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Affinity-chromatographic purification of sixteen cysteine-substituted maltoporin variants: thiol reactivity and cross-linking in an outer membrane protein of *Escherichia coli*

Greg Francis, Lyndall Brennan and Tom Ferenci

Department of Microbiology, University of Sydney, Sydney, N.S.W. (Australia)

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Wild-type and 16 variant maltoporins with site-directed cysteine substitutions at 14 sites were purified by a novel one-step affinity-chromatographic procedure. The trimer stability of purified proteins with C22S, C38S and G103C substitutions was reduced compared to wild-type maltoporin. Quantitative labelling with *N*-ethyl[^{14}C]maleimide, cross-linking with bifunctional bismaleimides and disulphide formation was used to test the reactivity of cysteines in the folded protein. The maleimide reactivity of the residues was in the order: 152 ≅ 153 > 265 > 30 ≅ 103 ≅ 120 ≅ 154 ≅ 382 > 57 ≅ 146, with the other sites (22, 38, 97, 184) poorly labelled. Only cysteines at 152 or 153 permitted the formation of inter-subunit disulphide bonds suggesting these residues are located within 0.5–0.9 nm of each other in homotrimers of maltoporin. S152C and S153C as well as S154C permitted the formation of inter-subunit cross-links using bifunctional bismaleimides. The cross-linkability and the high reactivity to *N*-ethylmaleimide of the 150 region was consistent with the current model of the structure of maltoporin in the outer membrane; the reactivity of the other sites is also discussed within the context of this model.

Introduction

Maltoporin (LamB protein) is a much-studied maltodextrin facilitator in the outer membrane of *Escherichia coli* [1–3]. Its structure is unknown at high resolution but is under investigation [4]. Maltoporin is a transmembrane protein whose average hydrophobicity is less that that of many soluble proteins and its secondary structure content is 50% β-sheet and 15% α-helix [5]. Maltoporin forms maltooligosaccharide-selective water filled channels across the outer membrane and is thought to contain three independent maltodextrin binding sites per trimer [6]. These sites bind starch from the outside of the bacterium [7]. Maltoporin also constitutes the receptor for the binding of bacteriophages Lambda and K10 (references in Refs. 8 and 9). Antibodies and mapping of proteolytic cleavage sites have also been used to probe the topology of maltoporin [10–13].

Maltoporin, as an entirely integral, β-structured membrane protein, must contain a large number of transmembrane segments (at least 15 according to the latest model [9] shown in Fig. 1). Recent data on the accessibility of additional parts of the protein, determined by Molla et al. [11], was fully consistent with the proposed orientation of four of the segments in the proposed model. The cysteine mutants described in this paper were constructed to study the reactivity of particular sites to further test the model and extend our knowledge of the accessibility of several regions of the protein. The sites of substitution are also shown in Fig. 1.

The cysteine mutagenesis approach was adopted recently for structural studies in other proteins [14–17] but there is no systematic evidence of the reliability of this approach in predicting membrane protein topography in the absence of detailed structural information. This study considers the accessibility of several cysteine substitutions in a single region of the protein assumed to be surface-located. The usefulness of thiols as topological markers was tested with substitutions in the 146–154 region, in segment (m) of Fig. 1. This loop was proposed to be external and accessible on the basis of previous genetic, immunological and proteolytic studies [9,13,18].

Correspondence: T. Ferenci, Department of Microbiology, University of Sydney, Sydney, N.S.W. 2006, Australia.

Cysteines also permit topological information to be derived from the possible cross-linking of residues within a maltoporin homotrimer. Thiols can be oxidised to disulphides, given the correct spacing [19] of particular cysteine residues, and several bifunctional maleimides are also available to study cross-linkability of residues at different sites. In this study, these aspects were investigated for cysteines at 14 positions in maltoporin.

Quantitation of the chemical reactivity of the thiol-substituted proteins required purification of a large number of variant proteins. Several methods of LamB purification are in the literature [1,20,21] but preliminary experiments suggested that methods involving just ion-exchange chromatography did not result in high enough purity for thiol quantitation. Some contamination problems in earlier channel reconstitution experiments using purified maltoporin preparations has also been reported [1]. So we developed a simple, one step purification of this membrane protein using affinity chromatography which yields pure protein and low backgrounds in thiol modification experiments. This rapid purification should find widespread use in studies with maltoporin including structural studies and crystallography [4], mutant analyses and channel reconstitutions.

Materials and Methods

Materials

N,N'-Bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine, iodoacetic acid, *N*-ethylmaleimide and 5,5'-dithiobis(2-nitrobenzoic acid), were purchased from Sigma Chemical Company, St Louis, MO, U.S.A.; 1,1'-(methylenedi-4,1-phenyiene)bismaleimide was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.; bismaleimidohexane and peroxide-free Triton X-100 were purchased from Pierce Chemicals, Rockford, IL, U.S.A.; bis(maleimido)methyl ether and phenylmethylsulphonyl fluoride (PMSF) was obtained from Boehringer Mannheim, North Ryde, N.S.W, Australia. Protein concentrations were determined using the Micro-BCA protein assay reagent kit purchased from Pierce, Rockford, IL, U.S.A. *N*-Ethyl[^{14}C] maleimide was from Amersham (Australia) Sydney.

Bacterial strains, plasmids and growth conditions

All bacterial strains used were derivatives of *Escherichia coli* K12. The following plasmids contained the *lamB* genes encoding the particular substitution(s) and were used as the source of mutant maltoporin: wild-type, pAM1520 [22]; C22S + C38S, pAM1611 [22]; C38S, pAM1610 [22]. The following mutations were

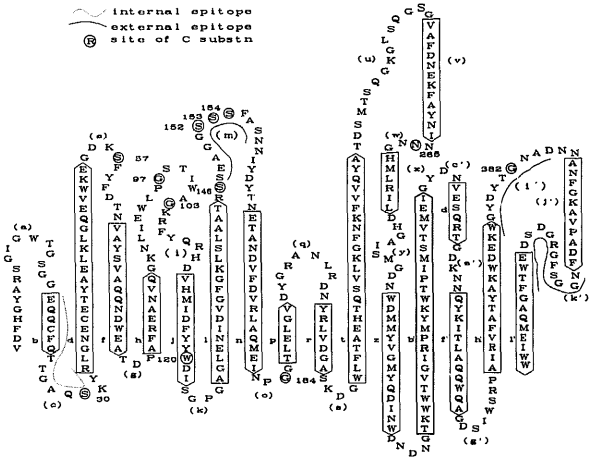

Fig. 1. A model of maltoporin folding across the outer membrane. The model derived in Ref. 9 is shown with the sites of cysteine substitution shown by circled, numbered residues. The segments of the protein (a)–(l') are shown as well as the epitopes studied in Ref. 11.

described by Francis, G., Brennan, L., Stretton, S. and Ferenci, T. ((1991) Mol. Microbiol., in press): S30C, pAM1701; C22S + S30C + C38S, pAM1707; S57C, pAM1600; P96A + G97C, pAM1601; G103C, pAM1602; W120C, pAM1504; S146C, pAM1618; S152C, pAM1603; S153C, pAM1616; S154C, pAM1617; G184C, pAM1604; N265C, pAM1704; G382C, pAM1605. These plasmids were present in strain pop6510 [23] and the bacteria were grown anaerobically in Minimal Medium A [24] containing 20% L-broth [24], 0.4% malto-oligosaccharide, 0.05% L-cysteine hydrochloride, 0.026 M $KHCO_3$ and 100 μg/ml ampicillin.

Maltoporin purification

Bacteria from 2–4 litre anaerobic cultures in the above medium were harvested by centrifugation and washed once in 10 mM phosphate buffer (pH 6.8) containing 15 mM mercaptoethanol. Harvested cells were broken by two passages through an ice-cold French Press in 10 mM phosphate, 15 mM mercaptoetanol, 10 μg RNase + 5 μg DNase, and 2 mM phenylmethylsulphonyl fluoride (PMSF) (pH 6.8). After addition of 1 mM $MgSO_4$, the lysate was centrifuged at 6370 × *g* for 15 min to remove whole cells. Membranes were sedimented by centrifugation at 48400 × *g* for 60 min. Membranes were resuspended using a homogeniser in 20 ml phosphate buffer (pH 6.8) (10 mM) containing 2% Triton X-100, 2 mM PMSF and 15 mM mercaptoethanol. Non-extracted outer membrane proteins were sedimented by centrifugation at 48400 × *g* for 60 min. The pellet was solubilized by homogenization in extraction buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 2% Triton X-100, 2 mM PMSF and 15 mM mercaptoethanol) and mixing occasionally at 37°C for 30 min. The supernatant, after centrifugation (48400 × *g* for 60 min), was applied to a 12 ml starch-Sepharose column containing 7–10 mg/ml immobilized starch, prepared as in Ref. 25. The column was pre-equilibrated with 50 ml 0.1 M sodium bicarbonate buffer (pH 8.5) containing 0.5% Triton X-100 and 15 mM mercaptoethanol. Unbound proteins were eluted by washing the column with 5 × 3 ml equilibration buffer. Loosely bound proteins were eluted with 5 × 3 ml equilibration buffer additionally containing 0.1% SDS, and further washing with 5 × 3 ml equilibration buffer without SDS. LamB protein was eluted with 4 × 3 ml equilibration buffer containing 20% maltose; these fractions were collected and analyzed by SDS-acrylamide gel electrophoresis. Purified protein in fractions was precipitated by the addition of 2 volumes ice-cold isopropanol and kept for 30 min at −20°C. Maltoporin was collected by centrifugation (3295 × *g* for 40 min) and washed in 70% ethanol prior to vacuum drying. Storage of the protein was in buffer containing 10 mM Tris-HCl (pH 7.5) 5 mM EDTA, 0.5% Triton X-100, 15 mM mercaptoethanol (or 10 mM dithiothreitol) at 4°C.

Reactivity of protein with N-ethylmaleimide

Mercaptoethanol was removed from approximately 50 μg purified protein in 100 μl storage buffer by a spun column gel filtration technique using 0.95 ml Sephadex G-25 in a 1 ml column equilibrated with 0.5% Triton X-100, 100 mM phosphate buffer (pH 7.4) (or pH 8.4 where indicated). Separation of the protein from the reducing agent was confirmed by using the Ellman assay for thiols [26]. Maltoporin was reacted with 5 mM *N*-ethyl[^{14}C]maleimide (specific activity, 1.4 μCi/mmol) at 37°C. At timed intervals, labelled protein was precipitated by the addition of 10 volumes ice-cold 10% trichloroacetic acid and storage on ice for 30 min. Any unincorporated radioactivity was removed by filtering the precipitate through a Whatman GF/C glass fibre disk and washing with 5 ml ice-cold acetone. Radioactivity on the filters was measured in a scintillation counter using ACS II (Amersham) scintillant.

Modification of protein variants with bi-functional maleimides

Mercaptoethanol was removed from approximately 50 μg protein in 100 μl storage buffer as described above. Maltoporin in 0.5% Triton X-100, 0.1 M phosphate buffer (pH 7.4) was treated for 90 min at 37°C with a one-tenth volume of 25 mM bismaleimide freshly dissolved in dimethyl sulphoxide/0.1 M phosphate, 0.5% Triton X-100 (pH 7.4) (1 : 1, v/v). Treated proteins were mixed after 90 min with an equal volume of electrophoresis sample buffer [27] but with a final SDS concentration of 4% and no β-mercaptoethanol.

Oxidation of maltoporin

After removal of reducing agents from storage buffer by gel filtration as above, autooxidation of maltoporin to form disulphide cross-links was achieved by a 90 min incubation at 37°C in the presence of 0.5% Triton X-100, phosphate buffer (pH 7.4). Samples were diluted 1 : 1 in electrophoresis sample buffer [27] with or without mercaptoethanol before electrophoresis. Oxidation of protein with copper-phenanthroline was as described in Ref. 14.

Results

Purification and properties of thiol-modified maltoporins

Fig. 2 describes the solubilization and chromatography of outer membrane proteins obtained by differential Triton X-100 solubilization of a whole-membrane fraction [29]. When applied to starch-Sepharose, Triton-solubilized maltoporin was retained whereas contaminating protein bound to the matrix or bound to maltoporin was eluted with 0.1% SDS; this step re-

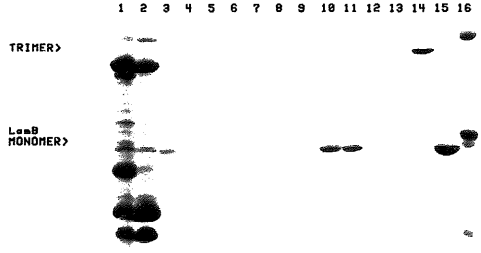

Fig. 2. Purification of maltoporin by chromatography on starch-Sepharose. An outer membrane protein preparation used as the starting point for the purification, as described in Methods, is shown in lane 1. Lane 3 shows proteins solubilized by extraction buffer and lane 2 shows the insoluble fraction after extraction. The solubilized proteins applied to starch-Sepharose were eluted with buffer containing 0.5% Triton X-100 (lanes 5, 6) and 0.5% Triton X-100 containing 0.1% SDS (lanes 7, 8); only the second and third of the five fractions is shown for these elutions. Finally, the starch-Sepharose was eluted with 0.5% Triton X-100 containing 20% maltose (lanes 9–12). Maltoporin concentrated by isopropanol precipitation is shown in lanes 14 and 15, both as monomer after boiling in SDS and as trimer without dissociation. Lane 16 contained molecular weight standards. Fractions were electrophoresed and stained as described in Ref. 27.

sulted in the loss of only minor amounts of maltoporin (lanes 5–8). Maltoporin was eluted with 0.5 M maltose in Triton X-100. Even from the poor outer membrane extract contaminated with cytoplasmic membrane proteins shown in lane 1, the maltose fractions show no contamination of maltoporin, even after concentration (lanes 14, 15). Maltoporin was routinely concentrated by propan-2-ol precipitation.The recovered protein was active in that over 89% of concentrated, purified wild-type protein was reversibly retained by starch-Sepharose under standard chromatographic conditions. The protein was also able to inactivate phage Lambda in vitro (results not shown). All these steps were carried out in the presence of mercaptoethanol to prevent

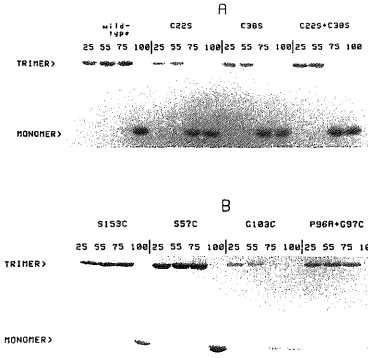

Fig. 3. Influence of temperature on the stability of maltoporin variants. Purified proteins were electrophoresed and stained as described in Ref. 27. Samples were heated in sample buffer containing 2% SDS for 5 min at the given temperature before loading on the gel. For gel A, purified wild-type maltoporin, variant C22S, variant C38S and C22S + C38S were heated at 25°C, 55°C, 75°C, and 100°C, respectively. In gel B, maltoporin variants S153C, S57C G103C and P96A + G97C were also heated for 5 min at the same four temperatures before electrophoresis.

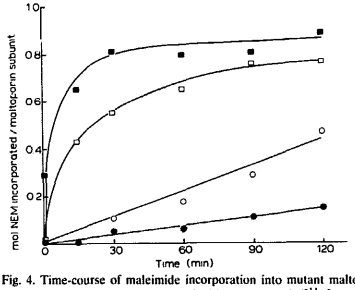

Fig. 4. Time-course of maleimide incorporation into mutant maltoporins. Purified protein was labelled with 5 mM *N*-ethyl[^{14}C]maleimide as described in Methods. Incorporation of label is shown for the C22S+C38S protein (●), the W120C protein (○), the S152C protein (□) and the S153C protein (■).

thiol oxidation of mutant proteins, but additional thiols are unnecessary for the purification of wild-type protein.

Maltoporin was purified from bacteria with site-directed thiols at 30, 57, 97, 103, 120, 146, 152, 153, 154, 184, 265 and 382. All these proteins were extracted from bacteria grown under reducing, anaerobic conditions; preliminary experiments showed that maltoporin extracted from cells grown aerobically had reduced thiol reactivity, due to partial oxidation of some of the readily accessible thiols in the outer membrane. For example, S152C showed only half the reactivity to labelling with *N*-ethylmaleimide from aerobic as against anaerobic cultures (see below). Protein from wild-type, as well as a cysteine-less double mutant (C22S + C38S) was also purified, along with S30C and S152C mutants that lacked the cysteines at residues 22 and 38. Maltoporin with individual cysteines at 22 or 38 was also purified in case the wild-type cysteines at 22 + 38 were inactive to thiol modification due to disulphide bonding in the native protein.

Trimer stability of thiol-modified maltoporins

The trimer stability of these purified proteins was tested by heating in the presence of SDS at different temperatures. Wild-type protein, but not the C22S, C38S, C22S + C38S and G103C proteins, was undissociated by heating at 75°C but not at 100°C (Fig. 3). These four proteins were fully dissociated at 75°C but not at 55°C, although G103C is significantly dissociated at 55°C. A previous study of the stability of the C22S + C38S mutant showed lower levels of dissociation at these temperatures when unpurified protein was heated embedded in the outer membrane [22]; the dissociation defect was much more marked with purified protein as shown in this study and recently in Ref. 29. The instability as a result of the 103 mutation, together with data on a temperature-sensitive trimerization mutation at 118/119 (Misra, R., Petersen, A., Ferenci, T. and

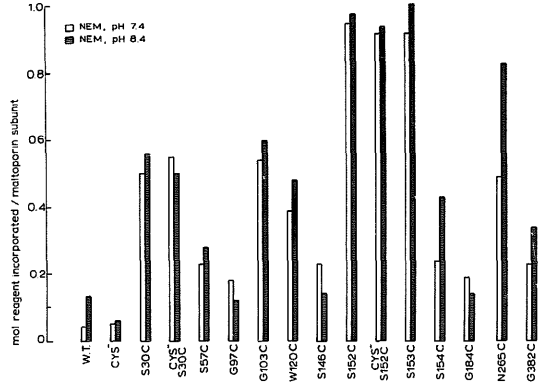

Fig. 5. Reactivity of cysteines at different positions in maltoporin. The purified proteins with the shown substitutions were assayed for 5 mM *N*-ethyl[^{14}C]maleimide incorporation as in Fig. 4. The figure shows the level of incorporation for all mutants after a constant 2-h period. The filled box shows the incorporation at pH 8.4. Cys$^-$ refers to proteins with C22S+C38S subsitutions.

Silhavy, T. (1991), J. Biol. Chem., in press), reinforces the importance of this region of the protein in trimer stability. All other mutant proteins behaved like wild-type, as exemplified by S57C, G97C and S153C in Fig. 3B.

Reactivity of thiols at different sites in maltoporin

The reactivity of residues was estimated by quantitating the number of thiol-specific reagent molecules covalently incorporated into purified, native protein. A time-course of *N*-ethylmaleimide incorporation for four representative proteins with different rates of reactivity is shown in Fig. 4. Slow incorporation of maleimide continued linearly over a 2 h period even into the cysteineless mutant, presumably into non-cysteine residues. A comparable, low reactivity with *N*-ethyl[^{14}C]maleimide was found for wild-type LamB with cysteines at residues 22 and 38, as summarized in Fig. 5. A mutant with a single cysteine at 22 was also unreactive under these conditions, with less than 0.06 mol maleimide/mol protein incorporated in 2 h, suggesting the unreactive nature of the thiol at the native position.

As also shown in Figs. 4 and 5, proteins containing an additional cysteine at either residue 152 or 153 was rapidly labelled. Incorporation never exceeded a 1:1 maleimide/polypeptide stoichiometry, suggesting ready accessibility of only one of the thiols in proteins with cysteines at 22 + 38 + 152/153. The same reactivity was found whether the two native cysteines at 22 + 38 were present or absent, as shown with the 30 or 152 substituted proteins (Fig. 5), hence the reactivity was due to the non-native cysteines. All other comparisons shown in Fig. 4 were for proteins with the native cysteines present, and the reactivity compared after a set 2-h reaction period. As also shown in Fig. 5, reactivity was tested at two pH values to see if maleimide incorporation was differentially affected by possible local pK variations of the thiols at different positions. All estimations were carried out in triplicate and the average value used in Fig. 5.

By the criterion of covalent labelling with *N*-ethylmaleimide over a 2-h period, only residues 152 and 153 are freely accessible and *N*-ethyl[^{14}C]maleimide only partially modified residue 154. Significant labelling was also found at residues 30, 103, 120, 265 and 382, with the level of incorporation still significantly above that at residues 22/38. There was some labelling at 57 and 146 but 97 and 184 were barely above control levels of incorporation with cysteine-less protein.

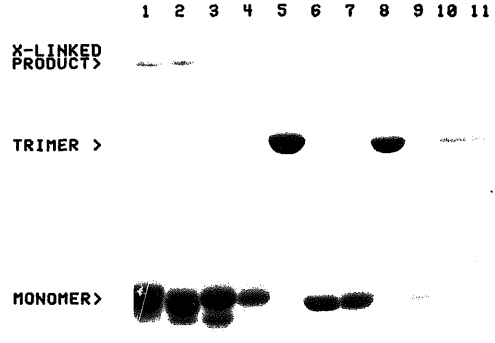

Fig. 6. Cross-linking of maltoporin variants by thiol oxidation and using bifunctional maleimides. Purified maltoporin from variant C22S + C38S + S152C was freed of mercaptoethanol by gel filtration. Lanes 1–3 contain the protein treated with 2.5 mM 1,1'-(methylenedi-4,1-phenylene)bismaleimide, bismaleimidohexane and bis(maleimido)methyl ether, respectively, at 37°C for 90 min and boiled for 5 min. Lanes 4 and 5 contain the same maltoporin variant treated with 2.5 mM *N,N'*-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine at 37°C for 90 min; the sample in lane 4 was boiled for 5 min before loading whereas that in lane 5 was not boiled. Lanes 6–8 contain maltoporin variant C22S + C38S + 152C allowed to autooxidize in the absence of thiol for 90 min at 37°C; lanes 6 and 7 contain oxidized protein boiled for 5 min in SDS in the presence and absence of mercaptoethanol, respectively, and lane 8 contains autooxidized trimer but not boiled in SDS and in the absence of mercaptoethanol. Lanes 9 and 10 contain untreated maltoporin monomer and trimer, respectively, and lane 11 contains molecular weight standards (170K, 97K, 55K).

As expected, maleimide reactivity was generally higher at pH 8.4 than at 7.4 but only the thiol at residue 265 significantly changed its reactivity relative to other sites, being fourth most reactive at pH 8.4 as against 7th most reactive at 7.4. Possibly the cysteine at this site has a higher pK, but this was not further investigated. However, the pH comparison indicates that the low reactivity is not obviously a pK effect at the other sites and more likely to be due to the steric exclusion of maleimide.

Cross-linking thiols within trimers

Topological information can be derived from the possible cross-linking of residues within a homotrimer. Thiols can be oxidised to disulphides, given the correct spacing [19] of particular cysteines. The purified maltoporin variants were hence oxidised (by autooxidation or with Cu-phenanthrolene [14]) to test for dimer formation. Also, four homobifunctional bismaleimides with different spacers (*N,N'*-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine, 1,1'-(methylenedi-4,1-phenylene)bismaleimide, bis(maleimido)hexane and bis(maleimido)methyl ether) were tested for their ability to form maltoporin dimers through covalent cross-links over different distances.

Of the proteins with individual thiols at the 14 sites tested, two variants were able to form dimers using either oxidation or cross-linkers. Cysteine residues at 152 and 153 were able to form both disulphides and maleimide-dependent cross-links between trimer subunits. As shown in Fig. 6, autooxidized 152 variant formed a slow-migrating species whose mobility in acrylamide gels is highly retarded, as found for other such cross-linked proteins [14]. As also shown in Fig. 6, these retarded species only appear once the treated trimers are dissociated by boiling in SDS; the dimers actually migrate more slowly than treated, undissociated trimers, which, as all porin trimers, exhibit an anomalously fast migration in acrylamide gels. These cross-links were also not dependent on the native cysteine residues at 22 or 38 as the 152 mutant missing these residues was able to form dimers. As expected for disulphides, the oxidation product could be dissociated into subunits by reduction with mercaptoethanol (Fig. 6). The bismaleimide cross-linking, as shown in Fig. 5 for the 152 mutant, also resulted in slow-moving dimers only upon boiling in SDS and dissociation of modified trimers, whose mobility was unaltered.

N,N'-Bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine, 1,1'-(methylenedi-4,1-phenylene)bismaleimide and bis(maleimido)hexane were equally good cross-linkers for the 152C protein. From scans of gels as in Fig. 6, the presence of S152C permitted 14–17% of protein to be cross-linked in 90 min. This compares with 4% cross-linking with bis(maleimido)methyl ether and 6% as a result of oxidation. The 153C protein only permitted 0.5–2.5% cross-linking with maleimides and 8% with oxidation (from scans of gels not shown). The 154C protein was not able to form detectable dimers by oxidation but was able to form small amounts (0.1–0.2%) of cross linked product with bis(maleimido) hexane and bis(maleimido)methyl ether. However, the low level of maleimide cross-linking at 154 and all other sites must be viewed in light of the low maleimide reactivity (Fig. 5) at sites other than 152 and 153.

Discussion

The maltoporin purified by affinity chromatography on starch-Sepharose is significantly less contaminated than earlier ion-exchange preparations [20] and is much simpler than the four-column method currently adopted to obtain maltoporin for crystallography [4]. This technique should find widespread use in studies of maltoporin structure and function and is simpler and probably more reliable than the application of immobilized maltose-binding protein for affinity-chromatographic maltoporin purification [21]. This ease of purification is not available for any other outer membrane protein or indeed probably any membrane transport protein.

The trimer stability of most of the mutant proteins was similar to wild-type. This was not unexpected as most tested substitutions were in predicted external loops according to the model in Fig. 1 and hence not expected to influence structure. One surprising finding was the greater instability of the purified C22S and C38S proteins as against unpurified protein previously shown to have limited stability defects in outer membrane preparations [22]. Presumably other outer membrane components contribute to trimer stability in the latter extracts. The 22 and 38 substitutions are in predicted transmembrane segments (Fig. 1) which may interact with other outer membrane components or affect subunit–subunit interactions directly.

In view of the recent demonstration of the role of the 118–119 region in trimer assembly (Misra, R., Petersen, A., Ferenci, T. and Silhavy, T. (1991) J. Biol. Chem., in press), it is tempting to suggest that the greatly reduced trimer stability of the G103C substitution is perhaps also due to a defect in subunit interactions. It is relevant that we have constructed an in-frame deletion of the residues in the 97–103 region and this resulted in complete lack of maltoporin assembly in the outer membrane (unpublished results). It will be important to analyze the role of this region in maltoporin trimerization and biogenesis in more detail.

The high *N*-ethylmaleimide reactivity of residues 152 and 153 is fully consistent with the proposed location of the 150 region at the surface of the protein [9]. The lower reactivity of the 154C mutant suggests however that full labelling is not to be expected for all residues in a surface loop. Indeed it would be surpris-

ing if all side-chains even in a flexible coil necessarily have the right steric or pK environment for reacting with maleimide. Hence the labelling comparable to that at 154 found with residues 30, 103, 120, 265 and 382 is also potentially significant and consistent with accessible locations of these residues. Indeed, only W120 of these residues was proposed to be in a transmembrane region (j) in Fig. 1 and the others sites were in membrane-external loops or turns. Even the labelling of 120 is consistent with the structural model, as this residue is between two residues (118 and 121) that have been proposed to be close to the transmembrane channel through the protein [3]. Hence 120 may get labelled inside the channel even though it is a transmembrane residue. However, it should also be kept in mind that *N*-ethylmaleimide shows significant hydrophobicity and may react with transmembrane regions covered by detergent in the purified protein, so absolute assignments of accesibility are not clearcut.

Residues 57, 97, 146 and 184 were in proposed turns but show low reactivity. Either the model in Ref. 9 and Fig. 1 is incorrect or local factors prevent access to these sites. The situation at 146 is particularly relevant in this regard, as epitope fusions inserted at residue 146 were buried to cross-reacting with antibodies, in contrast to epitopes inserted further along at 154/155 [18]. The maleimide reactivity of C146 as against C152–154 is in agreement with the relative accessibility to antibodies.

The 150 region in each of the subunits is capable of contributing to disulphide formation and also to be cross-linked by bismaleimides with 1.3–2.0 nm spacers. Disulphides in proteins are formed normally between cysteines 0.5–0.9 nm apart [19], so this is indicative of the distance between these regions in the trimer. The lack of cross-linking at other residues is however not conclusive evidence that these sites are further apart in trimers. It should be noted that the *N*-ethylmaleimide reactivity of sites outside the 150 region was not all that high so reactivity to bismaleimides is also likely to be poor.

In summary, we demonstrated the usefulness of maltoprin purification by affinity chromatography, identified residues affecting trimer stability, and the cysteine reactivity studies have contributed to our understanding of the 150 region and other sites in maltoporin. In addition, the type of studies in Refs. 14–17, and the usefulness of these mutants in mapping ligand binding sites (Francis et al. (1991) Mol. Microbiol., in press) makes the cysteine mutagenesis approach highly useful in the study of membrane proteins.

Acknowledgements

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References

1 Benz, R., Schmid, A., Nakae, T. and Vos-Scheperkeuter, G.H. (1986) J. Bacteriol. 165, 978–986.
2 Freundlieb, S., Ehmann, U. and Boos, W. (1988) J. Biol. Chem. 263, 314–320.
3 Ferenci, T. (1989) BioEssays 10, 3–7.
4 Stauffer, K.A., Page, M.G.P., Hardmeyer, A., Keller, T.A. and Pauptit, R.A. (1990) J. Mol. Biol. 211, 297–299.
5 Vogel, H. and Jaehnig, F. (1986) J. Mol. Biol. 190, 191–199.
6 Ferenci, T. and Lee, K.-S. (1989) J. Bacteriol. 171, 855–861.
7 Ferenci, T., Schwentorat, M., Ullrich, S. and Vilmart, J. (1980) J. Bacteriol. 142, 521–526.
8 Charbit, A., Clement, J.M. and Hofnung, M. (1984) J. Mol. Biol. 175, 395–401.
9 Charbit, A., Gehring, K., Nikaido, H., Ferenci, T. and Hofnung, M. (1988) J. Mol. Biol. 201, 487–496.
10 Gabay, J., Benson, S.A. and Schwartz, M. (1983) J. Biol. Chem. 258, 2410–2414.
11 Molla, A., Charbit, A., Le Guern, A., Ryter, A. and Hofnung, M. (1989) Biochemistry 28, 8234–8241.
12 Desaymard, C., Debarbouille, M., Jolit, M. and Schwartz, M. (1986) EMBO J. 5, 1383–1388.
13 Schenkman, S., Tsugita, A., Schwartz, M. and Rosenbusch, J.P. (1984) J. Biol. Chem. 259, 7570–7576.
14 Falke, J.J. and Koshland, D.E. Jr. (1987) Science 237, 1596–1600.
15 Milligan, D.L. and Koshland, D.E. Jr. (1988) J. Biol. Chem. 263, 6268–6275.
16 Flitsch, S.L. and Khorana, G.(1989) Biochemistry 28, 7800–7805.
17 Altenbach, C., Marti, T., Khorana, G., and Hubbell, W.L. (1990) Science 248, 1088–1092.
18 Charbit, A., Boulain, J.C., Ryter, A. and Hofnung, M. (1986) EMBO J. 5, 3029–3037.
19 Creighton, T.E. (1989) in Protein structure. A practical approach (Creighton, T.E., ed.), pp. 155–168, IRL Press, Oxford.
20 Schwartz, M. (1983) Methods Enzymol. 97, 100–112.
21 Nikaido, H. (1983) Methods Enzymol. 97, 85–100.
22 Ferenci, T. and Stretton, S. (1989) FEMS Microbiol. Lett. 61, 335–340.
23 Bouges-Bocquet, B., Villaroya, H. and Hofnung, M. (1984) J. Cell. Biochem. 24, 217–228.
24 Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25 Ferenci, T. and Lee, K.-S. (1982) J. Mol. Biol. 160, 431–444.
26 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
27 Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. and Van Alphen, L. (1975) FEBS Lett. 58, 254–258.
28 Schnaitman, C.A. (1971) J. Bacteriol. 108, 545–552.
29 Luckey, M., Ling, R., Dose, A. and Malloy, B. (1991) J. Biol. Chem. 266, 1866–1871.