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Recognition of ligands by SecB, a molecular chaperone involved in bacterial protein export

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SUMMARY

SecB is a molecular chaperone involved in protein export from *Escherichia coli*. It is a highly negatively charged, soluble, tetrameric protein with a monomer molecular mass of 16 400 kDa. It has two functions: it maintains precursors of some exported proteins in a conformation compatible with export, by preventing them from aggregating or from folding into their thermodynamically stable state in the cytoplasm, and it delivers both nascent and completed precursors to SecA, one of the components of the export apparatus that are on and in the plasma membrane. SecB recognizes completed precursors of soluble proteins, not by direct interaction with leader sequences but by virtue of the property, imposed by their leader sequences, that they fold slowly: i.e. there is a kinetic partitioning between folding and interaction with SecB. Only those polypeptides that fold slowly interact significantly with this molecular chaperone even though it is able to bind a wide variety of non-native proteins. Binding studies with purified peptides indicate that each SecB monomer has a binding site that can interact with flexible peptides having a net positive charge and a length of about ten residues, which may depend on the charge density. Binding of the hydrophobic fluorescent probe l-anilino-naphthalene-8-sulphonate (ANS) indicates that simultaneous interaction of multiple peptides causes a conformational change that exposes a hydrophobic site on SecB. This hydrophobic region is thought to contribute an extra binding site for physiological ligands of SecB. A model of SecB binding to nonnative precursors is presented.

1. INTRODUCTION

Export of selected proteins from the cytoplasm of *E. coli* where they are synthesized, to the periplasm or outer membrane where they have their function, occurs by a process believed to be very like that operating in eukaryotic cells, in which a similarly select group of proteins is transferred from the cytosol to the lumen of the endoplasmic reticulum. The most compelling reason for this belief is the striking similarity of the signal or leader sequences (von Heijne 1985). These sequences are amino-terminal extensions of the mature proteins that are essential for membrane translocation in both systems and that are removed during the process. Because these sequences are the topogenic sequences that are responsible for proteins entering the export pathway, it is not surprising in view of their similarity that eukaryotic leader sequences can function in bacterial protein export and that bacterial leader sequences can function in eukaryotic secretion. Despite this congruity, some important differences, perhaps less fundamental than they seem, occur between the processes of membrane translocation in the two kinds of cells. One such difference is that in *E. coli* many molecules of nearly all proteins so far examined traverse the membrane post-translationally (Josefsson & Randall 1981; Randall 1983) whereas in mammals, at least, it appears that mechanisms have evolved to ensure that translocation of most proteins across the endoplasmic reticulum membranes occurs strictly cotranslationally (Rapoport 1991).

Because precursors of exported proteins in bacteria are often completed before they are translocated, they have an opportunity to fold in the cytoplasm into their thermodynamically stable state. (We may note in passing that this opportunity is denied the precursors of secretory proteins in eukaryotes by their cotranslational mode of export, which ensures that stable folding is not possible until translocation has occurred.) A study, using loss of sensitivity to protease as an assay of folding of maltose-binding protein *in vivo*, showed that there was a clear inverse correlation between folding and export (Randall & Hardy 1986). Export of pulse-labelled maltose-binding protein only continued as long as there were unfolded molecules in the cytoplasm. Once all labelled precursor remaining in the cell had folded into the native conformation there was no further export. It appeared that to remain competent for export the protein had to remain in an unfolded or protease-sensitive state. This observation, together with similar results obtained in a cell-free system for import of proteins into mitochondria (Schleyer & Neupert 1985; Eilers & Schatz 1986), led to formulation of the idea that in order to cross membranes proteins must be in an unfolded state. The term unfolded in this statement is a

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misleading one, since it implies a complete lack of structure which is almost certainly not the case. Indeed there is evidence that proteins interacting with the export apparatus have considerable secondary structure (Lecker *et al.* 1990). A more accurate statement of the principle would be that proteins that have assumed their thermodynamically stable folded state are unable to cross membranes. This statement appears to be correct, since the attachment of stably folded bovine pancreatic trypsin inhibitor at the carboxyl terminus of an otherwise competent protein prevented membrane translocation *in vitro*. When the attached protein was unfolded by reduction of disulphide bonds, translocation readily occurred (Schiebel *et al.* 1991).

The requirement that precursors of exported proteins should remain unfolded in the cytoplasm means that there must be a component of the export apparatus whose function is to prevent folding. There may be several of these, but the one that seems to be most important and that is best understood, is SecB.

2. IDENTIFICATION AND PROPERTIES OF SecB

The gene *secB* was first identified from mutants of *E. coli* isolated in selections for general export defects, carried out in the laboratory of Jon Beckwith (Kumamoto & Beckwith 1983). The phenotype of the mutants was the accumulation of precursors of a subset of exported proteins, a subset that included all the common outer membrane proteins except lipoprotein, and one or two periplasmic proteins, among which was maltose-binding protein, the product of the *malE* gene (Kumamato & Beckwith 1985; Hayashi & Wu 1985). The mutations which were located in the gene named *secB*, had no effect on the export of the periplasmic proteins, ribose-binding protein and alkaline phosphatase or on that of the outer membrane lipoprotein. It was shown by total inactivation of the gene using a transposon (i.e. a *secB* null mutation), that under some conditions of growth in the laboratory the SecB function is not essential. However in this mutant only a fraction of maltose-binding protein and other affected proteins was exported (Kumamoto & Gannon 1988; Collier & Bassford 1989), the rest remaining as unprocessed folded precursor in the cytoplasm as would be expected if SecB is an antifolding factor. A further finding consistent with this role for SecB was that the export of slowly folding mutant forms of maltose-binding protein was less affected by the *secB* null mutation than was export of the wild type protein (Collier *et al.* 1988). However the observation that export of maltose-binding protein is strictly posttranslational in $secB^-$ mutants (Kumamoto & Gannon 1988; Collier & Bassford 1989) whereas in $secB^+$ cells it is both co and post-translational indicates that SecB interacts with nascent polypeptide chains which cannot stably fold, as well as with completed ones which can. Such interaction has in fact been directly demonstrated (Kumamoto 1989). SecB may therefore have another function in cotranslational export, for example that of facilitating productive interactions of nascent chains with the export apparatus on the membrane.

The *secB* gene has been cloned and sequenced (Kumamoto & Nault 1989), and SecB has been purified (Weiss *et al.* 1988; Kumamoto *et al.* 1989; Watanabe & Blobel 1989*a*). SecB is a negatively charged polypeptide of 16.4 kDa (Weiss *et al.* 1988) which exists in its active form as a soluble cytoplasmic oligomer, probably a tetramer (Watanabe & Blobel 1989*a*). SecB is required for efficient import of maltose-binding protein and other proteins, into everted cytoplasmic membrane vesicles *in vitro* (Weiss *et al.* 1988; Kumamoto *et al.* 1989; Watanabe & Blobel 1989*a*). It has been shown to bind to precursors of several exported proteins *in vitro* (Lecker *et al.* 1989) and *in vivo* (Kumamoto 1989), and also shown to maintain the precursor of maltose-binding protein in an unfolded protease-sensitive state both *in vivo* (Kumamoto & Gannon 1988) and in a cell-free system (Collier *et al.* 1988; Weiss *et al.* 1988). Furthermore when maltose-binding protein is diluted out of denaturant into a solution containing excess purified SecB, its folding, as measured by an increase in fluorescence of tryptophan, is completely blocked (Liu *et al.* 1989). Taken together, all these data compellingly indicate that SecB is a molecular chaperone for exported proteins. When it is acting as a chaperone for soluble proteins the illicit or non-productive interactions that it prevents are not primarily those of its ligand with other proteins, but are mainly normal folding interactions that would render the ligand unexportable. In contrast, when it is chaperoning outer membrane proteins, which are probably intrinsically insoluble, its main function may be to prevent aggregation.

SecB differs from other molecular chaperones in that it has not been possible to show any effect of ATP on binding or release of ligand. However, a specific binding interaction between SecB and SecA, the peripheral membrane protein on the cytoplasmic surface of the cytoplasmic membrane, has been demonstrated (Hartl *et al.* 1990). This interaction is enhanced if SecB is bound to a precursor. In contrast to SecB, SecA hydrolyses ATP in the presence of precursor and the remainder of the membrane translocation apparatus. Hydrolysis of ATP is necessary for translocation of the protein *in vitro* (Lill *et al.* 1989). Whether binding or hydrolysis of ATP is necessary for the transfer of precursor from SecB to SecA is not clear. The findings, savagely summarized above, indicate that SecB, as well as being a molecular chaperone for precursors of exported proteins, also acts to deliver them to SecA.

3. TARGET SELECTION BY SecB

There is no evidence that SecB functions in any process other than protein export. Therefore all the polypeptides that it should bind to *in vivo* have leader sequences, and one might expect that the possession of a leader sequence would be the distinguishing character of the targets of SecB. In fact this expectation is fulfilled, but in an indirect way, there being abundant

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evidence that SecB does not bind specifically with the leader sequence itself, but rather interacts directly with structures within the body of the unfolded precursor. The evidence consists of three types of investigation. First, when leader sequences are exchanged between a protein whose export is dependent on the function of SecB and one whose export is independent of it, the allegiance of SecB does not follow the leader (Gannon *et al.* 1989; Collier *et al.* 1990). Second, proteins that cannot be exported because of defects in their leader sequences, nevertheless interfere with the export of wild-type proteins from the same cell, by binding the available SecB and preventing it from acting on the nonmutant proteins (Bankaitis & Bassford 1984; Collier *et al.* 1988). This interference can be shown to be independent of the leader sequence on the interfering species but dependent on the presence of sequences within the body of the protein. Third, and most directly, stable complexes can be formed *in vitro* between SecB and either unfolded mature maltose-binding protein or unfolded precursor maltose-binding protein. When under conditions of limiting SecB, both mature and precursor maltose-binding protein were simultaneously presented for binding to SecB, there was no preferential formation of complex with precursor maltose-binding protein, indicating that the two forms of maltose-binding protein had very similar affinities for SecB, and therefore that the leader sequence did not contribute significantly to the binding (Randall *et al.* 1990). All these data indicate clearly that the binding sites for SecB do not lie within the leader sequence. It should be noted however that there is one report that appears to show that binding of SecB to maltose-binding protein is dependent on the presence of a leader sequence (Watanabe & Blobel 1989*b*).

If the leader sequence itself is not the binding site for SecB, how then does this molecular chaperone interact exclusively with polypeptides that contain leader sequences? We believe that the answer to this question is found in the influence of the leader sequences on the folding of the precursors that contain them. The possession of a leader sequence slows the folding of a precursor such that it remains in a state that allows interaction with SecB for much longer than other proteins which do not contain leaders, and this enables it to bind to the chaperone. In other words, on completion of a polypeptide that can initially interact with SecB, and as will become clear there are many of these, there is a kinetic partitioning between folding and interacting with SecB. Proteins lacking leader sequences fold very rapidly. Consequently only a minority of these molecules interact with SecB. Proteins possessing leader sequences fold slowly, and there is time before they fold and lose their ability to interact, for the great majority of molecules to bind to the chaperone and thus enter the protein export pathway.

The evidence for this model is first that SecB binds only to unfolded proteins and has no detectable affinity for native proteins (see below and Hardy & Randall, 1991), second that the possession of a leader sequence significantly retards the folding *in vitro* of precursor relative to the corresponding mature protein (Park *et al.* 1988; Laminet & Pluckthun 1989), and third that the ability of maltose-binding protein to interact with SecB *in vitro* and *in vivo* has been shown to depend on its rate of folding (Liu *et al.* 1989; Hardy & Randall 1991). The *in vivo* evidence was obtained by making use of the interference phenomenon described above, where proteins defective in export sequester the available SecB, thus disturbing the export of wild-type proteins that are SecB-dependent (Collier *et al.* 1988). In this case the interfering species of maltose-binding protein had the leader sequence completely missing, making it totally unexportable, as well as a mutation in the body of the protein that slowed its folding drastically. The expression of this protein interfered with the export of a SecB-dependent protein, but not with that of a SecB-independent protein. The expression of a control protein, which lacked the leader sequence but was otherwise wild-type, and which should therefore fold rapidly in the cytoplasm, did not interfere with export. The interpretation is that even without a leader sequence, a polypeptide can be a ligand for SecB, provided that it folds sufficiently slowly. A further implication is that one function of a leader sequence is to cause the precursor possessing it to fold slowly, thus ensuring that it enters the export pathway by binding SecB. That the leader sequence has other functions at the membrane is shown by the large number of studies of proteins with mutated leaders that can still bind to SecB *in vivo*, but are not exported (see Bassford (1990) for a review).

4. BINDING OF SecB TO DENATURED PROTEINS

When purified unfolded maltose-binding protein is rapidly diluted out of the denaturant used to unfold it, it regains its fully folded state with kinetics that can be monitored using the increase that occurs in the intrinsic fluorescence of tryptophanyl residues as the protein folds. The addition of excess SecB completely arrests the fluorescence increase characteristic of folding, indicating the formation of a stable complex between the chaperone and the incompletely folded maltose-binding protein (Liu *et al.* 1989). SecB thus blocks the folding of maltose-binding protein but it has no unfolding activity, since addition of excess SecB to folded maltose-binding protein causes no decrease in tryptophan fluorescence. When denatured maltose-binding protein is added to limiting quantities of SecB, only a fraction of the fluorescence increase observed in the absence of SecB is obtained, indicating the formation of a stable complex between the chaperone and a proportion of the molecules of denatured maltose-binding protein. The kinetics of folding of the remainder of the molecules remains unchanged (Hardy & Randall 1991). These data can be interpreted to mean that in conditions of limiting SecB, essentially all of the chaperone is tightly bound in a complex with maltose-binding protein.

From experiments of this kind, in which samples of denatured maltose-binding protein are added to dif-

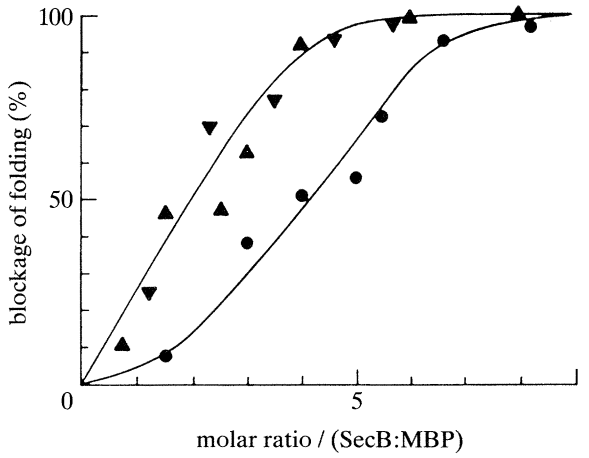

Figure 1. Blockage of folding of a slow-folding form of maltose-binding protein. The triangles show the data obtained when folding is very slow at 4°C, the circles when folding is much more rapid at 25°C. The molar ratio is that of monomer SecB to maltose-binding protein. Reprinted with permission from Hardy & Randall (1991); © AAAS.

ferent quantities of SecB and the residual folding of the uncomplexed molecules is measured, an estimate can be made of the proportion of the total fluorescence increase blocked by the presence of SecB, and this is taken as a measure of the proportion of the maltose-binding protein that forms a complex with SecB. Examples of such blockage of folding curves are shown in figure 1. Consistent with the model of kinetic partitioning, the quantity of SecB required to block a certain proportion of the molecules from folding increases as the rate of folding increases (figure 1). As the concentration of maltose-binding protein in this assay (50 nM) is more than tenfold higher than the dissociation constant of the complex with SecB (see below), the data in figure 1 can be used to give an estimate of the stoichiometry of interaction. For the blockage of folding carried out at 4°C, four moles of monomer SecB are required to block the folding of one mole of maltose-binding protein. The simplest interpretation is that one tetramer of SecB forms a complex with one molecule of this ligand.

The blockage of folding assay of the interaction between maltose-binding protein and SecB, of which an example is shown in figure 1, can be used to provide insight into the nature of proteins that bind to SecB. Proteins that interact with SecB should compete for binding with maltose-binding protein when SecB is limiting. Unfortunately the number of proteins that can be tested in this way is severely curtailed by two requirements, namely that any competitor protein should contain no tryptophan so that it does not interfere with monitoring the degree of folding of maltose-binding protein, and that it can be presented to the system in a stably unfolded state. Even when these criteria are met, interpretation of the competition data is quantitatively complicated, since if either is released from or never bound by SecB, maltose-binding protein folds and ceases to compete. Nevertheless the presence of any protein that can bind to SecB will reduce the blockage of folding of maltose-binding protein that is observed in its absence. Figure 2 shows four such competition experiments with the four different denatured proteins that were available to us, and that met at least the first requirement: two mammalian secreted proteins (reduced bovine pancreatic trypsin inhibitor (BPTI) and reduced pancreatic ribonuclease), and two *E. coli* proteins, an exported protein that is not dependent on SecB for export (ribose-binding protein) and a cytoplasmic protein (the α subunit of tryptophan synthase). The former two proteins were prevented from folding in the assay mix by use of a reducing agent dithiothreitol (DTT), but the latter two did not meet the second criterion and were free to fold in the assay. (The ribose-binding protein was a slow-folding mutant form.) However, in spite of these complications, it is clear that all four denatured proteins competed with maltose-binding protein for SecB, albeit with different concentration dependences. In contrast, the native proteins did not compete at the highest concentrations tested. Because there is no similarity in the sequences of amino acids among the four competitor proteins and the maltose-binding protein we conclude that SecB has very wide binding specificity for non-native proteins, and that its primary interaction must be with a structure or structures common to a wide variety of unfolded polypeptides. There is, however, a clear preference for some ligands, as denatured BPTI was a more effective competitor on a molar basis than were the other three proteins, whereas denatured ribose-binding protein was the least effective, being about tenfold less effective than reduced BPTI. Since a two to threefold excess of reduced BPTI over maltose-binding protein was required to decrease the blockage of folding by half, we estimate that the affinity of SecB for maltose-binding protein is two- to threefold higher than its affinity for reduced BPTI. We have estimated the dissociation constant of the complex of SecB and carboxamidomethylated BPTI to be approximately 5 nM (Hardy & Randall 1991). Therefore the dissociation constant of the complex between SecB and maltose-binding protein appears to be in the nanomolar range when estimated from the competition assay shown in figure 2.

It should be strongly emphasized, however, that a simple binding equilibrium between SecB and maltose-binding protein to form a complex with a dissociation constant of this magnitude cannot account for the blockage of folding data. This is because as the free ligand folds, its concentration will drop, and some of the complex will dissociate until finally no complex will remain. In order for the initial complex formed to appear stable in these experiments, that is for folding to appear to stop before all the maltose-binding protein is folded, we would need a dissociation constant of the complex several orders of magnitude lower than nanomolar. (In some experiments the complexes were incubated overnight without the occurrence of significant further folding of maltose-binding protein. Assuming that the protein was still able to fold after such a long incubation, this indicates that there was no appreciable dissociation of denatured maltose-binding protein from SecB, a finding

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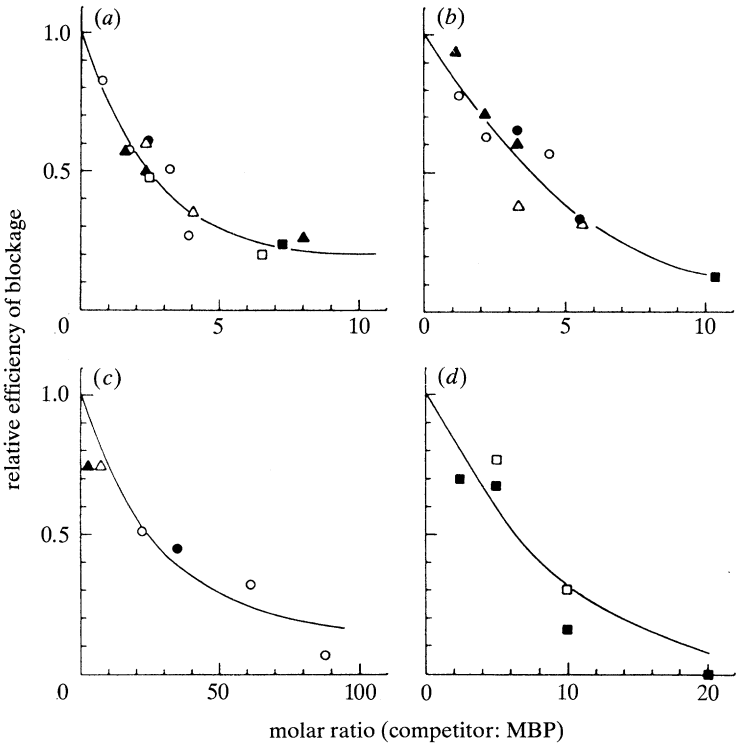

Figure 2. Competition for binding of SecB between unfolded mature maltose-binding protein and four other unfolded proteins. (*a*) Denatured BPTI; (*b*) denatured RNase; (*c*) denatured RBP; and (*d*) denatured α-subunit of tryptophan synthase. The ratio of SecB to maltose-binding protein was set so that blockage of folding was between 40 and 80% in the absence of competitor. The relative efficiency of blockage is the ratio of blockage obtained in the presence of competitor to that in its absence, expressed as a fraction. Different symbols within a panel indicate different experiments. Reprinted with permission from Hardy and Randall (1991); © AAAS.

that implies a very low off rate and thus a very stable complex.) Nevertheless, in competition experiments such as those shown in figure 2, the complex can be prevented from forming, or indeed be dissociated (since the competition appeared to be equally effective whether the competitor was added before, after, or at the same time as maltose-binding protein), by a tenfold excess of a competitor with a dissociation constant in the nanomolar range. It is as though there were an initial interaction followed by a conformational change that acts as a locking step which competitors are able to reverse. One further puzzling point about the blockage of folding studies is that nearly all experiments have indicated a cooperative effect of increasing quantitites of SecB, as shown by the resulting sigmoid curves (figure 1). A possible explanation may be that blockage of folding depends on an initial interaction of two SecB tetramers with maltose-binding protein to keep it unfolded long enough for the locking step to take place on one of them.

Whatever the detailed physicochemical explanation of these data, the clear implications are that denatured proteins can bind to SecB, whereas native proteins cannot, and that the binding is dependent on properties or structures shared by a large number of unfolded polypeptides. To elucidate the nature of the structures recognized by SecB, it was necessary to investigate its interaction with ligands simpler than the proteins used heretofore.

5. BINDING OF SecB TO PEPTIDES

An assay of binding was developed based on the protease sensitivity of SecB. At low ionic strength, SecB, when uncomplexed with ligand, is quantitatively cleaved by a low concentration of proteinase K to yield a large fragment, lacking about 50 residues from the carboxyl terminus. When bound to carboxamidomethylated BPTI (R-BPTI), SecB is resistant to the proteinase K. Native BPTI, which is not bound by SecB, does not confer protection. The protective effect of R-BPTI on SecB is shown in figure 3*a*. As the concentration of SecB in the assay (0.6 μM) is well above the dissociation constant of the complex formed with R-BPTI (5 nM), we are able to obtain an estimate of the stoichiometry of the interaction. Thus 0.3 μM R-BPTI protects approximately 0.3 μM SecB monomer, and we conclude that there is one binding site for R-BPTI per monomer or four per tetramer.

It appears therefore, that resistance of SecB to proteinase K under these conditions can be used as an assay of binding of a ligand. Once again however, an exact quantitative interpretation of the data cannot easily be made since it is not known (except in the case of R-BPTI (Randall 1992)) how susceptible the ligand itself may be to proteolysis. Nevertheless a large number of available peptides of known sequence were surveyed by this method. Three examples are shown in figures 3*b–d*. Those peptides that conferred 50%

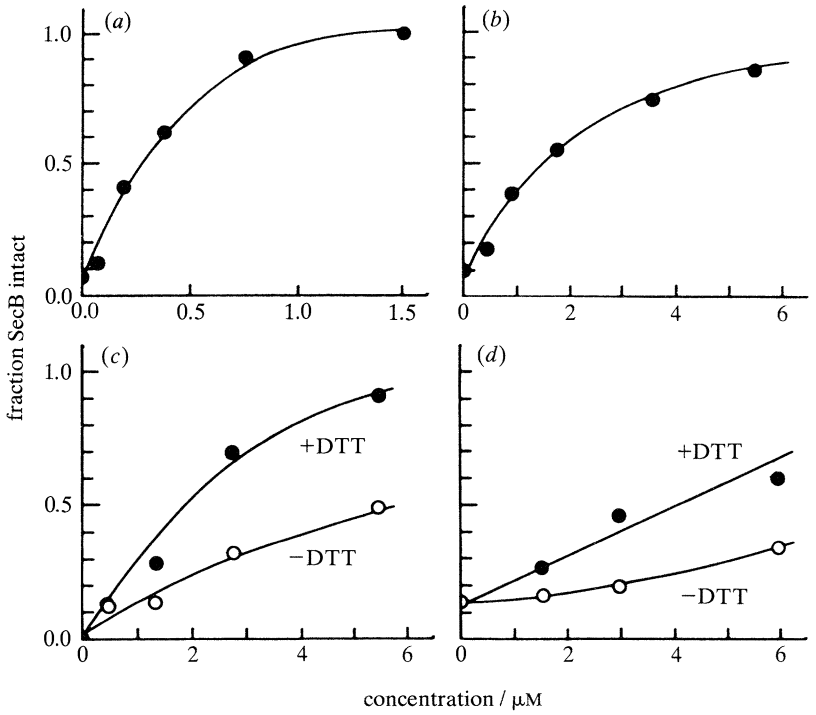

Figure 3. Binding of peptides to SecB. (*a*) R-BPTI; (*b*) S4; (*c*) defensin; and (*d*) somatostatin. The degree of protection of SecB from proteinase K by varying concentrations of individual peptides at pH 7.6 was used as an assay of binding as described in the text. The sequence of peptide S4 is shown in table 1. Reprinted with permission from Randall (1992); © AAAS.

protection of 0.6 μM SecB at a concentration of 15 μM or less were designated as ligands. Those that did not meet this criterion did not confer any protection even at the highest concentrations tested, which in some cases were much higher than 15 μM. A list of the ligands, and the non-binders is given in table 1, together with their sequences. The only common features shared by the ligands and missing from the non-binding peptides appear to be a net positive charge, together with a length greater than eleven residues (table 1). In addition, flexibility appears to be important, since the ligands confer better protection if they are not constrained by disulphide bonds (figure 3), or in the case of the zinc finger tested, by the presence of zinc ions. We conclude that at least part of the recognition site for SecB on its physiological

Table 1. *Interactions of peptides with SecB*

(In the sequences, Ac means acetyl. In the interaction column + means that the peptide protected SecB from proteinase K as described in the text; −, indicates no protection. See Randall (1992) for identification of peptides.)

peptide	sequence	approx. net charge (pH 7.6)	interaction
Pβ	RYFYNAKAGLCQTF	+2	+
S1	VIEVVQGAYRAIRHIPRRIR	+4	+
S1b	DRVIEVVQGAYRAIRHIPRRIRQG	+4	+
S4	NNNTRKSIRIQRGPGRAFVTIGKIG	+6	+
melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	+6	+
zinc finger (no Zn^{++})	RSFVCEVCTRAFARQEHLKRHYRSHTNEK	+4	+
defensin HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	+3	+
defensin NP-1	VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR	+9	+
defensin NP-5	VFCTCRGFLCGSGERASGSCTINGVRHTLCCRR	+4	+
somatostatin	AGCKNFFWKTFTSC	+2	+
mastoparan	INLKALAALAKKIL-NH_2	+4	+
bradykinin	RPPGFSPFR	+2	−
M-K bradykinin	MKRPPGFSPFR	+3	−
SO26-B	Ac-SLNAAKSELDKAIG-NH_2	0	−
Pα	NNFKSAEDCMRTAGGA	0	−
glucagon	HSEGTFTSDYSKYLDSRRAQDFVQWLMNT	−1	−
GCN4-p1	Ac-RMKQLEDKVEELLSKNYHLENEVARLKKLVGER	0	−
Fos-p1	Ac-CGGLTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAAY	−5	−

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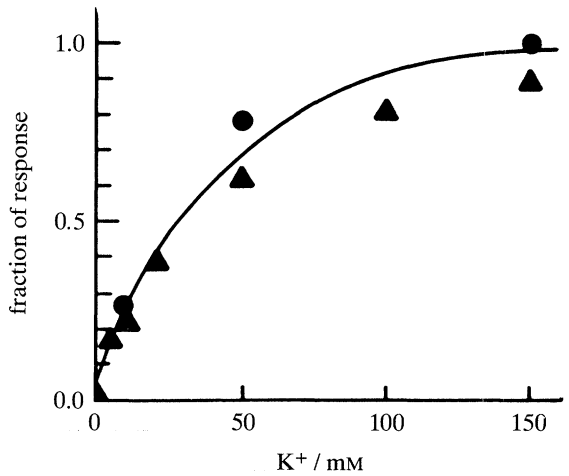

Figure 4. Effect of ionic strength on proteinase K resistance of, and ANS binding to, SecB in the absence of ligand. Circles show the fraction of SecB intact after proteinase K treatment. Triangles show increase in ANS fluorescence where maximal response is 5 units above a baseline of 9 units. Reprinted with permission from Randall (1992); © AAAS.

ligands is a flexible length of positively charged unstructured polypeptide.

The reduction in sensitivity to proteolysis of SecB, induced by its binding to a ligand could have either of two causes. The ligand could either mask the sensitive site directly or its binding could induce a conformational change in the SecB. That the latter is the case is suggested by the finding that at physiological ionic strength, or in the presence of low concentrations of divalent cations, SecB, even in the absence of ligand, is resistant to proteolysis (figure 4). Probably at low ionic strength the repulsion between the negative charges in SecB gives it a more open structure that is sensitive to proteinase K. Positively charged ions, or the binding of a positively charged ligand, would reduce the electrostatic interactions within the molecule and would allow the formation of a tighter, protease-resistant structure.

Because SecB is able to bind to positively charged peptides with little if any amino acid sequence specificity, it would be expected to bind polymers of basic amino acids. Indeed polylysine and polyarginine efficiently protect SecB from degradation by proteinase K, while polymers of glutamic acid and of prolyl glycyl proline do not. Figure 5*a* shows the protection of SecB by poly L-lysine of M_r 42 000, and figure 5*b* the protection by oligo L-lysine consisting of 15 residues. The polymer, while being thirty-fivefold more efficient than the oligomer on a molar basis, is only about twice as efficient on a mass basis. Since 0.02 μM polylysine protects approximately 0.3 μM SecB monomer, we can conclude that each molecule of polylysine of about 330 residues binds to about 15 monomers or four tetramers of SecB, so that on average a length of about twenty lysine residues protects each SecB monomer. Further experiments with shorter oligomers of lysine show that lys_7 provides no protection, while lys_8 to lys_{15} all confer about the same level of protection. This contrasts with the

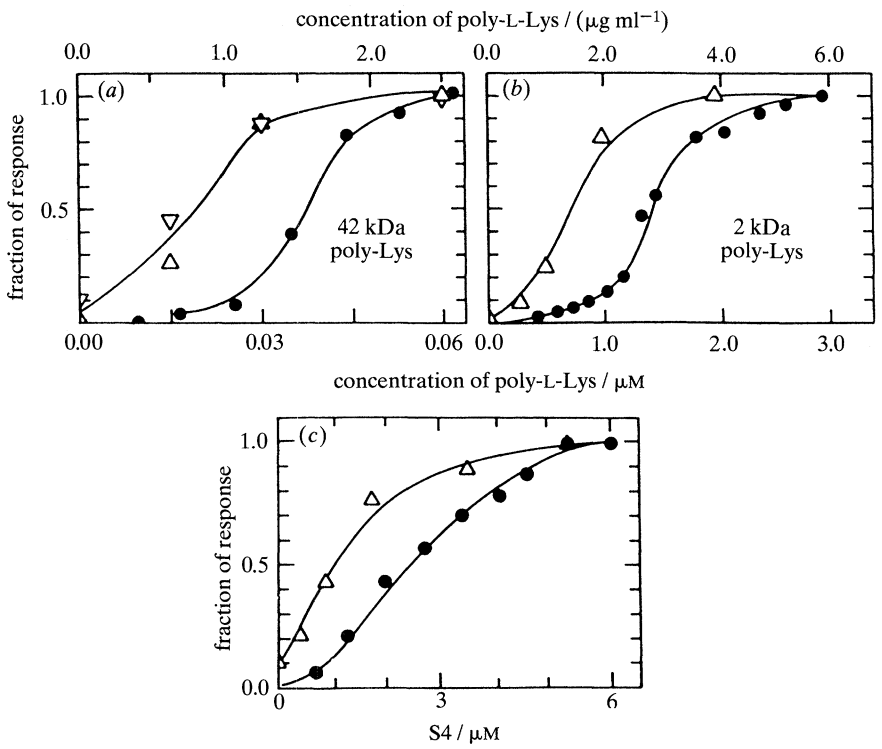

Figure 5. Effect of (*a*) polylysine, (*b*) oligolysine, (*c*) peptide S4, on proteinase K resistance of, and ANS binding to, SecB. Triangles show the binding of the ligands to SecB assayed by proteinase resistance. Closed circles show increase in ANS fluorescence where maximal increase is (*a*) 90 units; (*b*) 60 units; and (*c*) 20 units. Reprinted with permission from Randall (1992); © AAAS.

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finding that positively charged peptides of 9 and 11 residues do not confer protection (table 1). Perhaps the higher positive charge density of the lysine oligomers compensates for their lack of length.

6. INDUCED CONFORMATIONAL CHANGE IN SecB

Folded soluble proteins tend to have hydrophilic surfaces and hydrophobic interiors. Unfolded proteins, the targets of SecB, on the other hand, will have some hydrophobic regions exposed to the solvent. Thus it would not be surprising if a molecular chaperone that recognized unfolded structures contained a hydrophobic or non polar binding surface. One method of detecting a hydrophobic surface involves use of the fluorescent compound 1-anilino-naphthalene-8-sulphonate (ANS), which binds to hydrophobic patches and in so doing both increases its fluorescence intensity and shifts its emission maximum from 520 nm to 472 nm (Ptitsyn *et al.* 1990, Semisotnov *et al.* 1991). ANS fluorescence did not change on addition of SecB at low ionic strength, but as the ionic strength of the buffer was increased a small increase in fluorescence emission at 472 nm was observed. This increase matched the increase in resistance to proteinase K (figure 4), indicating that the assumption of a more resistant structure is paralleled by exposure of some hydrophobic surface.

A much greater increase in ANS binding is observed when SecB is bound to a peptide ligand, indicating a correspondingly greater exposure of hydrophobic surface. This was true for all the ligands investigated, namely R-BPTI, mastoparan, polyarginine, and as shown in figure 5, polylysine, oligolysine (lys_{15}) and peptide S4. In these studies the controls showed that ANS did not interact with either SecB or the free ligands (with the exception of polyarginine).

We conclude from these studies that the interaction of positively charged peptides with their binding sites on the surface of SecB induces a conformational change in the chaperone that exposes a hydrophobic surface to which ANS then binds. However, we cannot be sure that ANS is binding to SecB rather than to a conformation assumed by the ligand on its binding to the chaperone, or to sites that are constructed from a structural combination of both reactants. Indeed binding of ANS to the ligand was selected by Martin *et al.* (1991) as the most likely explanation of similar results obtained for binding of ANS to a complex of GroEL with either dihydrofolate reductase or rhodanese. However for the SecB ligand complex investigated here, it seems far more probable that the induced hydrophobic binding site for ANS is on the surface of SecB. The reasons are: first, that some of the ligands that induce the binding are small, and in some cases highly charged, making it unlikely that they could form, or contribute to, an area of hydrophobicity; second, it can be seen in figure 5 that the increase in fluorescence of ANS occurs at a higher concentration of ligand than does binding to SecB. If ANS were interacting with the bound ligand the increase in fluorescence should be coincident with binding. Rather the data are consistent with the interpretation that multiple peptide binding sites on a tetramer must be filled before ANS can bind, i.e. before the hydrophobic surface is exposed. Thus we believe that in these experiments ANS binds to a hydrophobic patch on SecB that appears only after induction of a conformational change in the tetramer, caused by the interaction of positively charged peptides with more than one site.

7. A MODEL

Although it is possible that the induced exposure of a hydrophobic area on the SecB tetramer functions in the interaction of the complex between SecB and its ligand with SecA, it seems more likely that this area serves as an additional binding site between unfolded polypeptides and SecB. The unfolded polypeptide, once held by interaction with SecB of several unstructured sequences having a net positive charge, would then be in an extremely favourable location for a further interaction of an available hydrophobic sequence with the newly exposed hydrophobic area on the chaperone. If this idea is correct we would expect that the hydrophobic patch would be unavailable for ANS binding when SecB is bound to a physiological ligand because it would already be filled by that ligand. This expectation is fulfilled for maltose-binding protein, since ANS fluorescence of the complex between maltose-binding protein and SecB at physiological ionic strength is less, not greater than, the sum of the ANS fluorescence obtained with the individual components, namely SecB and the relevant folding intermediate of maltose-binding protein (Randall 1992).

Our model for binding between SecB and its ligands is shown in figure 6. At low ionic stength the SecB tetramer is in an open structure with the carboxyl terminus accessible to cleavage by protease. Binding of flexible positively charged lengths of peptide or increase in ionic strength progressively tightens up the structure so that protease sensitivity decreases, and a

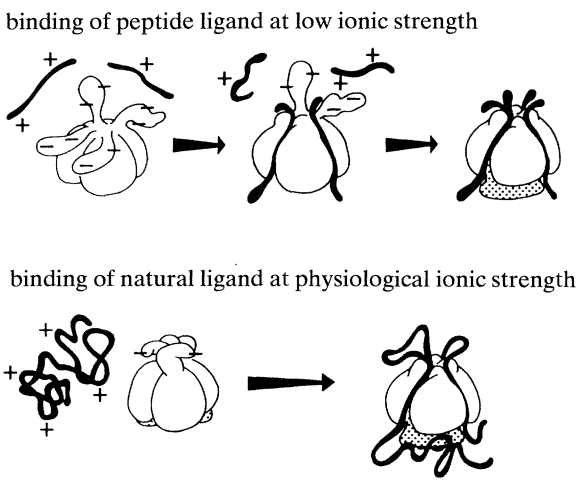

Figure 6. A model of interaction of peptides and nonnative polypeptides with SecB. See text for discussion. Reprinted with permission from Randall (1992); © AAAS.

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hydrophobic area is somewhat exposed. Once several lengths of peptide are bound in either condition the tetramer undergoes a change so that the hydrophobic area is fully exposed. When this happens there is an extra binding site for an available hydrophobic sequence in the ligand.

Multiple independently binding regions, each of rather low affinity, would lead to very stable binding of a single flexible molecule that could simultaneously interact with all or several of them. Moreover one would expect such a binding entity to have rather low specificity and thus bind a variety of flexible molecules. The requirement for flexibility in the ligands and the fact that the binding sites are for two different kinds of chemical moieties, one of which is only available appreciably in unfolded polypeptides, would ensure high specificity of interaction with just such molecules. Furthermore the appearance of the hydrophobic site only after several of the peptide sites are filled, would mediate against aggregation of SecB molecules, since, when the site did finally appear there would be a high probability of filling it with a stretch of the already bound polypeptide, whose effective concentration in the vicinity would be extremely high. This hydrophobic interaction might be equivalent to the locking mechanism suggested earlier as an explanation for the extreme stability of the complex between SecB and maltose-binding protein observed in the blockage of folding experiments.

Support for a model of binding that involves interaction of several different regions of the mature part of a physiological ligand with SecB comes from the recent study of de Cock *et al* (1992). They showed that newly synthesized outer membrane protein, PhoE, could be immune-precipitated from a cell-free translation system by antiserum raised against SecB, indicating the formation of a complex between the two proteins. A number of mutant forms of PhoE, each containing an internal deletion, were still precipitable in a complex with SecB but with lower efficiency. When non-overlapping deletions were combined the precipitation was reduced still further. The authors argue that at least four separate regions of PhoE contribute to interaction in the SecB, or that in other words there are a minimum of four sites to which SecB binds within the mature part of the protein.

Multiple binding sites for a single polypeptide, even if each is of rather low affinity, would result in a very high binding energy between chaperone and ligand. This makes the release step correspondingly difficult. However such a complex would 'breathe': each independently held sequence of the ligand would undergo intermittent temporary release before being rebound. It might be feasible for SecA, which apparently receives precursors from SecB, to exploit the breathing and to interact with the ligand one temporarily released sequence at a time, so that in effect the ligand is prised off SecB site by site in a piecemeal manner. Furthermore SecA *in situ* is an ATPase; it is possible that some of the energy released by hydrolysis of ATP could be used in removal of precursors from SecB.

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Discussion

J. Murphy (*Porton Down, Salisbury, U.K.*). What is the evidence that the SecB protein does not interact with the signal sequence?

S. J. S. Hardy. When the signal sequences are exchanged between SecB-dependent and SecB-independent precursor proteins the binding of SecB is not affected. Genetic evidence from deletion studies show that regions within the mature parts of the maltose binding protein and the PhoE protein are responsible for binding to SecB. Thirdly there is no preferential binding of the precursor of the maltose binding protein to SecB when this precursor is presented together with the mature maltose binding protein.

J. Murphy. Could it be that the binding of the precursor to SecB exposes the signal peptidase cleavage site?

S. J. S. Hardy. The cleavage of the signal peptide occurs on the other side of the cytoplasmic membrane from the binding of SecB so this peptide will not be cleaved until the protein has been released from SecB.

P. Lund (*School of Biological Sciences, University of Birmingham, U.K.*). The specificity of binding to SecB looks rather different from the specificity of binding to DnaK. A nascent chain emerging from a ribosome is presented with a number of molecular chaperones that it could bind, but SecB binds only a small subset of proteins. Is there evidence that the limited sequence similarities that you see in the peptides that bind to SecB account for this *in vivo* specificity?

S. J. S. Hardy. I am not aware that surveys of sequences of proteins emerging from ribosomes have been made in this context. However all four of the proteins we have tried do bind to SecB with some specificity so there may be nothing special about exported proteins.

P. Lund. Will maltose binding protein bind to DnaK or DnaJ *in vitro*?

S. J. S. Hardy. We have not tried that experiment.

W. J. Welch (*Department of Medicine and Physiology, University of California, San Francisco, U.S.A.*). If SecB will bind to mature protein with the signal sequence removed, how do the authors envisage that SecB

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discriminates between proteins that are to be secreted and those that are not?

S. J. S. Hardy. The signal sequence is involved indirectly since it slows down the rate of folding of the rest of the polypeptide and gives more time for SecB to bind. SecB will bind *in vivo* to proteins without signal peptides that fold slowly due to mutation.

P. Viitanen (*Du Pont de Nemours, Wilmington, U.S.A.*). The interpretation that more than one of the positively charged binding sites on SecB has to be occupied to get exposure of the ANS binding site is supported by the data for the 2 kDa polylysine, but why is the discrepancy even larger for the 42 kDa polylysine?

S. J. S. Hardy. This is probably an artefact due to the low ionic strength in the assay, which seems to allow ANS binding to the polylysine, but only in the presence of SecB.

At physiological ionic strength there is no cooperativity in the ANS fluorescence increase with concentration of polylysine; in fact the curve is strictly hyperbolic. In contrast, with oligolysine and small peptides at the higher ionic strength, the cooperativity in the fluorescence increase is maintained. This is what we would expect in our model. As the question implies, there should be no cooperativity with polylysine where a single molecule should by itself interact with all the binding sites on SecB and thus expose the putative hydrophobic patch, but there should be cooperativity with oligolysine where each molecule can only interact with a single binding site.

The reason for low ionic strength in these experiments was to allow the peptide binding assay to be carried out. That the ANS is binding to both polylysine and SecB at low ionic strength is indicated by the observation that increasing the ionic strength causes a several-fold reduction in the fluorescence to approximately the level seen when SecB is bound to a peptide. The cooperativity of the ANS binding with polylysine concentration, seen at low ionic strength, remains unexplained.

R. Jaenicke (*Department of Biophysics and Physical Biochemistry, University of Regensburg, F.R.G.*). Is it clear whether SecB is a tetramer or a monomer?

S. J. S. Hardy. It is definitely not a monomer; most people regard it as a tetramer on the basis of its elution from a gel sieving column.

R. Jaenicke. Is ligand binding to SecB independent or cooperative?

S. J. S. Hardy. Some of the binding curves show slight cooperative effects, but not all do, and there is no obvious cooperativity with the binding of the peptides.

R. Jaenicke. The authors said that SecB interacts with denatured proteins such as BPTI and RNase; were these proteins in the reduced state?

S. J. S. Hardy. Yes.

R. Jaenicke. Is there any relation between hydrophilic surface area, or accessible surface area, and binding to SecB?

S. J. S. Hardy. We have no information about that.

R. Jaenicke. In the authors' studies with maltose binding protein the binding to SecB at 4°C was hyperbolic, whereas the binding at 25°C was sigmoidal. How do they interpret that?

S. J. S. Hardy. Nearly all the binding curves have some degree of sigmoidicity and we first thought that this indicates that more than one SecB tetramer is involved in binding. But that interpretation conflicts with the kinetic data. A possible explanation is that two SecB tetramers are involved in the interaction, but that one of them helps the other to bind and does not bind itself.

F.-U. Hartl (*Memorial Sloan-Kettering Cancer Center, New York, U.S.A.*). With respect to the question as to whether DnaK and DnaJ are involved in protein export, C. Gross has published a study in which the export of outer membrane proteins becomes dependent on DnaK and DnaJ in a SecB-deletion strain of *E. coli*. This may indicate that normally the interaction with SecB is dominant, but that interactions with DnaK and DnaJ can act as a back-up system.

T. E. Creighton (*EMBL Laboratory, Heidelberg, F.R.G.*). I should like to raise the problem of what happens to charged proteins in the cytosol. Most cytosolic proteins are acidic, as is RNA, and most basic proteins are secreted from cells. Perhaps basic proteins would form large aggregates if they remained in the cytosol. So is one of the functions of SecB to protect basic proteins from aggregation before they are secreted?

S. J. S. Hardy. It is true that periplasmic proteins are positively charged compared to cytosolic proteins.

R. A. Laskey (*Wellcome CRC Institute of Cancer and Developmental Biology, University of Cambridge, U.K.*). It cannot be that secretion is a convenient way of handling basic proteins because ribosomal proteins are basic but have to be retained.

S. J. S. Hardy. In bacteria the ribosomal proteins will bind to RNA in the cytosol, whereas in eukaryotic cells these proteins combine with RNA in the nucleus. Even if ribosomal proteins bound to SecB in bacterial cells, they would not be secreted as they lack signal peptides.

T. E. Creighton. Perhaps a chaperone protects the ribosomal proteins in the cytosol in eukaryotes.

R. A. Laskey. In eukaryotic cells there is evidence for acidic proteins which shuttle back and forth between

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nucleus and cytosol. Such proteins are prime candidates for chaperoning ribosomal proteins.

G. H. Lorimer (*Du Pont de Nemours, Wilmington, U.S.A.*). It has been reported (Laminet *et al. EMBO J.* **9**, 2315–2319 (1990)) that native pre β-lactamase on long incubation with GroEL will form a binary complex with the GroEL, presumably as a result of GroEL binding to the very small amount of unfolded protein that is in equilibrium with the native state. Does this happen with maltose binding protein and SecB?

S. J. S. Hardy. We have not observed this effect in overnight incubations in the presence or absence of ATP, but perhaps this is not long enough.

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