Effect of Experimental Diabetes on the Susceptibility of Myelin Proteins to Proteolysis

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SUMMARY

To investigate abnormalities in peripheral nerve myelin in experimental diabetes, we studied the effects of the proteolytic enzymes trypsin and α-chymotrypsin on the proteins of this membrane, obtained from the sciatic nerves of normal rats and from animals made diabetic with streptozotocin. The dominant effect of the proteolytic enzymes was to incompletely degrade the major membrane protein (mol. wt. 28,000), with the appearance of new protein (mol. wt. 20,000). Using myelin isolated from the nerves of diabetic animals, the reaction was approximately one-half that of the controls ($P < 0.01$) for both enzymes. When, however, the myelin protein affected by trypsin and chymotrypsin was isolated from the membrane and then incubated with the proteolytic enzyme, its proteolysis was complete and took place at the same rate in the diabetic animals and controls. These findings suggest that, in this model of experimental diabetes, there is an alteration in the structure of peripheral nerve myelin that inhibits interaction between the protein in the membrane bilayer and two water soluble proteolytic enzymes. This alteration could not be demonstrated in protein isolated from the membrane, suggesting that the change relates to the interaction of the protein and other components of myelin, rather than to chemical alteration in the protein per se.

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Both neurophysiologic[1] and metabolic studies[2] suggest that diabetes induced in the rat by streptozotocin is accompanied by abnormalities in peripheral nerves. Standard histologic studies, however, have been inconsistent in showing abnormalities in this condition. While Sharma and Thomas found no morphologic changes in the sciatic nerves of these rats,[3] studies by Jakobsen and Lundbaek[4] suggest a loss in axonal mass. A clear-cut defect in myelin, such as the segmental demyelination that is characteristic of advanced diabetic neuropathy in man,[5] has not been seen in this model.

In this study we have searched for alterations in myelin from the sciatic nerves of animals made diabetic with streptozotocin, using a biochemical rather than a histologic approach. The effect of proteolytic enzymes on the proteins of membranes has been used to assess the characteristics of these proteins and their relationship to the lipid bilayer.[6] We have used trypsin and α-chymotrypsin to investigate myelin that was isolated from the peripheral nerves of control rats and those made diabetic with streptozotocin. We find that, under our experimental conditions, the major glycoprotein (P_o) of myelin is affected by proteolysis and that this effect in diabetes is approximately one-half of that seen in controls. This difference between the controls and diabetics is not seen when the P_o protein is isolated from the membrane and then subjected to proteolytic digestion.

MATERIALS AND METHODS

MATERIALS

Male albino rats were obtained from Zartman Farms, Douglassville, Pennsylvania, and diabetes was induced with streptozotocin (120 mg/kg given i.p.) when they were 6 wk old, as previously described.[2] The diabetes did not require insulin, and blood sugars ranged between 200 and 350 mg/dl. Experiments were carried out 4 wk later. Trypsin (type III) was obtained from Sigma and α-chymotrypsin from Worthington Biochemical Corporation.

METHODS

Myelin was isolated from sciatic nerve, by a series of discontinuous and continuous flotation steps as modified from

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the procedure of Autillo, Norton, and Terry,[7] that we have published previously for the isolation of myelin from human, rat, and rabbit peripheral nerves.[2,8]

Myelin was incubated with proteolytic enzymes in a final volume of 5.0 ml at 25°C for 30 min. In timed experiments, aliquots were removed at 2, 5, 10, 20, and 30 min.[2] The reaction mixture contained myelin from the sciatic nerves of four animals (total myelin, 10 mg; myelin protein, 2 mg) and trypsin or α-chymotrypsin (1 μg/ml in Tris-HCl buffer—10 mM, pH 8.2). At the end of the incubation, an aliquot of the reaction mixture was taken out and diluted with 10 ml of ice-cold Tris-HCl (10 mM, pH 8.2) and spun at 80,800 × *g* for 20 min. The pellet was washed once with the same buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Slab-gel electrophoresis on polyacrylamide in presence of sodium dodecyl sulfate was carried out by a modification of the method described by Studier.[9] In each experiment, 50 μg of myelin protein was applied to the gel. The details of this procedure have been described in our recent publication.[10] The gels stained with Coomassie blue were scanned at 550 nm using a Gilford 2400 spectrophotometer equipped with a linear transport.

Isolation of P_0 protein by slab-gel electrophoresis. Myelin protein (2 mg) was applied to the gel and the components were separated by electrophoresis as previously described.[10] For each experiment, proteins separated by two slab gels were used. At the end of the experiment, a strip of gel (1 cm wide) was cut, stained for 5 min with Coomassie blue, and then destained for 10 min. Using this stained gel as a guide, the part of the gel corresponding to P_0 protein was cut and finally dispersed in 5 ml of Tris-HCl buffer (10 mM, pH 8.2), using a polytron homogenizer, and was left at room temperature for 18 h. The acrylamide was then separated by centrifugation at 1000 × *g* and the supernatant containing the P_0 protein was freeze-dried. The freeze-dried material was then dissolved in 1.0 ml of water and dialyzed for 24 h against 2 L of water. After dialysis, the solution containing the P_0 protein was again freeze-dried and was then dissolved in 200 μl of 10% SDS. The purity of this material was established by analytic slab-gel electrophoresis, and this method showed mainly the one band corresponding to P_0 protein. For proteolytic digestion of P_0 protein, experimental conditions were the same as described above for myelin.

RESULTS

Effect of trypsin on rat peripheral nerve myelin. The densitometric scan (Figure 1) of myelin proteins incubated in the absence of trypsin is like that published previously[10] and is indistinguishable between diabetic and nondiabetic animals. The five major polypeptide components that are seen when this system is used are: (1) P_0 protein (mol. wt. 28,000), which is the major glycoprotein; (2) P_1 protein, which splits into two components (mol wt. 18,000 and 16,000); and (3) P_2 protein (mol. wt. 13,500), which also contains two closely moving components. After myelin is incubated with trypsin or chymotrypsin, a new polypeptide of approximate mol. wt. 20,000 appears (shown in shaded area in Figure 1). This proteolytic product appears within 2 min of incubation and does not increase further during a 30-min incubation. The reaction was also unaffected by altering the trypsin concentration from 1 to 5 μg/ml. The proteolytic product is completely absent in control experiments when myelin is incubated without the proteolytic enzymes. For several reasons, we believe that the polypeptide fragment (mol. wt. 20,000) is derived by proteolysis of P_0 protein (mol. wt. 28,000). First, the product, like P_0, stains for glycoprotein with periodic acid-Schiff reagent. Second, the area of the new peak is approximately 20% of the peak height of P_0, and this is comparable to the decrease in the area of P_0. Finally, as described below, after P_0 protein is isolated from myelin and then subjected to proteolytic digestion, the major product has the mobility and staining characteristics of the polypeptide that is formed when isolated myelin is digested with proteolytic enzymes.

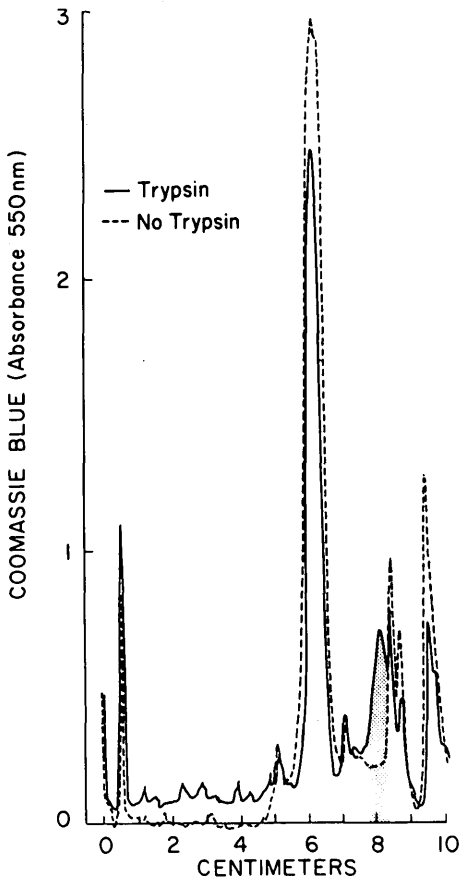


FIGURE 1. Densiometric scan of polypeptides obtained after digestion of myelin with trypsin. Myelin was isolated from rat sciatic nerves and then incubated with trypsin for 30 min. At the end of incubation, myelin treated with trypsin was separated from water-soluble digestion products by centrifugation. The water-insoluble products were partially delipidated, dissolved in SDS, and separated by SDS-polyacrylamide slab gel electrophoresis, then stained with Coomassie blue and scanned at 550 nm. The broken lines represent the incubations done in the absence of trypsin and the solid curve are those done in the presence of trypsin. The shaded area in the curve shows the product formed after proteolytic digestion. The mobilities of different polypeptides are: (1) P_0 protein (6.0 cm; mol. wt. 28,000); (2) P_1 protein (8.5 cm; mol. wt. 18,000 and 16,000); (3) P_2 protein (9.8 cm; mol. wt. 13,500); and (d) proteolytic digestion product (8.0 cm; mol. wt. 20,000).

Comparison of the extent of proteolysis of P_0 in myelin from diabetic and nondiabetic animals. To quantify the extent of the proteolytic reaction, we calculated the ratio of the peak height of the reaction product (polypeptide mol. wt. 20,000) to that of the P_0 in the densitometric traces of myelin proteins isolated from membranes incubated for 30 min with

TABLE 1
Effect of trypsin and α-chymotrypsin on myelin from diabetic and nondiabetic animals

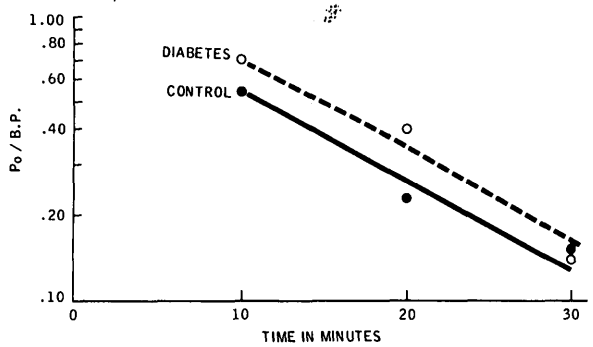
	Ratio: breakdown product/P_o	
	Nondiabetic	Diabetic
Trypsin	0.38 ± 0.03 (7)*†	0.23 ± 0.03 (7)
α-chymotrypsin	0.41 ± 0.08 (3)†	0.23 ± 0.03 (3)

* Mean ± SEM (number of different preparations of myelin prepared from at least four animals). Each sample was at least analyzed in duplicate and mean of the data was used.
† $P < 0.01$ between the diabetic and nondiabetic groups.

either trypsin or α-chymotrypsin. As indicated in Table 1, when myelin from control animals was incubated with either trypsin (seven incubations) or α-chymotrypsin (3 incubations), the ratio of reaction product to P_o approximated 0.4. This was nearly twice that seen in the membranes from diabetic animals. The difference for both enzymes was significant ($P < 0.01$) when tested by Student's *t* test.

Effect of trypsin on P_o protein isolated from myelin. To determine whether the relative resistance of P_o from myelin of diabetic animals to proteolysis reflected an abnormality of the protein per se, or a different interaction of the protein with the lipid bilayer of the membrane, we isolated P_o protein from myelin by preparative slab-gel electrophoresis and subjected the isolated protein to incubation with trypsin. When these experiments were compared with incubations with intact myelin, we found several differences. As indicated in Figure 2, the reaction was approximately linear for 30 min and led to almost complete digestion of the P_o protein. This is in contrast to the findings in whole myelin, in which, as noted above, the reaction was incomplete; in that experiment, digestion of P_o protein did not exceed 20% of its original peak height and was completed in under 5 min. In experiments in which P_o isolated from the membrane was used, as well as in those with intact membrane, the 20,000 mol. wt. fragment was the major product, although other products appeared, some transiently, during the incubation of the isolated P_o. Finally, as shown in Figure 2, the breakdown rate for the isolated P_o protein was the same for myelin obtained from the nondiabetic and diabetic animals, in contrast to the finding in the intact membrane.

FIGURE 2. Effect of trypsin on P_o protein isolated from myelin of diabetic and nondiabetic animals. P_o protein was isolated from the myelin of diabetic and nondiabetic animals by preparative polyacrylamide slab-gel electrophoresis. The isolated proteins were then subjected to proteolytic digestion and the products obtained at different time intervals were analyzed by electrophoresis. B.P., breakdown product obtained after digestion with proteolytic enzymes.



DISCUSSION

These studies indicate that in sciatic nerve myelin from both diabetic and control animals, the P_o protein is the major substrate for proteolytic attack, but that in the intact membrane, only a portion of the molecule is subject to proteolysis. This finding suggests that most of the protein is submerged in the lipid bilayer, consistent with the mosaic arrangement suggested by Singer and Nicolson[11] for proteins in membranes, and that this portion is protected from the action of the water-soluble enzymes, trypsin and chymotrypsin. In a study of the topography of proteins in spinal cord myelin from the cat spinal cord, Poduslo and Braun[12] also concluded, based on lactoperoxidase catalyzed iodination, that the proteins of higher molecular weight were exposed on the surface of the membrane. This concept is supported further by the complete and continuous susceptibility of the protein to the action of trypsin, when it is exposed to the enzyme, after it has been removed from the intact membrane.

The mechanism for the decreased susceptibility of the myelin protein obtained from diabetic animals to the action of the two proteolytic enzymes is not clear from this study. Alteration of the P_o protein by a reaction such as glycosylation, described for hemoglobin[13] and other proteins, could lead to steric interference at the site of action of these enzymes. The finding that the rate of proteolysis by trypsin of the P_o protein when removed from the membrane provides evidence against the hypothesis, since one would expect such a chemical effect to persist in the isolated protein. The exclusion of this possibility, however, would require a complete chemical analysis of the protein obtained from both sources, since it is possible that the extraction procedures used to isolate the P_o from the myelin of animals with diabetes might produce alterations that mask differences between it and the control material.

Other possibilities include alterations in the lipid bilayer in experimental diabetes that lead to decreased availability of the protein to the attack of proteolytic enzymes. Such an effect could result from changes in the bilayer at the site of the P_o protein that repel the enzymes, such as alterations in phosphoinositide or other phospholipid contents with changes in net charge.

In any case, these findings suggest differences in structure between myelin from diabetic animals and that of age-matched controls.

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