ACETYLCHOLINESTERASE OF THE HUMAN ERYTHROCYTE MEMBRANE[+]

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Received March 5, 1970

INTRODUCTION

Recent studies have revealed that a heterogeneous population of proteins is present in the erythrocyte membrane (Rosenberg and Guidotti, 1969; Lenard; Zwaal and van Deenan, 1968; Blumenfeld et al.; Maddy, 1965). Only in a few instances has identification of membrane function with any of these proteins been achieved. A glycoprotein with virus receptor and M and N blood group activities has been isolated by several investigators (Howe and Lee, 1969; Kathan et al., 1961; Springer et al., 1966). Marchesi et al. (1970) have reported the isolation of a high molecular weight fibrous protein. Harris (1969) obtained a protein component which appears to have ten subunits in the electron microscope. However, little is known of the role of these and other proteins in the organization of the native membrane or the manner in which they fulfill their function.

True acetylcholinesterase (EC 3.1.1.7) has been shown by Mitchell and Hanahan (1966) to be an integral part of the human red blood cell membrane. The high turnover number reported by Cohen and Warringa (1953) for this enzyme suggests that its quantitative level in the membrane is very small. By using tritiated diisopropylfluorophosphate (DIFP-^{3}[H]) to selectively label this enzyme, we have been able to elucidate some properties of this biologically active membrane bound protein.

[+]This work was supported by USPHS Grants #GM-16389, #HD-00674 and Training Grant #GM-563 from the National Institutes of Health.

[1]National Institutes of Health Development Award #GM-5998.

[2]Recipient of NIH Career Award #AM-19,435.

METHODS

Erythrocyte membranes were prepared from freshly drawn human blood by the method of Dodge et al. (1963). Membranes were pale white and intact in shape when viewed by phase contrast microscopy.

To selectively label acetylcholinesterase with DIFP-$^{3}[H]$, an aliquot, sample A, of membrane preparation suspended in pH 7.6 0.008 M sodium phosphate buffer was treated with 0.2 M butyrylcholine iodide for 30 minutes at room temperature, followed by non-radioactive 10^{-6} M DIFP for 30 minutes at room temperature. Butyrylcholine is not hydrolyzed by the enzyme, but protects the active site from reaction with DIFP (Cohen and Warringa, 1953). Membranes were then centrifuged at 5,900 x g for 40 minutes at 4° C to remove excess reagents, and washed three times with a two-fold volume of 0.15 M sodium phosphate buffer pH 7.6. They were resuspended in 0.15 M sodium phosphate buffer, pH 7.6 and incubated with 10^{-6} M DIFP-$^{3}[H]$ of specific activity 4.82 curies/μmole (Amersham-Searle, Des Plaines, Illinois) for 30 minutes at room temperature. At the end of this time they were centrifuged and washed as above until the membrane bound radioactivity became constant. Two other aliquots of membranes were similarly treated: with sample B reaction with butyrylcholine iodide was omitted; with sample C reaction with butyrylcholine iodide and non-radioactive DIFP were omitted. Another sample, D, served as control. It was subjected to no additions but was carried through the centrifugation and washing steps. Membrane bound acetylcholinesterase activity was measured by the hydroxamate assay of Hestrin (1949).

Disc electrophoresis on 5% polyacrylamide gels in 0.1% sodium dodecyl sulfate (SDS) was carried out according to the procedure of Maizel (1969). Preincubations were performed in three ways: 1) 1% SDS and 0.05 M iodoacetamide (IAA) at 37° C for three hour. 2) 1% SDS and 0.14 M 2-mercaptoethanol (2-ME) at 37° C for three hours; or 3) 1% SDS and 0.14 M 2-ME for 30 minutes at 37° C, followed by 0.23 M IAA for 30 minutes at 37° C followed by 0.33 M 2-ME for two hours at 37° C. Samples preincubated under condition 1 were dialyzed overnight against 0.1% SDS in 0.01 M sodium phosphate buffer, pH 7.1. Samples preincubated under conditions 2 and 3 were dialyzed overnight against 0.1% SDS in 0.01 M sodium phosphate buffer, pH 7.1, containing 0.1% 2-ME. Molecular

weight estimations were made using bovine serum albumin and immunoglobulin standards according to the method of Shapiro et al., (1967). The method of Tishler and Epstein (1968) was used for slicing of gels and radioactivity determinations, following staining of protein with Coomassie Blue.

RESULTS

As shown in Table I, the active site of human erythrocyte membrane acetylcholinesterase was effectively protected against DIFP inactivation by butyrylcholine, its nonhydrolyzable substrate analog. This result has previously been shown by Cohen and Warringa (1953) in the bovine erythrocyte. The unprotected sample B shows no acetylcholinesterase activity after DIFP inactivation, but the butyrylcholine protected sample A retains the enzymatic activity after removal of butyrylcholine. It can also be seen in Table I that the active site of acetylcholinesterase was selectively labelled with DIFP-^{3}[H] since sample B, which was treated with non-radioactive DIFP without protection by butyrylcholine, showed little tritium uptake after reaction with DIFP-^{3}[H]. That all nonspecific sites of phosphorylation were effectively blocked by pretreatment of membranes with non-radioactive DIFP is seen in a diminished uptake of radioactivity in butyrylcholine protected sample A, pretreated with non-radioactive DIFP, as compared to sample C which has not been previously reacted with non-radioactive DIFP.

TABLE I

Reaction of Acetylcholinesterase with DIFP-^{3}[H]

	μmoles acetylcholine hydrolyzed/min/ml		DPM/ml
Sample	after reaction with DIFP and washing	after reaction with DIFP-^{3}[H]	after final washing
A	1.52	none	31,700
B	none	none	4,950
C	1.94	none	64,600
D	2.53	2.12	6,025*

* In other experiments, cpm of this sample were not significant.

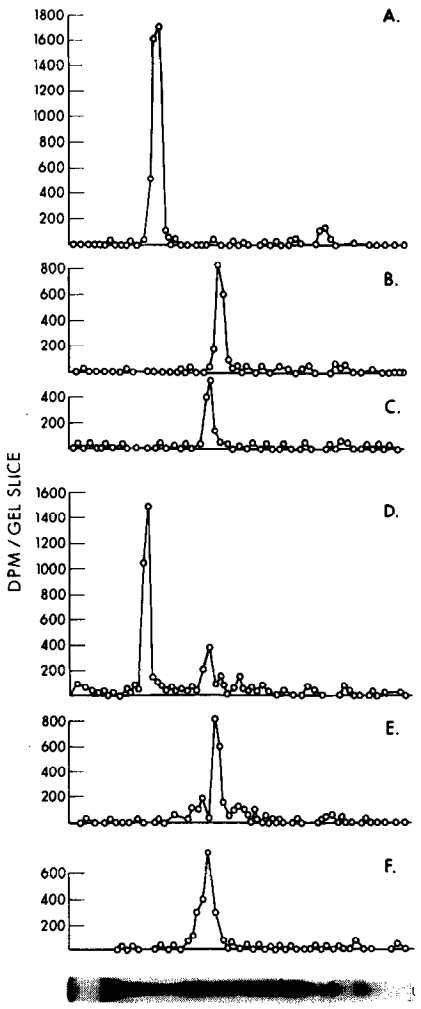

Figure 1: 5% polyacrylamide gels of sample A and sample C run at 11 volts/cm for 4 hours. A typical gel stained with Coomassie Blue is shown at the bottom of the figure. Gels were sliced in consecutive sections of approximately 2 mm. Migration was from left (-) to right (+).

1A) Sample A preincubated in SDS and IAA.

1B) Sample A preincubated in SDS and 2-ME.

1C) Sample A preincubated in SDS and 2-ME, followed by IAA, followed by 2-ME.

1D) Sample C preincubated in SDS and IAA.

1E) Sample C preincubated in SDS and 2-ME.

1F) Sample C preincubated in SDS and 2-ME, followed by IAA, followed by 2-ME.

The analysis of the radioactive protein components by polyacrylamide disc gel electrophoresis is shown in Figure 1. As expected, a multi-component protein band pattern is seen. Determination of radioactivity in consecutive gel slices shows that sample A, pre-incubated with 2-ME, either in the presence or absence of IAA shows one major peak of radioactivity (Fig. 1b and 1c). The molecular weight of this peak corresponds to approximately 90,000 (80,000 - 110,000). As seen in Fig. 1a, sample A preincubated in the absence of 2-ME also shows a major peak of radioactivity. Under these conditions the molecular weight is approximately 180,000 (170,000 - 187,000).

As seen in Figure 1d, 1e and 1f, similar peaks are present in sample C which was not protected with butyrylcholine and not pretreated with non-radioactive DIFP. This suggests that the major site of diisopropylphosphorylation, under the conditions of reaction with DIFP-^{3}[H] used here, is the acetylcholinesterase active site, and that little nonspecific reaction takes place. During dialysis 20% to 40% of the radioactivity of sample C was removed. This indicates that nonspecifically labelled components were of small molecular weight.

The turnover number calculated from radioactivity of the major peak and the measured enzymatic activity prior to DIFP-^{3}[H] treatment was 6 x 10^{5} moles/min/active site.

DISCUSSION

This study suggests that acetylcholinesterase of human erythrocyte membrane can be selectively labelled with DIFP-^{3}[H] and, then visualized by disc gel electrophoresis as a distinct radioactively labelled band which is distinct from the bulk of membrane protein.

Evidence presented here indicates that the diisopropylphosphorylated acetylcholinesterase of human erythrocyte membranes exists as a dimer of about 180,000 molecular weight. This dimer cleaves into smaller components of about 90,000 molecular weight in the presence of mercaptoethanol. This result is consistent with the observation of Lauf and Poulik (1968) that mild reduction and alkylation of human erythrocyte stroma resulted in a loss of acetylcholinesterase activity.

Many varying reports have appeared on the molecular weight of the most thoroughly studied acetylcholinesterase, derived from Electrophorus electricus. Recently reported results with a crystalline preparation indicate that this enzyme has a molecular weight of about 260,000 with two active sites, and is composed of four subunits of equal size (Nachmansohn, 1969). In our studies with the erythrocyte enzyme we have obtained as yet no evidence for the presence of subunits smaller than about 90,000 molecular weight, although we are observing only those components to which a tritiated diisopropylphosphoryl group is bound.

The turnover number determined in this study for the human erythrocyte membrane acetylcholinesterase was 6×10^5 moles/min/active site. This value is similar to that obtained by Cohen and Warringa (1953) for the bovine red cell enzyme. Assuming a molecular weight per active site of 90,000 or 180,000, on the order of 1 mg of active enzyme protein is present per unit of blood, containing about 600 mg of red cell membrane protein. The sensitivity and specificity of the technique described here permits study of some of the properties of an enzyme present in small amounts in the membrane. The availability of a selectively labelled enzyme, an integral constituent of the human erythrocyte membrane, and potentially capable of reactivation may be helpful in studies of membrane structure and function.

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