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Affinity partitioning of a poly(histidine)-tagged integral membrane protein, cytochrome bo_3 ubiquinol oxidase, in a detergent–polymer aqueous two-phase system containing metal-chelating polymer☆

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Abstract

A system has been developed for selective partitioning of membrane proteins. For the first time, an integral membrane protein, cytochrome bo_3 ubiquinol oxidase from *Escherichia coli*, has been affinity partitioned in an aqueous two-phase system. The systems used were different detergent/polymer aqueous two-phase systems containing a metal-chelating polymer, such as poly(ethyleneglycol)–iminodiacetic acid–Cu(II) as well as dextran–iminodiacetic acid–Cu(II). Many non-ionic detergents, such as alkyl(polyethyleneoxide) (C_mEO_n), Triton, Tween and alkylglucosides, form two-phase systems in mixture with polymers, such as dextran and poly(ethyleneglycol), i.e., a micelle-enriched phase in equilibrium with a polymer-enriched phase are formed. In general, membrane proteins partition strongly to the micelle phase. We show that it is possible to selectively partition a poly(histidine)-tagged integral membrane protein into the polymer phase by metal affinity partitioning, with a shift in the partitioning coefficient from 0.015 to 4.8 (300-fold). The affinity partitioning was characterized and the effects of ligand concentration, pH, time, salts, buffer type, imidazole and charged detergent are discussed. Thus, a fast and mild affinity procedure for the purification of integral membrane proteins can be developed in affinity detergent/polymer aqueous two-phase systems, and the method is especially suitable for the purification of labile integral membrane proteins, such as receptors. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Affinity partitioning; Detergent–polymer aqueous two-phase system

1. Introduction

The genome of *E. coli* encodes about 6000 proteins and of them, one third are predicted to be integral membrane proteins. In more biologically complex systems, the fraction of membrane proteins is likely to be even higher. Despite, the large abundance of membrane proteins, they have been sparsely studied. One reason is that it is difficult to isolate large amounts of pure membrane protein in a native and stable form with retained structural integrity [1–3]. The solubilization of membrane-bound proteins has been reviewed theoretically [4] and isolation of membrane proteins has been the subject for several practical reviews [1,5].

Extraction in aqueous two-phase systems is an attractive method for isolation of integral membrane proteins. Aqueous two-phase systems are formed

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when two hydrophilic polymers, such as poly(ethyleneglycol) (PEG) and dextran, are mixed over certain concentrations [6,7]. At equilibrium, both phases are aqueous and each phase is enriched in one of the polymers. Aqueous two-phase systems have been used for fractionation of a large number of biomolecules, such as cells, organelles, membrane fractions and proteins, and the systems have been applied both for analytical and preparative purposes [6,7]. The partitioning can be controlled and optimized with a judicious choice of phase system, pH, salt composition or by affinity partitioning.

Aqueous two-phase systems have been used in membrane research for the separation and subfractionation of membranes, for membrane domain analysis and for purification of membrane proteins [7–9]. Nonionic detergents with lower critical solution temperatures (cloud points), such as Triton X-114, also form aqueous two-phase systems over certain critical temperatures [10–12]. A two-phase system is formed where a detergent-enriched phase is in equilibrium with a detergent-depleted phase. The potential of this system to extract membrane proteins from cytosolic proteins was shown by Bordier [10]. The cloud-point extraction technique in detergent systems has since then been developed and applied as a fast initial purification step for the isolation of membrane protein from water-soluble proteins and insoluble particles prior to a subsequent high resolution purification method [11,12].

The cloud-point extraction technique has some drawbacks. First, the selectivity between different membrane proteins is low. Furthermore, only a few mild nonionic detergents can be utilized and a specific temperature needs to be exceeded for the formation of the two-phase system, both of which can lead to a decreased stability of some membrane proteins. By using a polymer–detergent aqueous two-phase system, it is possible both to increase the amount of available detergents and to use lower temperatures (0°C) [13–15]. Phase separation in detergent–polymer–water mixtures has been studied for a number of systems [16–23]. In all reported cases, mixtures of nonionic detergent, nonionic hydrophilic polymer and water segregate into two phases, one enriched in detergent and the other phase enriched in polymer. Both phases contain detergent over the critical micellar concentration (CMC), thus both phases contain micelles. A large range of commonly used nonionic detergents, in membrane protein isolation, can form two-phase systems, including the Triton series (polyoxyethylene alkyl phenol), alkyl poly(ethyleneoxide) (C_mEO_n), the Tween series (polyoxyethylene sorbitol esters) and alkylglucosides. Phase diagrams of these systems can be found in Sivars and Tjerneld [15]. Recently, we have characterized the mechanisms of phase behavior and protein in different detergent–polymer aqueous two-phase systems [15]. Polymers can be added either to the solubilized (by detergent) membrane proteins or be included into the solubilization buffer together with the detergent. An initial purification of membrane protein can thereby be achieved a few minutes after the solubilization of the membrane protein is completed. This is advantageous because a fast, partial purification lowers the contact time with proteases and detergent and the extreme partitioning of membrane proteins to the micelle phase makes it possible to reduce the volume without it resulting in low recovery. Thus, these systems are of interest for a mild and primary extraction of integral membrane proteins.

Some trials have been carried out to increase selectivity in detergent cloud-point extraction systems, most of them dealing with the selective separation of water-soluble proteins [24–26]. A few recent studies dealt with the selectivity problem for membrane proteins, but only small effects have been obtained [14,15,27].

In order to selectively separate integral membrane proteins, an affinity ligand was coupled to the polymer. The target protein is thereby partitioned to the polymer phase. The principles are shown in Fig. 1. An affinity purification procedure for integral membrane proteins can thereby be developed, due to the high partitioning of contaminating membranes proteins in the opposite micelle phase. We have utilized the affinity between immobilized metals and surface-exposed electron-rich amino acids such as histidine [28]. Iminodiacetic acid (IDA) was coupled to the polymer as a metal-chelating group. We used pure poly(histidine)-tagged cytochrome bo_3 ubiquinol oxidase from *E. coli* as a model protein in this study. This is a well-known integral membrane protein in the respiratory chain of some prokaryotes. Metal chelating affinity partitioning has been per-

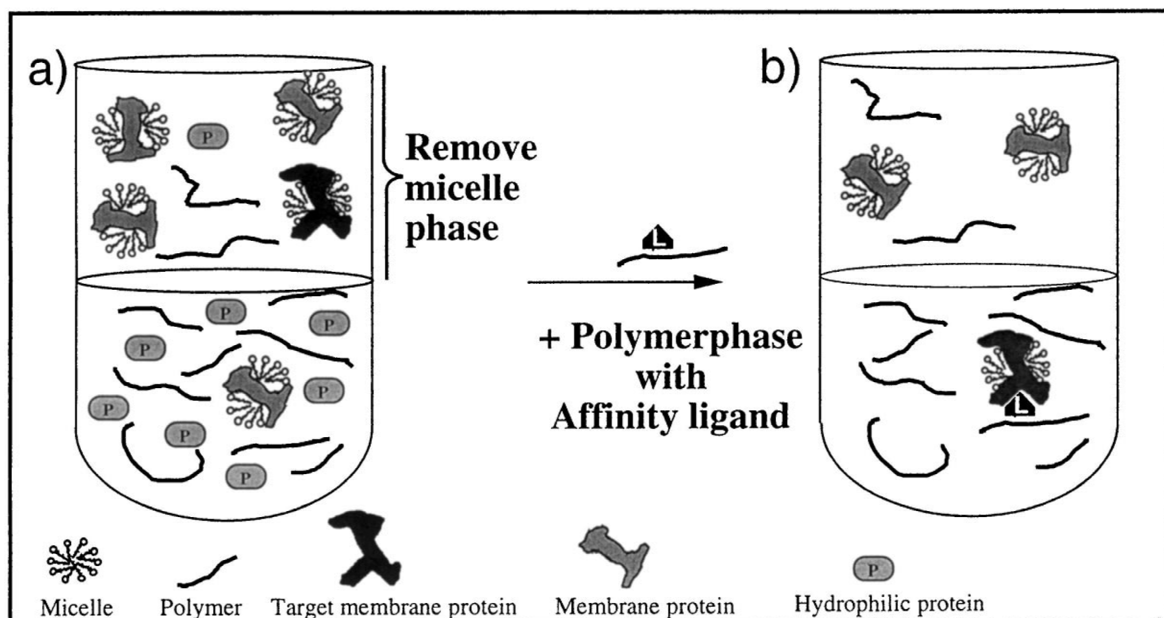

Fig. 1. Concept of the affinity partitioning of integral membrane proteins in detergent–polymer extraction systems. Mixtures of non-ionic detergents and hydrophilic polymer separate into a micelle phase in equilibrium with a polymer phase. (a) Integral membrane proteins can be solubilized and partially purified in the micelle phase. (b) Integral membrane proteins can be affinity partitioned to the polymer phase by including affinity ligand coupled to the polymer.

formed in aqueous two-phase systems for water-soluble proteins [7,29,30] and has been studied in great detail by Suh and Arnold [31]. Immobilized metal affinity chromatography (IMAC) has become a standard technique for purifying recombinant fusion protein [28]. We show here for the first time that it is possible to achieve an affinity partitioning of integral membrane proteins in aqueous two-phase systems. Cytochrome bo_3 ubiquinol oxidase could be partitioned into the polymer phase by the introduction of affinity polymer. This creates unique possibilities for achieving a fast selective purification of labile integral membrane protein in a mild environment.

2. Experimental

2.1. Chemicals

Dextran T500 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Poly(ethylene glycol) (PEG) 40 000 was purchased from Serva (Heidelberg, Germany). $C_{12}EO_5$ (penta ethylene glycol mono-*n*-dodecyl ether) was obtained from Nikko Chemicals (Tokyo, Japan). Triton X-100 [octylphenolpoly(ethyleneglycolether)9,6] was purchased from Sigma (St. Louis, MO, USA). SDS (sodium dodecyl sulphate) was purchased from Merck (Darmstadt, Germany). DTAB (dodecyltrimethylammonium bromide) was obtained from Tokyo Kasei (Tokyo, Japan). The detergents were used without further purification. All water used was of Millipore quality. All other chemicals were of analytical grade.

2.2. Synthesis of metal-chelating polymers

The chelating dextran was a kind gift from Amersham Pharmacia Biotech (Uppsala, Sweden). Allyl dextran T150 was used as a precursor. PEG 40 000-IDA was prepared by a modified procedure described by Chung et al. [32]. A 50-g quantity of PEG 40 000 was freeze-dried to remove excess water. A 100-ml volume of thionyl chloride was purified by distillation and the fraction at 69°C was collected. The dried PEG and the distilled thionyl

chloride were mixed and refluxed for 5 h. Excess thionyl chloride was removed by distillation. PEG 40 000 chloride (20 g), 5 g of iminodiacetic acid (IDA) and 0.5 g of NaOH were desolved in 100 ml of methanol and refluxed for 24 h. Methanol was removed by distillation and the mixture was resolved in 500 ml of water. Impurities were removed by ultrafiltration in a Minisette Membrane Cassettes system (cut-off 10 000) from Filtron Technology Corporation (Northborough, MA, USA). The mixture was lyophilized to give the product PEG 40 000–IDA. The degree of substitution (DS) was determined by titrating the chelating polymer with $CuSO_4$ and recording the shift in absorbance at 600 nm. The chelating polymer was loaded with Cu^{2+} by the addition of an excess amount of $CuSO_4$. Excess metal ion was removed by ultrafiltration in a Minisette Membrane Cassettes system (cut-off 10 000) to yield the final product, PEG 40 000–IDACu(II) (with a degree of IDA substitution of 0.22 mol of IDA per mol of PEG) and allyldextran T150–IDACu(II) (DS: 0.14 mol of IDA per mol of monomer sugar).

2.3. Proteins

Bacterial growth conditions and the preparation of membranes from *E. coli* cells were as described previously [33]. The bacterial strain used was GO105 containing the plasmid PJRHISA, which encodes for a genetically modified cytochrome bo_3 ubiquinol oxidase with a carboxyl-terminus histidine tag on subunit II. The purified membranes (10 g) were solubilized for 20 min in 100 ml of a solution containing 1% dodecylmaltoside, 20 m*M* Tris–HCl, pH 7.5, 300 m*M* NaCl and 5 m*M* imidazole and was centrifuged at 20 000 *g* for 20 min.

The cytochrome bo_3 oxidase was purified from the solubilized membranes by a one-step affinity chromatography system using Ni^{2+}–NTA as a column medium (Qiagen, Chatsworth, CA, USA). The solubilized membrane fraction was applied to a 65-ml bed volume that was equilibrated with 20 m*M* Tris–HCl buffer, pH 7.5, with 300 m*M* NaCl, 5 m*M* imidazole and 0.03% dodecyl maltoside. The column was washed with three bed volumes of the equilibration buffer to remove any non-specific binding enzyme. The sample was eluted with a linear imidazole gradient from 5 to 150 m*M*. The resulting chromatogram showed two clear populations of cytochrome bo_3 ubiquinol oxidase that were termed ''low'' and ''high'' imidazole, based on the elution concentration, where the ''low'' imidazole peak lacked the histidine tag.

The pure cytochrome bo_3 ubiquinol oxidase from the ''high'' imidazole fraction was used in the partitioning experiments after the detergent and buffer were exchanged as appropriate for each experiment. The detergent and buffer were exchanged by binding the protein to a metal chelating chromatography column, HiTrap™ chelating from Amersham Pharmacia Biotech (Uppsala, Sweden), loaded with Ni^{2+}. The bound protein was washed with four column-volumes of the new buffer containing appropriate detergent and was eluted from the matrix with 100 m*M* imidazole solution containing the new buffer and an appropriate detergent. Imidazole was removed by gel filtration on a PD-10 column from Amersham Pharmacia Biotech.

2.4. Phase systems for the partitioning experiments

Aqueous two-phase systems, with a total weight of 0.5 g, were prepared by mixing protein solution containing the appropriate detergent with a premixed stock phase system containing appropriate concentrations of detergent, polymer, buffer and additives. Thin glass test tubes (Ø 6 mm; length, 50 mm) were used. All concentrations used were calculated as weight percentages. The systems were incubated at 4°C for at least 15 min and were carefully mixed again. Phase separation was speeded up by centrifugation at 1800 *g* for 3–4 min in a table-top centrifuge at 4°C. The polymer and micelle phases were isolated using a syringe and were diluted as appropriate prior to assay.

2.5. Protein partition coefficient, yield and protein determination

The phases were analyzed for their cytochrome bo_3 ubiquinol oxidase content by measuring the absorbance at 406 nm against a reagent blank that contained a sample from an appropriately diluted phase from a system without protein.

The partitioning of a substance in an aqueous

two-phase system is described by the partition coefficient K, which is defined as $K=C_T/C_B$, where C_T and C_B are the equilibrium concentrations of the partitioned substance in the upper and lower phases, respectively. The total and phase volumes were determined by weighing distilled water to the same height in the test tube after cleaning and drying the tube. Protein recovery (mass balances) was determined by calculating the total protein amount added to the system and the amount of protein found in the different phases. A recovery of between 90 and 110% was accepted as satisfactory. All results are average values after partitioning of the protein in at least two equal systems.

3. Results and discussion

The pure poly(histidine) tagged cytochrome bo_3 ubiquinol oxidase from *E. coli* was used in this study as a model integral membrane protein. The protein was produced with a tag of six histidines at the C-terminus. Cytochrome bo_3 ubiquinol oxidase partitioned strongly into the micelle phase in all studied detergent–polymer systems when no affinity polymer was included in the system (Figs. 2 and 3). This is analogous to earlier findings for partitioning of membrane proteins both in detergent–polymer aqueous two-phase systems [15] and in cloud-point extraction systems [10,12]. In general, membrane proteins are extracted in the micelle phase due to non-specific hydrophobic effects. The one-sided partitioning of membrane proteins can be explained by the large detergent concentration difference between the upper and lower phase, respectively. Therefore, the extraction of membrane protein into the micelle phase can be increased by factors that increase the concentration difference between the phases and/or strengthens the interaction between membrane protein and detergent. The partitioning of membrane proteins has been found to be more extreme with factors such as increased concentration of the system (tie line length), decreased polymer molecular mass, increased temperature and shorter poly(ethyleneoxide) chain length of the detergent [15].

Selectivity in partitioning between different membrane proteins in micellar extraction systems is

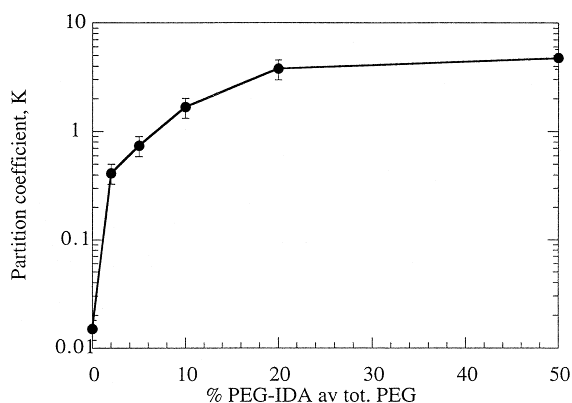

Fig. 2. Effect of the concentration of PEG 40 000–IDA chelated copper ions on affinity partitioning of the integral membrane protein poly(histidine) cytochrome bo_3 ubiquinol oxidase. System composition: 6.5% (w/w) total PEG concentration (i.e., PEG 40 000+PEG 40 000–IDACu(II) with a DS of 0.22 mol of IDA per mol of PEG), 13.0% (w/w) Triton X-100, 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 1.14). $K>1$ is equivalent to a preferred protein partitioning into the polymer phase in the PEG–Triton system.

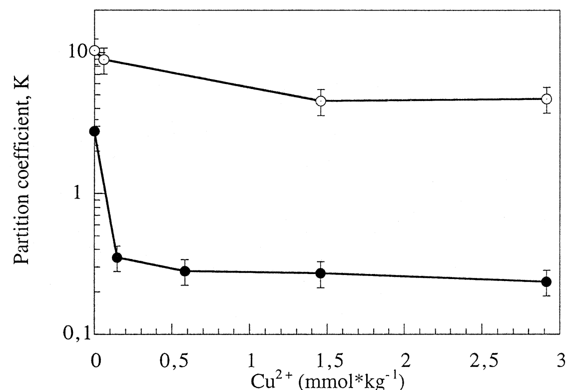

Fig. 3. Effect of the concentration of dextran–IDA chelated copper ions on affinity partitioning of the integral membrane protein poly(histidine)cytochrome bo_3 ubiquinol oxidase. The addition of 0.1 mol kg^{-1} $NaClO_4$ (●) enhanced the affinity partitioning into the polymer phase compared to the situation when no salt was added (○). System composition: 4.9% (w/w) $C_{12}EO_5$, 3.8% (w/w) total dextran concentration (i.e., dextran T500+dextran T150–IDACu(II) with a DS of 115 mol of IDA per mol of dextran), 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 0.48). $K<1$ is equivalent to a preferred protein partitioning into the polymer phase in the $C_{12}EO_5$–dextran system.

limited by the strong and non-specific hydrophobic interaction that will partition almost all membrane-bound proteins into the micelle phase. One exception is the membrane-bound acetylcholine receptor that was excluded from the Triton X-114 micelle phase [34].

Small amounts of a metal-chelating polymer were introduced into the system in order to obtain an affinity extraction method that can selectively separate polyhistidine-tagged membrane proteins from other membrane bound proteins (Fig. 1). Both PEG and dextran with iminodiacetic acid (IDA) coupled to the polymer were used. In this study, we used Cu^{2+} chelated to PEG–IDA and dextran–IDA, since the general binding strength of chelated metal ions towards histidines decreases in the order: $Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$ [28].

Pure poly(histidine)-tagged cytochrome bo_3 ubiquinol oxidase was increasingly partitioned into the polymer phase (upper phase) in a PEG−Triton X-100 system with increasing amounts of PEG-IDA-Cu(II) (Fig. 2). The partitioning was thereby shifted from a partitioning in the micelle phase (*K* value of 0.015) to a partitioning into the polymer phase (*K*-value of 4.8). Thus, the chelating polymer shifted the *K*-value of cytochrome bo_3 ubiquinol oxidase 300 times. This is, to our knowledge, the first affinity extraction of an integral membrane protein in an aqueous two-phase system. Qualitatively similar results were also obtained in other detergent−polymer systems: $C_{12}EO_5$−dextran with dextran-IDA-Cu(II) (Fig. 3), PEG−octylglucoside and PEG−dodecylmaltoside with PEG-IDA-Cu(II) (results not shown). An efficient affinity partitioning seems to require an excess amount of chelated copper ions, i.e., 50-100 times more mol of metal ion per mol of cytochrome bo_3 ubiquinol oxidase was needed.

In order to characterize the affinity partitioning, we examined the effects of pH, ligand concentration, salt effects, buffers and the addition of imidazole and ionic detergents. The affinity partitioning of cytochrome bo_3 ubiquinol oxidase to the polymer phase increased with pH (Fig. 4). At low pH, the membrane protein partitioned strongly into the micelle phase, while the partitioning towards the polymer phase was strongly increased at higher pH values and leveled off at around pH 7.5. The pH dependence

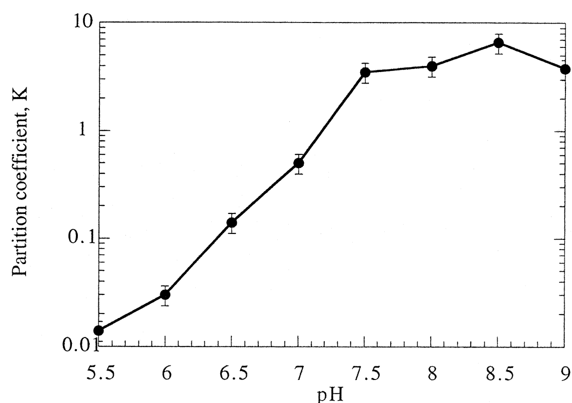

Fig. 4. Effect of pH on affinity partitioning of the integral membrane protein poly(histidine) cytochrome bo_3 ubiquinol oxidase. System composition: 0.071 mmol kg^{-1} PEG-IDA chelated copper ions, 6.5% (w/w) total PEG concentration [i.e., 5.2% (w/w) PEG 40 000+1.3% (w/w) PEG 40 000-IDACu(II) with a DS of 0.22 mol of IDA per mol of PEG], 13.0% (w/w) Triton X-100, 10 mmol kg^{-1} phosphate-borate buffer, pH 5.5–9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 1.14). $K>1$ is equivalent to a preferred protein partitioning into the polymer phase in the PEG–Triton system.

can be explained by the deprotonation of the imidazole group of the histidine, which is completely deprotonated at pH 7.5 [35,36]. The effect of pH was independent of the buffer used. The same effects were obtained in Tris–HCl buffer as in phosphate, phosphate–borate and sodium carbonate buffers for systems containing PEG-IDA-Cu(II). However, the partitioning of proteins in aqueous two-phase systems will be generally affected by the addition of different salts and buffer ions [6,7]. This pH dependence of metal affinity partitioning is convenient since the target protein can be back-extracted from the polymer phase into a micelle phase by decreasing the pH, and this can be done in suitable pH ranges for proteins. It also allows the possibility of removing and recycling the affinity polymer.

The effect on the partitioning of poly(histidine) cytochrome bo_3 ubiquinol oxidase by dextran-IDA-Cu(II) was very low in systems with no addition of salt, with *K*-values only shifted from ten to four in a $C_{12}EO_5$–dextran system (Fig. 3). Thus, the dextran could not pull the membrane protein sufficiently well into the polymer phase. However, we also noted that the dextran-IDA-Cu(II) seemed to partition almost

evenly between the phases (observed visually by the blue color of the copper ions). Thus, the chelating polymer metal–ion complex preferred the micelle phase rather than partitioning into the dextran-enriched phase. This could be due to a negative net charge on dextran-IDA-Cu(II). The original buffer used was a sodium phosphate borate solution (pH 9.0). Both phosphate ($HPO_4^{2-}/H_2PO_4^-$) and borate ion are relatively hydrophilic in the Hofmeister series and have been found to partition into the dextran phase in a PEG–dextran system [6]. The same behavior can be expected in a $C_{12}EO_5$–dextran system, due to a chemical resemblance between PEG–dextran and $C_{12}EO_5$–dextran systems. Thus, this uneven salt distribution will lead to the formation of an electrostatic potential difference between the two phases, which will influence the partitioning of molecules [37,38]. In a $C_{12}EO_5$–dextran system containing a phosphate/borate buffer, a negatively charged molecule will prefer the micelle phase. In order to prove this, an excess amount of sodium perchlorate (ten times the amount of phosphate/borate) was added to the system. The blue color of dextran-IDA-Cu(II) was strongly partitioned into the lower polymer phase (observed visually). Perchlorate ion (ClO_4^-) is chaotropic and relatively hydrophobic in the Hofmeister series and will therefore have the opposite effect on charged molecules to that of phosphate/borate ions [37]. The addition of sodium perchlorate also had a large impact on the affinity partitioning of cytochrome bo_3 ubiquinol oxidase (Figs. 3 and 5). A small amount of added salt (10 mmol kg^{-1}) could direct cytochrome bo_3 ubiquinol oxidase to the polymer phase (the *K*-value shifted with a factor of around 20). This effect is due to the shift in chelating polymer partitioning and not to the salt effect on the protein partitioning (Fig. 5). In order to increase the partitioning of the recombinant poly(histidine)-tagged integral membrane proteins into the polymer phase, various salts were tested (Table 1). The largest effect, i.e., the lowest *K*-value, was obtained with sodium perchlorate ($K=0.18$), which was slightly better than sodium chloride ($K=0.32$).

Protein partitioning can be shifted by the addition of charged phase components to the system. For example, the addition of ionic surfactants such as SDS or DTAB to the system creates a weakly charged mixed micelle that attracts oppositely charged proteins and repels similarly charged proteins to the opposite phase [26], with the effect being larger for hydrophilic proteins than for membrane

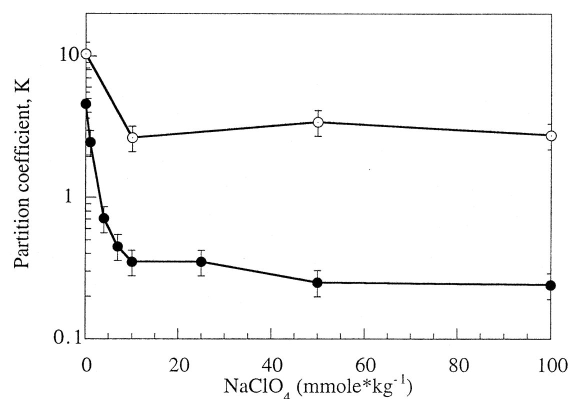

Fig. 5. Effect of $NaClO_4$ concentration on affinity partitioning of the integral membrane protein poly(histidine) cytochrome bo_3 ubiquinol oxidase in systems with an affinity ligand [1.90 mmol kg^{-1} dextran T150-IDA chelated copper ions, i.e., 0.76% (w/w)] (●) and without an affinity ligand (○). System composition: 4.9% (w/w) $C_{12}EO_5$, 3.8% (w/w) total dextran concentration [i.e., dextran T500+dextran T150-IDACu(II) with a DS of 115 mol of IDA per mol of dextran], 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 0.48). $K<1$ is equivalent to a preferred protein partitioning into the polymer phase in the $C_{12}EO_5$–dextran system.

Table 1
Effect of different salts on the affinity partitioning of cytochrome bo_3 ubiquinol oxidase[a]

Salt	$K_{additive}$
$NaClO_4$	0.12
NaCl	0.32
NaBr	0.31
NaSCN	0.37
$Na(H_2PO_4^-/HPO_4^{2-})$, pH 9.0	0.70
$N(C_4H_9)_4(H_2PO_4^-/HPO_4^{2-})$, pH 9.0	3.3
SDS	0.25
DTAB	60

[a] System composition: 1.90 mmol kg^{-1} allyldextran T150-IDA chelated copper ions [0.76% (w/w)], 4.9% (w/w) $C_{12}E_5$, 3.8% (w/w) total dextran concentration and 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 0.48). The addition of either 100 mmol kg^{-1} salt or 0.074% (w/w) ionic detergent. $K<1$ is equivalent to a partitioning into the polymer phase.

proteins [15]. Ionic detergents can also be used to direct the chelating dextran copper complex into the polymer phase and thereby the target protein. Adding small amounts of the anionic detergent SDS gave qualitatively similar results as adding NaCl and $NaClO_4$ (Fig. 6). However, a relatively strong effect on the partitioning of cytochrome bo_3 ubiquinol oxidase was obtained by the addition of SDS to systems without a chelating polymer, which could be partitioned into the polymer phase (K-value of 0.8). Most proteins are net negatively charged around the optimal pH range (7.5–8.0) for metal affinity partitioning. They will therefore tend to partition more into the polymer phase with the addition both of $NaClO_4$/NaCl and SDS, respectively. However, since the effect on membrane protein partitioning is larger following the addition of SDS rather than salt, it is advantageous to use salts to direct the partitioning of the metal chelating polymer into the polymer phase. Both the salt and ionic detergent experiments clearly indicate that the dextran-IDACu(II) complex is net negatively charged.

The extra addition of salt to the system may be avoided by the use of buffers that can direct the chelating dextran towards the polymer phase. Therefore, different buffers with buffering capacity in the appropriate pH range were screened (Table 2). Many buffers can direct the dextran-IDACu(II) and the target protein sufficiently well into the polymer phase with K-values of around 0.5, such as TES, Tris, MOPS, HEPES and piperzine. No significant differences were found between the buffers tested, with one exception, Tricine, which, like phosphate/borate buffers, was not able to partition cytochrome bo_3 ubiquinol oxidase into the metal-chelating polymer phase. Thus, the buffers tested, which are often referred to as ''good'' buffers are also good buffers for metal affinity partitioning in dextran containing detergent–polymer aqueous two-phase systems. However, the partitioning of cytochrome bo_3 ubiquinol oxidase to the polymer phase was enhanced by the addition of sodium perchlorate, even in the case of affinity systems containing MOPS and TES (Table 2).

Imidazole strongly competes with the binding between the protein and the metal chelate and high concentrations of imidazole are commonly used to elute the bound proteins in IMAC. This can also be used in metal chelating detergent–polymer systems to back-extract the protein from the polymer phase. A 1-mM solution of imidazole was enough to release

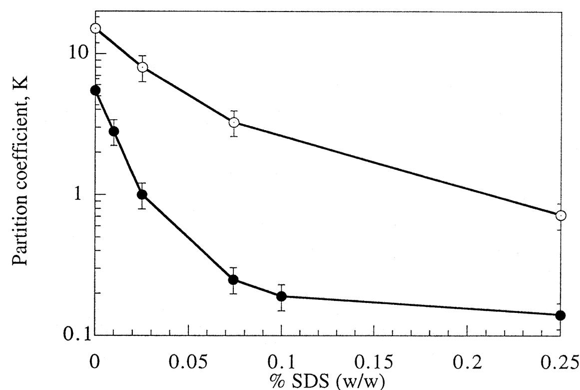

Fig. 6. Effect of SDS concentration on affinity partitioning of the integral membrane protein poly(histidine) cytochrome bo_3 ubiquinol oxidase in systems with an affinity ligand [1.90 mmol kg^{-1} dextran T150-IDA chelated copper ions, i.e., 0.76% (w/w)] (●) and without an affinity ligand (○). System composition: 5.2% (w/w) $C_{12}EO_5$, 6.6% (w/w) total dextran concentration [dextran T500+dextran T150-IDACu(II) with a DS of 115 mol of IDA per mol of dextran), 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 0.27). $K<1$ is equivalent to a preferred protein partitioning into the polymer phase in the $C_{12}EO_5$–dextran system.

Table 2
Effect of different buffers and the addition of $NaClO_4$ on the affinity partitioning of cytochrome bo_3 ubiquinol oxidase[a]

Buffer	Partition coefficient		
	$K_{no\ ligand}$	K_{aff}	$K_{aff}+NaClO_4$
Sodium phosphate	10.0	1.22	–
Sodium phosphate–borate	7.9	1.49	–
Tricine	–	3.9	–
MOPS	–	0.63	0.19
HEPES	–	0.60	–
TES	–	0.58	0.12
TRIS	–	0.46	–
Piperzine	–	0.39	–

[a] System composition: 4.9% (w/w) $C_{12}E_5$, 3.8% (w/w) total dextran concentration and 20 mmol kg^{-1} buffer, pH 7.5, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 0.48). The system with affinity ligand contained 0.48 mmol kg^{-1} allyldextran T150-IDA chelated copper ions [0.19% (w/w) of the affinity polymer]. The affinity system with salt added contained 100 mmol kg^{-1} $NaClO_4$ (K_{aff}+ $NaClO_4$). $K<1$ is equivalent to a partitioning into the polymer phase.

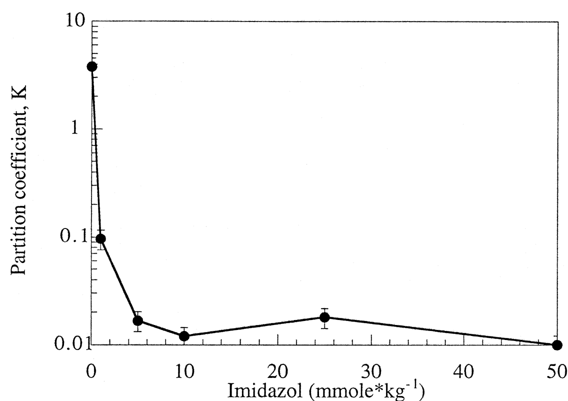

Fig. 7. Effect of imidazole concentration on affinity partitioning of the integral membrane protein poly(histidine) cytochrome bo_3 ubiquinol oxidase. System composition: 0.071 mmol kg^{-1} PEG-IDA chelated copper ions, 6.5% (w/w) total PEG concentration [i.e. 5.2% (w/w) PEG 40 000+1.3% (w/w) PEG 40 000-IDACu(II) with a DS of 0.22 mol of IDA per mol of PEG], 13.0% (w/w) Triton X-100, 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 1.14). $K>1$ is equivalent to a preferred protein partitioning into the polymer phase in the PEG−Triton system.

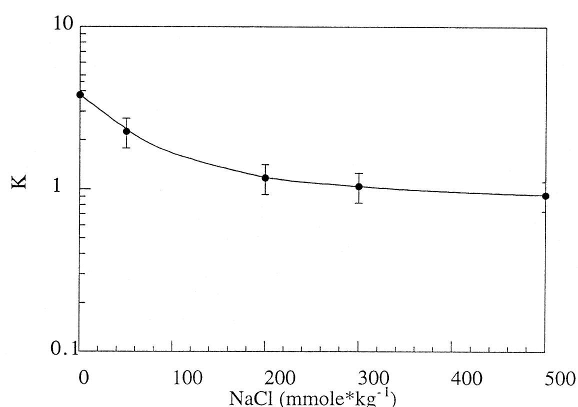

Fig. 8. Effect of NaCl concentration on affinity partitioning of the integral membrane protein poly(histidine) cytochrome bo_3 ubiquinol oxidase. System composition: 0.071 mmol kg^{-1} PEG-IDA chelated copper ions, 6.5% (w/w) total PEG concentration [i.e. 5.2% (w/w) PEG 40 000+1.3% (w/w) PEG 40 000-IDACu(II) with a DS of 0.22 mol of IDA per mol of PEG], 13.0% (w/w) Triton X-100, 10 mmol kg^{-1} phosphate–borate buffer, pH 9.0, ca. 0.02% (w/w) cytochrome bo_3 ubiquinol oxidase, at a temperature of 3–4°C (phase volume ratio, 1.14). $K>1$ is equivalent to a preferred protein partitioning into the polymer phase in the PEG−Triton system.

the bound protein in detergent–polymer aqueous two-phase systems (Fig. 7). With 10 m*M* imidazole, the protein was completely partitioned into the lower micelle phase, with $K=0.01$, i.e., with a 7000-fold excess of imidazole (mol) compared to protein. Thus, the addition of imidazole can be used in detergent–polymer systems for back-extraction of cytochrome bo_3 ubiquinol oxidase as an alternative to the use of low pH. The addition of low concentration of imidazole might also be used for reducing non-specific partitioning into the polymer phase in a similar fashion as in IMAC.

Non-specific electrostatic effects on protein partitioning from the charged metal chelating polymer may be reduced by the addition of large amounts of salt: commonly 300–500 m*M* NaCl is used in IMAC. Our results indicate that the selective partitioning between the poly(histidine) tag and the metal-chelating polymer is reduced only to a low degree, if at all, at high salt concentrations (Fig. 8). The slightly reduced effective affinity partitioning might be the result of general salt effects on the protein partitioning (see discussion above).

The kinetics of the affinity partitioning seem to be very fast. The partitioning was not shifted with increased mixing time or by the order in which the phase components were added (results not shown). The same *K*-values were obtained when the chelating polymer was added to the protein solution first, followed by the rest of the phase-forming components compared to when a preformed two-phase buffer solution, containing all phase-forming components including the metal chelating polymer, was added directly to the protein solution. This is convenient since it simplifies the handling of the system. Thus, a ready-prepared two-phase stock solution can be added to the target protein solution. A fast separation of the membrane protein can be achieved by a quick mixing of the system followed by a short low-speed centrifugation step.

4. Conclusions

A new highly selective purification procedure for the isolation of integral membrane proteins has been developed. Target membrane protein can be affinity partitioned and recovered from the polymer phase in

affinity detergent–polymer aqueous two-phase systems and can thereby be separated from contaminating membrane proteins with a preferred partitioning in the micelle phase. This study deals with metal affinity partitioning but the affinity purification procedure described should be generally applicable for other affinity systems. Integral membrane proteins can thereby be purified rapidly and selectively. Large systems can be handled and volumes can be reduced by affinity partitioning of target protein into a small phase volume. These systems should be mild towards the proteins since many mild non-ionic detergents can be used at low temperature under physiological conditions. A fast isolation procedure reduces the contact time with proteases/detergents, further contributing to the preparation of a homogeneous membrane protein fraction.

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References

[1] G. von Jagow, H. Schägger, A practical guide to membrane protein purification, Academic Press, New York, 1991.
[2] S. Hjertén, H. Pan, K. Yao, in: H. Peeters (Ed.), Protides of the biological fluids. 29th Colloquium 1981, Pergamon Press, New York, 1982, p. 15.
[3] J.E. Walker, M. Saraste, Curr. Opin. Struct. Biol. 6 (1996) 457.
[4] A. Helenius, K. Simons, Biochim. Biophys. Acta 415 (1975) 29.
[5] L.M. Hjelmeland, in: M.D. Deutcher (Ed.), Methods in Enzymology, Vol. 182, Academic Press, New York, 1990, p. 253.
[6] P.-Å. Albertson, Partition of Cell Particles and Macromolecules, Wiley, New York, 1986.
[7] H. Walter, G. Johansson (Eds.), Methods in Enzymology, Vol. 228, Academic Press, New York, 1994.
[8] P.-Å. Albertsson, B. Andersson, J. Chromatogr. 215 (1981) 131.
[9] P.-Å. Albertsson, Biochemistry 12 (1973) 2525.
[10] C. Bordier, J. Biol. Chem. 256 (1981) 1604.
[11] A. Sánchez-Ferrer, R. Bru, F. García-Carmona, Crit. Rev. Biochem. Mol. Biol. 29 (1994) 275.
[12] W.L. Hinze, E. Pramauro, Crit. Rev. Anal. Chem. 24 (1993) 133.
[13] T. Saitoh, H. Tani, T. Kamidate, T. Kamataki, H. Watanabe, Anal. Sci. 10 (1994) 299.
[14] H. Tani, T. Saitoh, T. Kamidate, T. Kamataki, H. Watanabe, Biotech. Bioeng. 56 (1997) 311.
[15] U. Sivars, F. Tjerneld, Biochim. Biophys. Acta (2000) in press.
[16] M. Yamazaki, M. Ohshika, T. Ito, Biochim. Biophys. Acta 1063 (1991) 175.
[17] S.M. Clegg, P.A. Williams, P. Warren, I.D. Robb, Langmuir 10 (1994) 3390.
[18] L. Piculell, K. Bergfeldt, S. Gerdes, J. Phys. Chem. 100 (1996) 3675.
[19] K. Zhang, G. Karlström, B. Lindman, J. Phys. Chem. 98 (1994) 4411.
[20] N.K. Pandit, J. Kanjia, Int. J. Pharm. 141 (1996) 197.
[21] N.K. Pandit, J. Kanjia, K. Patel, D.K. Pontikes, Int. J. Pharm. 122 (1995) 27.
[22] C. Wormuth, Langmuir 7 (1991) 1622.
[23] I.D. Robb, P.A. Williams, P. Warren, R. Tanaka, J. Chem. Soc. Faraday Trans. 91 (1995) 3901.
[24] N. Garg, I.Yu. Galaev, B. Mattiasson, Biotechnol. Appl. Biochem. 20 (1994) 199.
[25] T. Saitoh, W.L. Hinze, Talanta 42 (1995) 119.
[26] U. Sivars, K. Bergfeldt, L. Piculell, F. Tjerneld, J. Chromatogr. B 680 (1996) 43.
[27] H. Tani, T. Ooura, T. Kamidate, T. Kamataki, H. Watanabe, J. Chromatogr. B 708 (1998) 294.
[28] J. Porath, Protein Expression Purif. 3 (1992) 263.
[29] A. Otto, G. Birkenmeir, J. Chromatagr. 644 (1993) 25.
[30] D. Schustolla, W.-D. Deckwer, K. Schügerl, H. Hustedt, Bioseparation 3 (1992) 167.
[31] S.-S. Suh, F.H. Arnold, Biotech. Bioeng. 35 (1990) 682.
[32] B.H. Chung, D. Baily, F.H. Arnold, in: H. Walter, G. Johansson (Eds.), Methods in Enzymology, Vol. 228, Academic Press, New York, 1994, p. 167.
[33] J.E. Morgan, M.I. Verkhovsky, A. Puustinen, M. Wikström, Biochemistry 34 (1995) 15633.
[34] P.A. Maher, S.J. Singer, Proc. Natl. Acad. Sci. USA 82 (1985) 958.
[35] R.D. Johnson, R.J. Todd, F.H. Arnold, J. Chromatogr. A 725 (1996) 225.
[36] J.C. Cameselle, J.M. Ribeiro, A. Sillero, Biochem. Educ. 14 (1996) 131.
[37] G. Johansson, Acta Chem. Scand. B 28 (1974) 873.
[38] A. Pfennig, A. Schwerin, Ind. Eng. Chem. Res. 37 (1998) 3180.