INVESTIGATIONS ON THE BINDING OF SOLUBLE GAPDH TO THE MEMBRANE FRACTION OF RABBIT RETICULOCYTES

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1. Introduction

After hypotonic hemolysis of erythrocytes and reticulocytes a large part of the GAPDH is found in the membranal fraction [1–3]. The enzyme binding to the membranes is very firm. As already reported [3,4], it is possible to release the membrane-bound GAPDH of rabbit reticulocytes by different procedures. The reversibility of enzyme release and binding is a necessary condition for a possible regulatory importance of an enzyme binding.

Kant and Steck [2] reported that it is possible to rebind released GAPDH to the membranes of human erythrocytes. They represented a hyperbolic curve for the binding of the enzyme in dependence on the membrane concentration. The present paper deals with the reassociation of solubilized GAPDH with the membrane fraction of rabbit reticulocytes. It is shown that the kinetics of this process and its concentration dependence are strongly influenced by proteins released simultanously with the GAPDH.

2. Materials and methods

The starting material (bound GAPDH) was prepared by hypotonic hemolysis of rabbit reticulocytes [3]. Soluble GAPDH was obtained by incubation of bound GAPDH in 100 mM KCl containing 10 mM Tris (pH 8.2) for 10 min. After centrifugation (20 000 *g*, 10 min) the supernatant was dialyzed against 10 mM HEPES (pH 7.2) and used as soluble GAPDH (spec. act. about 1 U/mg protein). The sediment was resuspended in 10 mM HEPES (pH 7.2) with 20 mM KCl and 1 mM NADH to remove the last traces of bound GAPDH. The pellet of the subsequent centrifugation was washed twice with 10 mM HEPES (pH 7.2) and used as GAPDH-free membrane fraction. In several experiments (table 3) the GAPDH was more specifically solubilized by 10 mM HEPES (pH 7.2) containing 20 mM KCl and 50 μM NADH. The GAPDH fraction obtained by this solubilization had a spec. act. of 2.1 U/mg protein.

All rebinding incubations were carried out at 2°C. After centrifugation (20 000 *g*, 10 min) the GAPDH activity was measured in the pellet and in the supernatant by the method described in Bergmeyer [5]. The rabbit muscle GAPDH was a product of Boehringer, Mannheim.

3. Results and discussion

While the GAPDH release is a very quick process which is completed within one min [6], the reassociation of the solubilized enzyme with the GAPDH-free membrane fraction is very slow (table 1). After an incubation of about 6 hr the portion of the bound enzyme does not further increase. The decrease of the enzyme activity in the following time is a result of the GAPDH inactivation, which can be measured in the sediment as well as in the supernatant.

The time required for this rebinding process is about 500 times greater than that for other reassociation processes, as for example the recombination of the succinate dehydrogenase with the mitochondrial inner membrane [7]. A comparison of the two experiments in table 1 demonstrates that the amount of the bound enzyme depends on the enzyme addition, which means that in the case of the lower GAPDH

Table 1
Rebinding of released GAPDH to GAPDH-free particles (0.1 mg protein); dependence on the time of incubation

Incubation time (hr)		0.17	1	3	6	24	48
GAPDH addition 0.23 U	[U]	0.033	0.052	0.094	0.096	0.088	0.074
	%	14	22	41	42	38	32
GAPDH addition 0.46 U	[U]	0.081	0.166	0.232	0.266	0.216	0.208
	%	18	36	51	58	47	45

addition (0.23 U) free binding sites of the membrane are still present. In spite of this free capacity only 42% of the added enzyme activity is rebound. This incomplete rebinding is contradictory to the high enzyme affinity of the membrane fraction shown in repeated washing procedures [3]. The addition of more GAPDH causes an increase of the bound enzyme activity, which is higher than the proportional one (compare the percentages in table 1). It must be assumed that the bound enzyme molecules favour the binding of further ones. Therefore the membrane binding sites must be considered to depend on each other.

As the results in table 1 show, the enzyme concentration has a strong influence on the characteristics of the reassociation process. This effect was also observed in releasing experiments.

Under conditions of isotonic hemolysis the GAPDH-membrane-association is found to be unstable [8,9].

Table 2
The dependence of the GAPDH-membrane association on the addition of the GAPDH-membrane fraction (in the presence of 150 mM KCl)

	Protein [mg/ml]	GAPDH [U/ml]	GAPDH in the sediment U	GAPDH in the supernatant U	bound Activity %
1.	0.33	0.25	0.014	0.726	2
2.	0.67	0.42	0.050	1.2	4
3.	1.74	1.08	0.234	3.0	6
4.	3.33	2.7	0.75	7.3	10
5.	5.0	4.5	1.49	12.2	11
6.	6.67	5.9	1.95	15.7	11
7.	8.39	7.5	3.6	20.0	15
8.	30.0	14.9	5.2	9.7	35

As the data in table 2 indicate, the amount of the incubated GAPDH-membrane fraction has an evident influence on the stability of the binding against the solubilizing effect of the high ionic strength. It is possible to keep the GAPDH partly bound in presence of 150 mM KCl solution. These results agree with the findings of Clarke and Masters [10] for skeletal muscle.

Increasing amounts of bound GAPDH were incubated in 10 mM HEPES (pH 7.2) with 150 mM KCl (10 min; 2°C). After centrifugation (1–7, 20 000 *g*, 10 min, 8: 38 000 *g*, 30 min) the enzyme activity was measured in the pellet and in the supernatant.

The influence of the enzyme concentration on the rebinding of released GAPDH is represented in fig.1. The bound activity is plotted against the soluble one after a 12 hr incubation.

It must be concluded from the saturation characteristic of this curve that there is a limited number

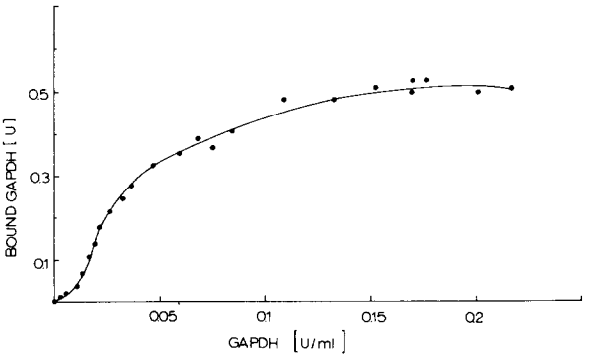

Fig.1. Dependence of GAPDH rebinding on the concentration of released GAPDH. GAPDH-free particles (0.13 mg protein) were incubated with increasing concentrations of solubilized GAPDH for 12 hr (2°C).

of binding sites on the membrane. Under these experimental conditions the membrane fraction can maximally bind 3.8 U/mg membrane protein. The binding curve is a sigmoidal one, which is characteristic for a co-operative interaction of the binding sites. Therefore an increase of the affinity of the binding sites due to the bound enzyme must exist. A transformation of this curve into the Hill plot yielded a straight line with a slope of $n_H = 1.7$. From the specific activity of the solubilized GAPDH (1 U/mg) it follows that this fraction contains further peripheral proteins of the reticulocyte membrane besides the enzyme, both released under preparation conditions. In parallel investigations with muscle GAPDH it was tested whether in the absence of these membrane proteins the binding behaviour is the same as in the case of the solubilized enzyme. Fig.2 shows a comparison of the time dependence of the rebinding of pure muscle GAPDH, of solubilized GAPDH and of a mixture of both. Within 10 min the muscle enzyme is nearly completely bound. The mixture of pure and released GAPDH shows a binding behaviour between that of both components. As demonstrated by the comparsion, the peripheral proteins, contained in the solubilized GAPDH-fraction, are responsible for the slow kinetics of the released GAPDH rebinding process.

Fig.3 shows the binding curves of rabbit muscle GAPDH with the membrane fraction in absence and in presence of 0.33 U released GAPDH. While

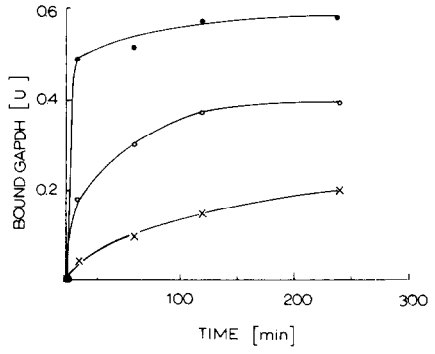

Fig.2. GAPDH rebinding to the GAPDH-free membrane fraction in dependence on the incubation time. (•) Pure muscle GAPDH (0.66 U). (○) Pure muscle GAPDH (0.33 U) and solubilized GAPDH (0.24 U). (×) Solubilized GAPDH (0.47 U).

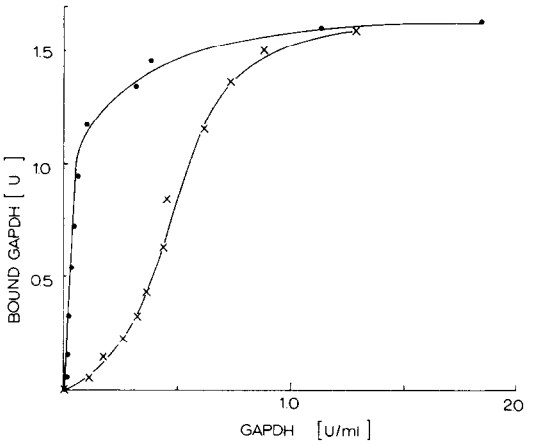

Fig.3. Depencence of GAPDH binding on the concentration of rabbit muscle GAPDH. Constant amounts of GAPDH-free membrane particles (0.1 mg protein) were incubated with increasing concentrations of pure muscle GAPDH (•) for 1 hr at 2°C. The parallel specimens (×) contained, in addition, 0.33 U released GAPDH.

the curve with the pure muscle GAPDH has a more hyperbolic shape ($n_H = 1.3$), the presence of the released GAPDH fraction produces a sigmoidal one ($n_H = 3.3$). The further proteins of the GAPDH fraction and the GAPDH evidently compete for the binding sites of the membrane. This competition is indicated by the shift of the binding curve to higher enzyme concentrations in the solution. It is necessary to use an essentially higher enzyme concentration to get the same saturation value (15.8 U/mg membrane protein) of the enzyme binding in the presence of these proteins, which are not further characterized. If GAPDH and the proteins are added in common (fig.1), the saturation value is only a quarter of the maximal enzyme binding in fig.3 as a result of the competition.

The quantity and the species of released membrane proteins depend on the conditions of hemolysis and release, respectively [11–13]. Therefore, we tried to release GAPDH more specifically by the effector NADH. The dependence of the rebinding of this enzyme fraction time is represented in table 3. Within 20 min the greater part of the GAPDH is rebound and only a small increase of the enzyme activity in the pellet takes place in the following time. Besides the faster kinetics the high degree of rebinding should be noted.

Table 3
Dependance of the rebinding of a specifically released (with 50 μM NADH) GAPDH on the incubation time

Time [min]	0	5	10	20	30	40	60	120
GAPDH in the sediment [U]	0.060	0.167	0.167	0.215	0.231	0.223	0.231	0.247
GAPDH in the supernatant [U]	0.225	0.096	0.078	0.051	0.045	0.046	0.039	0.033
Total activity [U]	0.285	0.263	0.245	0.266	0.276	0.269	0.270	0.280
Percentage of binding %	21	63	68	81	84	83	86	86

Constant amounts of a membrane fraction (0.25 mg protein) prepared by specific GAPDH release were incubated with the same amount of specifically released GAPDH (Materials and methods) for the indicated times.

In this enzyme fraction the concentration of disturbing proteins is so small that its behaviour is similar to that of the pure GAPDH. These investigations demonstrate that the binding of the GAPDH to the membrane matrix in rabbit reticulocytes also depends on peripheral [14] proteins, which can be released by higher ionic strength. These proteins provoke the cooperative interaction of the membrane binding sites and increase the affinity of them to the enzyme. It can be assumed that the reassociation of these peripheral proteins to the matrix involves extensive rearrangements of the membrane structure resulting in the slow kinetics of the rebinding process. For a deeper understanding of the GAPDH-membrane association the interaction of peripheral membrane proteins with the enzyme–membrane complex must be considered.

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