Journal of Chromatography, 646 (1993) 153–157
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2846

Separation of membrane-embedded tryptic peptides of Na,K-ATPase by size-exclusion chromatography

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ABSTRACT

Several attempts to separate hydrophobic tryptic and cyanogen bromide-digested short peptides from Na,K-ATPase, using HPLC and different acid–organic solvent gradients, failed because of the insolubility of the peptides in the initial or final solvents of the gradients used for elution. Therefore, we opted to use a detergent-containing mobile phase. For sodium dodecyl sulphate-solubilized tryptic peptides of M_r $7 \cdot 10^3$–$100 \cdot 10^3$, elution on a TSK-G3000SW size-exclusion column successfully separates families of peptides with a resolution of M_r $5 \cdot 10^3$–$10 \cdot 10^3$. Peptides in these size ranges can then be resolved completely by tricine–sodium dodecyl sulphate gel electrophoresis, and identified by microsequencing after transfer to polyvinylidene difluoride paper. For separation of smaller peptides a Biosep-SEC-S2000 column, eluted at slow flow-rates, was evaluated. Use of ammonium chloride buffer allows sensitive detection at 214 nm. The separated fractions are resolved and identified on 16.5% tricine gels. Reasonable resolution has been obtained with defined cyanogen bromide fragments of myoglobin. Resolution of small tryptic and cyanogen bromide fragments of Na,K-ATPase is less successful, but the experiments suggest ways of improving the resolution of peptides in the range M_r $2 \cdot 10^3$–$10 \cdot 10^3$.

INTRODUCTION

A well known problem in work with hydrophobic peptides is the difficulty in separating these peptides by reversed-phase HPLC owing to problems of solubility in the eluting solvents. In the course of our work with Na,K-ATPase we were confronted with this problem in connection with separation of hydrophobic tryptic peptides. Pig renal Na,K-ATPase, extensively digested by trypsin in the presence of Rb^+, produces membranes containing a stable M_r 19 000 fragment and smaller fragments (M_r $7 \cdot 10^3$–$11 \cdot 10^3$) of the α-chain and a largely intact β-chain [1]. We have made several attempts to separate these peptides and even smaller fragments of cyanogen bromide-digested M_r 19 000 fragment with a reversed-phase C_3 column eluted with a gradient of acidic water–2-propanol [2], or a C_{18} column eluted with a gradient of formic acid–ethanol [3], or a polyhydroxyethyl aspartamide column eluted with 50 m*M* formic acid [4]. All of these methods failed because of lack of solubility of the peptides in either the starting or ending solvent. After injecting radioactively labelled peptides {with 3-(trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine; [5]} we recovered in all cases less than 50% of the original radioactivity. Therefore, we opted for a detergent-containing mobile phase in order to ensure solubility of the peptides at all times. This publication describes results obtained by combining separation on size-exclusion columns in a sodium dodecyl sulphate (SDS)-containing buffer with electrophoretic separation of peptides using the tricine SDS–polyacrylamide gel electrophoresis (PAGE) system of Schagger and von Jagow [6].

These procedures provide a reliable method for separating hydrophobic peptides in the range

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0021-9673/93/$06.00

M_r $7 \cdot 10^3$–$100 \cdot 10^3$, and with some additional improvements, may become applicable for separating hydrophobic peptides in the range M_r $2 \cdot 10^3$–$10 \cdot 10^3$.

EXPERIMENTAL

Na,K-ATPase preparation, trypsin treatment, tricine–SDS-gel electrophoresis and transfer to polyvinylidene difluoride (PVDF) paper were done essentially as previously described [7].

Cyanogen bromide treatment

The M_r 19 000 peptide separated by TSK–HPLC (fraction 3, *ca.* 0.5 mg) was precipitated by addition of four volumes of ice-cold methanol and collected by centrifugation at 12 000 *g* for 30 min. The supernatant was discarded and the pellet was dissolved in 1 ml of 80% formic acid. Tryptophan was added to 1 μM and then cyanogen bromide, freshly dissolved in 80% formic acid, was added at 3 mg per mg of protein. The solution was purged with nitrogen and the tube sealed under nitrogen and incubated for 5 h in the dark at room temperature. Excess reagent and its products of reaction were removed by adding 1 ml of water and lyophilizing twice. A blank reaction was performed with all the components except the protein.

Size-exclusion HPLC separation of peptides

The HPLC system consisted of an injector (Rheodyne Model 7125, Cotati, CA, USA), a pump (Waters Model 501, Milford, MA, USA), a Waters 441 absorbance detector and a recorder (Omniscribe Series 5000, Houston Instrument, Bellaire, TX, USA).

For the separation of trypsinized membranes, a TSK-G3000SW column (600 × 7.5 mm, Toyo Soda Manufacturing, Tokyo, Japan) connected in series with a TSK-SW guard column (75 × 7.5 mm) was used. Elution was at 0.2 ml/min using a solution of 100 mM sodium acetate, pH 4.5, and 0.5% SDS. Detection was at 280 nm and fractions were collected manually.

For the separation of smaller peptides, a Biosep-SEC-S2000 column (600 × 7.8 mm, Phenomenex, Torrance, CA, USA) with a Biosep-SEC-S guard column (75 × 7.8 mm) was used. For analytical purposes the mixtures were eluted at 0.2 ml/min with a solution of 100 mM ammonium chloride, pH 4.5, plus 0.5% SDS, and peaks were detected by their absorption at 214 nm.

For preparative separations in non-reducing conditions the same column was eluted with 100 mM sodium acetate, pH 4.5, plus 0.5% SDS, and detection was at 280 nm.

For preparative separations in reducing conditions, the samples were dissolved in a solution of 2% SDS plus 20 mM dithiothreitol (DTT). Before injection, 5 mM DTT was added to the acetate buffer and the solution was purged with nitrogen before use.

Low-molecular-mass markers were cyanogen bromide fragments from myoglobin (M_r $2.5 \cdot 10^3$–$16.9 \cdot 10^3$) purchased from Merck (Cat. No. 15124).

RESULTS AND DISCUSSION

Size-exclusion separation of M_r 7000 and larger peptides

Fig. 1A shows a separation of SDS-solubilized trypsin-treated Na,K-ATPase into four well-resolved families of peptides, by size-exclusion HPLC on a TSK-3000 column under non-reducing conditions. Protein in each fraction was precipitated by addition of four volumes of ice-cold methanol and aliquots were applied to a 16.5% tricine gel (Fig. 1B, lanes 1–4) and, for comparison, an equivalent sample of the original tryptic digest is shown in Fig. 1B, lane T. The recovery of protein is essentially quantitative. Fraction 1 contains a mixture of aggregates and high-molecular-mass impurities. Fraction 2 contains the intact β-chain as well as both the M_r 50 000 and 16 000 fragments of it. These fragments are connected by an S–S bridge and therefore behave exactly like the intact β-chain on the column, but separate under the reducing conditions of the gel. Fraction 3 contains mainly the M_r 19 000 peptide and fraction 4 is a mixture of the seven smaller peptides of molecular mass 7600–11 700. The far right-hand lane shows concentrated peptides from fraction 4 of another experiment, next to marker peptides (lane M). The importance of this chromatographic method

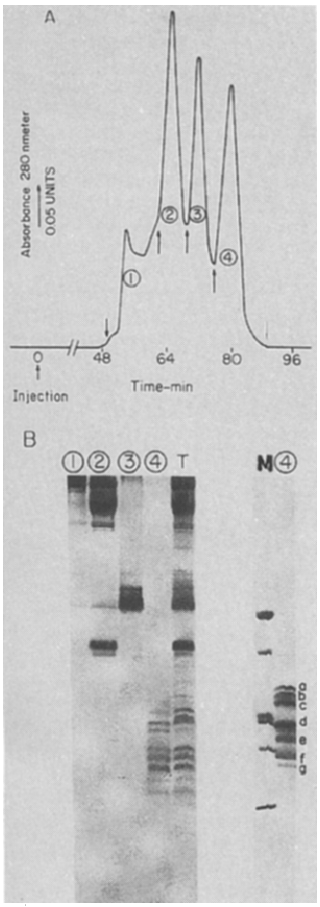

Fig. 1. Peptides of tryptic Na,K-ATPase separated by size-exclusion HPLC (A) and resolved on 16.5% gel (B). (A) A 1-mg aliquot of protein precipitated from the digest was dissolved in SDS and applied to the TSK-G3000SW columns. The arrows indicate times at which fractions were collected (2–4 ml per fraction). The protein in each fraction was precipitated again by adding four volumes of ice-cold methanol. (B) Equal proportions of fractions 1–4, equivalent to the amount of protein in 100 μg of the original digest, were applied to lanes 1–4 (taking into account the loss due to the precipitation) and, for comparison, 100 μg of the unfractionated digest were applied to lane T. Lane M shows marker peptides (M_r 2.5, 6.2, 8.2, 10.7, 14.4 and $16.9 \cdot 10^3$) next to the second lane 4 (70 μg of protein from fraction 4 of a separate experiment).

derives from its extremely high reproducibility, which makes it useful as a routine method for separating and concentrating individual peptides (β-chain and M_r 19 000) or families of shorter peptides of M_r $7.6 \cdot 10^3$–$11.7 \cdot 10^3$. By combining this HPLC method to partially separate and concentrate fraction 4 with the high resolution of 16.5% tricine gels we determined the identity of all the peptides in fraction 4. Stained bands from the 16.5% gel were cut out and placed in wells of a second 10% gel, run and (without fixing or staining) electroblotted onto PVDF paper [8]. Strips of this PVDF membrane were used to obtain the amino acid sequence of each peptide (a–g, in far right-hand lane). The knowledge of the N-terminal sequence and the molecular mass from the gel allowed location of the fragments in the primary sequence of the α-chain [7]. The same strategy was successfully used for the sequence determination of the cyanogen bromide fragments of the M_r 19 000 peptide [9].

Size-exclusion HPLC separation of small peptides

We have found that a size-exclusion Biosep-SEC-S2000 column is useful for the separation of short water-soluble peptides, as can be seen in Fig. 2A. A standard mixture of cyanogen bromide fragments of myoglobin (M_r $2.5 \cdot 10^3$, $6.2 \cdot 10^3$, $8.2 \cdot 10^3$, $10.7 \cdot 10^3$, $14.4 \cdot 10^3$ and $16.9 \cdot 10^3$) was clearly resolved. The peaks were collected, ice-cold methanol was added, and protein was precipitated by centrifugation. The pellets were dissolved in SDS and proteins separated on a 16.5% tricine gel (Fig. 2B). Fraction 1 contains only high-molecular-mass aggregates or impurities; fraction 2 is the clean M_r 16 900 peptide; fraction 3 is enriched in the M_r 14 400 peptide; fraction 4 is the combined M_r 8200 + 10 700 peptides; fractions 5 and 6, although clearly detected by 214 nm absorption in the HPLC detector, are undetectable in the gel and probably contain the two shortest peptides (M_r 6.2 and $2.5 \cdot 10^3$) which have been lost due to solubility in methanol. Measurement of absorption at 214 nm in analytical separations increases the sensitivity of detection and is possible with the use of an ammonium chloride buffer, which is transparent at this wavelength.

The application of this method for partial separation of peptides derived from Na,K-ATPase is demonstrated in Fig. 3. In this experiment, peptides that elute as a single peak in the fourth fraction from the TSK-G3000SW separation were applied to the SEC-S-2000 column. In

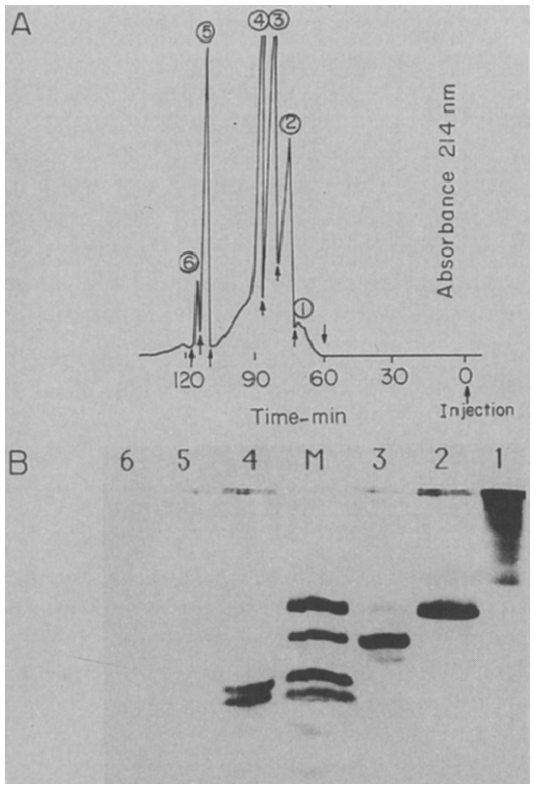

Fig. 2. Peptide markers separated by size-exclusion HPLC (A) and resolved on 16.5% gel (B). (A) A 150-μg aliquot of peptide markers (cyanogen bromide fragments from myoglobin, molecular mass 2512, 6214, 8159, 10 671, 14 404 and 16 949) was dissolved in the ammonium chloride running buffer and applied to the Biosep-SEC-S-2000 column. The arrows indicate times at which fractions were collected. The protein in each fraction was precipitated by addition of four volumes of ice-cold methanol. (B) The pellets were dissolved in 40 μl of sample buffer and applied to lanes 1–6. Lane M shows the mixture of marker peptides.

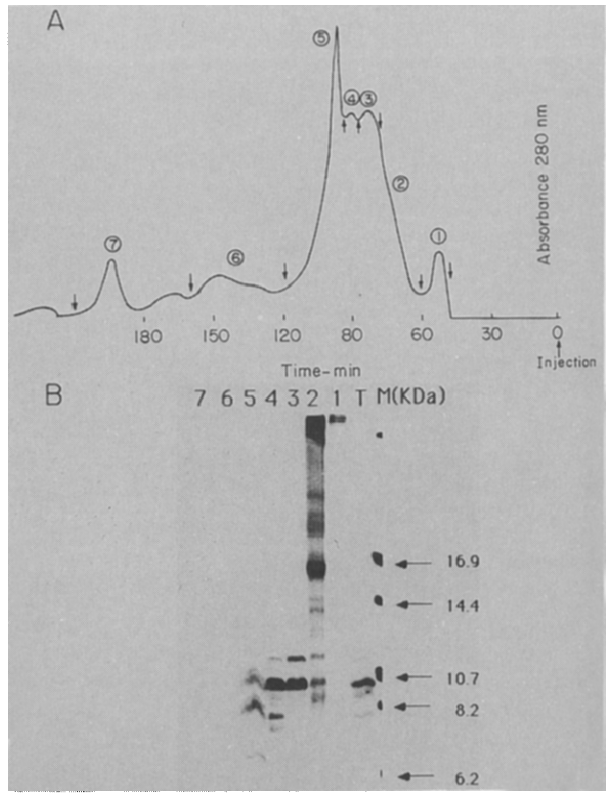

Fig. 3. Short peptides from trypsinized Na,K-ATPase separated by size-exclusion HPLC (A) and resolved on 16.5% gel (B). (A) A 225-μg aliquot of short peptides arising from trypsin-treated Na,K-ATPase, separated in a TSK-3000 column as a single peak (Fig. 1, fraction 4), were dissolved in SDS and applied to a Biosep-SEC-S-2000 column. The arrows indicate times at which fractions were collected. The protein in each fraction was precipitated by addition of four volumes of ice-cold methanol. (B) The pellets were redissolved in 40 μl of sample buffer and applied to lanes 1–7, and for comparison 100 μg of non-separated mixture were applied to lane T, and marker peptides to lane M (with the molecular mass as indicated by arrows). KDa = kilodalton.

spite of the very narrow window of molecular masses (seven peptides in a range of M_r $7.6 \cdot 10^3$–$11.7 \cdot 10^3$), several peaks were observed and were collected (Fig. 3A). In the 16.5% tricine gel it can be seen that a partial separation of the low-molecular-mass peptides is achieved (Fig. 3B). No single peptides are observed, but rather combinations arranged by molecular masses. Lane M contains the peptide markers, lane T is 100 μg of the total mixture before separation and lanes 1–7 are the fractions collected as indicated by arrows in Fig. 3A. Lane 2 shows more peptides that can be seen in the total (lane T) because it is highly concentrated.

We also attempted to separate cyanogen bromide fragments of the M_r 19 000 peptide (fraction 3 in the TSK column) with an SEC-S-2000 column (Fig. 4A). Peaks separating in the HPLC are observed, but SDS-PAGE analysis shows that these peaks contain "smears", proba-

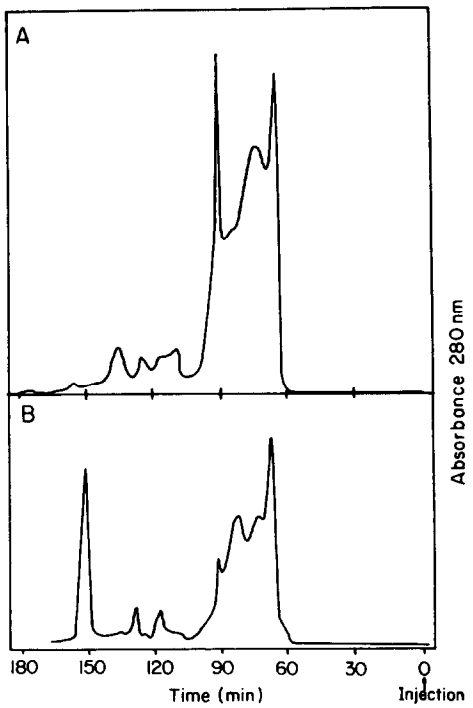

Fig. 4. Peptides from cyanogen bromide fragmented M_r 19 000 protein separated by size-exclusion HPLC in non-reducing (A) and reducing conditions (B). (A) A 225-μg aliquot of peptides arising from cyanogen bromide-treated M_r 19 000 protein (Fig. 1, fraction 3) was dissolved in SDS and applied to a Biosep-SEC-S-2000 column. The protein in each fraction was precipitated by addition of four volumes of ice-cold methanol. (B) The same mixture of peptides (225 μg) dissolved in 2% SDS plus 20 m*M* dithiothreitol (DTT) and run in the same column with the addition of 5 m*M* DTT to the eluent buffer.

bly due to cross-linkings, S–S bridges, etc. When the HPLC separation was done under reducing conditions (Fig. 4B), the shape of the chromatogram changed, but in the gel the "smears" can still be seen, indicating that reduction, although somewhat effective, was insufficient. Stronger reducing agents or conditions may be needed to separate these extremely hydrophobic peptides into single components by the SEC-S-2000 column. These experiments are in progress in our laboratory.

ACKNOWLEDGEMENT

This work was supported in part by United States Public Health Service Grant 322286.

REFERENCES

1 S.J.D. Karlish, R. Goldshleger and W.D. Stein, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 4566–4570.
2 A.K. Taneja, S.Y.M. Lau and R.S. Hodges, *J. Chromatogr.*, 317 (1984) 1–10.
3 Y. Takagaki, G.E. Gerber, K. Nihei and H.G. Khorana, *J. Biol. Chem.*, 255 (1980) 1536–1541.
4 P.C. Andrews and A.J. Alpert, presented at the *10th International Symposium on High-performance Liquid Chromatography, Wiesbaden, Oct. 29, 1990*, poster 110.
5 S. Hoving, D.M. Tal and S.J.D. Karlish, unpublished results.
6 H. Schagger and G. von Jagow, *Anal. Biochem.*, 166 (1987) 368–379.
7 J.M. Capasso, S. Hoving, D.M. Tal, R. Goldshleger and S.J.D. Karlish, *J. Biol. Chem.*, 267 (1992) 1150–1158.
8 P. Matsudaira, *J. Biol. Chem.*, 262 (1987) 10035–10038.
9 R. Goldshleger, D.M. Tal, J. Moorman, W.D. Stein and S.J.D. Karlish, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 6911–6915.