Analysis of protein structure in intact cells: Crosslinking in vivo between introduced cysteines in the transmembrane domain of a bacterial chemoreceptor

ANDREW G. HUGHSON,¹ GEOFFREY F. LEE,^{2,3} AND GERALD L. HAZELBAUER^{1,2}

¹Department of Genetics and Cell Biology, and ²Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660

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Abstract

Oxidative crosslinking of cysteines introduced by site-specific mutagenesis is a powerful tool for structural analysis of proteins, but the approach has been limited to studies in vitro. We recently reported that intact cells of *Escherichia coli* could be treated with Cu(II)-(o-phenanthroline)₃ or molecular iodine in a way that left unperturbed flagellar function or general chemotactic response, yet crosslinks were quantitatively formed between select cysteines in adjoining transmembrane helices of chemoreceptor Trg. This suggested that oxidative crosslinking might be utilized for structural analysis in vivo. Thus, we used our comprehensive collection of Trg derivatives, each containing a single cysteine at one of the 54 positions in the two transmembrane segments of the receptor monomer to characterize patterns of crosslinking in vivo and in vitro for this homodimeric protein. We found that in vivo crosslinking compared favorably as a technique for structural analysis with the more conventional in vitro approach. Patterns of crosslinking generated by oxidation treatments of intact cells indicated extensive interaction of transmembrane segment 1 (TM1) with its homologous partner (TM1') in the other subunit and a more distant placement of TM2 and TM2', the same relationships identified by crosslinking in isolated membranes. In addition, the same helical faces for TM1-TM1' interaction and TM2-TM2' orientation were identified in vivo and in vitro. The correspondence of the patterns also indicates that structural features identified by analysis of in vitro crosslinking are relevant to the organization of the chemoreceptor in its native environment, the intact, functional cell. It appears that the different features of the two functionally benign treatments used for in vivo oxidations can provide insights into protein dynamics.

Keywords: bacterial chemotaxis; helical bundles; transmembrane helices; transmembrane receptors

Oxidative crosslinking of specific cysteine pairs placed in a protein by site-specific mutagenesis is a powerful tool for structural analysis (Falke & Koshland, 1987; Falke et al., 1988; Milligan & Koshland, 1988; Karnik & Khorana, 1990; Francis et al., 1991; Carreaga & Falke, 1992; Stoddard et al., 1992; Lee MH et al., 1994; Scott & Stoddard, 1994; Zhang & Fillingame, 1995). The approach has been particularly productive when applied to transmembrane domains of integral membrane proteins (Lynch & Koshland, 1991; Pakula & Simon, 1992; Whitley et al., 1993; Duché et al., 1994; Lee GF et al., 1994; Chervitz et al., 1995; Lee et al.,

1995b; Maruyama et al., 1995; Chervitz & Falke, 1995). For two bacterial chemoreceptors, structures of entire transmembrane domains have been deduced using this strategy (Pakula & Simon, 1992; Lee GF et al., 1994). Since disulfide crosslinks would not be expected to be stable in the reducing environment of a living cell, such crosslinking studies of transmembrane domains have been performed with material from broken cells or with purified proteins. An exception was a characterization using whole cells of spontaneous disulfides formed between introduced cysteines at the periplasmic ends of the two transmembrane segments of leader peptidase (Whitley et al., 1993). In our studies of cysteine-containing forms of chemoreceptor Trg (Lee et al., 1995b), we found that in vivo crosslinking was possible in the transmembrane domain of a cytoplasmic membrane protein even at some distance from the membrane surface.

Trg is one of four homologous, transmembrane chemoreceptor proteins in *Escherichia coli* and *Salmonella typhimurium* (Bourret

Reprint requests to: Gerald L. Hazelbauer, Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660; e-mail: hazelbau@membrane.chem.wsu.edu.

³Present address: Department of Protein Engineering, Mail Stop 27, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080.

et al., 1991; Hazelbauer, 1992). It mediates chemotactic response to ribose and to galactose by recognition of the respective sugar-occupied, periplasmic binding proteins. The native form of the chemoreceptors is a stable homodimer (Milligan & Koshland, 1988) of two $\approx 60,000 M_r$ subunits, organized in three domains: a periplasmic ligand-recognition domain, a cytoplasmic signaling and adaptation domain, and a transmembrane domain that connects the two hydrophilic domains by means of four transmembrane segments, two from each subunit. The highly conserved cytoplasmic domain controls the activity of a histidine kinase with which it forms a noncovalent complex (Gegner et al., 1992; Schuster et al., 1993). Changes in kinase activity result in altered extents of phosphorylation of a response regulator that controls the rotational bias of the flagellar motors and thus motile behavior.

The structures of the periplasmic fragments of chemoreceptors Tar_S and Tar_E, from *S. typhimurium* and *E. coli*, respectively, have been determined by X-ray crystallography (Milburn et al., 1991; Yeh et al., 1993; Bowie et al., 1995). The two structures, essentially identical, are homodimers of two four-helix bundles in which two long helices of each bundle ($\alpha 1$ and $\alpha 4$) extend over 70 Å from the membrane surface to the ligand-binding site and two shorter helices pack on the membrane-distal segments of the longer helices. Subunit packing extends over much of the elongated structure and involves in large part interaction of $\alpha 1$ with $\alpha 1'$. The organizations of the transmembrane domains of chemoreceptors Tar_E and Trg_E were deduced from patterns of oxidative crosslinking in vitro between introduced cysteines. These deduced structures are very similar bundles of four helical segments that appear to be uninterrupted extensions of the four central helices of the periplasmic domain (Pakula & Simon, 1992; Lee GF et al., 1994).

In our study of the transmembrane domain of Trg, we used site-specific mutagenesis to create a family of 54 *trg* genes, each of which coded for a Trg polypeptide containing a single cysteine, at each one of the 30 possible positions in the first transmembrane segment (TM1) and the 24 possible positions in TM2 (Lee GF et al., 1994). In a native homodimer, such receptors contain two cysteines, located at comparable positions in the two subunits. If the two sulfhydryls of those cysteines were so positioned that a disulfide could be formed, then oxidation would create a covalently crosslinked dimer connecting TM1 to TM1' or TM2 to TM2'. Similarly, proteins with two cysteines per polypeptide, one in TM1 and the other in TM2, might be crosslinked within or between the subunits. We characterized crosslinking for the 54 single-cysteine forms and a set of double-cysteine forms using in vitro oxidation of receptors contained in isolated membranes, and found consistent patterns that allowed us to deduce a three-dimensional organization for the transmembrane domain (Lee GF et al., 1994). Subsequently, we explored the possibility that, in an intact bacterial cell, the hydrophobic region of the cytoplasmic membrane might provide sufficient insulation from cytoplasmic reducing power to allow stable formation of disulfides within that membrane. We found oxidation conditions in which intact cells of *E. coli* could be treated without significant effects on motility or chemotactic response, yet select cysteine pairs in the transmembrane domain of a chemoreceptor could be oxidatively crosslinked to essentially 100% (Lee et al., 1995b). This development suggested it might be possible to exploit oxidative crosslinking in vivo between introduced cysteines as a means of gaining information about the structure of the transmembrane domain of a protein contained in its natural environment: an intact, functionally unperturbed cell.

Results and discussion

Oxidative crosslinking in vivo and in vitro

In order to determine the efficacy of in vivo crosslinking as a technique for structural analysis, we submitted our collection of 54 single-cysteine forms of Trg to two alternative, functionally benign oxidation treatments for intact cells of *E. coli*, one with the catalyst Cu(II)-(o-phenanthroline)₃ (hereafter Cu-phenanthroline) and the other with the oxidant molecular iodine (Lee et al., 1995b). The two alternative treatments had been used for our initial in vivo crosslinking experiments because the experimental design for those experiments required essentially 100% crosslinking and, with one exception, a cysteine pair oxidized essentially completely by one functionally benign treatment was not as extensively crosslinked by the other. Because the basis for the differential effectiveness of the two oxidation treatments was not clear, it was important to examine the effects of both treatments on our entire collection of cysteine-containing proteins. In addition, we examined iodine-induced crosslinking in vitro using isolated membrane treated with the same reagent concentration and for the same time as used for the in vivo experiments. Oxidation was initiated by mixing a suspension of cells or membranes with a solution of molecular iodine or Cu-phenanthroline. Samples were taken into a stop buffer just before and at various times after mixing. The extent of oxidative crosslinking in each sample was analyzed by SDS-gel electrophoresis in the absence of reducing agent, immunoblotting with anti-Trg and quantification by densitometry of the electrophoretic species corresponding to unreacted monomer and crosslinked dimer (Lee GF et al., 1994).

Patterns of crosslinking

For positions exhibiting detectable crosslinking, we found that oxidation in vivo by molecular iodine reached a maximum within a few seconds, consistent with observations of in vitro oxidation of cysteine pairs by this reagent (Pakula & Simon, 1991), whereas in vivo oxidation catalyzed by Cu-phenanthroline increased over periods of a few to several minutes to reach a plateau level. The plateau value of percent crosslinking was roughly proportional to the initial rate, a pattern that can be understood in light of competing oxidation reactions that block the possibility of disulfide formation (Carreaga & Falke, 1992). For cysteine pairs exhibiting substantial crosslinking upon treatment with the functionally benign oxidation conditions, reducing the iodine concentration did not extend the time of reaction over which crosslinking occurred but instead simply reduced the extent of crosslinking. In contrast, reducing the Cu-phenanthroline concentration extended the time over which crosslinking increased to a plateau level. These properties are consistent with the difference between a reactant (iodine) that is consumed as it oxidizes and a catalyst (Cu-phenanthroline) that is not. We chose the extent of crosslinking 15 s after mixing with iodine or 10 min after mixing with Cu-phenanthroline as appropriate values for comparing crosslinking between different positions or crosslinking generated by different treatments. Average values for these measurements are shown in Figure 1. Crosslinking induced in the four conditions, treatments by iodine or Cu-phenanthroline in vivo, by iodine in vitro, and by Cu-phenanthroline in vitro as previously determined (Lee GF et al., 1994) are displayed as a function of residue position in TM1 and TM2. To facilitate comparisons between specific conditions or receptor environments, the data in Figure 1 are also displayed in pairwise

Fig. 1. Oxidative crosslinking between homologically placed cysteines in the two subunits of the transmembrane domain of the Trg homodimer. Trg proteins with a single cysteine per polypeptide chain (two cysteines per dimer) and contained in intact cells (c) or isolated membrane (m) were treated with molecular iodine (I) or Cu-phenanthroline (CP), and the extent of oxidative crosslinking between the subunits induced by these treatments was determined as described in Materials and methods. Average values from two to four experiments, with standard errors indicated by error bars, are plotted as a function of the position of the cysteine in the linear sequence of TM1 (A) and TM2 (B), both oriented with the periplasmic border on the left. Averages without error bars had standard errors too small to display. Crosslinking in vivo between cysteines at 218 and 218' could not be determined because the low cellular content of this cysteine-substituted protein precluded detection in the assay conditions used (Lee et al., 1995a).

grouping in Figures 2 and 3. We reasoned that crosslinking might vary as a function of the depth of residues in the lipid bilayer because of reagent accessibility, changes in functional redox potential, or other parameters related to the membrane environment, and thus it was useful to assess patterns of crosslinking not only by specific extents of reaction, as shown in Figures 1, 2, and 3, but also by identifying local maxima in crosslinking (Lee GF et al., 1994). Figures 4 and 5 show pairwise comparisons of such maxima displayed on helical net diagrams of the two transmembrane segments. As seen in the figures, there was extensive correspondence in the patterns of crosslinking as a function of residue position among the four different data sets. Although the absolute extent of crosslinking at a single position varied for different conditions and some conditions resulted in substantial crosslinking at a position for which

other conditions caused little reaction, whether crosslinks were induced in vivo or in vitro, with molecular iodine or Cu-phenanthroline, local maxima appeared at identical or adjacent positions. Since we have assessed crosslinking as the result of two different oxidation conditions as well as in two different environments, there were several viewpoints from which the data could be analyzed. In the following sections we consider first a technical one and then structural ones.

Comparing oxidation conditions

Our initial characterization of patterns of crosslinking between cysteines at homologous positions in the two subunits of Trg utilized Cu-phenanthroline treatment of isolated membrane in vitro

Fig. 2. Comparison of oxidation treatments. Data from Figure 1 are displayed in pairs to provide direct comparisons of oxidative crosslinking of Trg induced by treatment with molecular iodine (I) or Cu-phenanthroline (CP). Part A shows crosslinking of Trg contained in membranes and Part B of Trg contained in intact cells.

(Lee GF et al., 1994). In that study we observed a number of positions of substantial crosslinking between TM1 and TM1'. The positions of local maxima for crosslinking defined a distinct helical face, providing evidence for the helical organization of TM1

and identifying the putative packing interface between TM1 and TM1'. Treatment of Trg in isolated membrane with molecular iodine also induced substantial crosslinking at several positions between TM1 and TM1' (Fig. 2A). Positions of local maxima in

Fig. 3. Comparison of receptor environments. Data from Figure 1 are displayed in pairs to provide direct comparisons of oxidative crosslinking of Trg contained in intact cells (c) or in isolated membrane (m). Part A shows crosslinking induced by treatment with Cu-phenanthroline and Part B crosslinking induced by molecular iodine.

Fig. 4. Local maxima in homologous crosslinking generated by two different oxidation treatments. Local maxima in crosslinking (see Fig. 1) generated by Cu-phenanthroline (filled circles) or by molecular iodine (boxes) are displayed on helical net representations of TM1 and TM2 in which the position of each residue is marked by a circle. We considered local maxima as those positions at which percent crosslinking was greater than that for either neighbor by amounts greater than the sum of the respective standard errors (see Fig. 1). The two transmembrane helices are oriented as they would be in the cytoplasmic membrane with the periplasm at the top and the cytoplasm at the bottom. The vertical axes are labeled with residue numbers and the horizontal axes with rotational positions in degrees around the circumference of the helices. Part **A** shows crosslinking for Trg contained in isolated membranes (in vitro) and Part **B** for Trg contained in intact cells (in vivo).-

crosslinking catalyzed by iodine defined the identical interaction face to the one identified by Cu-phenanthroline crosslinking (Fig. 4A), even though the particular positions of maximal crosslinking were slightly different. A very similar correspondence between patterns created by the two oxidation treatments was observed for TM1-TM1' crosslinking in vivo (Figs. 2B, 4B) and for TM2-TM2' crosslinking in vitro (Figs. 2A, 4A). The remaining combination, TM2-TM2' crosslinking in vivo, exhibited such low levels of reaction in the presence of iodine that meaningful comparisons

Fig. 5. Local maxima in homologous crosslinking observed in vitro and in vivo. Local maxima in crosslinking (see Fig. 1) observed for Trg in isolated membranes (filled circles) or in intact cells (boxes) are displayed on helical net diagrams as in Figure 4. Part **A** shows crosslinking induced by Cu-phenanthroline and Part **B** induced by molecular iodine.

of the two oxidation treatments were not possible. Taken together the data indicate that, although the two oxidation treatments possess some different features (see below), the patterns of oxidative crosslinking between cysteines on neighboring helices were not significantly affected by the nature of the oxidation treatment. Thus in terms of structural analysis, the two oxidation conditions can be used to complement each other in comprehensive structural investigations.

Comparing receptor environments

To what extent can the unconventional procedure of in vivo oxidation provide internally consistent information about structural organization in a transmembrane domain, and to what extent would that information be consistent with the structural model deduced by the more conventional in vitro approach? In vivo crosslinking

was extensive for several cysteine pairs that spanned the TM1-TM1' interface but was more modest in extent and frequency across the TM2-TM2' interface (Fig. 2B), exactly the pattern observed in our *in vitro* study with Cu-phenanthroline (Lee GF *et al.*, 1994) as well as with our new *in vitro* experiments using molecular iodine (Fig. 2A). At the detailed level of comparing the positions of extensive crosslinking (Fig. 3) and of comparing local maxima along the sequences of the transmembrane segments (Fig. 5), crosslinking induced in intact cells was substantial at positions that also exhibited substantial crosslinking *in vitro*, although in some cases magnitudes of the crosslinking values differed (Fig. 3). In most cases, local maxima of crosslinking *in vivo* were at the same positions as local maxima observed in the corresponding *in vitro* studies. The remaining *in vivo* maxima were distributed along the same interaction faces identified by the maxima common to the *in vivo* and *in vitro* patterns (Fig. 5). Thus, as a technique for structural analysis, identification of patterns of oxidative crosslinking induced in intact cells compared favorably with the more conventional *in vitro* approach. The combined patterns of *in vivo* crosslinking induced by the two oxidation treatments (Fig. 2B) indicated that the structure of the transmembrane domain of Trg in intact cells involved close placement of TM1 and TM1' and a greater separation of TM2 and TM2', defined the specific face on which TM1 packed on its homologous partner TM1' (Fig. 4A), and identified, on the basis of limited information, the apposing faces of the more distantly placed TM2-TM2' pair (Fig. 4B). These were in essence the conclusions drawn from our original *in vitro* studies of crosslinking between homologically placed cysteines in TM1-TM1' and TM2-TM2' (Lee GF *et al.*, 1994). The detailed orientations of the two helical pair were identical for the two approaches. Our data can also address the converse issue: Is a structure deduced from *in vitro* experiments relevant to the chemoreceptor in its natural environment, an intact cell? The correspondence of *in vitro* and *in vivo* data documented in Figs. 3 and 5 provides strong support for the *in vivo* significance of the structure determined *in vitro*.

Domain dynamics

Patterns of oxidative crosslinking between introduced cysteines provide structural information because the rates and extents of such crosslinking are strongly dependent on the proximity of the reactive groups (Carreaga & Falke, 1992). However, reaction-relevant proximity involves at least two important factors: (1) the distance separating the two residues in the three-dimensional structure of the protein, and (2) the dynamic fluctuations by which the relevant segments of the protein deviate from that average structure. The particular features of disulfide crosslinking induced by the two oxidation conditions may reflect different relative contributions of average separation versus dynamic motion. Oxidation by iodine occurs rapidly for a brief time (see above and Pakula & Simon, 1991), whereas oxidation catalyzed by Cu-phenanthroline occurs more slowly over an extended period of time. Patterns of crosslinks induced by the two treatments exhibit systematic differences. Both *in vitro* (Fig. 2A) and *in vivo* (Fig. 2B) iodine treatment resulted in fewer crosslinked positions than Cu-phenanthroline and those positions corresponded precisely with helical periodicity whereas crosslinking induced by Cu-phenanthroline occurred at more positions and was more widely distributed. The cases of TM1 *in vitro* and *in vivo* are particularly striking (Fig. 2A, B). These patterns imply that rapid and brief oxidation by iodine captures in crosslinks

those cysteine pairs that are close to each other in the mean three-dimensional structure, whereas crosslinks observed with Cu-phenanthroline treatment include not only such nearby pairs but also some that come into reactive proximity as the result of substantial dynamic fluctuations from that mean structure. This differential sensitivity of the two oxidation treatments to dynamic fluctuations is plausible even though such movements would be expected to occur many orders of magnitude more rapidly than the time course of crosslinking, because the low probability of productive reaction of colliding sulfhydryls means that on the average many collisions occur before productive oxidative crosslinking (Carreaga & Falke, 1992). Considering the differences between patterns of crosslinking induced by the two reagents as a reflection of protein dynamics and the common features as a reflection of average structure provides some interesting implications. It reinforces the notion that TM1-TM1' are involved in intimate interactions and TM2-TM2' are not. Iodine induced more complete TM1-TM1' crosslinks than Cu-phenanthroline; the opposite is true for TM2-TM2' crosslinks. The magnitude and striking helical periodicity of TM1-TM1' crosslinking induced by iodine *in vitro* (Fig. 2A) and *in vivo* (Fig. 2B) strongly suggest that the two Trg helices interact as a helical coiled-coil, as postulated and modeled for TM1-TM1' of Tar (Scott & Stoddard, 1994). However, we observed a distinct gradient of crosslinking, highest toward the periplasmic end, lowest at the cytoplasmic end. For *in vivo* crosslinking, this gradient could well reflect the influence of the reducing cytoplasm, but the gradient of crosslinking was also observed in experiments performed with isolated membrane vesicles (Fig. 3B). Thus it appears that some feature of TM1 and TM1' interaction is not uniform across the width of the membrane. Conceivably, tight packing of the helices near the cytoplasm could reduce accessibility for iodine, but since Cu-phenanthroline, a larger reagent, induced greater crosslinking in this region than did iodine *in vitro* (Fig. 2A) or *in vivo* (Fig. 2B), this explanation is unlikely. Instead we favor the notion that the coiled-coil interaction of TM1 and TM1' of Trg dissipates toward the cytoplasmic side of the transmembrane domain, a pattern also suggested for Tar (Chervitz & Falke, 1995). For cysteine pairs that are well-positioned for crosslinking in the average structure, increased structural mobility might be expected to depress, not enhance crosslinking. Using this notion, we could speculate that the reduced iodine-induced crosslinking of TM1 to TM1' for Trg in membrane vesicles versus intact cells (Fig. 3A) implies that disruption of the cell and isolation of membrane increases dynamic movement in the receptor above its level *in vivo*.

Closing remarks

The data presented in this study demonstrate that crosslinking induced in intact cells by functionally benign treatments can provide sufficient information to deduce many features of the three-dimensional organization of the transmembrane domain of a chemoreceptor. Analyses *in vivo* and *in vitro* suggested essentially the same organization, thus providing mutual reinforcement for the significance of the two approaches. Our analysis of Trg structure in intact cells was not based on any unique feature of Trg or of chemoreceptors, but only on the availability of derivatives containing one or two cysteines per polypeptide. Thus it is likely that the same strategy would be productive for the analysis of other proteins located in the cytoplasmic membrane of *E. coli* and perhaps for proteins in the comparable membranes of other organisms.

Materials and methods

Strains and plasmids

CP177 (Park & Hazelbauer, 1986) and CP553 (Burrows et al., 1989) are strains of *E. coli* K-12 derived from OW1 (Ordal & Adler, 1974) that contain, respectively, a deletion of *trg* or deletions of the four chemoreceptor genes and of *cheR* and *cheB*. pGB1 (Burrows et al., 1989) carries wild-type *trg* under the control of a modified *lac* promoter/operator, producing a polypeptide containing a single cysteine (C23). Its derivatives code for Trg with a serine replacing the native cysteine (C23S) and for a cysteine at some position in one or the other transmembrane segment (Lee GF et al., 1994). Crosslinking results are reported for Trg produced from pGB1 or its derivatives harbored in CP177 (iodine—in vivo) or CP553 (all others). CP553 cells used in the in vivo Cu-phenanthroline experiments also contained pAI12 which carries the gene for ribose-binding protein (Iida et al., 1984).

In vivo crosslinking

Strains to be used for in vivo crosslinking experiments were treated in a manner designed to produce physiologically unperturbed cells. Cells were inoculated from a frozen glycerol stock into 2 mL tryptone broth containing 50 µg/mL ampicillin; incubated overnight with vigorous agitation at 35 °C; inoculated from that culture to 1.0×10^8 cells/mL in 4 mL H1 salts medium (Hazelbauer et al., 1969) containing required amino acids at 0.5 mM, 0.4% sodium succinate, 50 µg/mL ampicillin, 25 µg/mL chloramphenicol (for cells harboring pAI12), and 50 µM isopropyl β -D-thiogalactoside; incubated as above until reaching 2.5×10^8 cells/mL; inoculated from that culture to 2.5×10^7 cells/mL in the same minimal medium; incubated as above until reaching 2.5×10^8 cells per mL; and then rapidly cooled in an ice slurry to be kept for use that day. For experiments using Cu-phenanthroline, 0.5 mL cell suspension was placed in a 15 mL beaker, incubated at 35 °C with vigorous agitation to provide generous aeration and after 5 min an equal volume of water (for control cells) at 35 °C or solution of Cu-phenanthroline, prepared as described (Lee et al., 1995b), was added. Samples (40 µL) were removed just before, and 5 and 10 min after, addition of the catalyst, immediately mixed with 10 µL of stop solution/non-reducing sample buffer (20 mM Tris, 8 mM NaH_2PO_4 , pH 7.8, 12.5 mM EDTA, 12.5 mM N-ethylmaleimide, 1.25% SDS, 12.5% w/v sucrose and 2.5 µg/mL bromophenol blue), and then placed on ice. Samples were boiled for 4 min and stored at -20 °C until further processing. Experiments using molecular iodine were performed in essentially the same way (see Lee et al., 1995) except that 2.5 mL of culture were placed in a 10 mL beaker, incubated on a shaker at 25 °C for 5 min, and 2.5 mL of 0.5 mM iodine or water (for control cells) at 25 °C added. After 15 s at 25 °C, the cells were processed for analysis of crosslinking.

Preparation of membrane vesicles

Strains harboring pGB1 or its derivatives were grown in 10 mL Luria broth at 35 °C with agitation. At $\sim 2 \times 10^8$ cells/mL, isopropylthio- β -D-galactoside was added to 1 mM to induce the synthesis of Trg. After 4 h the cells were centrifuged, washed with H1 salts medium, suspended in 1 mL of 0.1 M sodium phosphate, pH 7.3, 0.5 mg/mL DNase, 10% (w/v) glycerol, 5 mM EDTA, 5 mM 1,10-phenanthroline, and transferred to 5 mL plastic scintillation

vials for storage at -20 °C. Cells were thawed on ice in the presence of 2 mM phenylmethylsulfonyl fluoride newly added from a 100 mM solution in ethanol, the vial placed in an ice-salt bath, and the cell suspension submitted to 5-sec pulses from a Tekmar TM-250 sonic disruptor equipped with a 9-mm diameter horn with 25 sec intervals between pulses. Four to six pulses were used to disrupt >95% of the cells as judged by examination under a phase-contrast microscope. Samples were centrifuged 5 min at $16,000 \times g$ and 4 °C in a microcentrifuge and the supernatant centrifuged at 4 °C, 100,000 rpm, 30 min in a TL100.2 rotor to pellet membrane vesicles. Pellets were suspended in 30 µL membrane buffer [50 mM sodium phosphate, pH 7.3, 10% (w/v) glycerol] and a sample taken for determination of protein by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard. Membranes were stored at -70 °C.

In vitro crosslinking using molecular iodine

Membrane preparations were diluted to 2.0 mg protein/mL in membrane buffer. 30 µL was equilibrated to room temp (21 °C) in a microcentrifuge tube, a 10 µL sample removed, immediately mixed with 30 µL non-reducing sample buffer, and boiled for 4 min. 20 µL of 1.0 mM molecular iodine was then added to the remaining 20 µL of suspension. After 15 sec, a 20 µL sample was removed, immediately mixed with 20 µL non-reducing sample buffer, and boiled for 4 min. Boiled samples were stored at -20 °C until further processing.

In vitro crosslinking using copper-phenanthroline

Samples at 2.5 mg protein/mL were prepared by addition of membrane buffer. 48 µL of this suspension in a microcentrifuge tube at 37 °C were mixed with 12 µL of 15 mM CuSO_4 , 45 mM of 1,10-phenanthroline, 50 mM NaH_2PO_4 , pH 7.3, 10% (w/v) glycerol. At various times 5 µL samples were withdrawn, added to 20 µL non-reducing sample buffer, boiled 4 min, and stored at -20 °C. See Lee GF et al. (1994) for additional information.

Quantification of crosslinking

Samples from crosslinking experiments were analyzed by SDS-polyacrylamide gel electrophoresis in the absence of reducing agents, immunoblotting using anti-Trg serum, and treatment with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) followed by 4-chloro-1-naphthol (Morgan et al., 1993). The extent of crosslinking for each sample was quantified by measuring the intensity of staining of the crosslinked and monomeric species using a Molecular Dynamics scanning densitometer and the analysis program ImageQuant (version 3.2). A value for percent crosslinking was calculated by dividing the intensity of the crosslinked species by the sum of intensities of the two species and multiplying by 100. Analysis was done using immunoblots in which staining intensities were linearly related to the amount of protein.

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