast periplasm, yet a similar amount is retained within the spheroplast. It is important to note that the localization and appearance of the AH(N378) polypeptide in subcellular fractionation experiments is the same regardless of whether it is expressed in combination with the ES(C211) or on its own (data not shown).

Enzymatic Activity. Among the six possible combinations of pairs of ER-targeted N- and C-terminal invertase fragments, only two exhibit enzymatic activity (Table 1). Cell extracts of S. cerevisiae DGY505 strains expressing fragment AH(N378) and either fragment ES(C211) or EX(C256) exhibit detectable enzymatic activity; however, the AH(N378)/ES(C211) combination displays a much higher activity than that of AH(N378)/EX(C256). Both cellular levels of activity and the amount of invertase polypeptides in strains expressing the AH(N378)/ES(C211) combination are much lower than the corresponding levels and amounts found in strains expressing the full invertase gene (Inv). In particular, the amounts of the

![Fig. 2. Immunodetection of glycosylated and deglycosylated invertase split gene products. Strains containing a chromosomal deletion of SUC2 and harboring the indicated plasmids were grown in glucose standard medium, and cell extracts were prepared. These were deglycosylated by treatment with endo H (Endo H +) or untreated (glycosylated, Endo H –) and subjected to SDS/PAGE and immunoblotting using anti-invertase antibodies. Gel order is according to invertase polypeptides expressed, as indicated above each lane. Arrows and letters on both sides of the gel indicate positions of deglycosylated and glycosylated forms of invertase polypeptides, respectively, as follows: Inv, a and a'; InvASP, b and b'; AH(N378), c and c'; and ES(C211), d and d'. Black dots at the center of the gel indicate the position of molecular mass markers: top, 84 kDa; middle, 48 kDa; and bottom, 27 kDa.](image1)

![Fig. 3. Cellular location of invertase polypeptides in S. cerevisiae. Cultures of S. cerevisiae (DGY505) expressing the combination AH(N378)/ES(C211) were grown in glucose medium, and the cells were fractionated into periplasm and spheroplasts. Fractions were analyzed by immunoblotting as in Fig. 2. Gel order was as follows: lane 1, total cell extract of the control culture (strain DGY505) that is devoid of invertase polypeptide expression; lane 2, spheroplast fraction of cells expressing the AH(N378)/ES(C211) combination; lanes 3, 4, and 5, periplasmic fragments of cells expressing the AH(N378)/ES(C211) combination prepared in the absence of protease inhibitors (lane 3), in the presence of antipain, pepstatin, aprotinin, leupeptin, and PMSF (lane 4), and in the presence of macroglobulin (lane 5); and lane 6, total extract of the strain expressing the AH(N378)/ES(C211) combination. Arrows indicate positions of the AH(N378) (a) and ES(C211) (b) polypeptides. Black dots to the right of the gel indicate 48-kDa (top), 36-kDa (middle), and 27-kDa (bottom) molecular mass markers.](image2)
Table 1. Invertase activity and growth on sucrose

<table>
<thead>
<tr>
<th>Polypeptide(s) expressed</th>
<th>Invertase activity*</th>
<th>Growth on sucrose†</th>
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<tr>
<td>Inv</td>
<td>+</td>
<td>+</td>
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<tr>
<td>InvΔSP</td>
<td>+</td>
<td>-</td>
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<tr>
<td>AH (N378) plus EH (C135)</td>
<td>-</td>
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<tr>
<td>AH (N378) plus EX (C256)</td>
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<td>AH (N378) plus ES (C211)</td>
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<tr>
<td>AH (N378) plus ES (C211)ΔSP</td>
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<tr>
<td>X (N258) plus any fragment</td>
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<td>Any single fragment</td>
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*Activity was measured by the Sommer method for determination of reducing sugars. A + indicates a >100-fold higher activity than a −. (See text for a description of the differences in the specific activity between full-length and fragmented invertases).
†A + indicates at least a 10-fold increase in culture optical density at 600 nm after 35-40 h of growth (see Fig. 4).

ES(C211) fragment in cells expressing this combination is much lower than the corresponding level of the full invertase. By determining enzymatic activities together with quantitative densitometric analysis of Western blots with varying amounts of cell extracts, the estimated specific activity of the AH(N378)/ES(C211) combination is ∼7% of that found for the full invertase.

As expected, the expression of mature invertase lacking a signal sequence (InvΔSP) results in high enzymatic activity in cell extracts. This activity is intracellular, whereas the activity exhibited by Inv (containing a signal peptide) is located mainly in the periplasm (see below). In contrast to the AH(N378)/ES(C211) combination, the expression of ES(C211)ΔSP together with AH(N378) does not result in detectable enzymatic activity, indicating that secretion of two functionally compatible fragments of a pair into the same compartment (ER) is a prerequisite for obtaining enzymatic activity. This conclusion is supported by the finding that the assembly is impaired in a kar2-159 temperature-sensitive mutant that is defective for protein translocation into the ER at the restrictive temperature. We find that the activity of the AH(N378)/ES(C211) combination induced at the restrictive temperature in a kar2-159 mutant is <35% of the activity found at the permissive temperature. This kar2-159 strain, and those described below, contain in addition to a plasmid with a constitutive promoter for expression of the large fragment (pRS-PGK-AH), a plasmid with an inducible GAL10 promoter for expression of the small fragment (YEp-GAL-ES). Induction is obtained by growth in galactose medium. In contrast to the effect of kar2-159 mutation on invertase fragment assembly, the corresponding sec18 (allows an ER to Golgi block) or sec1 (allows a secretory vesicle to periplasm block) mutant strains exhibit a wild-type degree of assembly at the restrictive temperature, which is higher than at the permissive temperature. Thus, while invertase fragment entry into the ER is required for assembly, transfer to the Golgi or the periplasm are not. These results indicate that the assembly of invertase fragments occurs in the ER.

To allow the direct demonstration of invertase activity in the periplasm of yeast cells expressing split genes, activity was measured in whole cells vs. total cellular extracts. The rationale behind this approach is that only periplasmic (and not intracellular) invertase should contribute to activity measured in cell extracts. Invertase lacking the signal peptide (InvΔSP) exhibits low activity in whole cells when compared with its activity in cell extracts, whereas invertase containing a signal peptide (Inv) exhibits similar levels of activity in the two. The combination AH(N378)/ES(C211) displays a significant level of activity in whole cells, although this activity is only 40-60% of that found in cell extracts. These results indicate that a significant portion of the invertase fragments expressed in yeast reach the periplasm and form an active enzyme complex.

**Growth on Sucrose.** To examine if complexes of invertase fragments in the periplasm were biologically functional, cultures were grown on sucrose as the single carbon source. Of the various possible combinations of fragments, only simultaneous expression of the AH(N378)/ES(C256) or the AH(N378)/ES(C211) pairs (which exhibit invertase activity) enable growth of yeast cells on sucrose (Table 1). Cells expressing the AH(N378)/ES(C211) combination were capable of efficient growth in sucrose medium, whereas cells expressing individual fragments or no fragments did not grow (Fig. 4). There was no difference in the growth of any of these strains in glucose medium (data not shown). The growth of the cells expressing the AH(N378)/ES(C211) combination was somewhat slower than the growth of cells expressing the unfragmented mature invertase, probably due to the much lower invertase activity of the combination in the yeast periplasm. Consistent with this notion is the finding that upon transfer into sucrose medium, yeast cells expressing mature invertase without a signal peptide (InvΔSP) show essentially no growth for 20 h and only very weak growth thereafter (Fig. 4). The level of enzymatic activity in extracts of cells expressing InvΔSP is at least 10-fold higher than cells expressing the AH(N378)/ES(C211) combination, yet they display poor growth when compared with the latter. Since it is without a signal peptide, the InvΔSP enzyme is not targeted to the periplasm and therefore cannot cleave extracellular sucrose. These results clearly indicate that a complex between AH(N378) and ES(C211) remains active during transit through the secretory pathway and release into the periplasm.

The possibility of producing a full-length invertase from a combination of split genes by genetic recombination can be ruled out, since extracts of cells, grown on sucrose, produce distinct AH(N378) and ES(C211) polypeptides (data not shown), and no protein species at the full mature invertase size can be detected (the pattern obtained is essentially identical to lane 3 of Fig. 2).

**Association Between Fragments.** In a control experiment, extracts prepared from cultures expressing either AH(N378) or ES(C211) were combined, and the mixture was assayed for invertase activity. No invertase activity was detected even though both the AH(N378) and ES(C211) fragments can be detected in the mixture at levels similar to those found in cells expressing both fragments simultaneously (Fig. 2, lanes 2-4). To obtain enzymatic activity, both fragments must therefore

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**Fig. 4.** Growth of *S. cerevisiae* strains on sucrose as the single carbon source. Yeast cells harboring the appropriate plasmids were incubated at 25°C in sucrose (1%) medium, and cell growth was monitored by optical density at 600 nm. Symbols indicating the invertase polypeptides expressed by the respective strains: △, Inv; ■, AH(N378)/ES(C211); ○, InvΔSP; ●, AH(N378); ○, ES(C211); and □, none (strain DGY505 with no plasmids).
be expressed simultaneously in the same cell, indicating that a complex is formed in vivo.

To examine the association between the split gene products, nondenatured extracts prepared from cells expressing the AH(N378)/ES(C211) combination were subjected to PAGE, with the detergent sarcosyl instead of SDS. The reason for this is that invertase activity of the combination is not destroyed in the presence of sarcosyl (0.1%), whereas even very low concentrations of SDS demolish its enzyme activity. After incubation of the gel in sucrose (0.1 M), invertase activity was assayed by staining with triphenyl tetrazolium chloride (14). As expected, the strains expressing mature invertase (Inv; Fig. 5A, lane 5) displayed invertase activity, whereas no activity was detected in strains expressing single fragments (lanes 3 and 4). For the combination, active enzyme was detected at a position corresponding to a high molecular weight (Fig. 5A, lanes 1 and 2). Electroelution of the active species from the gel, denaturation and deglycosylation of the eluates followed by SDS/PAGE, and immunoblotting detected both the AH(N378) and ES(C211) fragments, suggesting that they are in a high molecular weight active complex (Fig. 5B, compare lane 5 to lanes 2–4). Coomassie blue staining of a gel containing the combination before and after electroelution (corresponding to samples in lanes 4 and 5 in Fig. 5B) is shown in Fig. 5C (lanes 1 and 2, respectively).

After electroelution, the AH(N378) fragment appeared predominantly as a smaller cleaved species (Fig. 5B, lane 5). In this regard, the invertase fragments [AH(N378)/ES(C211)] are highly susceptible to proteolytic degradation in sharp contrast to unfragmented mature invertase, which is very stable (data not shown). Indeed, this observation is consistent with the suggestion that the complex is comprised of two polypeptide fragments, which are more sensitive to proteolytic degradation than the native mature protein.

The same is true with respect to the sensitivity of the complex to temperature as measured by changes in enzymatic activity. Extracts of cells expressing Inv, InvΔSP, and the combination AH(N378)/ES(C211) were assayed for invertase activity at different temperatures (Fig. 6). Whereas Inv and InvΔSP activity increased with temperature up to 60°C, the combination lost 50% of its activity at 50°C and essentially all the activity at 60°C (Fig. 6). In addition, in experiments not shown, a complete loss of enzyme activity was observed when extracts of cells expressing the combination AH(N378)/ES(C211) were exposed to low concentrations of SDS (e.g., 0.1%), in contrast to mature unfragmented invertase, whose activity is hardly affected. These results are consistent with the dissociation of the complex with increasing temperature or SDS concentrations and suggest diminished structural stability.

**DISCUSSION**

In this paper, we show that when overlapping fragments of invertase are simultaneously expressed in yeast, they (i) are independently translocated into the ER, (ii) are modified by glycosylation, and (iii) travel through the entire secretory pathway reaching the yeast periplasm. The AH(N378) fragment of invertase is translocated into the ER and reaches the periplasm, regardless of whether it is expressed simultaneously or separately with ES(C211). This conclusion is based on the finding that all the AH(N378) molecules are glycosylated and that we do not detect any unmodified fragments in samples not treated with endo H. In addition, fractionation experiments of cells expressing the AH(N378) alone or its combination with ES(C211) show that in both cases a significant portion of these polypeptides reaches the periplasm. In this regard, invertase fragments behave differently than other incorrectly folded mutant, truncated, or heterologous proteins, which are retained and/or degraded in the ER (27–31). For example, a study dealing with bacterial toxin EtxB assembly in yeast showed that all the toxoid oligomers are confined to the ER and essentially no oligomeric toxoid (or its activity) can be detected in the periplasm (19). It is also interesting to compare secretion of single invertase fragments, which does not depend on assembly, with immunoglobulin assembly and secretion, in which exit of the heavy chain from the ER depends upon its association with the light chain (32, 33). The molecular basis for this latter phenomenon has been worked out, showing that the heavy chain is complexed with the molecular chaperone BiP and is thereby retained in the ER until interaction with the light chain releases BiP and allows exit of the immunoglobulin from the ER (32, 33).

A major conclusion from this study is that simultaneously expressed complementing fragments of invertase are translo-
cated into the ER and assembled into an enzymatically active complex. Experiments analyzing secretory mutants strongly suggest that assembly of invertase fragments occurs in the ER. These results are reminiscent of those of Betton and Hofnung (10) in the prokaryotic system, in which they have demonstrated translocation of maltose binding protein fragments through the bacterial plasma membrane and their functional assembly in the periplasm of *E. coli*, a compartment which is functionally analogous to the ER. However, here we show that, in the eukaryotic system (represented here by yeast), split gene products traveling through the secretory pathway form an active complex that reaches its final subcellular destination, which is outside the cell plasma membrane. One question that remains to be resolved in the eukaryotic system is whether assembly of split invertase occurs before, during, or after glycosylation. We know that separately expressed fragments are glycosylated, but the question of its timing with respect to assembly remains open.

This study opens the way for examination of cellular factors required for the assembly of invertase fragments, a process that may also be related to the folding of the wild-type enzyme. In this regard, Bip/KAR2 (30, 34–36) was an obvious candidate, since it has been shown to be required in vivo for the assembly of multisubunit complexes, such as the bacterial EtxB toxoid in yeast (19) and immunoglobulin assembly in higher eukaryotes as discussed above. For that reason we have examined a kar2-1 temperature-sensitive mutant, which allows protein translocation into the ER and secretion at the restrictive temperature (and differs from kar2-159, which has a null phenotype). Surprisingly, in preliminary experiments analogous to those previously performed with EtxB, we find that in contrast to the toxoid assembly that is blocked at the restrictive temperature in the kar2-1 mutant strain, functional invertase fragment assembly is not affected by the kar2-1 mutation. Since invertase and its fragments are glycosylated, it will be intriguing to examine if other chaperones such as calnexin and calreticulin, which interact with glycoproteins, may be involved (37, 38).

The oligomers of invertase fragments and EtxB in yeast also differ with respect to stability: whereas the polypeptides of the AH(N378)/ES(C211) combination are extremely sensitive to proteinase K treatments (19), the fragments of invertase associate to obtain enzymatic activity, the compactness of the complex's structure is distinct from that of the native enzyme. This conclusion is also supported by the marked temperature and SDS sensitivity of the AH(N378)/ES(C211) complex vs. that of the unfragmented invertase.

Native invertase has been reported to exist in vivo as oligomers (dimers, tetramers, and octamers;refs. 39 and 40). It will be interesting to determine whether analogous oligomeric complexes are formed with split invertase fragments. In this regard, expression of the AH(N378) fragment separately caused its accumulation in a high molecular weight material (data not shown), similar to the AH(N378)/ES(C211) combination (Fig. 5). This is in contrast to ES(C211), which does not appear in high molecular weight form when it is expressed on its own. Thus, it is possible that sites responsible for invertase oligomerization may reside in its 378-aa N-terminal sequence.

Of particular importance is the finding that active split invertase complexes are actually secreted into the periplasm, enabling growth on sucrose of suc mutants. This phenotype can be exploited in the future for selecting mutants that are either enhanced or incapable of supporting the formation of active split invertase complexes, thus possibly leading to the identification of new cellular components required for the assembly process.

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