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OUABAIN-SENSITIVE INTERACTION BETWEEN HUMAN RED CELL MEMBRANE AND GLYCOLYTIC ENZYME COMPLEX IN CYTOSOL

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Summary

Binding of 2,3-diphosphoglycerate to monophosphoglycerate mutase, of which it is an obligatory cofactor, causes changes in the resonance positions of the ^{31}P nuclear magnetic resonance spectra of both phosphate groups. It had previously been shown that these resonances shift when other glycolytic enzymes, such as phosphoglycerate kinase, are added to form the 2,3-diphosphoglycerate · monophosphoglycerate mutase · phosphoglycerate kinase complex. In view of this association, we have examined the set of glycolytic enzymes from aldolase to pyruvate kinase and found evidence of direct communication between all of these enzymes. A multi-enzyme complex of $1—2 \cdot 10^6$ daltons has been separated from broken cell ghosts by Biogel column filtration and evidence has been presented to show that this complex exhibits aldolase, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase activity. The glycolytic multi-enzyme complex interacts with the outer face of inside-out vesicles prepared from human red cells and the interaction is suppressed by application of 10^{-6} M ouabain to the inner face of these vesicles. These studies show that the conformation of the enzymes comprising the megadalton complex are responsive to the application of ouabain to the outer red cell membrane surface.

Introduction

In a recent communication from this laboratory [1] we have put forward a hypothesis that the Mg-dependent ($Na^+ + K^+$)-ATPase used for the cation transport in the human red cell is linked through phosphoglycerate kinase (EC 2.7.2.3) to monophosphoglycerate mutase (EC 2.7.5.3). In the course of this investigation we found that glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) also could interact with the 2,3-diphosphoglycerate · mono-

phosphoglycerate mutase · phosphoglycerate kinase complex. In view of this association, we have examined the set of glycolytic enzymes stretching from aldolase (EC 4.1.2.13) through pyruvate kinase (EC 2.7.1.40) and found evidence of indirect communication between all of these enzymes. Furthermore, we have found that the red cell cytoplasmic glycolytic complex interacts with the inner membrane surface, and have shown that the conformation of the enzymes comprising the complex is responsive to the application of ouabain to the outer membrane surface.

Experimental methods

Nuclear magnetic resonance (NMR) experiments were either carried out as previously described [1] on a JEOL FX-60 Fourier transform NMR spectrometer (JEOL Analytical Instruments, Inc., Cranford, N.J.) operating at 24.16 MHz for ^{31}P, or on a Bruker HX-270 Fourier transform spectrometer (Bruker Instruments, Inc., Billerica, Mass.) operating at 109.3 MHz for ^{31}P in 10 mm (outer diameter) sample tubes. The sample was maintained at 25°C. The 90° resonance frequency pulse width was 21 μs. Pulse angles of 45°—60° were routinely used. Spectral widths of 3 KHz were employed with 8K data tables. The signal to noise ratio was improved by digital filtering (1.5 Hz line broadening). Broad band noise-modulated proton decoupling (2 KHz at 1.5 W) was used. In all cases, the spectrometer was locked on the deuterium signal of $^{2}H_2O$.

Experimental reproducibility is highly critical in our experiments. The normal half peak width at 109.3 MHz for the 2 phosphate of 2,3-diphosphoglycerate is 4.5 Hz and that for the 3 phosphate is also 4.5 Hz. We were able to place 6 data points over each peak and one data point clearly defined the maximum. Hence the resonance position was reproducible to 0.75 Hz on the HX-270. The presence of paramagnetic impurities can clearly be seen by peak broadening. The extensive dialysis used in the preparation of enzymes described below served to eliminate paramagnetic impurities and no broadening was observed, thus indicating that our dialysis procedure had been effective. Furthermore, 0.1 mM EDTA was occasionally added to the buffer in these experiments. The absence of any subsequent change in half-width confirmed that the preparations were free of paramagnetic impurities.

Multiple determinations of chemical shift values in the same sample were reproducible to one data point. However, when samples were prepared from different stock solutions on different occasions the mean values could differ by 1.75 Hz. Hence, the data in the tables are reported as differences from reference solutions measured at the same time and under the same conditions.

Virtually all the experiments reported in Tables I—IV were initially carried out on the JEOL FX-60 at 24.16 MHz for ^{31}P. At this frequency the resonance shifts observed are small, only 22% of those at 109.3 MHz. We felt it desirable to increase the observed frequency shifts and therefore all the experiments were repeated at 109.3 MHz to yield the results given in the tables. In each instance, the resonance shift increased appropriately for the higher frequency, thus indicating that the results were consistent on two different spectrometers in experiments carried out at different times.

All enzymes and phosphorus-containing molecules were purchased from

Sigma Chemical Co., St. Louis, Mo. and were dissolved or diluted to desired concentrations and then dialyzed for 3 × 3 h against the buffer in which the NMR experiments were to be conducted. The NMR buffer had the following composition (mM): NaCl, 150/KCl, 5/Tris, 17/20% ^2H_2O, pH 7.40. The pH of this NMR buffer containing 17 mM Tris was adjusted to 7.40 by titration with HCl for each experiment. In several instances pH was checked at the close of the experimental procedure and found to be unchanged. All the commercial enzymes were prepared from muscle, except phosphoglycerate kinase which was made from yeast. As previously discussed [1], preliminary experiments on human red cell phosphoglycerate kinase and monophosphoglycerate mutase show NMR resonance shifts similar to those of commercially available enzymes.

Inside-out and right side-out vesicles were prepared according to the technique of Steck and Kant [2] from bank blood, out-dated by no more than three days. As previously reported [1] this technique, in our hands, yields right side out vesicles about 97% pure and inside-out vesicles about 85% pure, measured by assay of acetylcholinesterase as an outside surface marker and glyceraldehyde 3-phosphate dehydrogenase as an inside marker. When inside-out vesicles were prepared to contain ouabain, the concentration was 10^{-6} M, unless otherwise specified, and the ouabain was added to the 0.5P8 vesiculation buffer (buffer notations as in ref. 2) but there were no other modifications to the preparation procedure.

Pink ghosts (partially washed so that some hemoglobin remains) were prepared following the initial steps in the procedure used to prepare vesicles, but the procedure was terminated after two washes in the 5P8 lysing buffer. The ghosts were then frozen and thawed through three cycles and added to a 120 × 2.5 cm Sepharose 6B column equilibrated with phosphate-buffered saline supplemented with 0.6 mM B mercaptoethanol. Fractionation was modeled after a similar experiment of Mowbray and Moses [3] on a similar complex from *Escherichia coli.*

Results and Discussion

Combination of glycolytic enzymes in cellular complex. Experiments were carried out with individual enzymes in the glycolytic chain to see whether combination of the enzymes, one at the time, with 2,3-diphosphoglycerate, produced any interactions, as measured by resonance shifts. Table I shows that the resonance shifts of both the 2-phosphate and the 3-phosphate were negligible at 109.3 MHz, except in the case of monophosphoglycerate mutase for which resonance shifts arise from 2,3-diphosphoglycerate binding to the enzyme as an obligatory cofactor. The next sets of experiments were carried out (at 109.3 MHz) by adding the enzymes sequentially until all six had been brought together, as shown in Table II. In the top portion of the table, the reference signal for the 2,3-diphosphoglycerate · monophosphoglycerate mutase complex was set at zero and the addition of phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase were found to produce resonance shifts, increasing with the addition of each enzyme. These results are in agreement with our previous report [1] and show that the environment of the 2,3-diphosphoglycerate, when complexed with monophosphoglycerate mutase is changed by the addition of

TABLE I

RESONANCE SHIFTS OF 2,3-DIPHOSPHOGLYCERATE IN SOLUTION IN PRESENCE OF SINGLE ENZYMES

All data in this and succeeding tables are averages of two experiments. The sign convention for the resonance shifts is that of the Bruker HX-270. A positive resonance shift corresponds to an increase in frequency.

Enzyme concn. (mg/ml)	2,3-Diphosphoglycerate (5 mM) + magnesium (5 mM)	Aldolase	Glyceraldehyde 3-phosphate dehydrogenase	Phosphoglycerate kinase	Monophosphoglycerate mutase	Enolase	Pyruvate kinase	Resonance shift	
								2-Phosphate (Δ Hz)	3-Phosphate (Δ Hz)
—	X							0.0 (reference)	0.0 (reference)
0.25	X	X						0.0	0.1
0.1	X		X					0.2	0.2
0.6	X			X				−0.2	−0.2
0.25	X				X			0.9	1.0
0.25	X					X		0.0	0.0
0.25	X						X	0.0	0.3

TABLE II

RESONANCE SHIFTS OF 2,3-DIPHOSPHOGLYCERATE IN SOLUTION IN PRESENCE OF SEVERAL ENZYMES

2,3-DPG, diphosphoglycerate; MPGM, monophosphoglycerate mutase; PGK, phosphoglycerate kinase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase

2,3-Diphosphoglycerate (5 mM) + magnesium (5 mM)	Aldolase	Glyceraldehyde 3-phosphate dehydrogenase	Phosphoglycerate kinase	Monophosphoglycerate mutase	Enolase	Pyruvate kinase	Resonance shift	
							2-Phosphate (Δ Hz)	3-Phosphate (Δ Hz)
Reference: 2,3-DPG · MPGM								
X				X			0.0 (reference)	0.0 (reference)
X			X	X			9.0	8.3
X		X	X	X			10.3	12.0
Reference: 2,3-DPG · MPGM · PGK · G3PDH								
X		X	X	X			0.0 (reference)	0.0 (reference)
X	X	X	X	X			1.5	0.6
X	X	X	X	X	X		1.9	1.1
X	X	X	X	X	X	X	2.5	2.0

each of the other two enzymes, thus indicating that conformational changes are transmitted between these three members of the glycolytic cycle *.

For the additional experiments shown in the bottom portion of Table II, the 2,3-diphosphoglycerate · monophosphoglycerate mutase · phosphoglycerate kinase · glyceraldehyde 3-phosphate dehydrogenase complex served as reference. Table II shows that the addition of aldolase causes an additional resonance shift, as do further additions of enolase (EC 4.2.1.11) and pyruvate kinase. These experiments indicate that there is a spontaneous association of all six of these enzymes in free solution to form a complex. The stability of the multi-enzyme complex was shown in an exploratory experiment using a Sephadex G-50 column. An equimolar mixture of enzymes was prepared (based on 10 mg/ml aldolase) containing: aldolase/glyceraldehyde 3-phosphate dehydrogenase/phosphoglycerate kinase/monophosphoglycerate mutase/enolase/pyruvate kinase. All protein was eluted in the void volume of the column. This means that less than 0.05 mg/ml of each protein remained unassociated; instead, the enzymes were combined in a stable complex, or complexes, with molecular weight greater than 200 000—400 000.

While these studies were in progress, our attention was called to a recent article by Mowbray and Moses [3] which presents evidence for a multi-enzyme complex of $1.6 \cdot 10^6$ daltons that comprises one copy each of all the glycolytic enzymes in *E. coli*, reaching the whole way from hexokinase through pyruvate kinase. Arese et al. [4] showed that the glycolytic enzymes remaining in white ghosts (washed so that all hemoglobin was removed) were able to synthesize 2,3-diphosphoglycerate from fructose 1,6-diphosphate and also to produce adenosine triphosphate and 3-phosphoglycerate. As some of the enzymes involved, particularly phosphoglycerate kinase, were thought to be bound to the membrane relatively loosely, the extensive washing required to produce white ghosts should have removed them. One possible explanation for the ability of these ghosts to synthesize 2,3-diphosphoglycerate would be that the glycolytic enzymes had been present in a multi-enzyme complex too large to escape through the apertures from which hemoglobin had diffused freely.

Following this line of reasoning, pink ghosts could be considered to be a rough sieve which could have retained much of the multi-enzyme complex, while allowing escape of the smaller hemoglobin molecule. Accordingly, we used pink ghosts as a starting material from which to attempt to isolate the

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* In fast exchange, as pertains to the present NMR experiments, the observed resonance frequency is the mean between the resonance frequency of the free and the bound form of the substrate. At 24.16 MHz, Fossel and Solomon [1] observed a shift of 0.2 Hz when 5 mM 2,3-diphosphoglycerate is bound to 0.25 mg/ml of monophosphoglycerate mutase in the presence of 5 mM Mg. Assuming that one molecule of 2,3-diphosphoglycerate mutase is bound to each monomer of monophosphoglycerate mutase we can compute that binding causes a shift of 110 Hz, equivalent to 495 Hz at 109.3 MHz. This is about what could be expected on the basis of an ionization change. When phosphoglycerate kinase was added to form the 2,3-diphosphoglycerate · monophosphoglycerate mutase · phosphoglycerate kinase complex, the observed shift increased to 2.3 Hz. This is equivalent to a resonance frequency shift of 5850 Hz at 109.3 MHz (54 ppm) when the complex is formed, which could be consistent with a change in the O-P-O bond angle (see discussion in ref. 5). A resonance shift of 5—10 Hz is, therefore, approximately equivalent to the binding of 0.1—0.2% of the 2,3-diphosphoglycerate. In Table III, the concentration of monophosphoglycerate mutase used was 0.25 mg/ml (M_r of dimer = 57 000). A resonance shift of 5 Hz is, therefore, roughly equivalent to the binding of 0.6 molecules of 2,3-diphosphoglycerate for each monophosphoglycerate mutase monomer.

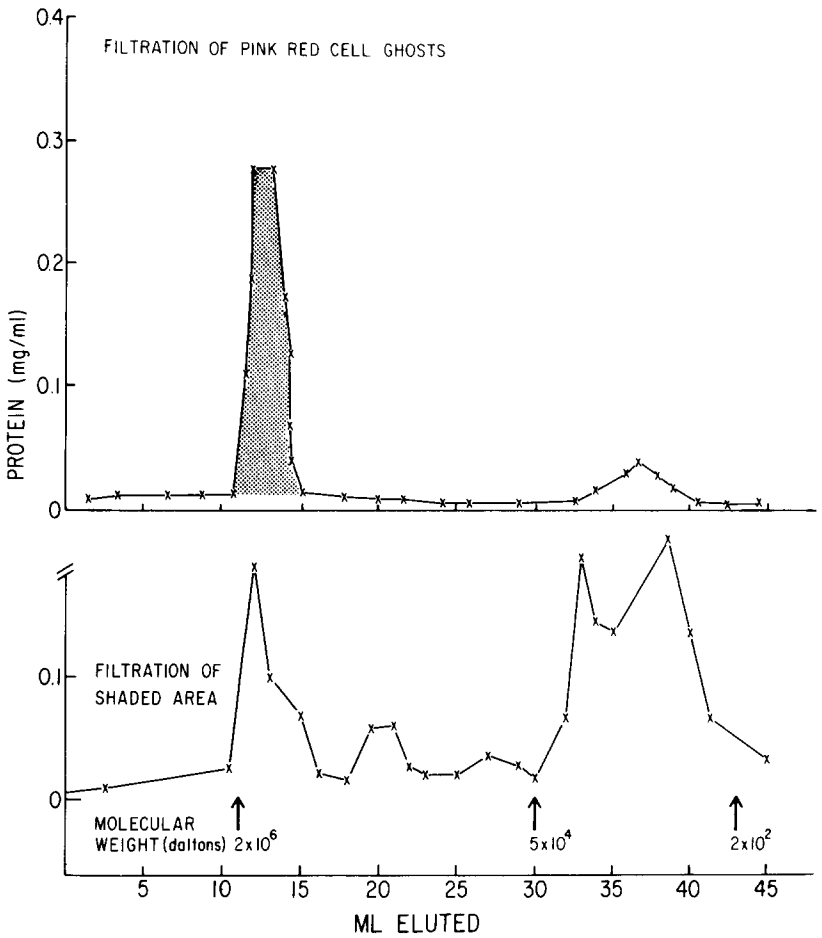

Fig. 1. Filtration of pink red cell ghosts on Biogel A-5M column. The molecular weight markers were blue dextran (2000 000), bovine serum albumin (68 000) and dinitrophenylalanine (200).

multi-enzyme complex. Following the technique of Mowbray and Moses [3], we passed broken pink ghosts through a Biogel A-5M column. The elution pattern is shown in Fig 1 in which a peak in the range of $0.5—2 \cdot 10^6$ daltons can be seen. The material in this peak was then separated and rechromatographed on the same column as shown in the bottom of Fig. 1. The megadalton peak can be seen together with a broad peak in the range of $0.1—5 \cdot 10^4$ daltons which could contain the separate enzymes from which the megadalton peak was assembled. These data would be consistent with an equilibrium between the megadalton complex and its constituents, very similar to that found by Mowbray and Moses [3] in *E. coli*. Thus, it would seem that the association between the glycolytic enzymes that we have found with separated enzymes, as shown in Table II, can also exist in the native red cell, or at least in ghosts prepared from the cells.

It would be possible for the mechanism of ghost preparation to have induced the complex formation in some manner. This possibility was obviated by

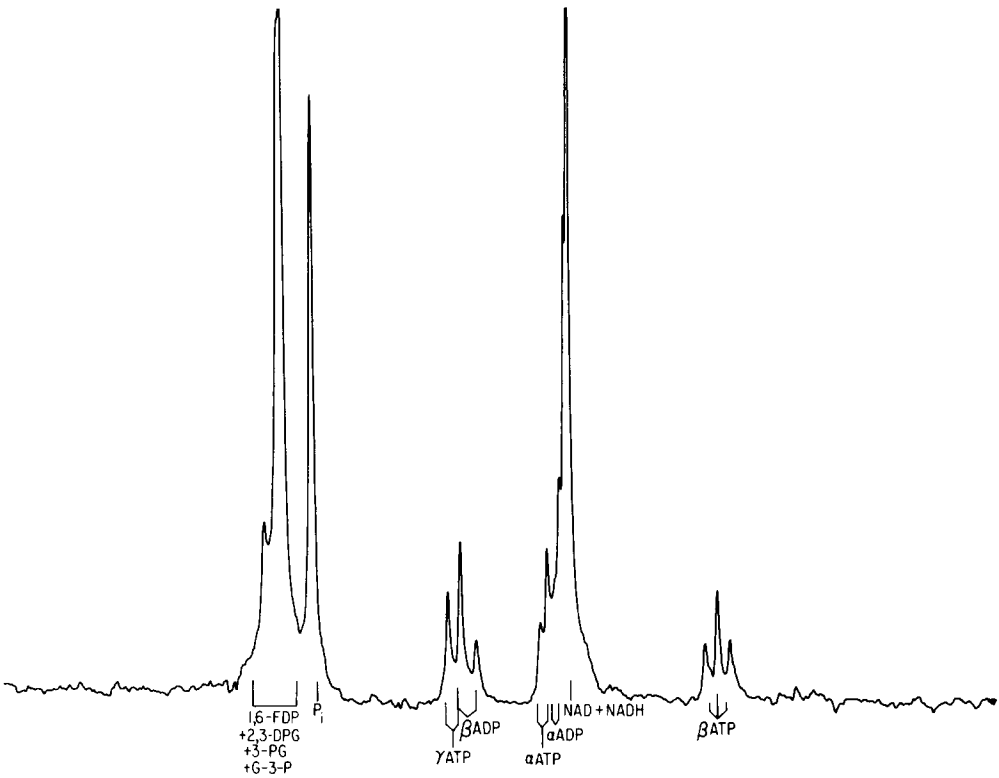

Fig. 2. Resonances of adenosine triphosphate produced from fructose 1,6-diphosphate (1,6-FDP) by megadalton complex prepared from pink red cell ghosts. The spectrum results from the accumulation of 2500 free induction decays from 8 μs pulses (36°) with repetition times of 1.0 s at 24.16 MHz. The resonances were identified by comparison of the chemical shifts with those of pure samples of the phosphate-containing molecules. Adenosine triphosphate production was measured by comparison of the area of the resonance of the β-phosphate of adenosine triphosphate with that from a standard solution of adenosine triphosphate run under the same conditions. 2,3-DPG, 2,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate; G-3-P, glyceraldehyde 3-phosphate.

carrying out a similar column filtration using broken red cells as the starting material rather than ghosts. In this case, the megadalton peak appeared in the same fractions as indicated in Fig. 1, and was dissociated into equilibrium with its components on rechromatography just as the complex prepared from pink ghosts.

These experiments show that there is a complex of the order of 10^6 daltons present in the human red cell, but they do not prove that the complex contains glycolytic enzymes. Accordingly, we carried out an experiment to demonstrate that glycolytic enzymes were present in the megadalton complex and could produce adenosine triphosphate from fructose 1,6-diphosphate. The enzyme complex was isolated from pink ghosts by Biogel column filtration and 0.03 mg protein were added to 2 ml of NMR buffer, to which had been added (mM): fructose 1,6-diphosphate, 10/nicotinamide adenine denucleotide, 10/adenosine diphosphate, 10/magnesium, 5/inorganic phosphate, 10. The production of adenosine triphosphate was measured by appearance of the ^{31}P phosphate peaks in the NMR signal as shown in Fig. 2. In 4 h, 10 mM

adenosine triphosphate was formed. When the initial protein concentration in the enzyme complex was increased to 0.06 mg, the adenosine triphosphate formation was complete at 10 mM in approximately 2 h. Similar results were obtained when the starting material was broken pink ghosts, rather than the enzyme complex isolated from the ghosts. In order to produce adenosine triphosphate from fructose 1,6-diphosphate, aldolase, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase are required. Hence, these experiments prove that these three enzymes elute from the Biogel column in the megadalton position.

Mowbray and Moses [3] estimated that an equimolar complex of all the enzymes of glycolysis would have a molecular weight of $1.6 \cdot 10^6$ and they presented evidence that all the enzymes from hexokinase through pyruvate kinase were present in their complex. In the case of the human red cell, published evidence (see ref. 5) shows that the glycolytic enzymes from aldolase to monophosphoglycerate mutase are present in concentrations between 10^4 and $2.7 \cdot 10^5$ copies/cell, based largely on indirect estimates which are not necessarily reliable. 10^5 copies/cell would equal $2.6 \cdot 10^{-13}$ g, 0.26% of the cell weight. If the entire megadalton complex were retained in the membrane fraction prepared for estimation of membrane protein content (see Guidotti review [6]), the megadalton complex could comprise up to 40% of the membrane protein. However, many of the cytoplasmic enzymes are loosely bound to the membrane (see ref. 5) and would probably have been removed in the protein isolation procedure so that 40% is a maximum on an order of magnitude scale. Nonetheless, these calculations serve to show that 10^5 copies/cell of the megadalton complex would not occupy an inordinate fraction of cell and membrane.

Interaction of enzyme complex with red cell membrane. Inside out vesicles were used to examine the interactions of the enzyme complex with the red cell membrane. Such interactions were to be expected in view of the binding sites for glyceraldehyde 3-phosphate dehydrogenase [7,8] and aldolase [9] on the cytoplasmic face of the membrane protein, band 3. Furthermore, De and Kirtley [10] have found a membrane-binding site for phosphoglycerate kinase. We have previously shown [1] that NMR shifts occur when inside-out vesicles are added to glyceraldehyde 3-phosphate dehydrogenase and the immediately contiguous enzymes in the glycolytic chain.

The resonance shifts (at 109.3 MHz) on adding inside-out vesicles to all the glycolytic enzymes from triose phosphate isomerase to pyruvate kinase are shown in Table III in the columns headed "Normal". As the top section of the table shows, when the number of enzymes in the combination is increased sequentially, going upstream from phosphoglycerate kinase to triose phosphate isomerase, increasing resonance shifts are induced in both phosphates of 2,3-diphosphoglycerate. Similarly, the bottom section of the table shows that the addition of enolase and pyruvate kinase also causes progressive increases in the resonance shifts. In the presence of inside out vesicles, the resonance shifts are negative as contrasted to the positive shifts when the enzymes are combined in the absence of vesicles (Table II). These experiments in Table III not only confirm that all the enzymes in this sequence interact with one another, but also that this interaction is modulated by the cell membrane.

As previously stated [1], inside-out vesicles can be considered as sided preparations of $(Na^+ + K^+)$-ATPase, with an ouabain site on the inside of the vesicle and the phosphorylation site on the outside. We have used inside-out vesicles prepared with 10^{-6} M ouabain on the inside to study interactions between the $(Na^+ + K^+)$-ATPase and the glycolytic enzyme complex. The columns marked "+ Ouabain" in Table III show that ouabain virtually abolished the conformation change causing the 2,3-diphosphoglycerate resonance shift and suppressed all evidence of interaction between the members of the glycolytic enzyme complex and the inside-out vesicle.

We have previously presented evidence [1] that the interactions of phosphoglycerate mutase with vesicles were confined to the cytoplasmic face of the membrane. Control experiments showed no resonance shifts when right side-out vesicles were substituted for inside-out vesicles. Addition of ouabain to either side of the right side-out vesicle membrane was without effect in these experiments.

In order to make sure that the ouabain inhibition could not be attributed to non-specific ouabain adsorption by the red cell surface, control experiments were carried out in which the inside-out vesicles were prepared from human red cells which had been washed * three times in ouabain-free buffer in order to remove loosely adherent ouabain. These measurements were carried out at 24.16 MHz so that the resonance shifts are less than those shown in Table III (measured at 109.3 MHz). Addition of inside-out vesicles to 2,3-diphosphoglycerate · monophosphoglycerate mutase · phosphoglycerate kinase produced resonance shifts of —1.8 Hz for the 2 phosphate and —1.3 Hz for the 3 phosphate. When the inside-out vesicles were prepared from red cells, exposed to 10^{-6} M ouabain and then washed as described above, the resonance shifts were reduced to —0.2 Hz for each phosphate in agreement with the results in Table III. Hence, the effects of ouabain shown in Table III are not to be attributed to binding of ouabain to non-specific sites. These experiments lead to two conclusions. They substantiate our previous finding that interactions with the $(Na^+ + K^+)$-ATPase on one side of the membrane can be transmitted to glycolytic enzymes on the other face and indicate that a signal transmitted to one or more members of the glycolytic complex is readily transferred to all the other members. This supports the view that the red cell membrane and the cytoplasmic glycolytic enzymes are attached to one another by a linkage so tight that a conformational change in one constituent can be passed to all the other enzymes in the complex.

Order of enzyme addition. The experiments reported in Tables II and III indicate that the enzymes are capable of self assembly into the multi-enzyme complex. In order to obtain information about the specificity of the assembly process, we carried out two experiments at 24.16 MHz in which we altered the order of addition of enolase and pyruvate kinase to inside-out vesicles in the presence of 2,3-diphosphoglycerate, magnesium, phosphoglycerate kinase and monophosphoglycerate mutase. When the enzymes were added in

* In Hoffman's [11] quantitative measurement of ouabain binding to the cation transport linked receptor in human red cell membranes, he washed the cells to remove loosely adherent ouabain but did not specify any number of washes beyond one. Gardner and Conlon's [12] results indicate that all the non-specifically-bound ouabain had been removed from human red cells after 5 washes with isosmotic saline.

TABLE III

RESONANCE SHIFTS OF 2,3-DIPHOSPHOGLYCERATE IN PRESENCE OF INSIDE-OUT VESICLES

400 μl of inside-out vesicles were used in each of these experiments and those in Table IV. This amount corresponds to 0.66 ± 0.14 (S.D., 5 determinations) units of acetyl cholinesterase activity (approx. 0.22 mg protein) and is the yield from 800 μl of washed red cells.

Triose phosphate isomerase *	Aldolase	Glyceraldehyde 3-phosphate dehydrogenase	2,3-Diphospho-glycerate + Magnesium + Phosphoglycerate kinase + Monophosphoglycerate mutase	Enolase	Pyruvate kinase	Resonance shift after IOV addition			
						2-Phosphate		3-Phosphate	
						Normal (Δ Hz)	+Ouabain (Δ Hz)	Normal (Δ Hz)	+Ouabain (Δ Hz)
			X			−5.8	−0.2	−3.6	−0.3
		X	X			−6.2	−0.2	−4.5	−0.2
	X	X	X			−7.1	−0.4	−5.7	−0.4
X	X	X	X			−7.6	−0.4	−6.0	−0.4
			X			−5.4	−0.3	−3.9	−0.3
			X	X		−6.3	−0.3	−4.6	−0.3
			X	X	X	−7.1		−5.0	

* Triose phosphate isomerase concn. 0.25 mg/ml

TABLE IV

EFFECT OF ORDER OF ENZYME ADDITION ON 2,3-DIPHOSPHOGLYCERATE RESONANCES

Time after enzyme addition (min)	Aldolase	Glyceraldehyde 3-phosphate dehydrogenase	2,3-Diphospho-glycerate + Magnesium + Phosphoglycerate kinase + Monophosphoglycerate mutase	Enolase	Pyruvate kinase	Resonance shift after IOV addition	
						2-Phosphate (Δ Hz)	3-Phosphate (Δ Hz)
G3PDH addition							
	X		X			−6.0	−4.7
0	X	X	X			−7.1	−5.6
30	X	X	X			−7.1	−5.7
Enolase addition							
			X		X	−5.5	−3.7
0			X	X	X	−5.7	3.7
30			X	X	X	−7.0	−5.0

the order enolase followed by pyruvate kinase, the resonance shifts were —0.6 Hz for the 2 phosphate and —0.3 Hz for the 3 phosphate. However, if pyruvate kinase were added first, followed by enolase, there was zero resonance shift during the first 10 min. After 30 min, the resonance shifts returned to —0.7 Hz and —0.3 Hz, respectively.

A variant of this experiment was designed in order to examine the phenomenon at 109.3 MHz. In these experiments, whose results are shown in the bottom section of Table IV, enolase and pyruvate kinase were added simultaneously to magnesium · 2,3-diphosphoglycerate · monophosphoglycerate mutase · phosphoglycerate kinase just prior to the addition of inside-out vesicles. Under these conditions, there was no effective resonance shift as shown by comparing lines 4 and 5 of Table IV. However, when 30 min had elapsed, the resonance shift had reappeared, as shown in the last line of the table. The resonance shifts are identical with those in the last line of Table III in which the enzymes were added in sequential order. These experiments, together with those in the paragraph above, not only indicate that enolase is required for pyruvate kinase to bind to its appropriate site in the complex, but also suggest that pyruvate kinase can bind in an alternative way which blocks the enolase site in the multi-enzyme complex. If we assume the complex to be in equilibrium with its constituents, it would seem reasonable both for pyruvate kinase and enolase to move on and off their respective sites. The free energy in the system reaches its minimum when the correct enzyme is on the correct site so that the proper configuration is achieved after sufficient time has elapsed. However, the energy difference must be relatively small to account for the ability of pyruvate kinase to inhibit the enolase interaction.

We were interested to see whether the order-of-addition effect was a general one that applied to many of the enzymes concerned. Consequently, we carried out a similar experiment to look for interactions between the aldolase and glyceraldehyde 3-phosphate dehydrogenase sites. However, no such interaction was observed, as shown in the top half of Table IV, in which it can be seen that there is no difference between the immediate effect of adding glyceraldehyde 3-phosphate dehydrogenase and that after 30 min. This is in contrast to the results of Strapazon (quoted in ref. 13) who reported that glyceraldehyde 3-phosphate dehydrogenase can block aldolase binding to the band 3 dimer. Our experiments indicate that the addition of aldolase after glyceraldehyde 3-phosphate dehydrogenase produces the same effect as when the order of addition is reversed. However, our experiments were carried out with inside-out vesicles in the presence of phosphoglycerate kinase, monophosphoglycerate mutase and 2,3-diphosphoglycerate, so that the conditions are not comparable to those of Strapazon.

Although aggregation of the red cell glycolytic enzymes in a membrane related multi-enzyme complex was, to us, unexpected, there are many biological examples of similar combinations of enzymes with related functions. Thus, in addition to Mowbray and Moses [3] finding of a glycolytic enzyme complex in *E. coli*, Mori et al. [14] have shown that the first three enzymes catalyzing the initial steps of pyrimidine biosynthesis in rat liver are present in a multi-enzyme complex in the cytosol. Mori et al. were able to dissociate the complex, but were unable to reassociate the separated components. Bates et al.

[15] have studied the properties of the pyruvate dehydrogenase multi-enzyme complex of *E. coli.* The complex consists of multiple copies of three different polypeptide chains responsible for three different enzyme activities. The catalytic activity is dependent upon the state of assembly, and the complex can self-assemble. It has a molecular weight of $8 \cdot 10^6$ when fully assembled. Meyer et al. [16] isolated a muscle glycogen particle from rabbit muscle. Though they were primarily interested in the phosphorylase, phosphorylase kinase and phophatase activities of this particle, they also observed that it contained all the enzymes of glycolysis, being able to produce lactic acid from glycogen in the presence of the proper cofactors.

Recently, Saks et al. [17] have shown that ouabain, interacting with (Na^+ + K^+)-ATPase on the outer face of the myocardial cell membrane, inhibits the intracellular activity of membrane-bound creatine phosphokinase. Many other examples of the modulation of intracellular activity by substances applied to the extracellular face of a membrane (and vice versa) have been discussed in Nicolson's review [18] so that our observation that the action of ouabain on one face of the membrane is reflected in conformational changes of the enzymes inside the cell is not without precedent.

Organization in the human red cell is at a very high level. At least seven, and probably all, of the glycolytic enzymes in the red cell are constituents of a multi-enzyme complex which is functionally linked to the membrane. Furthermore, the conformational changes induced when glycolytic enzymes are added to inside-out vesicles are entirely inhibited by the action of ouabain on the other face of the vesicle. These observations, taken together, indicate that the systems we are studying lie at the heart of the coupling between cation transport and its associated energy supply.

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