Journal of Chromatography, 296 (1984) 301–308
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 324

METHODOLOGY FOR PURIFICATION OF LARGE HYDROPHOBIC PEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

XAVIER GRANDIER-VAZEILLE and DANIEL TETAERT*
Unité INSERM No. 16 and L.A. CNRS 268, Place de Verdun, 59045 Lille Cédex (France)

SUMMARY

To aid in structural studies of pig cardiac myosin light chains (L_{27} and L_{28}), a procedure of ion-exchange chromatography (IEC) on Trisacryl M (noted for its high capacity) in combination with reversed-phase high-performance liquid chromatography (RP-HPLC) and volatile buffers has been developed. In contrast with other IEC methods (resins or HPIEC), the use of Trisacryl M facilitates subsequent peptide purifications by RP-HPLC. The advantage of the present combination of techniques is also that it enables the isolation of hydrophobic peptides in high yield, *e.g.*, the N-terminal chymotryptic peptide from L_{27} was thus purified and, after subdigestion with trypsin, its sequence has been established.

INTRODUCTION

Myosin is the principal contractile and structural protein comprising thick filaments in various muscle cells. It consists of two heavy chains ($\approx$200,000 daltons) and two pairs of light chains ($\approx$20,000 daltons), referred to as the essential (or alkali) pair and the regulatory pair, and denoted respectively as L_{27} and L_{18} for pig ventricular myosin[1,2]. The function of the light chains is not clearly understood, but there is a considerable body of evidence to suggest that they in some way determine the enzymatic characteristics of the actomyosin. More information about the role of the light chains could also be obtained by determining their amino acid sequence. However, chemical or enzymatic cleavages of L_{27} release large hydrophobic peptides, rich in proline and alanine residues: for instance, the N-terminal BrCN peptide (CB_1-peptide: residues 1–66) and the chymotryptic one (C_1-peptide: residues 1–20). These larger peptides from L_{27} often behave poorly in conventional chromatographic systems, because of insolubility, aggregation or irreversible adsorption to column materials, although high concentrations of glycerol, detergent or salt have been used[2,3]. In addition, the presence of proline residues in the N-terminal sequence causes significant decreases in the repetitive yields of the Edman degradation.

With the introduction of mobile phases capable of dissolving large denatured peptides, reversed-phase high-performance liquid chromatography (RP-HPLC) has been developed to optimize both resolution and yield[4]. However, not all peptide mixtures can be resolved by using only one RP-HPLC step[4,5]. A combination of a

0021-9673/84/$03.00

preparative technique and RP-HPLC has proved to be a more powerful separation method[5].

To facilitate the recovery of the hydrophobic CB_1 and C_1 peptides from L_{27} (excellent probes for testing purification procedures), we report here the combination of ion-exchange chromatography (IEC) on DEAE-Trisacryl M columns and RP-HPLC, using volatile buffers. Hence, the isolation and characterization of the hydrophobic C_1-peptide from pig ventricular myosin L_{27} light chain could be performed and we were then in a position to establish the N-terminal sequence of cardiac myosin alkali light chain, which has not previously been described for higher vertebrates.

EXPERIMENTAL

Chemicals

Bovine trypsin, treated with L-(1-tosylamido-2-phenylethyl) chloromethyl ketone (TPCK) and bovine α-chymotrypsin, treated with L-(1-tosylamido-2-lysylethyl) chloromethyl ketone (TLCK), were from Sigma. Sequanal grade trifluoroacetic acid (TFA) and reagents for sequence analysis were from Pierce. Ninhydrin and cyanogen bromide were obtained from Merck. Glass-distilled chromatography grade 1-propanol, 2-propanol, ammonia, acetic acid and formic acid were purchased from Carlo Erba. Pyridine was obtained from Merck and purified by refluxing for several hours with ninhydrin and then distilled: only the constant-boiling fraction (b.p. 115.2°C) was collected.

Sample preparations

Pig cardiac left ventricular myosin light chains (L_{27} and L_{18}) were purified as described earlier[2]. Following reduction and S-alkylation, the light chain L_{27} was dissolved in 70% formic acid and cleaved with cyanogen bromide[2]. Tryptic digestion was performed in 50 m*M* ammonium bicarbonate buffer pH 8.75 at 37°C for 4 h with an enzyme: substrate ratio of 2:100 (w/v). Chymotryptic digestion was performed in 50 m*M* ammonium bicarbonate buffer pH 8.0 (adjusted with formic acid) at 37°C for 2 h with an enzyme: substrate ratio of 1:100 (w/w).

High-performance liquid chromatography

The HPLC method was developed using a Varian Model 5000 system. Peptides were detected with an UV absorbance detector LKB 2238 Uvicord S_{II} and were collected with a 202 Gilson collector. The following RP-HPLC columns were used: from Waters Associates, semi-preparative μBondapak (two 60 × 7.8 mm), C_{18} μBondapak, phenyl (RØ) μBondapak (30 cm × 3.9 mm with 10-μm packing); from Beckman, 5-μm Ultrasphere ODS, Ultrasphere-Octyl (25 cm × 4.6 mm); from Varian, Micropak MCH_{10} and MCH_5 (25 cm × 4.6 mm); from Merck, LiChrosorb C_8 (25 cm × 4 mm with 5- and 7-μm packings).

L_{27} digests were introduced into C_8 and RØ-HPLC columns and eluted at a flow-rate of 1 ml/min. Measurements were made at 226 or 206 nm, in the sensitivity range of 0.5–2.0 absorption units full scale. The following gradient systems were employed: (a) 0.0013 *M* TFA in water to 60% 1-propanol–0.0013 *M* TFA or 2-propanol–TFA; 1b) ammonium formate pH 7.8 (1.6 ml of 25% ammonia + 0.25 of

TABLE I

DISCONTINUOUS PYRIDINE–FORMATE GRADIENT USED FOR IEC ON DEAE-TRISACRYL M

Time (h)	*Pyridine (M)*	*pH*
0 – 8	0.2	8.4
8 –18.5	0.2	8.4 –6.6
18.5–29.5	0.2–0.6	6.6
29.5–37.5	0.6–1.0	6.6
37.5–41.5	1.0	6.6 –5.25
41.5–55	1.0	5.25–4.0
55 –58.5	1.0	4.0 –3.5
58.5–60.5	1.0	3.5 –3.0
60.5–69	1.0–2.0	3.0

98% formic acid per 2 l water) to 60% 1-propanol. The buffers were filtered through a 0.22-μm Millipore membrane before use.

Ion-exchange chromatography

Separation of peptides by IEC was carried out on DEAE-Trisacryl M (from LKB) columns (25 × 2.5 cm) at a flow-rate of 150 ml/h. The column was preequilibrated with 0.2 *M* pyridine pH 8.4 and then developed in a discontinuous gradient of pyridine–formate buffer as shown in Table I. The column eluates were automatically monitored (Technicon Autoanalyzer) by a ninhydrin colorimetric assay.

Amino acid analysis

Amino acid compositions were determined using a Beckman 119 C amino acid analyzer after hydrolysis of the dried peptides with constant-boiling 6 *M* HCl at 110°C in evacuated sealed tubes for 24 and 72 h. Automated Edman sequence analyses were performed on a Beckman 890 C sequencer. The sequanator program and PTH amino acid determinations have been described[6].

RESULTS

Analyses and purity of pig ventricular myosin light chain L_{27}

The myosin light chains were isolated from pig heart left ventricle by preparative polyacrylamide gel electrophoresis as previously described[2]. The purity of L_{27} light chain was tested by one-dimensional gel electrophoresis and also by identification of the higher-molecular-weight BrCN peptide CB_1[2]. Because CB_1 failed to release any PTH amino acid in an Edman experiment, it contained the blocked NH_2 terminus of L_{27}. (The N-blocked amino acid residue is probably trimethylalanine.) In addition, the high content of proline and alanine residues essentially characterized the N-terminal sequence of L_{27} and the CB_1-peptide[2].

Separation of L_{27} chymotryptic and BrCN digests by direct RP-HPLC

The peptide mixtures obtained after chymotryptic digestion and cyanogen bromide cleavage from pig heart left venticular myosin light chain L_{27} have been

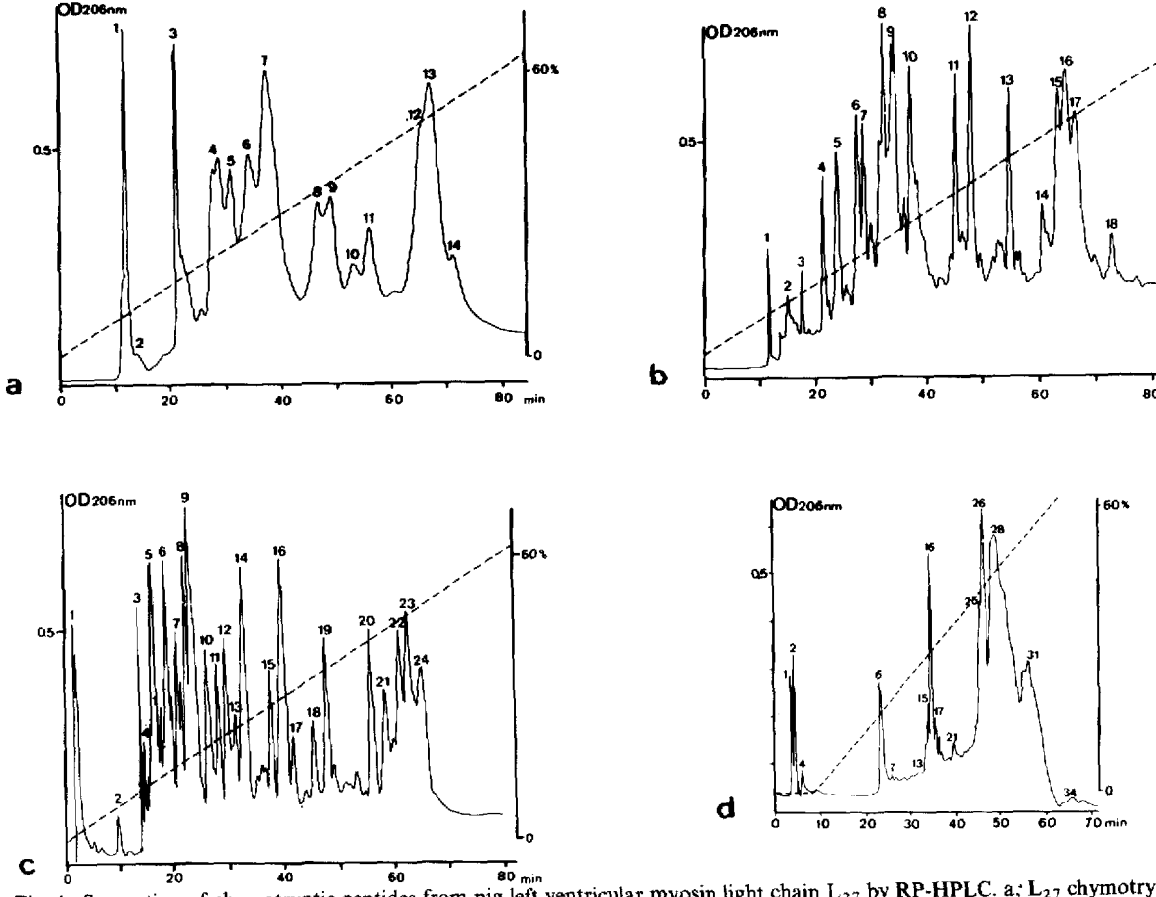

Fig. 1. Separation of chymotryptic peptides from pig left ventricular myosin light chain L_{27} by RP-HPLC. a; L_{27} chymotryptic digest (0.5 μmol) was applied to a Waters μBondapak C_{18} semi-preparative column (two 60 cm × 7.8 mm) and eluted with a 80-min linear gradient from 0 to 60% of 2-propanol in 0.0013 *M* TFA. Temperature: ambient. Flow-rate 3 ml/min. b, L_{27} chymotryptic digest (80 nmol) was applied to a Waters μBondapak C_{18} column (30 cm × 3.9 mm with 10-μm packing) and eluted with a 80-min linear gradient from 0 to 60% of 2-propanol in 0.0013 *M* TFA. Temperature: ambient. Flow-rate 1 ml/min. c, as in b but with a Merck (5-μm packing) C_{18} column (25 cm × 4 mm) and at a flow-rate of 0.5 ml/min. d, L_{27} cyanogen bromide fragments (100 nmol) was applied to a Waters μBondapak phenyl column (30 cm × 3.9 mm with 10-μm packing) and eluted with a 70-min linear gradient from 0 to 60% of 1-propanol in 0.0013 *M* TFA. Temperature: ambient. Flow-rate: 1 ml/min.

separated by RP-HPLC. Different experimental procedures were investigated to find optimal separation conditions for large hydrophobic peptides (C_1 and CB_1) on various RP-HPLC columns. Peptide elution was recorded at 206 and 226 nm and the peaks obtained were analyzed for amino acid composition and N-terminal residue.

The chymotryptic digest was first fractionated on the semi-preparative Waters columns; 0.5 μmol were applied and only fourteen peaks were obtained (Fig. 1a). The yield of the isolated fractions, based on the amount of L_{27} digested, was very low (5–10%). On the other hand, on analytical columns (Waters μBonpak, Merck C_8) about 30 peaks and shoulders were detected (Fig. 1b,c). The capacity of these analytical columns was low (40–80 nmol of digest) and the hydrophobic peptide C_1 was eluted as a well defined peak at the same gradient position (55% 1-propanol) whatever the analytical column used. However, recoveries of L_{27} chymotryptic peptides from the analytical columns were similar with about 1/10 of the injected sample. In addition, all the isolated fractions were not pure enough for direct sequence analysis, according to the amino acid compositions and PTH amino acid determinations. It should also be noted that, whith other supports tested, one or more of the peptides were not eluted (not shown).

Comparable results were also found for the RP-HPLC fractionation of BrCN peptides from L_{27} (CB-peptides). Only eight CB-peptides were released from L_{27}, but their separation was not complete even under optimal conditions (Fig. 1d). Moreover, drastic losses of the hydrophobic peptide CB_1 were observed.

Nevertheless, pure hydrophobic peptides CB_1 and C_1 were isolated by repeated RP-HPLC. Depending on the chromatographic behaviour of the peptide mixture, the elution gradient was optimized for each hydrolysate or rechromatography (not shown). The amount of peptide was not sufficient for subsequent amino acid analyses of the N-blocked sequences. In addition, the peptides were obtained at several retention times due to desamidation and/or the presence of homoserine lactone.

Isolation of the C_1-peptide

In performing IEC on DEAE-Trisacryl M, our objective was to purify quantitatively the N-blocked C_1-peptide generated by chymotrypsin digestion of L_{27}. The chymotryptic digest (5 μmol) was applied to the DEAE-Trisacryl M column and, using volatile buffers (pyridine–formate), the elution pattern obtained after ninhydrin reaction revealed nine major fractions (1–9) and nine minor fractions (A–K) (Fig. 2). These fractions were analyzed for amino acid composition and PTH amino acid determination. Fraction 3, well separated from the others, contained mainly the C_1-peptide. Because of the large amount (4 μmol) of fraction 3 isolated by IEC on DEAE-Trisacryl M, the C_1-peptide could be further purified by RP-HPLC. Despite the greater eluting power of 1-propanol relative to other solvents, the C_1-peptide was eluted at high concentration (55% 1-propanol) and emerged as a broad peak (Fig. 2B). Nevertheless, RP-HPLC was the most efficient produre for the final purification step of the C_1-peptide. The amount of sample isolated (1 μmol) was sufficient for subsequent amino acid analyses, different subdigestions and sequence analyses.

Determination of C_1-peptide amino acid sequence

To confirm the probable N-blocked covalent structure of L_{27} reported earlier using sequence homology[3], the C_1-peptide was subdigested with trypsin. Although

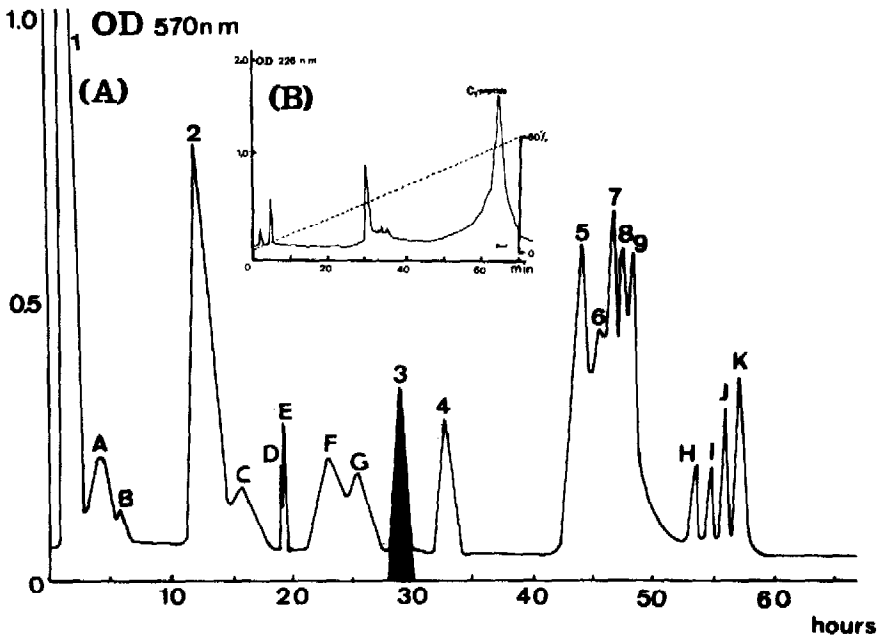

Fig. 2. Separation of the chymotryptic digest from pig left ventricular myosin light chain L_{27} on DEAE-trisacryl M and purification of the C_1-peptide by RP-HPLC. A, L_{27} chymotryptic digest (5 μmol) was applied to a DEAE-Trisacryl M ion-exchange (column (25 × 2.5 cm) and eluted with the discontinuous pyridine–formate gradient described in Table I at room temperature and with a flow-rate of 150 ml/h. The elution profile was obtained after ninhydrin reaction. B (inset), Fraction 3 from the IEC on DEAE-Trisacryl M was lyophilized, then dissolved in 0.2 ml water containing 0.0013 *M* TFA and applied to a Waters μBondapak phenyl column (30 cm × 3.9 mm). It was eluted with a 70-min linear gradient of 0 to 60% 1-propanol in 0.0013 *M* TFA at a flow-rate of 1 ml/min.

C_1-PEPTIDE

X-Pro-Lys-Lys-Pro-Glu-Pro-Lys-Ala-Ala-Pro-Ala-Pro-Ala-Glu-Ala-(Asp,Ser,Glu,Ile)-Phe

(1 → 20)

AMINO ACID COMPOSITION OF THE C_1-PEPTIDE

Asp	1,3	(1)
Ser	0.9	(1)
Glu	3.3	(3)
Pro	4.9	(5)
Ala	4,7	(5)
Ile	1,4	(1)
Phe	1,2	(1)
Lys	2,5	(3)

Fig. 3. Amino acid composition of the C_1-peptide from pig ventricular light chain L_{27}. The residues underlined with an arrow were determined by automatic Edman degradation. The composition was established after hydrolysis for 24 and 72 h with 6 *M* HCl. Amino acid residues are numbered according to Frank and Weeds[13]. X represents the N-blocked residue (trimethylalanine).

the Lys-Lys (residues 2 and 3) and the Lys-Ala (residues 7 and 8) sequences have been characterized, the C_1-peptide was only cleaved by trypsin at Lys-7 (Fig. 3): preliminary RP-HPLC fractionations led us to obtain two tryptic peptides and the uncleaved C_1-peptide. The tryptic digest from the C_1-peptide was thus submitted to automatic Edman degradation. Despite the presence of proline residues (positions 10 and 12), eight residues were identified. According to the amino acid composition and sequence determination, the N-terminal sequence of the alkali light chain L_{27} from pig ventricular myosin was identified with high accuracy (Fig. 3).

DISCUSSION

As a part of our interest in the structure of contractile proteins, we have reported on the primary structure of ventricular myosin light chains from higher vertebrates[2,7]. Attempts to determine the complete amino acid sequence of cardiac myosin light chain from higher vertebrates (human and beef[8,9]) were unsuccessful because of the hydrophobic and acidic character of L_{27}, particularly of its N-blocked terminal part.

Since the development of HPLC, new possibilities exist for purifying hydrophobic peptides[4,10]. Although RP-HPLC has proven to be a fast and efficient method, separations of large hydrophobic peptides can pose special problems. For example, the recovery of our pig L_{27} hydrophobic peptides from RP-HPLC was not great[11]. In addition, a mixture of about 30 chymotryptic peptides from L_{27} was not completely resolved in one RP-HPLC step. After repeated RP-HPLC the yields decreased and the elution of the same peptide often gave more than one HPLC peak (due to chemical changes).

We have developed a combination of IEC on Trisacryl M (a new support noted for its high capacity) with RP-HPLC and volatile buffers. Elution of the Trisacryl M column by pyridine and formic acid allowed the use of gradients with easily adjustable pH and/or ionic strength over a very wide range. The application of this chromatographic procedure had several advantages compared with other IEC methods of peptide purification: easy recovery of sample (without desalting steps) by evaporation or lyophilization, high yields, speed (using a high flow-rate at moderate pressure), flexibility, resolution, sensitivity (reactions with fluorescamine and *O*-phthaldehyde were also commonly performed) and chemical stability of the column material.

It should be noted that with other ion exchangers, *e.g.*, polystyrene resin, the particles of resin and peptides interacted and they were eluted in the same fractions[3]. This non-specific adsorption prevented any subsequent RP-HPLC fractionation. In contrast, combination of IEC on Trisacryl M and RP-HPLC has been used with excellent results for separation of hydrophobic and/or neutral peptides, for example, those generated from the pig cardiac myosin light chain L_{18} and using SP-Trisacryl M support (not shown).

In this novel combination of methods, HPLC remained the most suitable step in the purification procedure. It allowed us to isolate homogeneous peptide samples of unexpectedly high quality for sequence analysis. However, we have found our IEC procedure to be a useful and complementary addition to the limited set of methods currently available to purify large peptides. For example, the Trisacryl M chromato-

graphy enabled us to isolate the L_{27} N-terminal chymotryptic C_1-peptide in high yield. Despite the presence of charged amino acids (three Lys, three Glu and one Asp), this C_1-peptide had similar chromatographic behaviour to the very hydrophobic peptides (elution with 55% of 1-propanol). A special conformation and/or the core formed by the proline and alanine residues (8–13) in the C_1-peptide certainly must contribute to this unusual elution behaviour. Nevertheless, quantitative isolation of the C_1-peptide enabled us to establish its sequence. The determination of this N-terminal sequence of pig alkali light chain L_{27}, not previously reported for any higher vertebrate cardiac myosin light chain, was the most difficult part of the L_{27} structure. Moreover, this work now in progress has led us to compute, from the L_{27} primary structure, the secondary structure of the molecule as well as of its N-terminal part. Additional circular dichroism studies[12] confirmed the calculated secondary structure and explained the contribution of calcium ions to the conformation of the myosin alkali light chain.

REFERENCES

1 S. Sarkar, F. A. Srèter and J. Gergely, *Proc. Nat. Acad. Sci. U.S.A.*, 68 (1971) 946.
2 X. Grandier-Vazeille and D. Tetaert, *Biochimie*, 60 (1978) 409.
3 X. Grandier-Vazeille, D. Tetaert, B. Hémon, K. K. Han and G. Biserte, *Protides Biol. Fluids Proc. Colloq.*, 28 (1980) 75.
4 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
5 D. Tetaert, N. Takahashi and F. W. Putnam, *Anal. Biochem.*, 123 (1982) 430.
6 P. Sautière, G. Briand, M. Gusse and P. Chevallier, *Eur. J. Biochem.*, 119 (1981) 251.
7 D. Tetaert, X. Grandier-Vazeille, B. Hémon and G. Biserte, *J. Muscle Res. Cell Motil.*, 3 (1982) 474.
8 A. G. Weeds, *FEBS Lett.*, 59 (1975) 203.
9 C. Klotz, J. J. Leger and M. Elzinga, *Circ. Res.*, 50 (1982) 201.
10 G. E. Tarr and J. W. Crabb, *Anal. Biochem.*, 131 (1983) 99.
11 X. Grandier-Vazeille, D. Tetaert, B. Hémon and G. Biserte, *Protides Biol. Fluids Proc. Colloq.*, 30 (1982) 717.
12 D. Tetaert, X. Grandier-Vazeille, B. Grandier-Vazeille Hémon, J. P. Aubert and G. Biserte, *Proceedings of 15th FEBS Meeting, Brussels, July 24 29, 1983*, p. 202.
13 G. Frank and A. G. Weeds, *Eur. J. Biochem.*, 44 (1974) 317.