Samuel Barondes, Jacques Baenziger, and Edmond Crouch for helpful discussion.

Registry No. Lactose, 63-42-3.

REFERENCES

Blood, C. H., Sasse, J., Brodt, P., & Zetter, B. R. (1988) *J. Cell Biol. 107*, 1987-1993.

Cerra, R. F., Haywood-Reid, P. L., & Barondes, S. H. (1984) *J. Cell Biol. 98*, 1580-1589.

Hall, D. E., Frazer, K. A., Hann, B. C., & Reichardt, L. F. (1988) *J. Cell Biol. 107*, 687-697.

Hinek, A., Kawiak, J., Czarnowska, E., & Barcew, B. (1984) *Acta Biol. Hung. 35*, 245-258.

Hinek, A., Wrenn, D. S., Mecham, R. P. & Barondes, S. H. (1988) *Science 239*, 1539-1541.

Hynes, R. O. (1987) *Cell 48*, 549-554.

Indik, Z., Yeh, H., Ornstein-Goldstein, N., Sheppard, P., Anderson, N., Rosenbloom, J. C., Peltonen, L., & Rosenbloom, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5680-5684.

Jacob, M. P., Fulop, T. J., Foris, G., & Robert, L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 995-999.

MacDonald, R. G., Pfeffer, S. R., Coussens, L., Tepper, M. A., Brocklebank, C. M., Mole, J. E., Anderson, J. K., Chen, E., Czech, M. P., & Ullrich, A. (1988) *Science 239*, 1134-1137.

Mecham, R. P. (1987) *Methods Enzymol. 144 (D)*, 232-246.

Mecham, R. P., Lange, G., Madaras, J. G., & Starcher, B. C. (1981) *J. Cell Biol. 90*, 332-338.

Mecham, R. P., Madaras, J. G., & Senior, R. M. (1984a) *J. Cell Biol. 98*, 1804-1812.

Mecham, R. P., Griffin, G. L., Madaras, J. G., & Senior, R. M. (1984b) *J. Cell Biol. 98*, 1813-1816.

Peters, T., Jr. (1985) *Adv. Protein Chem. 37*, 161-245.

Ruoslahti, E., & Pierschbacher, M. D. (1987) *Science 238*, 491-497.

Senior, R. M., Griffin, G. L., & Mecham, R. P. (1982) *J. Clin. Invest. 70*, 614-618.

Senior, R. M., Griffin, G. L., Mecham, R. P., Wrenn, D. S., Prasad, K. U., & Urry, D. W. (1985) *J. Cell Biol. 99*, 870-874.

Varga, Z., Kovacs, E. M., Paragh, G., Jacob, M. P., Robert, L., & Fulop, T. J. (1988) *Clin. Biochem. 21*, 127-130.

von der Mark, K., & Risse, G. (1987) *Methods Enzymol. 144*, 490-507.

Wewer, U. M., Liotta, L. A., Jaye, M., Ricca, G. A., Drohan, W. N., Claysmith, A. P., Rao, C. N., Wirth, P., Coligan, J. E., Albrechtsen, R., Mudry, M., & Sobel, M. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7137-7141.

Wrenn, D. S., Griffin, G. L., Senior, R. M., & Mecham, R. P. (1986) *Biochemistry 25*, 5172-5176.

Wrenn, D. S., Parks, W. C., Whitehouse, L. A., Crouch, E. C., Kucich, U., Rosenbloom, J., & Mecham, R. P. (1987) *J. Biol. Chem. 262*, 2244-2249.

Wrenn, D. S., Hinek, A., & Mecham, R. P. (1988) *J. Biol. Chem. 263*, 2280-2284.

Zardi, L., Carnemolla, B., Balza, E., Borsi, L., Castellani, P., & Siri, A. (1985) *Int. J. Biochem. 146*, 571-579.

Structural and Functional Studies on the Sodium- and Chloride-Coupled γ-Aminobutyric Acid Transporter: Deglycosylation and Limited Proteolysis†

Baruch I. Kanner,* Shoshi Keynan, and Rodica Radian

Department of Biochemistry, Hadassah Medical School, The Hebrew University, P.O. Box 1172, Jerusalem 91010, Israel

Received August 31, 1988; Revised Manuscript Received November 16, 1988

ABSTRACT: The sodium- and chloride-coupled γ-aminobutyric transporter, an 80-kDa glycoprotein, has been subjected to deglycosylation and limited proteolysis. The treatment of the 80-kDa band with endoglycosidase F results in its disappearance and reveals the presence of a polypeptide with an apparent molecular mass of about 60 kDa, which is devoid of ^{125}I-labeled wheat germ agglutinin binding activity but is nevertheless recognized by the antibodies against the 80-kDa band. Upon limited proteolysis with papain or Pronase, the 80-kDa band was degraded to one with an apparent molecular mass of about 60 kDa. This polypeptide still contains the ^{125}I-labeled wheat germ agglutinin binding activity but is not recognized by the antibody. The effect of proteolysis on function was examined. The transporter was purified by use of all steps except that for the lectin chromatography [Radian, R., Bendahan, A., & Kanner, B. I. (1986) *J. Biol. Chem. 261*, 15437-15441]. After papain treatment and lectin chromatography, γ-aminobutyric transport activity was eluted with *N*-acetylglucosamine. The characteristics of transport were the same as those of the pure transporter, but the preparation contained instead of the 80-kDa polypeptide two fragments of about 66 and 60 kDa. The ability of the anti-80-kDa antibody to recognize these fragments was relatively low. The observations indicate that the transporter contains exposed domains which are not important for function.

The γ-aminobutyric acid (GABA)[1] transporter from rat brain is thought, just like other neurotransmitter transporters, to terminate the overall process of synaptic transmission (Iversen, 1971, 1973; Kuhar, 1973; Bennet et al., 1974). The protein which catalyzes cotransport of sodium, chloride, and GABA (Kanner, 1978, 1983; Kanner & Schuldiner, 1987; Radian & Kanner, 1983; Keynan & Kanner, 1988) has been purified to near homogeneity with a rapid reconstitution assay

† This work was supported by Grant 16708-01 from the National Institute of Neurology Communicative Diseases and Stroke.

[1] Abbreviations: GABA, γ-aminobutyric acid; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 135 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4; buffer A, 0.1 M NaP_i, pH 6.8, 300 mM NaCl, 0.2% Triton X-100, 50 mM octyl β-glucoside; buffer B, 25 mM sodium citrate, 50 mM NaP_i, pH 5, 25 mM NaCl, 0.01% SDS; buffer C, 0.2 M NaP_i, pH 6.1, 100 mM EDTA, 2% NP-40, 2% β-mercaptoethanol.

0006-2960/89/0428-3722$01.50/0

(Radian & Kanner, 1985; Radian et al., 1986). It is an 80-kDa glycoprotein which represents about 0.1% of the membrane protein, and polyclonal antibodies against the purified transporter have been prepared (Radian et al., 1986).

In this paper we provide further evidence that the 80-kDa polypeptide by itself represents the transporter, by analyzing immunoprecipitates from the ^{125}I-labeled transporter preparation. We also describe initial structural studies of the 80-kDa glycoprotein. The polyclonal antibodies against it have been used to monitor its deglycosylation and limited proteolysis. Furthermore, we demonstrate that a functional transporter can be isolated after papain[2] treatment. This preparation contains fragments of the 80-kDa polypeptide of apparent molecular masses of 66 and 60 kDa.

Experimental Procedures

Materials

Protein A–Sepharose CL-4B, WGA, all the proteases and protease inhibitors, neuraminidase, valinomycin, and DNase were purchased from Sigma. WGA–Sepharose CL-4B was from Makor Chemicals Ltd. Endoglycosidase F was from New England Nuclear. Octyl β-glucoside and nigericin were from Calbiochem, and Triton X-100 was from Packard Instruments Co. Soybean phospholipids (asolectin, Associated Concentrates) were partially purified (Kagawa & Racker, 1971). Crude bovine brain lipids were extracted as described (Folch et al., 1957). Cholic acid (Sigma) was recrystallized (Kagawa & Racker, 1971) and neutralized with NaOH to pH 7.4. Sephadex G-50 (fine) was from Pharmacia. Na^{125}I and ^{125}I-labeled protein A were from Amersham. [^{3}H]GABA was from the Nuclear Research Center, Negev. Standard proteins for SDS–PAGE were either from Sigma or from Pharmacia. All other reagents were obtained in the purest form commercially available.

Methods

Purification of the GABA Transporter. The GABA transporter was purified as described (Radian & Kanner, 1985; Radian et al., 1986) except that a few crystals of DNase were added to the membranes prior to extraction with cholate and the following protease inhibitors were present throughout the purification at the indicated final concentrations: PMSF (0.3 mM) and pepstatin, leupeptin, antipain, and aprotonin (all at 1 μg/mL). For the studies described in this paper we used either the transporter purified to homogeneity after the WGA–Sepharose step (Radian et al., 1986) or a less purified preparation, the peak fraction after the DEAE step, which contained the transporter after about 50-fold purification (Radian & Kanner, 1985). For the structural studies such as limited proteolysis and deglycosylation, the transporter after the WGA–Sepharose step was purified by preparative SDS–PAGE and electroeluted as described (Radian et al., 1986).

Antibodies against the GABA Transporter. Polyclonal antibodies were prepared against the GABA transporter as described (Radian et al., 1986).

Protein Iodination Using the Chloramine T Method. This was done basically as described (McConahey & Dixon, 1980). WGA was dissolved in PBS at 1 mg/mL. The pure transporter (after preparative gel electrophoresis and electroelution) and the partially pure transporter (the DEAE peak fraction) solutions at 0.025–0.040 and 0.12–0.18 mg of protein/mL, respectively, were adjusted to 0.15 M NaCl and 0.05 M NaP_i, pH 6.8. To 0.7–0.8 mL of the protein solutions 0.3–1.0 mCi of $^{125}I^-$ was added followed by 10 μL of chloramine T (1 mg/mL). After 10 min 10 μL of sodium metabisulfite (2 mg/mL) was added followed by 10 μL of sodium iodide (10 mg/mL). Subsequently, the free ^{125}I was separated from that bound to the protein as follows:

In the case of WGA it was passed over a 10-mL Sephadex G-50 (fine) column equilibrated in PBS, and the ^{125}I peak of the void volume was collected and dialyzed for 3 days against 2 L of PBS, three changes per day. In the case of the GABA transporter preparations, these were also passed over a similar column but equilibrated with 0.05 M NaP_i, pH 6.8, 0.15 M NaCl, and 0.1% SDS or with buffer A for the pure and partially pure transporter, respectively. The pure iodinated transporter after the gel filtration was dialyzed against two changes of 3 L of 5 mM NaP_i, pH 6.8, for 2.5 h each and then lyophilized, and the pure transporter band [80 kDa (Radian et al., 1986)] was isolated by preparative SDS–PAGE and electroeluted. The partially purified iodinated GABA transporter preparations were processed for immunoprecipitation as described below.

Immunoprecipitation. Immunoprecipitation of labeled transporter was done basically as described (Casadei et al., 1985). For the immunoprecipitation of labeled transporter, antitransporter serum (0.25 mL) or preimmune serum (0.42 mL) was incubated at 4 °C end over end with shaking for 4 h with 30 mg of protein A–Sepharose 4B-CL which had been washed twice with PBS. The beads were then washed twice with PBS and once with buffer A. Aliquots (1 mL) of the ^{125}I-labeled DEAE peak fraction were incubated with the immobilized sera for another hour. Both types of resin were then placed in a glass wool plugged pipet tip and were washed with 8–10 mL of buffer A. The bound material was then eluted with a solution containing 500 mM NaCl, 50 mM sodium citrate, pH 3.0, 0.2% Triton X-100, and 50 mM octyl β-glucoside. The counts were recovered in two fractions of 0.25 mL, and the pH was adjusted by addition of 1/8 volume of 0.4 M Na_2HPO_4. The samples were analyzed by SDS–PAGE and autoradiography.

Limited Proteolysis. Limited proteolysis of the purified GABA transporter in SDS was performed as described (Cleveland et al., 1977). The proteolytic fragments were separated by gel electrophoresis and analyzed by the techniques described below. For full details, see the legends to Figures 3–5.

Deglycosylation. The purified GABA transporter was treated with endoglycosidase F (Elder & Alexander, 1982) or with neuraminidase (Messner & Catterall, 1985). For full details, see the legends to Figures 3 and 4. During the long incubation necessary, the recovery of the bands was somewhat variable due to the sticking of the material to the reaction tubes. It was found that glass tubes are not suitable and better results were obtained in Eppendorf tubes, which were routinely used.

Effect of Papain Treatment on Function. To 4 mL of the DEAE peak fraction was added DTT followed by papain, such that the final concentrations were 1 mM and 0.45 mg/mL, respectively. After 3 min of incubation on ice, iodoacetamide was added to a final concentration of 3 mM. The mixture was immediately applied to a 2-mL WGA–Sepharose column which was run at 0–4 °C as described (Radian et al., 1986). After being washed with 50 volumes of the washing solution, the glycoprotein fraction was eluted exactly as described

[2] Enzymes: endoglycosidase F, endo-β-*N*-acetylglucosaminidase F (EC 3.2.1); neuroaminidase, sialidase (EC 3.2.1.18); α-chymotrypsin (EC 3.4.21.1); elastase, pancreatopeptidase E (EC 3.4.21.36); V_8, protease from *Staphylococcus aureus*, strain V_8; papain, papainase (EC 3.4.22.2); Pronase, Pronase E.

(Radian et al., 1986). Aliquots of the fractions were reconstituted and assayed for ($Na^+ + Cl^-$)-coupled GABA transport (Radian & Kanner, 1985; Radian et al., 1986). In order to determine the polypeptide composition of some of the fractions by SDS–PAGE and silver staining (see below), it was necessary to concentrate some of the fractions. This was done by dialyzing them at 0–4 °C against 5 mM NaP_i, pH 6.8, followed by lyophilization.

SDS–PAGE. SDS–PAGE (Laemmli, 1970) was done with a 4% stacking gel and a 10% separating gel unless stated otherwise in the figure legends. The gels were either silver stained (Merril et al., 1984) or blotted (see below). Standard proteins for SDS–polyacrylamide gels were as follows: phosphorylase *b*, M_r = 97 000; bovine serum albumin, M_r = 66 000; ovalbumin, M_r = 43 000; glyceraldehyde-3-phosphate dehydrogenase, M_r = 36 000; bovine carbonic anhydrase, M_r = 30 000; soybean trypsin inhibitor, M_r = 20 100; α-lactalbumin, M_r = 14 400.

Immunoblotting. Analysis of the specificity of the antibodies was done exactly as described (Radian et al., 1986; Towbin et al., 1979).

Identification of ^{125}I-Labeled WGA Binding Glycoproteins on Nitrocellulose Blots of SDS–Polyacrylamide Gels (WGA Blotting). This was done as described (Bartles & Hubbard, 1984). The periodate–aniline–cyanoborohydride treatment, which enhances the sensitivity of this method, was performed exactly as described by these authors.

Phosphate Determination. This was performed by the method of Ames (1966).

Protein Determination. This was done according to the Peterson (1977) method.

Results

Immunoprecipitation of GABA Transport Activity and the 80-kDa Polypeptide. Polyclonal antibodies were prepared against the predominant polypeptide present in the final purification step of the GABA transporter in three different rabbits. Most of the experiments described were done with all three antisera with basically the same results. We have already shown that the antibodies are able—in conjunction with protein A–Sepharose CL-4B beads—to remove the transport activity from partially pure transporter preparations (Radian et al., 1986). Analysis of the polypeptide(s) immunoprecipitated by the beads may provide information whether other polypeptides are associated with the 80-kDa polypeptide. Protein A–Sepharose beads treated with control or experimental sera were incubated with the DEAE peak fraction—a partially purified preparation—which had previously been radioiodinated. After being extensively washed the bound material was released with acid, and the presence of any labeled polypeptides was monitored after SDS–PAGE and autoradiography (Figure 1). It is apparent that one major labeled band was recovered from the antitransporter antiserum beads, namely, the 80-kDa band (Figure 1A, lane 2). No radioactive beads were recovered from the beads treated with control serum (Figure 1A, lane 1). Thus, the 80-kDa band, which does not represent more than 10% of this preparation (Figure 1B), is selectively immunoprecipitated by the antiserum (Figure 1A). The faint band at about 50 kDa is probably due to the major polypeptide in this region present in the DEAE peak preparation (Figure 1B). It can be seen that some of the aggregated material did not penetrate the gel (Figure 1A, lane 2). The results indicate that the antibody is very selective. It can be concluded from the experiment that the removal of the transport activity by the antibody is due to the removal of the 80-kDa polypeptide. Furthermore, there does not appear to be a specific association of other polypeptides to the 80-kDa polypeptide.

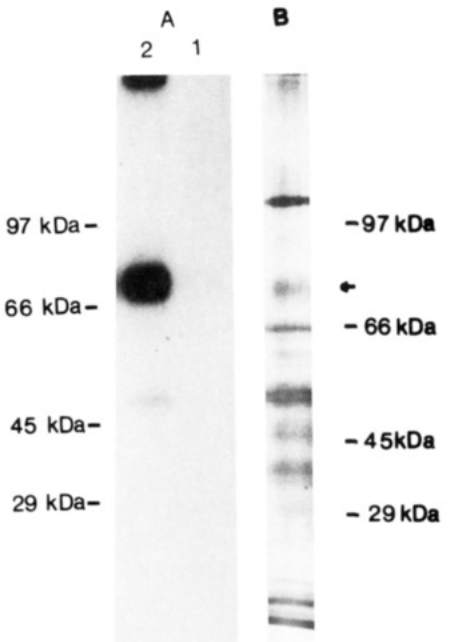

FIGURE 1: Immunoprecipitation of the ^{125}I-labeled GABA transporter. (A) The DEAE-cellulose peak fraction (Radian & Kanner, 1985), containing the partially purified GABA transporter, was labeled with ^{125}I (Experimental Procedures). The iodinated fraction was then incubated with protein A–Sepharose CL-4B beads, which had previously been incubated with (lane 1) preimmune serum or (lane 2) antitransporter serum. The proteins which were absorbed to the beads were eluted with sodium citrate containing buffer, pH 3 (Experimental Procedures). Samples of the eluted proteins were analyzed by SDS–PAGE and processed for autoradiography; 5 μL of the eluate in lane 2 gave 26 000 cpm. (B) A typical polypeptide pattern of the DEAE-cellulose peak fraction as visualized by silver staining is given (4 μg of protein). The localization of the GABA transporter is marked with the arrow.

Deglycosylation of the 80-kDa Polypeptide. The 80-kDa polypeptide was radioiodinated with ^{125}I. The preparation was then treated with endoglycosidase F, which is able to cleave "high-mannose" as well as "complex" carbohydrates linked through asparagine to the protein backbone (Elder & Alexander, 1982). Treated and control preparations were analyzed by SDS–PAGE and autoradiography. The apparent molecular mass of the 80-kDa band was reduced to about 60 kDa (Figure 2, lane 2) whereas its molecular mass in the control preparation was not changed (Figure 2, lane 1). Treatment with neuramidinase alone did not have any effect on the apparent molecular mass (Figure 2, lane 3), and the sequential treatment of neuramidinase and endoglycosidase F yielded the 60-kDa band (Figure 2, lane 4).

The ability of the polyclonal antibodies to recognize the deglycosylated polypeptide was checked by use of preparations which were not radioiodinated. After treatment with endoglycosidase F the preparation was run on SDS–PAGE, Western blotted, and reacted first with the antibody and then with ^{125}I-labeled protein A, followed by autoradiography. It can be seen that the deglycosylated GABA transporter can still be recognized by the antibody (Figure 3A, lane 2). The antibody also detects dimeric and trimeric forms of the transporter with (Figure 3A, lane 2) and without (Figure 3A, lane 1) deglycosylation. The extent of oligomer formation was variable from experiment to experiment, and probably one of the reasons for the variability is the protein concentration. Similar results were obtained when the 80-kDa band was subjected to a nonradioactive iodination and then treated as

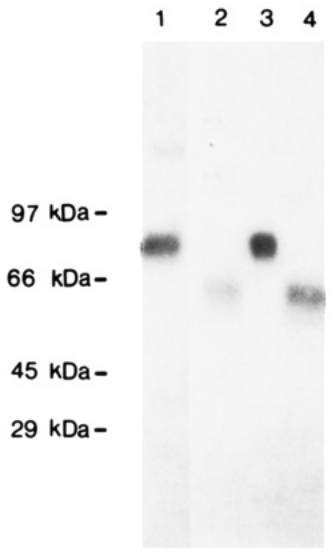

FIGURE 2: Effect of endoglycosidase F and neuroaminidase on the mobility of the ^{125}I-labeled GABA transporter. The purified 80-kDa band which represents the GABA transporter was iodinated as described under Experimental Procedures and treated as follows: (lane 1) 5 μL of ^{125}I-labeled 80-kDa band was incubated for 4 h at 37 °C with 5 μL of buffer B and then for another 24 h at room temperature upon addition of another 10 μL of buffer C; (lane 2) 2 μL of the ^{125}I-labeled 80-kDa band was incubated for 24 h at room temperature with 18 μL of buffer C plus 0.5 unit of endoglycosidase F; (lane 3) 5 μL of the ^{125}I-labeled 80-kDa band was incubated for 4 h at 37 °C with buffer B plus 0.2 unit of neuraminidase; (lane 4) 5 μL of ^{125}I-labeled 80-kDa band was first treated as in lane 3 and then as in lane 2, upon addition of equal volume of buffer C (5 μL of ^{125}I-labeled 80-kDa band gave 8000 cpm). The treated samples were analyzed by SDS–PAGE, stained with Coomassie blue, destained, and then processed for autoradiography.

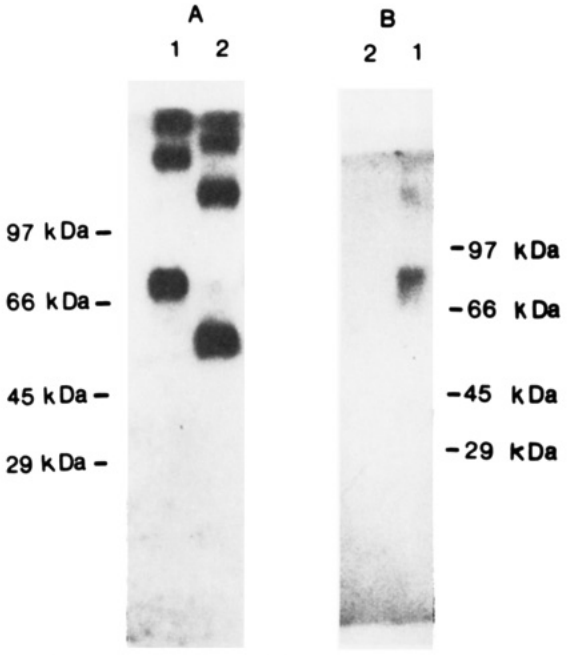

FIGURE 3: Immunoreactivity of anti-80-kDa antibodies with the deglycosylated GABA transporter. The 80-kDa polypeptide (25 μL, 1.2 μg of protein) was incubated for 2 days at room temperature with an equal volume of buffer C and the following additions: (lane 1) none; (lane 2) 0.25 unit of endoglycosidase F. Deglycosylation was stopped by addition of an equal volume of SDS sample buffer. The samples were then subjected to Western blotting. (A) Immunoblotting was performed as explained (Experimental Procedures) with a 1:100 dilution of the experimental serum. (B) WGA blotting was performed as described under Experimental Procedures.

above (data not shown). A control experiment was carried out to make sure that indeed under the condition of Figure 3A the polypeptide treated with endoglycosidase F had lost its sugar. In this experiment the bands on the blot were analyzed with ^{125}I-labeled WGA, rather than with antibody and ^{125}I-labeled protein A. The control preparation showed a major band capable of binding ^{125}I-labeled WGA with an apparent molecular mass of 80 kDa and a smaller amount of the dimer, and in addition a contaminant band, possibly due to proteolysis, at 40 kDa was visualized (Figure 3B, lane 1). The preparation treated with endoglycosidase F did not reveal any band with binding capacity for the lectin (Figure 3B, lane 2). This demonstrates that the deglycosylated band is still recognized by the antibody.

Limited Proteolysis of the 80-kDa Polypeptide. The radioiodinated 80-kDa polypeptide was subjected to limited proteolysis with a variety of proteases (Cleveland et al., 1977). It can be seen that polypeptides with decreased molecular mass, between 55 and 65 kDa, are generated with papain and Pronase (Figure 4, lanes 5 and 6). When V_8 is present (Figure 4, lane 4), there is only a slight effect on the mobility, whereas α-chymotrypsin or elastase has no effect (Figure 4, lanes 2 and 3). In addition, noniodinated 80-kDa polypeptide was treated as above with papain and Pronase, but the SDS–PAGE gel was Western blotted, and the blots were treated with the polyclonal antibody and then with ^{125}I-labeled protein A, and the generated bands were visualized by autoradiography (Figure 5A). It can be seen that only in the control sample are the bands of 80 kDa and its dimer and trimer forms observed (Figure 5A, lane 1). The treated preparations did not give rise to any visualized bands in the autoradiography (Figure 5A, lanes 2 and 3). The same result was obtained if the preparation was iodinated prior to proteolysis with non-radioactive iodine (data not shown). These results indicate that the proteolytic removal of only a small fragment of between 15 and 25 kDa causes the removal of the antigenic determinants recognized by the antiserum. It is of interest to note that the remaining polypeptides of 55–65 kDa still contain their sugar, since they preserved the ability to bind the ^{125}I-WGA (Figure 5B, lanes 2 and 3). The band at 40 kDa visualized after WGA blotting (Figure 5B) may be due to proteolysis. We could not recover the smaller fragments containing the antigenic determinants, in a controlled fashion. However, occasionally upon treatment with papain a 20-kDa fragment was detected which was able to bind the antibody (data not shown). This confirms our conclusion that the antigenic determinants are confined to a limited portion of the polypeptide.

Isolation of Functional Fragments of the Transporter by Proteolysis. In view of the structural data shown above, it was of interest to see if transporter function could be preserved after proteolysis. The transporter was purified through all steps but the last step (which is chromatography on WGA–Sepharose), and the material—the DEAE peak fraction—was used for the subsequent experiments. Transport activity upon reconstitution inactivated very quickly when the DEAE peak was incubated at 37 °C or at room temperature but not at 0 °C. Incubation with papain, Pronase, and trypsin was performed for 3–10 min. It was found that activity was best and most reproducibly preserved with papain, while the other proteases give more variability and varying extents of inactivation (data not shown). Thus papain was used for more detailed studies. Analysis of the papain-treated preparation by SDS–PAGE and silver staining did not reveal much since the pattern was more complex due to the presence of large amounts of the papain and its contaminants. Since the GABA transporter is the only glycoprotein in the DEAE peak fraction,

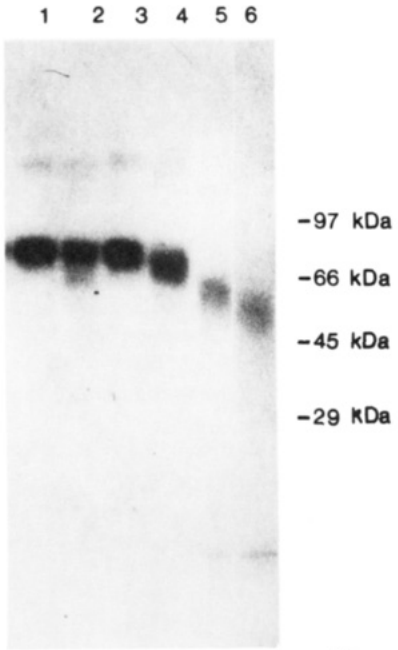

FIGURE 4: Effect of external proteases on the ^{125}I-labeled GABA transporter. The iodinated 80-kDa polypeptide was run on a SDS–polyacrylamide gel together with the indicated amounts of protease. At the interphase of the stacking and separating gel, the run was stopped for 30 min. Subsequently, the gel was cooled to 4 °C and run as usual. Cleavage products were identified by autoradiography after staining with Coomassie blue and destaining. In each lane the input was 8000 cpm. The proteolytic enzymes were as follows: (lane 1) none; (lane 2) 10 μg of α-chymotrypsin; (lane 3) 1 μg of elastase; (lane 4) 0.2 μg of V_8; (lane 5) 0.05 μg of papain; (lane 6) 0.05 μg of Pronase. Similar results were obtained with higher levels—up to 1 μg per lane—of the last three proteases (data not shown).

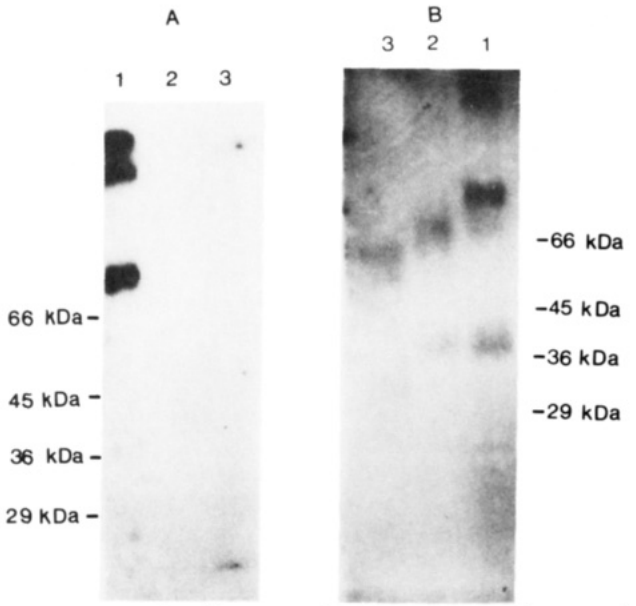

FIGURE 5: Reactivity of fragments of the 80-kDa polypeptide generated by limited proteolysis with anti-GABA transporter antiserum and ^{125}I-labeled WGA. The 80-kDa polypeptide was run on a 10%–20% SDS–polyacrylamide gel (continuous gradient) with the indicated amounts of proteases: (lane 1) no addition; (lane 2) 0.5 μg of papain; (lane 3) 1 μg of Pronase. At the interphase of the stacking and separating gel the run was stopped for 30 min. Subsequently, the gel was cooled to 4 °C and run as usual. After the run was completed, the gel was Western blotted. (A) Immunoblot, using a 1:100 dilution of experimental serum. (B) WGA blot (Experimental Procedures).

lectin chromatography after the papain treatment would be expected to simultaneously remove the papain and its contaminants and purify the fragment(s) of the GABA transporter. The papainized preparation was passed over a WGA–Sepharose column and then eluted with *N*-acetylglucosamine. It can be seen that indeed the activity was retained and eluted by the sugar (Figure 6). Analysis of the fractions by SDS–PAGE revealed that the flow through contains the papain and its impurities (Figure 7A, lane b). The active fraction is completely devoid of the 80-kDa polypeptide but contains two new bands, one of 66 kDa and one of 60 kDa (Figure 7A, lanes a and c). Never were bands other than the ones of 66 and 60 kDa observed in these active glycoprotein fractions. It can be observed that the untreated transporter purified by lectin chromatography contains the 80-kDa polypeptide and its dimer as the main components but that a

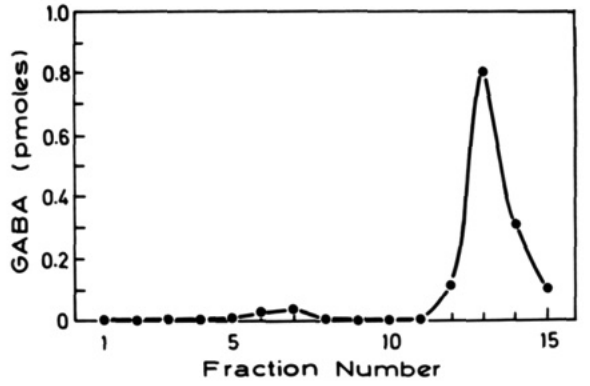

FIGURE 6: Purification of fragments of the GABA transporter by WGA chromatography. DEAE peak fractions (4 mL, 1.0 mg of protein) were treated with papain and run on a 2-mL WGA–Sepharose Cl-4B column as described under Experimental Procedure. After sample loading and washing, elution with the *N*-acetylglucosamine fraction started at fraction 11. The volumes of the fractions were as follows: (1–7) 2 mL; (8 and 9) 45 mL; (10 and on) 1 mL. Aliquots of 35 μL were reconstituted, and subsequently, [^{3}H]GABA transport was carried out for 10 min with 20 μL proteoliposomes.

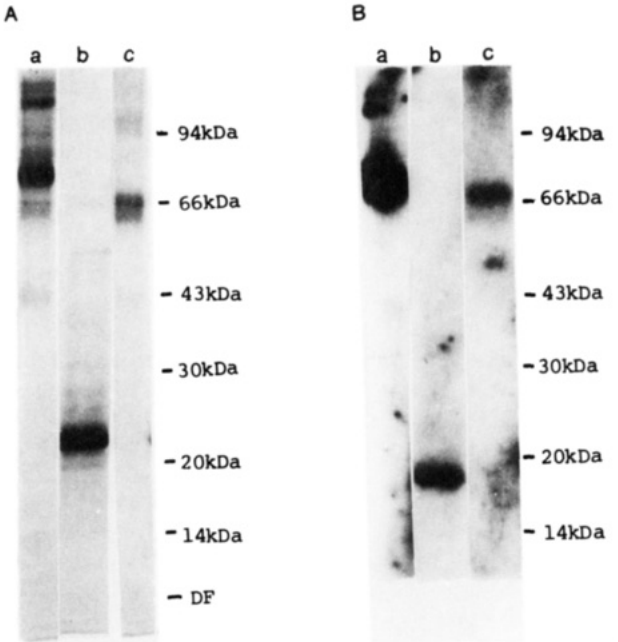

FIGURE 7: Polypeptide composition and immunoreactivity of fragments of the GABA transporter purified by WGA chromatography. Fractions 6 and 13 (lanes b and c, respectively) from Figure 6 were analyzed by SDS–PAGE and silver staining (A) or immunoblotting with the anti-80-kDa antibody (B). Lane a represents the GABA transporter purified by the lectin column, without previous papain treatment. The samples used in (A), lanes a and c, were first dialyzed against 5 mM NaP_i, pH 6.8, lyophilized, and resuspended in one-tenth of the original volume.

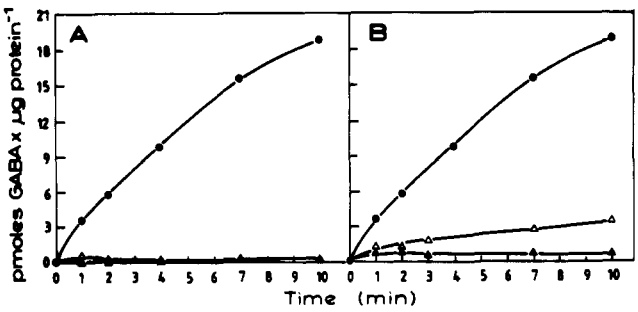

FIGURE 8: Effects of ionic composition of the medium and ionophores on GABA transport by the fragments of the GABA transporter. [^{3}H]GABA influx into proteoliposomes, obtained upon reconstitution of WGA–Sepharose peak fractions, was measured. (A) In the presence of 2.5 μM valinomycin. The external medium was 150 mM NaCl, containing 1 μCi of [^{3}H]GABA (●) or the same with 150 mM sodium glucuronate (△) or 150 mM LiCl (▲) replacing the NaCl. (B) The external medium was NaCl containing one of the following additions: (△) none; (●) 2.5 μM valinomycin; (▲) 5 μM nigericin + 2.5 μM valinomycin. For each time point 26.6 ng of protein was used.

small amount of a 63-kDa band is also present (Figure 7A, lane a). This is a well-known contaminant, which is not related to the transporter (data not shown). The amount of fragments is also much higher than the amount of 63 kDa in the "native" transporter preparation. Thus it appears that the bands of 66 and 60 kDa are fragments of the 80-kDa transporter, and not related to the contaminant. In some of the experiments, the polypeptide composition of the active fraction was somewhat different in the sense that only the 60-kDa band was visualized upon silver staining (data not shown). The immunoreactivity of the anti-80-kDa antibody toward the 66- and 60-kDa fragments was relatively low as compared with that toward the 80-kDa polypeptide obtained in parallel by lectin chromatography of the untreated DEAE peak (Figure 7B, lanes a and c). The film was overexposed in order to visualize the bands of 66 and 60 kDa. Thus, the 80-kDa polypeptide yields a broad band (Figure 7B, lane a). At shorter exposures a much narrower band, positioned at the center—at 80 kDa—was obtained (data not shown). It can be seen that the flow through contains a band of about 18 kDa which reacts with the anti-80-kDa antibody (Figure 7B, lane b). This reflects a fragment of the transporter since the antibody did not react with papain (data not shown). Thus, it seems that cleavage of the GABA transporter by papain is similar under denaturing (Figure 5) and "native" (Figure 7) conditions. GABA transport of proteoliposomes inlaid with the fragments is absolutely dependent both on sodium ions and on chloride ions (Figure 8A). The transport is strongly stimulated by the potassium ionophore valinomycin under conditions of an outward potassium gradient (Figure 8B). These conditions are expected to set up an interior negative membrane potential. The ionophore nigericin, which will effectively exchange external sodium for internal potassium, abolishes transport completely (Figure 8B). Thus the transport activity of the papainized transporter basically has the same characteristics as the pure native transporter (Radian et al., 1986) and the transporter in synaptic plasma membrane vesicles (Kanner, 1978).

Discussion

In this paper we have provided evidence that the 80-kDa polypeptide by itself represents the GABA transporter, using antibodies generated against this polypeptide. Furthermore, we have used this antibody for some structural studies in which the 80-kDa polypeptide was deglycosylated and subjected to limited proteolysis. Finally, we have provided evidence that fragments of the transporter—generated by papain—are fully functional in catalyzing ($Na^+ + Cl^-$)-coupled GABA transport.

Treatment of the 80-kDa polypeptide with endoglycosidase F, an enzyme which removes both "high-mannose" and "complex" carbohydrates, reduces its apparent molecular mass to about 60 kDa (Figure 2). This indicates that about 25% of the mass of this heavily glycosylated transporter is carbohydrate. This number however has to be interpreted with caution since it is well-known that glycoproteins have a nontypical mobility on SDS–PAGE as compared with that of the standard proteins. It is of interest to note that the deglycosylated polypeptide is still recognized by the antibodies (Figure 3). This, together with the data from Figure 5, indicates that the antigenic determinants reside in the protein part of the transporter.

Limited proteolysis reveals that—with a variety of proteases—fragments of 55–65 kDa are generated (Figure 4) and that these fragments are not recognized by the antibody (Figure 5A). These observations may be explained by assuming (1) that the transporter has—even in SDS—still a lot of secondary structure and (2) that the antibodies are generated against a relatively exposed part of the polypeptide. Limited proteolysis results in the digestion of the exposed part, thereby leaving the majority of the structure but destroying the antigenic determinants. It is also in harmony with the observation that the antibody is not able to inhibit transport activity (data not shown). The important functional parts involved in the binding of the substrates are most likely not exposed. The data on lack of recognition of proteolytic fragments by the antibody predict difficulties in their use to screen λgt11 libraries. However, other approaches to clone the transporter certainly seem feasible (Schofield et al., 1987; Hediger et al., 1987).

The effect of deglycosylation on function cannot be determined at present since it necessitates incubation at room temperature for extended periods under harsh conditions. Under these conditions transport activity is rapidly lost. The functional consequences of papain treatment indicate that a substantial part of the transporter polypeptide can be removed (Figure 7) without impairing function (Figures 6 and 8). It is likely that these are exposed parts of the transporter, since the ability of the anti-80-kDa antibodies to recognize the fragments is impaired (Figure 7B).

It appears that a considerable part of the GABA transporter is relatively exposed and is not important in the translocation function. Out of the 80 kDa, as much as 20 kDa may be carbohydrate, and up to 20 kDa represents protein which is not necessary for function. It is expected that the data here will give us additional important insights on structure–function relationships of the ($Na^+ + Cl^-$)-coupled GABA transporter once the gene for the 80-kDa glycopolypeptide has been cloned.

Acknowledgments

We thank Dr. Jonathan Gershoni for his helpful advice on the identification of ^{125}I-labeled WGA binding glycoproteins on nitrocellulose blots of SDS–polyacrylamide gels. We greatly appreciate the technical assistance by Annie Bendahan.

References

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.

Bartles, J. R., & Hubbard, A. L. (1984) *Anal. Biochem. 140*, 284–292.

Bennett, J. P., Jr., Mulder, A. H., & Snyder, S. M. (1974) *Life Sci. 15*, 1045–1056.

Casadei, J. M., Gordon, R. D., & Barchi, R. L. (1986) *J. Biol. Chem. 261*, 4318–4323.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102-1106.
Elder, J. H., & Alexander, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4540-4544.
Folch, J., Lees, M., & Sloane Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497-509.
Hediger, M. A., Coady, M. J., Ikeda, T. S., & Wright, E. M. (1987) *Nature 330*, 379-381.
Iversen, L. L. (1971) *Br. J. Pharmacol. 41*, 571-591.
Iversen, L. L. (1973) *Br. Med. Bull. 29*, 130-135.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477-5487.
Kanner, B. I. (1978) *Biochemistry 17*, 1207-1211.
Kanner, B. I. (1983) *Biochim. Biophys. Acta 726*, 293-316.
Kanner, B. I., & Schuldiner, S. (1987) *CRC Crit. Rev. Biochem. 22*, 1-38.
Keyan, S., & Kanner, B. I. (1988) *Biochemistry 27*, 12-27.
Kuhar, J. M. (1973) *Life Sci. 13*, 1623-1634.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
McConahey, P. J., & Dixon, F. J. (1980) *Methods Enzymol. 70*, 210-213.
Merril, C. R., Goldman, D., & Van Keuren, M. L. (1984) *Methods Enzymol. 104*, 441-447.
Messner, D. J., & Catterall, W. A. (1985) *J. Biol. Chem. 260*, 10597-10604.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346-356.
Radian, R., & Kanner, B. I. (1983) *Biochemistry 22*, 1236-1241.
Radian, R., & Kanner, B. I. (1985) *J. Biol. Chem. 260*, 11859-11865.
Radian, R., Bendahan, A., & Kanner, B. I. (1986) *J. Biol. Chem. 261*, 15437-15441.
Schofield, P. R., Darkson, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H., & Barnard, E. A. (1987) *Nature 320*, 221-227.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.

Transverse and Lateral Distribution of Phospholipids and Glycolipids in the Membrane of the Bacterium *Micrococcus luteus*

J. de Bony,[‡] A. Lopez,[‡] M. Gilleron,[‡] M. Welby,[‡] G. Lanêelle,[‡] B. Rousseau,[§] J. P. Beaucourt,[§] and J. F. Tocanne*[,‡]

Centre de recherche de Biochimie et de Gênêtique cellulaires du CNRS, 118, route de Narbonne, 31062 Toulouse Cedex, France, and Centre d'êtudes nucléaires de Saclay, 91191 Gif-sur-Yvette Cedex, France

Received November 9, 1988; Revised Manuscript Received January 4, 1989

ABSTRACT: The photodimerization of anthracene was used to investigate the transverse and lateral distribution of lipids in the membrane of the Gram-positive bacterium *Micrococcus luteus*. 9-(2-Anthryl)nonanoic acid (9-AN) is incorporated at a high rate into various membrane lipids of *M. luteus*. On irradiation of intact bacteria at 360 nm, anthracene-labeled lipids form stable photodimers which can be extracted and separated by thin-layer chromatography. We present here the results of a study on the distribution of two major lipids, phosphatidylglycerol (PG) and dimannosyldiacylglycerol (DMDG), within each leaflet of the membrane lipid bilayer. After metabolic incorporation of a tritiated derivative of 9-AN in *M. luteus*, the radioactivity associated with the photodimers issued from PG and DMDG was counted. In the bacterial membrane, the ratio of PG-DMDG heterodimer with respect to PG-PG and DMDG-DMDG homodimers is around half of what should be obtained for a homogeneous mixture of the two lipids. In order to find out whether this was due to an asymmetric distribution of the two lipids between the two membrane leaflets or a heterogeneous distribution of the two lipids within the same membrane leaflet, the transverse distribution of PG and DMDG was also investigated. This was carried out by following the kinetics of oxidation of the two lipids by periodic acid in the membrane of *M. luteus* protoplasts. PG predominated slightly in the outer layer (60%), while DMDG was found to be symmetrically distributed between the two leaflets. By itself, this lipid asymmetry cannot account for the lipid distribution determined from the photodimerization experiments. This indicates that PG and DMDG are not homogeneously distributed in the plane of the bacterial membrane.

One of the most challenging problems in membrane biology is to get a clear picture of lipid and protein organization and mobility at a microscopic and even molecular level. Surprisingly, biological membranes contain a large variety of lipids, and such a diversity is not required for maintaining the bilayer membrane assembly and fluidity. Despite extensive studies on the structure of biological membranes during the last decade, we have no direct information on the potential specificity of lipid distribution around membrane proteins or on the molecular organization of those lipids which are not in contact with proteins. A transverse asymmetry of lipids has been observed in red blood cells (Op den Kamp, 1979) and in various cytoplasmic (Sandra & Pagano, 1978) and intracellular membranes (Herbette et al., 1984). This asymmetry is related to the nature of the polar head group and the degree of unsaturation of the constituent fatty acids (Bick et al., 1987).

* To whom correspondence should be addressed.
[‡] Centre de recherche de Biochimie et de Gênêtique cellulaires du CNRS.
[§] Centre d'êtudes nucléaires de Saclay.

0006-2960/89/0428-3728$01.50/0