Current trends in α -helical membrane protein crystallization: An update

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Abstract: α -Helical membrane proteins (MPs) are the targets for many pharmaceutical drugs and play important roles in human physiology. In recent years, significant progress has been made in determining their atomic structure using X-ray crystallography. However, a major bottleneck in MP crystallography still remains, namely, the identification of conditions that give crystals that are suitable for structural determination. In 2008, we undertook an analysis of the crystallization conditions for 121 α -helical MPs to design a rationalized sparse matrix crystallization screen, MemGold. We now report an updated analysis that includes a further 133 conditions. The results reveal the current trends in α -helical MP crystallization with notable differences since 2008. The updated information has been used to design new crystallization and additive screens that should prove useful for both initial crystallization scouting and subsequent crystal optimization.

Keywords: membrane proteins; crystallization; crystallization additives

Abbreviations: Big CHAP, *N,N'*-bis-(3-*d*-gluconamidopropyl)-deoxycholamide; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate/*N,N*-dimethyl-3-sulfo-*N*-[3-[[3 α ,5 β ,7 α ,12 α)-3,7,12-trihydroxy-24-oxocholan-24-yl]amino]propyl]-1-propanaminium hydroxide; C₁₂E₈, polyoxyethylene(8)dodecyl ether; CYMAL, cyclohexyl- β -D-maltopyranoside; DDM, *n*-dodecyl- β -D-maltopyranoside; DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DM, *n*-decyl- β -D-maltopyranoside; FOS-Choline, phosphocholine; HEGA, *N*-hydroxyethylglucamide; LDAO, lauryldimethylamine-*N*-oxide; MEGA, *N*-methylglucamide; MPD, ($\pm$)-2-methyl-2,4-pentanediol; OG, *n*-octyl- β -D-glucopyranoside.

Additional Supporting Information may be found in the online version of this article.

Conflict of Interest: The data gathered in this study have been used by Molecular Dimensions Ltd. (UK) for the design and manufacture of a new sparse matrix crystallization screen (MemGold 2) and the additive screen (MemAdvantage) that are manufactured under license from the University of Oxford, Oxford UK.

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Introduction

Substantial progress has been made in tackling many of the hurdles faced in determining the crystal structures of α -helical membrane proteins (MPs),¹ including their production using recombinant systems,^{2,3} methods for engineering and screening stability,^{4,5} and in X-ray data collection using micro-focus beamlines.^{6,7} However, growing well-ordered three-dimensional crystals still represents a significant challenge that must be overcome. In 2008, we suggested the use of a rationalized sparse matrix type MP crystallization screen based on the crystallization conditions for 121 α -helical MPs deposited in the PDB at that time.⁸ Since our first analysis, however, the number of structures has more than doubled (see http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). In response to this increase in available crystallization data, an updated analysis is now presented that includes a total of 254 crystallization conditions from unique α -helical MP structures. Here, we present the summary of this analysis and show that the initial trends described in 2008 are broadly holding, but reveal intriguing new developments such as an increase in the number of

Figure 1. Successful detergents used for α -helical membrane protein crystallization. Numbers for each detergent class are shown, subdivided into the eight MP families used in the analysis. Respiratory complexes (brown), channels (black), transporters (green), photosynthetic and light-harvesting complexes (purple), GPCRs (red), ATPases (orange), bacterial rhodopsins (blue), and the “others” category (olive), those not fitting the seven main groupings. This color scheme is used throughout. Inset: Pie charts showing the change in the proportion of structures belonging to the eight MP families between 2008 and 2012, and the percentage contribution made by each family to the respective analyses is indicated.

cases where additional or mixed detergents have been required for structural determination. In addition to our analysis of crystallization conditions and the presentation of a complimentary crystal screen MemGold 2TM, an in-depth analysis of additives has also been possible with the increased database. The use of additional chemicals to optimize initial crystals to improve diffraction quality is well documented and many commercial kits are available.^{9,10} However, an additive screen targeted specifically for MPs has so far remained absent. A specific MP additive screen is therefore suggested to facilitate crystal optimization. As research groups start to tackle the more challenging eukaryotic MPs, we anticipate that these updated screens should enable more rational and successful approaches to crystallization and subsequent structural determination.

Results

Current trends in the number and types of α -helical MP crystal structures

Since 2008 an additional 133 novel α -helical MP structures have been added to our original crystalli-

zation database, bringing the total entries to 254. As before, these have been grouped into eight different families broadly divided by function (Fig. 1, inset). The data clearly show an increase in the determination of channel and transporter structures, with a concomitant decrease for the respiratory complexes. The number of additional structures for both the channel and transporter families has increased by 45 and 43, respectively, now making these two families the largest in the database, accounting for more than half of all the structures (alpha-MP-database-2012.xlsx). In 2008, respiratory complex structures accounted for 24% of the total; this has now dropped to 14%, signifying the addition of only seven new structures since this time. Another change has been the increase in the G-protein-coupled receptor (GPCR) family, which now makes up 7% of the database with 18 structures, up from 4% (or five structures) in 2008. Significant progress has been made in the structural determination of GPCRs due to a number of technological advancements in protein engineering and lipidic mesophase crystallization.^{11–13} The contribution from the photosynthetic and light-

harvesting complexes has also reduced, decreasing from 12 to 7%, with only three new structures. ATPases have increased from five structures in 2008 to 16, and now account for 6%, which is similar to the “others” category that includes α -helical MPs that could not be divided into the seven larger families described above.

Successful detergents

The majority of MP structures deposited in the Protein Data Bank have been determined using crystals grown from detergent-solubilized protein using traditional vapor diffusion experiments. In these experiments, the sample being crystallized is a mixture of both protein and associated detergent, making detergent selection a critical parameter for growing well-ordered, well-diffracting crystals.¹⁴ Significant progress has recently been made in the development of novel detergents for use in MP purification and crystallization.^{15,16} However, as in 2008, the alkyl maltopyranosides account for the majority of successfully used detergents, accounting for half of all structures in the database (Fig. 1) followed by the alkyl glucopyranosides (25%), amine oxides (7%), and polyoxyethylene glycols (7%). A full breakdown of individual detergents is given in Supporting Information Figure S1. Transporters still account for the majority of structures determined using alkyl maltopyranosides with 50 entries, followed by channels with 40. The most successful alkyl maltopyranoside detergent is *n*-dodecyl- β -D-maltopyranoside (DDM), which accounts for 22 transporter and 17 channel structures followed by *n*-decyl- β -D-maltopyranoside (DM), accounting for 13 transporter and 16 channel structures, respectively.

The choice of detergent depends on many different parameters, including solubilization efficiency, protein stability, and retention of function, such that some researchers may in practice have little “choice” at all! Nevertheless, considerable effort should be made to screen for crystals in shorter chain detergents as there is a greater probability of obtaining crystals that diffract to a higher resolution.¹⁷ An updated analysis of the resolution obtained here in the eight largest detergent classes further supports this conclusion [Fig. 2(A)], with the alkyl glucopyranoside detergent, *n*-octyl- β -D-glucopyranoside (OG), having both the highest resolution structure at 1.15 Å that of a yeast aquaporin Aqp1¹⁸ and highest mean resolution at 2.4 Å. The amine oxides, including *n*-lauryl dimethylamine-*N*-oxide (LDAO), gave the next most favorable resolution statistics followed by *n*-nonyl- β -D-glucopyranoside, both with mean resolutions of 2.7 Å. There is unlikely to ever be a single panacean detergent that can be applied to all types of MPs. Nevertheless, the data support the continued use of DDM, DM, OG, and LDAO as good first-choice

Figure 2. Analysis of reported resolution for α -helical membrane proteins. A: Box plots displaying the structural resolution for seven of the most successful crystallization detergent classes; the middle line in each box represents the mean value for each detergent class. Only structures crystallized using a single detergent were included. In red, the same analysis was used for all structures determined using lipidic phase methods. B: Reported high-resolution limit for the eight MP families analyzed in the study.

detergents when screening crystallization conditions using vapor diffusion methods.

Of course, a rational and intelligent approach should always be taken into account for detergent screening of MPs, which can now be accomplished

Figure 3. Analysis of precipitants. The different precipitants used in the successful crystallization of the eight MP families are shown. Salts, which include ammonium sulfate, sodium chloride, lithium sulfate, sodium phosphate, and trisodium citrate (green), and the organic molecule ($\pm$)-2-methyl-2,4-pentanediol (MPD) (purple) are indicated. The polyethylene glycols and other polymers that include Jeffamine and pentaerythritol propoxylate (5/4 PO/OH) are grouped into three classes depending on size, small MW (blue), medium MW (red), and large MW (yellow). Inset: The concentration of PEGs reported in the database are shown, revealing a broadly similar pattern to 2008.

easily using fluorescent-based methods early on in the structural determination process.^{19,20} A notable change since 2008 has been the increased success of detergent mixtures in the reported crystallization conditions. Interestingly, all families except the bacterial rhodopsins had at least one example where >1 detergent has been reported, suggesting that this should be a common approach to adopt early on in the screening process. However, no trend yet exists that may hint at whether certain detergent classes may be paired more successfully.

An important technical development in MP crystallization since our last analysis has been the increased use of lipidic phases, either in cubic phase,²¹ sponge phase²² or in the use bicelles.²³ As highlighted in Figure 2(A), the mean resolution for structures determined using these methods is 2.5 Å, almost half an ångström lower than for the alkyl maltopyranoside detergents and very close to the mean resolution obtained for OG, which in many cases is too “harsh” for α -helical MPs. These data add further support to the early adoption of lipidic phase crystallization in MP structure projects.

Precipitants, buffers, salts, and pH

Our analysis in 2008 of precipitants revealed a striking success for small MW PEGs in the crystallization of channels and transporters, with larger MW PEGs being more successful for respiratory complexes and MPs with large hydrophilic domains.⁸ These trends have remained in the updated dataset, with the notable appearance of small MW PEGs in the crystallization of the eukaryotic GPCR family (Fig. 3). The successful concentration ranges have also been maintained, with small MW PEGs being successful at concentrations between 20 and 40% and with larger MW PEGs being used at lower concentrations between 5 and 20% (Fig. 3, inset). However, the successful use of organic molecules such as MPD is still low, further confirming their unsuitability as general crystallization reagents for α -helical MPs; a situation that is dramatically different for outer MPs where organic molecules are clearly more successful.²⁴

Buffering chemicals and salts often have a significant impact on protein crystallization; in particular polyvalent cations and anions are often essential

Figure 4. Successful additives used for crystallization. The range of additives used for successful crystallizations are shown for each MP family. The additives have been grouped into more general classes for clarity and colored according to Figure 1.

for crystallization.^{25,26} Our data reveal a broadly similar pattern to 2008 (Supporting Information Fig. S1), with no clear correlation between different buffer types and MP family. Of note in the present data is that 37 different salts have been reported, covering many of the commonly found polyvalent cations and anions. The broad pH distribution in the conditions was also of interest, particularly for channels, with structures reported at pH values between 3.5 and 10 (Supporting Information Fig. S2). Screening over a wide range of pH values is therefore recommended to efficiently cover crystallization space.

Additives

For many projects, an initial crystal condition will require optimization often by the addition of small molecules, salts, and specific ligands. Figure 4 shows the range of different small molecules and salt additives that have been reported to improve initial crystallization conditions for α -helical MPs in the present literature. Of note is that all families are represented, suggesting that the use of additional crystallization additives is generally applicable and should be routinely explored. As observed previously, multivalent salts and polyalcohols appear prominently in the database, accounting for 10 and 15%, respectively. However, a notable difference is a substantial increase in the number of secondary deter-

gents and nonvolatile organic molecules that are now being recorded, which account for 19 and 12%, respectively, up from 10 and >1% in 2008. Structures of membrane transporters account for much of this increase, suggesting that screening secondary detergents for members of this family would be especially worthwhile. This observation supports the data presented in Figure 1, which shows the increased use of detergent mixtures for crystallization. Interestingly, the reported use of additional lipids as additives appears to be mainly isolated to channels, with monovalent salts being more successful for transporters.

Discussion

Recent advances in the use of crystallization robotics have substantially increased the number of different crystallization conditions that can be screened. A common conundrum for structural biologists is how many conditions are enough to adequately cover “crystallization space” for a given MP sample? This question is especially pertinent for eukaryotic MPs when amounts are likely to be far scarcer when compared with their prokaryotic counterparts. Many of the MPs analyzed in this study crystallized in similar precipitant conditions, with approximately two-thirds being crystallized in a low MW PEG. However, the pH, salt, and buffer components of the crystallization conditions differed substantially,

Table I. *MemAdvantage: A Targeted Additive Screen for α -Helical Membrane Protein Crystal Optimization*

	Additive	Classification	Suggested crystal drop concentration
1	10% (w/v) 1,2,3-Heptanetriol	Amphiphile	1.0%
2	20% (w/v) Benzamidine hydrochloride hydrate	Amphiphile	2.0%
3	40% (w/v) Sucrose	Carbohydrate	4.0%
4	30% (w/v) Trehalose	Carbohydrate	3.0%
5	0.1M EDTA	Chelating agent	0.01M
6	0.1M EGTA	Chelating agent	0.01M
7	30% (w/v) Butylated hydroxytoluene	Small molecule	3.0%
8	0.1M C-HEGA-11	Detergent	0.01M
9	0.009M C12E8	Detergent	0.1 mM
10	0.08M C8E4	Detergent	8.0 mM
11	0.08M CHAPS	Detergent	8.0 mM
12	3M CYMAL 1	Detergent	0.3M
13	1.2M CYMAL 2	Detergent	0.12M
14	0.08M CYMAL 4	Detergent	8.0 mM
15	0.05M CYMAL 5	Detergent	5.0 mM
16	0.005M CYMAL 6	Detergent	0.5 mM
17	0.002M CYMAL 7	Detergent	0.2 mM
18	0.002M <i>n</i> -Dodecyl- β -D-maltopyranoside	Detergent	0.2 mM
19	0.025M <i>n</i> -Decanoyl sucrose	Detergent	0.25 mM
20	0.014M Deoxy-Big CHAP	Detergent	1.4 mM
21	0.018M <i>n</i> -Decyl- β -D-maltopyranoside	Detergent	1.8 mM
22	0.0036M Decyl maltose neopentyl glycol	Detergent	0.036 mM
23	0.003M Dodecanoyl sucrose	Detergent	0.3 mM
24	0.015M FOS-Choline-12	Detergent	1.5 mM
25	0.395M FOS-Choline-9	Detergent	39.5 mM
26	0.014M HEGA 11	Detergent	1.4 mM
27	0.014M HEGA 10	Detergent	7.0 mM
28	0.012M <i>n</i> -Dodecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	Detergent	1.2 mM
29	0.001M Lauryl maltose neopentyl glycol	Detergent	0.01 mM
30	20% (v/v) MERPOL HCS	Detergent	2.0%
31	0.015M <i>n</i> -Dodecyl- <i>N,N</i> -dimethylglycine	Detergent	1.5 mM
32	0.065M <i>n</i> -Nonyl- β -D-glucopyranoside	Detergent	6.5 mM
33	0.06M <i>n</i> -Nonyl- β -D-maltopyranoside	Detergent	6.0 mM
34	0.2M <i>n</i> -Octyl- β -D-glucopyranoside	Detergent	20.0 mM
35	0.01M <i>N</i> -Octyl- β -D-maltopyranoside-fluorinated (OM-F)	Detergent	1.0 mM
36	0.01M Octyl glucose neopentyl glycol	Detergent	1.0 mM
37	0.003M <i>n</i> -Tridecyl- β -D-maltopyranoside	Detergent	0.03 mM
38	0.015M 3-Dodecylamido- <i>N,N'</i> -dimethylpropyl amine oxide	Detergent	1.5 mM
39	0.045M [(3-(3 Butyl-3-phenylheptanamido)- <i>N</i> , <i>N</i> -dimethylpropan-1-amine oxide)]	Detergent	4.5 mM
40	0.0059M <i>n</i> -Undecyl- β -D-maltopyranoside	Detergent	0.59 mM
41	0.002M <i>n</i> -Undecyl- β -D-thiomaltopyranoside	Detergent	0.2 mM
42	0.006M Anzergent 3-12	Detergent	0.6 mM
43	0.7M <i>n</i> -Heptyl- β -D-glucopyranoside	Detergent	70 mM
44	Deuterium oxide	Heavy water	—
45	0.3M Glycyl-glycyl-glycine	Linker	30.0 mM
46	0.1M Lithium citrate	Monovalent	10 mM
47	0.1M Lithium sulfate	Monovalent	10 mM
48	0.1M Potassium chloride	Monovalent	10 mM
49	0.1M Potassium fluoride	Monovalent	10 mM
50	0.1M Potassium silicate	Monovalent	10 mM
51	0.1M Rubidium chloride	Monovalent	10 mM
52	0.1M Sodium azide	Monovalent	10 mM
53	0.1M Sodium chloride	Monovalent	10 mM
54	0.1M Sodium fluoride	Monovalent	10 mM
55	0.1M Sodium phosphate	Monovalent	10 mM
56	0.1M Sodium silicate acetate	Monovalent	10 mM
57	0.1M Ammonium citrate	Multivalent	10 mM
58	0.1M Ammonium sulfate	Multivalent	10 mM
59	0.1M Cadmium chloride	Multivalent	10 mM
60	0.1M Calcium chloride	Multivalent	10 mM
61	0.1M Chromium chloride	Multivalent	10 mM
62	0.1M Cobalt chloride	Multivalent	10 mM
63	0.1M Copper chloride	Multivalent	10 mM

Table I. (Continued)

	Additive	Classification	Suggested crystal drop concentration
64	0.1M Gadolinium chloride	Multivalent	10 mM
65	0.1M Magnesium chloride	Multivalent	10 mM
66	0.1M magnesium sulfate	Multivalent	10 mM
67	0.1M Manganese chloride	Multivalent	10 mM
68	0.1M Osmium chloride	Multivalent	10 mM
69	0.1M Samarium chloride	Multivalent	10 mM
70	0.1M Strontium chloride	Multivalent	10 mM
71	0.1M Zinc nitrate	Multivalent	10 mM
72	0.1M Zinc sulfate	Multivalent	10 mM
73	30% (w/v) 1,6-Hexanediol	Organic, nonvolatile	3.0%
74	30% (w/v) 2-Methyl-2,4-pentanediol	Organic, nonvolatile	3.0%
75	30% (v/v) Dimethyl sulfoxide	Organic, nonvolatile	3.0%
76	0.08M Foscarnet	Organic, nonvolatile	8 mM
77	30% (w/v) Sodium phosphonoformate tribasic hexahydrate	Organic, nonvolatile	3.0%
78	Glutaric acid pH 6.0	Organic, nonvolatile	100 mM
79	0.01M L-Glutathione reduced	Reducing agent	1.0 mM
80	50% (v/v) PEG 400	Organic, nonvolatile	5.0%
81	5% (w/v) Polyvinylpyrrolidone K15	Organic, nonvolatile	0.5%
82	0.1M Spermidine	Polyamine	10 mM
83	50% (v/v) Jeffamine M-600	Organic, nonvolatile	5.0%
84	0.1M Taurine	Linker	10 mM
85	40% (v/v) 1,3-Propanediol	Organic, volatile	4.0%
86	40% (v/v) 1,3 Butanediol	Organic, volatile	4.0%
87	40% (v/v) <i>tert</i> -Butanol	Organic, volatile	4.0%
88	30% (v/v) Ethanol	Organic, volatile	3.0%
89	30% (v/v) Isopropanol	Organic, volatile	3.0%
90	10% (v/v) 1,2-Butandiol	Organic, volatile	1.0%
91	0.1M Triethylammonium phosphate	Organic, volatile	10 mM
92	30% (v/v) Ethylene glycol	Polyalcohol	3.0%
93	30% (v/v) Glycerol	Polyalcohol	3.0%
94	0.05M 2-Mercaptoethanol	Reducing agent	5.0 mM
95	0.05M DTT	Reducing agent	5.0 mM
96	0.05M TCEP hydrochloride	Reducing agent	10 mM

suggesting that successful crystallization screens should be more varied in these parameters rather than the precipitant type. Based on these data, a new sparse matrix style screen MemGold 2 is presented (Supporting Information Table S1). Our analysis of the available crystallization data suggests that MemGold and MemGold 2 provide a comprehensive set of initial screening conditions for α -helical MP crystallization.

Additionally, analysis of these data clearly shows that exploring the use of smaller micelle detergents, in many cases as additives, should be carried out as an avenue to improve diffraction quality following initial crystallization success. As in detergent selection, no single additive is likely to emerge as a panacea for optimization. Nevertheless, optimization is still likely to be required and to facilitate the identification of successful additives, we have designed a novel set of chemicals from the database into a 96-format screen, MemAdvantage, for implementation in robotic screening pipelines (Table I). We anticipate that this screen will prove valuable during crystal optimization and combined

with the present analysis and databases provide a useful resource for the MP structure community.

Materials and Methods

An analysis of the deposited structures for α -helical MPs was carried out as described previously.⁸ The original database (alpha-MP-database.xls) constructed in 2008 was complemented with additional unique α -helical MP structures to create the updated database (alpha-MP-database-2012.xlsx). Conditions from proteins crystallized using both vapor diffusion and lipidic mesophase crystallization are now included in the database and analysis. To avoid biasing the analysis, conditions from the same MP were excluded if they were identical. Reported conditions for each PDB entry were then divided into the different crystallization components: precipitant, buffer, pH, salts, additives, and detergents along with their respective concentrations. Chemicals were considered additives if their concentration was less than 20 mM, for example, zinc sulfate at 5 mM was counted as an additive and not as a salt unless otherwise indicated as such in the PDB deposition or

associated publication. These data were then analyzed by constructing a series of stacked bar charts showing the number of successful crystallizations for each α -helical MP family against the individual chemicals for each component of the crystallization experiments.

The MemGold 2 and MemAdvantage screens were designed by selecting 96 nonredundant conditions from this database. Concentrations suggested are guidelines and may be varied when preparing custom screens based on these reagents. However, both screens are currently commercially available through Molecular Dimensions Ltd. (UK).

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