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ACTIVATION OF ENZYMES IN RED BLOOD CELL MEMBRANES BY A BASIC PROTEIN ISOLATED FROM COBRA VENOM

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SUMMARY

Treatment of human red cell membranes with direct lytic factor, a basic protein isolated from cobra venom, resulted in a marked increase in glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), adenylate kinase (EC 2.7.4.3), 3-phosphoglycerate kinase (EC 2.7.2.3.) and aldolase (EC 4.1.2.7.) activities, while catalase (EC 1.11.1.6) activity was not affected. Direct lytic factor had no effect on the enzyme activities when added to stroma-free hemolysate. With rising direct lytic factor concentration activation of glyceraldehyde 3-phosphate dehydrogenase in the membrane reached maximum value prior to elution of the enzyme from the membrane. Increasing direct lytic factor concentrations applied to red cell suspensions induced leakage of catalase and hemoglobin in a constant ratio, while glyceraldehyde 3-phosphate dehydrogenase leaked in an increasing ratio to hemoglobin. Other basic proteins such as bee-venom melittin, protamine and histone mimicked the direct lytic factor effect, thus pointing to the significance of the net positive charge of the protein molecule in the activation of the enzymes in the membrane. The data are consistent with the assumption that for the red cell enzymes glyceraldehyde 3-phosphate dehydrogenase, adenylate kinase, 3-phosphoglycerate kinase and aldolase, those parts which are associated with the isolated membranes are actual membrane components. For catalase the membrane-associated part is unspecifically adsorbed.

INTRODUCTION

Cobra venom is known to contain a strongly basic, small-molecular-weight protein designated direct lytic factor in view of its ability to lyse erythrocytes, though to a limited extent[1,2]. It has been previously shown that by a synergistic action of direct lytic factor and phospholipase A, red cell phospholipids, unaccessible to phospholipase A alone, become hydrolysed and hemolysis is enhanced[1,3]. It has been shown that erythrocytes and osmotic ghosts bind direct lytic factor[4]. The present communication describes the effects of direct lytic factor on various enzymatic activities of the human red cell and discusses possible implications with regard to enzyme location.

Abbreviation: Hb, hemoglobin.

MATERIALS AND METHODS

Materials

Direct lytic factor was isolated from ringhals (*Hemachatus haemachatus*) venom and purified as described by ALOOF *et al.*[2]. Melittin from bee venom was kindly supplied by Dr. E. Habermann, Pharmakologisches Institut der Universität, Giessen. Protamine, histone and glyceraldehyde 3-phosphoric acid were purchased from Sigma Co. St. Louis, Mo. All other enzymes and substrates were obtained from Boehringer, Mannheim, Germany.

Stroma-free hemolysate

Red cells from freshly drawn normal human blood collected in sodium citrate were washed 3 times with cold isotonic saline. The packed cells were lysed in 4 vol. of distilled water and the stroma-free hemolysate was separated by centrifugation at 12000 $\times$ *g* for 30 min.

Red blood cell membrane suspension

Washed packed red cells were introduced in 4 vol. of cold 0.01 M Tris–HCl buffer, pH 7.4, and stirred for 30 min at 4°. The membranes were sedimented at 26000 $\times$ *g* for 30 min in a refrigerated centrifuge and washed repeatedly with the same buffer in volumes increasing up to 8 times the initial hemolyzing volume. After each centrifugation the button of hemoglobin (Hb)-rich ghosts was discarded and the procedure repeated until the sediment was creamy white. The membranes, which appeared intact under the phase microscope, were suspended in the Tris buffer and adjusted to $0.5 \cdot 10^9$–1.10^9 membranes per ml. Membrane counts with the aid of a celloscope (AB Lars Ljungberg and Co., Sweden, Model 101) were confirmed by camera counting. Residual Hb in the membrane preparations did not exceed 0.5 mg per 10^{11} membranes.

Enzyme assays

Enzyme assays, except for catalase, were carried out in a final volume of 3 ml at 25°, and the oxidation and reduction of NAD^+ was followed by recording absorbance at 340 nm in a Unicam Model SP. 800 A spectrophotometer equipped with an expanded scale. Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was assayed essentially according to SCHRIER[5]. The 3 ml reaction mixture contained 65 μmoles triethanolamine buffer, pH 7.4, 7 μmoles EDTA, 3 μmoles D-glyceraldehyde 3-phosphate, 18 μmoles sodium arsenate, 0.75 μmole NAD^+ and 0.05 ml membrane suspension or 0.02 ml of stroma-free hemolysate adjusted to 4 g/100 ml Hb. Adenylate kinase (EC 2.7.4.3.) was determined according to the method of SZEINBERG *et al.*[6] on 0.02 ml stroma-free hemolysate containing 0.4 g/100 ml Hb, or on 0.3 ml membrane suspension. Because of the high absorbance of the reaction mixture the adenylate kinase assay in the membranes was less sensitive than that of the other enzymes tested. 3-Phosphoglycerate kinase (EC 2.7.2.3) determinations were carried out according to the method of VALENTINE *et al.*[7] on 0.03 ml stroma-free hemolysate containing 0.4 g/100 ml Hb, or 0.3 ml membrane suspension. Aldolase (EC 4.1.2.7) was assayed according to the method of LOHR *et al.*[8] and lactate dehydrogenase (EC 1.1.1.27) according that of WROBLENSKY AND LADUE[9] with the minor modifi-

cations described by SZEINBERG *et al.*[6]. The amounts of membrane suspension used were 0.1 and 0.4 ml, respectively. Catalase (EC 1.11.1.6) was determined in stroma-free hemolysate as described by MILLER[10] or, using the same procedure, on 1 ml membrane suspension. The results were corrected for a small degree of permanganate consumption by the membrane suspension in absence of H_2O_2.

Other assays

Hb content in hemolysates was determined according to the method of ANDERSEN *et al.*[11] and in membrane suspensions by the benzidine method[12]. Protein was assayed according to the method of LOWRY *et al.*[13].

RESULTS

Effects of direct lytic factor on glyceraldehyde 3-phosphate dehydrogenase and catalase activities in erythrocyte suspensions

Saline-suspended red blood cells were incubated with graded amounts of direct lytic factor, and Hb, catalase and glyceraldehyde 3-phosphate dehydrogenase determinations were performed on samples of the red cell suspension and of the supernatants (Table I). While the catalase activity in the red cell suspension remained unchanged, glyceraldehyde 3-phosphate dehydrogenase activity increased with rising direct lytic factor concentration up to 50 μg/ml, higher direct lytic factor concentration producing no further increase. Hemolysis increased with rising direct lytic factor concentration reaching a maximum of about 2.8% already at 25 μg direct lytic factor per ml. Catalase was released from direct lytic factor-treated erythrocytes in a fairly constant catalase: Hb ratio. In contrast, the glyceraldehyde 3-phosphate dehydrogenase: Hb ratio in the supernatant continued to increase with augmenting direct lytic factor concentration.

Effect of direct lytic factor and other basic proteins on enzyme activities of red cell membranes

Table II presents the values for various enzyme activities in the red cell membranes along with standard values for the enzyme activities in stroma-free hemolysate found in this laboratory. The extent to which contamination by stroma-free hemolysate might possibly contribute to the enzyme activities found in the membrane preparation was calculated from the residual membrane Hb and the enzyme activity: Hb ratio in the stroma-free hemolysate. The percentage of membranal enzyme activity possibly due to contamination was negligible for glyceraldehyde 3-phosphate dehydrogenase and aldolase and did not exceed 15% for the other enzymes tested.

Different batches of red cell stromas exhibited variable levels of glyceraldehyde 3-phosphate dehydrogenase activity but in all cases incubation in presence of direct lytic factor induced a high degree of activation (Table III). In addition, direct lytic factor was found able to induce marked activation of aldolase, 3-phosphoglycerate kinase, and adenylate kinase. Lactate dehydrogenase was less affected and catalase activity of the membranes remained essentially unchanged (Table III).

A number of basic proteins tested as possible direct lytic factor substitutes, such as the bee-venom lytic protein melittin, protamine and histone, induced in the membranes a variable degree of enzyme activation which, except for lactate dehydro-

TABLE I

EFFECTS OF DIRECT LYTIC FACTOR ON ERYTHROCYTE GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE AND CATALASE

Assay system: 0.5 ml packed red blood cells suspended in 2 ml (final volume) phosphate-buffered saline, pH 7.4, were incubated with the amounts of direct lytic factor (DLF) indicated for 1 h at 37°. The red cells were sedimented at 1000 × *g* for 15 min, and the supernatant recentrifuged at 26000 × *g* for 30 min. GAPD, glyceraldehyde-3-phosphate dehydrogenase.

μg/ml DLF	*Red blood cell suspension*			*Supernatant*				
	Hb (*g* %)	*Catalase* (*units* · 10⁻³/ml*)	*GAPD* (*units** · 10³ ml*)	*Hb* (*g* %)	*Catalase*		*GAPD*	
					(*units* · 10⁻³/ml*)	(*units* · 10⁻³/mg Hb*)	(*units** · 10³/ml*)	(*units**/g Hb*)
0	5.1	607	1050	0.023	3.2	13.8	4.2	18.2
5	5.2	520	1050	0.054	5.9	10.8	16.1	24.8
12.5	5.1	570	1300	0.071	8.5	11.9	24.0	34.0
25	5.2	470	1540	0.113	10.7	9.5	34.4	30.4
50	4.65	550	1920	0.108	13.5	12.5	38.2	35.5
200	4.07	485	1820	0.111	12.5	11.3	51.0	46.0

* Units represent μl O_2 at standard temperature and pressure evolved from the H_2O_2 during the reaction.
** Units represent μmoles/min at 25°.

TABLE II

ENZYMATIC ACTIVITIES OF RED CELL MEMBRANES AND OF STROMA-FREE HEMOLYSATE

The results are expressed as mean ± S.D. When the number of experiments (indicated in parentheses) is less than 5, mean and range are given. Catalase units represent μl O_2 at standard temperature and pressure evolved from the H_2O_2 during the reaction. For all the other enzymes the units represent μmoles/min at 25°. GAPD, glyceraldehyde-3-phosphate dehydrogenase; 3-PGK, 3-phosphoglycerate kinase; AK, adenylate kinase; LDH, lactate dehydrogenase; SFH, stroma-free hemolysate.

Enzyme	*Activity of membranes* (*units/10¹¹ membranes*)	*Activity of SFH* (*units/g Hb*)	*Activity potentially due to contamination with SFH**	
			(*units/10¹¹ membranes*)	(% *of total membrane activity*)
GAPD	4.92 ± 3.6 (9)	6.46 ± 0.97 (43)	$2.3 \cdot 10^{-3} \pm 0.95 \cdot 10^{-3}$ (6)	0.031 ± 0.029 (6)
Aldolase	2.08 ± 0.99 (11)	2.34 ± 0.44 (26)	$0.77 \cdot 10^{-3} \pm 0.39 \cdot 10^{-3}$ (7)	0.03 ± 0.017 (7)
3-PGK	0.87 ± 0.57 (8)	62.4 ± 11.8 (38)	$23 \cdot 10^{-3} \pm 9.7 \cdot 10^{-3}$ (6)	4.85 ± 2.3 (6)
AK	0.41 ± 0.14 (8)	153 ± 28 (55)	$50.5 \cdot 10^{-3} \pm 27.5 \cdot 10^{-3}$ (7)	14.8 ± 9.4 (7)
LDH	0.64 ± 0.39 (5)	93.2 ± 22 (25)	$23.6 \cdot 10^{-3} \pm 10.3 \cdot 10^{-3}$ (5)	3.6 ± 0.43 (5)
Catalase	$31.7 \cdot 10^3$ (4)	$14.9 \cdot 10^6 \pm 2.1 \cdot 10^6$ (37)	$3.2 \cdot 10^3$ (4)	10.2 (4)
	$16.4 \cdot 10^3$–$42.5 \cdot 10^3$		$0.9 \cdot 10^3$–$6 \cdot 10^3$	2.5–14

* Calculated from residual membrane Hb and enzymatic activity: Hb ratio in stroma-free hemolysate.

TABLE III

EFFECT OF DIRECT LYTIC FACTOR AND OTHER BASIC PROTEINS ON ENZYMATIC ACTIVITIES IN RED CELL MEMBRANES

The incubation system contained $0.5 \cdot 10^9$–$1 \cdot 10^9$ membranes, one of the following basic proteins: direct lytic factor (DLF), melittin (125 μg), protamine, histone (50–125 μg), or as control albumin (125 μg), in 1 ml of 0.01 M Tris–HCl buffer, pH 7.4. Incubation was carried out at 37° for 1 h. The results are expressed as means of the ratio of the enzyme activity in treated membranes to the activity of incubated control membranes. Incubation caused a 35–50% decrease of activity for all enzymes tested. S.D. was calculated when the number of experiments (in parentheses) was 5 or larger. Otherwise the single results are given. For abbreviations see Table II.

Enzyme	*Ratio of enzymatic activity of treated membranes to that of control preparation*				
	DLF	*Melittin*	*Protamine*	*Histone*	*Albumin*
GAPD	9.21 ± 3.55 (8)	1.77	1.69	2.20	0.86 ± 0.25 (6)
		2.20	5.65	4.04	
		2.17	3.17	3.90	
			2.90	3.75	
Aldolase	4.6 ± 1.9 (5)	1.00	0.87 ± 0.62 (5)	1.20 ± 0.59 (6)	0.76
		1.00			1.32
		1.62			
3-PGK	1.76	1.30	2.10	1.67 ± 0.42 (6)	1.14
	8.35	1.75	1.18		
	2.49	1.40			
	3.15				
AK	7.3	4.00	4.89	1.31	
	3.9	3.20	2.67	3.33	1.00
		2.07	2.27	1.05	
LDH	0.81	2.50	1.06	1.68	
	1.21	1.60	1.10	0.73	1.15
	2.00	2.77	3.74	4.13	
Catalase	0.90	1.40	0.88	0.70	
	0.83	0.92	0.70	0.95	
	0.92				
	1.00				

genase, was consistently smaller than the direct lytic factor-induced activation. These basic proteins had little or no effect on aldolase activity and none on catalase activity. Among the enzymes tested, glyceraldehyde 3-phosphate dehydrogenase was activated to the largest degree. Albumin did not affect any of the enzyme activities. Neither direct lytic factor nor any of the other basic proteins, when added to the stroma-free hemolysate, had any effect on the activity of the enzymes.

The increase in glyceraldehyde 3-phosphate dehydrogenase activity in the membrane suspension and the release of enzyme from the stromas by direct lytic factor treatment is illustrated in Fig. 1. With increasing direct lytic factor concentration, up to 50 μg/ml, glyceraldehyde 3-phosphate dehydrogenase activity rose sharply. At higher direct lytic factor concentration the increase in activity slowed down and reached a plateau at 500 μg direct lytic factor per ml. No liberation of enzyme into the supernatant occurred with amounts of direct lytic factor up to 50 μg per ml that markedly activated glyceraldehyde 3-phosphate dehydrogenase. Substantial elution

of enzyme started at direct lytic factor concentrations which induced nearly maximal activation, and the elution continued until 90% of the enzyme activity was found in the supernatant.

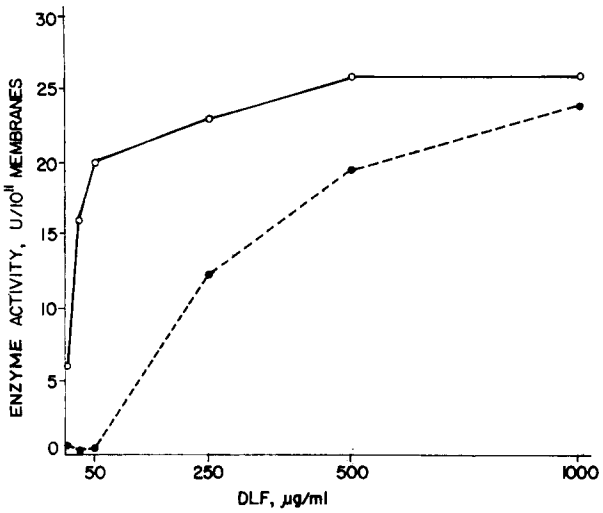

Fig. 1. Red cell membrane glyceraldehyde 3-phosphate dehydrogenase activation and release. $5 \cdot 10^8$ membranes and direct lytic factor (DLF) in the amounts indicated were incubated in 1 ml of 0.01 M Tris–HCl buffer, pH 7.4, for 1 h at 37°. The supernatants were separated after centrifugation at 26000 × *g* for 30 min. ○—○, total glyceraldehyde 3-phosphate dehydrogenase activity in the membrane suspension. ●---●, glyceraldehyde 3-phosphate dehydrogenase activity in the supernatant.

DISCUSSION

For a number of enzymes in human and beef erythrocytes such as glyceraldehyde 3-phosphate dehydrogenase, aldolase, 3-phosphoglycerate kinase and adenylate kinase, there is convincing evidence that part of the activity is intimately associated with the red cell membrane[14–16]. Furthermore, a distinction has been suggested as to the tightness of their binding. Thus, glyceraldehyde 3-phosphate dehydrogenase appears to be the most firmly bound[15,17], 3-phosphoglycerate kinase less strongly bound, and adenylate kinase is the easiest to elute[17]. According to SCHRIER[14] both glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase are organised in the membrane in a specific functional orientation. The association of lactate dehydrogenase activity with the human red cell membrane was postulated by BARLETT[18] and confirmed by KÄGI AND OTTOLENGHI[19]. However, membranes of digitonin-lyzed beef red cells were devoid of lactate dehydrogenase while retaining glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase and aldolase activities. The possibility of a non-specific adsorption of lactate dehydrogenase to the membranes has also been advanced[20]. In contrast to the above enzymes, there are as yet no data in favor of a specific association of catalase with the membrane. Moreover, the common synthetic pathway of Hb and catalase, as well as the role of catalase in the protection of Hb from oxidation[10,21], suggest their common intracellular location[22]. The presently found pattern of enzyme retention in human erythrocyte

membrane preparations compares well with the data on membranes obtained by others under similar conditions of pH and osmotic strength[5,14,19,20].

In the present study it was found that incubation of red cell membranes with direct lytic factor and with various other basic proteins, melittin, histone and protamine, resulted in a marked increase of enzyme activities. The ability of direct lytic factor and the other basic proteins to reveal additional enzyme activity in red cell membranes is not likely to represent a direct activation of the enzyme, since none of these agents had any effect when applied to stroma-free hemolysate or commercial enzyme preparations. Neither is the activation effect to be related to an increase in membrane permeability, since osmotic ghosts (prepared in the absence of Mg^{2+}) are freely permeable to the substrates used in the enzyme assays[14]. Thus, the activation effect may be attributed to a disorganisation of the membrane structure, possible resulting in uncovering of previously unavailable active sites of the enzyme. A parallel may be drawn to a reverse effect of direct lytic factor on the red cell membrane, rendering a phospholipid substrate situated in the membrane available to the action of exogenous phospholipase A^1. The marked augmentation by direct lytic factor of membranal glyceraldehyde 3-phosphate dehydrogenase, aldolase, 3-phosphoglycerate kinase and adenylate kinase activities may therefore be taken as an indication for these enzymes being membranal constituents, in agreement with the prevailing view[14–19]. In this context it is noteworthy that from all the enzymes tested, glyceraldehyde 3-phosphate dehydrogenase, considered to be the most tightly bound[15,17], was also the most strongly activated by the basic proteins. Conversely, the failure of direct lytic factor and the other basic proteins to increase stromal catalase activity supports the view that the catalase is non-specifically adsorbed to the membrane rather than being a membrane component. The inconsistent effects of direct lytic factor and the other basic proteins on lactate dehydrogenase activity in the red cell membranes precludes any interpretation as to lactate dehydrogenase location.

The assumption of different location of glyceraldehyde 3-phosphate dehydrogenase and catalase in the erythrocyte is supported by the data on the leakage of enzymes and Hb caused by direct lytic factor treatment. The degree of hemolysis induced by increasing concentrations of direct lytic factor did not exceed a maximum of 2.8 %. Similarly, the release of catalase from the direct lytic factor-treated erythrocytes reached a maximum of 2.4 % of total red cell catalase activity. Furthermore, while treatment of erythrocytes with rising concentrations of direct lytic factor caused catalase and Hb to leak in a constant ratio, the ratio of glyceraldehyde 3-phosphate dehydrogenase activity to Hb in the medium increased. Given its molecular weight of 137000[23], glyceraldehyde 3-phosphate dehydrogenase is not expected to escape from the erythrocyte to a greater extent than Hb. Conceivably, the increase in glyceraldehyde 3-phosphate dehydrogenase: Hb ratio in the medium of erythrocytes subjected to increasing direct lytic factor concentrations results from liberation of membrane-located glyceraldehyde 3-phosphate dehydrogenase. The reported presence of glyceraldehyde 3-phosphate dehydrogenase at the outer surface of the erythrocyte[17] supports this interpretation.

The degree of disorganisation of the erythrocyte membrane caused by direct lytic factor is reflected in the pattern of glyceraldehyde 3-phosphate dehydrogenase activation and elution when erythrocyte membranes are subjected to rising direct lytic factor concentrations. Thus, nearly maximal activation of glyceraldehyde

3-phosphate dehydrogenase was reached at a direct lytic factor concentration which left the enzyme activity still entirely associated with the membrane, for elution higher direct lytic factor concentrations being required. While the activation effect of direct lytic factor may reflect uncovering of active sites of the enzyme within the membrane, the elution points to a further loosening of the membrane structure.

Melittin, a basic protein isolated from bee venom[24], as well as protamine and histone, were found able to reproduce, although to a lesser extent, the effects of direct lytic factor on membranal enzymes. The net positive charge on the protein molecule seems therefore to be the necessary requirement in the production of this effect. It may be relevant that, as found by SCHRIER[14], sonication and lipid active agents such as digitonin and lecithinase failed to activate glyceraldehyde 3-phosphate dehydrogenase in red cell membranes prepared in the absence of Mg^{2+}.

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