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Interaction of NAD-Dependent Dehydrogenases with Human Erythrocyte Membranes

Evidence that D-Glyceraldehyde-3-Phosphate Dehydrogenase and Lactate Dehydrogenase Are Catalytically Active in a Membrane-Bound State

VLADIMIR I. MURONETZ,* NATALIA A. SHCHERBATOVA, AND NATALIA K. NAGRADOVA

A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899, Moscow, Russia

ABSTRACT

Interaction of D-glyceraldehyde-3-phosphate dehydrogenase (GPDH) and lactate dehydrogenase with human erythrocyte membranes was studied. Under the conditions of low ionic strength, both enzymes bound to the membranes with similar affinities ($Kd \approx 1\ \mu M$). The binding was accompanied by complete inhibition of GPDH and by a 65–75% inhibition of lactate dehydrogenase (LDH). Increasing the ionic strength to physiologically meaningful values (0.15*M*) completely abolished the inactivation of both dehydrogenases in the presence of erythrocyte membranes, but did not preclude their binding. These results suggest that different modes of enzyme–membrane interaction can be realized under the conditions of low and high ionic strength. They also indicate that GPDH and LDH are capable of functioning in a membrane-bound state.

Index Entries: D-glyceraldehyde-3-phosphate dehydrogenase; lactate dehydrogenase; erythrocyte membrane; band 3 protein.

*Author to whom all correspondence and reprint requests should be addressed. E-mail: muric@fac.genebee.msu.su.

INTRODUCTION

The binding of glycolytic enzymes, and in particular, D-glyceraldehyde-3-phosphate dehydrogenase (GPDH) and lactate dehydrogenase (LDH), with cell structural components has been observed in a number of studies (*1–3*). However, the relevance of these findings to a situation that may take place in vivo remains as yet unclear, and hypotheses on the physiological significance of the binding provoked serious criticism (*4,5*). The most detailed investigation has been carried out on the interaction between GPDH and the integral component of the erythrocyte membrane, the band 3 protein (*1,6,7*). The results of numerous studies performed by Steck and his group clearly demonstrated the inhibitory effect of this protein on GPDH activity (*6,7*). This led to the apoearance of hypotheses concerning the role of GPDH adsorption on erythrocyte membranes in the regulation of glycolysis (*8,9*).

In the work performed by one of us in collaboration with Srivastava and Knull, the GPDH interaction with another structural protein, tubuline, was investigated (*10*). A very strong binding of the enzyme with tubuline was observed under the conditions close to the conditions used by Yu and Steck (*6*) to demonstrate the strong binding of GPDH to the band 3 protein. A similar effect on the activity of bound GPDH (inhibition) was also observed. At the same time, a moderate increase of the ionic strength or even an addition of the substrates at concentrations normally used to measure the GPDH activity eliminated the inhibitory effect. It is noteworthy that the enzyme was not released from tubuline under these conditions, although the binding was markedly weakened.

The above observations stimulated our efforts to test experimentally the suggestion that an analogous situation might have a place in the case of GPDH interaction with other structural components of the cell. The present study was aimed at investigating the interaction of GPDH as well as of a closely related enzyme, LDH, with erythrocyte membranes under different conditions.

MATERIALS AND METHODS

NAD, NADH, D-fructose-6-phosphate, and pig muscle LDH were purchased from Reanal. Dithiothreitol (DTT), EDTA, and HEPES were the products of Serva. Pyruvate was obtained from Boehringer, and potassium phosphate and sodium chloride from Sigma. Sephadexes G-50 and G-100 were purchased from Pharmacia. GPDH was prepared according to the procedure of Szewczuk et al. (*11*). GPDH was purified from rabbit muscle by the method of Hill et al. (*12*). Human erythrocytes were obtained from a blood transfusion station.

Erythrocyte membranes were prepared according to Dodge et al. (*13*). Following washing of the erythrocytes by an isotonic buffer containing 5 m*M* potassium phosphate, 0.15 M sodium chloride (pH 7.5), they were subjected to lysis in 40 vol of the buffer containing 5 m*M* potassium phosphate, 2 m*M* EDTA, and 1 m*M* DTT (pH 7.5) under constant stirring for 15 min. To prepare erythrocyte ghosts, centrifugation of the hemolysate was performed (29,000*g* for 20 min), followed by several washings by the above buffer. The ghosts were then suspended in the desired buffer and used within 24 h. All procedures were carried out at 4°C.

The binding of the enzymes with erythrocyte membranes was investigated following the procedure described by McDaniel et al. (*15*). Solutions of GPDH or LDH were desalted on a Sephadex G-50 column (1 × 7 cm) equilibrated with 25 m*M* HEPES, 0.1 m*M* sodium arsenate, and 0.5 m*M* EDTA (pH 7.0) in the case of GPDH, and 10 m*M* HEPES, 0.5 m*M* DTT (pH 6.9) in the case of LDH. Washed erythrocyte membranes suspended in a buffer were used in the experiments with GPDH or LDH. A series of samples were prepared containing equal amounts of membrane suspension in each sample, as well as 0.1 m*M* NAD (in experiments with GPDH) or 0.075 m*M* NADH (in experiments with LDH). The titrations with increasing amounts of the corresponding enzymes were then performed. The procedure was as follows. Following addition of an aliquot of the enzyme solution, a sample was incubated for 20 min at 25°C on periodic stirring, with subsequent centrifugation (10,000*g* for 10 min at room temperature). The protein concentration in the supernatant was then measured to determine the amount of unbound enzyme. Analogous experiments were carried out under the conditions where incubation of the enzyme with erythrocyte membranes was performed in buffers supplemented with 0.15*M* NaCl.

The amounts of the membrane-bound GPDH and LDH were calculated from the data obtained. The results presented in Figs. 2 and 4 were fitted to the Eq. (1).

$$A = (A_{max}\,[E]) / (K_d + [E]) \quad (1)$$

Where A is the membrane-bound enzyme concentration, A_{max} is the absorbance capacity of the membrane protein, [E], the concentration of the free enzyme in solution, and K_d, the apparent dissociation constant of the enzyme–membrane protein complex.

Concentrations of GPDH, LDH, and erythrocyte membrane protein were measured according to Bradford (*14*) using the corresponding proteins as standards. The M_r values of 144,000 and 140,000 were used for GPDH and LDH, respectively.

GPDH activity was measured in 50 m*M* glycine, 50 m*M* potassium phosphate, 5 m*M* EDTA, 0.5 m*M* NAD, 0.5 m*M* GPDH (pH 8.9), 25°C, or in 25 m*M* HEPES, 0.1 m*M* sodium arsenate, 0.5 m*M* EDTA, 0.1 m*M* NAD, and 0.1 m*M* GPDH (pH 7.0) at 25°C. LDH activity was assayed in

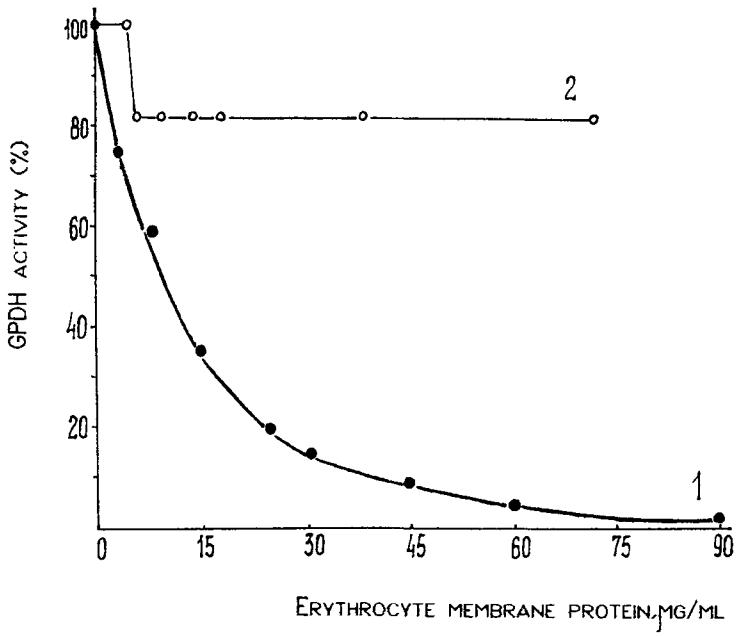

Fig. 1. Inhibition of GPDH activity by erythrocyte membranes. The activity of GPDH was measured in the presence of different concentrations of erythrocyte membranes in 25 m*M* HEPES, 0.083 μM enzyme, 0.1 m*M* glyceraldehyde-3-phosphate, 0.1 m*M* NAD, 0.1 m*M* sodium arsenate, and 0.5 m*M* EDTA (pH 7.0) at 25°C. Curve 1, no other additives; curve 2, 0.15*M* NaCl was added.

0.1*M* potassium phosphate, 1 m*M* EDTA, 0.4 m*M* NADH, and 0.4 m*M* pyruvate (pH 7.4) at 25°C or in 10 m*M* HEPES, 0.5 m*M* EDTA, 1 m*M* DTT, 0.025 m*M* NADH, and 0.2 m*M* pyruvate (pH 6.9) at 25°C.

RESULTS AND DISCUSSION

As shown in Fig. 1 (curve 1), incubation of GPDH with erythrocyte membranes results in the inhibition of the enzyme activity, which is nearly complete under appropriate experimental conditions. These results are consistent with the data on the inhibitory effect of the band 3 protein on GPDH (*1,6,7*), and can be explained by the interaction of this component of the membrane with the enzyme. Figure 2 illustrates the binding of GPDH with eyrthrocyte membranes measured under the experimental conditions employed to detect the inhibition of the enzyme (Fig. 1). As follows from the results obtained, GPDH was capable of binding to the membranes with a *Kd* of 1.1 ± 0.18 μM.

Next, we examined the effect of erythrocyte membranes on the activity of LDH. The results obtained (Fig. 3) indicate that this enzyme is also susceptible to the inhibition, but, in contrast to the situation observed with GPDH, no complete inactivation could be obtained even in the presence

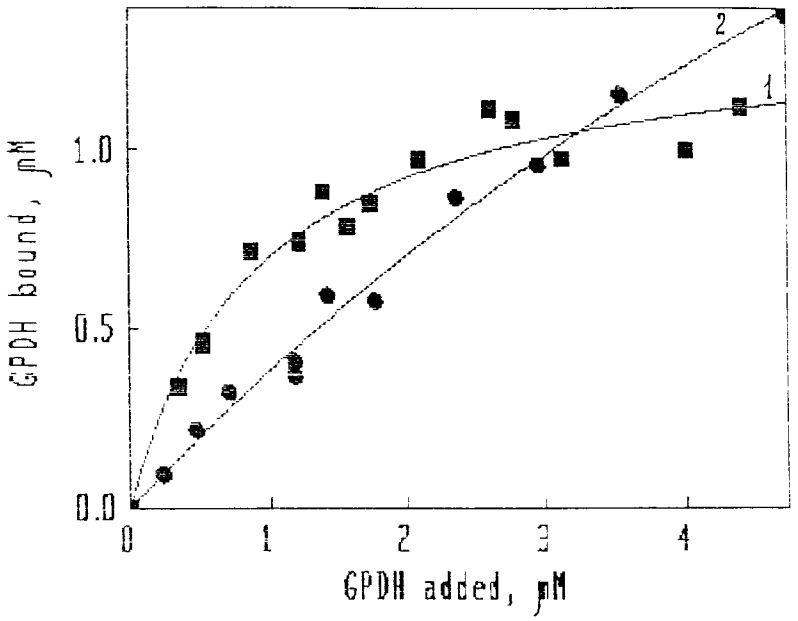

Fig. 2. The binding of GPDH with erythrocyte membranes at low ionic strength (curve 1) and in the presence of 0.15*M* NaCl (curve 2). The K_d values determined from these experiments were 1.1 ± 0.18 and 11.6 ± 3.36 μM in the absence and in the presence of 0.15*M* NaCl, respectively. The number of the enzyme binding sites/100 μg membrane protein was 1.27 ± 0.08 (without NaCl) and 4.38 ± 0.99 (in the presence of 0.15*M* NaCl).

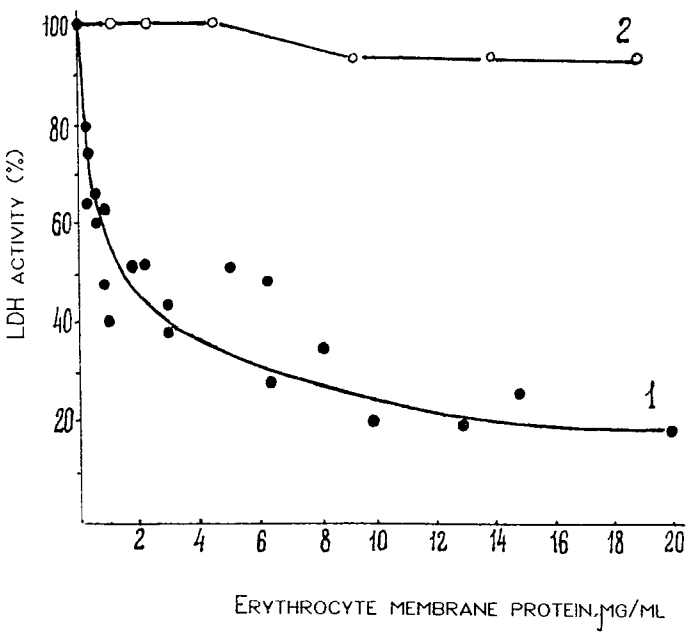

Fig. 3. Inhibition of LDH activity by erythrocyte membranes. The activity of LDH was measured in the presence of different concentrations of erythrocyte membranes in 10 m*M* HEPES, 0.00086 μM enzyme, 0.025 m*M* NAD, 0.2 m*M* pyruvate, 0.5 m*M* EDTA, and 1 m*M* DTT (pH 6.9) at 25°C. Curve 1, no additives: curve 2, 0.15*M* NaCl was added.

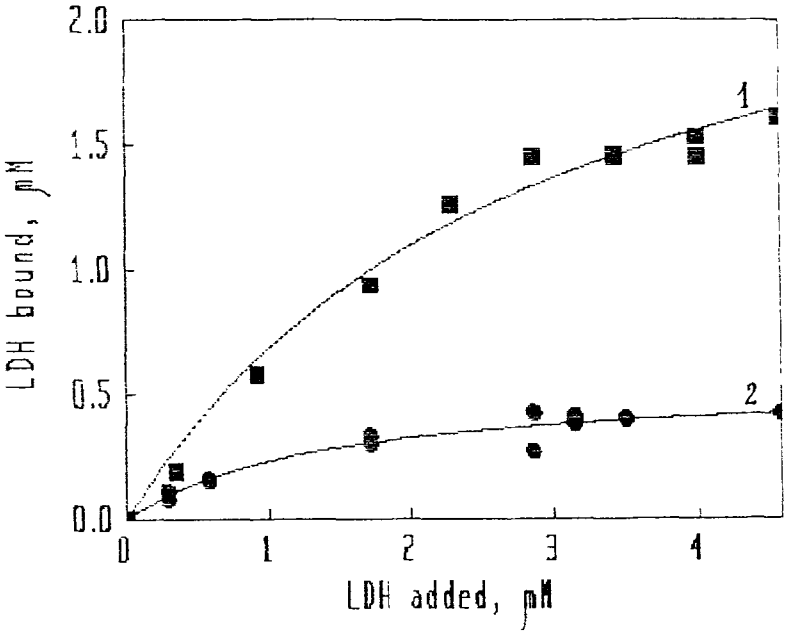

Fig. 4. The binding of LDH with erythrocyte membranes at low ionic strength (curve 1) and in the presence of 0.15*M* NaCl (curve 2). The K_d values determined from these experiments were 2.9 ± 0.77 and 1.2 ± 0.22 μM in the absence and in presence of 0.15 NaCl, respectively. The number of the enzyme binding sites/100 μg membrane protein was 2.81 ± 0.31 (without NaCl) and 0.52 ± 0.04 (in the presence of 0.15*M* NaCl).

of a considerable excess of the erythrocyte membranes. Figure 3 shows that the inhibitory effect could already be detected in the presence of rather low concentrations of membranes, but the maximal loss of activity did not exceed 70%. Figure 4, curve 1 illustrates the binding of LDH to erythrocyte membranes at low ionic strength. The K_d value obtained is close to that for GPDH. Taken together, the results obtained with GPDH and LDH demonstrated that both dehydrogenases are capable of forming complexes with some component(s) of the erythrocyte membranes (most likely, the band 3 protein).

A question then arose regarding whether the two enzymes are able to bind with membranes at physiological ionic strength and how this affects their functional properties. The experiments carried out with GPDH showed that the inhibitory effect of membranes disappeared in the presence of 0.15*M* NaCl. (Fig. 1, curve 2). However, the enzyme remained capable of binding to erythrocyte membranes under these conditions, although with less affinity than at lower ionic strength (Fig. 2, curve 2). These results suggest the existence of at least two types of the GPDH-binding sites on the membranes and are in accordance with proposed existence of the low- and high-affinity GPDH-binding sites on the erythrocyte membranes (*15*). Our present results point to different modes of

interaction between GPDH and the erythrocyte membrane at the high- and low-affinity binding sites, since only the interaction of the first type leads to enzyme inhibition.

Figure 3, curve 2, shows that the inhibitory effect of erythrocyte membranes on the activity of LDH disappears in the presence of 0.15*M* NaCl. The effect was similar to that observed with GPDH (Fig. 1, curve 2). At the same time, LDH displayed some peculiarities. Thus, despite the presence of 0.15*M* NaCl, the binding of LDH to erythrocyte membranes remained rather strong (Fig. 4, K_d 1.2 ± 0.22 μM). Taking into account the diminished number of the LDH-binding sites determined in the experiments with 0.15*M* NaCl, we suggest that there are probably two types of the LDH-binding sites on the membranes that differ in affinity. Only the binding sites with higher affinity exist at high ionic strength, and according to our data (Fig. 3), the LDH–membrane interaction in these sites does not affect the enzyme activity. It seems likely that incomplete inhibition of LDH by erythrocyte membranes observed in our study is owing to the presence of the high-affinity "noninhibitory" sites, since even in the presence of excess membranes, a portion of LDH bound at these sites remains active.

CONCLUSION

We have demonstrated that two NAD-dependent dehydrogenases operating in glycolysis are effectively inhibited by erythrocyte membranes at low ionic strenght (i.e., in the presence of low concentrations of substrates and NAD). It seems likely that the inhibition may result from an electrostatic interaction of the "acidic tail" of the band 3 protein with the positively charged regions of the NAD-binding site in the enzyme molecule. Similar interaction possibly occurs in the case of binding of these dehydrogenases to tubuline, which also contains an "acidic tail" (*16,17*).

A principally different interaction probably takes place between the enzymes and erythrocyte membranes under physiological conditions. Such a situation was simulated in our studies by addition of 0.15*M* NaCl. In this case, the enzyme-membrane interaction persists (although is weakened for GPDH), but the inhibitory effect disappears completely. Interestingly, analogous results have previously been obtained in the case of the GPDH-tubuline system. Taken together, these results support the idea about two types of enzyme-structural protein interactions differing in their functional tasks. Only the interaction occurring at a low ionic strength is accompanied by the enzyme inhibition. This indicates that enzymes bound to erythrocyte membranes under the conditions close to the physiological ones remain catalytically active. In the light of new information obtained in this study, the functional significance of the binding of GPDH to erythrocyte membranes should be reconsidered. Our

data support the idea that the inhibition of the enzyme on its binding to erythrocyte membranes at very low ionic strength cannot play any role in metabolic regulation. The mechanism of this phenomenon and its relevance to the enzyme functioning require further investigation.

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REFERENCES

1. Murthy, S. N. P., Liu, T., Kaul, R. K., Kohler, H., and Steck, T. L. (1981), *J. Biol. Chem.* **256,** 11,203–11,208.
2. Masters, C. J. (1984), *J. Cell. Biol.* **99,** 222–225.
3. Ryazanov, A. G., Ashmarina, L. I., and Muronetz, V. I. (1989), *Eur. J. Biochem.* **158,** 49–57.
4. Tsamaloukas, A. G., Kollecker, P., Maretzki, D., and Rappoport, S. (1978), *Acta Biol. Med. Germ.* **37,** 1273–1278.
5. Maretzki, D., Groth, J., Tsamaloukas, A. G., Grundel, M., Kruger, S., and Rappoport, S. (1974), *FEBS Lett.* **39,** 83–87.
6. Yu, J. and Steck, T. L. (1975), *J. Biol. Chem.* **250,** 9176–9184.
7. Tsai, Inn-Ho, Murthy, S. N. P., and Steck, T. L. (1982), *J. Biol. Chem.* **257,** 1438–1442.
8. Harrison, M. L., Rathinavelu, P., Arese, P., Geahlen, R. L., and Low, P. S. (1991), *J. Biol. Chem.* **266,** 4106–4111.
9. Low, P. S., Rathinavelu, P., and Harrison, M. L. (1993), *J. Biol. Chem.* **268,** 14,627–14,631.
10. Muronetz, V. I., Wang, Z.-X., Keith, T. J., Knull, H. R., and Srivastava, D. K. (1994), *Arch. Biochem. Biophys.* **313,** 253–260.
11. Szewczuk, A., Wolna, E., Wolny, M., and Baranowski, T. (1961), *Acta Biochim. Pol.* **2,** 201–207.
12. Hill, E. J., Meriwether, B. P., and Park, J. H. (1975), *Analyt. Biochem.* **63,** 175–182.
13. Dodge, J. T., Mitchell, C., and Hanahas, D. J. (1963), *Arch. Biochem. Biophys.* **100,** 119–130.
14. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
15. McDaniel, C. F., Kirtley, M. E., and Tanner, M. J. A. (1974), *J. Biol. Chem.* **249,** 6478–6485.
16. Wehland, J., Willingham, M. C., and Sandoval, I. V. (1983), *J. Cell. Biol.* **97,** 1467–1475.
17. Serrano, L., Avila, J., and Maccioni, R. B. (1984), *Biochemistry* **23,** 4675–4681.