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Review

Separation methods in the analysis of protein membrane complexes

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Abstract

The separation of membrane protein complexes can be divided into two categories. One category, which is operated on a relatively large scale, aims to purify the membrane protein complex from membrane fractions while retaining its native form, mainly to characterize its nature. The other category aims to analyze the constituents of the membrane protein complex, usually on a small scale. Both of these face the difficulty of isolating the membrane protein complex without interference originating from the hydrophobic nature of membrane proteins or from the close association with membrane lipids. To overcome this difficulty, many methods have been employed. Crystallized membrane protein complexes are the most successful example of the former category. In these purification methods, special efforts are made in the steps prior to the column chromatography to enrich the target membrane protein complexes. Although there are specific aspects for each complex, the most popular method for isolating these membrane protein complexes is anion-exchange column chromatography, especially using weak anion-exchange columns. Another remarkable trend is metal affinity column chromatography, which purifies the membrane protein complex as an intact complex in one step. Such protein complexes contain subunit proteins which are genetically engineered so as to include multiple-histidine tags at carboxyl- or amino-termini. The key to these successes for multi-subunit complex isolation is the idea of keeping the expression at its physiological level, rather than overexpression. On the other hand, affinity purification using the Fv fragment, in which a *Strep tag* is genetically introduced, is ideal because this method does not introduce any change to the target protein. These purification methods supported by affinity interaction can be applied to minor membrane protein complexes in the membrane system. Isoelectric focusing (IEF) and blue native (BN) electrophoresis have also been employed to prepare membrane protein complexes. Generally, a combination of two or more chromatographic and/or electrophoretic methods is conducted to separate membrane protein complexes. IEF or BN electrophoresis followed by 2nd dimension electrophoresis serve as useful tools for analytical demand. However, some problems still exist in the 2D electrophoresis using IEF. To resolve such problems, many attempts have been made, e.g. introduction of new chaotropes, surfactants, reductants or supporting matrices. This review will focus in particular on two topics: the preparative methods that achieved purification of membrane protein complexes in the native (intact) form, and the analytical methods oriented to resolve the membrane proteins. The characteristics of these purification and analytical methods will be discussed along with plausible future developments taking into account the nature of membrane protein complexes.
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1. Introduction

The membrane system is an interface between the outer and inner worlds across the membrane. Every level of the biological system, such as mitochondria, chloroplasts, cells and organs, needs to communicate with the outer world. The membrane system is one of the most important interfaces in biological systems. Such a membrane system contains many kinds of receptor proteins, transporter proteins and channel proteins (Table 1) which have critical roles for the biological activity. For example, aquaporins, such as AQP1 [1,2], work as channels to exclusively transfer water molecules across cell membranes. P-type ion transporting ATPases (such as Ca^{2+}-ATPase) work to establish ion gradients across biological membranes [3,4]. Furthermore, the proteins associated with energy transducing electron transport chains in mitochondria and chloroplasts are located in the membrane system. Accordingly, these proteins associated with the membrane systems, which are called membrane proteins, are also important from the clinical point of view. For example, alteration of human ClC Cl^{-} channels is known to be closely related to several kidney-associated diseases [5]. It has been reported that the catalytic activity of NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport chain is reduced in Parkinson's disease [6]. Potassium channels suffer interaction with toxins from scorpion venom [7].

Recent advances in genetic information should support research on these membrane proteins. The whole genome sequence of humans [8,9] as well as other many organisms including mice [10] have been reported, and a list of species whose genomes have been completely analyzed can be obtained at, e.g., http://www.genome.ad.jp/kegg/catalog/org_list.html. Furthermore, analysis of the whole genome is still being intensively developed for many species (e.g. http://www.genome.ad.jp/dbget-bin/get_htext?Genome_Projects+-n). According to these genome sequences, the numbers of genes encoding proteins can be estimated, e.g., around 30,000–40,000 for humans [8,9]. Using such available genome information (11 eubacteria, three archaea and one eukaryotic organism, *Saccharomyces cerevisiae*), Mitaku et al. reported that 15–20% of ORFs coded for membrane proteins irrespective of the species and the genome size [11]. They obtained this value using the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) developed by their group [12]. Wallin and von Heijne also reported a similar value (20–30%) for the ratio of membrane protein genes to the total ORFs using a slightly different genome set (but including *Caenorhabditis elegans* and *Homo sapiens*) and another program [13]. Despite the relatively smaller variety of membrane proteins compared to soluble proteins, they are very important for the development and maintenance of life for all organisms, as described above.

Membrane proteins have close contact with membrane lipids and form protein membrane complexes. Furthermore, in many cases, such membrane proteins form another level of complex which is composed of subunit proteins and cofactors; membrane protein complexes. The functional platform of the membrane protein (complex) is the membrane, which is composed of lipids. There is a mutual relationship between the membrane proteins and the membrane lipids.

The difficulties in the investigation and separation of membrane protein complexes originate from their nature as membrane proteins. (1) They are very hydrophobic and have single or several transmembrane parts, or closely associate with the membrane. (2) In the functional form, many of them comprise (homologous or heterologous) multi-subunit complexes. (3) Such membrane protein complexes contain many cofactors and, inevitably, lipids. (4) Some membrane protein complexes have several peripheral proteins which are functionally important but easily detached during the isolation process.

The separation methods for research on membrane proteins (complexes) contain two categories: preparative and analytical separation. Keeping the membrane protein complex intact is a prerequisite for preparative separation, but not for analytical separation. In spite of the difficulties described above, there are many successes, and challenges to overcome these difficulties. To date, several membrane protein complexes have been purified and their structures and functions have been analyzed in detail (e.g. Table 1). Such crystallized protein complexes are good examples which achieved high quality for the crystallization samples as well as the usual research on membrane proteins: high purity, high homogeneity, monodispersity, etc. Although the number of successes is limited compared to the soluble proteins, investigation into membrane protein complexes is growing

Table 1
The crystallized membrane protein complexes and the related membrane complexes

Chromatographic purification	Source	Detergents for solubilization	Detergents for post-solubilization	Additive	Column	No. of unique subunits	Formation	Size (10^3 rel. mol. mass units)	Remarks	Status, resolution	Refs.
NADH:ubiquinone oxidoreductase	Bovine heart mitochondria	1% (w/v) DDM at 12 mg protein/ml, then 1.6% Na-cholate+ammonium sulfate precipitation	0.1% DDM	10% (v/v) ethylene glycol	Mono Q HR 10/10, then ammonium sulphate precipitation	27					[44]
(complex I)	Bovine heart mitochondria	1% (w/v) DDM at 12 mg protein/ml, then 1.6% Na-cholate+ammonium sulfate precipitation	0.1% DDM	10% glycerol, 50 m*M* sucrose	Hiload Sephacryl S-300 HR, Hiload Q-Sepharose HP, Mono-Q HR and Hiload Sephacryl S-300 HR	43		1279			[45]
	Bovine heart mitochondria	1% (w/v) DDM at 12 mg protein/ml, then 1.6% Na-cholate+ammonium sulfate precipitation	0.1% DDM	10% (v/v) ethylene glycol	Hiload Q-Sepharose HP, then ammonium sulphate precipitation	42		890		(EM), 22Å	[147]
	Bovine heart mitochondria	1.75% Triton X-100 with 600 m*M* NaCl at 35 mg protein/ml, then, 2% Triton X-100 with 600 m*M* NaCl at 40 mg protein/ml	0.1% DDM		Hydroxyapatite, DEAE Biogel A, TSK G 4000 SW	43		944			[33]
	Neurospora crassa	4.0% Triton X-100 (at 50 mg protein/ml), then 10.6% Triton X-100	0.1% Triton X-100		DEAE-Sepharose CL 6B, TSK G 4000 SW	~25	Monomer	610		(EM), 39Å	[148]
	Neurospora crassa	3.3% Triton X-100	0.1% Triton X-100		DEAE Sepharose CL-6B, hydroxyapatite (Bio-Gel HTP), then sucrose density gradient	35		1120		(EM), 35Å	[149]
Succinate dehydrogenase	*Escherichia coli*	4% Lubrol PX at 10 mg protein/ml	1% Lubrol PX		DEAE-Sepharose CL-6B	4					[150]
(complex II)	*Escherichia coli*	4% Lubrol PX at 10 mg protein/ml	1% Lubrol PX		DEAE-Sepharose CL-6B	4	Trimer	360	Overexpressed in *E. coli*	Crystallized, 2.6Å	[151]
Cytochrome *b*/*c*1 (complex III)	*Rhodobacter sphaeroides*	0.66 mg DDM/mg protein	0.01% DDM	25% glycerol	Ni–NTA agarose (eluted by 200 m*M* histidine)	4	Dimer				[69]
	Potato tuber mitochondria	1.5% DDM at 10 mg protein/ml	0.01% DDM, Brij 35		DEAE Sepharose CL-6B, Hydroxyapatite	>10					[34]
	Vertebrate heart mitochondria	1.5% DDM at 10 mg protein/ml			DEAE Sepharose CL-6B, Sepharose CL-6B	11	Dimer	2×243		Crystallized, 3Å	[152]
	Bovine heart mitochondria	1.5% DDM at 10 mg protein/ml	0.01% DDM, Brij 35		DEAE Sepharose CL-6B, Hydroxyapatite		Dimer	~480		Crystallized, 4.0Å	[86]
	Paracoccus denitrificans	DDM, LDAO, OG at 1–1.5 g/g			Strep tag affinity column						[74]
	Paracoccus denitrificans	protein at 10 mg protein/ml			Strep tag affinity column	4				Crystallized, 2.8Å	[75]

Table 1 (*Continued*)

Chromatographic purification	Source	Detergents for solubilization	Detergents for post-solubilization	Additive	Column	No. of unique subunits	Formation	Size (10^3 rel. mol. mass units)	Remarks	Status, resolution	Refs.
Cytochrome *b*/*c*1 with cytochrome *c*	*Saccharomyces cerevisiae*	1.5% DDM at 10 mg protein/ml	0.01% DDM		Strep tag affinity column					Crystallized, 2.97Å	[77]
Cytochrome *aa*3	*Paracoccus denitrificans*	LDAO			Strep tag affinity column (Streptavidin Sepharose column)	2				Crystallized, 2.7Å	[76]
Cytochrome *ba*3	*Thermus thermophilus*	5% Triton X-100	0.1% Triton X-100, 0.05% DDM, 0.1% DDM		DEAE-Biogel, Fractogel EMD TMAE-650 (S), Superdex 200	3		85		Crystallized, 2.4Å	[153–155]
Cytochrome *bo*3	*Escherichia coli*	1% Triton X-100+1.25% OG	0.03% DDM, 1% OG		Ni–NTA (eluted by imidazole), MonoQ 10/10	4	Monomer		C-t His on subunit II	Crystallized, 3.5Å	[156,157]
Fumarate reductase (QFR)	*Escherichia coli*	Thesit			Anion-exchange, perfusion, and gel filtration chromatography	4		121		Crystallized, 3.3Å	[158]
Photosystem I	*Thermosynechococcus elongatus*	0.6% DDM at 1 m*M* Chl	0.02% DDM		Q-Sepharose HP	11	Trimer	3×356		Crystallized, 4Å	[159]
	Thermosynechococcus elongatus	0.6% DDM at 1 m*M* Chl	0.02% DDM		Q-Sepharose HP	12	Trimer	3×356		Crystallized, 2.5Å	[38]
	Synechococcus sp.	2.22% Triton X-100	0.03% Triton X-100		Sepharose-CL-6B	~10				2D crystal, 19Å	[160]
Photosystem II	*Thermosynechococcus elongatus*	DDM	0.03% DDM		Toyopearl 650	>17	Dimer	500		Crystallized, 3.8Å	[49]
	Thermosynechococcus elongatus	1.2% DDM+0.5% Na Cholate at 1 mg Chl/ml	0.03% DDM	1.65 *M* ammonium sulphate	POROS ET, Uno-Q		Dimer	500		Crystallized, 4.3Å	[55]
	Thermosynechococcus elongatus	1.0% DDM at 1 mg Chl/ml	0.03% DDM	10% glycerol, 25% glycerol	ProBond (eluted by 200 m*M* imidazole)				C-t His on PsbC		[63]
	Thermosynechococcus vulcanus	1.2% DDM at 1 mg Chl/ml	0.05% DDM	25% glycerol	Mono-Q	>17	Dimer	580		Crystallized, 3.7Å	[50]
	Synechocystis sp. PCC 6803	1.0% DDM at 1 mg Chl/ml	0.03% DDM	25% glycerol	DEAE-Toyopearl 650S						[59]
	Synechocystis sp. PCC 6803	1.0% DDM at 1 mg Chl/ml	0.04% DDM	25% glycerol	Ni–NTA agarose (eluted by 50 m*M* histidine)	(>20)			C-t His on PsbB		[64]
	Chlamydomonas reinhardtii	2% DDM at 2 mg Chl/ml	0.03% DDM	10% glycerol	ProBond (eluted by 200 m*M* imidazole)		Dimer	580	C-t His on PsbD		[60]
Photosynthetic reaction center	*Rhodobacter sphaeroides*	0.5% LDAO	0.06% LDAO		DEAE-Sepharose, Fractogel TSK HW-55 (S)	3				Crystallized, 2.65Å	[161]
	Rhodobacter sphaeroides	0.3% LDAO	0.1% LDAO		DE52 column, Sepharose Q column, Superdex 200	3				Crystallized, 2.6Å	[162]
	Rhodobacter sphaeroides (mutant)	0.3% LDAO	0.1% LDAO		DE52 column, Sepharose Q column, Superdex 200	3				Crystallized, 2.1Å	[39]
	Rhodopseudomonas viridis	5% LDAO	0.1% LDAO		TSK 3000 SW	4		132		Crystallized, 2.3Å	[163]
	Thermochromatium tepidum	0.25% LDAO	0.05% LDAO		DEAE-Sephacel (twice), DEAE-Toyopearl	4		133		Crystallized, 3.0Å	[164]
	Thermochromatium tepidum	0.25% LDAO	0.05% LDAO		DEAE-Sephacel (twice), DEAE-Toyopearl	4				Crystallized, 2.2Å	[40]
B800-820 LHC (LH2)	*Rhodopseudomonas acidophila*	2% (v/v) LDAO	0.1% LDAO		RESOURCE Q					Crystallized, 2.8Å	[165]
Bacteriorhodopsin	*Halobacterium salinarum*	1.2% OG	1.2% OG		BioGel A-0.5 m	1				Crystallized, 2.5Å	[166]
Sensory rhodopsin II	*Natronobacterium pharaonis*	1.6% OG at 300 m*M* NaCl			Ni-affinity column, (eluted by 300 m*M* imidazole)	1			C-t His-tag	2D crystal, 6.9Å	[167]

Ca^{2+} ATPase	Rabbit, skeletal muscle sarcoplasmic reticulum	10% C12E8 at 5 mg protein/ml	0.1% C12E8	20% glycerol	Reactive Red 120 affinity column (elution by 2 m*M* ADP)	1	Monomer	110		Crystallized, 2.6Å	[3]
Ca^{2+} ATPase w/o Ca^{2+}	Rabbit, skeletal muscle sarcoplasmic reticulum	10% C12E8 at 5 mg protein/ml	0.1% C12E8	20% glycerol	Reactive Red 120 affinity column (elution by 2 m*M* ADP)	1	Monomer	110		Crystallized, 3.1Å	[4]
K^{+} channel (KcsA)	*Streptomyces lividans*	40 m*M* DM	5 m*M* LDAO		Co-affinity, gel filtration	1	Homotetramer		C-t His tag, overexpressed in *E. coli*	Crystallized, 3.2Å	[168]
K^{+} channel (MthK)	*Methanobacterium thermoautotrophicum*	40 m*M* DM	5 m*M* LDAO		Co-affinity (TALON) (eluted by imidazole), gel filtration (Superdex-200)	1	Homotetramer		C-t His tag, overexpressed in *E. coli*	Crystallized, 3.3Å	[169]
Na^{+}/H^{+} antiporter (NhaA)	*Escherichia coli*	1.0% DDM	0.1–0.02% DDM	30% glycerol	Ni–NTA (eluted by pH shift to pH 4)	1	Homodimer	2×42	Overexpressed in *E. coli*	Crystallized, 7Å	[66]
ClC Cl^{-} channel	*Salmonella typhimurium*, *Escherichia coli*	50 m*M* DM	45 m*M* OM		Co-affinity (TALON) (eluted by imidazole), gel filtration (Superdex-200)	1	Homodimer	2×50	C-t His tag, overexpressed in *E. coli*	Crystallized, 3.0Å	[5]
Cl pump Halorhodopsin	*Halobacterium salinarum*	5% cholate at 4 *M* KCl	1% OG		Phenyl-Sepharose CL-4B	1	Homotrimer	3×27	Overexpression in *H. salinarum*	Crystallized, 1.8Å	[170]
Mechanosensitive channel of large conductance (MscL)	*Mycobacterium tuberculosis*	1.0% DDM	0.1% DDM		Ni-affinity, anion-exchange, size exclusion	1	Homopentamer	15×5	N-t His tag, overexpressed in *E. coli*	Crystallized, 3.5Å	[171]
Mechanosensitive channel of small conductance (MscS)	*Escherichia coli*	1% Foscholine-14	0.05% Foscholine-14		Ni-affinity, anion-exchange, size exclusion	1	Homoheptamer		His-tag, overexpressed in *E. coli*	Crystallized, 3.9Å	[172]
Anion-selective porin (Omp32)	*Comamonas acidovorans*	OPOE	0.6% OPOE		Superdex-200, Q-Sepharose FF medium	1	Homotrimer	3×35		Crystallized, 2.1Å	[173]
Aquaglyceroporin, glycerol facilitator (GlfP)	*Escherichia coli*				Ni-affinity, anion-exchange, size exclusion	1	Homotetramer		N-t His tag	Crystallized, 2.2Å	[174]
Aquaporin-1 (AQP1)	Human red cell	1% *N*-lauroylsarcosine, then 4% Triton X-100	1.2% OG		POROS Q/F	1	Homotetramer	4×28		Crystallized, 3.8Å	[1]
Aquaporin-1 (AQP1)	Bovine erythrocytes	NG	13 m*M* NG		DEAE Sephacel	1	Homotetramer	4×28		Crystallized, 2.2Å	[2]
Maltoporin (LamB)	*Salmonella typhimurium*	2% LDAO	0.08% LDAO		Amylose resin	1	Homotrimer	3×48		Crystallized, 2.4Å	[175]
ABC transporter (MsbA)	*Escherichia coli*	1% α-DDM	0.05% α-DDM		Ni-chelate chromatography, ion-exchange chromatography	1	Homodimer	129	N-t His tag, overexpressed in *E. coli*	Crystallized, 4.5Å	[176]

Table 1 (*Continued*)

Chromatographic purification	Source	Detergents for solubilization	Detergents for post-solubilization	Additive	Column	No. of unique subunits	Formation	Size (10^3 rel. mol. mass units)	Remarks	Status, resolution	Refs.
ABC transporter (BtuCD)	*Escherichia coli*	1% LDAO			Ni–NTA, gel filtration	2	Two copies of each subunit		N-t His tag in BtuC, BtuC and BtuD were coexpressed from a single plasmid in *E. coli*	Crystallized, 3.2Å	[72]
Bacterial multidrug efflux transporter (AcrB)	*Escherichia coli*	2% DDM	0.2% DDM	10% glycerol	Chelating Sepharose with Ni^{2+}	1	Homotrimer	3×114	C-t His tag, overexpressed in *E. coli*	Crystallized, 3.5Å	[177]
Translocon at the outer-envelope membrane of chloroplasts (Toc34)	Pea chloroplast outer envelope				Fast-flow Ni–NTA	1	Homodimer	2×34	C-t His tag, overexpressed in *E. coli*	Crystallized, 2.0Å	[178]
Ferric enterobactin receptor (FepA)	*Escherichia coli*	2% Triton X-100,	1% OG		DE-52 (twice), then PBE 94 chromatofocusing column,			80		Crystallized, 2.4Å	[78]
FhuA, the siderophore receptor	*Escherichia coli*	1.0% LDAO	0.10% LDAO		DE-52, Sephadex G100 Ni–NTA agarose	1	Monomer	79	Overexpressed in *E. coli*	Crystallized, 2.5Å	[179]
Electrophoretic purification											
Fumarate reductase (QFR)	*Wolinella succinogenes*				DEAE CL-6B column (twice), then, preparative IEF	3	Dimer			Crystallized, 2.2Å	[82]
Photosystem I	*Synechococcus* sp. PCC 7002	1.44% DDM at 1.2 mg Chl/ml			Preparative IEF, then, Q-Sepharose		Trimer	690–760		Crystallized	[80]
Ammonium acetate fractionation											
Cytochrome *b*/*c*1	Bovine heart mitochondria	Cholate			Ammonium sulfate fractionation	13	Dimer			Crystallized, 2.8Å	[180]
	Bovine heart mitochondria	Cholate			Ammonium sulfate fractionation	13	Dimer			Crystallized, 2.8Å	[36]
	Bovine heart mitochondria	Deoxycholate			15 step ammonium acetate fractionation; recovery was from 18.5 to 33.5% saturation	11				Crystallized, 2.9Å	[181]
Cytochrome *b*/*c*1 with famoxadone	Bovine heart mitochondria	Deoxycholate		0.66 *M* sucrose	15 step ammonium acetate fractionation; recovery was from 18.5 to 33.5% saturation	11	Dimer	~500		Crystallized, 2.4Å	[182]
Cytochrome *b*/*c*1 with inhibitor	Bovine heart mitochondria	Deoxycholate			15 step ammonium acetate fractionation; recovery was from 18.5 to 33.5% saturation	11				Crystallized, 3.0Å	[183]
Phase partitioning											
Rhodopsin	Bovine rod outer segment	HTG or NG with 80 m*M* divalent cation			Slow rate centrifugation	1		40		Crystallized, 2.8Å	[184]
Sucrose density gradient											
Photosystem I	*Thermosynechococcus vulcanus*	2.0% OG			Sucrose density gradient	12				Crystallized, 6Å	[185]

C12E8, octaethylene glycol dodecyl ether; DDM, *n*-dodecyl-β-D-maltopyranoside; α-DDM, *n*-dodecyl-α-D-maltopyranoside; DM, *n*-decyl-β-D-maltoside; HTG, *n*-heptyl-β-D-thioglucoside; LDAO, lauryldimethylaminoxide; NG, *n*-nonyl-β-D-glucoside; OG, *n*-octyl-β-D-glucoside; OM, *n*-octyl-β-D-maltoside; OPOE, octyl-polyoxyethylene. C-t and N-t, carboxy and amino termini, respectively.

more important. This is true for clinical biochemistry of medical necessity. Analysis of the structure through the crystallization work will enable clarification of the precise function of the membrane protein complexes. Furthermore, the determined structure will lead to the effective design of new drugs.

The analytical separation of membrane proteins is quite important for clinical research. Based on the genome information, a proteomic approach has been developed, which aims to detect whole expressed proteins to analyze the function of such proteins and the functional linkage between them. This proteomic approach is one of the important clinical analyses. Analysis of the subunit components in an isolated membrane complex is also necessary for the full understanding of the function of the membrane protein complex. For this analytical aim, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [14] and/or 2-dimensional electrophoresis in conjunction with isoelectric focusing (IEF) [15] or blue native (BN) [16] electrophoresis are frequently employed. 2D electrophoresis combined with IEF is widely performed for membrane protein samples [17,18]. This method makes it easy to find differences in the expression levels of known or unknown proteins between normal and diseased samples. However, the application of membrane protein complexes to these electrophoreses still has some problems originating from the nature of the membrane protein complexes, as will be discussed later.

This review will focus on two points based on the problems described above. One main section will describe the methods for isolating membrane protein complexes. Since the category of membrane protein complexes is too wide to cover all of them here, this review will discuss the reports which described or oriented the analysis of the crystal structure of membrane proteins and the related reports. The other main section will describe the methods for resolving each protein for analytical use, such as SDS–PAGE and 2D electrophoresis. These methods will be discussed in view of plausible application to medical (clinical) biochemistry as well as my experiences in overcoming the difficulties of hydrophobic membrane proteins.

2. Preparative separation of membrane protein complexes

The crystallized membrane protein complexes and the related membrane protein complexes are listed in Table 1 with key information on the purification methods. Among these methods, notable topics will be picked up and discussed in detail.

2.1. Solubilization by detergents

Before discussing individual separation methods, it is useful to glance over the usage of detergents. In many cases, the first important step in purifying membrane protein complexes from any membrane system is to solubilize them from their environment surrounded by lipids (Fig. 1). The success of the purification relies greatly on the choice of detergents and their concentrations, especially when one wants to purify the membrane protein complexes in their intact (native)

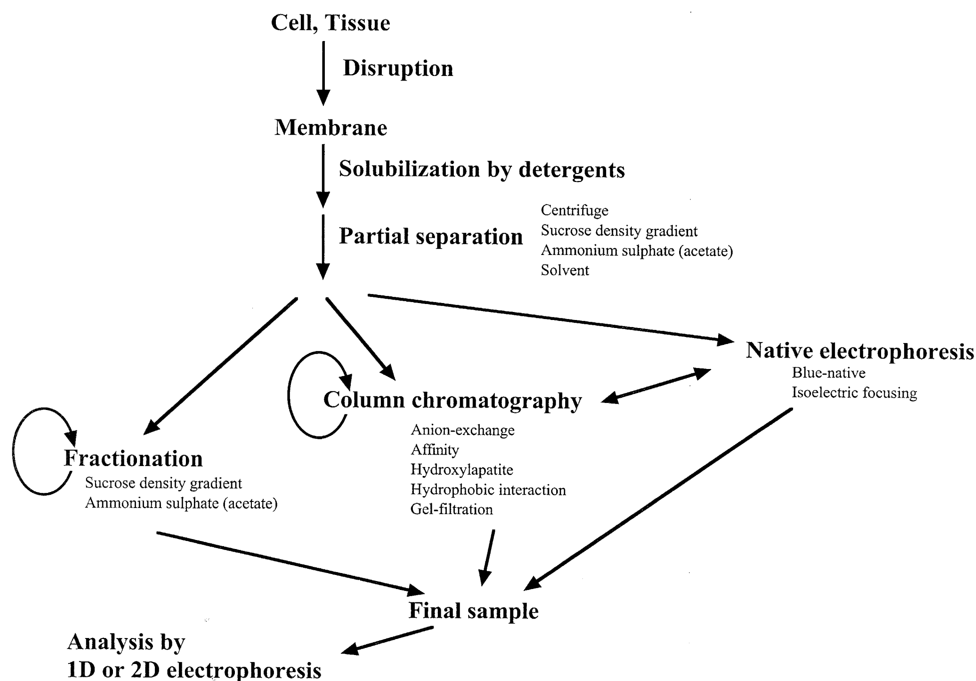

Fig. 1. General isolation scheme for membrane protein complexes.

form. Although there have been many successes in membrane protein purification, it is still a very difficult problem to identify the proper detergents. Recently, we have been able to use many new detergents. Nonetheless, and accordingly, we have to understand the nature of such detergents. For recent advances on the matter of detergents for membrane proteins, see the review by Garavito and Ferguson-Miller [19].

Triton X-100 was successfully applied to solubilize the photosystem II (PS II) complex from chloroplasts in higher plants [20]. *n*-Heptyl-β-D-thioglucoside (HTG) was effective in removing the light-harvesting chlorophyll *a*/*b* protein complex (LHCII), which associates with the PS II complex in a large amount, from this PS II preparation [21,22]. Recently, the structure and function of the PS II complex have been frequently analyzed using cyanobacteria. Triton X-100 is not so effective for isolating the cyanobacterial PS II complex (personal experience). In early studies on the cyanobacterial PS II complex, *n*-octyl-β-D-glucoside (OG) was used to solubilize the PS II complex from the thermophilic cyanobacterium, *Thermosynechococcus elongatus* [23]. OG, HTG [24] and sucrosemonolaurate [25] at proper concentrations (and the ratio to the proper amount of proteins) preferentially solubilized PS II complex rather than PS I complex from thylakoid membranes. This selective solubilization was seen at around 20 °C but not at low temperature [23,24]. Okada et al. found similar selective solubilization [26]. They solubilized rhodopsin selectively from bovine rod outer segment (ROS) membranes using alkyl(thio)glucoside (HTG or *n*-nonyl-β-D-glucoside) in the presence of a high concentration (80 m*M*) of a divalent cation. These complicated phenomena may not only depend on the detergent nature but also partly on micro-environmental differences in the lipids around the complex, as seen in the bacteriorhodopsin–lipid complex [27] or the asymmetrical distribution of lipids in biomembranes such as the outer- and inner-leaflet of the thylakoid membrane system [28,29]. Accordingly, it is better to consider the combination of detergents not only with the membrane type (from bacteria, mammals and so on) but also with the target membrane protein complex.

The concentration of detergents is another point which should be considered. Recently, *n*-dodecyl-β-D-maltoside (DDM) has been favorably used for the solubilization of PS II from cyanobacterial thylakoid membranes. The most common concentration used is 1–2%, as shown in Table 1. The selectivity of this detergent against the PS II complex seems to be less when compared to the detergents described above. Higher concentrations of DDM can solubilize more proteins. However, in the case of the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803, higher concentrations seem to cause some unexpected effects on the PS II complex. Fig. 2 shows the effect of the DDM concentration on the fluorescence emission spectra at 77 K from thylakoid membranes. Various concentrations of DDM were added to thylakoid membranes and the fluorescence emission spectra at 77 K were monitored. The fluorescence at around 720 nm is from the PS I complex while the emissions around 685 and 695 nm are from the PS II complex. It is remarkable that, when DDM exceeded 1%, the emission from PS II markedly increased leaving the emission from PS I at the same level. A similar effect was observed for PS II in a thermophilic cyanobacterium, *T. elongatus* (Kashino and Aminaka, unpublished data). The precise mechanisms of this phenomenon are unknown, but some kind of loosening of the conformation in the PS II complex, which results in alteration in the energy transfer between chlorophylls, could happen at higher concentrations of DDM even though DDM is a mild detergent. It has been reported that some detergents such as Triton X-100 and SDS affect the apparent differential absorption coefficient of P700 (reaction center chlorophyll) in PS I depending on their concentration [30–32]. Okun et al. examined the effect of varying the concentration of several detergents on the specific activity and inhibitor sensitivity of complex I from bovine heart mitochondria [33]. These detergents included Triton X-100, Brij-35, Thesit, Chaps, K-Cholate and DDM. It was shown that all three polyoxyethylene-ether detergents (Triton X-100, Brij-35 and Thesit) could act as specific inhibitors of complex I even at low concentrations. In addition, they also suggested the possibility of nonspecific delipidation or disintegration of the protein complex by higher concentrations of the detergents. The inhibitory effect of detergent was also reported in the cytochrome $b/c1$ complex (complex III) purified from potato tuber mitochondria [34]. The activity of the isolated complex depended on the concentration of DDM and was

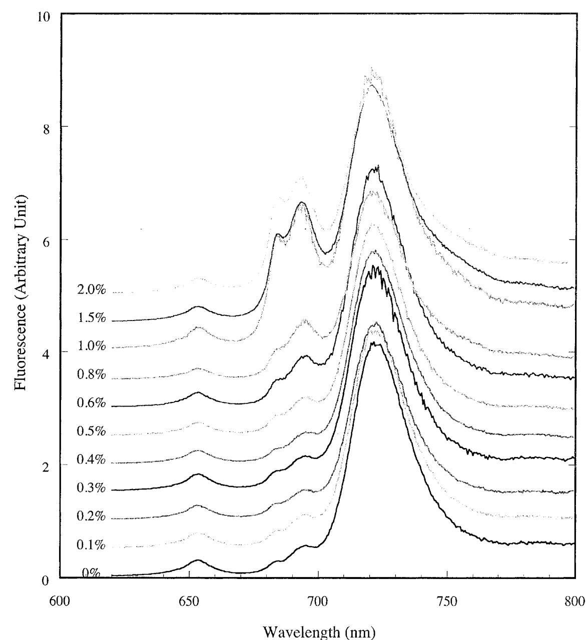

Fig. 2. Effects of DDM on the yield of fluorescence from PS I and PS II at 77 K. Various concentrations of DDM were added to a thylakoid membrane suspension (1 mg Chl/ml) and incubated for 20 min, after which the fluorescence was recorded at 77 K. Chlorophyll was excited at 420 nm (Kashino, unpublished data).

inhibited when DDM was over 0.01%. In summary, to purify a membrane protein complex in its native form, the conditions (concentration of detergents, temperature, coexisting salts, etc.) of the solubilization are important as well as the choice of detergent. The detergents (and their concentrations if available) which were used for the purification of the crystallized proteins are included in Table 1.

Detergents are also necessary to keep the complexes intact after solubilization and purification, and during column chromatography and preparative electrophoresis, but at lower concentrations. There are also problems here. The complex III of mitochondria is thought to be present in a dimeric form in the intact membrane system [35,36]. However, it is difficult to maintain the dimeric form after solubilization. Musatov et al. [35] tested the effect of several detergents on the solubilization and maintenance of the dimeric form. Triton X-100 was most effective for solubilizing the complex, but it destabilized the dimeric form. Under their experimental conditions, they summarized the effectiveness of the detergents for solubilizing the complex III as Triton X-100 > DDM ≃ *n*-undecyl-β-D-maltoside > *n*-decyl-β-D-maltoside ≃ octaethyleneglycolmonododecyl ether > Tween 20 ≃ cholate ≃ deoxy-cholate ≃ CHAPS ≃ CHAPSO. Unexpectedly, most of these detergents destabilized the dimerization of the complex. Among those tested, only low concentrations of DDM around neutral pH maintained the dimeric form of the complex without leading to aggregation [35]. In general, to maintain the solubilized form, detergent at twofold the critical micelle concentration (CMC) is sufficient. However, considering the results of Musatov et al. [35], we need to keep in mind the effect of detergents on the maintenance of the multimeric form of membrane protein complexes.

Several crystallized examples have been reported where trimers or dimers are thought to be stabilized by lipid mediation [27,37]. The crystallized PS I complex also has four lipids per unit whose functional importance is speculated [38]. The crystallized photosynthetic reaction center from photosynthetic bacteria also contained lipids [39,40]. From the point of view of the multimeric form of protein complexes, good detergents may leave some specific lipids in the complex to keep the complexes intact.

Table 2
Columns found in Table 1

Columns
Anion-exchange columns
DEAE-Biogel, DEAE Biogel A [Bio-Rad]
DEAE Sephacel [Amersham Bioscience]
DEAE-Sepharose CL-6B [Amersham Bioscience]
DEAE-Toyopearl 650S [Tosoh]
DEAE-Toyopearl [Tosoh]
DE52 [Whatman]
Mono-Q [Amersham Bioscience]
Mono-Q HR [Amersham Bioscience]
POROS Q/F [PerSeptive]
Q-Sepharose [Amersham Bioscience]
Q-Sepharose HP [Amersham Bioscience]
Q-Sepharose FF [Amersham Bioscience]
RESOURCE Q [Amersham Bioscience]
Uno-Q [Bio-Rad]
Fractogel EMD TMAE-650 (S) [Merck]
Affinity columns
Amylose resin [New England Biolabs]
Chelating Sepharose with Ni^{2+} [Amersham Bioscience]
Ni–NTA agarose (Ni-affinity) [Qiagen]
Fast-flow Ni–NTA (Ni-affinity) [Qiagen]
ProBond (Ni-affinity) [Invitrogen]
Reactive Red 120 affinity column [Sigma]
Streptavidin CH Sepharose 4B column [Amersham Bioscience]
TALON (Co-affinity) [Clontech]
Hydrophobic interaction chromatography (HIC) columns
POROS ET [PE Biosystems]
Phenyl-Pepharose CL-4B [Amersham Bioscience]
Chromatofocusing column
PBE 94 [Amersham Bioscience]
Hydroxyapatite column
Bio-Gel HTP [Bio-Rad]
Gel permeation columns
BioGel A-0.5 m [Bio-Rad]
Fractogel TSK HW-55 (S) [Merck]
Sephacryl S-300 HR [Amersham Bioscience]
Sephadex G100 [Amersham Bioscience]
Superdex 200 [Amersham Bioscience]
Sepharose-CL-6B [Amersham Bioscience]
TSK 3000 SW [LKB]
TSK G 4000 SW [Tosoh]

2.2. Chromatographic separation

2.2.1. Purification of membrane protein complexes through ion-exchange columns

It is remarkable that all the ion-exchange columns in Table 1 are anion-exchange columns (Table 2). The process for enriching membrane protein complexes prior to the column chromatographic work is quite important in the purification of such complexes. Many successful methods paid great attention to the process preceding the column chromatography steps.

Complex I of mitochondria is one of the largest multi-subunit membrane protein complexes in the biological system, and it comprises 43 subunit proteins forming a 944×10^3 rel. mol. mass in bovine heart mitochondria [33]. Complex I is the entry point of electrons into the mitochondrial respiratory chain [41]. Due to its importance for biological activity, including humans, extensive efforts were made to purify this complex. Other than complex I, there are four major membrane protein complexes in the mitochondrial membrane system: succinate dehydrogenase (complex II), complex III, cytochrome *c* oxidase (complex IV) and F1F0 ATPase (complex V) [42]. The first task in purifying complex I is to separate it from the other complexes. For this aim, ammonium sulfate or ammonium acetate fractionation is frequently used [43]. The method described by Finel et al. [44] was developed from such methods. They solubilized the membrane fraction first

using 1% DDM at 12 mg protein/ml in the presence of 20 m*M* Tris–HCl (pH 8.0). The solubilized fraction which was obtained after centrifugation was further subjected to 1.6% sodium cholate and ammonium sulfate at 40% saturation (final concentration). Complexes I and V were recovered in the fraction sedimented by ammonium sulfate at 52% saturation while most of the other respiratory complexes (complexes II–IV) remained in the supernatant. The precipitated fraction containing complexes I and V was suspended in a solution containing both 1.5% cholate and 1% DDM.

After further purification by a 2nd ammonium sulfate fractionation, complexes I and V were solubilized again by 1% DDM and applied to a Mono Q HR 10/10 anion-exchange column (Amersham Bioscience, Buckinghamshire, UK) in the presence of 0.1% DDM. Complex I was eluted at 250–320 m*M* NaCl in a highly purified form. The polypeptide profile of this purified complex I showed high purity (but with partial loss of a 42×10^3 rel. molecular mass subunit). However, the purified complex I lost some of its activity to transfer electrons from NADH to ubiquinone-1, and became insensitive to the inhibitor rotenone.

To overcome these problems, they modified the method [45]. Complexes I and V were obtained in the 40–60% saturated ammonium sulfate fraction in the above method. This pellet was suspended in a solution containing 100 m*M* sodium cholate and 1.0% DDM followed by desalting and delipidation using a HiLoad 26/60 Sephacryl S-300 HR gel filtration column (Amersham Bioscience). Subsequently, complexes I and V were subjected to a HiLoad 26/10 Q-Sepharose HP anion-exchange column (Amersham Bioscience). Complex I, which was eluted by 300 m*M* NaCl, was further purified using a Mono-Q HR 10/10 column and a HiLoad 26/60 Sephacryl S-300 HR gel permeation column. The purified complex I preparation showed a monodisperse feature and rotenone-sensitive activities. Furthermore, phospholipid was not detected in the purified preparation. The delipidation was quite important because phospholipid could contribute to the enzyme heterogeneity.

Okun et al. extended a different approach [33]. As described in the previous section, they first tested the effect of detergents on the activity. They found that Triton X-100 was the most suitable to selectively solubilize complex I, and that the inhibitory effect of Triton X-100 on the activity was reversible. The mitochondrial membranes (35 mg protein/ml) were solubilized by 1.75% Triton X-100 in the presence of 600 m*M* NaCl at pH 7.4. Centrifugation yielded a pellet containing complexes I and III, while the matrix proteins, cytochrome *c*, and complexes II and V remained in the supernatant. The pellet was homogenized into a buffered solution to 40 mg protein/ml, and solubilized by 2.0% Triton X-100 in the presence of 600 m*M* NaCl. Subsequent centrifugation yielded a supernatant which contained most of the complexes I and III. With an additional 1% Triton X-100, the supernatant was applied to hydroxyapatite for the aim of desalting and further delipidation (the column was washed with one volume of a solution containing 0.1% Triton X-100). The fraction containing complexes I and III, obtained by elution with 200 m*M* K-phosphate (pH 7.4) and 0.5% Triton X-100, was subjected to DEAE Biogel A (Bio-Rad, Hercules, CA, USA) and the detergent was replaced with 0.1% DDM (at pH 7.0) which had not shown inhibitory effects on the complex I activity. Complex I was eluted by 200 m*M* NaCl in the presence of 0.1% DDM at pH 7.4 and further purified by a TSK G 4000 SW gel-filtration column (Toso-Haas, Stuttgart, Germany).

The use of a hydroxyapatite column enabled them to avoid ammonium sulfate fractionation because the hydroxyapatite step was efficient for delipidation and reduction of the salt concentration. The exchange of the detergents at the step of the DEAE Biogel A column, which kept the complex I activity and the inhibitor sensitivity, was also important, as discussed in the previous section. The resulting purified complex I showed the typical polypeptide profile on SDS–PAGE without contamination. Although the procedure included an extensive delipidation step, the preparation retained phospholipid but showed the monodisperse feature, ~500 nmol/mg protein after Biogel A column, and ~100 nmol/mg protein after TSK gel column. The retained phospholipid might be effective in keeping the complex I intact.

Here is a simple thought on the purification methods for mitochondrial protein complexes: are there any peripheral proteins which are closely associated but easily removed by a high salt concentration? Many methods include steps with a high salt concentration (ionic strength). In the case of PS II complex of higher plants and cyanobacteria, the peripheral proteins, which are important in catalytic reactions, are easily removed by a high salt concentration, such as 1 *M* NaCl or 1 *M* $CaCl_2$ [46–48]. Accordingly, special attention is paid to not losing such peripheral proteins in preparing the PS II complex, which will be described later. When a high salt concentration is used during the preparation for the isolation of the PS II complex, the peripheral proteins are then restored by reducing the salt concentration through dialysis [21,22]. The preferential conditions for retaining such lumenal proteins in intact PS II complexes contain low salt (10 m*M* NaCl, 5 m*M* $MgCl_2$ and 5 m*M* $CaCl_2$) with 25% (or 10%) glycerol (or comparable concentrations of sucrose) at weak acidic pH.

The PS II reaction center complex in plants, algae and cyanobacteria is another example of a very complicated membrane protein complex. It is made of 16 or more transmembrane proteins, the number of which is more than that of the cytochrome $b/c1$ complex (complex III) of bovine mitochondria (13 subunit proteins), three or four peripheral water-soluble proteins, and other cofactors (e.g. around 40 chlorophyll *a* molecules, two plastoquinones, two heme, 4 Mn, etc), whose molecular mass ranges over 500×10^3 rel. molecular mass units in a dimeric form [48–50]. The PS II complex has a unique function. It oxidizes water to produce molecular oxygen, which is necessary for the respiration of most organisms, using light energy [49]. The PS II complex is embedded in the thylakoid membrane, which is a major

membrane system of the cyanobacteria, and the chloroplasts of algae and higher plants. Other than the PS II complex, PS I complex, cytochrome $b6/f$ complex, and ATPase are also present in the thylakoid membrane system, which are also multi-subunit membrane protein complexes [51]. In the cyanobacterial thylakoid membrane system, the most abundant membrane protein complex is the PS I complex. The ratio of the PS II reaction center to the PS I reaction center is around 0.2. Usually, to purify the PS II complex, the first effort is applied for enriching the PS II complexes in the thylakoid membrane sample. The second point is to separate phycobilisomes, which are tightly associated with the PS II reaction center complexes and play a role in collecting light energy, mainly for the PS II reaction centers [52]. The phycobilisomes are water-soluble and most of them can be removed by salt-washes such as 1 *M* $CaCl_2$. The amount of them is considerably large, and they are recognized as a major component of the thylakoid membrane fraction even if the PS II subunit proteins are not easily recognized as distinct bands on the SDS–PAGE profile (lane 1 in Fig. 4, although the major part of the phycobiliproteins were lost in advance in this sample). However, the procedures to remove phycobiliproteins also easily remove peripheral proteins which are located on the opposite side (lumenal side) of the thylakoid membrane and play important roles in evolving molecular oxygen. So, the second effort is applied to remove phycobilisomes without the loss of these peripheral proteins (such as the 33×10^3 rel. molecular mass manganese-stabilizing protein (PsbO), 12×10^3 rel. molecular mass protein (PsbU) and cytochrome *c*550 (PsbV)).

Many purification methods for the PS II complex consist of the following two processes: enrichment of the PS II fraction and subsequent column chromatography. Enrichment is usually performed by solubilization of the membrane fraction with detergents and subsequent centrifugation. The frequently performed chromatographic methods are ion-exchange chromatography, hydrophobic interaction chromatography or affinity chromatography after genetical transformation (Table 1). The presence of detergents during the purification process is critically important as described above.

The first crystal of the PS II complex was reported by Zouni et al., and was purified from the thermophilic cyanobacterium, *T. elongatus* [49] (optimum growth temperature is around 55 °C). They separated the PS II fraction by using a sucrose density gradient after extraction of the PS II complex from thylakoid membranes. The first sucrose density gradient centrifugation contained 10–40% sucrose in a buffer (20 m*M* MES-NaOH (pH 6.5), 10 m*M* $MgCl_2$, 20 m*M* $CaCl_2$) and a detergent, sulfobetain 12 (SB 12), which was kept below its CMC (0.1%). The addition of SB 12 to the sucrose gradient decreased the amounts of phycobilisome in the PS II fraction, which may be caused by solubilization of phycobiliproteins. In addition, the intended lower concentration of SB 12 allowed the PS II complex to aggregate during the centrifugation to make a sharper band. Then, this fraction enriched in PS II complexes was applied to a second sucrose gradient centrifugation, which contained DDM at 3–5-fold the CMC. This second sucrose gradient centrifugation yielded a PS II complex fraction totally free from phycobiliproteins. PS II complexes were further purified by anion-exchange chromatography using an FPLC Mono-Q HR 5/5 column, which was equilibrated with MES buffer (pH 6.5) in the presence of 0.03% DDM. The bound PS II complexes were eluted by a gradient of $MgCl_2$ (5–200 m*M*). This chromatography separated active PS II dimers and inactive PS II monomers [53]. Improvement of the purification has been achieved by using a Toyopearl 650 column [54]. The resulting PS II complexes were precipitated twice in the form of small crystals by polyethyleneglycol (PEG) for further purification. This purification method successfully produced PS II complex crystals suitable for X-ray analysis [49]. According to their structural model of PS II, it is remarkable that the complex, purified as above, retained lumenal peripheral proteins but no phycobiliproteins.

Kuhl et al. also obtained crystals from the same organisms [55] with a different purification method. Thylakoid membranes were first washed with 0.05% DDM, which was higher than the CMC (around 0.009%) but does not solubilize membrane proteins in this condition. This might release the excess amount of phycobiliproteins and other weakly associated proteins. After resuspension in medium (20 m*M* HEPES (pH 7.5), 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 0.2 *M* $(NH_4)_2SO_4$), membrane proteins were solubilized at 1 mg Chl/ml, which is approximately equal to 8 mg protein/ml [56], with 1.2% DDM and 0.5% sodium cholate at room temperature for 30 min. Following centrifugation, the supernatant was diluted by a solution containing 3 *M* ammonium sulfate to give a final concentration of 1.65 *M* ammonium sulfate. PS II complexes were trapped in a hydrophobic interaction chromatography (HIC) column (POROS ET, PE Biosystems, Foster City, CA, USA) and eluted by a gradient of 1.65–0 *M* ammonium sulfate. After dialysis, the PS II fraction was applied to an anion-exchange column (UNO Q-6, Bio-Rad) and the dimeric and monomeric forms of the PS II complex were separately eluted with a gradient of $MgCl_2$. During this process, the concentration of DDM was kept at 0.03%.

Kamiya and Shen crystallized the PS II complex from a similar thermophilic cyanobacterium, *T. vulcanus* [50,57]. They obtained crude PS II by using lauryldimethylamine *N*-oxide (LDAO) prior to the purification step. The crude PS II particles (1 mg Chl/ml) were solubilized with 1.2% DDM [57] in the presence of 25% glycerol and 20 m*M* NaCl at pH 6.0 (40 m*M* MES) and 0 °C for 5 min. The PS II complex was applied to a Mono-Q column (an anion-exchanger) in the presence of 0.05% DDM at pH 6.0 and 18 °C. The co-existing phycobiliproteins and other proteins were removed by 200 m*M* NaCl. The dimeric form of the PS II complex was eluted by 330 m*M* NaCl [50,57,58].

Tang and Diner [59] purified the PS II complex from the mesophilic cyanobacterium *Synechocystis* 6803 (optimum growth temperature is around 30 °C). They solubilized the membrane proteins with 1% DDM at 0 °C from thylakoid membranes (1 mg Chl/ml), which were suspended in MES buffer (pH 6.5) containing lower concentrations of salts and 25% glycerol. After centrifugation, the supernatant was loaded onto a DEAE-Toyopearl 650S column (a weak anion-exchanger; Toso Haas). Following a subsequent wash in the presence of 0.03% DDM, the PS II complex was eluted by a linear gradient of $MgSO_4$ (from 20 to 30 m*M*). The purified intact PS II complex was completely free of phycobiliproteins.

These examples show that DDM is effective for solubilizing PS II complexes at 0 °C irrespective of the difference in growth temperature (i.e. the composition of lipids in thylakoid membranes). Furthermore, anion-exchange columns are effective for purifying the PS II complex. Since the PS II complex is stable and has high activity between pH 6 and 7, the purification process is performed in this pH range. The lower p*I* of many of the subunit proteins might create a lower p*I* for the total complex, which makes anion-exchange columns effective in the weak acidic pH range.

The use of glycerol (or sucrose) in the PS II purification procedure is one of the notable points. Although it is not so common for other membrane protein complexes, glycerol (or sucrose) can work to retain peripheral proteins. Furthermore, it may have the effect of reducing the concentration of water, which will help to keep the hydrophobic proteins intact.

2.2.2. *Affinity tagging*

An alternative approach for purifying the PS II complex was developed by Sugiura et al. [60]. They introduced a hexa-histidine tag (His-tag) into one of the subunit proteins. Purification of overexpressed protein with a genetically introduced His-tag [61] is frequently employed in many laboratories. The key point of the idea was to introduce the His-tag into one of the subunit components in the original organism and allow the physiological expression level, rather than overexpression. Accordingly, the PS II components, as many as 20 subunits and cofactors, assemble into the normal complex. Sugiura et al. showed that this idea is effective for the PS II complex of the green alga *Chlamydomonas reinhardtii* [60]. The His-tag was introduced at the carboxy terminus of the D2 protein (PsbD), because Goldsmith and Boxer [62] successfully introduced the His-tag to the carboxy terminus of the M subunit in the photosynthetic reaction center complex in *Rhodobacter sphaeroides* (a smaller complex composed of four subunit proteins), which is the counterpart of the PsbD protein. The mutant cells grew somewhat more slowly than the wild type, but showed almost the same character.

Thylakoid membranes of the mutant cells were solubilized using 2% DDM in the presence of 10% glycerol at pH 7.5 and 0 °C. After centrifugation, the supernatant was applied to a Ni^{2+} affinity column (ProBond, Invitrogen, San Diego, CA, USA). After a subsequent wash at pH 6.0, the PS II complexes were eluted by 200 m*M* imidazole. During this procedure, 0.03% DDM and 10% glycerol were present in the medium. The obtained fraction contained PS II subunit components other than the PsbD protein, including peripheral proteins, and showed high oxygen evolving activity. This means that the His-tagging method is very useful for easy purification of the PS II complex. This method is very rapid and simple, which allowed the supermolecular complex to remain intact. Although the yield of PS II was around 3% on the basis of chlorophyll, they estimated that 75% of the initial amount of the PS II complex was recovered. They also tried introducing a His-tag into the amino terminus of the D1 protein (PsbA), another reaction center protein of PS II. However, the cells did not grow autotrophically. There are also unexpected factors which affect the success, although we can design the position of the His-tag so that the spatial topology and function of the complex are disturbed as little as possible.

The same investigators extended this technique to the thermophilic cyanobacterium *T. elongatus* [63]. The His-tag was introduced to the carboxy terminus of the CP43 protein (PsbC), whose carboxy terminus was expected to locate at the stromal surface. Since several functional peripheral proteins and functional manganese clusters are present in the lumenal side of PS II, the stromal side of PS II may be better for inserting the His-tag. They added a thrombin recognition site preceding the His-tag to reserve the availability of His-tag removal after purification. From this mutant, they could constantly purify highly stable and active PS II through a similar process to the case of *C. reinhardtii*. They used ProBond (Invitrogen) for the Ni^{2+} affinity column chromatography and 200 m*M* imidazole as the eluting agent. Analytical gel filtration using Superdex 200 (Amersham Bioscience) showed that the major part of the purified PS II was in the dimeric form.

Bricker et al. applied the same technique to the mesophilic cyanobacterium *Synechocystis* 6803 to introduce a His-tag at the carboxy terminus of the CP47 protein (PsbB), and purified the PS II complex free from phycobiliproteins [64] (see also Fig. 4, lanes 1 and 2). Kashino et al. refined the isolation method and demonstrated that the PS II complex really is intact. The purified PS II complex enabled detection of whole subunit components, including novel proteins, which had not been previously recognized as PS II components [48]. It showed high oxygen evolving activity and maintained this high activity for over 2 weeks at 4 °C in the dark [65].

Several Ni^{2+} affinity resins were tested (personal experience). Ni^{2+} affinity resin supported by Superflow such as TALON Superflow (Clontech, Palo Alto, CA, USA) and Ni–NTA (nitrilotriacetic acid) Superflow (Qiagen, Valencia, CA, USA), which are suitable for application to large scale preparation using an FPLC system, showed severely low yield (far less than 1% based on the initial chlorophyll content). They could not effectively retain the PS II com-

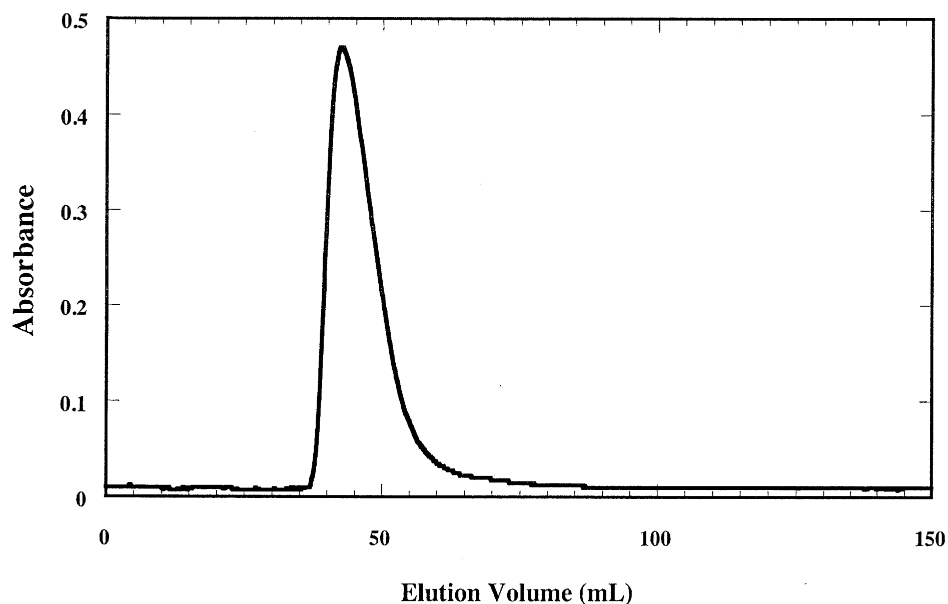

Fig. 3. Elution pattern during gel permeation chromatography of His-tagged PS II complex purified through Ni^{2+}–NTA agarose. The PS II complex was purified as described in Ref. [48]. Gel permeation was performed using a HiPrep 16/60 Sephacryl S-300 HR column at a flow-rate of 1 ml/min (FPLC, Pharmacia Biotech) in the presence of 0.04% DDM. Elution was monitored at 280 nm (Aminaka, Sugahara and Kashino, unpublished data).

plexes. This is quite different from the description in the manufacturer's handbook, which says that the Ni-affinity Superflow resin has a capacity of 5–10 mg protein/ml. This discrepancy may come from the spatial conflict between supermolecules of the PS II complex and the Superflow resin. The agarose-based resins have good performance for such supermolecules. The yield of PS II complex from *Synechocystis* 6803 was 4 to 8% based on chlorophyll when Ni–NTA agarose (Qiagen), which is composed of Ni–NTA coupled to Sepharose CL-6B, was used. Considering that the ratio of chlorophyll molecules belonging to PS II in cyanobacterial thylakoid membranes is around 10%, the recovery is satisfactory. Sugiura et al. obtained a higher yield of PS II complexes using ProBond rather than Ni–NTA agarose (Dr. Sugiura, personal communication). Other metals, such as Co^{2+}, can be used instead of Ni^{2+} (e.g. TALON, Clontech)

Supercomplexes with a His-tag can be highly purified with high activity in a one-step procedure using a Ni^{2+} affinity column (Fig. 4, lane 2). Note that the proteins contained in the PS II complex (Fig. 4, lane 2) are hard to recognize in the initial solubilized thylakoid membrane fraction (Fig. 4, lane 1). However, a further process may be effective, e.g. gel filtration. Fig. 3 shows the elution pattern of the His-tagged PS II complex from *Synechocystis* 6803 by gel filtration using Sephacryl S-400 HR (Amersham Bioscience) in the presence of 0.04% DDM and 25% glycerol (Fig. 3). The elution pattern showed a single peak at around 450×10^3 rel. mol. mass units, which demonstrated the purity and dimeric form of the isolated PS II complex. Some of the minor components which were recognized in the PS II complex after just the affinity purification (Fig. 4, lane 2) disappeared through this gel filtration (Fig. 4, lane 3). Such a further purification step is frequently found in other publications (Table 1).

Most of the His-tagged proteins were eluted with either histidine or imidazole in the reviewed papers. One exception is NhaA, which was eluted by lowering the pH [66,67]. To elute the PS II complex from metal-chelating resins, high EDTA concentrations or high pH are unsuitable because the peripheral proteins, which have important functions in oxygen evolution, will be dissociated under such conditions. Sugiura et al. used 200 m*M* imidazole to elute the PS II complex from the metal affinity column [60,63]. The optimum concentration was checked by a gradient of imidazole (Dr. Sugiura, personal communication). His-tagged FhuA, a siderophore receptor in the outer membrane of *E. coli*, was also found to elute at an imidazole concentration of around 200 m*M* [68]. In contrast, Bricker et al. used 50 m*M* histidine [64], since they thought that the larger size (M_w 209.6 vs. 68.08 for imidazole) and the zwitterionic charge of histidine would be less likely to interact with the PS II reaction centers [64].

Although imidazole can retain the high activity of the PS II complex [60,63], it causes problems if the protein complexes have cytochromes [69]. Guergova-Kuras et al. obtained highly active His-tagged cytochrome $b/c1$ complex from *Rhodobacter sphaeroides* by using 200 m*M* histidine as the eluting agent, but its activity was severely low when 200 m*M* imidazole was used instead of histidine. They speculated that a kind of ligand substitution took place in cytochrome $c1$ in the cytochrome $b/c1$ complex when a high concentration of imidazole was used in the purification step. Accordingly, histidine may be better as an eluting agent if the protein complex contains cytochromes. Rumbley et al. indicated

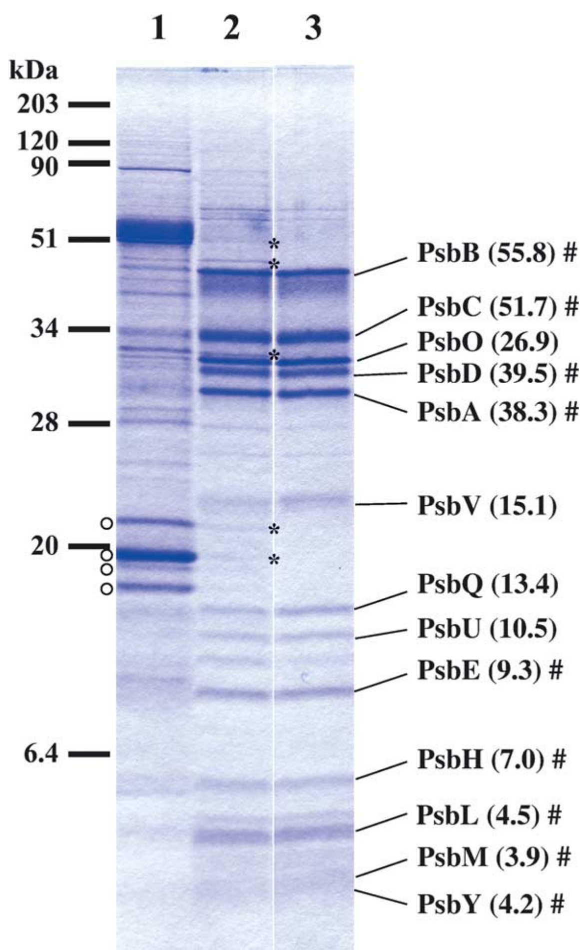

Fig. 4. Polypeptide patterns of the purified PS II complex before and after the gel permeation step shown in Fig. 3. Electrophoresis was performed according to Ref. [91], with a gel containing an 18–24% linear acrylamide gradient and 6 *M* urea. Lane 1, solubilized thylakoid membranes; lane 2, PS II complex before gel permeation; lane 3, PS II complex after gel permeation (sample in the peak in Fig. 3). Each sample contained 5 μg Chl (∼40 μg protein). The samples were not heated and delipidated for denaturation. The notable bands which disappeared after the gel permeation are indicated by asterisks. The dots on the left of lane 1 represent phycobiliproteins. The hydrophobic membrane proteins are indicated by "#". The numbers in parentheses are the predicted molecular masses of the mature proteins. The molecular mass standards were from Bio-Rad (prestained SDS–PAGE standards, broad range) (Aminaka, Sugahara and Kashino, unpublished data).

that removal of the imidazole used for elution is essential because a high concentration of imidazole would denature the enzyme over time in the cytochrome *bo*3 complex [70].

Vrettos et al. extended this His-tagging technique to purify peripheral proteins closely associated with the PS II complex [71]. The purification of cytochrome *c*550 (PsbV) is usually disturbed by phycobiliproteins whose molecular mass is comparable to cytochrome *c*550 and which are also closely associated with the PS II complex as described above. They obtained highly purified cytochrome *c*550 from previously purified PS II complexes whose PsbB proteins were His-tagged.

The His-tag can be introduced without interference with the function in the membrane protein complexes. The same purification method is used for many other membrane protein complexes including multi-subunit complexes (Table 1). For successful purification, the position of the His-tag may have to be tested so as not to interfere with the function or the expression level. Locher et al. [72] purified the BtuCD transporter (an ABC transporter mediating vitamin B_{12} uptake) from *E. coli* by introducing a His-tag into the subunit. Prior to this success, they subcloned 28 distinct ABC transporters originating from different biological sources, and systematically tested the location of the His-tag (amino terminus or carboxy terminus) in all combinations for each subunit to examine the expression level from a single plasmid. Rumbley et al. individually introduced a His-tag at the carboxy terminals of subunits I, II and III of the cytochrome *bo*3 complex, and successfully isolated complexes which contained either His-tagged subunit I or II [70]. Most of the examples described above used a His-tag at the carboxy terminus or amino terminus. However, an exception was found. Ferguson et al. introduced a His-tag in the middle of FhuA, the siderophore receptor in the outer membrane of *E. coli*. The protein consists of 714 amino acids, and has a surface-exposed loop. They inserted the His-tag genetically into the *fhuA* gene after amino acid 405 which is located in the loop. The insertion of this His-tag did not interfere with the FhuA function, and enabled them to isolate the intact FhuA protein.

In summary, His-tagging is applicable to membrane protein complexes for purification in the native (intact) form whether they are major or minor fractions in the membrane system. This method seems to be very useful, especially if the target membrane protein complex is a minor component in that membrane system. The following points must be considered to achieve success. (1) The subunit and the position of the His-tag to be introduced. The carboxy terminus may be better to avoid the problem of N-terminal processing. However, to keep the function intact, the position might need to be tested. (2) The physiological expression level. The multi-subunit complex might not be assembled if only one of the subunit proteins is overexpressed. If it is possible to introduce the genetically engineered gene of the subunit protein into cultured cells and keep the expression at the physiological level, the method will be successful for purifying the membrane protein complex. (3) The selection of the metal affinity resin. Some kinds of resins may not be suitable for supercomplex purification, such as resins for FPLC in the case of the PS II complex. (4) The eluting agent. If the complex contains cytochromes, imidazole may not be suitable. If the sample contains a metal ion as the active center, EDTA and EGTA are not suitable because such metal cofactors will be removed. If there are some components that are labile at higher or lower pH, then a pH shift is not suitable as the elution method. (5) The detergents. Suitable detergents at suitable concentrations (maybe around twofold the CMC) should be added throughout the affinity column process.

2.2.3. Affinity columns

Affinity separation of complex I from *Neurospora crassa* using an antiserum has been reported [73]. The solubilized membrane fraction was subjected to an antiserum raised against complex I, and the resulting antibody-associated complex I was separated using Protein A-Sepharose CL-4B (Amersham Bioscience). However, it is hard to elute the protein complex in a native form from such a column. Kleymann et al. resolved this problem [74]. They cloned the cDNAs encoding the variable domains of hybridoma-derived antibodies raised against complex III or complex IV. The Fv fragments were genetically engineered to include a *Strep tag* at the carboxy terminus of the VH chain. These engineered Fv fragments act as bifunctional agents, since they would bind to the antigen proteins to form combined complexes and the resulting combined complexes will be immobilized on a streptavidin CH Sepharose 4B column via the *Strep tag*. The immobilized complex can be eluted in a pure and highly active form with bound Fv fragments by using the mild competitor, diaminobiotin. The corresponding Fv fragments were overexpressed in *E. coli* and applied to the purification of complexes III and IV from *Paracoccus denitrificans*. Crystallization studies using this purification method have been reported [75–77]. In contrast to the His-tagging technique, this method does not include any alteration of subunit proteins in the target complex.

Complex III (cytochrome $b/c1$ complex in mitochondria) was also successfully purified by either 15-step ammonium acetate fractionation, or anion-exchange column chromatography (DEAE Sepharose CL-6B) (Table 1).

2.2.4. Chromatofocusing

Chromatofocusing was used to purify FepA (a ferric enterobactin receptor) from *E. coli* [78,79]. This chromatofocusing was employed exclusively to remove phospholipids after purification through an anion-exchange column.

2.3. Electrophoretic separation

The PS I reaction center complex was purified from the thermophilic cyanobacterium *Synechococcus* sp. PCC 7002 using preparative IEF [80]. At the first step, the trimeric PS I complex was separated by a sucrose density gradient after solubilization by 1.4% DDM at 1.2 mg Chl/ml (13.2 mg protein/ml). The PS I preparation was subjected to the preparative electrofocusing in the presence of 0.05% DDM. The purified trimeric PS I complex was obtained at the pH 4.6–4.8 position, and eluted by diffusion into the desired buffer solution. Subsequent anion-exchange chromatography and gel permeation chromatography removed the remaining ampholytes. The authors estimated the p*I* of the trimeric PS I complex by analytical IEF in advance, so that a narrow pH range (pH 4–6, Servalyte 4-6) could be used at the purification step. This enabled the separation of very closely positioned individual complexes. Crystals were obtained using this purified preparation. It was only after the IEF separation that the PS I complex formed crystals. Due to this, the authors speculated that this IEF step removed a minor form of the PS I complex with a slightly different p*I* [81].

By applying this preparative IEF system, Lancaster et al. purified and crystallized fumarate reductase (QFR) from *Wolinella succinogenes* [82]. The membranes, solubilized by 0.05% Triton X-100, were loaded onto a DEAE CL-6B column and the fraction containing QFR was obtained. After the detergent was replaced with 0.05% DDM/0.20% DM using a second DEAE CL-6B column, the enzyme was separated by using preparative IEF (but using 0.01% DDM/0.1% DM in Refs. [83,84]). The preparative IEF was conducted according to the methods described by Tsiotis et al. as above [80], changing the detergents and the pH as suitable for the QFR enzyme.

These successes in purifying large supercomplexes (around 260×10^3 for the dimeric form of QFR [82], and around 700×10^3 rel. mol. mass for the trimeric form of PS I [80]) may rely partly on the use of a dextran matrix, Ultrodex (Pharmacia). In this purification, the stabilizing matrix for electrofocusing was essential. Since the PS I complex in the trimeric form has a large molecular mass, the larger pore size realized by this matrix may be one of the important factors.

3. Analytical separation of membrane protein complexes

For clinical analysis, separation and determination of each membrane protein is important. Furthermore, it is also necessary to determine the subunit components after the isolation of membrane protein complexes. For this aim, electrophoresis is frequently used.

3.1. 1D electrophoresis

In the reviewed reports on membrane protein crystals, the most frequently used electrophoresis is that based on Laemmli's system [14]. To obtain optimal resolution by SDS–PAGE, one has to choose a suitable acrylamide concentration, a guide for which is given in Ref. [85]. However, in some cases, Laemmli's system is not suitable. Some membrane protein complexes, such as complex III [86,87], PS I [38] and II [24,48,49,88] complexes and cytochrome $b6/f$ complex [89,90], contain low molecular mass proteins smaller than 10×10^3 rel. mol. mass units. These low molecular mass proteins are not clearly resolved by conventional SDS–PAGE even if the acrylamide concentration is raised [91]. To resolve such low molecular mass proteins, the Tris/Tricine system is the most suitable [85,92] (Fig. 5, panel IV). However, it takes a long time to run (Fig. 5, panel IV and Fig. 6, panel V). Furthermore, if the sample has proteins of a wide molecular mass range to over 100×10^3 rel. mol. mass, the Tris/Tricine system has a disadvantage be-

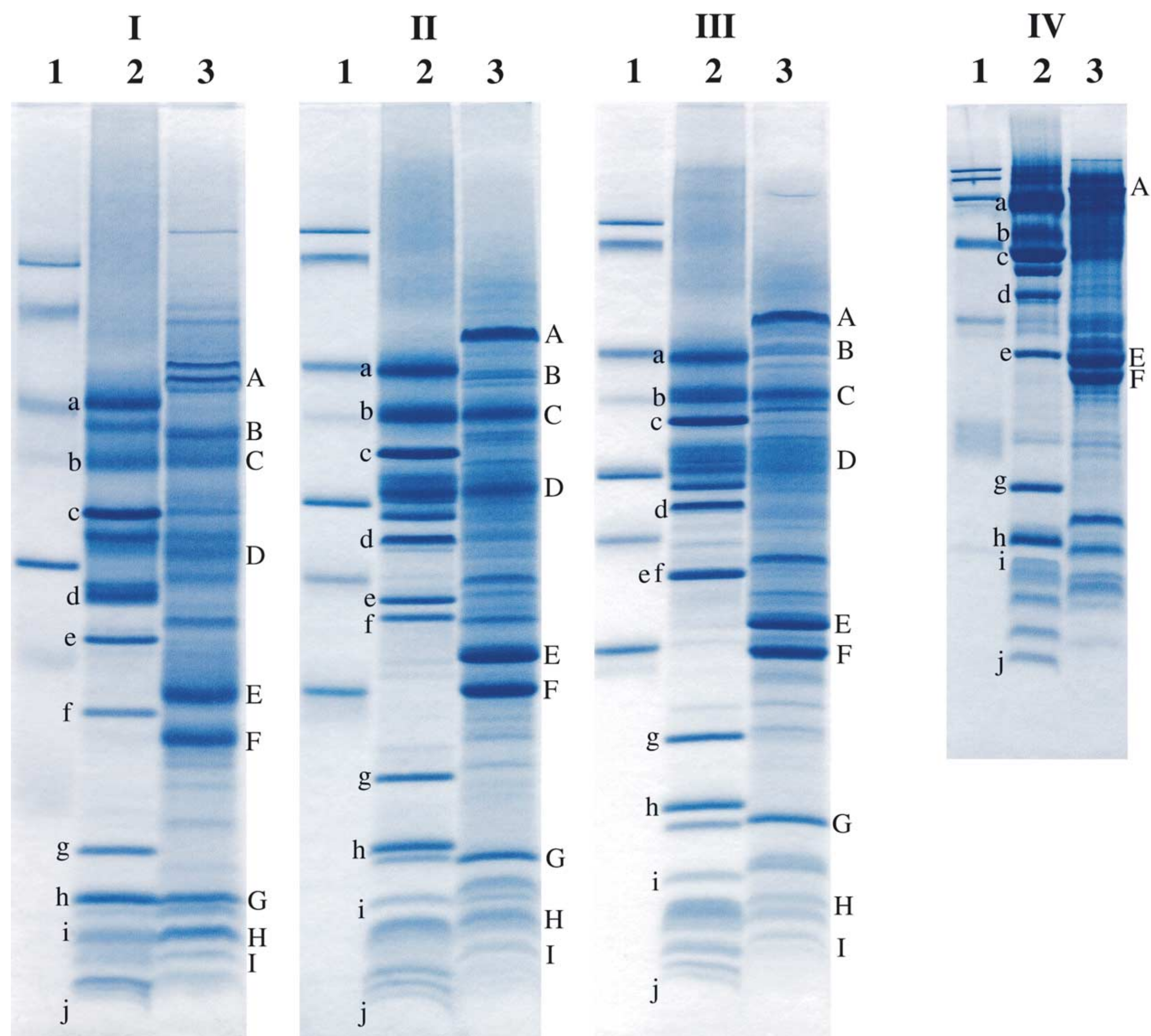

Fig. 5. Effects of urea on the resolution of membrane proteins. The gels contained 2 *M* (panel I), 6 *M* (panel II) and 7.5 *M* (panel III) urea. The acrylamide concentration was an 18–24% linear gradient in all gels and the buffer system was the same as in Fig. 4 for panels I to III. Panel IV was performed in a Tris/Tricine buffer system according to the method described by Ref. [92] (16.5% T and 6% C acrylamide) using the same samples and the same gel size as other panels (but shrunk after the de-staining step). In each panel: lane 1, molecular mass standards (14.4, 20.1, 30, 43, 67 and 94×10^3 rel. mol. mass units); lane 2, PS II complex purified from spinach according to Ref. [22]; lane 3, PS II-enriched fraction prepared from the thermophilic cyanobacterium *T. vulcanus* according to Ref. [24]. Electrophoresis was performed at 15 mA for 13 h in the Tris/MES system, and at 90 V for 24 h to separate the proteins in the Tris/Tricine system. For ease of recognition, some key protein bands are marked (a, b, c,... and A, B, C,...) for the corresponding bands in each panel: a, PsbB (56.2×10^3); b, PsbC (51.8×10^3); c, PsbO (26.7×10^3); d, PsbP (20.2×10^3); e, PsbQ (16.5×10^3); g, PsbR (10.2×10^3); h, PsbE (9.3×10^3); j, PsbK (4.3×10^3) (Kashino, unpublished data; values in parentheses refer to relative molecular mass units).

cause the separation of protein bands over 20×10^3 rel. mol. mass is apparently decreased (Fig. 5, panel IV). To overcome this problem, Ikeuchi and Inoue [88] and Kashino et al. [91] presented other choices, which provided convenient resolution from the lower to higher molecular mass region at lower cost (comparable to Laemmli's system). The two systems (Tris–HCl system in Ikeuchi's and Tris/MES system in Kashino's) give preferable resolution from the lower (around 3×10^3 rel. mol. mass) to higher (over 100×10^3 rel. mol. mass) molecular mass region, although better resolution can be obtained for the proteins smaller than 10×10^3 rel. mol. mass in Shägger's Tris/Tricine system. Fig. 5 shows the resolution pattern of PS II complexes isolated from spinach and a thermophilic cyanobacterium, *T. vulcanus*, in the Tris/MES system in comparison with the resolution pattern of Shägger's Tris/Tricine system. The gel containing 6 *M* urea of the Tris/MES system can resolve many protein bands over a wide molecular mass range (Fig. 5, panel II). Kashino et al. separated and determined the subunit protein components of over 30 proteins in the PS II complexes from a cyanobacterium, *Synechocystis* 6803, using the Tris/MES system with 6 *M* urea [48]. From this analysis, they found novel proteins which were not previously known as photosystem II components from the wide molecular mass region.

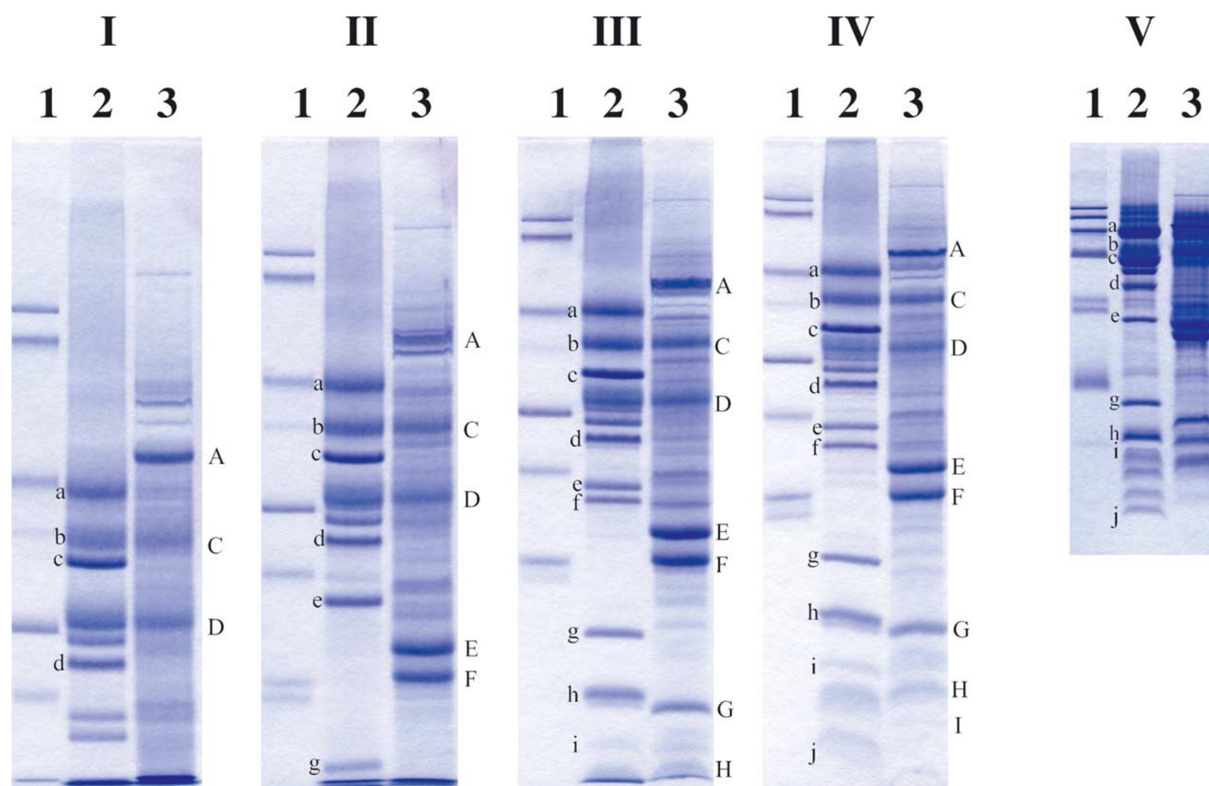

Fig. 6. Differences in the separation patterns according to the acrylamide concentration using the Tris/MES buffer system [91] with a mini-gel. The gel contained 12% (panel I), 15% (panel II), 18% (panel III) and 20% (panel IV) acrylamide as well as 6 *M* urea. Panel V was performed in the Tris/Tricine buffer system according to the method described by Shägger [92] (16.5% T and 6% C acrylamide) using the same size mini-gel. The samples are the same as in Fig. 5. Electrophoresis was performed at 25 mA for 2 h in the Tris/MES system, and at 105 V for 6 h to separate proteins in the Tris/Tricine system. For ease of recognition, some key protein bands are marked (a, b, c, . . . and A, B, C, . . .) for the corresponding bands in each panel (Kashino, unpublished data).

The benefit of the use of urea should be emphasized for the separation of membrane proteins by SDS–PAGE. Although urea is now widely used for immobilized pH gradient (IPG) isoelectric focusing in combination with thiourea (see below), it is not so often used in 1D SDS–PAGE and the 2nd dimensional SDS–PAGE for 2D electrophoresis. Generally, the presence of urea makes the bands sharper, while many hydrophobic proteins will not form clear bands on conventional SDS–PAGE without urea. Higher concentrations of urea are generally more effective at making the bands clearer, and many more protein bands can be easily recognized in the presence of 6 *M* urea than in the presence of 2 *M* urea (compare lanes 3 of panels I and II in Fig. 5). Furthermore, different concentrations of urea in the gel result in differences in the migration patterns of proteins even when the acrylamide concentration is the same (Fig. 5). Some proteins happen to migrate the same distance at some urea concentrations and to be separated at other urea concentrations. Typical examples are bands e and f in Fig. 5. The migration distances of these are quite different in the presence of 2 *M* urea. The difference between them became smaller at 6 *M* urea, and they became the same at 7 *M* urea. This indicates that one should try several gel conditions including different urea and acrylamide concentrations and gel buffer systems to obtain the optimal gel system for the membrane protein complexes being investigated.

Although the Tris/MES system is useful for separating proteins over a wide molecular mass region, the original system is not very convenient for many laboratories. The original system was performed in a full-size slab-gel containing an acrylamide concentration gradient. The Tris/MES system can be operated in a mini-gel of uniform acrylamide concentration. When a 20% acrylamide gel containing 6 *M* urea is used (Fig. 6, panel IV), it gives preferable resolution from the smaller to larger molecular mass region with a resolution comparable to the original slab-gel containing the acrylamide concentration gradient (Fig. 5, panel II). The mini-gel also allows a reduction in the amount of sample required for analysis.

Accordingly, it is better to consider several gel systems to obtain a precise protein profile. For Laemmli's system and Shägger's Tris/Tricine system, practical guidelines for the choice of gel system are presented in Ref. [85].

Ikeuchi and Inoue [88] and Kashino et al. [91] included a delipidation process to improve the resolution in the low molecular mass region. Usually, isolated membrane protein complexes contain high levels of lipids, detergents and, in the case of photosystems, chlorophylls, which run faster than the

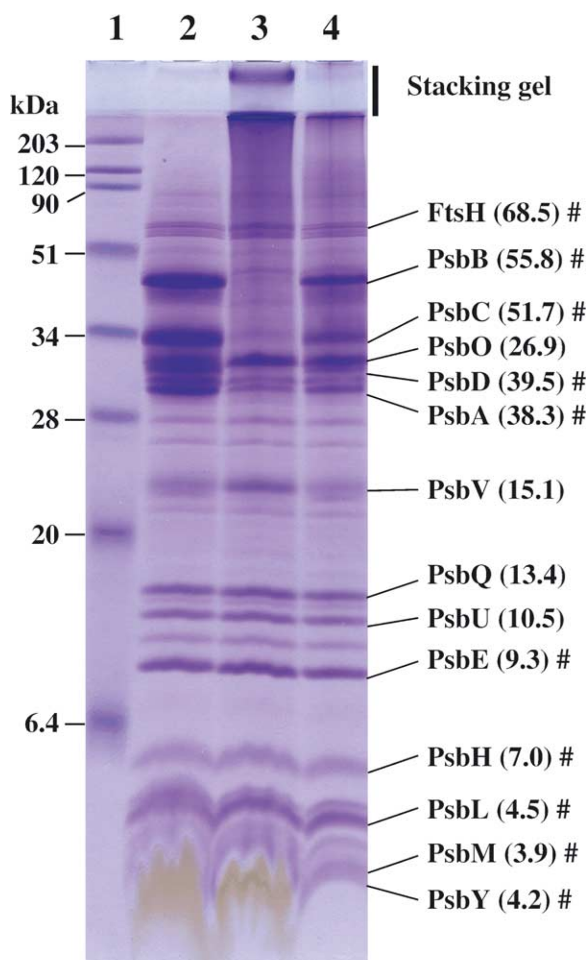

Fig. 7. Effects of the denaturing method on the separation patterns of membrane proteins. Lane 1, molecular mass standards (6.4 (aprotinin), 20 (lysozyme), 28 (soybean trypsin inhibitor), 34 (carbonic anhydrase), 51 (ovalbumin), 90 (bovine serum albumin), 120 (β-galactosidase) and 203 (myosin)$\times 10^3$ rel. mol. mass, Bio-Rad); lane 2, unheated and non-delipidated; lane 3, heated; lane 4, delipidated. The same amounts of the samples (purified His-tagged PS II, 7 μg Chl equivalent) were loaded in each lane. The denaturing conditions were as follows. For the unheated and non-delipidated sample, the same volume of denaturing solution was added to the sample and incubated for 20 min on ice. For the heated sample, the same volume of denaturing solution was added to the sample, and the sample was incubated in boiling water for 5 min. For the delipidated sample, 100 μl of methanol and 1 ml of diethyl ether were sequentially added to the sample (below 30 μl) followed by centrifugation, and the resulting pellet was dissolved in the denaturing solution. The denaturing solution contained 5.2% LDS, 172 m*M* Tris–HCl (pH 8.0), 40 m*M* dithiothreitol, 0.5 *M* sucrose (for high density to aid loading), 0.01% pyronine Y (for visualization of the migration front during electrophoresis). The hydrophobic membrane proteins are indicated by "#". The numbers in parentheses are the predicted molecular masses of the mature proteins (Kashino, unpublished data).

migration front and disturb the resolution of the low molecular mass region (Fig. 7, lane 2). Note that the electrophoretic front region (smaller than 6×10^3 rel. mol. mass units) in lane 2 is severely disturbed by many chlorophylls and lipids (yellowish color). This difficulty arises from the fact that membrane protein complexes have close interaction with lipids and hence, many lipids remain associated with such complexes even after the complexes are isolated. The removal of lipids by using a solvent, diethyl ether, improves the resolution of the low molecular mass region (Fig. 7, lane 4). However, at the expense of this improvement by delipidation, some of the highly hydrophobic proteins of somewhat higher molecular mass become insoluble or form large aggregates (Fig. 7, lane 4). In lane 4 of Fig. 7, the bands of highly hydrophobic proteins such as PsbA, PsbB, PsbC and PsbD became smaller, while other hydrophilic proteins such as PsbO and PsbU, and low molecular mass hydrophobic proteins (e.g. PsbE (large subunit of cytochrome *b*559) and PsbH (PS II H protein)) were not altered by the delipidation. Carboni et al. found that delipidation by acetone–methanol (8:1, v/v) or acetone–methanol–tributyl phosphate (12:1:1, v/v/v) reduced the spot intensity on a 2D map from rat brain tissue without improved resolution of the proteins, and concluded that delipidation is unnecessary for brain samples [93].

The separation is also decreased by boiling the sample (Fig. 7, lane 3) which is frequently performed with the aim of full denaturation [94], and the effective inactivation of proteinases [94,95]. This heat treatment causes heavy aggregation of hydrophobic membrane proteins, which can be recognized by the appearance of a smear in the stacking gel and the boundary of the stacking and resolving gels, which results in the loss of actual protein bands (Fig. 7, lane 3). This effect is especially prominent for hydrophobic membrane proteins of relatively higher molecular mass. The bands of the hydrophobic PsbA, PsbB, PsbC and PsbD were decreased. However, the same amounts of proteins were retained in the bands for hydrophilic proteins and low molecular mass hydrophobic proteins, such as those described above. We can avoid this problem by omitting the heating step. To prevent proteinase activity, it is recommended to keep the sample cold (on ice) even after the addition of denaturing buffer solution. This low temperature will cause precipitation of the SDS which is present at higher concentration in the denaturing solution. It has been reported that incubation in SDS solution at a temperature below 20 °C is ineffective for denaturing proteins [85]. Lithium dodecyl sulfate (LDS) as a replacement for SDS, and loading the samples as quickly as possible, are recommended. LDS at a high concentration (as high as 5.2% (w/v)) will not precipitate, even at 0 °C. A denaturing solution comprising 5.2% LDS, 172 m*M* Tris–HCl (pH 8.0), 40 m*M* dithiothreitol (DTT), and 0.5 *M* sucrose (for high density to aid loading) works very well [91] (lane 2 in Fig. 7). Sonication using a bath-type sonicator will help the denaturation if necessary.

Although it is hard to recognize significant changes in the mobility of individual proteins in Fig. 7 irrespective of the presence or absence of a heating step (if resolved in the gel), we can find examples where the mobility was changed by heating, e.g. FepA (79.9×10^3 rel. mol. mass) showed an apparent molecular mass of 81×10^3 after heating and 62×10^3 without heating with a denaturing solution containing 4% SDS and 10% β-mercaptoethanol [79].

Castellanos-Serra and Paz-Lago [95] demonstrated the effectiveness of thiourea for preventing proteolysis during sample preparation for 2D electrophoresis. The addition of a proper concentration of thiourea may help prevent proteinase attack when proteolysis is a problem. It is also true for membrane proteins that trials of several methods of sample preparation are generally desirable, as pointed out by Weber et al. [94].

As is easily recognized in Figs. 4 and 7, the molecular masses of membrane proteins are far from agreeing with the molecular mass standards. This discrepancy might come from the values for pre-stained molecular mass marker proteins, which were calibrated with Laemmli's system by the vendor (Bio-Rad). However, in general, hydrophobic membrane proteins migrate faster than hydrophilic proteins when proteins of the same molecular mass are compared. This feature results in confusion when a larger hydrophobic protein happens to migrate faster than smaller hydrophilic proteins. Typical examples are the PsbD and PsbO proteins, where the PsbD protein (hydrophobic, 39.5×10^3 rel. mol. mass) migrated faster than the PsbO protein (hydrophilic, 26.9×10^3 rel. mol. mass) (Figs. 4 and 7). This suggests that hydrophobic membrane proteins may retain some conformation even in the presence of SDS and/or urea. This kind of discrepancy also occurs between hydrophobic proteins. The apparent molecular mass of the PsbE protein (9.4×10^3 rel. mol. mass) from *T. vulcanus* was much smaller (around 2×10^3 rel. mol. mass) than the homologous PsbE protein (9.3×10^3 rel. mol. mass) in *Synechocystis* 6803 although the predicted molecular masses are almost the same [24]. In this case, it was suggested that the difference of the mobility came from the relatively large difference in the intrinsic net electric charges between these proteins.

3.2. 2D electrophoresis with isoelectric focusing

2D electrophoresis is a powerful tool for visualizing protein components of a sample. In fact, it is widely used for proteome analysis. It is expected that this tool will contribute to the analysis of the precise protein components of purified membrane protein complexes. However, it is known that there is difficulty in obtaining a high quality of resolution for hydrophobic membrane proteins [18,96]. Wilkins et al. estimated that around 30% of the total hydrophobic proteins (proteins with positive grand average hydropathy (GRAVY) scores [97], which can be calculated at http://us.expasy.org/tools/protparam.html or http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) did not make spots on 2D electrophoresis of *Escherichia coli*. In particular, hydrophobic proteins whose GRAVY scores are larger than 0.1, 0.15, and 0.3 in *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *E. coli*, respectively, have never been detected by 2D electrophoresis. In contrast, membrane proteins which have high GRAVY scores are resolved by SDS–PAGE. Examples are PsbA and PsbD (see Fig. 4), whose GRAVY scores are 0.41 and 0.36, respectively. Accordingly, the limit of separation of hydrophobic proteins in 2D electrophoresis comes from the limit of the IEF used in the first dimension to separate the hydrophobic membrane proteins. Some researchers prefer conventional fractionation by sucrose density gradient centrifugation or column chromatography rather than IEF because of the separation limit of membrane proteins by IEF. Some of these researchers separate all the components from each fraction using SDS–PAGE, and then determine the total components of visible protein bands in every fraction using modern mass spectrometry (e.g. Ref. [98]), namely, an alternative 2D analysis.

On the other hand, many efforts have been made to improve the analytical quality of membrane proteins using 2D electrophoresis. These include the use of thiourea as a chaotrope [99], amidosulphobetain 14 (ASB 14) as a surfactant [100], and tributyl phosphine (TBP) instead of DTT as a reducing agent [101] in the sample buffer. Although both chaotropes and surfactants are necessary to resolve membrane proteins in IEF, there is incompatibility between some reagents [102]. Rabilloud et al. carefully tested the solubilizing efficiency on microsomal and nuclear proteins with several combinations of chaotropes and surfactants. They recommended a solubilization mixture containing 2 *M* thiourea, 5 *M* urea, 2% CHAPS and 2% sulfobetain 3-10 (SB 3-10) for proteins which require an efficient detergent, and a mixture containing 2 *M* thiourea, 7 *M* urea, and 4% CHAPS for proteins which require a high concentration of chaotropes (e.g. tubulin). This formula may be the first choice to be considered for the membrane protein samples.

Since zwitterionic amphiphilic compounds such as CHAPS or SB 3-10 improved the solubility of membrane proteins, Rabilloud and his group accordingly synthesized new such compounds (amidosulphobetain 14 (ASB 14) and 16 (ASB 16)) [100]. They found that the combination of amidosulphobetain with thiourea plus urea was efficient for increasing the solubility of membrane proteins. These new surfactants are now commercially available (Calbiochem, Darmstadt, Germany). Carboni et al. also compared the extraction efficiency between CHAPS, ASB 14, and Nonidet P-40 (NP-40) using rat brain tissue [93], and found good resolution and a large number of spots when CHAPS or ASB 14 were used. They also recognized less streaking on the 2D map when CHAPS was used rather than ASB 14. Alkylation is also effective for reducing streaking. Herbert et al. [103] emphasized that the reduction of –SH groups should be followed by alkylation prior to electrophoresis. If a sample does not undergo alkylation after reduction, then artefactual spots due to scrambled disulfide bridges among polypeptides may appear. Acrylamide is a good reagent for the alkylation [103].

Barent and Elthon [104] reported that the addition of the protein sample to the IEF acrylamide solution prior to polymerization made it possible to load large amounts of protein without spoiling the resolution. This technique was applied to 2D electrophoresis with IPG [105]. Pasquali then reported that this technique was also useful for preparative

2D electrophoresis of membrane proteins [106]. The addition of the membrane protein sample into the swelling buffer for IPG gel strips makes it easy to increase the amount of (membrane) proteins, which enables the detection of minor components. When the sample is applied from the edge of the IEF gel or IPG strip, many membrane proteins will not enter the gel because they associate easily with each other. Therefore, application of the membrane protein sample to the gel solution prior to polymerization or to the swelling solution will improve the separation of proteins.

Although these new techniques refine the quality of 2D electrophoresis, they are still not sufficient to separate very hydrophobic proteins. Molloy et al. indicated the possibility that hydrophobic proteins are simply not extracted from the sample prior to 2D electrophoresis [107]. Then, they introduced an extraction method using organic solvents. They used a chloroform–methanol (1:1, v/v) solution to extract the hydrophobic proteins from the membrane fraction and detected five proteins by 2D electrophoresis with positive GRAVY scores in *E. coli*, which had not previously been detected in 2D electrophoresis. Seigneurin-Berny et al. [108] and Ferro et al. [109] applied the same extraction method to extract the highly hydrophobic proteins from the plant chloroplast envelope and thylakoid membranes. They demonstrated effective recovery of membrane proteins by this method with classical 1D SDS–PAGE. This is one of the alternative strategies for proteome analysis without using 2D electrophoresis. However, we have to keep in mind that, as described in the previous section, organic solvents do not provide good results for the resolution of membrane proteins (Fig. 7 and Ref. [93]), even if the solvent is effective in enriching hydrophobic proteins. We need to consider the balance of the advantages and disadvantages of the solvent extraction method.

While it is true that there are some problems with the extraction methods, the main problem seems to be elsewhere. From my experience using purified and “solubilized” PS II complexes, the hydrophobic membrane proteins such as PsbA, PsbB, PsbC and PsbD, whose GRAVY scores are 0.41, 0.12, 0.27 and 0.36, respectively, did not make any spots on 2D electrophoresis using an IPG gel and incorporating the refined methods described above. This may be partly because of the membrane protein characteristics which tend to associate together to form larger multimeric protein “complexes”. For example, even in the presence of SDS, hydrophobic membrane proteins such as PsbA and PsbB are also found as hetero- or homo-multimers in a higher molecular mass region than that of their major populations [48]. Furthermore, membrane protein complexes behave as complexes under mild conditions, which can be seen in preparative IEF [80,82] as described above and BN-PAGE [110] as described later. Oh-Ishi et al. [111] reported an alternative, refined IEF system using agarose gels. They successfully improved the separation of high molecular mass proteins larger than 150×10^3 rel. mol. mass, up to 500×10^3 rel. mol. mass, in the 1st dimensional IEF. The system can also be loaded with as much as 1.5 mg of proteins, which is suitable for preparative 2D electrophoresis. The future development of this technique may contribute to the analytical methods for membrane protein complexes. Henderson et al. [112] also used agarose gels to separate large supercomplexes in BN electrophoresis, which enabled the separation of complexes greater than 1×10^6 rel. mol. mass units. They resolved the pyruvate dehydrogenase complex of $\sim7\times10^6$ rel. mol. mass units (a mitochondrial matrix enzyme) [112,113]. Agarose gels seem to be suitable for resolving large molecular mass complexes.

Taking these things into account, we (Harayama and Kashino) are now currently developing an IEF electrophoresis system using agarose gels especially for the separation of membrane protein complexes. In this system, hydrophobic proteins whose GRAVY values are between 0.11 and 0.41 made distinct spots, which implies that the system would provide better resolution for very hydrophobic membrane proteins. After further refinement, this system will be reported soon.

3.3. 2D electrophoresis with blue native electrophoresis

BN-PAGE was originally developed for the analysis of mitochondrial respiratory complexes [16,110,114]. This excellent gel system can resolve membrane protein complexes according to their molecular mass in the native form while retaining their individual subunit proteins, which enables estimation of the molecular mass of the complexes. The system can separate complexes with higher resolution than gel filtration or sucrose density gradients. By applying the 1st dimensional BN-PAGE gel to the 2nd dimensional SDS–PAGE, the individual subunit proteins of the complex are disclosed. The multimerization of the complexes can be obtained from the resulting two pieces of information: the molecular mass of the separated complex (1st dimension) and the total subunit proteins of the complex (2nd dimension). Since the BN-PAGE system was originally designed to separate membrane protein complexes, it seems to be free from the problems found in 2D electrophoresis with IEF (or IPG).

The recent improved method and increasing application to other complexes are described in Refs. [110,113,114]. This method has been applied to the analysis of Parkinson’s disease [6], Alzheimer’s disease [115] and the diagnosis of oxidative phosphorylation defects [116]. Jung et al. evaluated the usefulness for assessing the functional changes of mitochondria in neurodegenerative disorders [117].

BN-PAGE can also be applied to preparative use. However, from written reports, it takes several hours to elute the complexes from the gel after BN-PAGE. If this inconvenience can be resolved, this method will become more useful for preparative purposes. Considering preparative IEF [80,82,111], the introduction of agarose to this system as a supporting matrix [112] might help to resolve this problem. Furthermore, the recent and future advances of the synchrotron radiation technique will reduce the amount of pro-

tein sample required for the analysis of crystallized complexes. Then, BN electrophoresis may be able to provide homogeneous membrane protein complexes for the crystallographic approach.

To study the interaction between proteins, such as the formation of complexes, some kind of analytical methods should be developed similar to the yeast two hybridization method [118], since the yeast two hybridization method is not suitable for hydrophobic proteins. 2D electrophoresis in combination with BN electrophoresis is one of the useful candidates for this demand applicable to any organisms as well as His-tagging and TAP (tandem affinity purification [119] which was not described in this review).

3.4. Detection and determination

Coomassie Blue R250 (CBB) is the most popular dye used for staining gels after electrophoresis. The detection limit by CBB is from 8 to 16 ng depending on the proteins, and the linear dynamic range of this staining is 125–1000 ng [120]. Silver staining [121] is also widely used especially when the amounts of proteins are quite small. The sensitivity is high with detection limits of around 0.5 [122] to 8 ng [120] depending on the proteins [123]. However, even though the sensitivity of silver staining is very high, the dynamic ranges are not as wide, e.g. 8–60 ng linear relationship for the alkaline/silver diamine stain and 4–60 ng for the acidic/silver nitrate stain [120]. This becomes a problem when one wants to quantify proteins in a sample containing proteins whose amounts are widely different between proteins.

Recently, alternative staining systems have become available to avoid the intrinsic problems of silver staining. The fluorescence-based SYPRO Ruby (Molecular Probes, Eugene, OR, USA) [120,122–126] is one of these methods, whose sensitivity is comparable to that of silver staining, and whose detection limit ranges from 0.5 to 5 ng [122]. The dynamic range is much larger than that for silver staining, a 1–1000 ng linear relationship, which enables reliable quantification [120,122]. Although the sensitivity depends on the proteins, as in the case of CBB and silver staining [123], SYPRO Ruby binds to the basic amino acids in proteins non-covalently [122] and it is applicable to membrane proteins without any problems. This method is quick and simple. When the available sample is very limited, sensitive staining methods with a high dynamic range seem quite valuable. Malone et al. [124] modified the staining procedure to raise the contrast without effects on its sensitivity. The only disadvantage of SYPRO Ruby seems to be that it is very costly compared with silver staining, including the detection system.

Negative staining methods (zinc [127,128] and copper [129] staining) are also available. The characteristics (detection limits and linear dynamic ranges) of these staining methods are summarized in Ref. [120] for gels and in Ref. [130] for electroblotting.

When the protein sample contains *c*-type cytochromes, heme-staining is inevitably performed. Vargas et al. applied the enhanced chemiluminescence (ECL) technique to detect the *c*-type cytochrome [131]. This method is very useful because the sensitivity is very high, it is convenient, and after the detection, it can be smoothly used for Western blotting. Following the usual transfer onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes after SDS–PAGE, the *c*-type cytochrome bands are visualized using ECL (e.g. with FemtoPico and WestPico supplied by Pierce (Rockford, IL, USA), [132]) in conjunction with a chemiluminescence detection imaging analyzer or X-ray film. This method is based on the peroxidase activity of the cytochrome heme. After transfer onto the membrane, the band can be visualized within minutes, which is quite short compared to the conventional heme-staining procedure with 3,3′-diaminobenzidine (DAB) [133] or 3,3′,5,5′-tetramethylbenzidine (TMBZ) [134]. Vargas et al. [131] reported that 0.2 μg of bovine heart cytochrome *c* can easily be detected by ECL with high linearity and without any background. The sensitivity is comparable to the method with TMBZ. In my recent experience, the use of WestFemto (Pierce) gives much more sensitivity than WestPico (Pierce).

This method is recommended because it does not use hazardous chemicals like TMBZ. However, one should keep in mind that the relative amounts of signals detected can only be compared between the same *c*-type cytochromes in different samples because the intrinsic peroxidase activity can vary significantly between cytochrome species [131]. Furthermore, the calibration standard for the cytochrome should be from the same source for precise quantification (this problem is also true for the conventional detection methods using DAB and TMBZ). Actually, cytochrome *c*550 from a mesophilic cyanobacterium, *Synechocystis* 6803, showed quite different stainability from that of a thermophilic cyanobacterium, *T. elongatus* (Kashino and Pakrasi, unpublished data). Delipidation using diethyl ether may sometimes induce the deletion of heme (Kashino and Pakrasi, unpublished data). 2D electrophoresis is also not suitable for detecting *c*-type cytochromes because the iron may be removed by the high voltage during the first dimensional IEF (Kashino, unpublished data).

Considering the cost of ECL reagents, plastic wrap is sometimes used to incubate the PVDF or nitrocellulose membrane in the ECL reagents. However, this is not good for the quality of the image because the reagent solution will not be evenly dispersed, which results in a smear. In this case, a transparency (OHP) sheet is useful for incubating the PVDF or nitrocellulose membrane with the reaction mixture, which markedly reduces the cost, and is ready to be loaded into the detector. After removal of the reaction solution, the membrane is ready for the subsequent procedures of Western blotting.

Wu et al. [135] reported a modified washing procedure to improve Western blotting detection. A biotinylation-based procedure to detect proteins at the 40 pg level has been

reported by Schamel [136]. Application of this detection method to BN-PAGE has also been reported [137].

Mass-spectrometry is widely used to determine proteins. Matrix-assisted laser desorption ionization mass spectroscopy (MALDI) is one of the most frequently used methods. Peptide mapping using MALDI is quite powerful, especially if the genome information is available. However, if the molecular mass of the protein is small (less than about 10×10^3 rel. mol. mass), the probability of the presence of cleavage sites for trypsin, which is commonly used for this analysis, is decreased. Then, it becomes hard to determine the identity even if the genome information is available. In this case, N-terminal amino acid sequencing is effective [48]. Kashino et al. determined another 11 proteins of less than about 10×10^3 rel. mol. mass which were not determined by MALDI methods [48]. N-terminal micro-sequencing is also used for the determination of protein spots on 2D-gels (e.g. Ref. [138]).

MALDI is a useful tool for identifying proteins, as described above. However, the disadvantage is the time duration required to determine the identity. When the samples are composed of numerous proteins, this becomes a problem. Recently, many attempts have been aimed at developing high-throughput analysis. A new method developed by Smith and co-workers is one of these methods [139–141]. This method can be applied to membrane protein complexes.

When proteins are scheduled to be analyzed by mass-spectrometry, one may have to pay attention to the protein staining after electrophoresis. Conventional silver-staining is not suitable for the mass-spectroscopic analysis. Scheler et al. [142] and Gharahdaghi et al. [143] modulated the method to overcome this problem. They refined the destaining and washing processes before the mass-spectrometric analysis to improve the detectability of the proteins. This method does not affect the sensitivity of the staining because the refined point is in the destaining process prior to the mass-spectrometric analysis. Later, Yan et al. [144] reported a modified silver staining protocol using a commercial kit (Silver Stain PlusOne, Amersham Bioscience), and demonstrated a high compatibility with the subsequent mass spectrometry. Staining using SYPRO Ruby is compatible with mass-spectrometric analysis [145] and N-terminal sequencing [130].

4. Concluding remarks

The methods which have been discussed here are very limited. However, in some papers, there are several impressive words for the motivation to improve the purification methods, which indicated avoidance of the time consumption for purifying membrane protein complexes, and of the possibility of damaging the purified proteins (e.g. Refs. [70,74]). Based on such ideas, they manipulated the methods to be as simple as possible and obtained highly active and highly purified complexes with simpler methodology. This is reasonable because the complexes no longer had to experience environments which were extreme for them. Considering the successful examples focused on in this review, it is not too extreme to say that a method which is easy for the researcher is also good for the membrane protein complexes. More simple methods with fewer steps, which are developed with deep consideration, will serve to retain the complex in the native form. In this context, the shortening of the procedure with consideration could produce better results. The CIM (convective interaction media) monolith supported column (BIA Separations, Ljubljana, Slovenia) may be such a possibility as an alternative method to the usual ion-exchange column [146]. Using such a column, intact rhodopsin is constantly separated (Dr. Inoue-Ashida, personal communication). However, it will also be good to keep in mind that, in many cases, combining two or three methods will yield higher purity.

The use of 2D electrophoresis using IEF as the 1st dimensional electrophoresis is beneficial for determining defects in proteins by comparing the patterns of protein spots between the normal and defective samples. In particular, the current availability of genetic information for humans will contribute to the determination of such proteins. Once the protein is determined, then the relationships to other proteins can be detected by using 2D electrophoresis with BN electrophoresis if it is associated with other proteins to form a complex. This procedure may become a convenient method in the research on membrane proteins, which is an alternative to the yeast two hybridization method.

5. Nomenclature

ASB14	amidosulphobetain 14
BN	blue native
C12E8	octaethylene glycol dodecylether
CBB	Coomassie Blue R250
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid
Chl	chlorophyll
CIM	convective interaction media
CMC	critical micelle concentration
complex I	NADH:ubiquinone oxidoreductase
complex II	succinate dehydrogenase
complex III	cytochrome *b*/*c*1 complex
complex IV	cytochrome *c* oxidase
complex V	F0F1-ATPase
DAB	3,3′-diaminobenzidine
DDM	*n*-dodecyl-β-D-maltoside
α-DDM	*n*-dodecyl-α-D-maltoside
DM	*n*-decyl-β-D-maltoside
DTT	dithiothreitol
ECL	enhanced chemiluminescence
GRAVY	grand average hydropathy
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid

His-tag	hexa (or multiple)-histidine tag
HTG	*n*-heptyl-β-D-thioglucoside
IEF	isoelectric focusing
IPG	immobilized pH gradient
LDAO	lauryl-*N*,*N*-dimethylamine-*N*-oxide
LDS	lithium dodecyl sulphate, lauryl sulphate lithium salt
MALDI	matrix-assisted laser desorption ionization mass spectroscopy
MES	2-morpholinoethanesulfonic acid, monohydrate
NG	*n*-nonyl-β-D-glucoside
NP-40	Nonidet P-40
OG	*n*-octyl-β-D-glucoside
OPOE	*n*-octylpolyoxyethylene
ORF	open reading frame
PEG	polyethyleneglycol
PAGE	polyacrylamide gel electrophoresis
PS I and PS II	photosystems I and II
PVDF	polyvinylidene difluoride
SB 3-10	sulfobetain 3-10
SDS	sodium dodecyl sulfate, lauryl sulphate sodium salt
TAP	tandem affinity purification
TBP	tributyl phosphine
TMBZ	3,3′,5,5′-tetramethylbenzidine
Tricine	*N*-[tris(hydroxymethyl)methyl]glycine

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