Short communication

Adsorption of Bovine Muscle Lactate Dehydrogenase to Erythrocyte Membranes

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A number of reports have suggested that the lactate dehydrogenase (LDH) isozymes are to be classiefied among glycolytic enzymes which are able to reversibly associate with subcellular membrane structures (Masters 1971). The binding of LDH is believed to be controlled by non-specific electrostatic interactions since the association is strongly affected by pH and concentraction changes of salts, ionic substrates and metabolites. Thus the subcellular localization of the enzymes may be influenced by the metabolic state of a cell. To emphasize their capacity for distribution between both locations, the cytosol and membranes, Wilson (1978) termed them "ambiquitous". The chemical nature of the binding site (s) for LDH isozymes on cell membranes of various tissues remains unknown (Dąbrowska and Gutowicz 1986). Another, still unresolved, problem concerns the different effects of the oxidized and the reduced form of the coenzyme on the enzyme adsorption to membranes. In the present work we examined the adsorption and the effects of NAD and NADH on the adsorption of bovine skeletal muscle lactate dehydrogenase to human and bovine erythrocyte membranes. To measure adsorption, the method of "ghost" centrifugation in the presence of the enzyme was employed.

Bovine skeletal muscle lactate dehydrogenase was prepared from bovine muscle according to Pesce et al. (1964), purified by CM-Sephadex column chromatography and by subsequent repeated recrystallization from ammonium sulfate. Its specific activity was about 300 U/mg and the preparation gave a single band on polyacrylamide gel electrophoresis (LDH-5).

Bovine heart lactate dehydrogenase was obtained from Department of Biochemistry. According to Boehringer's criteria the purity of the preparation was characterized by a single band on PAGE and by a contamination by other enzymes less than 0.01 % in relation to the specific activity of LDH. Before the experiments the lactate dehydrogenase suspension in ammonium sulfate solution was centrifuged and the pellet was dissolved in 15 mmol/l Tris-HCl, 1 mmol/l EDTA, pH = 7.5 and subsequently dialyzed for 48 hours 5 °C against this buffer. The final protein concentration was adjusted to 1 mg/ml. This

Table I. The dependence of skeletal muscle lactate dehydrogenase adsorption to human and bovine erythrocyte membranes on salt concentration in the presence of 2×10^{-4} mol/l NAD, pH = 6.5

Salt concentration (mol/l)	LDH activity in supernatant fraction (%)					
	NaCl		$CaCl_2$		$MgCl_2$	
	Human	Bovine	Human	Bovine	Human	Bovine
0	40	35	40	35	40	35
0.03	68	85	80	75	90	90
0.1	99	100	100	100	100	100

procedure yielded the apo-form of the enzyme with a A_{280}/A_{260} ratio of about 1.8.

The lactate dehydrogenase in the supernatant was determined by the enzyme activity assay according to the method of Bergmeyer at al. (1965). The assay sample (3 ml) contained 0.2 mmol/l NADH and 10 mmol/l sodium pyruvate in 100 mmol/l phosphate buffer (pH = 7.5). One unit of the enzyme activity (U) is defined as the amount of the enzyme which converts 1 μmol of the coenzyme per 1 min at room temperature.

Unsealed bovine and human erythrocyte "ghosts" were prepared from fresh and outdated blood, respectively, by the method of Fairbanks et al. (1971). The erythrocytes were washed three times with 150 mmol/l NaCl, 5 mmol/l sodium phosphate (pH = 8.0). Then, the cells were suspended for lysis in 40 volumes of 5 mmol/l sodium phosphate solution. After pelletting the membranes were washed with the same solution several times. The membranes were pelletted by centrifugation at $37\,000 \times g$ and then dialyzed for a day against 17 mmol/l Tris-HCl, 0.1 mmol/l EDTA buffer (pH = 7.5). The membrane suspension was stored in a refrigerator.

The membrane concentrations were estimated by determining total protein using the method of Lowry et al. (1951). For hemoglobin determination in the "ghosts" they were solubilized in 1 % sodium lauryl sulfate and the content of cyanmethemoglobin was determined by measuring the optical density at 540 nm.

The enzyme — erythrocyte membranes mixture was dialyzed, after 30 min incubation, against the appropriate solution for 4 hours and then centrifuged at 40,000 rpm (MSE-50 ultracentrifuge). The lactate dehydrogenase content in the pellet was calculated from the difference between the initial activity and the activity in the supernatant. Control centrifugation of the enzyme alone gave no pellet.

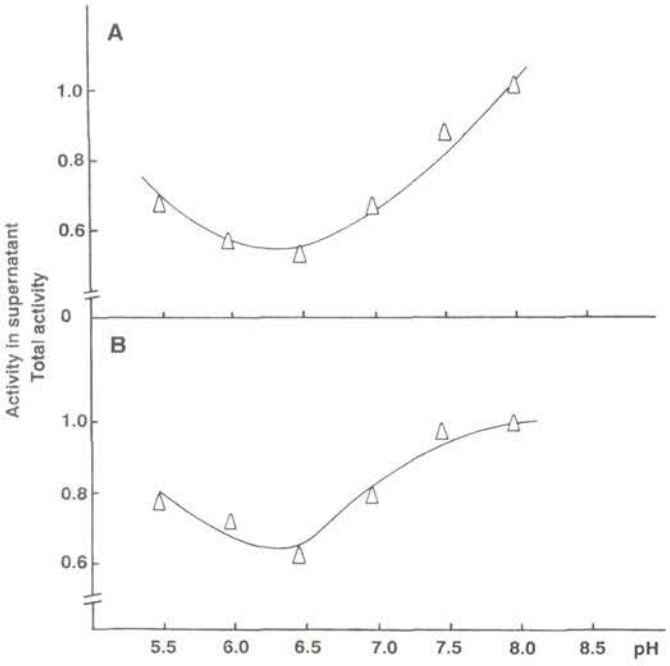

Fig. 1. The pH dependence of bovine skeletal muscle lactate dehydrogenase adsorption to human (*A*) and bovine (*B*) erythrocyte membranes. Initial enzyme concentration 100 μg/ml. In all experiments, 17 mmol/l Tris-HCl, 1 mmol/l EDTA buffer of appropriate pH was used. Each point represents mean value of three parallel determinations.

Centrifugation of the human erythrocyte membranes in the presence of bovine skeletal muscle or bovine heart lactate dehydrogenase reducted the amount of the skeletal muscle enzyme in the supernatant, where as there was no change in the supernatant content of the heart muscle enzyme. The same results were obtained with bovine erythrocyte membranes (data non shown). The binding of the skeletal muscle enzyme was very sensitive to the ionic strenght (Table I) and pH (Fig. 1). Both dependences strongly suggest that nonspecific interactions of ionic type are responsible for the adsorption. The occurrence of an adsorption pH optimum is an interesting observation. A similar optimum was observed for the adsorption of skeletal and heart muscle lactate dehydrogenase to liposomes, which represent a less specific adsorption system (Dąbrowska et al. 1989). Skeletal muscle isozymes of lactate dehydrogenase have a positive net electric charge at pH = 6.5 (Lluis 1985) and the surfaces of

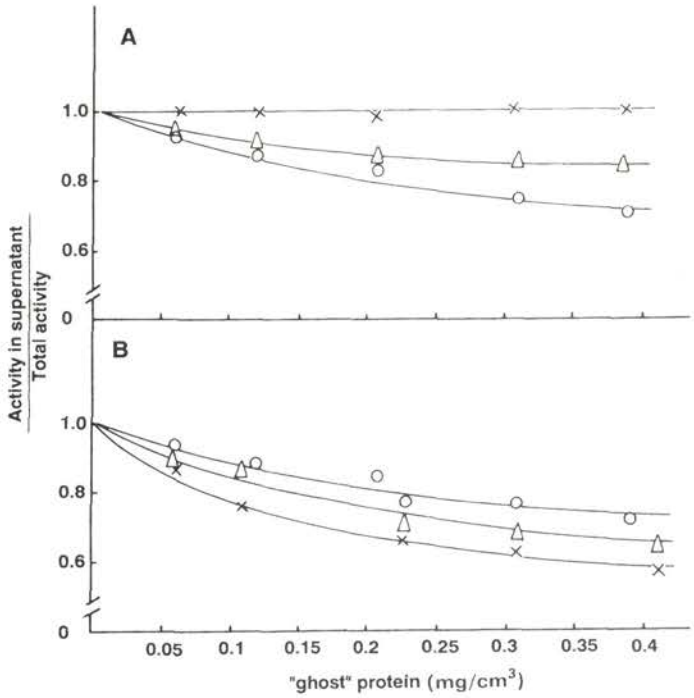

Fig. 2. Effects of NADH (*A*) and NAD (*B*) concentrations on the adsorption of bovine skeletal muscle lactate dehydrogenase to bovine erythrocyte membranes at pH 6.5 *A* — NADH concentrations: 0.5×10^{-8} mol/l (circles); 8×10^{-7} mol/l (triangles); 2×10^{-5} mol/l (crosses). *B* — NAD concentrations (circles); 4.5×10^{-5} mol/l (triangles); 2.0×10^{-4} mol/l (crosses). Other conditions as in Fig. 1.

erythrocyte membranes and those of liposomes bear negative charge over a wide pH range. A simple overall electrostatic attraction between the two systems would be expected to cause monotonic increase of the enzyme adsorption with decreasing pH. The occurrence of the pH optimum for adsorption, in our opinion, suggests the existence of a complex adsorption site on the enzyme molecule. The differences in the nature of the adsorption sites between various isozymes are likely to be responsible for the specifity of adsorption.

Fig. 2 *A* and *B* clearly shows that the adsorption of the enzyme to both membrane types is very sensitive to the presence of the coenzyme; the oxidized and the reduced coenzyme form have quite opposite effects on the adsorption. The addition of NAD enhances the binding, where as the presence of NADH promotes the dissociation. At NADH concentration of afflux 10^{-4} mol/l the binding is completely dissociated. A similar adsorption pattern and quantitatively similar effects of NAD and NADH were observed with the enzyme

adsorption to liposomes prepared of erythrocyte lipids. Different effects of NAD and NADH on the adsorption to liposomes were also shown for heart muscle lactate dehydrogenase (Dąbrowska and Gutowicz 1986). Opposite effects of NAD and NADH have been reported also for the binding of phosphoglycerate kinase to erythrocyte membranes (De and Kirtley 1977). Our results show that the nonspecific erythrocyte membranes can, in some conditions, be an efficient adsorber for skeletal muscle, but not for heart muscle type, of lactate dehydrogenase. Obviously, the differences in the nature of binding sites between the isozymes are responsible for the specificity. Also, Hultin et al. (1972, 1974) and Lluis (1985) could not find any binding capacity of chicken H-type lactate dehydrogenase to chicken skeletal muscle and heart particulate matter. Since they found that the kinetics of the skeletal muscle enzyme was modified upon the binding they proposed that the M-type, but not H-type lactate dehydrogenase, was a regulatory enzyme to glycolysis. The complex nature of the enzyme binding to the membranes is also strongly supported by the quite opposite action of the reduced and the oxidized form of the coenzyme. Since both forms are anionic (considering the net charge) the simple explanation of the phenomenon by the difference in electrostatic interactions and by block of the adsorption site by competitive binding, seems insufficient. Two following possible mechanisms seem more probable: 1) Binding of NAD and NADH modify the conformation of the binding site (s) differently; 2) NAD and NADH interact differently with membranes, changing their adsorption characteristics in a different manner. Both mechanisms may combine to contribute to the phenomenon. Such a mechanism would have important biological implications for in vivo functioning of membranes.

Acknowledgements. The authors wish to thank Prof. J. Kwiatkowska for valuable comments on the manuscript. This work was supported by grants C.P.B.04.01.1.12 and C.P.B.04.01.2.06. from Polish Academy of Sciences.

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Final version accepted March 13, 1990