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THE ALLOSTERIC TRANSITIONS FROM MEMBRANE-BOUND ENZYMES: BEHAVIOR OF ERYTHROCYTE ACETYLCHOLINESTERASE FROM FAT-DEFICIENT RATS*

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SUMMARY

The allosteric behavior of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from red cell ghosts of rats fed fat-sufficient and fat-deficient diets was investigated. Allosteric type kinetics with $n = -1.6$ have been obtained for the inhibition by F^- in rats fed a fat-sufficient diet. In animals fed a fat-deficient diet the values of n changed from -1.6 to -1.0. When these animals were then fed a fat-sufficient diet the values of n shifted from -1.0 to -1.6. This *in vivo* reversion was obtained after 8 days of refeeding. Two types of changes in the values of n were obtained *in vitro* in fat-deficient rats: (1) from -1.0 to -1.6 by solubilization of the membrane-bound enzyme with Triton X-100, (2) from -1.6 to -1.0 by resconstitution of the membrane-like structure from the soluble enzymatic preparation. The possibility that the structure of the membrane could be responsible for the changes in the phenomenon of phenotypic allosteric desensitization in the membrane-bound enzymes is discussed

INTRODUCTION

The allosteric properties of some membrane-bound enzymes have been reported by our laboratory (a) the activation by Na^+ and K^+ of the $(Na^+–K^+)$-ATPase[1], (b) the inhibition by F^- of the $(Na^+–K^+)$-ATPase, Mg^{2+}-ATPase[2] and *p*-nitrophenylphosphatases[3] from rat erythrocytes, and (c) the inhibition by Na^+ of the Ca^{2+}-ATPase from an unsaturated fatty acid auxotroph of *Escherichia coli*[4]. The allosteric behavior of these enzymes has been shown to change under conditions in which the fatty acid composition of the membrane is modified[1–4].

In the studies reported here, this biological phenomenon has been extended to membrane-bound acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and a new insight into the molecular mechanisms involved is offered.

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In the acetylcholinesterase of erythrocytes from rats fed a fat-sufficient diet, the values of n for the inhibition by F^- were found to be -1.6. When the fatty acid composition was changed by feeding the rats a fat-deficient diet values of n close to -1.0 were found. After disruption of these erythrocyte membranes by detergent the values of n shifted from -1.0 to -1.6. The original value of n (-1.0) could be recovered by the reconstruction of the membrane. The value of n (-1.6) for the acetylcholinesterase from erythrocytes of rats fed a fat-sufficient diet remained unchanged during similar treatment.

The allosteric desensitization which occurred in acetylcholinesterase bound to the recombined membrane from deficient animals is consistent with the hypothesis that the differences observed between the two groups of animals could be due to modifications in the binding between the enzyme and the effector resulting from the alteration of lipid–protein interactions in the intact membrane.

METHODS

Animals, diet and acetylcholinesterase assay

Details of experimental animals and diets have been published previously[2]. In the fat-sufficient diet, only corn oil was used as the fat supplement. Red cell ghosts were prepared by the method of Dodge *et al.*[5] with the addition of 1 mM EDTA in 20 mosM sodium phosphate (pH 7.4). The determination of the acetylcholinesterase activity was performed with a suspension of freshly prepared red cell ghosts or with preparations which had been kept at 4 °C for 1–2 weeks. The standard reaction mixture included 100 mM sodium phosphate (pH 8.0), 0.87 mM $MgCl_2$, 0.5 mM acetylthiocholine iodide and 20–40 μg ghost protein; final volume, 3 ml. The amount of thiocholine was determined spectrophotometrically by the method of Ellman *et al.*[6]. The mixture was incubated at 30 °C for 30 min. Inhibition by F^- was investigated by measuring the enzymatic activity in the presence of concentrations of F^- from 0.5 to 3.0 mM.

Solubilization and reaggregation procedures

The solubilization of the acetylcholinesterase with Triton X-100 and the formation of membrane-like material from membrane components of erythrocyte ghosts solubilized in detergent were carried out by a method similar to that described by Miller[7]. After adding Triton X-100 to membrane suspensions in 20 mM sodium phosphate (pH 7.4) and letting the preparation stand for 15 min at room temperature, samples were centrifuged for 1 h at 100000 $\times$ g at 4 °C and any pelleted material was discarded. The ratios Triton: membrane protein and the membrane concentration are indicated in Table V and VI. The resulting supernatant was dialyzed for 96 h at 4 °C against 7–8 changes of 500 ml of 20 mM sodium phosphate (pH 7.0) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The fraction of dialyzed material which then pelleted during centrifugation at 30000 $\times$ g for 20 min at 4 °C was defined as aggregated material. This material was resuspended, homogenized in 20 mM sodium phosphate (pH 7.0) and assayed for acetylcholinesterase activity.

Other methods

Protein was determined by the method of Lowry *et al.*[8]. The fatty acid analysis

of ghosts was carried out according to the method of Phillips *et al.*[9]. Lipid phosphorus was determined by the method of Marinetti[10] in aliquots extracted according to the method of Folch *et al.*[11].

RESULTS

Deficiency in essential fatty acid

Table I shows the fatty acid composition of the total lipids of red cells from animals fed deficient and supplemented diets. An increase of 20:3 (*n*-9) and a reduction in the *n*-6 acids in the fat-deprived rats were observed. The ratio 20:3 (*n*-9)/20:4 (*n*-6) clearly shows that the rats fed a fat-free diet were deficient in essential fatty acids[12].

Kinetic constants and reversibility of the inhibition by F^-

The values of K_m, n and V for acetylcholinesterase with acetylthiocholine as substrate (in the absence and presence of 1 mM F^-) from both groups of animals are presented in Table II. The Lineweaver–Burk plots of the data from these experiments

TABLE I

FATTY ACID COMPOSITION (%) OF ERYTHROCYTES FROM RATS FED FAT-SUFFICIENT AND FAT-DEFICIENT DIETS

The analysis of fatty acids by gas chromatograhpy was carried out as described under Materials and Methods.

Fatty acid	*Diet*	
	Fat-sufficient	*Fat-deficient*
Saturated	25.4	25.7
18:1 (*n*-9)	12.5	10.9
18:2 (*n*-6)	12.0	4.3
20:3 (*n*-9)	0.4	13.5
20:4 (*n*-6)	30.0	20.6
Unsaturated (C_{22}–C_{24})	12.9	16.2
Ratio $\frac{20:3\ (n\text{-}9)}{20:4\ (n\text{-}6)}$	0.01	0.66

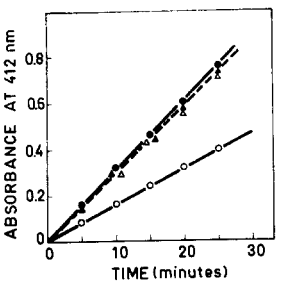
TABLE II

KINETIC PARAMETERS OF THE INHIBITION BY F^- OF ACETYLCHOLINESTERASE FROM RATS FED FAT-SUFFICIENT AND FAT-DEFICIENT DIETS

The incubation medium and experimental conditions were as described under Materials and Methods.

Parameter	*F^- (mM)*			
	Fat-sufficient diet		*Fat-deficient diet*	
	0	*1*	*0*	*1*
$K_m \times 10^4$ (M)	0.66	0.66	0.90	0.85
n	1.0	1.0	1.0	1.0
V (μmoles/h per mg protein)	14.8	11.6	15.1	12.9

showed that the acetylcholinesterase was not competitively inhibited by F^-. The non-allosteric nature of the enzyme–substrate interaction was revealed by the values of n which were close to 1.0. No difference was observed between the kinetic constants of the acetylcholinesterase from rats fed fat-sufficient and fat-deficient diets. The reversible character of the inhibition by F^- is shown in Fig. 1. After dialysis, the enzymatic activities in preparations previously incubated in the presence of 2 mM F^- or without F^- were similar.



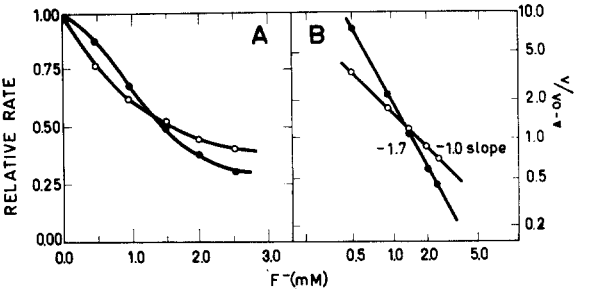


Fig. 1. The effect of dialysis on the inhibition by F^- of acetylcholinesterase. Reaction rate as a function of time in the absence (●—●) and in the presence (○—○) of 2 mM F^-. These pre-incubated preparations were dialysed against 100 mM sodium phosphate (pH 8.0) for 4 h. The curves indicated by (▲—▲) and (△—△) represent the preincubated sample after dialysis in the absence and in the presence of 2 mM F^-, respectively.

Fig. 2. Effect of F^- on the acetylcholinesterase from rats fed a fat-sufficient (●—●) and a fat-deficient (○—○) diet. (A) Direct plot of relative reaction rate as a function of $[F^-]$. (B) Plot of $v/v_0 - v$ as a function of $[F^-]$ on logarithmic coordinates. The slope of each line is indicated in the figure.

Effect of fat deprivation on the allosteric inhibition by F^-

Acetylcholinesterase from several sources was inhibited by F^- as shown by Cimasoni[13] who made no claim as to the allosteric nature of the inhibition. As can be seen from Fig. 2A, when the relative rate of the enzymatic activities was plotted against the concentration of F^-, curves of different shapes were found for both groups of rats. In Fig. 2B the values of n were determined graphically by using the following equations: $\log v/(v_0-v) = n \log I - \log K_i$ (ref. 14). Slopes of -1.7 and -1.0 were obtained for animals fed fat-sufficient and fat-deficient diets, respectively. No overlapping of the values of n between the two groups of animals has ever been found. The values of K_i were similar for animals fed deficient and supplemented diets. Table III summarizes the data obtained for n and K_i of the acetylcholinesterase from rat erythrocytes under these experimental conditions.

Modification of the values of n for the acetylcholinesterase with change of diet in fat-deficient animals

The sequential changes which take place in the fatty acid composition of different tissues from rats deficient in essential fatty acids when these animals are fed a diet containing corn oil are well documented[15,16]. Feeding a diet supplemented with corn oil to the fat-deficient animals results in the rapid incorporation of linoleic acid by erythrocytes, the normal level being approached within 6–9 days[15–17]. The time-dependent reversion of the values of n for the acetylcholinesterase from rats

TABLE III

VALUES OF n AND K_i FOR THE INHIBITION BY F^- OF ACETYLCHOLINESTERASE FROM RATS FED FAT-SUFFICIENT AND FAT-DEFICIENT DIETS

The results are expressed as the mean ± S.E. (N) = number of experiments and n.s. = not significant.

Diet	(N)	n	K_i (mM)
Fat-sufficient	(12)	-1.64 ± 0.02	1.50 ± 0.04
Fat-deficient	(12)	-1.00 ± 0.01	1.76 ± 0.06
		$P < 0.001$	n.s.

grown on a fat-deficient diet is shown in Table IV. On the days indicated, rats refed a diet containing corn oil and their respective controls were analyzed. The results show that after 8 days the values of n were similar to those of animals grown on a fat-sufficient diet.

TABLE IV

EFFECT OF THE CHANGE FROM FAT-DEFICIENT TO FAT-SUFFICIENT DIET ON THE VALUES OF n FOR ACETYLCHOLINESTERASE

At the beginning of the experiment 1 ml of blood was extracted from fat-deficient animals, numbered I to VI, and then 1 ml more was extracted at variable times after the change in diet as indicated. The fat-deficient rats, numbered VII and VIII, were put on the fat-sufficient diet without previous extraction. Other experimental details are described under Materials and Methods.

Rat No.	*Diet*	*Values of n* *Time on the diet (days):*			
		0	*4*	*8*	*12*
I	Sufficient	−1.1	−1.0		
II	Deficient	−0.9	−1.0		
III	Sufficient	−1.0		−1.6	
IV	Deficient	−1.0		−1.0	
V	Sufficient	−1.1			−1.6
VI	Deficient	−1.1			−1.0
VII	Sufficient				−1.4
VIII	Sufficient				−1.7

Effect of solubilization with Triton X-100 on n values of acetylcholinesterase from rats fed normal and fat-deficient diet

The unexpressed allosteric nature of the F^- inhibition seen in fat-deficient rats was unmasked by releasing the acetylcholinesterase from the membrane. Treatment of the particulate enzyme with Triton X-100 and the modifications of kinetic behavior which were observed in both groups of animals are summarized in Table V. The recovery of the solubilized enzyme was 96 % and 83 % in the 100000 × g supernatant of fat-sufficient and fat-deficient rats, respectively. The specific activity of the supernatants obtained by treatment of the particulate preparations with Triton X-100 was not altered significantly. Although the values of n for the acetylcholinesterase decreased from −1.0 to −1.6 in rats fed a fat-deficient diet during the transition

TABLE V

COMPARISON OF THE KINETIC PARAMETERS OF ACETYLCHOLINESTERASE AND THE LIPID PHOSPHORUS: PROTEIN RATIO OF BOTH TYPES OF ANIMALS AFTER TREATMENT OF THE ERYTHROCYTE MEMBRANE WITH TRITON X-100

Treatment with Triton X-100 of the erythrocyte membrane was performed using a 1 ml mixture of 20 mM sodium phosphate (pH 7.4) containing 3.0 mg ghost protein and 10 ml 0.8% Triton X-100 in 100 mM sodium phosphate (pH 8.0). After 15 min at room temperature an aliquot of homogenate was assayed. The supernatant of the ghost treated with Triton X-100 was obtained by centrifugation at 100000 × *g* for 1 h at 4 °C. Other experimental details are described under Materials and Methods. A, erythrocyte membrane; B, erythrocyte membrane treated with Triton X-100; C, supernatant.

Enzyme preparation	*Total activity* (*μmoles/h*)	*Specific activity* (*μmoles/h per mg protein*)	*Yield* (%)	*Lipid* PO_4^{3-}*/protein* (*μg* PO_4^{3-}*/mg protein*)	*n*	K_i (*mM*)
Fat-sufficient diet						
A	28.5	9.5	100	111.3	−1.7	1.4
B	29.1	8.8	98	—	−1.6	1.6
C	27.7	10.5	96	84.9	−1.6	1.6
Fat deficient diet						
A	36.6	12.2	100	88.9	−1.0	1.8
B	35.1	12.1	98	—	−1.6	1.8
C	30.4	13.8	83	85.4	−1.6	1.6

from particulate to solubilized enzyme, no change was observed for control rats. In both cases the values of K_i remained unchanged after solubilization.

The lipid phosphorus:protein ratio was similar in cell ghosts from both groups of animals and no change appeared between particulate and solubilized enzymes. The fatty acid analysis of the total lipids of ghosts and supernatant showed no apparent difference. These facts support the possibility that the allosteric behavior of the particulate enzyme from fat-deficient rats was masked or occluded by a bond between the enzyme and the membrane structure, since the decrease in the values of *n* was found in the presence of the same lipid phosphorus:protein ratio and fatty acid composition.

Aggregation of solubilized membrane components and kinetic behavior of the acetylcholinesterase from deficient animals

The reconstitution of membranes after solubilization by detergent and organic solvents has been described and also demonstrated by the use of electron microscopy[7,18–20]. During dialysis of the supernatant of the membrane solubilized by detergent against the aqueous buffer, the solution in the bag became opalescent and the turbidity increased with time. After 96 h of dialysis the precipitate contained full activity of acetylcholinesterase. At this step the yield of acetylcholinesterase activity was 80–100 %. The specific activity increased four-fold throughout the procedure. A typical experiment of reconstitution of membranes and the changes in the values of *n* from the acetylcholinesterase of rats fed a fat-deficient diet are described in Table VI. The recombined membrane closely resembles the original stroma in terms of the values of *n*. In these preparations the same values of *n* were obtained by inhibition by F^- of the acetylcholinesterase. Since in membrane-like material the value of *n* =

TABLE VI

EFFECT OF FORMATION OF MEMBRANE-LIKE STRUCTURE ON VALUES OF n FROM THE ACETYLCHOLINESTERASE OF FAT-DEFICIENT ANIMALS

Treatment of the membrane with Triton X-100 was performed by mixing 1 ml of 20 mM sodium phosphate buffer (pH 7.4) containing 4.25 mg of ghost protein with 9 ml of 0.2% detergent solution in distilled water. The reaggregated material was obtained as described under Materials and Methods. Conditions for obtaining the supernatant are described in the text.

Enzyme preparation	*Total activity* (*μmoles/h*)	*Specific activity* (*μmoles/h per mg protein*)	*Yield* (%)	n	K_i (*mM*)
Erythrocyte membrane	65.7	15.4	100	−1.0	1.8
Material reaggregated by dialysis	58.8	28.9	89	−1.0	1.2
Supernatant after treatment of reaggregated material with Triton X-100	50.9	58.5	77	−1.7	1.6

−1.0 might reflect an allosteric desensitization of the acetylcholinesterase for inhibition by F^- during the dialysis, the insoluble material was resolubilized with 0.2% Triton X-100 and centrifuged at 100000 × *g* for 1 h. In this preparation the acetylcholinesterase recovered the value of $n = -1.7$. Although the results obtained with preparations from fat-sufficient animals indicated the same pattern, the values of $n = -1.6$ were maintained throughout the whole process.

DISCUSSION

Conformational changes in the acetylcholinesterase have been previously suggested by Changeux *et al.*[21-24] and other investigators[25,26].

The sigmoidal shape of the concentration curves for F^- activation in membrane-bound adenyl cyclase has been reported[27]. This cooperative characteristic has been questioned recently since the activation process was not easily reversed by dilution, extensive washing or dialysis[28]. This is not the case with the effect of F^- on acetylcholinesterase since it was reversed by dialysis. In addition, the allosteric nature of the inhibition by F^- of the acetylcholinesterase from rat red cells is supported by the following facts: (a) Sigmoidal curves and values of $n < -1.5$ were obtained and (b) F^- behaves like a non-competitive inhibitor. The latter observation indicates that the catalytic site of this enzyme is entirely different from its inhibition site for F^-.

As observed with ATPase[1-3] a similar response of allosteric behavior was evident with acetylcholinesterase, of which the allosteric character was reduced when rats were fed a fat-free diet. This fact would indicate the general nature of this biological phenomenon for membrane-bound enzymes.

The results of the *in vivo* reversion through changes in the diet of fat-deficient animals indicated that the normal values of n were obtained when no more than 10% of the total population of erythrocytes had been renewed. This fact is a strong argument against the necessity of *de novo* acetylcholinesterase synthesis for the changes in allosterism to occur. This is similar to our previous study on ATPases[2].

The reversible allosteric alteration induced in the enzyme when it binds the

F^- is lost in the membrane-bound acetylcholinesterase from rats fed a fat-deficient diet. It seems that the release of this enzyme from the membrane provokes a reorientation in it, such that the stereospecific interactions for the allosteric effector are recovered. This did not seem to be due to changes in the lipid: protein ratio or the fatty acid composition of the solubilized preparation.

It is generally believed that the physicochemical properties of membrane lipids play an important role in the structure and function of the membrane[29,30].

Enzymes associated with the intact membrane differ in several aspects from the enzymatic preparations obtained after treatment by detergents, organic solvents or phospholipases. The restoration of catalytic functions and other properties was achieved after recombination of solubilized or treated enzymes with lipids[32-39]. Our results for the aggregation of membrane-like material on allosteric transition also indicated this pattern, although it should be noted that the acetylcholinesterase from deficient animals lost the normal allosteric transition in this step of reaggregation. This fact and the evidence that the allosteric behavior of membrane-bound ATPase systems can be altered by modification in the fatty acid composition of the membrane[1-4] suggest that the membrane structure could be an important physiological regulator for the normal allosteric changes *in situ* of the enzyme associated with the membrane.

The effect presented by the allosteric behavior of the membrane-bound acetylcholinesterase from fat-deficient animals was remarkably similar in its reaction to the desensitization phenomenon described for regulatory enzymes[40]. Regulatory enzymes which have been desensitized do not respond allosterically to their effector but are still fully active, that is: the interaction between the sites where the regulatory effector binds can be modified in such a way that it no longer recognizes this allosteric ligand. Several molecular mechanisms have been invoked to account for the desensitization of allosteric enzymes. In our case, the phenotypic allosteric desensitization phenomenon is interpreted by the uncoupling of the interaction without modifying the site of the effector, since the same K_i for F^-, but different for the allosteric behavior were observed for the two groups of animals.

The structural requirements of the membrane lipid composition for the "normal" allosteric transitions of a membrane-bound enzyme are not known at present, but it is tempting to suggest that in those cases where the binding of the enzyme to the membrane provokes a conformational alteration in the protein due to changes in the membrane lipid composition, "abnormal" allosteric behavior might be found.

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