Biochimica et Biophysica Acta, 649 (1981) 587–594
Elsevier/North-Holland Biomedical Press

BBA 79499

LOCALIZATION OF 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHODIESTERASE IN HUMAN ERYTHROCYTE MEMBRANES

CHARLES E. DREILING

Division of Biochemistry, School of Medicine, University of Nevada, Reno, Reno, NV 89557 (U.S.A.)

(Received May 7th, 1981)

Key words: Cyclic nucleotide; Phosphodiesterase localization; Membrane asymmetry; (Human erythrocyte)

The location of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in human erythrocyte membranes was determined. This was accomplished by comparing the enzyme's accessibility with that of glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic surface marker) and acetylcholinesterase (external marker) in sealed and unsealed ghosts and normal and inverted membrane vesicles. The results showed that 2′,3′-cyclic nucleotide 3′-phosphodiesterase, like glyceraldehyde-3-phosphate dehydrogenase, meets several criteria for an inner (cytoplasmic) membrane location: (1) the enzyme was accessible to substrate in unsealed ghosts and inside-out vesicles but not in sealed or right-side-out vesicles, (2) latent activity in sealed ghosts could be exposed with detergent (Triton X-100), (3) activity in unsealed ghosts was gradually sequestered during resealing and could be re-exposed with detergent, and (4) the enzyme was susceptible to trypsin proteolysis only in unsealed ghosts. These results demonstrate that the active site of 2′,3′-cyclic nucleotide 3′-phosphodiesterase faces the cytoplasm of erythrocytes and that the enzyme may not span the lipid bilayer of the membrane. The localization of the phosphodiesterase on the inner membrane surface of erythrocytes suggests that the similar enzyme of myelin may be embedded within the major dense line of the compact lamellae.

Introduction

2′,3′-Cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37) catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to form 2′-nucleoside monophosphates [1]. High levels of activity were first described in brain and spinal cord white matter and the enzyme was subsequently shown to be an integral myelin protein [2–4]. Studies on the enzyme during development [5] and certain myelin deficiencies [6] lead to its use as a quantitative and qualitative myelin marker. Virtually nothing is known about the function and natural substrate of the phosphodiesterase.

2′,3′-Cyclic nucleotide 3′-phosphodiesterase is thought to be a basic hydrophobic protein due to its extractability from myelin with high ionic strength buffers [7] and non-ionic detergents [1] and its amino acid composition [4]. It has been suggested that the enzyme does not occupy a site deep within the myelin lipid bilayer but has a rather shallow membrane location intermediate between that of the extrinsic myelin basic protein and the deeply buried (intrinsic) proteolipid protein [8]. It is not known whether the phosphodiesterase is located on the cytoplasmic (major dense line) or external (interperiod line) surface of the myelin lamellae. Determination of the enzyme's precise location in these membranes would do much toward identifying its function.

Studies on the localization of proteins in myelin generally rely on covalent and immunohistochemical probes and freeze-fracture techniques [9]. These techniques are difficult to perform and their results must be interpreted with caution due to the inaccessibility of compact myelin to probes, the poor resolution of freeze-fracture and the lack of established reference points for the two halves of the myelin lipid

Abbreviation: DTNB, 5,5′-dithiobis-2(nitrobenzoic acid).

bilayer. These difficulties were circumvented in the present study by locating 2′,3′-cyclic nucleotide 3′-phosphodiesterase in a less complex membrane system. Low but significant levels of activity have been found in the plasma membranes of several non-neural tissues including the spleen, lung, adrenal gland, and heart and skeletal muscles [1,10,11]. In addition, we have found the enzyme in both the inner and outer membranes of liver mitochondria [12]. More important, however, 2′,3′-cyclic nucleotide 3′-phosphodiesterase has been shown to be an integral protein of human erythrocyte membranes where it is similar with respect to pH optimum, substrate specificity and effect of metal ions to that found in myelin [13]. Because erythrocyte membranes are easily studied and have been well characterized, we have proposed that the enzyme be located in this membrane. Extrapolating the results to myelin assumes that myelin and erythrocyte membranes are organized, at least with respect to the phosphodiesterase, in a structurally similar manner. It is known that myelin and red blood cell membranes differ markedly with respect to lipid and protein composition. However, myelin is a continuum of regular (glial) membranes [14] and the apparent ubiguity of the 2′,3′-cyclic nucleotide 3′-phosphodiesterase suggests that the enzyme may play a fundamental role in several membranes and therefore may have a similar distribution within membranes of different origins.

In the present study, the location of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in the erythrocyte membrane was determined by comparing the accessibility of the enzyme in sealed and unsealed ghosts and in normal and inverted visicles. Glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase were used as inside and outside markers, respectively. The results obtained for the phosphodiesterase paralleled those for glyceraldehyde-3-phosphate dehydrogenase and indicated that the enzyme is located on the cytoplasmic surface of the erythrocyte membrane.

Materials and Methods

Human blood in acid/citrate/dextrose was obtained from the Nevada Blood Services blood bank and used within 2 weeks of the withdrawal date. The chemicals purchased from Sigma Chemical Co. were: adenosine-2′,3′-cyclic monophosphoric acid (sodium salt); acetylthiocholine chloride; 5,5′-dithiobis-(2 nitrobenzoic acid), DTNB; DL-glyceraldehyde-3-phosphoric acid (diethyl acetyl monobarium salt); β-nicotinamide adenine dinucleotide (Grade III), β-NAD; Triton X-100; trypsin (Grade III). All other chemicals used were reagent grade.

Erythrocyte membrane preparations

Erythrocytes from whole blood were sedimented and thoroughly washed (2500 × *g*; 10 min) with isotonic buffer (5 mM sodium phosphate/150 mM NaCl, pH 8.0). The plasma, buffy coat and supernatant were removed by aspiration. Unsealed hemoglobin-free ghosts were prepared by lysing washed erythrocytes in 40 vol. hypotonic buffer (5 mM sodium phosphate, pH 8.5) according to the method of Dodge et al. [15]. The membranes were washed three times (12000 × *g*, 10 min) until creamy white. Sealed ghosts were made by incubating unwashed ghosts in 40 vol. prewarmed isotonic buffer (37°C) followed by three washes [16]. Sealed inside-out vesicles were prepared by incubating freshly isolated ghosts in 40 vol. buffer (0.5 mM sodium phosphate, pH 8.5) for 30–60 min. The ghosts were observed by phase microscopy to undergo endocytosis and were pelleted at 20000 × *g* and aged at 0–4°C for 5 days. The pellets were homogenized and inverted vesicles were released after several passages of the undiluted pellets through a No. 27-gauge needle followed by additional transfers through a Hamilton syringe and a narrow gauge (0.0045 inch internal diameter) needle. Tightly sealed right-side-out vesicles were prepared by incubating washed ghosts in low concentrations of trypsin (0.05 mg/ml HCl, 1 mM) at 37°C for 30 min [17].

Enzyme assays

2′,3′-Cyclic nucleotide 3′-phosphodiesterase was assayed by the spectrophotometric method previously described by Dreiling and Mattson (1980) [18]. The reaction mixture contained 0.02% phenol red/7.5 mM 2′,3′-cyclic AMP/ 150 mM NaCl; total volume = 0.8 ml, pH 7.0. 20 μl (100 μg protein) of the membrane preparation were used to initiate the reaction. The pH decrease accompanying the enzymic hydrolysis of 2′-3′-cyclic AMP was followed by recording the decrease in absorbance of phenol red at 550 nm.

Titration of the reaction mixture with 1 mM HCl showed that a decrease of 1.0 absorbance unit was equivalent to 0.133 μmol substrate hydrolyzed.

Acetylcholinesterase was assayed by a modification of the procedure of Ellman et al. [19]. Equal volumes (20 μl) of membranes (2–10 μg protein) and buffer (5 mM sodium phosphate, pH 8.5) were mixed with 0.7 ml sodium phosphate (100 mM, pH 7.5), 50 μl DTNB (10 nM DTNB, 100 mM sodium phosphate, pH 7.0 and 3 mg $NaHCO_3$ per 8 mg DTNB). Acetylthiocholine chloride (50 μl 12.5 mM) was added at t_{zero}. The increase in absorbance at 412 nm was recorded. An increase of 1.0 absorbance unit corresponded to 0.0588 μmol product formed.

Glyceraldehyde-3-phosphate dehydrogenase was assayed by following the reduction of β-NAD by glyceraldehyde-3-phosphate at 340 nm [20]. Equal volumes (20 μl) of membrane protein (10–100 μg) and buffer (5 mM sodium phosphate, pH 8.5) were mixed with 0.82 ml sodium pyrophosphate (30 μM, pH 8.4), 30 μl sodium arsenate (0.4 M) and 50 μl β-NAD (20 nM). The reaction was initiated with 100 μl freshly prepared DL-glyceraldehyde 3-phosphate (15 mM, pH 7.0). The change in absorbance was recorded and an increase of 1.0 absorbance unit corresponded to 1 μM NADH generated.

Enzyme assays were performed at room temperature with a Beckman DU monochromator and Gilford Photometer (No. 252) and Recorder (No. 6051). Specific enzyme activities are reported as μmol product formed/min/per mg protein. Protein was determined by the method of Lowry et al. [21].

Assays of sidedness and sealing

The orientation (sidedness and sealing) of the erythrocyte ghosts and vesicles were determined as follows. Acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase were taken to represent markers of the outer and inner membrane surfaces, respectively [22,23]. The accessibility of these enzymes to exogenous substrate and their susceptibility to proteolytic digestion were determined. Typically, each membrane preparation was assayed under normal conditions for 2 min followed by the addition of 20 μl Triton X-100 (10%; final concn. = 0.20–0.25%). Enzyme activity was recorded for an additional 2 min and the increase in activity after Triton represented latent (inaccessible) enzyme in sealed membranes. Sidedness and sealing were also determined by examining the susceptibility of both marker enzymes to proteolytic digestion. In this case, the ghosts were incubated with trypsin (2.5 mg/ml sodium phosphate, 5 mM, pH 8.5; final concn.) at 37°C. Aliquots of this mixture were periodically assayed for activity in the presence and absence of Triton X-100. Decreased activity in the trypsin-treated preparations was interpreted as proteolytic digestion of exposed enzyme. The location of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in the erythrocyte membrane was determined by repeating these experiments and assaying each preparation (±Triton X-100 and ±trypsin). The results for the phosphodiesterase were compared with those obtained for acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase.

Results

The asymmetrical distribution of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase in erythrocyte membranes was used to determine the sidedness and sealing of the ghost preparations. It also served as the basis for determining the location of 2′,3′-cyclic nucleotide 3′-phosphodiesterase. Table I shows that acetylcholinesterase was highly accessible to its substrate in both sealed and unsealed ghosts. The specific activity in these preparations was 0.368 ± 0.160 and 0.963 ± 0.302, respectively. The 2.6-fold difference in activities was not statistically significant and probably resulted from their 2.4-fold difference in protein content (due to trapped hemoglobin in the sealed ghosts). These results are consistent with acetylcholinesterase's outer membrane location. The glyceraldehyde-3-phosphate dehydrogenase activity was low in the sealed ghosts (0.0104 ± 0.0024) but high in the unsealed (0.459 ± 0.131) reflecting this enzyme's inner surface location on the erythrocyte membrane. The 44.1-fold difference in activities was highly significant ($P < 0.001$) although the actual difference may be inflated by the 2.4-fold protein factor. The unsealed/sealed ratios for acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase, corrected for the differing protein content of the two ghosts preparations, were 1.1 and 18.4, respectively (corrected ratios not shown in Table 1). The data for these enzymes indicate that the sealed

TABLE I

COMPARISON OF ACETYLCHOLINESTERASE (AChase), GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (G3PD) AND 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHODIESTERASE (CNP) ACTIVITY AND PROTEIN CONTENT OF SEALED AND UNSEALED HUMAN ERYTHROCYTE GHOSTS

Enzyme activity presented as specific activity (μmol S → P/min per mg protein). Data expressed as mean ± S.E.

	Sealed ghosts (n = 8)	Unsealed ghosts (n = 6)	Ratio	P [c]
AChase	0.368 ± 0.160	0.963 ± 0.302	2.6 [a]	n.s.
G3PD	0.0104 ± 0.0024	0.459 ± 0.131	44.1	<0.001
CNP	0.0044 ± 0.002	0.270 ± 0.060	61.4	<0.001
Protein (mg/ml)	11.32 ± 1.28	4.73 ± 0.54	2.4 [b]	<0.01

[a] Ratio = unsealed/sealed.
[b] Ratio = sealed/unsealed.
[c] Paired t statistical test.

ghosts were nearly 100% sealed and that they were normally (right-side-out) oriented. The results for 2′,3′-cyclic nucleotide 3′-phosphodiesterase show that this enzyme was 61.4-times more accessible in the unsealed than sealed ghosts (i.e., 0.270 ± 0.060 vs, 0.0044 ± 0.02 μmol/min per mg). When corrected for protein, the unsealed/sealed ratio was 25.1. These data indicate that the selaed ghosts were impermeable to the phosphodiesterase substrate (i.e., 2′,3′-cyclic AMP). More importantly, however, they suggest that 2′,3′-cyclic nucleotide 3′phosphodiesterase, like glyceraldehyde-3-phosphate dehydrogenase, is located on on the cytoplasmic surface of the erythrocyte membrane.

The data in Table I suggest an inner location for 2′,3′-cyclic nucleotide 3′-phosphodiesterase, however, the enzyme may be partially buried within the lipid bilayer and therefore may be incompletely exposed in unsealed ghosts. Thus, another series of experiments were performed in which the sealed and unsealed ghosts were assayed with Triton X-100 added to the reaction mixture midway through the assay. This addition served to expose latent activity and allowed each enzyme to be assayed in a single ghost preparation that was first sealed and then unsealed. This eliminated the problem created by differing protein concentrations. The results are shown in Table II. As can be seen, Triton had no effect on any of the enzymes in the unsealed ghosts; however, the detergent did produce marked increases (2.35- and 25.9-fold, respectively) in the accessibility of glyceraldehyde-3-phosphate dehydrogenase and the phosphodiesterase. These increases were similar to those shown in Table I for the unsealed and sealed ghosts, especially if the latter are corrected for their protein differences (see above). These results suggest that 2′,3′-cyclic nucleotide 3′-phosphodiesterase is not sequestered in the lipid bilayer and that the enzyme is fully exposed in unsealed ghosts preparations. The data also confirm the normal orientation of the sealed ghosts.

The results shown in Tables I and II are consistent with an inner location of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in the erythrocyte membrane. This was substantiated by reversing the experiment described in Table II. That is, unsealed ghosts were first assayed for each enzyme and then gradually resealed with dilute trypsin according to the method of Avruch et al. [17]. During resealing, the ghosts were assayed before and after the addition of Triton X-100. The results of this experiment are shown in Table III. The data show that incubating unsealed ghosts in trypsin (0.05 mg/ml 1 mM HCl) resulted in a gradual but permanent decrease in acetylcholinesterase. The decrease (25% after 40 min) was not reversed by Triton and probably represented proteolysis of the enzyme. Table III also shows that incubating unsealed ghosts with dilute trypsin resulted in a precipitous drop in glyceraldehyde-3-phosphate dehydrogenase and 2′,3′-cyclic nucleotide 3′-phosphodiesterase activity that could be recovered by Triton. This indicated that typsin induced sealing of the unsealed ghosts; sequestering the inner membrane surface from the assay medium. The parallel drop and

TABLE II

COMPARISON OF ACETYLCHOLINESTERASE (AChase), GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (G3PD) AND 2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHODIESTERASE (CNP) ACTIVITY IN UNSEALED AND SEALED HUMAN ERYTHROCYTES BEFORE AND AFTER TREATMENT WITH TRITON X-100

Enzyme activity presented as specific activity (μmol S → P/min per mg protein). Data expressed as mean ± S.E.

	n	−Triton X-100	+ Triton X-100	Ratio [a]	*P* [b]
Unsealed ghosts					
AChase	(6)	0.963 ± 0.302	0.887 ± 0.258	0.9	n.s.
G3PD	(5)	0.459 ± 0.131	0.654 ± 0.216	1.4	n.s.
CNP	(6)	0.270 ± 0.060	0.270 ± 0.050	1.0	n.s.
Sealed ghosts					
AChase	(5)	0.368 ± 0.160	0.334 ± 0.143	0.9	n.s.
G3PD	(8)	0.0104 ± 0.0024	0.245 ± 0.054	23.5	<0.01
CNP	(10)	0.004 ± 0.002	0.114 ± 0.024	25.9	<0.01

[a] Ratio = +Triton/−Triton.
[b] Paired *t* statistical test.

subsequent recovery of glyceraldehyde-3-phosphate dehydrogenase and the phosphodiesterase suggests that both enzymes are on the same (inner) side of the erythrocyte membrane.

This experiment was repeated but with sufficient trypsin to digest the major surface proteins of unsealed erythrocyte membrane [24]. The purpose of this experiment was to determine if trypsin would digest only the outer acetylcholinesterase in the sealed preparation while degrading both inner and outer surface enzymes in the unsealed ghosts. Sealed and unsealed ghosts were assayed before and during the trypsin treatment as well as in the presence and absence of Triton. The results are shown in Fig. 1. Incubating unsealed ghosts in high concentrations of trypsin resulted in sharp decreases in each enzyme, i.e., to 5–20% of control activity within 60 min. These decreases were not reversed by Triton. From this it was concluded that all three enzymes in the unsealed ghosts were accessible to, and degraded by, trypsin. When the sealed ghosts were treated with trypsin, only acetylcholinesterase was digested. Glyceraldehyde-3-phosphate dehydrogenase and the phosphodiesterase were spared appreciable hydrolysis and up to 75% of the control activity was recovered after 3 h by adding Triton to the assay medium. Digestion of all three enzymes in the unsealed ghosts but degradation of only the outer enzyme (i.e., acetylcholinesterase) in the sealed preparation indicates that both glyceraldehyde-3-phosphate dehydrogenase and 2',3'-cyclic nucleotide 3'-phosphodiesterase are located on the inner face of the membranes.

Final evidence for an inner location of 2',3'-cyclic nucleotide 3'-phosphodiesterase on the erythrocyte

TABLE III

EFFECT OF RESEALING AND SUBSEQUENT LYSIS ON THE ACCESSIBILITY OF ACETYLCHOLINESTERASE (AChase), GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (G3PD) AND 2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHODIESTERASE (CNP) IN ERYTHROCYTE GHOSTS

Unsealed erythrocyte ghosts were prepared as described in the text but were washed only once with buffer (5 mM sodium phosphate, pH 8.5). Resealing was induced by incubating the unsealed ghosts with 0.05 mg/ml trypsin dissolved in 1 mM HCl (temp. 20°C). CNP was assayed at timed intervals. The resealed ghosts were lysed after 20 min by adding 20 μl Triton X-100 (10%, v/v) to the assay mixture (final Triton concn. = 0.25%). Results for both resealed and lysed ghosts are expressed as percentage of control.

	Unsealed ghosts (Control, spec. act.)	Resealed with trypsin at (min)				Lysed with Triton
		5	10	15	20	
AChase	1.129	95	89	78	75	75
G3PD	0.967	44	32	18	8	94
CNP	0.269	48	20	18	11	101

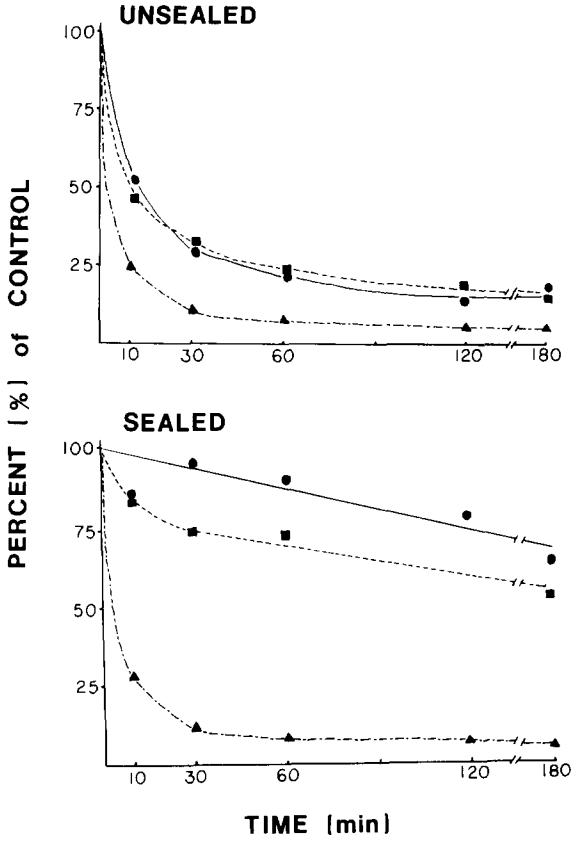

Fig. 1. Proteolysis of surface enzymes of sealed and unsealed erythrocyte ghosts. Freshly prepared ghosts were incubated with 5 mM sodium phosphate buffer (pH 8.5) or with trypsin (final concn. = 2.5 mg/ml phosphate buffer) at 37°C and assayed for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (●———●), glyceraldehyde-3-phosphate dehydrogenase (■- - - - -■) and acetylcholinesterase (▲– – –▲) activity at timed intervals. All assays were performed in the presence of Triton X-100 (final concn. = 0.25%). There was no significant loss of 2′,3′-cyclic nucleotide 3′-phosphodiesterase or acetylcholinesterase activity in the control group during the incubation period; however, there was a slight decrease in glyceraldehyde-3-phosphate dehydrogenase by the end of the 3rd h.

membrane was obtained by comparing the accessibility of the enzymes in inside-out vesicles. In these experiments, acetylcholinesterase should be sequestered within the inside-out vesicles, whereas the other two should be exposed on the inverted membrane surface. The accessibility of each enzyme was measured first in the absence then in the presence of Triton X-100. The data, shown in Table IV, indicate that approx. 25% of the total acetylcholinesterase activity was inaccessible in the inside-out preparations (that is, 25% of the vesicles were sealed inside-out and 75% were either unsealed and/or sealed right-side-out). The data for glyceraldehyde-3-phosphate dehydrogenase show that approx. 50% of the vesicles were sealed right-side-out. From this it was calculated that the vesicle population was a mixture of approx. 25% inside-out sealed, 50% right-side-out sealed, and 25% unsealed. By comparison, the accessibility of 2′,3′-cyclic nucleotide 3′-phosphodiesterase in the inverted vesicle preparation did not differ significantly from that for glyceraldehyde-3-phosphate dehydrogenase but was highly different ($P < 0.001$) from that for acetylcholinesterase. The increased accessibility of 2′,3′-cyclic nucleotide 3′-phosphodiesterase and glyceraldehyde-3-phosphate dehydrogenase in the inside-out vesicles is consistent with our earlier conclusion that both enzymes are on the same (inner) surface of the membrane.

Discussion

Sudo et al. [13] were the first to describe 2′,3′-cyclic nucleotide 3′-phosphodiesterase activity in human erythrocyte membranes. The present study extends this observation and demonstrates that the enzyme is located on the inner (cytoplasmic) surface of these membranes. This study was facilitated by the fact that 2′,3′-cyclic AMP did not penetrate the sealed membrane preparations and therefore could serve to probe the sidedness of the enzyme. That 2′,3′-cyclic nucleotide 3′-phosphodiesterase meets several criteria for an inner membrane location is seen by the fact that the phosphodiesterase was: (1) accessible to exogenous substrate in unsealed ghosts and inverted vesicles but not in sealed ghosts or right-side-out vesicles; Tables I and IV; (2) inaccessible in sealed ghosts and right-side-out vesicles, but was fully exposed after treatment with Triton X-100; Table II; (3) sequestered during resealing of erythrocyte ghosts with full activity being recovered with detergent; Table III; and (4) susceptible to proteolysis in unsealed but not sealed ghosts; Fig. 1. Finally, the results for 2′,3′-cyclic nucleotide 3′-phosphodiesterase paralleled those for glyceraldehyde-3-phosphate dehydrogenase in every case. This study not only indicates that the active site of the phosphodiesterase is directed toward

TABLE IV

ACCESSIBILITY OF ACETYLCHOLINESTERASE (AChase), GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (G3PD) AND 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHODIEASTERASE (CNP) IN INSIDE-OUT VESICLES PREPARED FROM HUMAN ERYTHROCYTES

Data expressed as specific activity (μmol S → P/min per mg protein). Mean ± S.E. (n = 4). Date in parenthesis is percent of control activity. Latent activity = total − exposed activity (i.e., (+Triton) − (−Triton)).

	Total activity (+Triton)	Exposed activity (−Triton)	Latent activity (Calcd.)	P
AChase	0.688 ± 0.113 (100)	0.522 ± 0.087 (75.7 ± 1.11)	0.166 ± 0.027 (24.3 ± 1.11)	<0.001
G3PD	0.217 ± 0.018 (100)	0.117 ± 0.011 (53.8 ± 1.49)	0.100 ± 0.008 (46.2 ± 1.49)	n.s.
CNP	0.106 ± 0.011 (100)	0.044 ± 0.001 (43.0 ± 3.45)	0.062 ± 0.010 (57.0 ± 3.54)	

the cytoplasm but it also provides evidence that the enzyme is fixed in position and is not reoriented during harsh manipulation of the membranes (i.e., hemolysis, resealing and inversion). The data also suggest but do not prove that the enzyme does not span the erythrocyte lipid bilayer. Although treatment of sealed ghosts with sufficient trypsin to degrade the outer acetylcholinesterase did not reduce the amount of the phosphodiesterase recovered with Triton, a hydrophobic portion of the enzyme could anchor the enzyme in the membrane and could even reach the outer protein layer. Enzymatic cleavage of the tail would go undetected provided the active site was preserved.

The finding of 2′,3′-cyclic nucleotide 3′-phosphodiesterase only in the inner surface of the erythrocyte membrane is important for several reasons. It confirms the absolute asymmetry of proteins in these membranes [16]. It also implies that the natural (endogenous) phosphodiester substrate has access to, and is hydrolyzed on, the cytoplasmic side of the membrane. Of greater significance here, however, is the possibility that 2′,3′-cyclic nucleotide 3′-phosphodiesterase may have a similar (inner surface) location in other membranes, such as myelin. The precise location of the enzyme in myelin has been given considerable attention; however, there has been no definitive study on the subject. It has been reported to be associated with both the outer and inner surfaces of the myelin sheath [25], however, Mikoshiba et al. [26] concluded that the enzyme could not be present on the inner (cytoplasmic) surface because the myelin of shiverer mutant mice possesses normal phosphodiesterase levels but is devoid of the major dense line. The work of Gruener and Peterson [27], on the other hand, suggests that basic proteins (such as the 2′,3′-cyclic nucleotide 3′-phosphodiesterase) are located on the cytoplasmic side of myelin while other major proteins are on the outer surface. This and the present results are consistent with the proposal [28,29] that the phosphodiesterase is similar, if not identical, to the Wolfgram proteins W1 and W2; the first of which has been localized on the cytoplasmic side of the oligodendrocyte plasma membrane [30]. Unfortunately, it is not possible to make a general statement regarding the precise location of proteins in membranes from various tissues. It is reasonable to conclude from studies on protein hormone receptors, glycosylated membrane proteins and transport proteins, however, that proteins are located according to their metabolic function and that each has a similar location in plasma membranes regardless of the tissue's origin. On the basis of this assumption it is proposed that 2′,3′-cyclic nucleotide 3′-phosphodies-

terase, like the encephalitogenic myelin basic protein, is present on the inner surface of myelin and is embedded within the major dense line of the lamellae.

Acknowledgements

This reasearch was supported in part by grants from the Research Advisory Board, UNR, and by NIH Grant No. RR-09035. The author wishes to thank Drs. R.C. Reitz and W.H. Welch for their helpful discussions during the course of this research, and to express gratitude to Michelle DiRienzo and Sherry Stevens for preparation of the typescript.

References

1 Drummond, G.I., Iyer, N.T. and Keith, J. (1962) J. Biol. Chem. 237, 3535–3539
2 Olafson, R.Q., Drummond, G.I. and Lee, J.F. (1969) Can. J. Biochem. 47, 961–966
3 Kurihara, T. and Tsukada, Y. (1967) J. Neurochem. 14, 1167–1174
4 Drummond, R.J., Hamill, E.B. and Guha, A. (1978) J. Neurochem. 31, 871–878
5 Kurihara, T. and Tsukada, Y. (1968) J. Neurochem. 15, 827–832
6 Kurihara, T., Nussbaum, J.L. and Mandel, P. (1970) J. Neurochem. 17, 993–997
7 Guha, A. and Moore, S. (1975) Brain Res. 89, 279–286
8 Waehneldt, T.V. (1978) Adv. Exp. Med. Biol. 100, 117–133
9 Crang, A.J. and Rumsby, M.G. (1978) Adv. Exp. Biol. Med. 100, 235–248
10 Kurihara, T., Nussbaum, J.L. and Mandel, P. (1971) Life Sci. 10, 421–429
11 Konings, A.W.T. and Pierce, D.A. (1974) Life Sci. 15, 491–499
12 Dreiling, C.E., Schilling, R.J. and Reitz, R.C. (1981) Biochim. Biophys. Acta 640, 114–120
13 Sudo, T., Kikuno, M. and Kurihara, T. (1972) Biochim. Biophys. Acta 255, 640–646
14 Davison, A.N. (1971) Neurosci. Res. Prog. Bull. 9, Pt. 4, 465–470
15 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
16 Steck, T.L. (1974) Methods Membrane Biol. 2, 245–281
17 Avruch, J., Price, H.D., Martin, D.B. and Carter, J.R. (1973) Biochim. Biophys. Acta 291, 494–505.
18 Dreiling, C.E. and Mattson, C. (1980) Anal. Biochem. 102, 304–309
19 Ellman, G.L., Courtney, K.D., Valentino, A., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
20 Cori, G.T., Slein, M.W. and Cori, C.F. (1948) J. Biol. Chem. 173, 605–618.
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
22 Firkin, B.G., Beal, R.W. and Mitchell, G. (1963) Aust. Annu. Med. 12, 26–29
23 Kant, J.A. and Steck, T.L. (1973) J. Biol. Chem. 248, 8457–8464
24 Triplett, R.B. and Carraway, K.L. (1972) Biochemistry 11, 2897–2903
25 Engel, E.L. and Wood, J.G. (1976) Neurosci. Abs. I., 582
26 Mikoshiba, K., Nagaike, K. and Tsukada, Y. (1980) J. Neurochem. 35, 465–470
27 Gruener, W. and Peterson, R.G. (1977) Tex. Soc. Electr. Microsc. Newslett. 8, 35–36
28 Sprinkle, T.J., Wells, M.R., Carver, F.A. and Smith, D.B. (1980) J. Neurochem. 35, 1200–1208
29 Drummond, R.J. and Dean, G. (1980) J. Neurochem. 35, 1155–1165
30 Roussel, G., Delaunoy, J.P., Mandel, P. and Nussbaum, J.L. (1978) J. Neurocytol. 7, 155–163.