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ALKALI CATION-ACTIVATED AMP DEAMINASE OF ERYTHROCYTES: SOME PROPERTIES OF THE MEMBRANE-BOUND ENZYME

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SUMMARY

1. Human erythrocyte membranes were prepared by several different methods. All preparations, including hemoglobin-free membranes, contained some alkali cation-activated AMP deaminase (AMP aminohydrolase, EC 3.5.4.6).

2. Various washing procedures and fragmentation of membranes by sonication showed that although part of the activity of some membrane preparations could be released, a portion of the enzyme activity was tightly bound to the membrane.

3. Intact erythrocytes did not deaminate AMP which was added to the incubation medium. This suggested that, unlike the situation in muscle, AMP deaminase was not on the outer surface of the membrane.

4. Substrate–velocity curve of the membrane enzyme was hyperbolic in contrast to the sigmoid curve of the soluble enzyme.

5. The effects of ouabain and ethacrynic acid, which are inhibitors of $(Na^+ - K^+)$-activated ATPase, on the membrane AMP deaminase were studied. Ouabain did not affect the enzyme. Ethacrynic acid was found to be a potent inhibitor of the enzyme.

INTRODUCTION

Our previous studies on AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) of erythrocytes[1–3] have primarily been concerned with the requirement of the soluble enzyme for monovalent cations, the specificity of the enzyme for various alkali cations, and the mechanism of changes in the cation-specificity of the enzyme under the influence of ATP and other anions. In an earlier report[1] we indicated that part of the AMP deaminase of human erythrocytes is associated with the cell membrane. In this paper we present the results of studies on the membrane-bound enzyme and comparison of some of its properties with those of the soluble enzyme.

MATERIALS AND METHODS

Nucleotides and ouabain were purchased from Sigma Chemical Co., St. Louis,

Mo. Ethacrynic acid was obtained from Merck Institute for Therapeutic Research, West Point, Pa. Methods for the assay of AMP deaminase have been described[2].

RESULTS AND DISCUSSION

Our first object was to find if several different preparations of human erythrocyte membranes, previously used by other investigators for various purposes, contained AMP deaminase activity. Activities of the following preparations are shown in Table I. A, hemoglobin-free ghosts[4]. B, ghosts which are still able to transport Na^+ activity,

TABLE I

AMP DEAMINASE ACTIVITIES OF HUMAN ERYTHROCYTE MEMBRANES PREPARED BY FOUR DIFFERENT METHODS

All preparations were made from freshly drawn citrated blood according to the methods referred to in the text. The same volume of packed cells was used for all preparations. In each procedure a portion of the original hemolysate was saved, and enzyme activities of the hemolysate and membrane fraction were determined[2]. ATP and K^+ were added to obtain maximum rate of activity[2]. The total deaminase activities of the various hemolysates were the same ($-\Delta A_{265}$/ml of cells per h = 0.72). The total activity of each membrane fraction is expressed as per cent of this value.

	Membrane preparation:			
	A	*B*	*C*	*D*
Total activity as % of the total activity of the hemolysate	8.6	43.0	58.5	29.0

and produce lactate from appropriate substrates[5]. C, ghosts prepared by hemolysis of cells in water, and repeated washings of the membranes in 0.5 mM Tris–HCl (pH 7.1)[6]. D, ghosts which are first prepared by Method C and are then washed in 0.1 M Tris–glycylglycine (pH 8.1)[7]. These ghosts have a high ratio of ($Na^+ + K^+$)-activated ATPase to Mg^{2+}-activated ATPase[7]. As evident from the results all of the tested preparations contain AMP deaminase activity. Because different methods of preparation yielded membranes with various amounts of deaminase activities, the question

TABLE II

RELEASE OF ENZYME FROM MEMBRANES BY VARIOUS WASHING PROCEDURES

A fixed volume of Preparation C of Table I was suspended in 10 volumes of the cold wash solution, stirred for 15 min at 4°, and centrifuged at 20 000 × *g* for 0.5 h. Supernatant was decanted and the pellet was washed in the same manner four more times. After each washing a portion of the pellet was used for enzyme assay by the same procedure described for Table I.

Wash solution	*Total activity of membranes as % of the total activity of the original hemolysate*					
Number of washes	*0*	*1*	*2*	*3*	*4*	*5*
0.08 M Tris–HCl (pH 7.4)	58.0	29.0	21.0	16.5	17.0	16.2
0.15 M KCl	58.0	34.0	20.0	15.9	16.0	16.3
0.15 M NaCl	58.0	40.0	28.5	19.0	16.5	16.0
0.01 M Tris–glycylglycine (pH 8.1)	58.0	28.5	23.0	17.5	17.0	16.6

arose as to whether membranes free of enzyme activity could be prepared. Preparation C of Table I was washed by various procedures to see if the enzyme activity could be released from it. The results presented in Table II show that although the first few washings release a good portion of the enzyme activity, further repeated washings do not release a fraction of the activity which seems to be bound to the membrane. It is also interesting to note that the fraction which can be released, is washed off more easily by some of the washing solutions than by others. These differences between the various washing procedures were not further studied. Comparison of the data of Tables I and II shows that the tightly bound portion of the activity of Preparation C is still higher than the activity of the hemoglobin-free ghosts (Preparation A). Because of this observation it was thought that further washings of the hemoglobin-free membranes with the same solution that had been used in the original preparation might result in membranes free of the activity. However, in five different preparations of hemoglobin-free ghosts the enzyme activities were not significantly affected by such repeated washings. To determine if the enzyme activity of the ghosts were due to soluble enzyme entrapped within the ghosts, or due to binding of the enzyme to the membrane, the washed Preparation C of Table II was sonicated according to the procedure described before[6]. The suspension of broken membranes was centrifuged at 20 000 × *g* for 0.5 h, and the enzyme activities of the pellet and the supernatant were determined. All of the enzyme activity remained with the fragmented membranes. The combined results of Tables I and II, and the effects of sonication, clearly show that a portion of AMP deaminase of erythrocytes is tightly bound to the isolated membranes. The results also show that the degree of binding is dependent on the conditions of hemolysis and washings. Therefore, whether this phenomenon is due to the binding of the enzyme to the membrane of the intact cell, or to the binding of the soluble enzyme to the membrane after hemolysis cannot be decided.

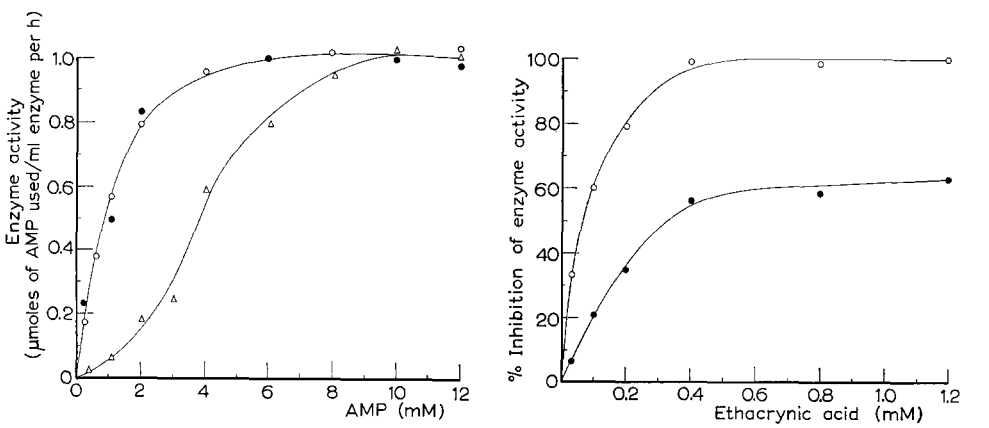

Fig. 1. Effect of varying substrate concentration on the soluble and membrane-bound AMP deaminase activities. △, soluble enzyme in the absence of added modifier; ○, soluble enzyme in the presence of 2 mM ATP; ●, membrane enzyme in the absence of added modifier. In each reaction the concentration of K^+ was 100 mM. Other conditions and procedures as described before[2,3].

Fig. 2. Effect of varying concentration of ethacrynic acid on AMP deaminase. ○, in the presence of 100 mM Na^+; ●, in the presence of 100 mM K^+. Assay conditions the same as described for Table I. Enzyme activity in the absence of ethacrynic acid was 0.210 ($-\Delta A_{265}$/ml per h).

Because of recent reports[8,9] which indicate that AMP deaminase of muscle is at least partly associated with the outside of the muscle surface, we determined the fate of AMP added to a suspension of intact erythrocytes. No deamination, under a variety of conditions could be detected. Therefore, the erythrocyte enzyme does not seem to be located on the outside surface of the membrane.

Fig. 1 shows the substrate–velocity curves of the membrane-bound enzyme and the soluble enzyme. We have already shown[3] that the curve of the soluble enzyme is sigmoidal in shape, and that it becomes hyperbolic only in the presence of ATP. As evident from Fig. 1 the curve of the membrane-bound enzyme is hyperbolic in the absence of ATP. This suggests that either the modifying site for ATP is absent in the membrane-bound enzyme, or that the site is already occupied within the membrane. It should also be mentioned that the membrane-bound enzyme, like the soluble enzyme, has an absolute requirement for an alkali cation.

The finding of AMP deaminase in various membrane preparations prompted us to determine the effects of ouabain and ethacrynic acid on the enzyme. These drugs are known to be good inhibitors of another alkali cation-activated enzyme (ATPase) of the membrane[7,10]. Ouabain at concentrations as high as 10^{-3} M did not affect the enzyme. Ethacrynic acid, however, was found to be a potent inhibitor of both the soluble and the membrane-bound deaminase (Fig. 2).

The demonstration of apparent binding of AMP deaminase to the membrane raises the question of the relevance of the findings to the previous suggestion on the possible role of this enzyme in the process of alkali cation selectivity of erythrocyte membrane[2]. Briefly, it can be said that although the association of the enzyme with membrane may be a necessity for considering such a role for the enzyme, it does not constitute proof of the suggested function. In this context, and in view of the recent report[11] which suggests that a component of Na^+ pump in erythrocytes is sensitive to ethacrynic acid but not to ouabain, it is tempting to attach some significance to our findings on the effects of ethacrynic acid on AMP deaminase. However, since this drug may have a variety of as yet unknown effects on other metabolic processes of erythrocytes, the suggested functional role of AMP deaminase must still remain a conjecture.

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