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Detection and Identification of Stable Oligomeric Protein Complexes in *Escherichi coli* Inner Membranes

A PROTEOMICS APPROACH*

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In this study we present a new technology to detect stable oligomeric protein complexes in membranes. The technology is based on the ability of small membrane-active alcohols to dissociate the highly stable homotetrameric potassium channel KcsA. It is shown via a proteomics approach, using diagonal electrophoresis and nano-flow liquid chromatography coupled to tandem mass spectrometry, that a large number of both integral and peripheral *Escherichia coli* inner membrane proteins are part of stable oligomeric complexes that can be dissociated by small alcohols. This study gives insight into the composition and stability of these complexes.

Approximately one of four open reading frames encodes for a protein that resides in or on a membrane (1). This abundance signifies the importance of membrane proteins for cellular function. For several classes of these proteins it is known that they function as oligomeric complexes. Examples are solute channels (2) and protein translocators (3). Although extensive networks have been established of interaction partners of water-soluble proteins in for example *Escherichia coli* (4) and yeast (5), very little is known about the interaction partners of membrane proteins or of the stability of the complexes that are formed in membranes. This lack of insight is largely the result of the difficulties in handling membrane proteins and the lack of suitable tools to study them.

The bacterial potassium channel protein KcsA is a well studied example of a protein that functions as a stable oligomeric complex. It is a small homotetrameric protein of known structure (6) that is representative of many related channel proteins (2, 7–9). When KcsA-containing membranes are treated with SDS, the protein retains its tetrameric form, even at elevated temperatures (2). Very recently it was discovered that small alcohols like trifluoroethanol (TFE)[1] can dissociate the KcsA tetramer into monomers in a manner that is dependent on both the concentration and the type of alcohol (10, 11). This observation suggests that small alcohols may be used as a generic tool to detect and characterize stable oligomeric membrane protein complexes. Here we explored this possibility for proteins in *E. coli* inner membranes. For this we developed a new proteomics approach using diagonal SDS-based electrophoresis. In this approach non-heated protein samples are first separated by SDS-polyacrylamide gel electrophoresis followed by *in gel* exposure to the alcohol and subsequent electrophoresis in a second dimension. All proteins or protein complexes that are not affected by the alcohol are expected to migrate on the diagonal of the gel, whereas protein complexes that are stable in room temperature SDS but are dissociated by the alcohol will migrate off the diagonal. Off-diagonal spots are then digested by trypsin and identified by nano-flow liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Using this method we observed many off-diagonal spots that contained membrane proteins, either integral with one or more transmembrane segments or peripheral with a membrane-interactive domain. We thus readily identified a subproteome consisting of membrane proteins that are part of very stable homo- or hetero-oligomeric complexes. The method is expected to be applicable to a variety of membrane preparations of different cells or organelles because it does not require labeling of the proteins with specific tags, as used in other studies to detect interaction partners of proteins (4). The method is complementary to traditional two-dimensional electrophoresis methods of blue-native PAGE in combination with SDS-PAGE (12), which allows detection of protein complexes that are not stable in SDS.

EXPERIMENTAL PROCEDURES

Materials—Electrophoresis setups and electrophoresis buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) sodium dodecyl sulfate) were purchased from Bio-Rad. Other sources of materials were: Ni^{2+}-nitrilotriacetic acid (Qiagen Benelux N.V.), *N*-dodecyl-β-D-maltoside (Anatrace Inc.), TFE (Merck), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Acros Organics), Coomassie Brilliant Blue G-250 (ICN Biomedicals Inc.), Bocillin FL (Invitrogen), enhanced chemiluminescence detection kit (Amersham Biosciences) and bovine trypsin, sequencing grade (Roche Diagnostics). SecA antibodies were a kind gift of Dr. Hans de Cock. The pHK2414 plasmid was a kind gift of Dr. Martine Nguyen-Distèche.

Isolation of Membrane Vesicles and Purification of KcsA—Crude *E. coli* BL21(DE3) membrane vesicles from cells overexpressing KcsA were prepared as described previously (13). Membranes of HMS174 cells overexpressing penicillin-binding protein 1B from the pHK2414 plasmid were obtained similarly. Vesicles were either resuspended in 100 mM NaCl, 5 mM KCl, and 10 mM HEPES, pH 7.5, for use in the two-dimensional gel-system or used for KcsA purification according to Ref. 14. Resuspended membrane vesicles were stored at −20 °C. Purified KcsA was stored at 4 °C in buffer containing 100 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.5, and 1 mM *N*-dodecyl-β-D-maltoside.

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[1] The abbreviations used are: TFE, 2,2,2-trifluoroethanol; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; LC, nano-flow liquid chromatography; MS/MS, tandem mass spectrometry; PTS, phosphotransferase system.

Purified inner membrane vesicles from late-exponential BL21(DE3) *E. coli* cells were prepared as described previously (15). Cells were collected and lysed in a French press. Inner and outer membrane vesicles were separated via sucrose density centrifugation. The purified inner membrane vesicles were stored at −80 °C.

Fluorescence Detection of Penicillin-binding Protein 1b—A crude preparation of membrane vesicles from HMS174 cells expressing PBP-1b was labeled with 10 μM Bocillin FL for 30 min at 35 °C. Vesicles were subsequently dissolved in SDS gel-loading buffer (12 mM Tris/HCl, pH 6.8, 5% (v/v) glycerol, 0.4% (w/v) SDS, 14 mM dithiothreitol and 0.02% bromphenol blue) and kept at room temperature, heated in sample buffer for 5 min, or incubated with 20% TFE for 1 h. The samples were then run on regular one-dimensional 11% SDS-polyacrylamide gels. After electrophoresis the gels were rinsed with water. The labeled proteins were visualized by scanning the gels on a Typhoon 9400 imaging system (Amersham Biosciences) using an excitation wavelength of 488 nm and an emission wavelength of 526 nm.

Immunodetection of SecA—Preparations of inner membrane vesicles were solubilized in SDS gel-loading buffer and kept at room temperature, heated in sample buffer for 5 min, or incubated with 25% TFE for 1 h. These samples were run on an 11% SDS-polyacrylamide gel. Proteins were blotted onto a nitrocellulose membrane. SecA was detected by a specific antibody and by the use of the enhanced chemiluminescence detection kit.

SDS-PAGE in the First Dimension—The membranes were solubilized at room temperature in SDS gel-loading buffer, and the samples were run in electrophoresis buffer in two different setups. A Bio-Rad mini-Protean II setup was used for gels for either purified KcsA or a crude *E. coli* inner membrane preparation. These samples were run on a lane of a regular 0.75-mm thick 11% SDS-polyacrylamide gel of 7.2 × 8.2 cm at a voltage of 180 V. Purified *E. coli* inverted inner membrane vesicles were run on a Bio-Rad Protean II setup on a 0.75-mm thick 11% gel of 16 × 18 cm at a current of 30 mA.

In Gel TFE Exposure and SDS-PAGE in the Second Dimension—Lanes of either 5.5 × 1 cm or 15 × 2 cm were excised from the first dimension gels and run on a Bio-Rad mini-Protean III or -Protean II setup, respectively. To dissociate membrane protein complexes the excised gel strips were incubated for 1 h in electrophoresis buffer containing the indicated percentage (v/v) of TFE or HFIP at room temperature. After incubation the gel strips were washed twice for 5 min with water and twice with electrophoresis buffer to reduce the amount of TFE, which interferes with electrophoresis.

The gels for the second dimension consisted of a 5 × 8.2-cm separating gel with a 0.8 × 8.2-cm stacking gel on top for use in the Bio-Rad mini-Protean III setup or a 15 × 18-cm separating gel with a 1.2 × 18-cm stacking gel on top for the Bio-Rad Protean II system. Room was left on top of the gels to fit in the TFE-treated gel strips. An overlay of isopropanol was used during polymerization of the stacking gel, as this gave a smoother surface than a watery overlay. After polymerization the isopropanol was decanted, and the surface of the stacking gel was rinsed with water to remove traces of isopropanol. The gel strips were placed on top of the second dimension gels. Empty regions between the excised gel strips and the glass plates were filled with 0.3% agarose in electrophoresis buffer. The gels were run at the same voltage or current as the first dimension gels. Gels were stained using either Coomassie Brilliant Blue G250 or silver staining.

In Gel Tryptic Digestion of Off-diagonal Spots—Off-diagonal protein spots were excised and *in gel* digested with trypsin with a protocol slightly modified from the one described by Wilm *et al.* (16). In brief, the gel pieces were destained using 50% (v/v) methanol followed by reduction (6.5 mM dithiothreitol in 50 mM ammonium bicarbonate, pH 8.5) and alkylation (54 mM iodoacetamide in 50 mM ammonium bicarbonate, pH 8.5). Subsequently, the gel pieces were washed and dehydrated using 50 mM ammonium bicarbonate and acetonitrile, respectively. Proteins were digested overnight at 37 °C by adding trypsin at a concentration of 10 ng/μl. The digestion was stopped by the addition of 1 μl of acetic acid.

LC-MS/MS—LC-MS/MS analysis was performed by coupling an Agilent 1100 Series LC system (vacuum degasser, auto sampler, and one high-pressure mixing binary pump without static mixer) to a LCQ™ Classic quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA) basically as described by Meiring *et al.* (17). Briefly, peptide mixtures were delivered to a trap column (Aqua™ C18RP (Phenomenex); 15 mm × 100 μm, packed in-house) at 5 μl/min 100% eluent A (0.1 M acetic acid). After reducing the flow to ~150 nl/min by using a splitter, the peptides were transferred to the analytical column (PepMap C18 (LC Packings); 15 cm × 75 μm, packed in-house) with a linear gradient from 0 to 50% eluent B (0.1 M acetic acid in 80% acetonitrile) for 60 min.

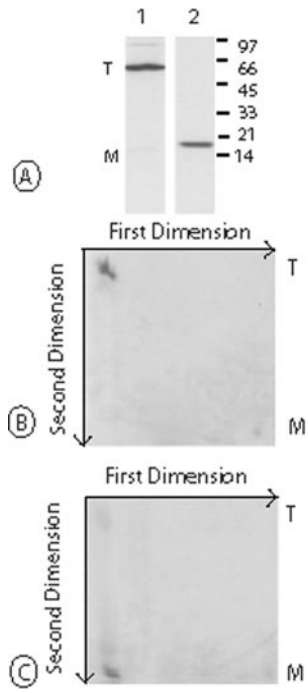

FIG. 1. **TFE-induced dissociation of the KcsA tetramer as shown on SDS-PAGE.** *A*, shows a one-dimensional (10) SDS-polyacrylamide gel of KcsA before (*lane 1*) and after (*lane 2*) exposure to 25% TFE. Monomeric (*M*) and tetrameric (*T*) KcsA are indicated, and a protein size marker (in kDa) is shown on the *right*. *B* and *C*, show a two-dimensional SDS-polyacrylamide gel of KcsA without exposure to TFE (*B*) and with exposure to 25% TFE after running the first dimension (*C*). Monomeric and tetrameric KcsA are indicated as well as the direction of electrophoresis in the first and second dimensions.

The column eluent was sprayed directly into the electrospray ionization source of the mass spectrometer via a butt-connected nano-electrospray ionization emitter (New Objectives). The LCQ operated in positive ion mode, and peptides were fragmented in data-dependent mode. One mass spectrometry survey scan was followed by three data-dependent MS/MS scans.

Identification of MS/MS Spectra by Data Base Searches—The SEQUEST algorithm (18) was used to interpret the obtained MS/MS spectra. The SEQUEST algorithm was run against the complete nonredundant proteome data base in the FASTA format of *E. coli* from the European Bioinformatics Institute (EBI). The algorithm was set to use trypsin as enzyme, allowing at maximum for two missed cleavages and assuming carbamidomethyl as a fixed modification and oxidized methionine as a variable modification. The program DTAselect (19) was used to select the peptide identifications and to assemble the peptides into proteins. The following selection criteria were used to filter the peptide identifications: the minimum Xcorr was set at 1.9, 2.2, and 3.75 for 1+, 2+, and 3+ peptides, respectively, and the ΔCn was 0.1 or higher for each peptide.

RESULTS

The principle of the proteomics approach presented is shown in Fig. 1 for purified KcsA. One-dimensional SDS-PAGE (Fig. 1*A*) confirms that KcsA migrates as a stable tetramer (2), which is dissociated into monomers by incubation with 25% TFE (v/v). In the absence of TFE only a minor band of the monomer is observed. When the sample is run on a two-dimensional gel under identical conditions in both dimensions and in the absence of TFE, the major spot of the tetramer and the minor spot of the monomer are expected to lie on the diagonal of the gel, as is indeed observed (Fig. 1*B*). However, when after running the gel in the first dimension, the gel strip is exposed for 1 h to 25% TFE, the tetramer dissociates and migrates off-diagonally on the monomer position in the second dimension (Fig. 1*C*). The same behavior of KcsA is observed for a crude *E. coli* membrane preparation obtained from cells over-

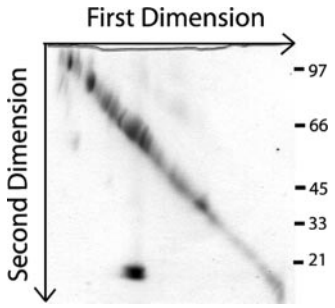

FIG. 2. **TFE-induced dissociation of protein complexes in a crude *E. coli* inner membrane preparation of cells overexpressing KcsA, as shown on SDS-PAGE.** The *directions* of migration in the first and second dimension are indicated. The intense protein *spot* under the *diagonal* corresponds to the KcsA monomer.

expressing KcsA (Fig. 2). A prominent KcsA spot that runs off-diagonal at its monomer position shows up in addition to a heavily stained diagonal corresponding to *E. coli* proteins that are not affected by TFE. Interestingly, some other spots at higher molecular weight also run off-diagonally, indicating that besides KcsA several other *E. coli* proteins also form stable complexes in SDS that are dissociated by the alcohol. To better visualize these off-diagonal proteins, purified inner membrane vesicles obtained from wild-type *E. coli* cells were applied to larger gels. A representative gel, stained with Coomassie, is shown in Fig. 3*A*. An intensely stained diagonal is observed as well as a number of off-diagonal spots. The stain above the diagonal is most likely the result of smearing. This interpretation is supported by the observation that analysis of such an off-diagonal spot that corresponded to an approximate molecular mass of 70 kDa was found to contain protein with molecular masses ranging from 20 to 55 kDa (not shown). Furthermore, when such a gel was silver-stained the smearing was even more obvious (Fig. 3*B*). The spots under the diagonal were excised and *in gel* digested with trypsin, after which the resulting peptides were analyzed by mass spectrometry. In this way 58 proteins could be identified (Table I). The large majority (39 proteins) consisted of proteins that are known to be present in or are associated with the inner membrane. Five outer membrane proteins were found that most likely originate from the remnants of outer membranes known to be present in these preparations (15). The remaining proteins were four ribosomal proteins, possibly originating from ribosomes that are attached to the membrane, and 10 proteins of unknown location.

From an analysis of the monomeric molecular weights of the proteins that we identified and from the apparent molecular weight of the corresponding protein complex on the diagonal, several homo-oligomeric protein complexes could be identified. For instance, the penicillin-binding protein 1b that is encoded by the *mrcB* gene (Fig. 3*A*, *spot 1*) undergoes an apparent molecular mass shift from ~200 to 100 kDa, suggesting that in room temperature SDS the protein is at least partially present as a dimer that becomes dissociated by TFE. This is indeed the case as shown by one-dimensional gel electrophoresis of *E. coli* inner membranes obtained from cells overexpressing PBP-1b that are solubilized in cold SDS and stained with a fluorescent penicillin analogue (Fig. 4). Clearly, two bands are visible with molecular weights corresponding to the dimer and monomer (Fig. 4, *lane 2*). In the absence of the plasmid carrying the *PBP-1b* gene no staining is observed (Fig. 4, *lane 1*). When the SDS-solubilized sample is either heated (Fig. 4, *lane 3*) or treated with 20% (v/v) TFE (Fig. 4, *lane 4*) the dimer band vanishes, and only a monomeric band remains.

Next to homo-oligomeric integral membrane proteins we identified homo-oligomers of proteins that do not contain trans-

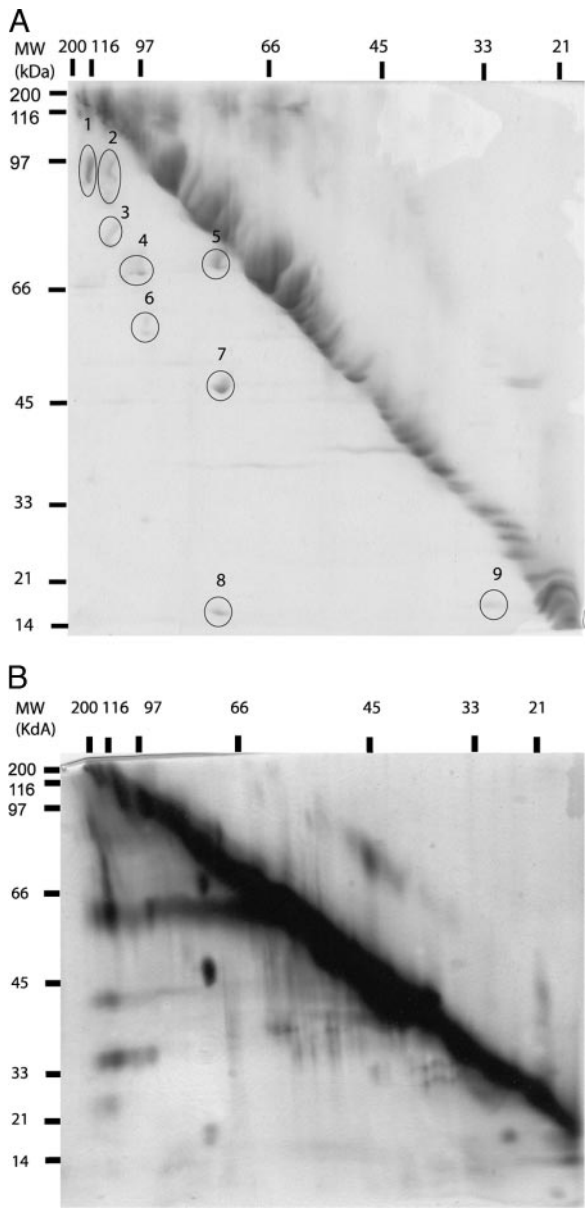

FIG. 3. **Dissociation of protein complexes from a preparation of *E. coli* inverted inner membrane vesicles by 25% TFE as shown on SDS-polyacrylamide gel stained with Coomassie (*A*) or silver-stained (*B*).** The *numbers* in *A* correspond to the spot numbers shown in Table I. Molecular mass markers are shown in both the first and second dimension.

membrane helices but that are more peripherally bound to the membrane. For instance, we identified the SecA protein in an off-diagonal spot (Fig. 3, *spot 2*). The off-diagonal position of SecA on the two-dimensional gel corresponds to a TFE-induced dimer-monomer transition. We verified this using one-dimensional gel electrophoresis, identifying the SecA protein with a SecA antibody (Fig. 5). In wild-type *E. coli* inner membranes dissolved in cold SDS, two spots are visible in Fig. 5, *lane 1*, one corresponding to the monomer (molecular mass of 100 kDa) and one to the dimer (molecular mass of 200 kDa). The band at 200 kDa ran at exactly the same position as a chemically linked dimer (data not shown). Either heating the solution (Fig. 5, *lane 2*) or incubation of the sample with 25% TFE (Fig. 5, *lane 3*), results in disappearance of the dimeric band fully consistent with an interpretation for the two-dimensional experiment that some SecA protein is present as a dimer that becomes dissociated by TFE. The implication from the one-dimensional

TABLE I
E. coli inner membrane proteins that are dissociated from complexes in SDS at room temperature by TFE

Type[a]	Spot[b]	Gene	Swissprot accession number	TMs[c]	Gene product	No. of peptides[d]	Coverage[e]	Molecular mass
								kDa
Integral	3	*nagE*	Q8X9H9	10	PTS system, *N*-acetylglucosamine-specific enzyme IIABC	1	2%	68
	6	*ptsG*	P05053	10	PTS system, glucose-specific IIBC component	4	9%	51
	6	*treB*	Q8XCE0	10	PTS system enzyme II, trehalose specific	4	9%	51
	4	*mtlA*	Q8XDH1	8	PTS system, mannitol-specific enzyme IIABC components	7	9%	68
	1	*mrcB*	Q8X903	1	Penicillin-binding protein 1b	26	32%	94
	2	*mrcA*	Q8X809	1	Penicillin-binding protein 1a	7	10%	94
	3	*mrdA*	P08150	1	Penicillin-binding protein 2	3	6%	71
	4	*secD*	P19673	6	Protein export membrane protein secD	8	17%	67
	9	*secG*	P33582	2	Protein export membrane protein secG	1	16%	11
	4, 5	*cydA*	Q8X979	9	Cytochrome *d* terminal oxidase, polypeptide subunit I	11	17%	58
	1	*mdoH*	P62517	6	Glucans biosynthesis glucosyltransferase H	3	5%	97
	4	*cydD*	Q8X5I1	5	ATP-binding component of cytochrome-related transport, Zn-sensitive	6	10%	65
	5	*oxaA*	Q8FBV4	4	Inner membrane protein oxaA	17	23%	62
	1	*yggB*	P11666	3	MscS mechanosensitive channel	5	18%	31
	3, 4, 6	*cyoA*	Q8XE63	3	Cytochrome *o* ubiquinol oxidase subunit II	3	18%	35
	5	*hflB*	Q8X9L0	2	Cell division protease ftsH	15	23%	71
	4	*tolA*	Q8X965	1	Membrane-spanning protein TolA	8	20%	41
	5	*pqiB*	Q8XDA7	1	Paraquat-inducible protein B	6	15%	60
	5	*yfgA*	Q8XAA6	1	Putative membrane protein	4	13%	36
	6	*yibP*	Q8XDE2	1	Putative membrane protein	7	14.4%	47
	6	*yrbD*	P45391	1	Hypothetical protein yrbD precursor	10	57%	20
	8	*yibN*	P37688	1	Hypothetical protein yibN - *E.* coli	12	62%	16
	9	*yhcB*	P39436	1	Hypothetical protein yhcB	6	52%	15
Peripheral	3	*nuoC*	Q8XCW9	0	NADH dehydrogenase I chain C, D	48	65%	69
	6	*nuoF*	Q8XCX1	0	NADH dehydrogenase I chain F	4	13%	49
	2	*nuoG*	Q8XCX2	0	NADH dehydrogenase I chain G	7	10%	100
	4	*atpA*	P00822	0	ATP synthase α chain	28	51%	55
	9	*atpH*	P00831	0	ATP synthase δ chain	10	55%	19
	5	*atpD*	P00824	0	ATP synthase β chain	5	12%	50
	3	*dld*	Q8X666	0	D-Lactate dehydrogenase, FAD protein, NADH independent	4	4%	65
	7	*lldD*	Q8XDF7	0	L-Lactate dehydrogenase	9	29%	43
	5	*frdA*	Q8XDQ0	0	Flavoprotein subunit of fumarate reductase FrdA	2	5%	66
	2	*plsB*	P58130	0	Glycerol-3-phosphate acyltransferase	24	29%	91
	4, 5	*glpD*	Q8X6Y5	0	*sn*-glycerol-3-phosphate dehydrogenase (aerobic)	46	59%	57
	7	*manX*	P08186	0	PTS system, mannose-specific IIAB component	34	73%	35
	4	*hybC*	P37181	0	Hydrogenase-2 large chain precursor	3	9%	62
	4	*mglA*	Q8X5D9	0	ATP-binding component of methyl-galactoside transport system	4	10%	56
	2	*lepA*	P60787	0	GTP-binding protein lepA	4	8%	67
	2	*secA*	Q8X996	0	Preprotein translocase SecA	65	52%	102
Outer membrane	1	*ompF*	Q8XDF1	OM	Outer membrane protein 1a (Ia,b,F)	12	27%	39
	2	*imp*	Q8XA13	OM	Organic solvent tolerance protein precursor	7	13%	90
	1, 2	*lamB*	Q8X5W7	OM	Maltoporin precursor	8	20%	50
	4, 5	*TolC*	Q8XBP7	OM	Outer membrane channel TolC	20	42%	54
	4	*z2268*	Q8X9X1	OM	Putative outer membrane receptor for iron transport	2	4%	77
Ribosomal	9	*rp1I*	P02418	0	50 S ribosomal protein L9	5	36%	16
	9	*rp1J*	P02408	0	50 S ribosomal protein L10 (L8)	7	47%	16
	9	*rp1O*	P66071	0	50 S ribosomal subunit protein L15	3	26%	15
	9	*rplQ*	P02416	0	50 S ribosomal protein L17	3	21%	14
Unknown	1	*sucA*	P07015	0	2-Oxoglutarate dehydrogenase E1 component	19	24%	105
	4, 5	*sucB*	P07016	0	Dihydrolipoyllysine residue succinyltransferase component of 2-oxoglutarate dehydrogenase	6	15%	44
	1	*aceE*	P06958	0	Pyruvate dehydrogenase E1 component	53	54%	100
	4, 2	*aceF*	Q8X966	0	Pyruvate dehydrogenase dihydrolipoyltransacetylase component	14	23%	66
	2	*malP*	Q8X708	0	Maltodextrin phosphorylase	5	10%	91
	2	*speA*	Q8XCX9	0	Biosynthetic arginine decarboxylase	3	6%	74
	6	*srmB*	Q8XA21	0	ATP-dependent RNA helicase	8	18%	50
	2	*ydiJ*	Q8X5Y8	0	Putative oxidase	6	8%	113
	9	*ybjP*	Q8X6N7	0	Putative enzyme	2	14%	19
	2	*ECs3386*	Q8XA93	0	Orf, hypothetical protein	9	8%	181

[a] Type of protein or subcellular localization.
[b] Number of the spot in Fig. 3*A*.
[c] Number of transmembrane segments as calculated from the primary structure of the proteins by the TMHMM 2.0 program (38). The designation OM indicates an outer membrane protein.
[d] Total number of peptides used for protein identification.
[e] Percentage of the amino acid sequence covered by the detected peptides.

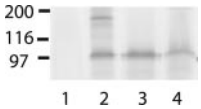

FIG. 4. **Fluorescence image of an 11% SDS-polyacrylamide gel with Bocillin FL-labeled PBP-1b.** The figure shows membrane vesicles from HMS174 without the pHK2414 plasmid (*lane 1*) as well as membrane vesicles from HMS174 carrying the pHK2414 plasmid, run without heating the sample in loading buffer (*lane 2*), after heating to 100 °C in loading buffer for 5 min (*lane 3*), and after exposure to 20% TFE in loading buffer for 1 h (*lane 4*). Molecular mass markers are shown on the *left-hand side*.

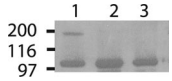

FIG. 5. **Western blot with anti-SecA antibodies of an 11% SDS-polyacrylamide gel.** The figure shows purified *E. coli* inner membrane vesicles run without heating the sample (*lane 1*), after boiling in sample buffer for 5 min (*lane 2*), and after being exposed to 25% TFE for 1 h in sample buffer (*lane 3*). Molecular mass markers are shown on the *left-hand side*.

gel experiment on SecA in Fig. 5 is that in the two-dimensional gel experiment of Fig. 2 next to the off-diagonal SecA spot, SecA should be present as monomer on the diagonal. We could confirm this with immunoblotting (data not shown).

In this study we concentrated only on protein spots that were visible after staining with Coomassie. However, some gel pieces outside the Coomassie-stained regions were found to contain additional membrane proteins (data not shown). Moreover, most spots were found to contain several different proteins. How can we explain this? The most likely interpretation is that only a selected number of proteins are detected by Coomassie blue staining, whereas a much larger number of proteins can be detected by mass spectrometry. This would imply that the proteins that we detect in the spots are a significant underestimation of the total number of proteins that are stable in SDS but become dissociated by TFE. In four cases (CydA, GlpD, SucB, and TolC) the protein was detected in two spots that were well separated in the first dimension, suggesting that these proteins were present in a larger complex that was dissociated by SDS in different hetero- or homo-oligomeric complexes. Finally, it should be noted that some proteins were also detected in neighboring spots, suggesting some smearing as is clearly visible on the silver stained gel (Fig. 3*B*). This smearing occurred mostly in the second dimension as we also observed for KcsA (*e.g.* Fig. 2). This would imply that the molecular weights of some of the proteins, as determined from this second dimension, may be somewhat overestimated.

It should be noted that all off-diagonal spots we identified are caused by the treatment with TFE. In a control experiment in which the gel strip that was excised after electrophoresis in the first dimension was incubated in alcohol-free buffer, no Coomassie-stained spots below the diagonal spot were observed. Moreover, when five spots that corresponded in position to those of the Coomassie-stained spots in Fig. 3 were analyzed by LC-MS/MS none of them was found to contain a protein.

By varying the concentration of the alcohol, it was possible to gain insight into the stability of membrane protein complexes by analyzing their resistance against alcohol-induced dissociation. Thus, differences in stability were investigated by monitoring the off-diagonal appearance as a function of the alcohol concentration of selected proteins for which it was established they were solely or dominantly present in particular spots. As shown in Fig. 6, dissociation of KcsA occurs at 15% TFE. Less TFE is needed to dissociate the complex containing ManX, a non-membrane-spanning inner membrane protein of the PTS family, and more TFE is needed to dissociate the complex

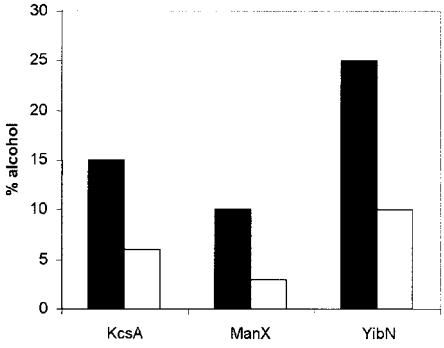

FIG. 6. **Minimum concentration of TFE (*filled bars*) and HFIP (*open bars*) required to visualize dissociation of different *E. coli* inner membrane proteins from oligomeric complexes as off-diagonal spots.**

containing YibN, a single membrane-spanning protein of unknown function. Fig. 6 also shows that dissociation of *E. coli* inner membrane proteins is not a specific effect of TFE but that it can also be induced by the alcohol HFIP with similar protein specificity but with much higher efficiency.

DISCUSSION

Although the search for protein interaction partners has received much attention recently and although extensive interaction networks have been established for water-soluble proteins (4, 5), little attention has been focused so far on the search for interaction partners of membrane proteins. In this study we developed a new and relatively simple membrane protein proteomics approach using diagonal electrophoresis to identify a subproteome consisting of membrane protein complexes that are stable in SDS and become dissociated by small membrane-active alcohols. Next to KcsA, close to sixty *E. coli* inner membrane proteins were identified that became dissociated from larger complexes by TFE (Table I). Because the analysis was limited to protein spots visible after staining with Coomassie, this is probably a gross underestimation of the total number of TFE-dissociable membrane protein complexes. Thus we can conclude that a large number of *E. coli* membrane proteins are part of highly stable oligomeric complexes that withstand solubilization into monomers by room temperature SDS.

We have a number of arguments that support our view that the protein complexes we detect in our assay are complexes that reside in the *E. coli* membrane and are not artifacts of the procedure we apply. First, we have detected a substantial number of proteins that are already known from published data to be present in complexes in membranes. This applies not only to the KcsA tetramer on which we based the technology but also to the large number of subunits of members of the PTS family that are known to occur as dimers (20, 21) in membranes and which we identified as such. For instance, the trehalose-specific enzyme II encoded by *treB* (Fig. 3*A*, *spot 6*) undergoes a molecular mass shift from ~100 to 60 kDa upon TFE treatment. We also identified a dimer from the structurally very different penicillin-binding protein 1b in room temperature SDS consistent with the suggested dimeric state in the membrane (22, 23). It should also be realized that within the range of uncertainty of determining the molecular weight of a membrane protein from SDS-PAGE it is clear that most of the proteins that we identified in off-diagonal spots run at their monomeric position. This demonstrates that TFE fully dissociates these proteins from a complex of which they were part. This makes it extremely unlikely that TFE causes the formation of artificial oligomeric membrane protein complexes.

The apparent molecular weight of proteins on an SDS gel is influenced by many factors. For example, proteins that form

stable homo-oligomeric complexes will often run at a position that is different from that expected from their monomeric position. Nevertheless, inspection of the differences in apparent molecular weight before and after treatment with alcohol led to several interesting observations for different membrane protein complexes.

Next to homo-oligomeric membrane proteins, proteins were also identified that seemed to undergo only a small shift in molecular weight upon TFE treatment, such as the penicillin-binding protein 1a encoded by MrcA (Fig. 3*A*, *spot 3*). This protein undergoes an apparent shift in molecular mass from 110 to 90 kDa, suggesting it is part of a hetero-oligomeric complex. Consistent with this, several candidate interacting proteins have been reported with a molecular mass of ~20 kDa (23).

We furthermore observed four outer membrane proteins that run off-diagonal and most likely originate from the small amount of outer membranes that contaminate the purified inner membranes (15). These proteins form oligomeric β-barrels, which run in the second dimension at their oligomeric molecular masses of about 100 kDa (LamB) or 80 kDa (OmpF). Their off-diagonal positions on the gel suggest that they are present as oligomers in larger complexes that are stable in room temperature SDS but become dissociated by TFE. This interpretation is consistent with the finding that in room temperature SDS the porins OmpF and LamB remain associated with other proteins and cell wall components (24). Apparently they are liberated out of these complexes by TFE as stable oligomers. We could substantiate this interpretation for OmpF using one-dimensional SDS-PAGE and OmpF-specific antibodies (data not shown). This sharply contrasts with their behavior upon heating in SDS, which dissociates the oligomers into monomers (25, 26). The resistance of the oligomers to alcohol-induced dissociation demonstrates an interesting difference in stability between β-barrel and α-helical membrane protein complexes.

Strikingly, many proteins that also do not contain transmembrane segments are released from larger complexes. These include the SecA component of protein translocase (Fig. 3*A*, *spot 2*), several components of the PTS system, different subunits of the ATPase, and many components of large *E. coli* inner membrane complexes involved in bioenergetics.

The result for SecA is particularly intriguing. The off-diagonal position of SecA on the two-dimensional gel corresponds to a TFE-induced dimer-monomer transition of part of the proteins. Experimental evidence has been presented that the protein indeed is a dimer (27), but recently the oligomeric state of SecA has been questioned (28, 29). Our results demonstrate that there are two pools of SecA that behave differently. What are these two pools? SecA is an integral part of the membrane-embedded protein translocase, but it is present in a great excess over the translocase (30). This pool of excess SecA will be in interaction with the membrane lipids and is expected to migrate as monomer because purified SecA migrates on SDS-PAGE (non-boiled) as a monomer, both by itself and in interaction with lipids (data not shown). The simplest interpretation then for the off-diagonally localized SecA is that it represents a pool of SecA, which is part of the translocase complex to which it thus must be bound as a dimer. SDS would dissociate this hetero-oligomeric SecYEG complex, thereby liberating the SecA dimer that after TFE exposure runs at its monomeric molecular weight.

The examples illustrated above demonstrate that alcohol-induced dissociation can occur for a large structural variety of integral membrane proteins. How can we understand the strong effects that small alcohols have on all these different membrane protein complexes? One possibility is that the alcohols perturb protein-protein contacts. For soluble proteins it is known that TFE can cause weakening of such contacts (31). However, alternative possibilities may be related to the effects on lipid packing in the SDS micelles. For KcsA we provided evidence that the perturbation of the lipid packing in the membrane is responsible for dissociation of the membrane-embedded protein by alcohols (11). These alcohols partition into the head group region and loosen the acyl chain packing, thereby altering the lipid-protein interaction, resulting in tetramer dissociation. For KcsA we proposed that in particular it is the change in lateral pressure profile in the membrane that is sensed by the tetramer (11). It is possible that a similar mechanism occurs for dissociation of the different *E. coli* inner membrane protein complexes in SDS by alcohols. The alcohols are expected to partition into the water-micelle interface and thereby to alter the interactions of the oligomeric protein with the surrounding SDS and/or the endogenous membrane lipids that will be present in the micelle. This interpretation is consistent with the observation that HFIP is much more efficient than TFE in dissociating a particular protein because it is known that HFIP perturbs lipid packing more strongly than TFE (10, 11). Furthermore, for several of the protein complexes that were found to become dissociated by TFE such as the mannitol PTS system (32, 33) and protein translocase (30, 34, 35), it was demonstrated that lipid-protein interactions are important for function. Our results suggest that this may be related to the effects of the lipids on oligomer stability, possibly via effects on the lateral pressure profile. We propose that the resistance of the various protein complexes against alcohol-induced dissociation in SDS micelles reflects the stability of these complexes in intact membranes. This proposition is supported by our finding that when *E. coli* inner membranes that contain penicillin-binding protein 1b are incubated with increasing concentrations of TFE prior to solubilization in SDS, a dimer-monomer transition around 20% TFE could be observed in subsequent SDS-PAGE in room temperature SDS (data not shown).

According to theory (36), membrane proteins can be sensitive to changes in lateral pressure profile only when destabilization or dissociation would be accompanied by a change in shape of the protein, as we also proposed for KcsA (37). In this respect the results that we obtained with the β-barrel-forming porins are particularly intriguing. We observed a lack of effect of TFE on the stability of these trimeric proteins, whereas heating clearly dissociates the trimers. Because these rigid β-barrel-forming proteins are not expected to be able to change their shape, these results are fully consistent with an important role for lateral pressure profiles in the stabilization of membrane proteins.

In conclusion, we have shown that small alcohols dissociate many *E. coli* inner membrane protein complexes that are stable in cold SDS. This technology provides a powerful and simple tool to detect and characterize stable membrane protein complexes. In principle, it is possible to obtain similar information by boiling the samples prior to running the second dimension to dissociate complexes. However, the use of small alcohols offers several important advantages. First, varying the concentration and nature of the alcohol allows insight into differences in stability of the membrane protein complexes (see *e.g.* Fig. 6). Second, because the alcohols will act differently on the proteins and the lipids (10), insights may be obtained on the importance of protein-protein and protein-lipid interactions that hold the complexes together. Third, because these alcohols act as local anesthetics and because for KcsA the potency to dissociate the tetramer was shown to correlate with the anesthetic potency (10), it may offer new insights into the mode of action of anesthesia in relation to its effect on lateral pressure profiles.

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