Rabbit peritoneal polymorphonuclear neutrophils reduced inorganic \([^{35}\text{S}]\)sulfate to \([^{35}\text{S}]\)sulfite in vitro, concomitant with incorporation of \(^{35}\text{S}\) into a 10.68-kDa cytosolic protein as a \(\text{S-}^{[35}\text{S}]\)sulfo-derivative. Amino-terminal sequencing of the purified protein identified calgranulin C, a member of the S100 protein family. cDNA clones of calgranulins B and C were isolated using oligonucleotide primers based on the established amino acid sequences of other mammalian calgranulins. The complete amino acid sequence of rabbit calgranulin C was deduced from the nucleotide sequence of the corresponding cDNA. It comprises 91 amino acid residues, has a calculated molecular mass of 10.52 kDa, has 74% identity with porcine calgranulin C, and shows high homology with other S100 calcium-binding proteins. Rabbit calgranulin C has a single cysteine residue at position 30, which we believe to be modified to \(\text{S-}^{35}\text{S}\)sulfo-cysteine as a consequence of sulfate reduction by neutrophils. The formation of \(\text{S-}^{35}\text{S}\)sulfo-calgranulin C appears to be a reaction specific to neutrophils. The specific radioactivity of calgranulin C from the neutrophil culture medium was 50-fold greater than that of the calgranulin C within the cells, suggesting that \(\text{S-}^{35}\text{S}\)ulfation of calgranulin C might be associated with its secretion.

Sulfate reduction to sulfite has been well characterized in microorganisms (1, 2) where the reaction is catalyzed by an enzyme, \(3'-\)phosphoadenosine-5' -phosphosulfate-reductase, and at least one carrier protein (1). Sulfate reduction in mammalian tissues is less common (3, 4), and little is known about the mechanism. In a previous paper (5) we reported that rabbit polymorphonuclear neutrophils (PMN) \(^3\) reduce inorganic sulfate to inorganic sulfite. We have proposed that PMN may form sulfite as an anti-bacterial agent, as an agent for cleaving disulfide bonds in proteins to facilitate proteolysis, and even as an agent for changing the antigenicity of proteins for recognition by the immune system (5). Although a large proportion of the reduced \(^{35}\text{S}\)sulfate was released in the form of inorganic \(^{35}\text{S}\)sulfite, polyacrylamide gel electrophoresis (PAGE) revealed the presence of a component that migrated with an apparent molecular mass of less than 14 kDa. \(^{35}\text{S}\) label could be displaced from this macromolecule by treatment with sodium sulfite, consistent with the presence of an \(\text{S-}^{35}\text{S}\)sulfo-derivative.

Because this reaction is reversible, the detection and isolation of \(\text{S-}^{35}\text{S}\)sulfo-proteins requires elimination of free thiols from the reaction mixture. This has been achieved by addition of excess N-ethylmaleimide (NEM), which reacts rapidly with free thiols and with inorganic sulfite (7).

The formation of a small protein associated with the majority of the protein-bound \(^{35}\text{S}\)sulfite in PMN cells raises the possibility that this protein might be involved in the sulfite formation mechanism. In this paper we describe the isolation and purification of this protein and its identification as the rabbit homolog of the porcine granulocyte protein calgranulin C, a member of the S100 protein family (8). Calgranulins A, B, and C are specifically expressed in neutrophils and monocytes, although calgranulins A and B are also expressed in certain cells of epithelial lineages (9).

**EXPERIMENTAL PROCEDURES**

Materials—\(^{35}\text{S}\)Sulfate (1000 Ci/mmol) and N-\(\text{C}^4\)ethylmaleimide (40 Ci/mmol) were from DuPont NEN, and \(^{45}\text{Ca}^2+\) (19.1 Ci/g) was from Amersham Corp. Staphylococcus aureus V8 protease, N-ethylmaleimide, low melt agarose, sodium EDTA, phenylmethylsulfonyl fluoride, benzamidine hydrochloride, 6-aminohexanoic acid, N-dansylaziridine, and N-lauroylsarcosine were from Sigma, polyvinylidene difluoride (Immobilon-P) membranes from Millipore; Hema IEC BioQ resin (10 \(\mu\)m) was from Alltech; PepRPC (C\(_{18}\)) HR 5/5 and Mono-Q HR 5/5 columns, and Sephadex G-25 and G-50 were from Pharmacia Biotech Inc.; and molecular mass standards for electrophoresis were from Novex (New South Wales, Australia).

Cell isolation and culture—PMN were harvested from the peritoneal cavity of adult female laboratory rabbits of mixed breeds as described by Cohn and Hirsch (10). Rabbit PMN cells (10\(^2\)-10\(^3\) cells/ml) were incubated at 37 °C with 0.1 \(\mu\)m \(^{35}\text{S}\)sulfate (1000 Ci/mmol) in Hank's balanced salt solution (11) containing 0.5 \(\mu\)m MgCl\(_2\) in place of MgSO\(_4\) and 19802
supplemented with 5.5 mM glucose as described previously (5).

For identification of S-S-[35S]Sulfito-proteins, rabbit PMN were incubated with [35S]sulfate for 1 h and then disrupted by sonication. The lysate was divided into two equal portions; one was treated immediately with excess NEM (final concentration 50 mM), while the other was treated with 25 mM Na2SO3 at room temperature for 30 min before the addition of NEM. These preparations were analyzed for [35S]-labeled components as described below.

Identification of Inorganic [35S]Sulfite—NEM (30–50 mM) was used to trap inorganic [35S]Sulfite and free thiols in order to prevent disulfide reoxidation in the presence of amine-inhibitors (final concentrations, 10 mM sodium EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5.0 mM benzamidine hydrochloride, 10 mM 6-aminopenicillanic acid, and 50 mM NEM). After centrifugation at 5,000 rpm for 10 min at 4°C, the PMN extract was fractionated by gel filtration on a column (2 × 105 cm) of Sephadex G-50 (Pharmacia, fine grade) equilibrated with 0.1 M NaHCO3. Elution was performed at a flow rate of 20 ml/h. Eluted fractions (5 ml) were assayed for radioactivity, and protein content was determined using the Bradford protein assay (Bio-Rad). Specific fractions were analyzed by SDS-PAGE. Fractions containing the major [35S]-labeled protein (see Fig. 3A, fractions 21–25) were pooled, diluted 2-fold, and subjected to anion-exchange chromatography on a Mono Q HR 5/5 column (Pharmacia). Equilibration with 0.1 M NaHCO3, pH 8.0, in the column was eluted at a flow rate of 1.0 ml/min with a linear concentration gradient of 0.01–1.0 M NaHCO3 as indicated in the legend of Fig. 4. Fractions of 1.5 ml were collected and assayed for radioactivity and protein content. The major peak of [35S]-labeled protein (see Fig. 4A, fractions 52–56) was pooled and desalted by reverse-phase chromatography using a PepRPC (C18) HPLC column (Millipore) as described by Scha¨gger and von Jagow (12). Samples for nonreducing conditions on a 16% gel in a Mini-PROTEIN II apparatus were dissolved in 68 mM Tris-HCl buffer, pH 8.8, containing 2 mM MgCl2, 1.25 units of Taq polymerase (Promega), 200 μM mixed dNTP (GTP, CTP, A TP, and TTP) together with each primer at a concentration of 1 μM. Optimal reaction conditions were: initial cycle of denaturation at 95°C for 2 min, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min, followed by 29 cycles of denaturation at 95°C for 40 s, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min. This was then followed by a single 5-min extension cycle at 72°C.

RESULTS

Detection of S-Sulfo-cysteine—A sample of the [35S]-labeled protein, recovered from anion-exchange chromatography on Mono Q, was digested with 10 mg of Pronase at 50°C for 24 h in 1.0 ml of 0.1 M Tris-HCl, pH 8.0, containing 1.0 mM CaCl2. The aliquot of the digested sample was mixed with standard S-sulfo-cysteine and subjected to anion-exchange HPLC on a column (4.6 × 300 mm) of Hema IEC BioQ and eluted with a gradient of triethylamine acetate in 0.2 M acetic acid. Components as described below.

Enzymic Digestion and Peptide Purification—A sample of purified [35S]-labeled protein, recovered from reverse-phase chromatography on PepRPC (Pharmacia), was lyophilized and resuspended in 0.1 mM NH4HCO3, 2 ml EDTA, pH 8.0. Digestion was carried out with 12 μg (10 units) of S. aureus V8 protease (Sigma), at 37°C for 24 h. The reaction was terminated by freezing and lyophilizing the reaction mixture.

Electrophoresis—SDS-PAGE in Tricine buffer was performed under nonreducing conditions on a 16% gel in a Mini-PROTEIN II apparatus (Bio-Rad) as described by Schagger and von Jagow (12). Samples for electrophoresis were dissolved in 68 mM Tris-HCl buffer, pH 6.8, containing 0.2% (w/v) SDS, 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue. The resultant gels were stained with Coomassie Brilliant Blue or silver and were analyzed for radioactivity with a PhosphorImager (Molecular Dynamics) or by fluorography. Fluorography was carried out by exposure to Kodak X-Omat AR film (Kodak, Melbourne, Australia) for 7–8 days at –80°C.

Fluorescence Measurements—Proteins labeled with the fluorescent reagent N-dansylaziridine were detected with a Hitachi (Tokyo, Japan) Fluorescence Spectrophotometer Model F-4000 using excitation at 345 nm and detection of emission at 510 nm (13). Fluorescent proteins were detected on gels after SDS-PAGE using a Spectroline UV transilluminator (Bio-Rad) as described by Schagger and von Jagow (12). Samples for nonreducing conditions on a 16% gel in a Mini-PROTEIN II apparatus were dissolved in 68 mM Tris-HCl buffer, pH 6.8, containing 2 mM MgCl2, 1.25 units of Taq polymerase (Promega), 200 μM mixed dNTP (GTP, CTP, ATP, and TTP) together with each primer at a concentration of 1 μM. Optimal reaction conditions were: initial cycle of denaturation at 95°C for 2 min, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min, followed by 29 cycles of denaturation at 95°C for 40 s, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min. This was then followed by a single 5-min extension cycle at 72°C.

Mass Spectrometry—The molecular mass of calgranulin C was determined using an API III LC/MS system equipped with a triple quadrupole iontrap mass spectrometer (Perkin-Elmer Scieks). Samples were run in positive ion mode with 59.0% argon, 0% oxygen, and 41% helium as the gas (N2) flow rate of 0.6 ml/min, and a liquid flow rate of 30 μl/min.
Formation of S-[\textsuperscript{35}S]Sulfo-proteins in PMN—Rabbit PMN (4 × 10\textsuperscript{7} cells/ml) were incubated with [\textsuperscript{35}S]sulfate for 1 h and treated with NEM and sulfite as described under “Experimental Procedures.” The amount of NEM-[\textsuperscript{35}S]sulfite formed was calculated to be 1.02 × 10\textsuperscript{3} and 1.36 × 10\textsuperscript{3} dpm/10\textsuperscript{6} cells in the untreated and sulfite-treated preparations, respectively (Fig. 2A), equivalent to an overall rate of sulfite formation of 61 pmol/h/10\textsuperscript{6} cells.

Proteins in the two preparations were separated by SDS-PAGE and subjected to fluorography (Fig. 2B). A number of 35S-labeled proteins was observed in the untreated preparation (Fig. 2B, lane 2); however, densitometric scanning of the fluorogram showed that 83% of the radioactivity was associated with a protein that migrated with an apparent molecular mass of 8 kDa. After sodium sulfite treatment, this protein and another protein of 16 kDa were shown to have lost their \textsuperscript{35}S label (Fig. 2B, lane 3), whereas a 31-kDa protein and other proteins of even larger molecular size retained their label.

The ability of neutral sodium sulfite to displace the \textsuperscript{35}S label suggested that the two proteins were S-[\textsuperscript{35}S]sulfo-derivatives in which [\textsuperscript{35}S]sulfite was bonded as a thiol-sulfate ester with a cysteine residue. The increase in NEM-[\textsuperscript{35}S]sulfite detected in the sulfite-treated sample could thus be attributed to the displacement of \textsuperscript{35}S sulfite bound to these proteins by unlabeled sodium sulfite.

Purification of the \textsuperscript{35}S-Labeled Protein—Rabbit PMN (6.3 × 10\textsuperscript{7} cells/ml) were incubated with \textsuperscript{35}S sulfate for 4 h at 37 °C, sonicated, treated with excess NEM, and centrifuged. The supernatant was fractionated by gel filtration on a Sephadex G-50 column, which resolved the \textsuperscript{35}S-labeled macromolecules into two major peaks (Fig. 3A), well separated from unincorporated labeled. SDS-PAGE of the two peaks of protein (Fig. 3B, lanes 2 and 3) and analysis of the resultant gels for radioactivity using a PhosphorImager established that the highly radioactive 8-kDa protein was associated with the second peak (data not shown).

Fractions from Sephadex G-50 containing the \textsuperscript{35}S-labeled 8-kDa protein were combined and subjected to ion-exchange chromatography on a Mono Q-HR 5/5 column as described under “Experimental Procedures.” Three major peaks of protein (A, B, and C) were eluted by the salt gradient (Fig. 4A), but a single major radioactive peak was eluted with 0.4 M NaHCO\textsubscript{3}, just after the last peak of protein (peak C). The peak of radioactivity (fraction 54) did not coincide with the peak of protein (fraction 52), but SDS-PAGE (Fig. 4B) revealed that the \textsuperscript{35}S-labeled 8-kDa protein eluted in peak C (fractions 52-56; Fig. 4B, lanes 4, 5, and 6). The two-fraction displacement of the protein profile and the radioactivity profile in the peak C protein (Fig. 4A) was assumed to indicate the slightly more anionic behavior of the S-[\textsuperscript{35}S]sulfated protein compared with the nonsulfated protein. This difference could not be detected after SDS-PAGE of fractions 52-56; the Coomassie Blue staining bands and the PhosphorImager bands coincided and migrated the same distance in each lane.

The fractions containing the 8-kDa protein were pooled and subjected to reverse-phase chromatography on a PepRPC column to remove minor contaminating proteins and salt. Fractions containing the single sharp peak of \textsuperscript{35}S-labeled protein, which eluted with 50% acetonitrile (data not shown), were pooled and lyophilized for further characterization and sequence analysis. SDS-PAGE analysis of this preparation revealed a single radioactive band migrating with an apparent mass of 8 kDa.

Protein estimation showed that the labeled protein constituted 8.6% of the total cytosolic (soluble) protein of PMN. The precise molecular mass of the purified protein, determined by
mass spectrometry, was 10.68 kDa, in contrast to the apparent value of 8 kDa calculated by comparison with the Novex standards on SDS-PAGE.

Primary Structure of the 35S-Labeled Protein—The lyophilized protein from the PepRPC purification step was subjected to amino-terminal sequencing. The sequence of the first 25 residues was found to be TKLEDHLEGIINIFHQYSVRTGHYD. Amino acid comparison with the available data bases revealed a high degree of homology between this protein and several proteins of the S100 family. The best alignment was with a porcine granulocyte protein, calgranulin C with 96% homology within the first 25 amino acid residues (Fig. 5B).

In order to obtain further amino acid sequences of rabbit calgranulin C, fragments were generated by protease V8 digestion of the purified protein. The digest was resolved into three peaks of 35S label by reverse-phase chromatography on PepRPC. SDS-PAGE revealed that the first peak contained no detectable protein, the second peak (fraction 29) contained two peptides of approximately 3.0 and 4.0 kDa (designated V1 and V2), and the third peak (fraction 40) contained undigested material together with a partially digested fragment, whereas fraction 41 contained a single fragment of the same size as that in fraction 40 (designated V3). The amino acid sequences of these peptide fragments were determined by Edman degradation. The amino-terminal residues of V3 (TKLEDHLEGIINIFHQYSVRTGHYD) were identical to those of undigested calgranulin C; peptide V3 thus results from digestion at the carboxyl end of calgranulin C. The full sequences of V1 (GIINIFHQYSVRTGHYD) and V2 (LVNTIKNTDQATVDRIFRDLDEGDH) are shown. B, SDS-PAGE analysis of PMN lysate (lane 1); protein peak 1, fractions 18–20 (lane 2), and protein peak 2, fractions 22–24 (lane 3) from Sephadex G-50. Each lane was stained with Coomassie Blue.

Identification of S-[35S]Sulfo-cysteine in 35S-Labeled Calgranulin C—To confirm the presence of S-sulfo-cysteine, exhaustive Pronase digestion of purified 35S-labeled calgranulin C was performed. The digest was subjected to anion-exchange HPLC. 35S label (45% of the total) co-eluted with the S-sulfo-cysteine standard (data not shown). Thus [35S]sulfite formed by PMN could become bonded to cysteine residues through a covalent, S-sulfate, or thiol-ester bond.

Cloning of Rabbit Calgranulin C and Calgranulin B—An oligonucleotide primer was prepared based on the predetermined amino-terminal sequence of purified rabbit calgranulin...
C. This primer was used, together with a R1T primer, to amplify specific rabbit PMN cDNA by PCR. Two PCR products having the approximate sizes of 400 and 500 base pairs were cloned into the pGEM-T vector by T-A ligation. Positive clones were selected by restriction enzyme digestion, and the nucleotide sequence of the insert was determined. The resultant partial nucleotide sequences, when compared with nucleotide sequences in GenBank/EMBL data bases, indicated that the cDNA clones were of calgranulin C and calgranulin B. The partial nucleotide sequences determined and the deduced amino acid sequences of calgranulin C and B are shown in Fig. 5A. The nucleotide sequence was confirmed using clones obtained from independent PCR trials.

The partial cDNA sequence of rabbit calgranulin C, when combined with the amino-terminal sequence obtained by Edman degradation (Fig. 5A), indicated that the cDNA had an open reading frame of 273 nucleotides, predicting a protein of 91 amino acids with a calculated molecular mass of 10,520 Da. A comparison of the complete amino acid sequence with the porcine counterpart showed an overall amino acid identity of 74%. Notably, residue 30 in rabbit calgranulin C, the site postulated to form a S-sulfato-derivative during sulfate reduction by PMN, was confirmed to be cysteine. The molecular mass of 10.52 kDa calculated for rabbit calgranulin C is less than the value of 10.68 kDa determined by mass spectrometry. The discrepancy of 160 Da most probably arises from the attachment of NEM to the single cysteine residue because the elution profile from Mono Q (Fig. 4A) indicates that nonsulfated calgranulin C is the major form present. A less likely possibility is bound Ca$^{2+}$ because, using the $^{45}$Ca$^{2+}$ overlay technique of Maruyama et al. (15), we have demonstrated (data not shown) that purified rabbit calgranulin C binds calcium, even after reaction with NEM and SDS-PAGE.

The partial nucleotide sequence of rabbit calgranulin B had 73, 71, and 70% identity to the nucleotide sequences of human (16), mouse (17), and rat (18) calgranulins B, respectively. The putative amino acid sequence thus determined for rabbit calgranulin B (Fig. 5A) had 70, 65, 61, and 57% identity with the corresponding proteins of bovine, human, mouse, and rat granulocytes, respectively. Fig. 5B shows the alignment of the rabbit sequences with those of some other mammalian calgranulins B and C.

Distribution of $^{35}$S-Labeled Calgranulin C in Cultured Cell and Medium—Rabbit PMN were incubated with Hanks' balanced salt solution containing 0.1 mM $[^{35}$S]sulfate (1000 Ci/mol) for 4 h at 37°C. At the end of the incubation, the cells were separated from the medium by centrifugation. The cell pellet was resuspended in Hanks' balanced salt solution containing 0.1 mM sulfate and disrupted by sonication. Excess NEM (50 mM) was added to both the cell lysate and the medium. Purification of $^{35}$S-labeled calgranulin C was then carried out as described above.
groups in the PMN proteins. Insoluble material was then removed by centrifugation at 5,000 rpm for 10 min at 4 °C, and the PMN extract was fractionated by gel filtration on Sephadex G-50 followed by anion-exchange chromatography on Mono Q as described under “Experimental Procedures.” Fig. 6 shows the elution profile from Mono Q revealing three peaks of protein coincident with peaks of fluorescence at 510 nm and a single sharp peak of 35S radioactivity corresponding to the labeled calgranulin C. SDS-PAGE of fractions 26 and 30, the peak fluorescence fractions, revealed the presence of two major protein components (both fluorescent) with apparent molecular masses of 8 and 15 kDa. These bands were electrophoresed onto a polyvinylidene difluoride membrane and subjected to amino-terminal sequencing. The 8-kDa component yielded the sequence PTDLENSLSI5VYHYKSL, which has 75% identity with the amino-terminal sequence of human calgranulin A; a protein that contains cysteine residues (20). The amino terminus of the 15-kDa component seemed to be blocked, and this component remains unidentified. Separate experiments established that both of these components were labeled by reaction with [35S]NEM (data not shown). A purification procedure similar to that described above was used to isolate the 14C-labeled macromolecules. SDS-PAGE revealed two major [35S]-labeled components of 8 and 15 kDa. Although the 14C-labeled 8-kDa component was not readily resolved from calgranulin C by SDS-PAGE, it behaved quite differently during anion-exchange chromatography and reverse-phase chromatography (data not shown).

In addition to these proteins, a separate 13-kDa protein with amino-terminal sequence homology to rabbit lysozyme C was also detected in the PMN lysate. Lysozyme C contains four disulfide bonds. These results indicate that apart from calgranulin C, rabbit PMN contain other cysteine-containing, disulfide-bonded proteins. Yet during sulfate-reduction in PMN, calgranulin C appears to be the major protein that becomes labeled in association with the formation of sulfate.

**DISCUSSION**

Rabbit PMN reduce [35S]sulfate to [35S]sulfite over a period of 24 h in vitro (Fig. 1), at a rate of 64 ± 22 pmol/h per 106 cells (mean ± standard deviation for 7 separate measurements). Over the same period, PMN macromolecules became labeled with 35S. The majority of the 35S label was associated with a protein of 10.68 kDa in the form of a S-[35S]sulfo-protein. Pronase digestion of the purified 35S-labeled protein yielded 5-[35S]sulfo-cysteine as a major radioactive product.

Amino-terminal sequence analysis of the labeled protein identified it to be the rabbit homolog of a porcine PMN protein identified as calgranulin C by Dell’Angelica et al. (8). No cysteine residue, however, was present in porcine calgranulin C. To confirm the presence of and locate the cysteine residue(s) in rabbit calgranulin C, molecular cloning techniques were applied to generate a cDNA clone of calgranulin C by PCR. The nucleotide sequence of the cloned cDNA confirmed and extended the amino acid sequence determined by amino-terminal sequencing (Fig. 5A). Residue 30 was shown to be cysteine, and this was the only cysteine residue within the entire calgranulin C structure.

The sequence of rabbit calgranulin C is consistent with the features exhibited by other S100 calcium-binding proteins, which have two structurally conserved and well defined Ca2+-binding sites: the “EF-hands” (19, 20) at amino acid positions 18–31 and 61–72 (Fig. 5B). The least conserved regions of rabbit calgranulin C are at the carboxyl-terminal region and in the hinge region that connects the two EF-hands; yet even these regions are highly conserved between porcine and rabbit calgranulins C (Fig. 5B). The divergence of the amino acid

**FIG. 6.** Ion-exchange chromatography of PMN proteins labeled with N-dansylaziridine. A, fluorescent, [35S]-labeled PMN proteins were recovered from gel chromatography on Sephadex G-50 and fractionated by ion-exchange chromatography on a Mono Q-HR 5/5 column as described in the legend of Fig. 4. Fractions 1.5 ml were collected and assayed for protein concentration (○). Fluorescence at 510 nm with excitation at 345 nm (dashed line), and radioactivity (●). B, SDS-PAGE analysis of Novex standards (lane 1) and fraction 26 (lane 2), fraction 30 (lane 3), and fraction 34 (lane 4) from ion-exchange chromatography. Each lane was stained with Coomassie Blue.

The specific radioactivity of the purified calgranulin C isolated from the cell lysate and from the medium was calculated to be 8.87 × 104 and 4.40 × 105 dpm/mg protein, respectively, corresponding to a 50-fold increase in S-sulfation of the secreted calgranulin C compared with that which remained within the cell. The specific radioactivity of the extracellular calgranulin C was equivalent to 0.021 mol of [35S]sulfite bound/mol of calgranulin C. The specific radioactivity of the peak (fraction 54) of labeled calgranulin C eluted from ion-exchange chromatography (Fig. 4A) indicated a similarly low level of sulfation (0.017 mol of [35S]sulfite bound/mol). These calculations have made no correction for dilution of the [35S]sulfate with endogenous sulfate but are consistent with the elution profile from Mono Q (Fig. 4A), which indicated that the 35S-labeled calgranulin C was a minor component of the calgranulin C. The proportion of calgranulin C recovered in the medium accounted for 23% of the total calgranulin C in the preparation (2.6 μg/10⁶ cells).

Specific S-[35S]Sulfo-labeling on the Cysteine Residue of Calgranulin C by PMN—Rabbit PMN (6.3 × 10⁷ cells/ml) were incubated for 2 hours at 37 °C with 0.1 mM [35S]sulfate (1000 Ci/mol), and then the incubation mixture was treated with 0.1 mM N-dansylaziridine in isopropanol to yield a final concentration of 30 mM N-dansylaziridine. The resultant mixture was then incubated at room temperature for 16 h to label free thiol groups in the PMN proteins. Insoluble material was then removed by centrifugation at 5,000 rpm for 10 min at 4 °C, and the PMN extract was fractionated by gel filtration on Sephadex G-50 followed by anion-exchange chromatography on Mono Q as described under “Experimental Procedures.” Fig. 6 shows the elution profile from Mono Q revealing three peaks of protein coincident with peaks of fluorescence at 510 nm and a single sharp peak of 35S radioactivity corresponding to the labeled calgranulin C. SDS-PAGE of fractions 26 and 30, the peak fluorescence fractions, revealed the presence of two major protein components (both fluorescent) with apparent molecular masses of 8 and 15 kDa. These bands were electrophoresed onto a polyvinylidene difluoride membrane and subjected to amino-terminal sequencing. The 8-kDa component yielded the sequence PTDLENSLSI5VYHYKSL, which has 75% identity with the amino-terminal sequence of human calgranulin A; a protein that contains cysteine residues (20). The amino terminus of the 15-kDa component seemed to be blocked, and this component remains unidentified. Separate experiments established that both of these components were labeled by reaction with [35S]NEM (data not shown). A purification procedure similar to that described above was used to isolate the 14C-labeled macromolecules. SDS-PAGE revealed two major [35S]-labeled components of 8 and 15 kDa. Although the 14C-labeled 8-kDa component was not readily resolved from calgranulin C by SDS-PAGE, it behaved quite differently during anion-exchange chromatography and reverse-phase chromatography (data not shown).

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sequences at the hinge and carboxyl-terminal regions may confer functional specificity to each S100 protein (19).

The sequence of the S'-oligonucleotide primer used to isolate a cDNA encoding calgranulin C was based on the highly conserved α-helix region common to calgranulins B and C (8), so it came as no surprise when rabbit calgranulin B cDNA was also amplified in the PCR process. The calgranulins B in each species have highly conserved α-helix and Ca\(^{2+}\)-binding regions (Fig. 5B). The hinge region of these proteins also shows a high degree of homology. The carboxyl terminus of rabbit calgranulin B has a repeated GHGHGHSH tail not observed in the other mammalian homologs. The nucleotide sequence corresponding to this repeated tail is not a single repeating structure, which makes the possibility of error in the sequence determination seem unlikely. Whether the repeated carboxyl-terminal GH-rich tail conveys any particular function of rabbit calgranulin C has yet to be determined. It is noteworthy that the amino acid sequence for rabbit calgranulin B contained no cysteine residues.

Comparison of the rabbit calgranulin C with sequences in protein data bases revealed a maximal homology with calgranulin B, which is also referred to as cystic fibrosis antigen, L1 protein, or MRP-14 (16, 21, 22). Human calgranulin B occurs as a heterodimer with calgranulin A (MRP-8). The formation of the biologically active complex is Ca\(^{2+}\)-dependent (23, 24). Although the precise function of calgranulin B and its complex forms has yet to be defined, it has been suggested that the calgranulin A/B complex plays a role in the differentiation and maturation of myeloid cells (17, 25, 26).

Rabbit calgranulin C comprises about 8.6% of the total granulocyte cytosolic protein, consistent with the value of 8% reported for porcine granulocytes (8). In contrast, a human calgranulin C homolog (27) constitutes just 5% of the total PMN cytosolic protein, far less than human calgranulins A and B, which together constitute 45– 49% (21, 23). Porcine calgranulin C has been shown to undergo conformational change upon calcium binding and has been implicated in Ca\(^{2+}\)-dependent signal transduction pathways (8), whereas human calgranulin C has been shown to translocate to the PMN plasma membrane, together with calgranulins A and B during stimulation of the cell by Ca\(^{2+}\)-dependent stimuli (27–29). Because we have been unable to purify S-sulfo-calgranulin C from the unmodified protein, we have no evidence about the effect of S-sulfation on its calcium binding.

Dell'Angelica et al. (8) have used cross-linking experiments to demonstrate that porcine calgranulin C occurs as a homodimer. They proposed that the association in the homodimer was noncovalent in nature. Many S100 proteins have been reported to exist as dimers, both in disulfide-bonded (30, 31) or noncovalently associated forms (23). Because rabbit calgranulin C does contain a cysteine residue, it could form a disulfide-bonded homodimer. There is, however, no direct evidence for disulfide bonding, but the formation of a S-sulfo-derivative is consistent with that possibility. Furthermore the result of fluorography on SDS-PAGE in Fig. 2B suggests that two S-[\(^{35}\)S]sulfo-proteins were formed by PMN: the major band of calgranulin C with an apparent mass of 8 kDa and a minor band at about 16 kDa. If the latter band was a dimeric form of calgranulin C with \(^{35}\)S label bonded through a S-sulfo-linkage, then the formation of this dimer does not necessarily involve disulfide bond formation.

S-Sulfation of calgranulin C is a novel reaction, the biological significance of which has yet to be determined. Rabbit calgranulin C appears to act as a major sulfite acceptor in the sulfate reduction mechanism of PMN (Fig. 2B). Whether this protein is directly involved in the sulfate-reducing mechanism, acting perhaps as a sulfite-carrier protein, or the S-sulfation of calgranulin C occurs as the primary event that then leads to sulfite formation as a result of thiol-exchange has yet to be determined. S-Sulfation of calgranulin C in rabbit PMN does, however, appear to be a specific reaction. Lysozyme, which contains disulfide bonds, was also detected in the cytosol of rabbit PMN together with rabbit calgranulin C, which contains a free thiol group; yet these proteins did not become labeled with \(^{35}\)S in these experiments. In separate experiments we have shown that exogenous proteins, including bovine serum albumin (5) and egg white lysozyme, do become labeled with \(^{35}\)S when incubated with PMN, but in cell-free experiments egg white lysozyme does not react with 1.0 mM inorganic \(^{35}\)Sulfite. These results suggest that PMN products, such as calgranulin C, may be involved in the labeling mechanism. Because we have not purified S-sulfo-calgranulin C, we have no evidence about its ability to transfer \(^{35}\)S label to other proteins.

During incubation of PMN with \(^{35}\)Sulfate, 23% of the total calgranulin C was recovered in the medium of the culture. The specific radioactivity of the \(^{35}\)S-labeled calgranulin C in the medium was found to be 50-fold greater than that which remained in the cell. This result could reflect the reducing environment, maintained by the ratio of reduced to oxidized glutathione, within the PMN cytoplasm. A reducing environment would displace \(^{35}\)Sulfite from cytoplasmic S-[\(^{35}\)S]sulfo-calgranulin C, yet the same protein could be more stable within the endoplasmic reticulum and the vesicles of the secretory pathway. Amino-terminal sequencing of calgranulin C isolated separately from the cell pellet and from the culture medium revealed no evidence of posttranslational modification at the amino terminus of the protein, indicating that the protein lacks a leader sequence for translocation into the endoplasmic reticulum. Because the oligonucleotide probe used to isolate calgranulin C cDNA by PCR was based on the amino-terminal sequence of the protein, we cannot exclude the involvement of a leader sequence during its secretion. Calgranulins A and B have also been reported to occur extracellularly (20, 26), yet neither contains a signal peptide sequence required for membrane translocation. Intracellular transport of the calgranulins has been claimed to follow a pathway different from the endoplasmic reticulum-Golgi route (32), and it is tempting to speculate that the S-sulfation of calgranulin C might be involved in its secretion. Extracellular calgranulin C could well exhibit cystostatic activity like the calgranulin A and B complex (33–35).

REFERENCES

1. Schwenn, J. D., Krone, F. A., and Husmann, K. (1988) Arch. Microbiol. 150, 313–319
2. Kielzyn, A. (1989) J. Bacteriol. 171, 1663–1664
3. Wortman, B. (1963) Biochem. Biophys. Acta 77, 65–72
4. Robinson, H. C., and Pasternak, C. A. (1964) Biochem. J. 93, 487–492
5. Gardiner, E. R., Robinson, H. C., Sriratana, A., Mok, S. S., Lowther, D. A., and Handley, C. J. (1992) Biochem. J. 288, 577–583
6. Celli, R. (1963) In The Proteins (Neurath, H., ed) 2nd Ed., pp. 379–476, Academic Press, New York
7. Ellis, R. J. (1966) Nature 211, 1266–1268
8. Dell’Angelica, E. C., Schneider, C. H., and Santamoe, J. A. (1994). J. Biol. Chem. 269, 28292–28296
9. Goeber, R., Kuzn, R., van den Bos, C., Ader, G., and Sorg, C. (1995) Biochem. J. 310, 419–424
10. Cob, Z. A., and Hirsch, J. G. (1960) J. Exp. Med. 112, 993–1004
11. Nakas, J. H., and Wallace, R. F. (1969) Proc. Soc. Exp. Biol. Med. 121, 196–200
12. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
13. Stouf, W. H., Lubber, R., and Baughman, W. (1974) Biochem. Biophys. Acta 336, 421–426
14. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
15. Maruyama, K., Mikawa, T., and Ebashi, S. (1984). J. Biochem. (Tokyo) 95, 511–519
16. Ondik, K., Cereletti, N., Brügger, J., Clerc, R. G., Tarscay, L., Zawadlo, G., Gerhardis, G., Schlegel, R., and Sorg, C. (1987) Nature 330, 80–82
17. Lagasse, E., and Weisman, J. L. (1993) Blood 79, 1907–1915
18. Imamichi, T., Uchida, I., Wahl, S. M., and McCartney-Francis, N. (1993) Biochem. Biophys. Res. Commun. 194, 819–825
19. Kligman, D., and Hilt, D. C. (1988) Trends Biochem. Sci. 13, 437–443
20. Hessian, P. A., Edgeworth, J., and Hogg, N. (1993) J. Leukocyte Biol. 53, 197–204
21. Fagerhol, M. K., Andersson, K. B., Naess-Andresen, C.-F., Brandtzaeg, P., and Dale, I. (1990) in Stimulus-Response Coupling (Smith, V. L., and Dedman, J. R., eds) p. 187, CRC Press, Inc., Boca Raton, FL
22. Andersson, K. B., Sletten, K., Bernstzen, H. B., Dale, I., Brandtzaeg, P., Jellum, E., and Fagerhol, M. K. (1988) Scand. J. Immunol. 28, 241–245
23. Edgeworth, J., Gorman, M., Bennet, R., Freemon, P., and Hogg, N. (1991) J. Biol. Chem. 266, 7706–7713
24. Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hager, G., Karas, M., and Sorg, C. (1991) J. Biol. Chem. 266, 13462–13467
25. Lagasse, E., and Clerc, R. G. (1989) Mol. Cell. Biol. 9, 2402–2410
26. Murao, S. (1994) Acta Histochem. Cytochem. 27, 107–116
27. Guignard, F., Mauel, J., and Markert, M. (1995) Biochem. J. 309, 395-401
28. Lemarchand, P., Vaglio, M., Mauel, J., and Markert, M. (1992) J. Biol. Chem. 267, 19379–19382
29. Roth, J., Burwinkel, F., van den Bos, C., Goebeler, M., Vollmer, E., and Sorg, C. (1993) Blood 82, 1875–1883
30. Kligman, D., and Marshak, D. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7136–7139
31. Pedrocchi, M., Schäfer, B. W., Durussel, I., Cox, J. A., and Heizmann, C. W. (1994) Biochemistry 33, 6732–6738
32. Bhardwaj, R. S., Zott, C., Zwaldo-Klarwasser, Roth, J., Goebeler, M., Mahnke, K., Falk, M., Meinardus-Hager, G., and Sorg, C. (1992) Eur. J. Immunol. 22, 1891–1897
33. Steinbäkel, M., Naess-Andresen, C., Lingaaas, E., Dale, I., Brandtzaeg, P., and Fagerhol, M. K. (1990) Lancet 336, 763–765
34. Murphy, A. R. K., Lehrer, R. I., Harwig, S. S. L., and Miyasaki, K. T. (1993) J. Immunol. 151, 6291–6301
35. Yui, S., Mikami, M., and Yamazaki, M. (1995) J. Leukocyte Biol. 58, 307–316
36. Dianoux, A. C., Stasia, M., Garin, J., Gagnon, J., and Vignals, P. V. (1992) Biochemistry 31, 5889–5905