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USE OF A SMALL TSK GSW HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMN FOR LARGE-ZONE CHROMATOGRAPHIC STUDIES OF MONOMER–OLIGOMER EQUILIBRIA OF MEMBRANE PROTEIN

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SUMMARY

A TSK GSWP size-exclusion high-performance liquid chromatography "pre-column" (75 mm × 7.5 mm) was used for large-zone chromatographic studies of the effect of ligands on the monomer–oligomer equilibrium in sarcoplasmic reticulum Ca-ATPase solubilized in a non-ionic detergent. The monomer–dimer association constants determined for the Ca^{2+}-occluded $E_1P[Ca_2]$ and vanadate-reacted E_2V forms were 2–3 fold lower than the association constant of the E_1Ca_2 form (bound Ca^{2+} is freely exchangeable). With the TSK GSWP column, large-zone chromatography was rapid and required only small quantities of protein. This column was found to be useful also for studies in the presence of phospholipid, provided that the influence of lipid binding, *per se*, on the partition coefficient of the protein in the column was taken into consideration. This opens new possibilities for studies of membrane proteins under non-denaturing conditions.

INTRODUCTION

The self-association state of membrane-transport proteins has attracted widespread interest, because of the possible functional rôle of protein–protein associations. One example is the sarcoplasmic reticulum calcium-pump protein (Ca-ATPase). There is evidence that this enzyme may exist as an oligomer of two or more 110 000 dalton polypeptide chains[1–3], and the implications of the quaternary structure for the transport mechanism of Ca^{2+} is currently being debated[4–6]. Translocation of Ca^{2+} involves its occlusion in the phosphoprotein, *ie.*, formation of an $E_1P[Ca_2]$ form and transition between two major conformational states, E_1 and E_2[7,8]. An intriguing question is whether changes occur in the nature and extent of the protein–protein contacts during these steps. A major hindrance to elucidation of this problem has been the lack of suitable methods for characterization of interpeptide associations within a short time period. In a recent report we suggested that size-exclusion high-performance liquid chromatography (HPLC) can be used for this purpose under conditions where the membrane protein is solubilized in a low concentration of non-ionic detergent[8]. However, when HPLC is conducted as small-zone experiments as was done previously, the ligand-induced changes in the elution volume

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of the soluble protein cannot easily be evaluated quantitatively, in terms of a change in the monomer–oligomer association constant. Furthermore, the results are difficult to interpret, because a change in the elution volume may be brought about by a protein conformational change and/or a change in detergent binding without an accompanying shift in the monomer–oligomer equilibrium. This has prompted us to develop a microscale version of the large-zone chromatographic technique worked out by Ackers and associates[9,10] for soluble proteins. In large-zone experiments, ligand-induced changes in the elution volume can be studied at various protein concentrations, and thereby it is possible to determine the association constants for oligomerization of the protein. We used a small TSK GSWP HPLC "precolumn", which permitted large-zone chromatography to be carried out within a short time and with a sample volume of only 2 ml. In this way, we were able to demonstrate that, for soluble Ca-ATPase, ligand-induced changes in the elution volume do result from changes in the monomer–oligomer equilibrium. Furthermore, the small HPLC column has proved to be useful for chromatography in the presence of exogenous phospholipid in the eluent at a relatively high concentration.

EXPERIMENTAL

High-performance liquid chromatography

Large-zone experiments were conducted in a TSK GSWP size-exclusion column (75 mm × 7.5 mm, Toyo Soda, Japan; manufactured through LKB as an "Ultropac Precolumn"). The column was operated at 20°C at a flow-rate of 0.1 ml/min (pump pressure less than 1 bar). An LKB 2150 precision HPLC pump was used. The absorbance of eluted fractions was read continuously at 226 or 280 nm depending on the protein concentration. Ca-ATPase solubilized in the non-ionic detergent octaethylene glycol monododecyl ether ($C_{12}E_8$, Nikko Chemicals) was injected through a 2.0- or a 10-ml loop. The eluent contained 20 m*M* N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (Tes) (pH 7.0), 0.1 *M* sodium chloride, 1.0 m*M* ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 m*M* magnesium chloride, 5 m*M* dithiothreitol (DTT), 0.15 or 2.0 mg $C_{12}E_8$/ml with further additions of calcium chloride (total concentration 1.5 m*M*), 0.25 m*M* CrATP and 100 μ*M* Na_3VO_4 as indicated. In some experiments, 1 mg egg lecithin per ml was added to the sample and eluent.

For delipidation, soluble Ca-ATPase was subjected to small-zone HPLC in a TSK G4000SW column (600 mm × 7.5 mm, Toyo Soda) at 0.8 ml/min with the same eluent as described above. The major peak containing Ca-ATPase protein was collected and diluted in eluent for direct use in large-zone experiments.

The large-zone elution profiles were analyzed by determination of centroid boundary positions by planimetry, and calculation of the apparent association constants for dimerization according to[9]

$$K_a = \frac{1 - f}{2f^2 C_T} \tag{1}$$

where $f = (V_e - V_D)/(V_M - V_D)$ is the fraction of Ca-ATPase molecules which is found in the monomeric state and C_T is the total molar concentration of Ca-ATPase

calculated by assuming a protomer molecular weight of 110000[11]; V_D and V_M are the elution volumes of pure dimeric and monomeric Ca-ATPase, respectively, determined, with stable dimers prepared by EGTA treatment of soluble Ca-ATPase as described below, and with monomers solubilized at 0.5 μg protein/ml in 2 mg $C_{12}E_8$/ml.

Preparation of reversibly and irreversibly self-associated Ca-ATPase

Purified Ca-ATPase in membranous form was prepared from rabbit skeletal muscle as previously[12]. The protein (10 mg/ml) was solubilized in 25 mg $C_{12}E_8$/ml, 20 m*M* Tes (pH 7.0) and 0.1 *M* sodium chloride with further additions as described below, and centrifuged in a Beckman airfuge at 130000 *g* for 20 min to remove insoluble residue. The supernatant was used directly for studies on reversible monomer–oligomer reactions as described above, or it was incubated with an excess of EGTA (1.5 m*M* added) for 10 min at 20°C to obtain the disulphide-linked dimer used for calibration of elution positions[13]. The irreversible aggregation induced by removal of Ca^{2+} with EGTA was terminated by readdition of Ca^{2+} in excess of EGTA, and the dimer was isolated by small-zone HPLC in the TSK G4000SW column.

Stabilization of the various functional states of Ca-ATPase (E_1Ca_2, $E_1P[Ca_2]$, E_2V)

For studies of the E_1Ca_2 form in which the bound Ca^{2+} is free exchangeable, all buffers contained 0.5 m*M* calcium chloride in excess of EGTA. $E_1P[Ca_2]$ (the Ca^{2+}-occluded phosphorylated state) was formed by incubation of the membrane-bound enzyme with 10 m*M* magnesium chloride, 0.5 m*M* calcium chloride and 0.8 m*M* CrATP for 6 h, before solubilization[5]. The eluents used in delipidation and large-zone chromatography of $E_1P[Ca_2]$ contained 0.5 m*M* calcium chloride in excess of EGTA together with 0.25 m*M* CrATP (a β,γ-bidentate complex of Cr(III) with ATP). The form E_2V, stabilized by binding of vanadate, was obtained by incubation of the membrane with 0.5 m*M* Na_3VO_4, 10 m*M* magnesium chloride and 1.0 m*M* EGTA for 1 h before solubilization. The eluents used in the delipidation and large-zone chromatography of E_2V contained 0.1 m*M* Na_3VO_4 and no Ca^{2+}. Due to the protecting effect of vanadate[8], there was no formation of irreversibly associated dimers, despite the absence of free Ca^{2+}.

Other methods

Protein and phospholipid concentrations were determined as previously[13]. Sedimentation equilibrium studies were performed in a Beckman Model E analytical ultracentrifuge at 9000 and 12 000 rpm[12,13]. Equilibrium was reached after approximately 20 h.

The buoyant molecular weight, $M(1-v\rho)$, was obtained from the slopes of equilibrium plots of ln C *vs.* r^2. The molecular weight of the protein moiety of the soluble particles, M_p, was calculated according to the equation

$$M(1-v\rho) = M_P[(1-v_P\rho) + \delta_D(1-v_D\rho) + \delta_L(1-v_L\rho)] \quad (2)$$

where δ_D and δ_L are the amounts of detergent and lipid bound (in g/g of protein), v_P, v_D and v_L are the partial specific volumes of the protein (0.74 cm^3/g), of the bound detergent (0.97 cm^3/g) and of the bound lipid (0.98 cm^3/g) and ρ is the solvent density.

The last two terms in eqn. 2 can be neglected since $v_D\rho$ and $v_L\rho$ are both equal to 1.0.

RESULTS

Fig. 1 shows large-zone experiments with soluble delipidated Ca-ATPase at low detergent concentration (0.15 mg/ml) in three different functional states, E_1Ca_2, $E_1P[Ca_2]$ and E_2V. A stable Ca^{2+}-occluded $E_1P[Ca_2]$ form was obtained by reaction of E_1Ca_2 with CrATP[5], whereas the Ca^{2+}-free E_2 state was stabilized by binding of vanadate. For both of these reaction intermediates, the elution volume corresponding to the centroid position of the leading boundary is significantly larger than that for the E_1Ca_2 state. The trailing boundary is broad, but unimodal as expected for a reversible monomer–dimer equilibrium[9,10].

The flow-rate and sample volume used in the experiments of Fig. 1 were 0.1 ml/min and 2.0 ml, respectively. We found that the flow-rate could be increased to 0.4 ml/min without seriously affecting the high resolution of this column. When the injected volume was varied between 2.0 and 10.0 ml, no change in the leading boundary centroid position was detected. At lower sample volumes the retention times increased, indicating that the minimum sample volume required for large-zone experiments is about 2.0 ml.

A series of experiments was conducted as in Fig. 1, at various protein concentrations. Fig. 2A shows that the elution volumes corresponding to the leading boundary centroid positions decreased with increasing protein concentrations. Furthermore, the $E_1P[Ca_2]$ and E_2V forms were eluted at significantly higher volumes than was E_1Ca_2, except at the lowest protein concentrations where the elution volumes converged towards a common value of 1.92 ml ("M"), independently of the functional state of the protein.

The same upper limit of elution volumes was observed at an higher $C_{12}E_8$ concentration (2 mg/ml. Fig. 2B). In this case the elution volume was constant up to about 0.5 mg protein/ml. We have previously shown that under conditions where the elution volume is 1.92 ml, the soluble Ca-ATPase consists of single peptide chains of M_r 110000[13]. The elution volume of dimeric Ca-ATPase ("D") was determined with

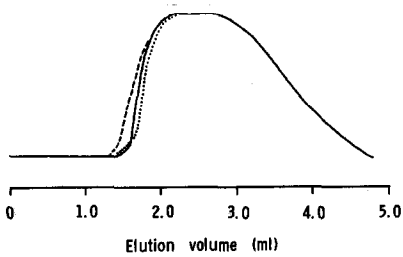

Fig. 1. Large-zone elution profiles of soluble Ca-ATPase in three different functional states. A 2-ml volume of delipidated protein (0.58 mg/ml) was injected into the small TSK GSWP column and eluted at 0.1 ml/min. Buffer composition: 20 m*M* Tes (pH 7.0), 0.1 *M* 10 m*M* magnesium chloride, 5 m*M* DTT, 1.0 m*M* EGTA and 0.15 mg $C_{12}E_8$/ml with 1.5 m*M* calcium chloride (– – –) or 1.5 m*M* calcium chloride plus 0.25 m*M* CrATP (———) or 0.1 m*M* Na_3VO_4 (· · · ·). Absorbance (ordinate scale) was read at 280 nm.

a covalently linked dimer isolated by small-zone HPLC after treatment of soluble Ca-ATPase with EGTA[13]. The assignment of the elution volumes of pure monomers and dimers of Ca-ATPase permits calculation of the apparent association constant of the reversible monomer–dimer equilibria from the data of Fig. 2 (see Discussion).

We also examined the possibility of using the small HPLC column for large-zone chromatography in the presence of phospholipid. This is of importance, since soluble Ca-ATPase in the delipidated form loses activity rapidly in the absence of protecting ligands such as Ca^{2+} or vanadate[14], whereas high stability is maintained when phospholipid is present. Fig. 2B shows that phospholipid induced a decrease in the upper limit of the elution volumes, which depended on the concentration of lipid added. Below 50 μg protein/ml the elution position in the presence of 1 mg phospholipid/ml was 1.78 ml ("M_L") regardless of the functional state of the protein (E_1Ca_2 and E_2V forms shown). Above 50 μg/ml the E_2V form was eluted at higher volume than was E_1Ca_2, consistent with an effect of the functional state on the monomer–oligomer equilibrium.

We did not notice any influence of lipid on the elution positions of water-soluble standard proteins (the Pharmacia kit consisting of thyroglobulin, ferritin, catalase and aldolase was tested, data not shown). Calibration of the column with these proteins using Ackers plot of R_s as a function of $\text{erf}^{-1}(1-K_D)$ (*cf.*, refs. 15 and

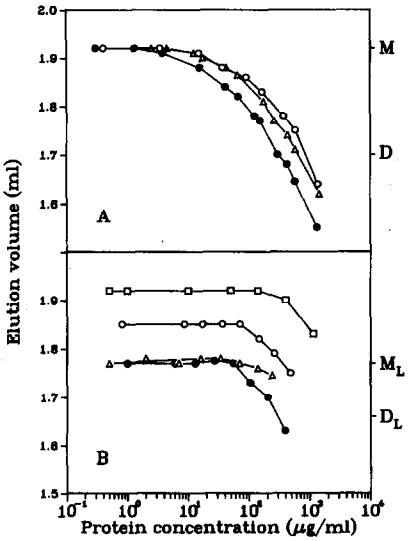

Fig. 2. Effects of ligand binding and phospholipid on centroid boundary elution volumes of large-zone experiments. (A) Delipidated Ca-ATPase chromatographed as in Fig. 1 at 0.15 mg $C_{12}E_8$/ml. ●, E_1Ca_2 form (1.5 m*M* calcium chloride added); △, $E_1P[Ca_2]$ form (1.5 m*M* calcium chloride plus 0.25 m*M* CrATP added); ○, E_2V form (0.1 m*M* $NaVO_4$ added). M and D indicate the elution positions of pure monomer and irreversibly associated dimer, respectively. (B) Ca-ATPase at 2.0 mg $C_{12}E_8$/ml and various phospholipid concentrations added to both the sample and eluent. Other conditions were similar to those in (A). □, E_1Ca_2 form in the absence of phospholipid. ○, E_1Ca_2 form in the presence of 0.6 mg egg lecithin/ml; ●, E_1Ca_2 form in the presence of 1.0 mg egg lecithin/ml; △, E_2V form in the presence of 1.0 mg egg lecithin/ml. M_L and D_L indicate the respective elution positions of pure monomer and irreversibly associated dimer in the presence of 1.0 mg egg lecithin/ml.

16) indicated that the reduction of the elution volume from 1.92 to 1.78 ml observed for Ca-ATPase corresponds to an increase in the apparent Stokes radius from 5.7 to 6.4 nm.

Equilibrium centrifugation studies in the analytical ultracentrifuge were performed in the presence of lipid to see whether the effect of lipid on the elution position was due to dimerization. Results obtained after centrifugation for 24-36 h at various speeds (9000–12 000 rpm) and protein concentrations (50–200 μg/ml) gave an average buoyant molecular weight, $M(1-v\rho)$ of 28 500 (S.D. = 1800, n = 4). The calculated protein molecular weight was 110 000 ± 6800, consistent with a single peptide chain[11]. Thus it can be concluded that the protein eluting at 1.78 ml in the presence of 1 mg egg lecithin/ml consists of monomeric Ca-ATPase. In accord with this conclusion we found that the elution volume of covalently linked dimer in the same condition was as low as 1.66 ml ("D_L").

DISCUSSION

Using the small TSK GSWP HPLC "precolumn" for large-zone chromatography of Ca-ATPase solubilized in active form, we have demonstrated that at high protein concentrations (relative to detergent), Ca^{2+}-occluded $E_1P[Ca_2]$ and vanadate-reacted E_2 forms are eluted at higher volumes than E_1Ca_2, whereas at low protein concentration the elution volumes of these three states are identical.

Previous column calibration data have indicated that the apparent Stokes radii of membrane proteins solubilized in detergent are higher than their true values, both when determined by classical gel filtration techniques and by size-exclusion HPLC[13,15,16]. For delipidated monomeric Ca-ATPase in $C_{12}E_8$, the Stokes radius obtained by hydrodynamic measurements is 5.1–5.6 nm, whereas this complex is eluted at a value corresponding to a radius of 5.6–5.8 nm from TSK G3000SW columns[13,15]. In the light of these results, one may ask whether an increase in the elution volume, as observed after formation of the Ca^{2+}-occluded $E_1P[Ca_2]$ form or the vanadate-reacted E_2 state, could reflect a change in the shape and detergent-binding properties of the soluble complexes without an accompanying change in the monomer–oligomer equilibrium. In this case one would expect a significant difference between the elution volumes of the various functional states, also at the low protein

TABLE I

ASSOCIATION CONSTANTS FOR THE MONOMER–DIMER EQUILIBRIUM OF SOLUBLE Ca-ATPase

Functional state	*$C_{12}E_8$ concentration (mg/ml)*	*K_a (M^{-1})**
E_1Ca_2	0.15	$1.35 \cdot 10^6$
$E_1P[Ca_2]$ ("occluded")	0.15	$0.62 \cdot 10^6$
E_2V (vanadate-reacted)	0.15	$0.44 \cdot 10^6$
E_1Ca_2 with phospholipid (1 mg/ml)	2.0	$0.72 \cdot 10^6$
E_1Ca_2	2.0	$< 10^5$

* Calculated according to eqn. 1, K_a being identical to the reciprocal of the Ca-ATPase concentration, for which half of the Ca-ATPase molecules are found in the monomeric state.

concentrations, where the protein is monomeric. Since this was not observed, it seems safe to conclude that we are in fact dealing with an effect on the monomer–oligomer equilibrium.

Assuming that the elution position of irreversibly associated dimer (covalently linked) is identical to that of reversibly associated dimer, the apparent monomer–dimer association constants can be calculated for the soluble Ca-ATPase protein under the various conditions studied, as shown in Table I. The changes in K_a, elicited by CrATP-induced Ca^{2+} occlusion and by formation of E_2V, are relatively small (2–3 fold decrease in K_a), but clearly significant (note the large number of data points in Fig. 2). A similar ligand-induced change in K_a as for delipidated Ca-ATPase was observed in the presence of phospholipid (Fig. 2B).

The apparent Stokes radius of the Ca-ATPase monomer in either the E_1Ca_2 or E_2V form was found to be significantly higher with phospholipid (6.4 nm) than in the delipidated form. This indicates that the protein *does* bind the lipid, despite the simultaneous presence of detergent. The bound lipid seems to interfere even more with the partition of the protein into the pores of the column material than does bound detergent. Interestingly, the R_s of 6.4 nm is comparable to the value of 6.3 nm measured on Sephacryl S-300 columns, in the absence of lipid added to the eluent, but with Ca-ATPase samples containing endogenous lipid[16].

Table I also indicates that lipid *per se* increases the association constant relative to that measured in a delipidated sample containing the same total concentration of detergent. This effect may be due to the formation of mixed micelles of lipid and detergent, reducing the concentration of pure detergent micelles. Since the critical micelle concentration (CMC) of $C_{12}E_8$ is 0.05 mg/ml, the difference between the K_a values of delipidated Ca-ATPase at 0.15 and 2.0 mg $C_{12}E_8$/ml suggests the involvement of micellar detergent in dissociation of the Ca-ATPase oligomer to monomers.

The use of the small TSK GSWP HPLC precolumn for large-zone chromatography as described here should prove useful also for studies of monomer–oligomer equilibria of other membrane proteins. In cases where the membrane protein under study is unstable in detergent solution, the short duration of a chromatographic experiment and the possibility of including lipid in the eluent may be of importance. With longer HPLC columns we found it difficult to avoid development of an high pressure in the presence of the high concentration of lipid used. Generally, since monomer–oligomer equilibria of proteins may be pressure dependent[17], the low pressure existing in the small column (less than 2 bar) is advantageous. Furthermore, only small quantities of protein are required for large-zone chromatography in this column, and many enzymes can be studied under conditions where substrate is being utilized. For Ca-ATPase we have found[18] the elution volume during hydrolysis of ATP to be identical to that observed for the CrATP-stabilized $E_1P[Ca_2]$ state. Since the resolution obtained with TSK GSWP is high, despite its small dimensions, it may be useful also in small-zone experiments, if only minimum quantities of protein are available.

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