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Glycosylation of extracellular superoxide dismutase studied by high-performance liquid chromatography and mass spectrometry

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ABSTRACT

Extracellular superoxide dismutase, EC-SOD, the main superoxide dismutase in biological fluids, is known from its lectin binding to be a glycoprotein. We have characterized the glycosylation of recombinant EC-SOD. A tryptic digest of the protein contained only one glycosylated peptide. This peptide was specifically bound to lectins and stained by periodic acid–Schiff stain. Although appearing very large on size-exclusion chromatography, it was shown to be glycosylated at only one site, asparagine-89, by specific cleavage with glycanases followed by mass spectrometry of the resulting peptide. Based on the binding properties of the peptide to concanavalin A and lentil lectin and the elution profile of N-glycanase-treated glycopeptide on ion-exchange chromatography, the carbohydrate appears to be of the complex biantennary type with a core fucose.

INTRODUCTION

Extracellular superoxide dismutase, EC-SOD (EC 1.15.1.1), is the major SOD isoenzyme in extracellular fluid. The enzyme was first described by Marklund *et al.* [1] and has been found to consist of four identical subunits and has an apparent molecular weight of 135 000 [2]. The cDNA clone from human placenta coding for the protein has been isolated and characterized [3]. According to this, the subunit consists of 222 amino acids corresponding to a molecular weight of 24 200. The middle part of the protein shows strong homologies with the C-terminal portion of CuZn-SOD. The C-terminal portion of EC-SOD consists of a great number of charged amino acids and is also responsible for its heparin affinity [3]. Each subunit of EC-SOD binds one copper and one zink atom [4]. EC-SOD bound to concanavalin A, lentil lectin and wheat germ lectin indicating that EC-SOD is glycosylated at least at the possible asparagine site [4]. The difference between the apparent molecular weight and that expected from the amino acid sequence indicates a large carbohydrate moiety.

In the present paper we have further characterized the glycosylation by isolat-

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ing the glycosylated, nineteen-amino acid-long, tryptic peptide followed by enzymatic release of the carbohydrate portion. The resulting peptide was characterized by mass spectrometry and the released carbohydrate was studied by high-performance anion-exchange chromatography at high pH. From the results we conclude that each subunit of recombinant human EC-SOD has one single carbohydrate moiety attached to asparagine-89. Based on the binding to lectins and the elution profile on anion-exchange chromatography we suggest that the carbohydrate moiety is of the complex biantennary type having a core fucose.

EXPERIMENTAL

Protein purification

Human recombinant EC-SOD produced in Chinese hamster ovary cells was purified to at least 98% purity [according to reversed-phase chromatography and sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)] by a three-step purification scheme at Symbicom AB, (Umeå, Sweden). The purified EC-SOD was stored at −70°C in 50 m*M* sodium phosphate buffer, pH 7.4.

Carboxymethylation of EC-SOD

EC-SOD (5 mg) in 200 μl of sodium phosphate buffer, pH 7.4, was mixed with 1.5 ml of 0.5 m*M* ammonium acetate, 6 *M* guanidine hydrochloride, pH 8.0, and flushed with nitrogen. The protein was thereafter reduced with 15 μl of 1 *M* dithiothreitol, flushed with nitrogen, and incubated for 1 h at room temperature. Iodoacetic acid (30 μl) was added and the sample was flushed with nitrogen and incubated in the dark for 2 h. Finally, the reaction was terminated by addition of 30 μl of 2-mercaptoethanol and 525 μl of glacial acetic acid.

Trypsin cleavage

Carboxymethylated EC-SOD was dialysed against 0.1 *M* NH_4HCO_3, pH 7.8, and mixed with trypsin (EC 3.4.21.4, Boehringer-Mannheim) at a mass ratio of 50:1. The mixture was then incubated overnight (16–20 h) at room temperature. Cleavage was terminated by addition of 1 m*M* phenylmethylsulphonyl fluoride (PMSF).

Lectin chromatography

The tryptic digest of EC-SOD was applied to concanavalin A–Sepharose 4B or lentil lectin–Sepharose 4B (Pharmacia, Uppsala) equilibrated with 5 m*M* sodium acetate, pH 6.9, 1 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$. After the column was thoroughly washed, it was eluted with the same buffer containing 0.5 *M* α-methylmannoside. Bound and unbound material were collected as single fractions. Both glycopeptide and undigested EC-SOD could be eluted at 0.15 *M* α-methylmannoside but to keep the volume of the pooled, bound material low, the batch elution was made at higher molarity.

Glycopeptide isolation

The glycopeptide was isolated by reversed-phase chromatography on a Beckman System Gold liquid chromatography system using an Ultrapore C_8 (250 × 4.6 mm, Beckman Instruments). The glycopeptide was eluted at approximately 30%

acetonitrile in 0.1% trifluoroacetic acid at 38°C. Prior to glycanase cleavage, it was further purified by gel filtration on TSK-3000SW, 375 × 7.6 mm, equilibrated with 10 m*M* sodium phosphate, pH 7.4 and 0.25 *M* NaCl.

Enzymatic cleavage of glycopeptide

For cleavage of asparagine-linked carbohydrate, 50 nmol of glycopeptide in 0.25 *M* sodium phosphate buffer were mixed with 2 U of *N*-glycanase [peptide-N^4-(N-acetyl-β-glucosaminyl)asparagine amidase; EC 3.5.1.52, Genzyme, Boston, MA, USA] and incubated at 37°C. Samples were withdrawn at distinct times for reversed-phase liquid chromatographic (RP-LC) analysis. The reaction was terminated by immersing the samples in boiling water or by immediate RP-LC. For cleavage of serine/threonine-linked carbohydrate the peptide was first subjected to hydrolysis of sialic acid residues by incubation at 80°C for 1 h in 0.05 *M* sulphuric acid. The peptide was thereafter cleaved with O-glycanase (endo-α-N-acetylgalactosaminidase, EC 3.2.1.97, Genzyme) at several enzyme/peptide ratios ranging well above the recommended values in 20 m*M* sodium cacodylate, 10 m*M* calcium acetate, pH 6.5. Samples were then analyzed by RP-LC.

Amino acid analysis

For the amino acid analysis we used the Waters (Bedford) Pico-Tag system. Samples were hydrolyzed and derivatized with phenyl isothiocyanate according to the instructions supplied. The following exceptions were made. The chromatographic system was Beckman System Gold and instead of the supplied column we used a Beckman Ultrasphere C_{18} (150 × 4.6 mm) with an elution program starting at 3% Pico-Tag eluent B, 3–15% B curve 4 (concave) in 6 min, 15–28% B in 1 min, 28–33% B in 5.5 min, 33–40% B in 1.5 min, 40–100% B 1 min, 100% B for 5.5 min and then re-equilibration to 3% B in 1 min. The flow-rate started at 1 ml/min, increased to 1.5 ml/min in 0.5 min at 14 min and decreased to 1 ml/min in 0.5 min at 24 min. The cycle of the program was 24.5 min and all amino acids were well separated and eluted within 17 min.

Periodic acid–Schiff (PAS) staining of blotting matrices

Immobilon-P, polyvinyl difluoride transfer membranes (Waters) were stained with Schiffs reagent (Sigma, St. Louis, MO, USA) using a modification [5] of the method of Glossmann and Neville [6].

Mass spectrometry

A ZAB HF 2F mass spectrometer connected to a VG Analytical 11/250 computer system and equipped with a Xenon FAB gun was used for peptide analysis. Samples were dissolved in distilled water and thioglycerol was used as matrix. The instrument was scanned from high to low masses, m/z 2600–90, 15 s per decade. Acceleration voltage was 8 kV and the target was bombarded with xenon at 8 keV. A cesium iodide spectrum was used for calibration.

High-performance anion-exchange chromatography

The N-glycanase-cleaved glycopeptide was analyzed at high pH on a Dionex (Sunnyvale, CA, USA) CarboPac PA-1 column (250 × 4.6 mm) using a Dionex Bio

LC gradient pump and a Model PAD 2 detector [7]. A CarboPac PA guard column (25 × 3 mm) was also used. The elution was done by 2 min of isocratic elution with 100 m*M* NaOH, followed by a linear gradient to 150 m*M* sodium acetate in 100 m*M* NaOH during 63 min, and a flow-rate of 1 ml/min. A 300 m*M* solution of NaOH was added to the column effluent via a mixing tee at a flow–rate of 1 ml/min. Detection was accomplished by triple-pulse amperometry on the PAD detector using a gold working electrode as described [7].

RESULTS

To identify the glycosylation of EC-SOD, the lectin-binding properties of tryptic peptides were studied. When a tryptic digest of EC-SOD was applied to concanavalin A–Sepharose or lentil lectin–Sepharose the bound material contained one heterogeneous main peak according to RP-LC (Fig. 1). The peptide was identified by

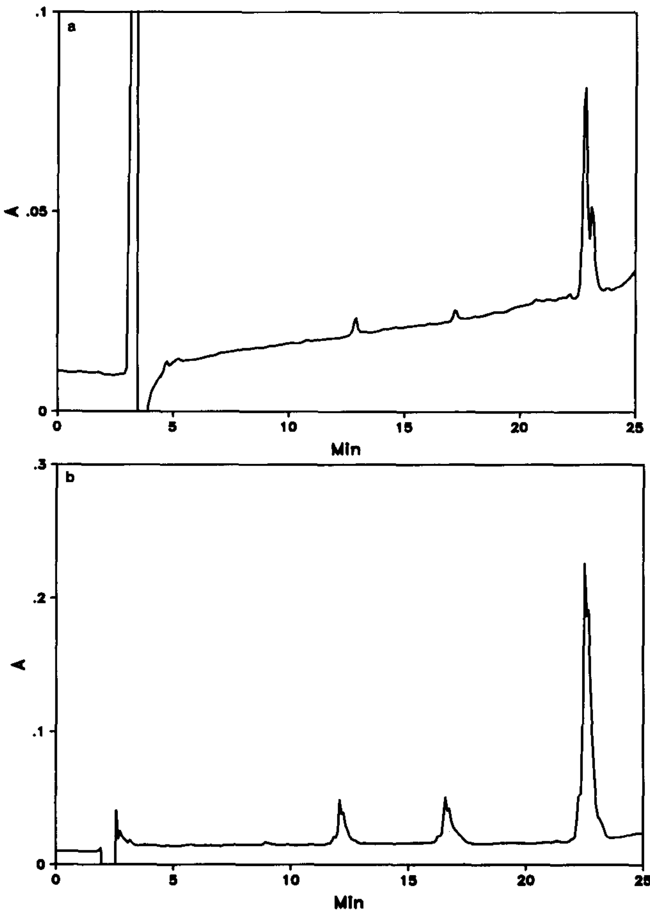

Fig. 1. RP-LC of tryptic fragments of EC-SOD bound to (a) concanavalin–Sepharose 4B and (b) lentil lectin–Sepharose 4B. Conditions as given under Experimental.

TABLE I

THEORETICAL AND OBSERVED AMINO ACID COMPOSITION AND MOLECULAR MASSES OF LECTIN-BOUND MATERIAL, PEPTIDES T8 AND T9, AND THE TWO PEAKS COLLECTED FROM N-GLYCANASE-CLEAVED GLYCOPEPTIDE.

All amino acids not listed in the table had a concentration corresponding to <0.2.

Amino acid	Peptide T8	Peptide T9	Peptide T8 + 9	Lectin-bound		N-glycanase	
				LL[a]	Con A[b]	Peak 1	Peak 2
Asx	0	2	2	2.0	2.2	2.0	2.0
Glx	0	2	2	2.2	1.9	1.9	1.9
Ser	0	3	3	3.5	3.0	2.6	2.6
Gly	0	1	1	1.5	1.9	1.1	1.1
Arg	0	1	1	1.0	1.0	1.1	1.1
Thr	0	1	1	0.9	0.7	1.0	1.0
Ala	1	2	3	2.2	1.7	2.8	2.1
Pro	0	2	2	2.3	2.3	1.8	1.9
Leu	0	2	2	2.1	1.4	2.1	2.1
Phe	0	3	3	2.6	2.4	2.8	2.9
Lys	1	0	1	0.3	0.8	1.0	<0.2
Molecular mass	217	2085	2284	N.D.[c]	N.D.[c]	2279	2084

[a] LL = lentil lectin.
[b] Con A = concanavalin A.
[c] N.D. = not determined.

its mobility on RP-LC and by amino acid analysis (Table I) to be the nineteen-amino acid-long peptide Leu-75–Arg-93 (T9). Small amounts of two other fragments also bound but these were later found by amino acid analysis to be unspecifically cleaved fragments both composed of the main part of T9.

The glycopeptide was purified by collecting peptide T9 from RP-LC of a tryptic digest and by size-exclusion chromatography of the collected fraction (Fig. 2). The carbohydrate-containing peptide was easily separated from its impurities as it migrated as if had a molecular weight between 15 000 and 20 000 (Fig. 2b). The peak was slightly broader than protein peaks of approximately the same apparent molecular weight (myoglobin) probably due to its heterogeneity in the glycosylation.

The peptides separated by RP-LC were also slot-blotted and stained by PAS. Peptide T9 stained strongly and fractions containing the two smaller fragments that bound to lectins also stained but weaker. No other peptides stained, showing that no other parts of EC-SOD contained any carbohydrate stainable with PAS.

The high apparent molecular weight of both the native protein and the glycopeptide raised the question of whether the glycosylation really was restricted only to the single N-glycosylation site of T9 or if some O-linked carbohydrate also existed. To answer this question we tried to cleave the peptide with O-glycanase at serveral enzyme-to-peptide ratios. However, RP-LC of the cleavage product showed no difference with that of an undigested stample. Secondly, we cleaved the peptide with N-glycanase and separated the cleavage products by RP-LC. In this case the chromatography resulted in the appearance of one major and one minor new peak in the

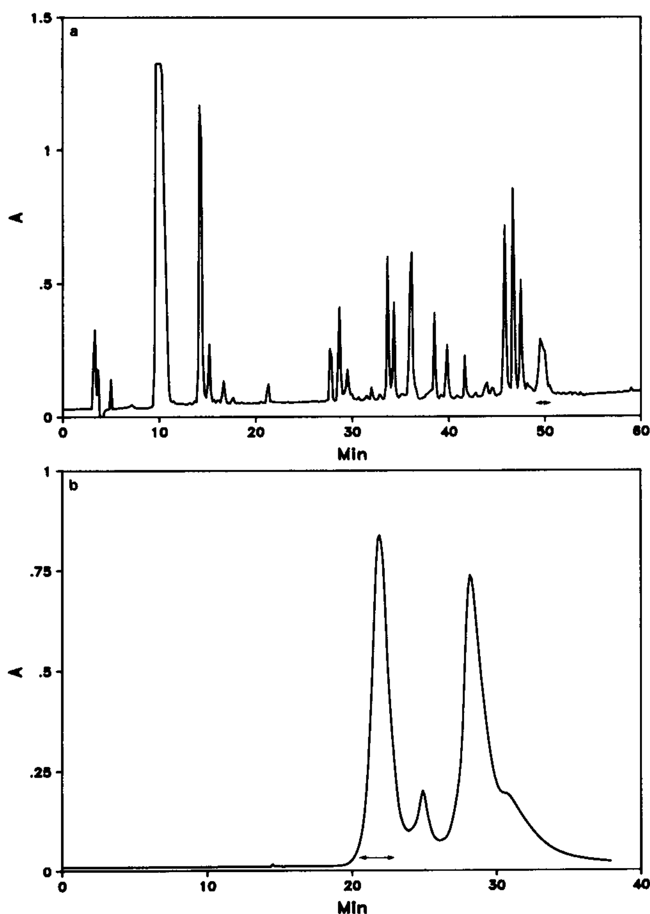

Fig. 2. Purification of the glycosylated peptide. (a) The tryptic peptides of EC-SOD separated on RP-LC. The pooled material is indicated by the arrow. (b) Size-exclusion chromatography of the pooled fraction from RP-LC. The collected fraction is indicated by the arrow.

chromatogram and at the same time the original peak decreased (Fig. 3). The two new peaks were collected and subjected to further analysis. First the amino acid composition was determined and then the molecular weight was established by mass spectrometry.

The results proved that the new peaks were the result of a complete deglycosylation of the peptide. Amino acid analysis showed that the composition of the main peak was the expected for the glycopeptide (Table I). It was very pure, which was not surprising as it now had a completely different mobility from its contaminants. The minor peak was a result of an incomplete tryptic cleavage of EC-SOD by trypsin. It contained, in addition to the amino acids of T9, one alanine and one lysine corresponding to the composition of tryptic fragment T8 (Table I). Apparently T9 and T8 + T9 was not separated by RP-LC when glycosylated due to the dominant carbohydrate portion, but when deglycosylated the difference in hydrophobicity resulted in

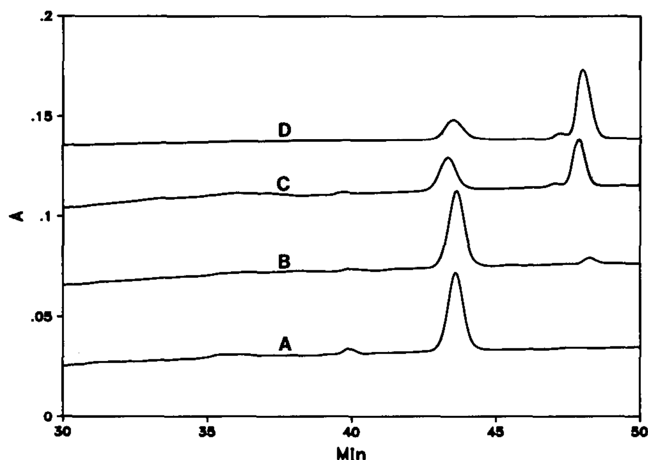

Fig. 3. RP-LC of the N-glycanase-cleaved glycopeptide. The glycopeptide was cleaved as described under Experimental. Samples were withdrawn from the reaction mixture at (A) 0 h, (B) 1 h, (C) 8 h and (D) 18 h.

a separation of the two peptides. The increase in hydrophobicity upon deglycosylation of the two fragments is in accordance with what is expected when the hydrophilic carbohydrate moiety is removed. Mass spectrometry supported these results and gave molecular weights of 2084 and 2279 for the two peaks respectively (Table I).

After N-glycanase cleavage, the glycopeptide was analyzed by anion-exchange chromatography at high pH. As seen in Fig. 4, several peaks were detected. The most retarded peak has a retention time very similar to the reference compound (indicated by an arrow in Fig. 4), the disialylated biantennary structure from human serotransferrin (Fig. 5). Other peaks most probably correspond to peptide and carbohydrate with missing terminal sialic acids. Variations in the grade of sialylation is also supported by inhomogeneity of recombinant EC-SOD upon isoelectric focusing.

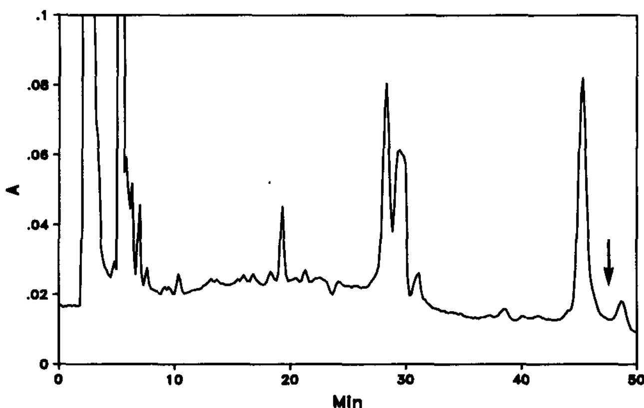

Fig. 4. Ion-exchange chromatography of N-glycanase-cleaved glycopeptide. Conditions as given under Experimental. Retention of the biantennary carbohydrate standard (Fig. 5) is indicated by the arrow.

Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)
Man(β1-4)GlcNAc(β1-4)GlcNAc
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)

Fig. 5. Biantennary carbohydrate standard (from human serotransferrin) used for comparison of retention on ion-exchange chromatography (Fig. 4).

DISCUSSION

The glycosylation of EC-SOD is interesting both from the scientific and the clinical point of view. As defence against oxygen radicals EC-SOD is highly interesting for medical use. EC-SOD is, compared to CuZn-SOD, in many ways more interesting for clinical purposes as it has a much longer half-life in the vasculature [8]. The explanation for that is probably the size of the protein. Marklund [2] reported the molecular weight to be 135 000 on gel filtration and we have observed somewhat higher values on a TSK 3000SW size exclusion column. According to the amino acid sequence [3], EC-SOD should have a molecular weight of about 97 000. This large difference indicates the presence of a large carbohydrate portion on the protein. SDS-PAGE of EC-SOD also resulted in a higher molecular weight than expected. We have found the subunit to have a molecular weight up to 32 000 (due to the incomplete glycosylation by the CHO cells several bands could be seen). However, with glycoproteins, SDS binding is not strictly proportional to molecular weight and therefore interpretation of the results is not straight forward.

Deduced from the primary structure, EC-SOD has one potential site for N-linked sugar. It seemed unlikely that this single site was responsible for the observed large difference between the theoretical and the observed molecular weight. Therefore we isolated the carbohydrate-containing material from a tryptic digest. We found that the only carbohydrate containing part of the protein was the nineteen-amino acid-long peptide containing the N-glycosylation site. The peptide gave an apparently high molecular weight upon gel filtration. Its mobility indicated a molecular weight considerably higher than insulin (6000) and close to that of myoglobin (17 600). As this peptide also contained several serines and one threonine, we had to find out whether some of these were also glycosylated. To achieve this we cleaved the peptide with different glycosidases. We found that N-glycanase cleaved the peptide whereas O-glycanase did not. After N-glycanase cleavage, the deglycosylated peptide bound more strongly to a reversed-phase column and could therefore be isolated and studied separately. The amino acid content was not changed and the peptide could not be stained with PAS. Finally, to prove that this peptide was free from carbohydrate we determined its molecular weight by mass spectrometry and the results clearly showed that the peptide was completely free from carbohydrate (Table I).

The data on the lectin-binding properties of the glycopeptide give us some information concerning the structure of the carbohydrate moeity. Binding to concanavalin A–Sepharose indicates either a high mannose or a biantennary type of N-linked sugar. Since it elutes from the column at 0.15 *M* α-methylmannoside, known to leave high-mannose type sugars bound to the column, it is reasonable to assume that it is of the biantennary type [9]. Binding of the glycopeptide to lentil lectin suggests the presence of a core fucose residue [9]. Analysis of the N-glycanase-released carbo-

hydrate moiety by anion-exchange chromatography also supported this proposed structure as the fully glycosylated carbohydrate moiety had a retention time very similar to the disialylated biantennary reference compound from human serotransferrin.

A mutant of the protein with asparagine-89 exchanged to glutamine has a mobility on size-exclusion chromatography that corresponds completely to what is expected (results to be published). One explanation for this behaviour is that the carbohydrate is highly hydrated and thereby interacts with water molecules and orders them into a structure that makes the migrating protein a much larger molecule on SEC. This is not a single phenomenon only restricted to EC-SOD. Several other examples of this behaviour are known and one example is human bile salt-stimulated lipase that migrates on gel filtration as if it had a molecular weight of more than 300 000 but only about 100 000 on SDS-PAGE [10].

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REFERENCES

1 S. L. Marklund, E. Holme and L. Hellner, *Clin Chim. Acta*, 126 (1982) 41–51.
2 S. L. Marklund, *Proc. Natl. Acad. Sci. USA*, 79 (1982) 7634–7638.
3 K. Hjalmarsson, S. L. Marklund, Å. Engström, and T. Edlund, *Proc. Natl. Acad. Sci. USA*. 84, (1987) 6340–6344.
4 L. Tibell, K. Hjalmarsson, T. Edlund, G. Skogman, Å. Engström and S. L. Marklund, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 6634–6638.
5 M. Strömqvist and H. Gruffman, submitted for publication.
6 H. Glossmann and D. M. Neville, Jr., *J. Biol. Chem.*, 246 (1971) 6339–6346.
7 M. R. Hardy and R. R. Townsend, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 3289–3293.
8 K. Karlsson and S. L. Marklund, *Biochem. J.*, 242 (1987) 55–59.
9 R. K. Merkle and R. D. Cummings, *Methods Enzymol.*, 138 (1987) 232–259.
10 L. Bläckberg and O. Hernell, *FEBS Lett.*, 157 (1983) 337–341.