Slowing Bacterial Translation Speed Enhances Eukaryotic Protein Folding Efficiency

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Introduction

Misfolding of eukaryotic proteins upon recombinant production in bacteria has placed great limitations on their biochemical and structural analyses and their therapeutic utilization.1,2 In bacteria, folding of polypeptide nascent chains can be delayed relative to their synthesis (“posttranslational” folding), a process that may promote misfolding of certain recombinant proteins.3,4 In contrast, the eukaryotic cytosol appears to be highly capable of efficiently folding protein domains as they emerge from the ribosome (“cotranslational” folding).3–5 Kingdom-specific molecular chaperones have been demonstrated to support each of these distinct folding regimes, including trigger factor (TF) in bacteria3,6 and the ribosome-associated complex in fungi.7 In addition to their different chaperone complements, a major difference between bacteria and eukaryotes is their translation speed. In Escherichia coli, polypeptide elongation rates vary from ∼10 amino acids per second (aa/s) during slow growth to ∼20 aa/s during fast growth.8,9 In contrast, elongation rates in eukaryotes are thought to be fairly constant and considerably slower (3–8 aa/s).10 Although ribosomal pausing at rare codons along mRNAs encoding particular proteins has been shown to affect their activities,11,12 the effect of general variations in polypeptide synthesis rates on protein folding efficiency has remained largely unexplored. In this work, we aimed to study the impact of global changes in protein synthesis rates on de novo protein folding by utilizing streptomycin (Sm)-pseudodependent (SmP) ribosomes of E. coli,13 whose polypeptide elongation rates can be modulated by varying the concentration of Sm present in the growth medium.
Results

Polypeptide elongation rates can be modulated in SmP bacteria with no detrimental effects on the folding of endogenous proteins or activation of stress response

SmP ribosomes contain mutations in protein S12 of their decoding center (see Materials and Methods). In the absence of Sm, bacteria harboring these ribosomes display a “hyperaccurate” phenotype, with considerable reduction in translation rates (~5 aa/s) and ~20-fold increase in the accuracy of amino acid incorporation compared to wild type. Addition of Sm relieves this phenotype in a concentration-dependent fashion, restoring translation speed to nearly wild-type levels, as reflected by restoration of growth rates (which correlate directly with protein synthesis speed; Fig. 1a), albeit with a concomitant ~7-fold increase in misincorporation rates compared to wild type.

Utilization of SmP ribosomes allowed us to focus on the effects of decreased polypeptide elongation rates on protein folding, since for every comparison between bacteria harboring slow ribosomes (without Sm) and bacteria harboring fast ribosomes (with Sm), all other experimental parameters were identical and constant. We also wished to ascertain that a general and constant decrease in translation speed did not lead to upregulation of molecular chaperones due, for example, to misfolding and aggregation of certain endogenous E. coli proteins. Thus, we began our analysis by comparing the levels of aggregated proteins present in the SmP strain grown under different concentrations of Sm (Fig. 1b). We observed no major differences in the patterns or levels of proteins present in the insoluble fraction of lysates prepared under native conditions. We next analyzed whether slow translation led to activation of bacterial stress response and accumulation of molecular chaperones. We performed immunoblot analyses of the steady-state levels of the major chaperone systems known to influence nascent protein folding in E. coli (Fig. 1c). We observed no major differences between cultures grown in the absence of Sm and cultures grown in the presence of Sm in the accumulation of the Hsp70 homolog DnaK, the chaperonin GroEL, or TF. Thus, we concluded that a general reduction in translation speed in E. coli does not result in major alterations in the folding efficiency of its endogenous proteins.

Slow translation speed enhances the de novo folding of firefly luciferase

Having established that there were no major differences in the molecular chaperone capacities of SmP bacteria under different antibiotic concentrations, we wished to assess whether alterations in polypeptide elongation rates per se influenced the folding efficiency of firefly luciferase (FL; 64 kDa).
a model protein whose in vivo folding and in vitro refolding requirements have been extensively characterized. Production of FL in E. coli is characterized by very poor folding yields, with the majority present as aggregated inactive material. In contrast, heterologous production of FL in the yeast Saccharomyces cerevisiae, whose ribosomes are slower than those of E. coli, results in nearly 100% of the protein being soluble active species. Remarkably, the bacterial chaperone system DnaK/DnaJ/GrpE is highly capable of assisting the in vitro refolding of FL upon dilution from a denaturant, as evidenced by the high yields (∼90%) of native luciferase obtained in its presence compared to its spontaneous refolding (<10%). These and other results have suggested that the misfolding of luciferase during its de novo production in bacteria occurs, at least partially, from a cotranslational misfolding event that the DnaK/DnaJ/GrpE system is incapable of resolving. Thus, we wished to determine whether production of FL by bacteria with slower translation rates (more closely resembling those of eukaryotes) led to beneficial effects on its folding yield. Faster ribosomes synthesize more FL chains than slower ones in the same amount of time. In order to assess FL solubility at equivalent levels of accumulation bet-

Fig. 2. Reduced translation rates enhance the de novo folding of FL. (a and b) Assessment of solubility (top) and quantification of the activity (bottom) of FL produced in Sm7 bacteria in the absence (slow) or in the presence (fast) of Sm (500 μg/ml) under conditions of low (a) or high (b) levels of accumulation (see the main text for details). The presence of FL in the total (T), supernatant (S), and pellet (P) fractions after lysis under native conditions was determined with an antibody against a c-myc tag (a) or with SDS-PAGE (b) (see Materials and Methods). The arrow indicates the position of FL in (b). FL activity in the total (T) and supernatant fractions (S) was determined from three separate experiments, with the highest value in each experiment set to 100%. Error bars represent SEM. (c) Quantification of total luciferase activity upon induction in Sm7 bacteria grown in the absence (gray bars) or in the presence (black bars) of Sm (500 μg/ml) at the time points indicated. Error bars represent SEM. Inset depicts slower growth rates in the absence of Sm, as reflected by the lower A600 values. (d) Time course of refolding of the chemically denatured FL isolated from inclusion bodies of Sm7 bacteria grown in the presence (squares) or in the absence (circles) of Sm (500 μg/ml) into a refolding buffer supplemented with the DnaK/DnaJ/GrpE chaperone system. RLU: relative light units.
ween bacteria containing slow ribosomes (in the absence of Sm) and bacteria containing fast ribosomes (in the presence of Sm), we set up the following experiment (Fig. 2a and b) (see Materials and Methods). A starter culture of Sm⁺ bacteria transformed with a plasmid encoding FL under the control of an arabinose promoter (grown in the absence of Sm) was diluted into equal volumes of

**Fig. 3 (legend on next page)**
growth media containing arabinose. One volume contained Sm (fast translation), and the other one did not (slow translation). Aliquots of each culture were harvested at regular intervals (15 min for “low accumulation” and 1 h for “high accumulation”). The content of the total FL chains produced was monitored by SDS-PAGE, followed by densitometry of the band corresponding to FL in immunoblots (for “low accumulation”) or Coomassie-brillian-blue-stained gels (for “high accumulation”). For solubility assessment, cell pellets containing equivalent amounts of the total FL protein were lysed under native conditions and separated into supernatant and pellet fractions by centrifugation. We consistently found that a larger fraction of luciferase, when translated by slow ribosomes, was present in the supernatant, and the pellet contained less aggregated material (Fig. 2a and b). This effect did not depend on the concentration of the recombinant protein produced, since very similar results were obtained with shorter or longer induction times (Fig. 2a and b). We next examined whether this increase in solubility corresponded to increased enzymatic activity, which would confirm that a greater fraction of FL indeed reached its native state under slow translation conditions. Equal volumes from the total fraction and the supernatant fraction used for solubility determination were assayed for luciferase activity (see Materials and Methods). Fractions containing FL synthesized by slow ribosomes displayed ~2-fold greater activity than those from faster ribosomes, and this effect was also independent of the levels of FL accumulation (Fig. 2a and b).

The absence of molecular chaperone induction and the higher activity of FL produced in Sm− bacteria in the absence of Sm (irrespective of levels of accumulation) suggest that the folding of FL nascent chains is enhanced by slower polypeptide elongation rates. An alternative explanation would be that, in the absence of Sm, misfolded FL chains are more efficiently degraded and, thus, lesser amounts of insoluble material accumulate under this condition than in the presence of the antibiotic. To rule out this possibility, we set up an experiment to compare the total activity of FL produced under each condition within a defined time period, irrespective of the number of FL nascent chains produced. Recombinant expression of FL was initiated in cultures with and without Sm and allowed to proceed for the same amounts of time. Equal culture volumes were harvested, and the yield of total enzymatic activity was determined for each. We observed a higher accumulation of active enzyme in the culture grown without Sm, in spite of the fact that this culture had fewer cells than the one with Sm (Fig. 2c). Since slower ribosomes could not have synthesized a greater number of total nascent chains than the faster ones within the same period of time, a higher fraction of total nascent chains must have folded correctly in the culture synthesizing FL more slowly. Thus, enhanced degradation alone cannot account for our findings.

A different scenario that could also explain our findings involves the higher rate of amino acid misincorporation by Sm− ribosomes in the presence of Sm, which could render nascent chains simply incapable of folding. If this were the case, misfolded FL produced by error-prone ribosomes should be less capable of refolding to the native state after denaturation. To test this possibility, we denatured FL isolated from inclusion bodies from bacteria grown in the presence and in the absence of Sm in urea and allowed it to refold into a buffer supplemented with the DnaK/DnaJ/GrpE chaperone system. The kinetics and refolding yields of FL translated by slow or fast ribosomes were essentially identical (Fig. 2d). Thus, we concluded that amino acid misincorporation cannot solely account for the increased misfolding of FL produced in the presence of Sm. Taken together, the above findings strongly suggest that a reduction in translation rates per se leads to significantly higher yields of native FL upon recombinant production in E. coli.

The folding of diverse aggregation-prone eukaryotic proteins is promoted by decreased bacterial translation rates

Having established that FL synthesized at slower speeds was more capable of acquiring its native state, we wished to determine whether this effect was generally applicable to eukaryotic proteins prone to aggregation when synthesized in bacterial systems. The green fluorescent protein (GFP; 27 kDa) from Aequorea victoria 20 is a single-domain protein composed mostly of β-strands that does not depend on assistance from molecular chaperones for in vitro refolding. 21 However, it displays considerable misfolding and aggregation upon recombinant production in E. coli. 5,21 A variant of GFP selected for...
efficient maturation in bacteria at 37 °C, the so-called “Cycle3” mutant\(^\text{20}\) (utilized in this study), displays soluble yields of \(<50\%\).\(^\text{21}\) In order to analyze the behavior of GFP under fast and slow translation conditions, we set up experiments similar to those described above for FL. We observed that the fraction of fluorescent GFP (indicative of the correct acquisition of its native state), when synthesized by slower ribosomes, was \(\sim 2\)-fold higher than when translated at faster rates (Fig. 3a). A similar, yet more modest, behavior was observed for its solubility.

GFP and its derivatives have been extensively utilized as reporter domains in a wide variety of fusion proteins.\(^\text{25}\) It has been shown that the GFP moiety can impose significant constraints on the de novo folding of fusion proteins in bacteria, probably as a result of intramolecular interference with the folding of adjacent domains.\(^\text{5}\) In order to assess whether interference of GFP on its fusion partner could be ameliorated by decreased translation speed, we conducted similar experiments with the previously characterized model fusion proteins GFP–enolase (74 kDa) and maltose-binding protein (MBP)–GFP (70 kDa). Both enolase and MBP are produced as soluble native species even when recombinantly expressed to very high levels in bacteria.\(^\text{5}\) In contrast, the resulting proteins, when fused to GFP, are present mainly in the insoluble fraction, and their fluorescence emission is drastically reduced.\(^\text{5}\) We observed that both GFP fusion proteins displayed \(\sim 3\)-fold higher fluorescence emission when produced by the \(\text{Sm}^\text{P}\) strain under slower translation conditions (Fig. 3b and c). Similar to GFP alone, the amount of recombinant proteins present in the soluble fraction was only moderately enhanced. We next performed an experiment to explain the observed discrepancy between the considerably higher changes in fluorescence versus solubility observed for GFP and the changes observed in its fusion proteins. We hypothesized that some amount of the recombinant proteins produced could be misfolded (and thus nonfluorescent), yet has remained in the soluble fraction under our centrifugation conditions (22,000g for 10 min). To test this possibility, we performed a gel-filtration experiment on the supernatant fraction of MBP–GFP synthesized by \(\text{Sm}^\text{P}\) bacteria in the presence of \(\text{Sm}^\text{P}\) (Fig. 3d). We analyzed each of the eluted fractions for native protein content by fluorescence emission and for total protein content by immunoblotting. We found that the native MBP–GFP peak accounted for only a small fraction of the total MBP–GFP content present in the supernatant, the majority of which eluted at earlier fractions, corresponding to higher apparent molecular weights. These results confirm our idea that the supernatants of our GFP fusions contain misfolded recombinant proteins that do not sediment under our experimental conditions. Thus, experiments involving biochemical activities, rather than solubility, more accurately reflect the folding behaviors of the proteins being studied.

We next investigated whether this approach could be successfully applied to large multidomain eu-karyotic proteins previously shown to be inefficiently folded to their native state upon recombinant production in bacteria. We selected the telomere-binding protein Cdc13 from \(S.\ cerevisiae\) (105 kDa), a protein essential for telomere maintenance that protects chromosome ends from damage and recruits the telomerase complex.\(^\text{24}\,\text{25}\) Cdc13 contains three distinct regions: an N-terminal telomerase recruitment domain, a central DNA binding domain, and a C-terminal capping region.\(^\text{26}\,\text{27}\) The central DNA binding domain of Cdc13 has been expressed as a soluble and active species in \(E.\ coli\), which has facilitated its biochemical and structural analyses.\(^\text{28}\) However, these analyses for the full-length protein have been hindered by the inability of wild-type bacteria to yield native material. Similar to our results with FL and the GFP fusion proteins, we observed that most of the full-length Cdc13 protein was present in the soluble fraction when synthesized by \(\text{Sm}^\text{P}\) ribosomes under slow translation conditions (Fig. 3e). To assess whether the full-length Cdc13 produced in this manner was indeed native, we purified it from the soluble fraction (see Supplementary Information) and compared its DNA binding activity to that of the central DNA binding domain alone by electromobility shift assay (EMSA)\(^\text{29}\) (Fig. 3f). We found that the purified full-length Cdc13 displayed DNA binding affinity and selectivity (i.e., 11-base length requirement) comparable to the well-characterized properties of the central DNA binding domain\(^\text{30}\) (Fig. 3f). Furthermore, examination version of various Cdc13 derivatives produced in the \(\text{Sm}^\text{P}\) strain, including the full-length version, shows that the additional domains of this protein are produced in native form, as the N-terminal domain stimulates telomerase activity and the C-terminal domain associates with additional telomere proteins to cap telomeric DNA \(\text{in vitro}\).\(^\text{31}\)

Discussion

In this study, we set out to investigate whether the fast polypeptide elongation rates of the bacterial ribosome could be responsible, at least partially, for the poor capacity of \(E.\ coli\) to fold proteins of eu-karyotic origin, normally translated at the considerably slower speed of the eu-karyotic ribosome. We have found that, indeed, decreasing bacterial polypeptide elongation rates to rates similar to those of eu-karyocytes promotes the folding of a diverse set of heterologous proteins. How do slower translation rates favor correct eu-karyotic protein folding? The folding pathways of eu-karyotic proteins evolved in the context of slower translation rates.\(^\text{3}\,\text{33}\,\text{32}\) However, when eu-karyotic proteins emerge from the bacterial ribosome at faster rates, longer nascent chains are exposed to greater conformational possibilities, some of which may result in misfolded species that were never originally selected against. Slower emergence from the ribosome may thus temporally restrict the number of incorrect conformations that an elongating nascent chain can adopt. Additionally,
the chaperone complement of the bacterial cytosol may be incompatible with the folding regimes of certain eukaryotic proteins.35

Diverse methodologies have been previously attempted to increase the yield of correctly folded aggregation-prone eukaryotic proteins produced in bacterial systems, with varying degrees of success. A widely utilized approach is to decrease temperature during the induction period, which has proven to be beneficial for a diverse set of proteins.3,34 However, the folding yield of a considerable number of proteins appears not to improve even at induction temperatures as low as 18 °C (such as Cdc13, discussed above). These different behaviors may be explained, at least partially, by the varying extent to which decreased temperature affects cellular processes and parameters,32 including protein synthesis and folding rates, as well as hydrophobic interaction. For proteins that misfold even at reduced temperatures, the beneficial effects of lowering temperature (e.g., slower translation rates and decreased hydrophobic interactions) might be masked by adverse effects (e.g., a strong reduction in intrinsic folding rates and the biochemical activities of molecular chaperones). Since the strategy outlined in this study targets translation speed exclusively, all other cellular processes are maintained constant. Thus, a nascent chain emerging slowly from the ribosome may benefit from unaltered folding rates and full chaperone assistance. In cases where the adverse effects of low temperature have no major repercussions on the folding properties of the protein being produced, reducing translation rate may further enhance its folding yields. Similarly, utilization of Sm ribosomes may be beneficial for the production of proteins whose de novo folding regimes depend on overexpression of or supplementation with molecular chaperones,35 a strategy that, by itself, has so far proven only of limited success.

In summary, we have found that decreasing translation rates in bacteria per se promotes the folding of a diverse set of proteins of eukaryotic origin. We found that reduced protein synthesis rates led in no case to detrimental effects on the folding of recombinant proteins. Slower translation did not result in endogenous protein misfolding or activation of bacterial stress response. We believe that our findings provide a general strategy for the production of recombinant proteins that does not rely on the individual manipulation of coding sequences or on the introduction of specific accessory factors.

### Materials and Methods

#### Strains and growth conditions

The *E. coli* Sm3 strain utilized here was CH184 (a W3110 derivative), a kind gift from Prof. D. Hughes (Uppsala University). It contains two mutations in the *rpsL* gene (see the text below), C256A and C272A, resulting in R86S and P91Q substitutions in protein S12, corresponding to *rpsL1204* in Kurland *et al.*14 and thus corresponding to strain SM3 in Zengel *et al.*13 For recombinant protein production (see the text below), this strain or a ΔDE3-lysogenized derivative (Novagen) was transformed with arabinose-controlled promoter-based plasmids36—pBAD-Luc (encoding FL with a C-terminal e-νyc-His6 epitope tag),3 pBAD-GFPuv (encoding the Cycle3 variant of GFP22), and pBAD-GFP-Eno and pBAD-MBP-GFP (encoding the fusion proteins of GFP fused to *E. coli* enolase or MBP via a 16-aa flexible linker)—or with the T7-driven promoter-based plasmid pET28-Cdc13 (encoding amino acids 1–924 of Cdc13 with an N-terminal His6 tag) or pET6H-Cdc13-DDD (encoding amino acids 451–694 of Cdc13 with an N-terminal His6 tag). Cells were grown in LB broth at 37 °C with 250 rpm of orbital shaking in volumes that occupied, at most, one fourth of the total vessel volume in the presence of ampicillin (100 μg/ml). For fast translation rates, Sm was added to a final concentration of 500 μg/ml, unless indicated otherwise. Sm cells consistently grew faster in the presence of Sm. For the experiments in Fig. 1, volumes of cells containing equivalent *A*400 values were harvested by centrifugation and lysed by spheroplasting under native conditions.37 Similar amounts of total protein in the resulting lysates were verified by the Bradford assay (BioRad).

#### Recombinant protein production

Starter cultures were grown as described above and diluted into two equal volumes (for experiments with and without addition of Sm). Protein induction was carried out when the cell density had reached *A*400 = 0.8 with 0.2% (wt/vol) arabinose or 1 mM IPTG, harvested at either 15-min intervals (for low-protein-accumulation experiments) or 1-h intervals (for high-protein-accumulation experiments). For experiments in Figs. 2a and b and 3, the total amounts of recombinant protein produced during each interval were assessed by examining equivalent amounts of cells (equal *A*400 values), which were subsequently lysed, ran on SDS-PAGE, and either immunoblotted (for low-protein-accumulation experiments) or stained with Coomassie brilliant blue (for high-protein-accumulation experiments). Aliquots harvested at time points containing equivalent levels of each recombinant protein produced in the presence and in the absence of Sm (as assessed by band densitometry) were then lysed under native conditions as described,7 and their solubility and activity or fluorescence emission were assessed (see the text below). For the experiments in Fig. 2c, equal culture volumes were harvested at the time points indicated and lysed under native conditions, and total luciferase activity was determined (see the text below).

#### rpsL sequencing

The entire rpsL gene from strain CH184 was amplified by PCR with *Pfu* turbo DNA polymerase (Stratagene) with oligonucleotide primers RPSLup (5′-CAG ACT TAC GGT TAA G-3′) and RPSLdn (5′-CAG GAT TGT CAA AAA C-3′) and sequenced with an ABI Prism 3730 capillary sequencer (Sequiserve).

#### Determination of protein solubility

Cells were harvested by centrifugation, and spheroplasts were prepared as described previously.37 Spheroplasts were lysed by dilution into an equal volume of...
native lysis buffer [5 mM MgSO\(_4\), 0.2% (vol./vol) Triton X-100 (Sigma), Complete EDTA-Free Protease Inhibitors (Roche), 100 U/ml Benzonase (Roche), and 50 mM Tris–HCl (pH 7.5)]. Aliquots were centrifuged into supernatant and pellet fractions (20,000 g for 10 min) and analyzed by SDS-PAGE, followed by either staining with Coomassie brilliant blue or immunoblotting with the anti-DnaK 8E2/2 monoclonal antibody (Stressgen), the anti-GroEL 9A1/2 monoclonal antibody (Stressgen), an anti-TF polyclonal antibody (a kind gift from Dr. P. Genevaux, Cologne), the anti-c-myc 9E10 monoclonal antibody (Roche), the anti-His\(_6\) tag monoclonal antibody HIS6.H8 (Abcam), or the anti-GFP JL8 monoclonal antibody (Clontech).

### Determination of luciferase activity and green fluorescence

Total and supernatant fractions from cells expressing FL, GFP, and GFP fusion proteins were prepared as described above, and dilutions equivalent to those used for solubility assessment were utilized. FL activity was determined using the Luciferase Assay System (Promega) in a Sirius luminometer (Berthold), as described previously. Green fluorescence was measured in a Fluorolog 3 fluorescence spectrometer (Horiba/Jobin Yvon) with excitation at 398 nm and emission at 508 nm, as described previously.

### Gel-filtration experiments

A supernatant fraction from Sm\(^{a}\) bacteria grown in the presence of Sm was prepared by native lysis, as described above. It was applied to a Superdex 75 column (GE) preequilibrated in phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), and 1.8 mM KH\(_2\)PO\(_4\) (pH 7.4)]. Fractions were collected, and equivalent volumes were immediately assayed for fluorescence emission (as described above) or ran on SDS-PAGE and immunoblotted with the anti-GFP antibody (as described above).

### Electromobility shift assays

Indicated Cdc13 protein versions utilized in EMSAs were used in TMG-30 buffer supplemented with 200 μg/ml bovine serum albumin, 200 μg/ml poly[d(C–C)], and end-radiolabeled oligonucleotide. The single-stranded telomeric oligonucleotides were 5′-GTG GTG TTG ATG TG-3′, 5′-GTG GTG TTG TG-3′, 5′-GTG GTG GTG TTG TG-3′, and 5′-GTG GTG GTG TTG TG-3′. Following a 20-min incubation at 22 °C, the samples were resolved on a 6% native polyacrylamide gel in 1× GTG buffer (29 mM taurine, 0.7 mM ethylenediaminetetraacetic acid, and 90 mM Tris), which was subsequently dried. The products were visualized with a PhosphoImager instrument (Molecular Dynamics).

### In vitro refolding assays

FL (10 μM) from inclusion bodies of the Sm\(^{a}\) strain grown in the presence (500 μg/ml) or in the absence of Sm was denatured in a denaturation buffer [6 M Gdm–HCl, 5 mM DTT, 5 mM magnesium acetate, 50 mM potassium acetate, and 25 mM Hepes–KOH (pH 7.4)] at 25 °C for 30 min. Refolding was started by 100-fold dilution into a refolding buffer [5 mM ATP, 10 mg/ml bovine serum albumin, 1 mM DTT, 5 mM magnesium acetate, 50 mM potassium acetate, and 25 mM Hepes–KOH (pH 7.4)] and allowed to proceed at 25 °C in the presence of DnaK (10 μM), DnaJ (4 μM), and GrpE (6 μM). Luciferase activity was determined as described above.

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### Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.12.042

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