Transglutaminase from Rat Coagulating Gland Secretion

POST-TRANSLATIONAL MODIFICATIONS AND ACTIVATION BY PHOSPHATIDIC ACIDS*

(Received for publication, October 30, 1995, and in revised form, July 29, 1996)

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Structural and biochemical characteristics of transglutaminase purified by a rapid chromatographic procedure from the rat coagulating gland (anterior prostate) secretion are reported. Fast atom bombardment mapping and automated Edman degradation experiments allowed us to verify that at least 85% of the entire transglutaminase amino acid sequence is identical to that derived from the cDNA of the major androgen-dependent rat prostate protein called DP1. The enzyme was found NH₂ terminally blocked and largely post-translationally modified, since the presence of N-linked oligosaccharides, as well as of complex lipidic structures, was observed. Mass spectral analysis showed that Asn-408 and -488 are the glycosylated sites, the N-linked structures identified belonging to both high-mannose and complex type glycans. The presence of myo-inositol, of glycerol bound fatty acids, and the high content of mannose residues, are in agreement with previous observations suggesting that a lipid anchor is bound to coagulating gland secretion transglutaminase. Furthermore, two tightly bound calcium ions per molecule of enzyme were detected. Finally, a strong stimulation of the enzyme activity in vitro by both SDS and a variety of phosphatidic acids was observed. The reported structural and functional peculiarities should definitively lead to consider the prostate enzyme as a new member (type IV) of the transglutaminase family.

Transglutaminases (TGase, EC 2.3.2.13) are Ca²⁺-dependent enzymes that catalyze the formation of covalent isopeptide bonds between the γ-carboxamide group of protein-bound glutaminyl residues and various primary amines, including the ε-amino group of lysyl residues present in the same or in a different polypeptide chain (1–3). In vertebrates, TGases form a large protein family and have a wide distribution among tissues and body fluids. Sequence comparisons revealed that these enzymes are structurally related and derive from a common ancestral gene. The most studied TGases were recently classified, with the exception of the well characterized blood coagulation factor XIIIa (4), into type I (TGaseC, occurring as a membrane-bound form in the keratinocytes) (5, 6), type II (TGaseE, widely distributed in the cytosol of many cells and tissues) (7, 8), and type III (TGaseK, occurring as a proenzyme in the epidermis) (9).

Recently, by molecular cloning, a complementary DNA coding for a major secretory protein (DP1) of rat anterior and dorsal prostate (10), has been isolated by Ho et al. (11). Sequence analysis of the protein indicated that DP1 resembles other TGases at the 30% level. These findings suggested that DP1 may account for the TGase activity previously described in rat prostate.

TGase produced by the prostate could be historically considered the first discovered enzyme in the TGase family. In fact, in 1896, Camus and Gley (12) recognized that the secretions of the rodent seminal vesicle were coagulated by an enzyme, called vesiculase, present in the prostatic secretion. This catalytic protein was found to be produced in the anterior lobe of the prostate, also termed coagulating gland (13). The basic biochemical characteristics of the enzyme and the formation of glutamine-lysine intermolecular cross-links of the vesicular secretory proteins were described first by Lorand and Williams-Ashman (14). Recently, rat prostate TGase was reported to be a highly glycosylated polypeptide chain (M₀ = 65,000) and to possess a lipid anchor that is retained during secretion (15).

Concerning its biological significance, it was generally accepted that the TGase-catalyzed coagulation of rodent semen occurring in the vagina after coitus, and resulting in the formation of a copulatory plug, should aid fertilization by facilitating the passage of spermatozoa through the uterine cervix (13). A series of studies of our group on one of the major protein secreted from the rat seminal vesicles (16–18) suggested an additional and more intriguing biological role for the prostate TGase. It was demonstrated that the enzyme produces polymeric forms of this protein which, in this way, acquires the capability of both binding to the epididymal sperm cells and suppressing their immunogenicity. The role of prostate TGase to avoid the rejection of sperm cells by the immunocompetent elements present in the female genital tract has been hypothesized also in humans, since we detected TGase activity both free in the human seminal plasma and bound on the spermatozoon surface (19).

In the present paper we report several structural and biochemical characteristics of the enzyme purified from the rat...
coagulating gland secretion (CGS) by a rapid chromatographic procedure.

**MATERIALS AND METHODS**

N,N-Dimethylated casein (DMC), guinea pig liver TGase, spermidine trihydrochloride, diethiobiotid (DTT), 1-,a-phosphatidic acid, lauric acid sodium salt, myristic acid sodium salt, phosphatidic acid sodium salt, lauroyl lysine, L-[120 m M Tris-HCl buffer, pH 8.0, 10 m M DTT, 50 n M [3H]spermidine] aliquots of the diluted sample, containing about 5 mg of protein, onto a 30 cm column (30 m × 0.25-mm inner diameter, 0.25-mm film thickness) was from Supelco. HPLC grade solvents and reagents were obtained from Sigma. L-o-phosphatidylethanolamine (dilauryl) and L-o-phosphatidylserine (dilauroyl) were from Calbiochem (La Jolla, CA). [3H]Spermidine (specific activity 15 Ci/mmol) was obtained from Amersham (Buckinghamshire, United Kingdom). Peptide N-glycosidase F (PNGase F), endoproteinase Glu-C, and “Glycan differentiation kit” were from Boehringer Mannheim. N,O-Bis(trimethylsilyl)acetamide was obtained from Fluka. Phenylisothiocyanate was from Pierce. Pre-packed PD-10 gel filtration cartridges were from Pharmacia. Pre-packed C18 Sep-Pak cartridges were purchased from Waters. Immobilon membranes were from Applied Biosystem (Perkin Elmer). A SPB-1 fused silica capillary column (30 m × 0.25-mm inner diameter, 0.25-mm film thickness) was from Supelco. HPLC grade solvents and reagents were obtained from Carlo Erba. Reagents for electrophoresis and immunoblot analysis were purchased from Bio-Rad.

**TGase Assay**—Enzyme activity was assayed by measuring the incorporation of [3H]spermidine into DMC. The assay mixtures (0.1 ml), containing 120 mM Tris-HCl buffer, pH 8.0, 10 mM DTT, 50 n M [3H]spermidine, and 0.2 mg of DMC, were incubated at 37°C for 1 h. At the end of the incubation, 1.0 ml of 10% trichloroacetic acid containing 2 mM unlabeled spermidine was added to the assay mixtures, which were then centrifuged. The resulting precipitates were washed twice by suspension in the above solution, dissolved in 1.0 ml of 0.1 N NaOH, and finally counted in 5 ml of Pico-Fluor 40 scintillation mixture (Packard). One enzyme unit (arbitrary) was defined as that amount of enzyme that catalyzes the incorporation of 1 pmol of [3H]spermidine into DMC under the described conditions.

**Purification of TGase from Rat CGS**—Sexually mature male Wistar rats (250–300 g), lightly anesthetized with diethyl ether, were killed by cervical dislocation. The coagulating glands were dissected and the secretions were removed by gentle pressure. Insoluble fragments were removed by centrifugation at 10,000 rpm for 10 min. The resulting precipitates were washed twice by nitrogen, redissolved in 50 mM sodium bicarbonate, pH 8.0, at 40°C for 20 h, using 1:50 (w/w) enzyme to substrate ratio. The resulting sample was immediately concentrated by ultrafiltration since the excess of solid reagents. The hexane supernatant (1:50) was used for the GC-MS analysis. The dried sample was dissolved in 0.5 ml of 1 M methanolic-HCl at 80°C for 16 h. The re-N-acetylation was achieved by adding 0.5 ml of methanol, 10 µl of pyridine, and 50 µl of acetic anhydride and incubating at room temperature for 15 min. The trimethylsilylation was carried out in 0.2 M sodium acetate at 70°C for 30 min. The sample was then centrifuged, dried in 50 µl of hexane and centrifuged to remove the excess of solid reagents. The hexane supernatant (1:50) was used for the GC-MS analysis. The GC-MS analysis was performed on a VG Trion 2000 quadrupole mass spectrometer equipped with a Fisons Instruments 8060 gas chromatograph by using a SPB-1 fused silica capillary column (30 m × 0.25 mm). The oven temperature was held at 90°C for 1 min before increasing to 140°C at 25°C/min, and then to 200°C at 5°C/min and finally to 300°C at 10°C/min. Electron ionization mass spectra were recorded by continuous quadruple scanning at 70 eV ionization energy. Quantitative analysis was performed by using arabinol as internal standard.

**Enzymatic Digestion and Deglycosylation of Purified Rat CGS TGase**—0.5 mg of freeze-dried purified rat CGS TGase was hydrolyzed for 5 h at 30°C in 1 ml of 1.5 M NaOH. After acidification with 2 M HCl, liberated substituents were extracted with chloroform/water (1:2, v/v); the organic phase was dried down, dissolved in water, extracted twice in ethyl acetate (1:1, v/v), and then freeze-dried. The acyl residues were derivatized first to methyl ester by reaction with diazomethane (0.3 ml, room temperature, 5 min) and then to Me,Si derivatives using 0.3 ml of Me,Si acetamide at 70°C for 15 min. Samples were dried down under nitrogen, redissolved in 50 µl of dichloromethane and 1 µl was analyzed using the GC-MS apparatus described above. The temperature gradient of the temperature gradient was as follows: from 60°C to 150°C at 15°C/min and then to 280°C at 3°C/min.

**Enzymatic Digestion and Deglycosylation of Purified Rat CGS TGase**—0.5 mg of freeze-dried purified rat CGS TGase was reduced and carboxymethylated as described (22). The modified TGase was hydrolyzed with trypsin in 50 mM ammonium bicarbonate, pH 8.5, at 37°C overnight using a 1:50 (w/w) enzyme to substrate ratio and an aliquot of the tissue mixture was analyzed by fast atom bombardment (FAB)-MS. The trypptic peptide mixture was deglycosylated by incubation with 0.3 units of PNGase F in 0.4% ammonium bicarbonate buffer, pH 8.5, at 37°C for 16 h. The sample was freeze-dried, the dry powder dissolved in 5% acetic acid and then loaded on a C18 Sep-Pak cartridge. The oligosaccharides were eluted with 5% acetic acid, permethylated, and analyzed by FAB-MS. An aliquot of this fraction was subjected to acid hydrolysis and the released sugar residues were Me,Si derivatized and analyzed by GC-MS. The deglycosylated peptides, eluted from the C18 Sep-Pak cartridge with 40% 1-propanol containing 5% acetic acid, were desalted with endoproteinase Glu-C in 50 mM ammonium bicarbonate, pH 8.0, at 40°C for 20 h, using 1:50 (w/w) enzyme to substrate ratio. The deglycosylated trypptic and trypptic/Glu-C peptide mixtures were analyzed by FAB-MS.

The putative O-linked oligosaccharides were released from the peptides by the sodium borohydride (NaBH₄) procedure (23). Oligosaccharides were permethylated using the sodium hydroxide procedure (24) and then analyzed by FAB-MS.

**FAB-MS Analysis**—FAB-MS mass spectra were recorded on a VG ZAB SE double focusing mass spectrometer fitted with a VG cesium gun operating at 25 kV (2 µA). Samples were dissolved in 5% acetic acid or methanol and loaded onto a glycerol-coated probe tip; thiglycerol was added just before inserting the probe into the ion source. Spectra were recorded on ultraviolet-sensitive paper and manually counted. Mass signals recorded in the spectra were assigned to the corresponding...
peptides on the basis of their expected molecular weight with the aid of a suitable computer program (25). Manual Edman degradation steps were performed on the whole peptide digests, followed by FAB-MS analysis of the truncated peptides in order to confirm the assignment as already described (26).

HPLC Separation of Tryptic Peptides—An aliquot of the tryptic digest of TGase was fractionated, before deacylosylation, by HPLC with a μBondapack C18 column equilibrated with 0.1% trifluoroacetic acid/acetoneitrile (95:5, v/v). The elution was performed by a linear gradient of acetonitrile from 5 to 40% over 90 min (flow rate 1.0 ml/min). Five well separated peptides eluted from the column were manually collected, dried down, dissolved in 0.1% trifluoroacetic acid, and submitted to automated N-terminal sequencing, as previously reported (22).

RESULTS

Purification

The purification of rat CGS TGase was carried out by a fast protein liquid chromatography procedure on a Superose 12 column. TGase activity was recovered in a single peak which resulted in the only positive to the immunoreaction carried out with the antiserum prepared with the purified enzyme preparation. The pool of the active fractions was concentrated by ultrafiltration and rechromatographed on the same column. The obtained elution pattern showed a better resolved peak of both protein and enzyme activity. Analysis of purified TGase protein and enzyme activity. Analysis of purified TGase.

The obtained elution pattern showed a better resolved peak of both protein and enzyme activity. Analysis of purified TGase was carried out by both SDS-PAGE and Western blotting. Fig. 1 compares a Coomassie Blue-stained SDS-polyacrylamide gel (
panel A) with a Western blot (
panel B) of an equivalent gel. By Coomassie staining, the purified TGase appeared as a single band at an apparent MW of 75,000. The Western blot analysis confirmed that only the single band seen in the purified TGase preparation reacts with the obtained antiserum. Furthermore, the same single protein band with MW of 75,000 can be also detectable in the CGS by both SDS-PAGE and Western blotting. A typical experiment of enzyme purification is summarized in Table I.

Post-translational Modifications

Sugar Analysis—Rat CGS TGase samples were preliminarily analyzed for the presence of oligosaccharides by immunological assays using lectins with different specificity. The enzyme was specifically recognized by GNA, DSA, and AAA lectins which bind to terminal mannose, galactosyl β(1,6)N-acetylglucosamine, and fucose of α(1,6) residues, respectively (Fig. 1C). Immunoblotting experiments with SNA, MAA, and PNA gave negative results, thus showing the absence of both sialylated complex type glycans and O-linked oligosaccharide chains containing the structure galactosyl β(1,6)N-acetylglucosamine at their reducing end. Because of the lectins specificity, these results suggested the presence of both high-mannose and non-sialylated complex type glycans.

The oligosaccharide composition of rat CGS TGase was determined by GC-MS analyses of the Me3Si derivatives of the sugar components. The total monosaccharides were released from the protein after its decylation of the putative fatty acids (15) in basic conditions followed by mild acid hydrolysis. The GC-MS analysis of the total sugar components is shown in Fig. 2A. The high mannose-to-galactose ratio, unusual for complex type glycans, supports the lectin assay data indicating the occurrence of high mannose structures. Moreover, since lectin binding assays ruled out the occurrence of O-linked structures, the presence of N-acetylgalactosamine together with that of myo-inositol and the high content of mannose residues suggests the existence of a glycosylphosphatidylinositol anchor in rat CGS TGase, as previously hypothesized (15).

A further GC-MS analysis was performed on the N-linked oligosaccharides released from the enzyme tryptic digest following PNGase F incubation and mild acid hydrolysis (Fig. 2B). Quantitative analysis of the N-linked monosaccharides using arabinol as internal standard yielded the following sugar composition: mannose 8.0, galactose 1.7, N-acetylglucosamine 6.1, fucose 1.0. These results are consistent, in agreement with lectin binding experiments, with the presence of two N-linked structures: a high mannose oligosaccharide consisting of five mannose residues and a fucosylated biantennary complex glycan.

The intact N-linked oligosaccharides were released from the peptide backbone by PNGase F deacylosylation of the tryptic peptides. After a Sep-Pak C18 purification step, the N-glycans, eluted in the 5% acetic acid fraction, were methylated (24) and then analyzed by FAB-MS (Fig. 2C). The major signal occurring at m/z 1557 was assigned to a glycosidic chain with composition Hex2HexNAc consistent with a high-mannose type N-linked chain having two mannose residues linked to the pentasaccharide core. This interpretation was confirmed by the signal at m/z 1280 originated from a fragment ion due to an A-type cleavage occurring at the reducing end of the glycosidic bond between the two HexNAc residues of the core. The other signals recorded in the spectrum at m/z 842, 688, and 464 were correlated to the fragment ions FucHex2HexNAc+, Hex3HexNAc+, and HexHexNAc+, respectively, originated from a complex type structure containing the epitope galactose α(1,3)-galactose and fucose on the antennae.

In order to evidenciate the presence of O-linked oligosaccharides, the Sep-Pak 40% 1-propanol fraction was submitted to reductive elimination (23). The putative oligosaccharides were permethylated and then analyzed by FAB-MS; however, the spectra did not show any mass signal, ruling out the presence of this type of glycans and thus confirming previous results from the lectins assays.

Lipid Analysis—Lipid components released from rat CGS TGase by basic hydrolysis were first methyl esterified by diazomethane treatment and then the hydroxyl moieties were derivatized to Me3Si adducts. The GC-MS analysis of the methyl esterified fatty acids shown in Fig. 3A demonstrated the presence of four different components in almost equal amount (lauric, myristic, palmitic, and stearic acids), together with traces of linoleic acid. The Me3Si derivatives (Fig. 3B) showed
the occurrence of glycerol as well as two incomplete hydrolysis products, namely 1-palmitoylglycerol and 2-stearoylglycerol. The presence of low amounts of palmitic and stearic acids in this analysis is very likely due to previous incomplete methyl esterification of these fatty acids.

Primary Structure Analysis

The only data reported so far on rat CGS TGase amino acid sequence derive from homology studies between factor XIIIa, TGasec, and the primary structure of rat prostate DP1 derived from cDNA sequence. These studies, in fact, suggested that DP1, a protein with a molecular mass of 75,000, might be indeed the TGase produced by the rat coagulating gland and intended to be secreted in its lumen (11).

To verify the structural similarity between DP1 and the TGase occurring in the rat CGS, extensive primary structure studies on the CGS enzyme were carried out. Preliminary sequence analysis of the intact enzyme yielded negative results, thus indicating that CGS TGase is a NH2 terminally blocked protein. Therefore, FAB mapping experiments were performed on the reduced and carboxymethylated enzyme digested with trypsin, or both trypsin and endoproteinase Glu-C before and after deglycosylation with PNGase F. The results of FAB-MS analyses are reported in Table II. Each signal recorded in the spectra was associated with the corresponding peptide along the amino acid sequence on the basis of its mass value and the enzyme specificity. All the assignments were confirmed by submitting a portion of the digest to a single step of Edman degradation as described previously (26) followed by mass analysis of the truncated peptides. Separation of the tryptic peptide mixture was also carried out by HPLC and the sequence of five peptides best resolved chromatographically was determined by automated Edman degradation (Table III).

The combination of mass spectral analyses from different hydrolysates and sequence data allowed us to verify that at least 85% of the entire DP1 cDNA sequence is identical to CGS TGase amino acid sequence (Fig. 4). No information could be
obtained on the N terminus of TGase leaving the N-blocking group unidentified.

The mass signals at \( m/z \) 2426 and 1405, obtained only with the samples previously treated with PNGase F, were attributed to the expected peptides 481–502 and 408–419 where the Asn-488 and Asn-408 have been converted into Asp residues by PNGase F reaction (\( \Delta m = 51 \) Da), thus indicating the occurrence of N-glycosylation on these sites (Fig. 4). However, Asn-488 resulted to be only partially modified as demonstrated by the presence of a mass signal at \( m/z \) 2425 corresponding to the fragment 481–502 carrying Asn at position 488. Three more putative N-glycosylation sites inferred on the basis of the consensus sequence (Asn-X-Ser/Thr) at Asn-151, Asn-226, and Asn-472 were found unmodified. The region from Leu-174 to Trp-220 escaped mass spectral analysis despite the different proteolytic procedures employed possibly because of the occurrence of other post-translational modifications. This region contains the last putative N-glycosylation site at Asn-219 which remains to be verified.

**Table II**

| Trypsin before and after PNGase F | Trypsin, PNGase F, and Glu-C |
|-----------------------------------|-----------------------------|
| Peptide                           | MH<sup>a</sup>              | Peptide                           | MH<sup>a</sup>  |
| 2745                              | 59–81                       | 2287                               | 248–267         |
| 2638                              | 512–534                     | 1833                               | 334–349         |
| 2637<sup>a</sup>                  | 455–478                     | 1709                               | 144–157         |
| 2436                              | 360–380                     | 1622                               | 312–323         |
| 2426<sup>a</sup>                  | 481–502                     | 1482                               | 97–111          |
| 2005<sup>a</sup>                  | 45–58                       | 1317                               | 467–478         |
| 1982                              | 92–111                      | 1438                               | 45–56           |
| 1953                              | 308–322                     | 1435                               | 582–593         |
| 1852                              | 17–32                       | 1340                               | 128–138         |
| 1842                              | 252–287                     | 1339                               | 455–466         |
| 1679<sup>a</sup>                  | 45–58                       | 1341                               | 333–342         |
| 1653<sup>a</sup>                  | 112–125                     | 1223                               | 594–604         |
| 1527                              | 232–244                     | 1141                               | 559–568         |
| 1521                              | 288–300                     | 1118                               | 512–521         |
| 1493                              | 337–349                     | 1101                               | 33–41           |
| 1464                              | 275–287                     | 1016                               | 334–342         |
| 1450                              | 628–639                     | 1005                               | 337–345         |
| 1435                              | 582–593                     | 1000                               | 503–511         |
| 1405<sup>a</sup>                  | 408–419                     | 996                                | 350–359         |
| 1429                              | 33–44                       | 876                                | 609–616         |
| 1389<sup>a</sup>                  | 163–173                     | 872                                | 504–511         |
| 1377                              | 582–593                     | 866                                | 291–297         |
| 1338                              | 605–616                     | 864                                | 628–634         |
| 1257                              | 617–627                     | 842                                | 393–398         |
| 1249                              | 535–545                     | 821                                | 351–359         |
| 1238                              | 559–604                     | 809                                | 303–307         |
| 1173                              | 419–427                     | 803                                | 387–392         |
| 1145                              | 221–231                     | 800                                | 662–668         |
| 1138                              | 291–300                     | 775                                | 552–558         |
| 1060                              | 503–511                     | 763                                | 435–440         |
| 996                               | 350–359                     | 738                                | 5–10            |
| 965                               | 573–581                     | 705                                | 413–418         |
| 872                               | 504–511                     | 689                                | 11–16           |
| 821                               | 381–388                     | 683                                | 268–274         |
| 809                               | 301–307                     | 672                                | 640–645         |
| 738                               | 5–10                        | 659                                | 281–286         |
| 689                               | 11–16                       | 656                                | 33–37           |
| 688                               | 394–398                     | 649                                | 546–551         |
| 683                               | 268–274                     | 617                                | 84–88           |
| 672                               | 640–645                     | 583                                | 658–662         |
| 663–668                            |                             |                                    |                 |
| 630                               | 446–450                     | 522                                | 650–653         |
| 522                               | 650–653                     | 506                                | 646–649         |
| 506<sup>a</sup>                   | 646–649                     | 500                                | 33–36           |
| 500                               | 33–36                       | 490                                | 542–545         |

**Table III**

| Amino acid sequence of five peptides separated by HPLC from tryptic digest of rat CGS TGase |
|-------------------------------------------------------------------------------------------|
| Numbering was inferred from the cDNA-derived DP1 sequence.                                 |

| Amino acid sequence | Sequence position |
|---------------------|-------------------|
| L I F N T G H N M P F Y V E L D         | 59–75             |
| S V T N F E S A H T E K                   | 275–287           |
| N F H M W T D A W K          | 312–322           |
| R Q D L P Q G H D G W Q V L D S T P Q E I | 323–343           |
| K T N L G V I Q K                  | 503–511           |

**Fig. 4.** Amino acid sequence of DP1 derived from cDNA sequence (11). Black boxes indicate the protein regions identical to those detected to occur in the rat CGS TGase by FAB-MS analyses and automated Edman degradation. Possible sequences for N-glycosylation are underlined; the sites modified are indicated by solid arrows; an additional putative site is indicated by the question mark. The asterisk indicates the active site cysteine residue.

**Effect of Ca<sup>2+</sup> and Zn<sup>2+</sup>**

Rat CGS TGase, similarly to other TGases, is a Ca<sup>2+</sup>-dependent enzyme even though both the purified enzyme and that occurring in the crude CGS were only slightly activated in vitro by the addition of CaCl<sub>2</sub> at concentrations ranging between 0.5 and 1.0 mM (Fig. 5). Since the presence of 50 μM EGTA in the assay caused, both in the presence and absence of exogenous calcium, a complete loss in the enzyme activity, we suspected the occurrence of calcium ions tightly bound to the polypeptide chain. This hypothesis was confirmed by atomic absorption spectrophotometry experiments that demonstrated the existence of the purified enzyme of two Ca<sup>2+</sup> ions per molecule. Attempts to deplete the enzyme from calcium by dialysis in the presence of EGTA were unsuccessful since CGS TGase was inactive following this treatment also when the assay mixture was enriched with high calcium concentrations. Finally like other TGases, the CGS enzyme was also found to be inhibited by Zn<sup>2+</sup> in a dose-dependent manner (Fig. 5).

**Activation by SDS and Phosphatidic Acids**

It was previously reported that the bulbourethral gland secretion and certain macromolecular polyanions, such as poly-
glutamate and polyaspartate, enhanced the enzymatic coagulation of rat vesicular secretion proteins (27), and that small organic compounds, like tosylglycine, caused activation of the non-secretory TGase of the guinea pig prostate gland (2). We observed that TGase purified from rat CGS was strongly activated by SDS and that the maximal increase of its activity (about 11-fold) was detected when SDS was present in the assay at a concentration of 1.5 mM (Fig. 6). It is also noteworthy increasing SDS concentrations reverted the observed activating effect. Similar results were obtained by using as enzyme source the crude CGS (data not shown). In contrast, guinea pig liver TGase was found to be dose-dependently inhibited by increasing concentrations of the detergent (Fig. 6).

Therefore, we found it of interest to investigate if some physiological analogues of SDS could be able to activate CGS TGase. Since neither sodium laurate nor myristate were found to stimulate the enzyme activity (data not shown), we concluded that the sulfate polar head occurring in the SDS molecule was important in the activation mechanism and could not be substituted with the carboxylic group. We passed, thus, to test amphipatic biomolecules containing phosphate instead of sulfate moiety. The first experiment was performed with a mixture of phosphatidic acids obtained from egg yolk phosphatidylcholine by hydrolysis with phospholipase D. Fig. 7 shows that the phosphatidic acids strongly stimulated the enzyme activity and that this activating effect was a selective feature of CGS TGase, since the liver enzyme was found to be markedly inhibited.

To investigate the role played by different fatty acids bound to glycerophosphatidylcholine we tested the effect of various phosphatidic acids selectively containing lauric, myristic, palmitic, stearic, or oleic acids. Even though all the phosphatidic acids assayed were found able to activate the enzyme at micromolar concentrations, those containing the shorter fatty acid chain (particularly the dilauroyl-phosphatidic acid) were found to be the most effective (Fig. 8). It is noteworthy that glycerophosphate, as well as glycerol and orthophosphate, were completely unable to activate the enzyme (data not shown) and that the addition of ethanolamine, choline, or serine to a phosphatidic acid molecule led to a marked decrease of enzyme activation (Fig. 9).

**DISCUSSION**

TGase isolated from the secretion of rat coagulating gland has been reported as a single highly glycosylated polypeptide chain ($M_r = 65,000$) with a lipid anchor retained during the enzyme apocrine secretion process (15, 28, 29). The only data reported so far on rat CGS TGase amino acid sequence derive from homology studies between different molecular forms of TGase and the cDNA-derived primary structure of the major androgen-dependent secretory protein of rat coagulating gland (DP1) (11). The results of these studies led us to suggest that DP1 is the prostate TGase originally referred to as vesiculase (30). Here we report further biochemical characteristics of TGase secreted from rat CGS and we provide further evidence that CGS TGase is post-translationally modified and its amino acid sequence is identical to that derived from DP1 cDNA.

FAB mapping and automated Edman degradation experiments confirmed that the enzyme is NH$_2$-terminally blocked (10, 11) and allowed to verify that about 85% of the sequence derived from DP1 cDNA is identical to the amino acid sequence of the rat CGS TGase. TGase active site was recognized both in the 252–267 fragment of the trypsin map and in the 248–267 fragment of the tryp tic and endoproteinase Glu-C map.

On the basis of the DP1 sequence, six putative N-glycosylation sites were inferred at Asn-151, Asn-219, Asn-226, Asn-408, Asn-472, and Asn-488. Mass spectral analysis showed that Asn-408 was fully glycosylated, Asn-488 was only partially glycosylated, whereas Asn-151, -226, and -472 were found to be unmodified. The remaining putative N-glycosylation site, Asn-
with the existence of a glycosylphosphatidylinositol anchor. Therefore, all these results are consistent with the hypothesis of Seitz et al. (15) that the enzyme contains not only oligosaccharides but also lipid components bound to the polypeptide chain. The presence of N-linked glycans was inferred on the basis of the lectin binding assays and demonstrated by both the GC-MS analysis of the Me3Si monosaccharides and the FAB spectra of the intact permethylated oligosaccharides released from the protein by PNGase F hydrolysis. The N-linked structures identified belong to the high-mannose and complex type glycans with the former containing five mannose residues and the latter showing microheterogeneity as judged by the occurrence of different antennae fragments in the spectra. The presence of such glycosidic structures actually suggests the existence of different antenna fragments in the spectra. The presence of different concentrations of diacylglycerol (A), dimyristoyl (B), dipalmitoyl (C), diacetylgalactosamine which are known to be components of the biological significance of such activation deserve further investigations.

GC-MS analyses of lipid components showed the presence of similar amounts of lauric, myristic, stearic, and palmitic acids together with traces of linoleic acid. Furthermore, some incomplete hydrolysis products such as 1-monopalmitoylglycerol and 2-monostearoylglycerol were detected, thus suggesting that these fatty acids might be involved in a complex lipid anchor structure around the fatty acid(s) bound to the enzyme and to its possible interaction either with the sperm surface or with some specific seminal component(s) originating from the different accessory sexual glands at the moment of ejaculation.

![Figure 8](image1.png)

**FIG. 8.** Effect of different phosphatidic acids on rat CGS TGase activity. 0.1 μg of purified enzyme was incubated at 37 °C for 1 h in 0.1 ml of 120 mM Tris-HCl buffer, pH 8.0, containing 10 mM DTT, 1.0 mM CaCl2, 50 mM L-histidinamide, and 0.2 mg of DMC, in the presence of different concentrations of diacylglycerol (A), dimyristoyl phosphatidylethanolamine (B), dipalmitoyl phosphatidylcholine (C), and diacylglycerol phosphatidyserine (D). Further experimental details are given in the text.

![Figure 9](image2.png)

**FIG. 9.** Effect of diacylglycerol phosphatidic acid derivatives on rat CGS TGase activity. 0.1 μg of purified enzyme was incubated at 37 °C for 1 h in 0.1 ml of 120 mM Tris-HCl buffer, pH 8.0, containing 10 mM DTT, 1.0 mM CaCl2, 50 mM L-histidinamide, and 0.2 mg of DMC, in the presence of different concentrations of diacylglycerol phosphatidic acid (A), diacylglycerol phosphatidylethanolamine (B), diacylglycerol phosphatidylcholine (C), and diacylglycerol phosphatidyserine (D). Further experimental details are given in the text.
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