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Engineering the quaternary structure of an exported protein with a leucine zipper

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The leucine zipper of the yeast transcriptional factor GCN4 was grafted to the C-terminal amino acid of the maltose binding protein (MalE) by fusing the *malE* gene of *Escherichia coli* to a synthetic gene coding for the leucine zipper. The hybrid protein, MalE-Lzp, was synthesized in large amounts from multicopy plasmids and efficiently exported into the periplasmic space of *E.coli*, up to 200 000 molecules per cell. Unlike hybrids between MalE and other proteins, MalE-Lzp was quite stable exhibiting only minimal degradation. The hybrid was purified from a periplasmic extract in one step by affinity chromatography on cross-linked amylose. Sedimentation velocity and gel filtration experiments showed that MalE-Lzp existed as a dimer in conditions where MalE was a monomer, at all concentrations tested down to 0.1 μM. Thus, it was possible to engineer the quaternary structure of an exported, monomeric protein by using a structural motif taken from a natural protein.

Key words: Escherichia coli/hybrid protein/maltose binding protein/protein design/protein dimer

Introduction

Protein design takes different approaches, according to its goals. When designing active sites, to obtain new functions or understand the mechanisms of catalysis, one generally uses existing structural scaffolds, as in the conversion of one enzyme into another by mutagenesis or the selection of catalytic antibodies. When creating three-dimensional structures *de novo* to test the principles that govern the stability or folding of proteins, one uses typical structural patterns, e.g. coiled coils or bundles of α-helices, β- or α/β-barrels (Richardson and Richardson, 1989).

A third approach to protein design, close to the one that evolution follows (Go and Nosaka, 1987), seems feasible. In this approach, structural or functional motifs coming from natural proteins are assembled to create new structures or combine functions. As a prerequisite to this approach, one has to demonstrate that such motifs can be extracted from their natural environment, while maintaining their properties. In this work, we tested this modular approach by using a leucine zipper (Lzp) to dimerize a periplasmic protein of *Escherichia coli*.

We have chosen the leucine zipper of protein GCN4 because it is well characterized structurally. GCN4, an activator of transcription in yeast, binds DNA as a dimer (Hope and Struhl, 1987). The DNA binding domain consists of two regions. A region of 29 amino acid residues, which is located at the C terminus of GCN4 and includes a leucine every seven residues, is responsible for dimer formation and has been termed the 'leucine zipper'. Equilibrium sedimentation experiments have shown that a synthetic peptide 33 residues long, corresponding to the leucine zipper, can form dimers in a concentration range from 10 μM to 2 mM. CD and NMR experiments have shown that this peptide forms a coiled coil of two parallel α-helices (O'Shea *et al.*, 1989a; Oas *et al.*, 1990). The region responsible for specific DNA binding is immediately NH_2-terminal to the leucine zipper (Talanian *et al.*, 1990). This region has a characteristic sequence rich in basic amino acids and has been termed the basic region. The leucine zipper and the basic region are linked together by a peptide of six residues. It has been proposed that the two polypeptide chains diverge, producing a fork upstream of the leucine zipper (Vinson *et al.*, 1989; O'Neil *et al.*, 1990; Weiss *et al.*, 1990).

We have developed a protein vector which is based on the maltose binding protein (MalE) of *E.coli* (Bedouelle and Duplay, 1988; Blondel and Bedouelle, 1990). MalE is an abundant, medium-sized (370 residues) protein which is encoded by the *malE* gene (Duplay *et al.*, 1984). Its expression is controlled by *malEp*, a strong promoter which is activated by protein MalT in the presence of maltotriose and repressed by glucose (Bedouelle *et al.*, 1982; Bedouelle, 1983; Raibaud *et al.*, 1989). It is exported into the periplasmic space by means of an N-terminal signal peptide which is cleaved during the export process (Bedouelle *et al.*, 1980). MalE binds maltose and maltodextrins with dissociation constants (K_d) around 1 μM and can be purified by affinity chromatography on cross-linked amylose. The elution is performed by competition with free maltose (Kellermann and Ferenci, 1982). MalE is normally a monomeric protein (Richarme, 1982).

In this paper, we report the construction and characterization of a hybrid between MalE and the leucine zipper of GCN4. We show that the hybrid protein, MalE-Lzp, was efficiently exported into the periplasmic space of *E.coli* and present in a dimeric state at all concentrations tested (0.1–30 μM).

Materials and methods

Media, vectors and strain

Plasmids pBR322 (Backman, 1986), pEMBL8$^+$ (Dente and Cortese, 1987), pPD1 (Duplay *et al.*, 1984), pAB1 and the media (Blondel and Bedouelle, 1990) have been described. The complete genotype of strain PD28 (*malT^{c}1, ΔmalE444, recA*) can be found in Duplay *et al.* (1987).

Construction of the lzp gene fragment

A double-stranded DNA fragment coding for the leucine zipper of GCN4 was assembled from six overlapping oligonucleotides. They were prepared using an Applied Biosystems DNA synthesizer and purified by PAGE as described (Labouze and Bedouelle, 1989). Four internal oligonucleotides (100 pmol each) were individually phosphorylated in 20 μl reaction mixtures containing 50 mM Tris–Cl, pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP and 10 units of T4-polynucleotide kinase at 37°C for 45 min. The kinase was then heat inactivated at 70°C for 10 min. The four phosphorylated oligonucleotides

and the two terminal unphosphorylated ones (10 pmol each) were annealed by heating to 95°C and slow cooling to room temperature over 3 h in 100 mM NaCl. The oligonucleotides were ligated in a reaction (20 µl) containing 25 mM NaCl, 50 mM Tris−Cl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol-8000, 2.5 pmol of each oligonucleotide, 0.1 pmol of pBR322 vector, digested with *Bam*HI and *Sal*I, and 1 unit of T4-DNA ligase (1 unit/µl; BRL). The mixture was incubated at 24°C for 4 h, then, after addition of 1 more unit of ligase, overnight at 16°C. After transformation of *E.coli* with the ligated DNA, 66% of the colonies were tetracyclin sensitive, which indicated the insertion of a foreign DNA fragment into the vector. Eleven out of 12 recombinant plasmids had the correct size insert and had acquired restriction sites for *Sac*I and *Bss*HII.

Purification on cross-linked amylose

The PD28 derivatives harbouring the recombinant plasmids were grown in the presence of ampicillin (100 µg/ml). They were kept in conditions where promoter *malEp* is repressed, i.e. on plates of LB agar supplemented with 2% glucose. For the expression of proteins MalE and MalE-Lzp, the strains were grown at 30°C in LB broth without added surgar, and harvested at $A_{600\ nm}$ = 1.0/cm. The preparation of periplasmic and shocked cell extracts, and the purification of proteins on a column of cross-linked amylose were performed as described (Bedouelle and Duplay, 1988), except that we included 1 mM phenylmethylsulfonyl fluoride in the buffers. We adjusted the periplasmic extract to 50 mM Tris−HCl, pH 7.5, 2.5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (buffer A) immediately after the osmotic shock and the column was eluted with this buffer.

Protein assays

The concentrations of MalE and MalE-Lzp were estimated using the extinction coefficient determined for the MalE protein: 1.47/cm/g/l at 280 nm (Kellermann and Ferenci, 1982). The protein concentrations were also determined using the BioRad protein assay with bovine serum albumin as a standard. The values obtained by the two methods were very close. SDS−PAGE was performed as described (Bedouelle and Duplay, 1988). For their immunological detection, the proteins were subjected to electrophoresis in two identical gels. One of the gels was stained with Coomassie blue. Proteins in the other gel were transferred to nitrocellulose and incubated with rabbit serum directed against MalE. Staining was with goat anti-rabbit IgG coupled to alkaline phosphatase (Sambrook *et al.*, 1989).

Centrifugation

The zonal centrifugations were conducted using buffer B (50 mM Tris−Cl, pH 7.5, 50 mM NaCl, 2.5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM maltose) and calibrated as described (Blondel and Bedouelle, 1990). Each protein sample contained bovine liver catalase as a marker. After centrifugation, the gradients were pumped through a UV detector and the positions of the protein peaks determined by absorbance at 280 nm. The peaks were collected manually and their content identified by PAGE and reaction with oxygen peroxide. The sedimentation coefficient at 20°C in water ($s_{20,w}$) of MalE-Lzp was determined from the values for catalase (11.10 S) (Sober and Harte, 1968) and MalE (3.30 S) (Blondel and Bedouelle, 1990), using a linear relationship between $s_{20,w}$ and the distance from the protein peaks to the meniscus of the gradients on the absorbance profiles.

Gel filtration chromatography

The Stokes radii (R_S) of MalE and MalE-Lzp were determined by gel filtration through a Superose 12 HR 10/30 column (Pharmacia) eluted with buffer B as described (Blondel and Bedouelle, 1990). The effluent of the column was continuously monitored at 280 nm. Fractions were collected manually and their content identified by PAGE. We used a linear relationship between the Stokes radii and the elution volumes of the proteins. This relationship was calibrated with horseradish peroxidase (3.02 nm), hen egg white ovalbumin (3.05 nm), horse haemoglobin (3.11 nm), the β_2 subunit of *E.coli* tryptophan synthase (4.0 nm), rabbit muscle phosphorylase *b* (4.8 nm) and catalase (5.22 nm) (Sober and Harte, 1968).

Molecular mass calculations

The molecular mass of MalE-Lzp was deduced from the equation:

$$m = 6\pi\eta R_S N_A s_{20,w}/1-\varrho\bar{v}$$

derived from Svedberg's and Stokes' laws, where $\bar{v}$ is the specific volume of the molecule, N_A, the Avogadro's number, ϱ = 1 g/ml and η = 1 mPa.s, the density and viscosity of water at 20°C (Marshall, 1978). From the amino acid sequence of MalE (Duplay *et al.*, 1984) and sedimentation equilibrium data (Kellermann and Szmelcman, 1974), we calculated $\bar{v}$ = 0.764 ± 0.015 for MalE and took the same value for MalE-Lzp. The ratio of the molecular masses of MalE and MalE-Lzp calculated in this way is independent of the value of $\bar{v}$.

Results

Construction of hybrid MalE-Lzp

We have constructed a gene fragment, *lzp*, coding for the leucine zipper of GCN4 from six chemically synthesized oligonucleotides (Materials and methods). The main features of *lzp* are shown in Figure 1. This fragment encoded the 35 C-terminal residues of GCN4, including the whole leucine zipper and the six amino acid residues that link it to the upstream regions of GCN4

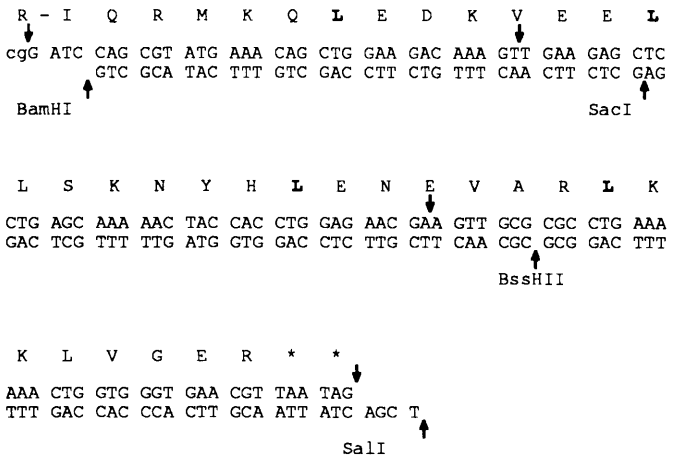

Fig. 1. A synthetic leucine zipper gene (*lzp*). The double-stranded DNA fragment was assembled from six synthetic oligonucleotides, 33−40 nucleotides long (delimited by vertical arrows on the figure). Its sequence is bracketed by *Bam*HI and *Sal*I restriction sites and codes for the 35 C-terminal residues of the yeast protein GCN4. The fusion point between the *malE140* allele (lower-case letters) and the gene fragment (upper-case letters) is also shown. *malE140* carries a *Bam*HI restriction site inserted into the 3′-terminal codon of *malE*. Due to the insertion of the *Bam*HI site, the C-terminal residue of MalE is changed from Lys to Arg and the N-terminal residue of the leucine zipper, from Leu to Ile.

(Hinnebusch, 1984), except that the N-terminal residue of the linking peptide was changed from Leu to Ile. The DNA fragment had *Bam*HI and *Sal*I restriction sites at the 5′ and 3′ ends respectively and contained internal sites for *Sac*I and *Bss*HII. All codons employed are frequent in *E.coli*. Recombination between the *Bam*HI sites of *lzp* and *malE140*, a mutant allele of *malE* in which a *Bam*HI linker had been inserted into the 3′-terminal codon (Duplay *et al.*, 1987), created an in-frame fusion. The synthetic *lzp* was cloned between the *Bam*HI and *Sal*I sites of plasmid pBR322, to give plasmid pHB16 (Figure 2).

We constructed a hybrid *malE140-lzp* between the *malE* gene and the *lzp* gene fragment by inserting the synthetic fragment between the *Bam*HI and *Sal*I sites of plasmid pAB1, which carries the mutant gene *malE140* under control of the *malE* promoter. We named the resulting recombinant plasmid pAB7. This plasmid encoded the desired hybrid protein, MalE-Lzp. We also inserted the *Eco*RI−*Sal*I fragment that carried the *malE140-lzp* hybrid gene of pAB7 between the *Eco*RI and *Sal*I sites of phagemid pEMBL8$^+$ (Figure 2). Using the single-stranded form of the resulting plasmid, pAB8, we checked the nucleotide sequence of the *lzp* gene fragment.

Expression and purification of hybrid MalE-Lzp

To avoid toxicity problems during the constructions, the recombinant plasmids were propagated in *malT* strains, in which promoter *malEp* is inactive (Bedouelle and Duplay, 1988). For expression studies, plasmids pAB7 and pAB8 were introduced into PD28, a strain which constitutively expresses the maltose regulon but cannot ferment maltose because it harbours deletion Δ*malE444* (Duplay *et al.*, 1984). Strains PD28(pAB7) and PD28(pAB8) could ferment maltose on McConkey indicator plates. These genetic complementation experiments and appropriate controls (Bedouelle and Duplay, 1988) suggested that MalE-Lzp was exported into the periplasm and was active for maltose transport.

To determine the cellular location of MalE-Lzp, we submitted cultures of strain PD28(pAB7) and PD28(pAB8) to the cold osmotic shock procedure (Neu and Heppel, 1965) and analysed the contents of the periplasmic and shocked-cell extracts by PAGE. The periplasmic extracts contained a protein with an apparent molecular mass of 45 kd, corresponding to the theoretical value for MalE-Lzp, calculated from its amino acid sequence. This protein was the major species in the periplasmic extracts and was absent from the periplasm of the control strain (Figure 3, lanes 4 and 5).

Hybrids between MalE and foreign proteins can be purified by affinity chromatography on cross-linked amylose (Bedouelle and Duplay, 1988; Blondel and Bedouelle, 1990). We applied this technique to the purification of MalE-Lzp. We purified 2.6 mg of MalE-Lzp from a periplasmic extract of 1 l of strain

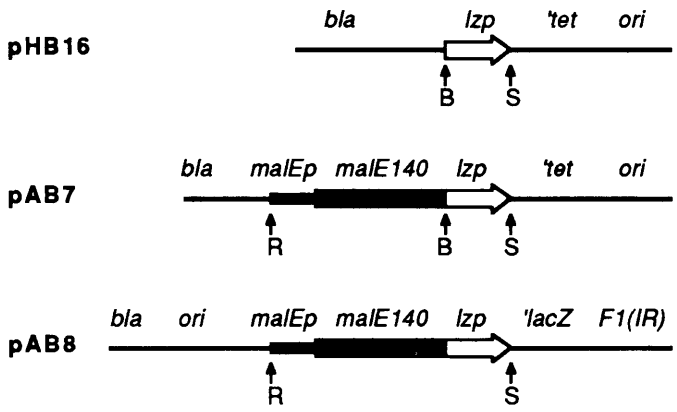

Fig. 2. Construction of hybrid *malE-lzp*. B, *Bam*HI; R, *Eco*RI; S, *Sal*I. The *lzp* gene fragment was inserted between the *Bam*HI and *Sal*I restriction sites of plasmid pBR322 to give the recombinant plasmid pHB16. It was subsequently excised from pHB16 and inserted between the *Bam*HI and *Sal*I sites of pAB1. The resulting plasmid, pAB7, codes for a hybrid protein between MalE and the leucine zipper. The *malE140-lzp* hybrid gene was excised from pAB7 as an *Eco*RI−*Sal*I fragment and inserted into the polylinker of plasmid pEMBL8$^+$ giving plasmid pAB8.

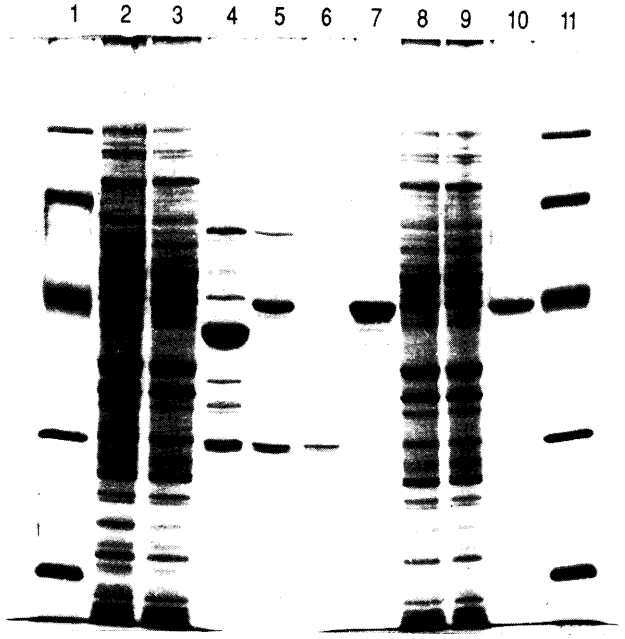

Fig. 3. Cellular location and purification of MalE-Lzp. Cellular location: the figure compares shocked-cell extracts (**lanes 2** and **3**) and periplasmic extracts (**lanes 4** and **5**) of strains PD28(pPD1), overproducing the wild-type MalE (**lanes 2** and **4**), and PD28(pAB7), producing MalE-Lzp (**lanes 3** and **5**). Purification of MalE-Lzp: **lanes 5−7**, from a periplasmic extract; **lanes 8−10**, from a shocked-cell extract. For each purification, we analysed the initial material (**lanes 5** and **8**), the fraction flowing through the amylose column (**lanes 6** and **9**), and a fraction purified on the amylose column (**lanes 7** and **10**). **Lanes 1** and **11**, molecular mass markers: phosphorylase *b*, 97.4 kd; bovine serum albumin, 66.2 kd; hen egg white ovalbumin, 42.7 kd; bovine erythrocyte carbonic anhydrase, 31.0 kd; soybean trypsin inhibitor, 21.5 kd. We loaded amounts of the extracts and flow-through fractions originating from 200 μl of a culture at $A_{600\ nm}$ = 1.0/cm.

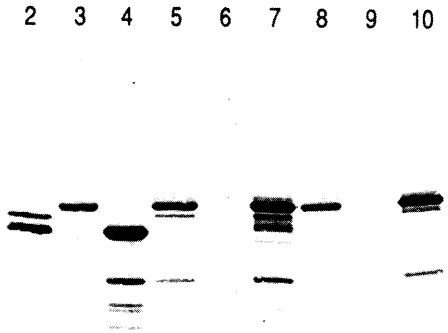

Fig. 4. Immunological detection of MalE-Lzp. The protein gel was run and the lanes are numbered as described in the legend to Figure 3, except that we loaded only one-tenth as much material in each lane. The proteins were revealed with an anti-MalE antiserum.

PD28(pAB7), harvested at $A_{600\,nm}$ = 1.0/cm (Figure 3, lanes 5–7), and 0.18 mg from a soluble extract of the shocked cells (Figure 3, lanes 8–10). MalE-Lzp was only slightly degraded during its synthesis, processing and purification (Figure 3, lanes 7 and 10) contrary to what was found for hybrids between MalE and other proteins (Bedouelle and Duplay, 1988; Blondel and Bedouelle, 1990). Only trace amounts of the MalE-Lzp chains in the periplasmic and shocked-cell extracts flowed through the amylose columns during loading, as shown by immunological analysis of the fractions with an anti-MalE polyclonal antibody (Figure 4, lanes 6 and 9). Thus, each cell of PD28(pAB7) produced ~76 000 molecules of MalE-Lzp in a soluble form, 93% being exported into the periplasm. Most of the hybrid chains bound amylose. Plasmid pAB7 was derived from pBR322 and pAB8 from pEMBL8$^+$, which has ~10 times more copies than pBR322 (Dente and Cortese, 1987). To test the effect of an increased copy number of the plasmid on the production of MalE-Lzp by the cell and on its location in the cell, we also purified MalE-Lzp from PD28(pAB8). We obtained respectively 6.4 and 1.2 mg of hybrid protein from periplasmic and shocked-cell extracts of this strain. Thus, the yield of MalE-Lzp purified from PD28(pAB8) was 2.5 times higher than from PD28(pAB7) and 2.5 times more hybrid remained with the cells following osmotic shock.

Oligomerization of periplasmic MalE-Lzp

The sedimentation coefficient ($s_{20,w}$) of MalE-Lzp was determined by zonal centrifugation through a sucrose gradient in the presence of 1 mM maltose, taking MalE (3.30 S) and catalase (11.10 S) as standard proteins (Figure 5; Materials and methods). Pure MalE-Lzp was layered on top of the gradients at concentrations between 0.65 and 26 μM. Following centrifugation, the maximal local concentration of MalE-Lzp in the gradients was between 0.12 and 4.5 μM. Under these conditions, we determined $s_{20,w}$(MalE-Lzp) = 4.95 ± 0.05 S.

The Stokes radius (R_S) of MalE-Lzp was determined by gel filtration in the presence of 1 mM maltose (Figure 6). Pure MalE-Lzp was injected onto the chromatography column at concentrations between 0.1 and 30 μM. Following elution, the corresponding maximum local concentrations were between 15 nM and 3.5 μM. We found R_S(MalE-Lzp) = 4.0 and 4.4 ± 0.3 nm for the lowest and highest concentrations tested. In the hypothesis of an equilibrium between a monomeric and a dimeric form of MalE-Lzp, the difference between these values is very low and indicates that the dissociation constant of the MalE-Lzp dimer was below 0.1 μM. In the same experiment, we found R_S(MalE) = 2.6 ± 0.3 nm.

From the above values of $s_{20,w}$ and R_S, we calculated values for the molecular mass of MalE as 41.5 ± 7 kd and that of MalE-Lzp as 105 ± 15 kd, which are about the theoretical molecular masses for the MalE monomer, i.e. 40.3 kd, and for the MalE-Lzp dimer, i.e. 90 kd.

Discussion

We have shown previously that the MalE protein can be used as a vehicle for the periplasmic localization and facile purification in a functional state of a variety of proteins, including cytoplasmic, secreted, monomeric and dimeric ones (Bedouelle and Duplay, 1988; Blondel and Bedouelle, 1990). In the present work, we have shown that hybrid MalE-Lzp was synthesized and exported in large quantities in *E.coli*. The hybrid was purified using cross-linked amylose, and it formed stable dimers. Thus, our new results extend the use of MalE as a vehicle to produce protein motifs and study their features.

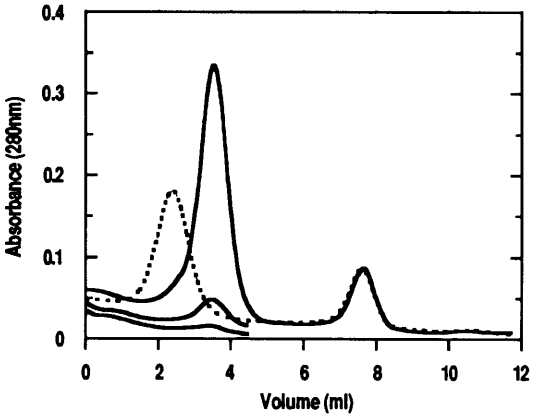

Fig. 5. Determination of the sedimentation coefficient of MalE-Lzp by zonal centrifugation. Each of the protein samples (0.2 ml) contained catalase as a marker. They were layered on top of preformed linear gradients (11.5 ml) of 5–20% sucrose and sedimented in a Beckman SW41 rotor at 40 000 r.p.m. and 4°C for 18 h. All the buffers contained 1 mM maltose (Materials and methods). After centrifugation, the gradients were pumped through a UV detector. The volumes are plotted from the top of the gradients. The peak of catalase was recovered around 7.6 ml. Continuous lines: MalE-Lzp at initial concentrations of 0.65, 2.6 and 26 μM. Dashed line: MalE at 12 μM. For clarity, portions of the profiles were omitted and the profile corresponding to the lowest concentration of MalE-Lzp was uniformly translated downward by 0.015 $A_{280\,nm}$ units.

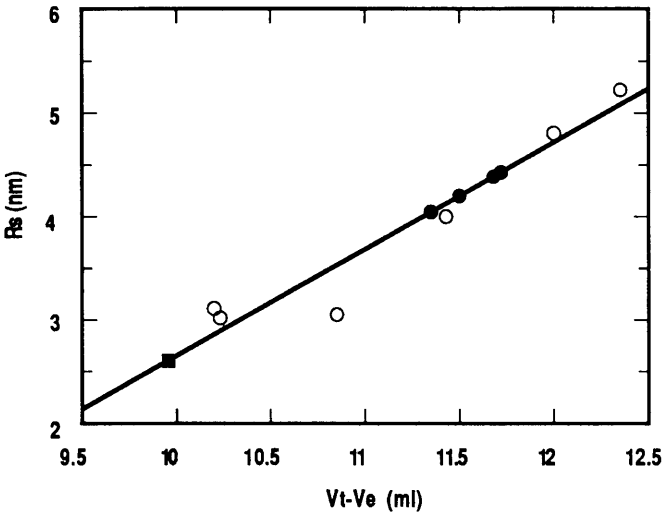

Fig. 6. Determination of the Stokes radius of MalE-Lzp by gel filtration. The Superose 12 HR 10/30 column was run at 1 ml/mn at room temperature and the samples loaded in a volume of 0.1 ml. All the buffers contained 1 mM maltose (Materials and methods). V_t, total volume of the column; V_e, elution volume. R_S, Stokes radius. ●, MalE-Lzp at initial concentrations of 0.1, 0.3, 3.0 and 30 μM; ■, MalE at 10 μM; ○, standard proteins (haemoglobin, peroxidase, ovalbumin, β_2 subunit of tryptophan synthase, phosphorylase and catalase). The protein samples that are represented by the same symbol are listed in the order of increasing $V_t - V_e$ values.

Hybrid MalE-Lzp was produced in large amounts and in a soluble form. Roughly 75 000 molecules/cell were produced from a plasmid derived from pRB322 and 190 000 molecules/cell from a plasmid derived from pEMBL8$^+$. The increase in yield was not proportional to the copy number. This difference could be due to the limiting amount of MalT, the positive activator of transcription at the *malE* promoter, within the cell (Bedouelle, 1984).

Most of hybrid MalE-Lzp was exported to the periplasm. The efficiency of export decreased from 93 to 84% when the

production of hybrid increased 2.5-fold. This decrease could be due to a partial jamming of the cellular export machinery, as was observed with other periplasmic or hybrid proteins (Ito *et al.*, 1981; Pagès *et al.*, 1984). The capacity of MalE-Lzp to form very stable dimers (see below), did not prevent its efficient translocation through the membrane. It will be interesting to study the role of the *E.coli* protein SecB, which slows the folding kinetics or prevents the aggregation of exported proteins (Lecker *et al.*, 1990), in the export of MalE-Lzp. Our results suggest that the signal peptide of hybrid MalE-Lzp was cleaved since point mutations in MalE that abolish the cleavage of its signal peptide also prevent the release of the precursor into the periplasmic space (Fikes and Bassford, 1987).

MalE-Lzp could be purified by affinity chromatography on cross-linked amylose. This result demonstrated that the MalE portion of the hybrid bound maltose and maltodextrins efficiently. Indeed, mutant derivatives of MalE, whose dissociation constants for these sugars are increased 20 times relative to the wild-type protein, are not retained on a cross-linked amylose column (Martineau *et al.*, 1990). The result obtained with MalE-Lzp is consistent with previous data showing that the fusion of polypeptides to the C-terminal end of MalE does not alter its dissociation constant for maltose nor its kinetic parameter, K_M, for the transport of this sugar inside the cell (Duplay *et al.*, 1987).

MalE is normally a monomeric protein (Kellermann and Ferenci, 1982). However, a dimeric species can be obtained when it is produced and purified in special conditions. This dimeric form does not detectably bind maltose and it dissociates into monomers upon addition of this sugar (Richarme, 1982, 1983). We therefore performed all our sedimentation and gel filtration experiments in the presence of a large excess of maltose (1 mM, corresponding to 300 times the normal K_d) to ensure that the MalE portions of the MalE-Lzp hybrid chains were saturated with maltose and did not associate. The values of the molecular masses that we determined from these experiments showed that MalE-Lzp dimerized in conditions where MalE was a monomer, i.e. in the presence of maltose, and thus that the dimerization was due to the Lzp portion of the hybrid. By fusing the leucine zipper, a short polypeptide of 35 residues, to MalE, which is 370 residues long, we could study the dimerization of the leucine zipper at low concentrations while maintaining a good sensitivity of detection. This allowed us to determine an upper limit for the dissociation constant of the dimer, 0.1 μM, which is 100 times lower than the previous estimate made with a synthetic peptide of 33 residues (O'Shea *et al.*, 1989a).

Several mutations have already been constructed in leucine zippers (reviewed in Hu *et al.*, 1990). MalE-Lzp can be used to study the interface between the two α-helices by site-directed mutagenesis experiments. Hybrids between MalE and other leucine zippers will enable a detailed analysis of the interactions that are responsible for the specificity of association. Also, crystallization of MalE-Lzp could facilitate characterization of the structure of the leucine zipper, inasmuch as the crystal structure of MalE is known (Spurlino, 1988).

Our results show that it was possible to create stable homodimers of a foreign globular protein by fusion to the leucine zipper of GCN4. More generally, our results suggest that the leucine zipper of the Fos/Jun system (Kouzarides and Ziff, 1989; O'Shea *et al.*, 1989b) could be used to specifically create heterodimers of proteins to which the two complementary α-helices would be fused. Such heterodimers could constitute an alternative to protein fusion, to combine within one molecule functions that are normally carried out by different molecules.

Leucine zippers have been found in regulatory proteins but also as structural elements of proteins with various functions (Webber and Malkin, 1990; Delwart *et al.*, 1990). The use of the leucine zipper of GCN4 to cross link microtubule filaments has been reported then retracted (Lewis and Cowan, 1990). To our knowledge, our results constitute the first report of the use of a leucine zipper to engineer the quaternary structure of a non-regulatory protein.

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