ELSEVIER

Journal of Chromatography A, 712 (1995) 141–147

JOURNAL OF CHROMATOGRAPHY A

Identification of phosphocysteine by electrospray mass spectrometry combined with Edman degradation☆

Christiane Weigt[a], Horst Korte[a], Rembert Pogge von Strandmann[b], Wolfgang Hengstenberg[b], Helmut E. Meyer[a,*]

[a]*Institut für Physiologische Chemie I, Abteilung Biochemie Supramolekularer Systeme, Protein-Sequenzlabor, MA 2/143, Ruhr-Universität Bochum, 44780 Bochum, Germany*

[b]*Physiologie der Mikroorganismen, NDEF 06/744, Ruhr-Universität Bochum, 44780 Bochum, Germany*

Abstract

Phosphocysteine is an intermediate in the phosphoenolpyruvate-dependent phosphotransferase system (PTS) and in the dephosphorylation of phosphotyrosine residues by protein tyrosine phosphatases. A method is described for the direct and non-radioactive localization of phosphocysteine. The phosphorylated Glu-C peptide of the EII^{Mtl} protein of the PTS of *Staphylococcus carnosus* was identified by LC–electrospray MS and isolated for further analysis. Following chemical modification with alkaline ethanethiol, S-ethylcysteine was identified during Edman degradation, demonstrating that phosphocysteine reacts like phosphoserine. As a control, the unphosphorylated cysteine could not be modified by the alkaline ethanethiol.

1. Introduction

Cysteine phosphorylation is an essential step in the uptake and transport of carbohydrates by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) in bacteria [1–3]. A second system with a phosphocysteine intermediate is the dephosphorylation of phosphotyrosine residues catalysed by protein tyrosine phosphatases, an important element of many signal transduction pathways [4]. So far, only indirect methods have been available pointing to phosphorylated cysteine, e.g., site-directed mutagenesis or chemical modification with Ellman's reagent, maleimide or iodoacetamide. Additionally, most of these methods required ^{32}P labelling.

We have developed a non-radioactive method allowing the localization of phosphocysteine in the lower picomolar range by sequence analysis. As a test peptide, we isolated the phosphopeptide from EII^{Mtl} protein of the PTS of *Staphylococcus carnosus* which presumably contains phosphocysteine [5]. This peptide was modified with alkaline ethanethiol and subjected to Edman degradation [6]. To prove that S-ethylcysteine was formed from phosphocysteine, we sequenced the non-phosphorylated peptide after alkaline ethanethiol treatment and demonstrated the presence of cysteine by modification with 4-vinylpyridine.

* Corresponding author.

☆ Dedicated to Professor Harald Tschesche on the occasion of his 60th birthday.

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00457-2

2. Experimental

2.1. Protein digestion and HPLC

As starting protein we used the hydrophilic region (amino acids 345–515) of the EII^{Mtl} protein of *Staphylococcus carnosus*, an enzyme of the phosphoenolpyruvate-dependent phosphotransferase system. It was purified and phosphorylated as described previously [5,7].

A 20-nmol (400-μg) amount of phosphorylated or unphosphorylated EII^{Mtl} fragment was dissolved in 25 m*M* ammonium carbonate buffer (pH 7.8) and digested with 33 μg of endoprotease Glu-C in presence of 5% acetonitrile. Incubation was carried out for 18 h at 25°C.

The protein digest was separated on a reversed-phase C_{18} column (250 mm × 4 mm I.D., 250GL4-ODS2-30/5 from SGE, Weiterstadt, Germany) using an ammonium acetate gradient system [8]. The identified phosphocysteine- or cysteine-containing peptides were modified as described below and subjected to Edman degradation.

2.2. Mass spectrometry

All spectra were recorded on a TSQ 7000 triple quadrupole mass spectrometer from Finnigan MAT (Bremen, Germany) equipped with a Finnigan electrospray ion source.

Mass spectrometry of the intact proteins

A 150-pmol amount of the phosphorylated or unphosphorylated EII^{Mtl} fragment was dissolved in water and subjected to reversed-phase chromatography on a C_4 column (50 mm × 2 mm I.D., 50GL2-C4-30/5 from SGE). The protein was eluted with a gradient (10% B/min) of 0.6% formic acid (A) vs. 0.6% formic acid–acetonitrile (B) (16:84) at a flow-rate of 80 μl/min and injected on-line into the electrospray ionization source.

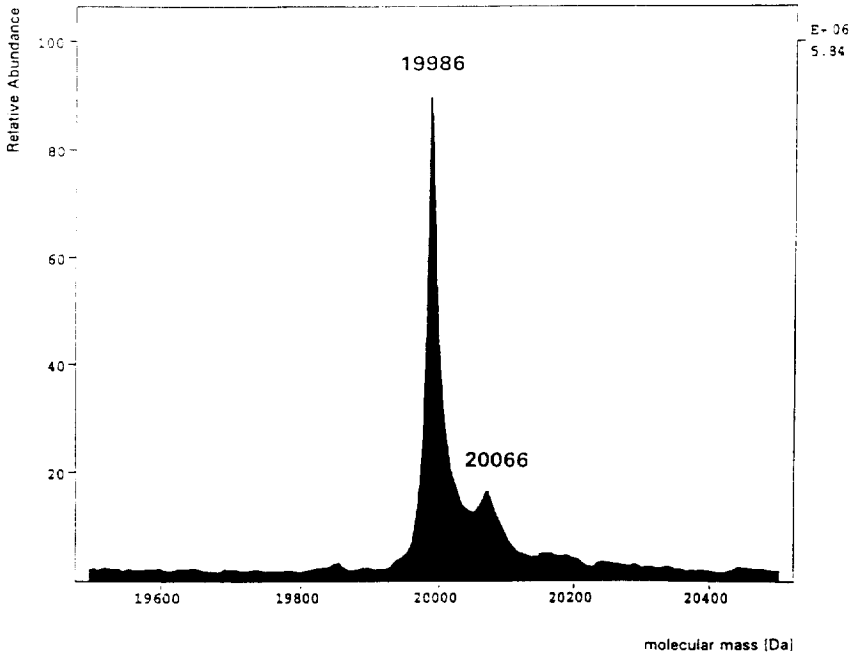

Fig. 1. Deconvoluted ESI mass spectrum of the unphosphorylated EII^{Mtl} fragment. A 150-pmol amount of EII^{Mtl} fragment was dissolved in water and separated using a C_4 column connected on-line to the electrospray source. For other conditions, see Experimental. The calculated mass of 19 986 corresponds to the mass determined from the amino acid sequence. A small amount of the phosphorylated fragment with M_r 20 066 is also present.

Mass spectrometry of the protein digests

For analysing the Glu-C digest, 500 pmol of the peptide mixture were separated using a C_{18} column (100 mm × 2 mm I.D., 100MGL1-ODS2-30/5 from SGE) connected on-line to the electrospray source. The peptides were eluted with a gradient (2% B/min) of 0.6% formic acid (A) vs. 0.6% formic acid–acetonitrile (B) (16:84) at a flow-rate of 50 μl/min.

The mass spectrometer was operated under unit-mass resolution in the positive-ion mode. Total ion current chromatograms were obtained by scanning the mass range corresponding to m/z values between 200 and 2500 every 2 s.

2.3. Sequence analysis

Sequence analysis was performed using an Applied Biosystems Model 476A pulse-liquid sequenator or a Model 470A gas-phase sequenator. The S-ethylcysteine modification followed the method described for phosphoserine in [6]. Modification of cysteine with 4-vinylpyridine was carried out according to a procedure as described in Refs. [9 and 10] and modified in Ref. [11]. In detail, the sample was applied to a biobrene-coated glass filter disc and dried. A 20-μl volume of a reduction–alkylation 'cocktail' containing 2 μl of tri-*n*-butylphosphine and 5 μl of 4-vinylpyridine in 100 μl of acetonitrile was added to the sample, the cartridge was immediately reassembled and the sequencing of the modified peptide was started.

2.4. Materials

Acetonitrile of HPLC grade was obtained from Scharlau (Barcelona, Spain); water and analytical-reagent grade ethanol, sodium hydroxide and acetic acid were purchased from Merck (Darmstadt, Germany) and ethanethiol, tri-*n*-butylphosphine and 4-vinylpyridine from Aldrich (Steinheim, Germany). All sequencer chemicals and sample discs were obtained from Applied

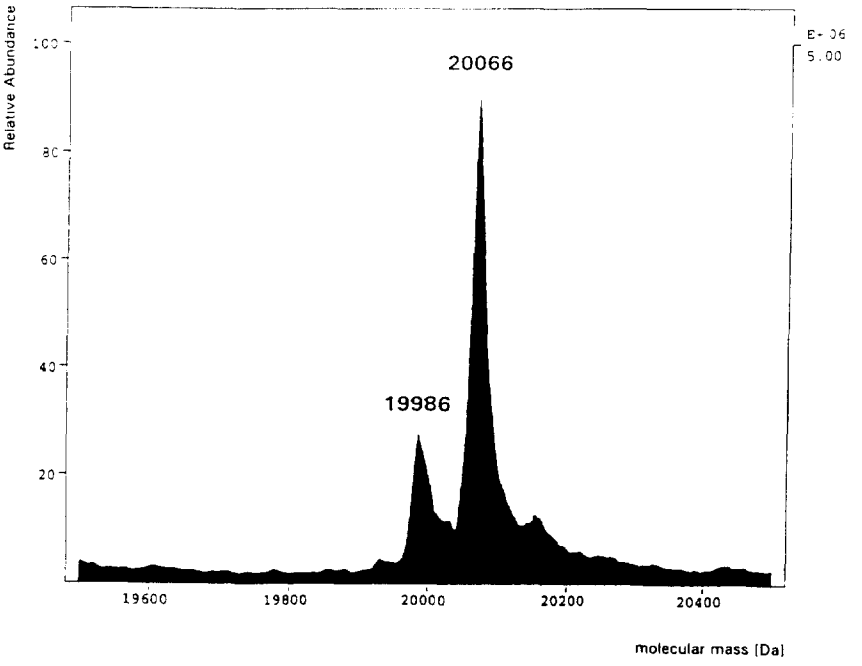

Fig. 2. Deconvoluted ESI mass spectrum of the phosphorylated EII^{Mtl} fragment. A 150-pmol amount of phosphorylated EII^{Mtl} fragment was dissolved in water and separated using a C_4 column connected on-line to the electrospray source. For other conditions, see Experimental. The phosphorylated form of the EII^{Mtl} fragment with a calculated mass of 20 066 predominates over the unphosphorylated form of the fragment.

Biosystems (Weiterstadt, Germany) and endoprotease Glu-C, sequence grade, from Boehringer (Mannheim, Germany). All other chemicals were of the highest purity available.

3. Results and discussion

Figs. 1 and 2 show the electrospray mass spectra of the unphosphorylated and phosphorylated form of the EII^{Mtl} fragment, respectively. From these spectra, the M_r of the unphosphorylated protein was calculated to be 19 986 and that of the phosphorylated protein to be 20 066. These calculated masses correspond to the mass determined by the amino acid sequence with one bound phosphate group in the case of the M_r 20 066 species.

For the EII^{Mtl} fragment the phosphorylated amino acid was indirectly established by several experiments to be the single cysteine residue in the amino acid sequence [7]. To prove this hypothesis, we subjected both the phosphorylated and unphosphorylated form of the EII^{Mtl} fragment to Glu-C digestion to obtain the phosphopeptide and its unphosphorylated counterpart.

The Glu-C digest was analysed using LC–MS. In Fig. 3 the total ion current chromatogram of the Glu-C digest of the phosphorylated EII^{Mtl} fragment is depicted. Additionally, the extracted traces of m/z values 875–876 and 891–892 are shown. As illustrated in Fig. 4A, the m/z value 875–876 represents the 5-charged ion $[M+5H]^{5+}$ of a peptide with a M_r 4372. The m/z value 891–892 belongs to a peptide with M_r 4452 (Fig. 4B). These calculated masses agree with the mass of the expected Glu-C peptide 80–120 in the unphosphorylated or phosphorylated state containing a single cysteine in position 17. By this method, it is possible to identify rapidly the phosphopeptides and to subject them to Edman degradation.

The phosphopeptide was sequenced without

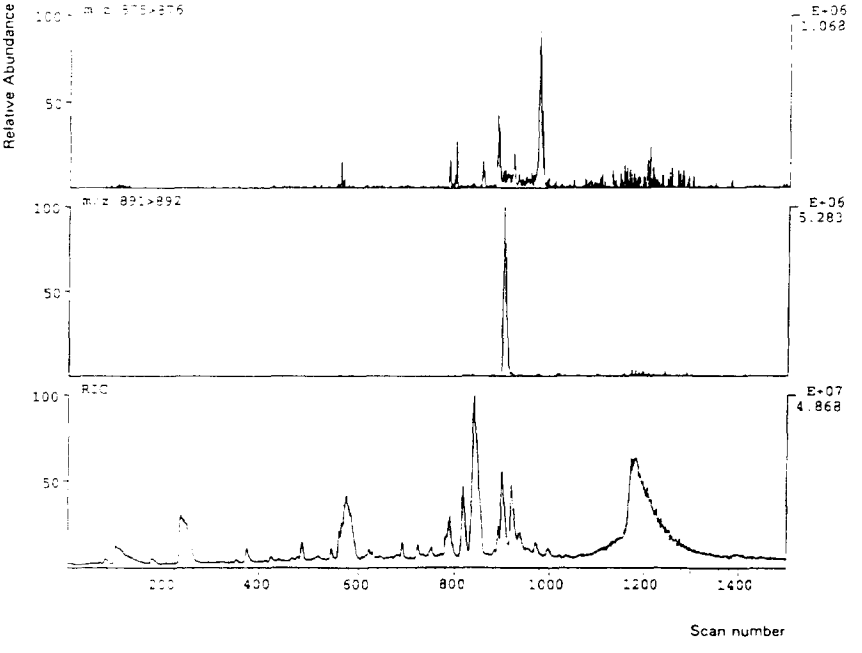

Fig. 3. Total ion current chromatogram of the Glu-C digest of the phosphorylated EII^{Mtl} fragment A 500-pmol amount of the Glu-C digest was separated using a C_{18} column connected on-line to the electrospray source. For other conditions, see Experimental. Additionally, the extracted traces of the m/z 875–876 and 891–892 ions are depicted. The former is the 5-charged ion of a peptide with M_r 4372 (see Fig. 4A) and the latter is the 5-charged ion of a peptide with M_r 4452 (see Fig. 4B).

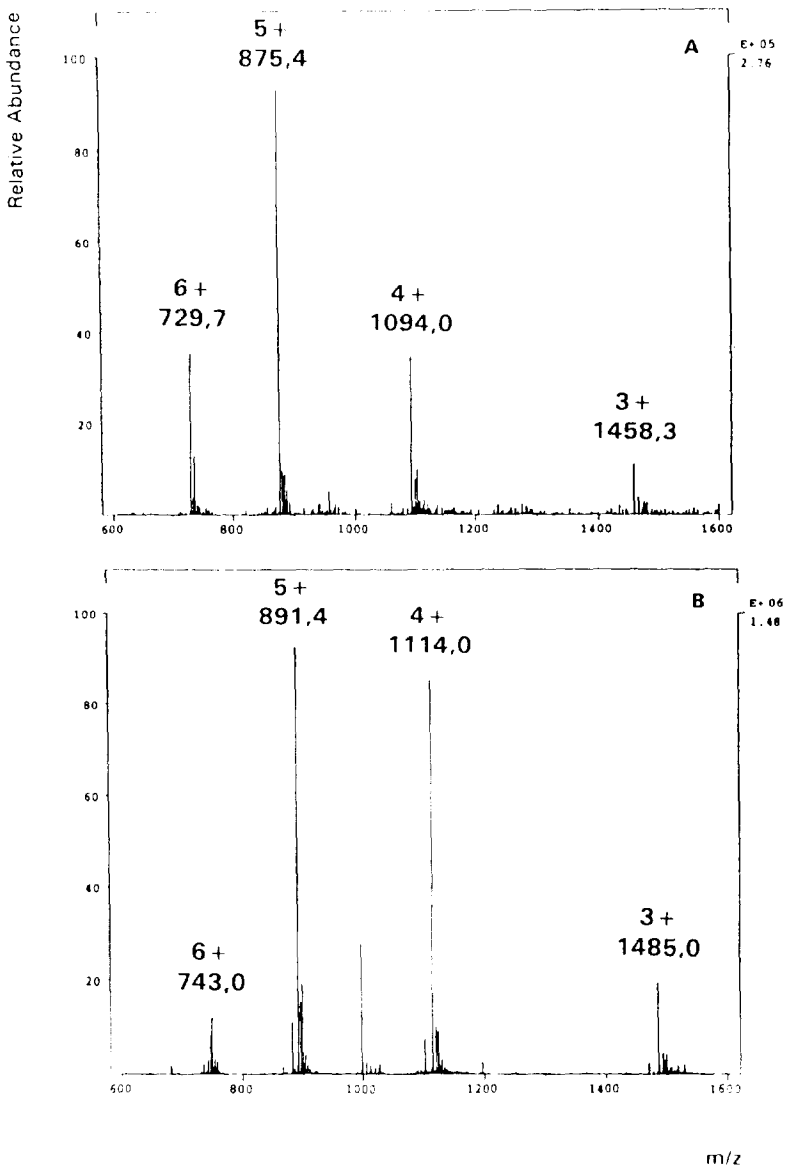

Fig. 4. Extracted ESI mass spectra of peptides with molecular masses of (A) 4372 and (B) 4452. The mass spectra were obtained by summing the scans (A) 967–980 and (B) 894–910 from the total ion chromatogram (Fig. 3) after subtracting the background.

modification or with modification using the alkaline ethanethiol reagent. In Fig. 5, three cycles of the corresponding Edman degradation steps are depicted. Fig. 5A shows the results obtained with the phosphopeptide without further treatment. In cycle 17 only a little DTT-serine appears. This result is expected for a cysteine residue. However, sometimes a small amount of serine is also detectable, indicating partial hydrolysis of thiophosphate. Fig. 5B depicts the results obtained using the phosphopeptide modified with the alkaline ethanethiol reagent. By this treatment, the cysteine residue in position 17 is transformed into S-ethylcysteine, which is detectable during Edman degradation in cycle 17.

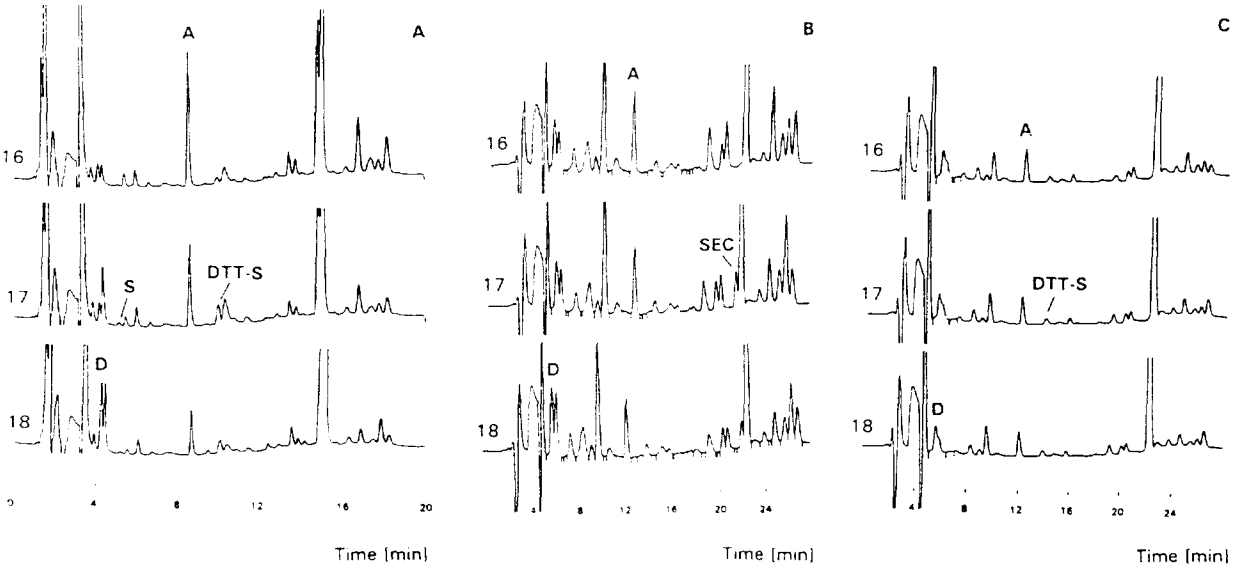

Fig. 5. Sequence analysis of the phosphorylated and unphosphorylated Glu-C peptide 80–120 with a single cysteine in position 17. (A) Results obtained with the phosphopeptide without further modification; (B) results of Edman degradation of the phosphopeptide after treatment with alkaline ethanethiol; (C) Edman degradation of the unphosphorylated peptide after ethanethiol treatment, as a control. The sequence analysis and modifications of the peptides were carried out as described under Experimental. DTT-S denotes DTT-serine and SEC S-ethylcysteine.

As a control, Edman degradation of the unphosphorylated peptide after ethanethiol modification is shown in Fig. 5C. Only a slight increase in DTT-serine in cycle 17 is visible, and no S-ethylcysteine can be detected. This result is expected for a cysteine not post-translationally modified. As an additional control, we subjected the unphosphorylated peptide without ethanethiol modification to Edman degradation. As expected for cysteine, we found only a small amount of DTT-serine in cycle 17 and no serine (data not shown).

To demonstrate the presence of cysteine in the unphosphorylated peptide, we alkylated it with 4-vinylpyridine and obtained S-pyridylethylcysteine in degradation cycle 17 (data not shown).

The results of Edman degradation and of the LC–MS experiments on the differently treated phosphorylated and unphosphorylated forms of the Glu-C peptides reveal some important characteristics of phosphocysteine residues, as follows.

(1) Phosphocysteine is relatively stable in the presence of dilute formic acid and remains intact during the isolation by reversed-phase HPLC, as could be demonstrated. This allows the application of LC–MS methods for the identification of phosphocysteine-containing peptides in a proteolytic digest.

(2) Phosphocysteine itself is not stable during Edman degradation, but is hydrolysed to cysteine and phosphate and, to a less extent, to serine and thiophosphate. This is in contrast to phosphoserine. The latter is stable during Edman degradation and undergoes β-elimination as soon as the phosphoserine residue is in the N-terminal position and is modified with PITC, yielding DTT-serine quantitatively [12].

(3) Phosphocysteine reacts like phosphoserine [6] or farnesylcysteine [13] during the ethanethiol modification, resulting in the formation of S-ethylcysteine. This means that phosphocysteine undergoes β-elimination followed by the addition of ethanethiol. Hence it can be detected without radioactive labelling by sequence analysis after chemical modification.

In conclusion, LC–MS allows the rapid identi-

fication of phosphocysteine-containing peptides and chemical modification of phosphocysteine to S-ethylcysteine localizes the site of phosphorylation by Edman degradation. The different behaviour during Edman degradation of the unmodified phosphopeptide allows us to distinguish between phosphoserine and phosphocysteine. Hence phosphocysteine can be clearly identified.

Acknowledgement

This study was supported financially by the Deutsche Forschungsgemeinschaft, Me 765/3-2.

References

[1] P.W. Postma, J.W. Lengeler and G.R. Jacobson, Microbiol. Rev., 57 (1993) 543–594.
[2] G.T. Robillard, H. Boer, R.P. van Weeghel, G. Wolters and A. Dijkstra, Biochemistry, 32 (1993) 9553–9562.
[3] H.H. Pas and G.T. Robillard, Biochemistry, 27 (1988) 5835–5839.
[4] K.L. Guan and J.E. Dixon, J. Biol. Chem., 266 (1991) 17026–17030.
[5] R. Fischer and W. Hengstenberg, Eur. J. Biochem., 204 (1992) 963–969.
[6] H.E. Meyer, E. Hoffmann-Posorske, H. Korte and L.M.G. Heilmeyer, Jr., FEBS Lett., 204 (1986) 61–66.
[7] R. Pogge von Strandmann, C. Weight, R. Fischer, H.E. Meyer, H.R. Kalbitzer and W. Hengstenberg, Eur. J. Biochem., in press.
[8] M. Serwe and H.E. Meyer, in R. Kellner, F. Lottspeich, H.E. Meyer (Editors), Microcharacterization of Proteins, VCH, Weinheim, 1994, pp. 29–45.
[9] P.C. Andrews and J.E. Dixon, Anal. Biochem., 161 (1987) 524–528.
[10] U.T. Ruegg and J. Rudinger, Methods Enzymol., 47 (1977) 111–116.
[11] D. Hawke and P. Yuan, Applied Biosystems User Bull., December (1987) 28.
[12] H.E. Meyer, E. Hoffmann-Posorske and L.M.G. Heilmeyer, Jr., Methods Enzymol., 201 (1991) 169–185.
[13] L.M.G. Heilmeyer, Jr., M. Serwe, C. Weber, J. Metzger, E. Hoffmann-Posorske and H.E. Meyer, Proc. Natl. Acad. Sci. U.S.A., 89 (1992) 9554–9558.