Biochimica et Biophysica Acta, 464 (1977) 82—92

BBA 77568

MEMBRANE MEDIATED LINK BETWEEN ION TRANSPORT AND METABOLISM IN HUMAN RED CELLS

E.T. FOSSEL and A.K. SOLOMON

Biophysical Laboratory and Department of Biological Chemistry, Harvard Medical School, 25 Shattuck Street, Boston, Mass. 02115 (U.S.A.)

(Received June 18th, 1976)

Summary

When 10^{-6} M oubain is added to human red cells that have been incubated without glucose for two hours, there is a significant shift in the ^{31}P nuclear magnetic resonances of both phosphate groups of cellular 2,3-diphosphoglycerate, which is not found in control cells incubated with glucose. This means that an effect induced by ouabain on the outside of the red cell membrane is transmitted through the membrane to alter the environment of an intracellular metabolite. Experiments with glycolytic cycle inhibitors have indicated that the intracellular ligand responsible for the resonance shifts is monophosphoglycerate mutase which requires 2,3-diphosphoglycerate as a cofactor for the reaction it catalyzes. To account for this finding a hypothesis is presented that the $(Na^+ + K^+)$-ATPase in human red cells is linked to monophosphoglycerate mutase through the agency of phosphoglycerate kinase. Evidence is presented for the existence of phosphoglycerate kinase/monophosphoglycerate mutase in solution. It is shown that this complex can interact with the cytoplasmic face of $(Na^+ + K^+)$-ATPase at the outside surface of inside out red cell vesicles, and that this interaction is inhibited when 10^{-6} M ouabain is contained within the vesicle. Neither monophosphoglycerate mutase nor phosphoglycerate kinase is significantly bound to the inside surface of the intact human red cell, but glyceraldehyde 3-phosphate dehydrogenase is; it is shown that this enzyme also interacts with the cytoplasmic face of the $(Na^+ + K^+)$-ATPase and that the interaction is inhibited by 10^{-6} M ouabain.

---

^{31}P nuclear magnetic resonance (NMR) was used by Heustis and Raftery [1], Moon and Richards [2] and Henderson et al. [3] in studies of 2,3-diphosphoglycerate in whole red cells and hemolysates. More recently Fossel and Solomon [4] used ^{31}P NMR in dog red cells to show that the molecular environment of 2,3-diphosphoglycerate could be affected by small perturbations in membrane

shape; in this species cell volume had previously been shown [5—8] to produce dramatic alterations in Na^+ flux. Fossel and Solomon were unable to specify the ligand responsible for the observed changes in the 2,3-diphosphoglycerate NMR resonances, although they did rule out a number of the more obvious possibilities such as hemoglobin binding and intracellular pH. In order to carry these studies further, we have turned to human red cells in which, unlike the dog, cardiac glycosides have specific effects on the ion transport process. The high concentration of 2,3-diphosphoglycerate normally found in human red cells (4.3 mM) [9] and the pattern of its two NMR resonances make this substrate a very useful probe of the metabolic process in the living cell, and we have utilized it to examine the link between metabolism and K^+ transport in these cells.

Experimental methods

The NMR experiments were carried out either on a Varian XL-100-15 Fourier transform spectrometer (Varian, Inc., Palo Alto, Calif.) operating at 40.5 MHz for ^{31}P or on a JEOL FX-60 Fourier transform spectrometer (JEOL Analytical Instruments, Inc., Cranford, N.J.) operating at 24.16 MHz for ^{31}P. The sample temperature was 25—26° C. 90° rf pulses were routinely used in the whole cell experiments. On the XL-100-15 the 90° pulse was 32 μs and on the FX-60 it was 20 μs. For the in vitro experiments the pulse angles were 45°—60°. Typically, data accumulations of 8—15 min were required for the observation of 2,3-diphosphoglycerate in whole cells; the times were proportional to 2,3-diphosphoglycerate concentration for in vitro experiments. Digital filtering was applied to the accumulated free induction decay to improve signal to noise ratios. Broad band noise modulated proton decoupling (1500 Hz at 3 W on the XL-100-15 and 1000 Hz at 12.5 W on the FX-60) was used. In all cases the spectrometer was locked on the deuterium signal of ^2H_2O. Resonance peak shifts are given in Hz; they have all been converted to equivalent values at 24.16 MHz, the frequency used for almost all the experiments.

Whole red cells were observed at 50% hematocrit suspended in the following NMR buffer (mM): NaCl, 150; KCl, 5; Tris, 17 plus 20% ^2H_2O, pH 7.40. In experiments with solutions containing enzymes alone, 0.1 mM EDTA was frequently added to the buffer. Concentrated enzyme solutions were dialyzed against NMR buffer prior to use. Solutions of 2,3-diphosphoglycerate, adenosine triphosphate, glyceraldehyde 3-phosphate, and β,γ-methylene-adenosine triphosphate were prepared in this buffer and pH was re-adjusted to 7.40. Mg (usually 5 mM) was added in some cases, as noted in the descriptions of individual experiments.

Isotope flux experiments were carried out at 37° C as described by Poznansky and Solomon [10] except that ^{86}Rb (New England Nuclear Co., Boston, Mass.) was used instead of ^{42}K. Inside out vesicles and right side out vesicles were produced from dated bank blood by the method of Steck and Kant [11].

All enzymes and phosphorus containing molecules were purchased from Sigma Chemical Co., St. Louis, Mo.

Experimental reproducibility is highly critical in our experiments as some of the reported chemical shift changes are small. Multiple determinations of

chemical shift values on the same sample were reproducible to one computer data point (usually one data point equaled 0.25 Hz). This extremely good reproducibility is due in part to the fact that the peaks measured were very sharp, so that we were able to place 12—25 data points over the upper half of the peak and one data point clearly defined the maximum (see Fig. 3). The reproducibility is made evident by the right side out vesicle experiment reported in Table III. 600 μl of right side out vesicles were added in 200 μl increments to 5 mM, β,γ-methylene-adenosine triphosphate. The chemical shift change for each increment was less than one data point (0.25 Hz) and totalled 0.0 Hz after all three increments had been added. However, differences between stock solutions made up on separate occasions varied as much as ±1 Hz, and reached 2—4 Hz for the chemical shift values of 2,3-diphosphoglycerate in red cell samples of different origin. In order to avoid difficulties on this score, each experiment began with measurements of a control sample to which no reagents were added. All differences were expressed relative to the control in each experiment, so that the reproducibility of 0.25 Hz under these conditions applies to the results reported in the tables.

Results and Discussion

Effect of ouabain on 2,3-diphosphoglycerate NMR resonances

When ouabain, a cardiac glycoside inhibitor of cation transport, is added to human red cells at a concentration of 10^{-6} M, the cation flux is reduced by about 80%, but there is only a small effect on the ^{31}P NMR spectrum of 2,3-diphosphoglycerate as shown in the top two lines of Table I. However, when human red cells are incubated in the absence of glucose for 2 h at 37°C to remove all the glycolytic intermediates above the triose phosphate level [12], 10^{-6} M ouabain not only reduces cation flux by 80%, but also causes a shift of 1.6 Hz in the ^{31}P NMR resonance of 2,3-diphosphoglycerate, as shown in the next two lines in Table I. 2,3-Diphosphoglycerate is known to be a cytoplasmic component and the cells were washed twice before suspension in NMR buffer, so that these resonance shifts result from intracellular interactions, caused by an action on the outside face of the membrane where the ouabain site is

TABLE I

RESONANCE SHIFTS OF 2,3-DIPHOSPHOGLYCERATE IN HUMAN RED CELLS

	Resonance shift	
	2 Phosphate Δ Hz	3 Phosphate Δ Hz
Red cells (control)	0.0	0.0
$+10^{-6}$ M ouabain (3)	+0.2	+0.2
Substrate depleted red cells (control)	0.0	0.0
$+10^{-6}$ M ouabain (4) *	−1.6 ± 0.4	−1.7 ± 0.5
$+10^{-2}$ M sulfate (3)	−0.5	−0.1
$+8 \cdot 10^{-3}$ M fluoride (2)	+2.5	+0.6

* Errors are S.E.M. Number of experiments is given in parenthesis.

located. Hence, these experiments indicate that the configurational shift in the $(Na^+ + K^+)$-ATPase induced by ouabain (see Kyte [13]) is transmitted through the membrane to affect the environment of intracellular 2,3-diphosphoglycerate. Since 2,3-diphosphoglycerate is neither a substrate nor a co-factor for the $(Na^+ + K^+)$-ATPase reaction, other steps in the metabolic cycle are also necessarily involved.

Ligand concerned with 2,3-diphosphoglycerate resonance shift

In the 2-h depleted red cells, the metabolic cycle is truncated and adenosine triphosphate is produced at the expense of 2,3-diphosphoglycerate as indicated in Fig. 1. In the depleted cells there is essentially no glyceraldehyde 3-phosphate; it is included in Fig. 1 because glyceraldehyde-3-phosphate dehydrogenase will play a role in the subsequent discussion. Since there is no production of reduced nicotinamide adenosine dinucleotide in the absence of glyceraldehyde 3-phosphate, the truncated cycle ends with pyruvate which diffuses out of the cell. The ligand whose interaction is reflected in the NMR resonance shifts would be expected to be one of the enzymes concerned with 2,3-diphosphoglycerate metabolism, either the combined [14,15,16] enzyme diphosphoglycerate mutase (EC 2.7.5.4)/diphosphoglycerate phosphatase (EC 3.1.3.13) which catalyzes the production of 2,3-diphosphoglycerate and its dephosphorylation to 3-phosphoglycerate, or monophosphoglycerate mutase (EC 2.7.5.3) which requires 2,3-diphosphoglycerate as a cofactor for the production of 2-phosphoglycerate from 3-phosphoglycerate.

We used anionic metabolic inhibitors to study the role of these three enzyme activities in K^+ transport in two hour depleted cells. $^{86}Rb^+$ was used as a tracer for K^+ since the flux of these two cations has been shown to be nearly the same [17]. Exposure of glucose-free human red cells for 2.5 h to 10 mM sulfate, to

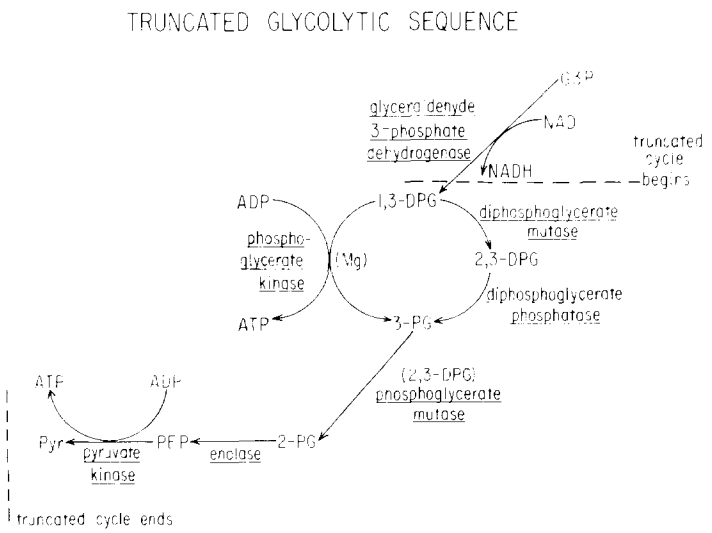

Fig. 1. Schematic diagram showing the truncated glycolytic cycle in human red cells that have been incubated without glucose for 2 h at 37°C.

which red cells are known to be permeable [18], has been shown by Duhm et al. [19] to inhibit 2,3-diphosphoglycerate phosphatase activity. We found that exposure of depleted human red cells to 10 mM sulfate for 2.5 h had virtually no effect on Rb^+ flux, increasing it by an average of 3% in three experiments. This observation indicates that 2,3-diphosphoglycerate phosphatase is not directly involved in cation flux in depleted human red cells. Duhm et al. have also shown that 10 mM bisulfite stimulates the production of 3-phosphoglycerate in glucose free cells. In two experiments 10 mM bisulfite increased Rb^+ influx by 21%. Fluoride inhibits enolase and hence causes an increase in 2-phosphoglycerate concentration. In four experiments this anion, at a concentration of 8 mM, reduced Rb^+ influx by 58%. These results are explicable * in terms of an effect on monophosphoglycerate mutase which catalyzes the formation of 2-phosphoglycerate from 3-phosphoglycerate. Since an increase in the concentration of the substrate of this enzyme promotes Rb^+ transport and an increase in its product inhibits Rb^+ transport, it would appear that the action of monophosphoglycerate mutase is related to cation transport in depleted human red cells.

We next used NMR to study the effect of sulfate and fluoride on 2,3-diphosphoglycerate, the required cofactor for the monophosphoglycerate mutase reaction. The anions were added 30 min prior to a 2 h incubation of the red cells at 37° C as was done in the Rb^+ flux experiments. As the bottom section of Table I shows, 10 mM sulfate had a small effect on the NMR resonance peaks, shifting both resonances slightly upfield, whereas 8 mM fluoride shifts both resonances in the other direction. The differential changes between the two resonances may probably be ascribed to differences in their microenvironment. In the cation flux experiments, sulfate had little effect, whereas fluoride inhibited the flux. The general similarity between these data and the resonance shifts provides further support for our hypothesis that the NMR resonance shifts are related to ion flux, probably by way of monophosphoglycerate mutase.

Linkage of monophosphoglycerate mutase to phosphoglycerate kinase

Monophosphoglycerate mutase is known to be a soluble enzyme located in the red cell cytoplasm, but it has not previously been directly linked to ion transport. Parker and Hoffman [21] and Proverbio and Hoffman [22] have suggested that the active transport process in the human red cells is linked to phosphoglycerate kinase (EC 2.7.2.3) by a compartmentalized form of adenosine triphosphate. Segel et al. [23] and Schrier et al. [24] have also suggested that phosphoglycerate kinase plays an important role in cation transport, and Elford and Solomon [8] have implicated the same enzyme in dog red cell cation transport. The present hypothesis proposes that monophosphoglycerate mutase is linked to the ouabain sensitive ($Na^+ + K^+$)-ATPase via phosphoglycerate kinase as shown in Fig. 2. Phosphoglycerate kinase is only loosely linked to the cytoplas-

* Hoffman (private communication) has recently informed me that fluoride also inhibits the ($Na^+ + K^+$)-ATPase in red cells ghosts as mentioned in a 1962 review article [20]. Had we known this at the time these experiments were done, it would have weakened the evidence on the basis of which we examined monophosphoglycerate mutase behavior. However, Hoffman's observation does not vitiate our conclusions with respect to the role of monophosphoglycerate kinase since they are substantiated by the addition of this enzyme to preparations of inside out vesicles.

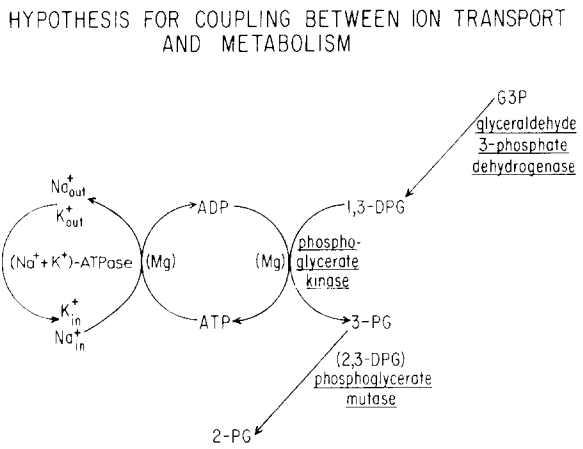

Fig. 2. Schematic diagram showing our hypothesis for the coupling between cation transport and metabolism in human red cells.

mic face of the membrane, 1% remaining attached under conditions in which more than 50% of the cellular glyceraldehyde phosphate dehydrogenase is membrane associated [24]. These considerations led us to look for a soluble complex of phosphoglycerate kinase and monophosphoglycerate mutase.

Preliminary experiments to show that the complex existed were carried out with commercially available enzymes on Sepharose 6B columns. The monophosphoglycerate mutase had been prepared from rabbit muscle and the phosphoglycerate kinase from yeast *. Both enzymes were pure as judged by sodium dodecyl sulfate gel electrophoresis and showed single peaks when eluted on Sepharose 6B columns. However, when both enzymes were mixed in three experiments (monophosphoglycerate mutase with an equal weight of phosphoglycerate kinase) a third peak was found, whose position was consonant with a complex consisting of one molecule of each enzyme. Analysis of the third peak by sodium dodecyl sulfate gels indicated that both the original polypeptides were present.

It was also possible to demonstrate the existence of the complex by NMR. First it is necessary to show that the reaction between 2,3-diphosphoglycerate and monophosphoglycerate mutase is specific to the enzyme and not a general property of all phosphotransferases. The half width of the 2 phosphate resonance of 1 mM 2,3-diphosphoglycerate is 1.2 Hz. Addition of 0.25 mg/ml monophosphoglycerate mutase increased the half-width of this resonance to

* Though the molecular weights of human red cell phosphoglycerate and that of the muscle and yeast enzymes are similar, Hass et al [25] report that there are significant differences in the sulfur containing residues between yeast and muscle enzymes. Comparison of their amino acid analyses with that of Yoshida and Watanabe [26] on the human red cell enzyme indicates a closer similarity between red cell and muscle than between red cell and yeast enzymes. Rose et al. [27] have argued that the properties of yeast and muscle phosphoglycerate mutase are similar, and Harkness et al. [28] have shown that the K_m for the effect of 2,3-diphosphoglycerate on human red cell monophosphoglycerate mutase metabolism is similar to that of the yeast enzyme. We have carried out preliminary experiments with enzymes prepared from human red cells and have observed NMR resonance shifts similar to those presented here.

4.1 Hz. When the 2,3-diphosphoglycerate concentration was increased to 2 mM, the half-width decreased to 2.6 Hz and then to 2.3 Hz at 4 mM. This indicates that we are observing an equilibrium that is rapid on the NMR time scale. The resonance is an average between the bound and unbound substrate. The broadening is specific to this enzyme substrate interaction since there is no broadening when phosphoglycerate kinase (0.6 mg/ml) replaces monophosphoglycerate mutase. There is essentially no resonance shift when 2,3-diphosphoglycerate is added to either enzyme alone as Table II (lines 2 and 3) shows. However when both enzymes are together, both resonances shift by about 2 Hz. These experiments indicate that a soluble complex of these two enzymes exists because the binding of 2,3-diphosphoglycerate to its site on monophosphoglycerate mutase is altered by phosphoglycerate kinase.

Interaction of enzyme complex and red cell membrane

To show that this soluble enzyme complex is responsible for the NMR resonance shifts observed in whole red cells, as proposed in Fig. 2, it is necessary to show that the complex interacts with the red cell membrane. For this purpose, we made use of Steck and Kant's [11] preparations of right side out and inside out vesicles. We have prepared right side out vesicles about 97% pure and inside out vesicles about 85% pure, as measured by assay of acetyl cholinesterase as an outside surface marker and glyceraldehyde 3-phosphate dehydrogenase as an inside surface marker.

Since red cell ouabain-sensitive ($Na^+ + K^+$)-ATPase has been shown to extend through the membrane, the vesicles may be considered as sided ATPase preprations, with the ouabain binding face exposed in right side out versicles and the adenosine triphosphate interactive site exposed in inside out vesicles. Hence

TABLE II

RESONANCE SHIFTS OF 2,3-DIPHOSPHOGLYCERATE IN SOLUTIONS

2,3-Diphosphoglycerate concentration was 5 mM in each experiment, except for the bottom line when it was 2 mM in one experiment and 5 mM in the other. All data are averages of three experiments in the top four lines and two in the remainder. The concentration of Mg^{2+} was 5 mM, and the enzyme concentrations were in mg/ml sample: phosphoglycerate kinase 0.6 (in one experiment 0.45), monophosphoglycerate mutase 0.25 and glyceraldehyde-3-phosphate dehydrogenase 0.1

	Resonance shift	
	2 Phosphate Δ Hz	3 Phosphate Δ Hz
2,3-Diphosphoglycerate + Mg (control)	0.0	0.0
+ phosphoglycerate kinase	+0.1	+0.1
+ monophosphoglycerate mutase	+0.2	0.0
+ phosphoglycerate kinase and monophosphoglycerate kinase	+2.3	+2.0
+ glyceraldehyde 3-phosphate dehydrogenase	+0.2	0.2
+ phosphoglycerate kinase and monophosphoglycerate mutase and glyceraldehyde-3-phosphate dehydrogenase	+2.5	+3.2

these preparations make possible a definitive test of our hypothesis. The hypothesis requires: (1) that ouabain applied to the outside of the red cell cause a configurational change that is transmitted through the membrane by the ouabain sensitive ($Na^+ + K^+$)-ATPase and is reflected in the binding of 2,3-diphosphoglycerate to monophosphoglycerate mutase; and (2) that the interactions between the ATPase and monophosphoglycerate mutase be mediated by phosphoglycerate kinase.

We first established that the action of ouabain is transmitted through the inside out vesicle membrane by the ($Na^+ + K^+$)-ATPase as is known to be the case for intact human red cells [29]. For this purpose we examined the NMR resonance of β,γ-methylene-adenosine triphosphate, a non-metabolizable analog of adenosine triphosphate. As shown in the upper part of Table III, the addition of inside out vesicles to this compound causes a ^{31}P resonance shift of the γ-P which is inhibited when 10^{-6} M ouabain is placed inside the inside out vesicle.

We next showed that this configurational change is related to the phosphoglycerate kinase/monophosphoglycerate mutase/2,3-diphosphoglycerate system by adding these three compounds (plus 5 mM Mg) to inside out vesicles. Fig. 3 shows a typical ^{31}P NMR spectrum of 2,3-diphosphoglycerate under these conditions and Fig. 4 shows that the addition of inside out vesicles to the enzyme complex causes a shift in the 2,3-diphosphoglycerate resonance which increases as the inside out vesicle concentration is increased. This demonstrates that the enzyme complex interacts with a site on the inside surface of red cells. As a control, right side out vesicles were added instead of inside out vesicles.

TABLE III

EFFECT OF INSIDE OUT VESICLES ON ^{31}P RESONANCES

Results of duplicate experiments. The glyceraldehyde 3-phosphate concentration was 2.5 mM; that for β,γ-methylene-adenosine triphosphate was 5 mM. The inside out vesicle concentration may be estimated as follows. 10 ml of washed red cells yield about 0.5 ml of inside out or right side out vesicles which are suspended in 5 ml of 10 mM Tris. 100 µl of inside out vesicles correspond to the yield from 200 µl of washed red cells.

	Resonance shift	
	Δ Hz	$+10^{-6}$ M ouabain Δ Hz
β,γ-Methylene-adenosine triphosphate (γ-P) + 5 mM Mg (control)	0.0	0.0
+ 200 µl inside out vesicles	−2.2	0.0
+ 400 µl inside out vesicles	−8.7	−0.2
+ 600 µl inside out vesicles	−12.9	−0.8
+ 600 µl right side out vesicles	0.0	—
Glyceraldehyde 3-phosphate (control)	0.0	0.0
+ 100 µl inside out vesicles *	+1.2	+0.3
+ 300 µl right side out vesicles	+0.4	—

* Results of a single experiment; in the duplicate experiment we used inside out vesicles depleted of glyceraldehyde-3-phosphate dehydrogenase by 1 mM reduced nicotinamide adenosine dinucleotide, to which stock glyceraldehyde-3-phosphate dehydrogenase was added to reach a concentration of 0.2 mg/ml. The shifts were 1.5 Hz for the glyceraldehyde 3-phosphate resonance which was reduced to 0.0 Hz upon the addition of 10^{-6} M ouabain.

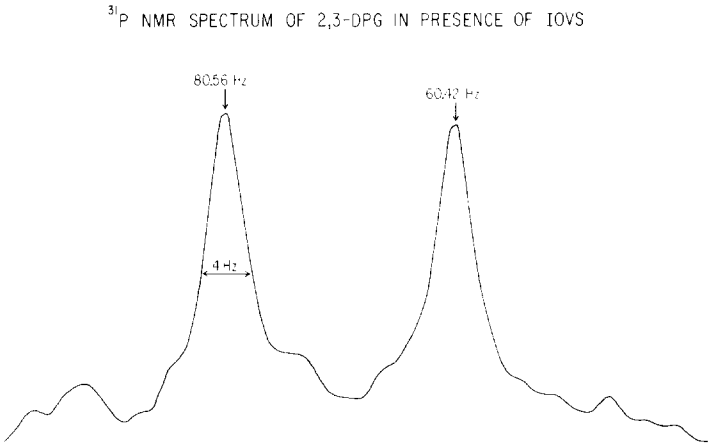

Fig. 3. ^{31}P NMR spectrum at 24.16 MHz of 2,3-diphosphoglycerate (5 mM) when 200 μl of inside out vesicles are added to 0.25 mg/ml monophosphoglycerate mutase plus 0.6 mg/ml phosphoglycerate kinase in NMR buffer in the presence of 5 mM Mg. Under the conditions of data accumulation used in this typical experiment, there are 16 data points over the 4 Hz half width of the peaks. The peaks are identified by their resonance shifts from phosphoric acid.

These produced a negligible shift * (5 experiments, up to 900 μl right side out vesicles) indicating that the interacting membrane site is specific to the inside surface of the cell. Fig. 4 also demonstrates that the normal ^{31}P resonance shifts do not take place when 10^{-6} M ouabain has been added to the inside of the inside out vesicles. Control experiments show that 10^{-6} M ouabain has no effect on the resonances when added to the outside of inside out vesicles or on either

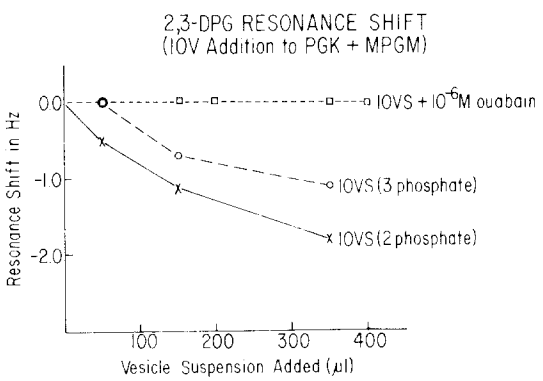

Fig. 4. Resonance shifts for the resonances of 2,3-diphosphoglycerate (5 mM) when inside out vesicles (IOVS) are added to 0.25 mg/ml monophosphoglycerate mutase plus 0.6 mg/ml phosphoglycerate kinase in NMR buffer in the presence of 5 mM Mg. In the experiments with 10^{-6} M ouabain, the ouabain was added to the inside of the inside out vesicle.

* The resonance shifts were 0 Hz for each phosphate with 200 and 500 μl right side out vesicles. When 900 μl right side out vesicles were added, the shift was 0 Hz for the 3 phosphate and —0.6 Hz for the 2 phosphate resonance. On the basis of 3% contamination of right side out vesicles with inside out vesicles according to our assay, 900 μl right side out vesicles should contain 30 μl inside out vesicles. According to Fig. 4, 30 μl inside out vesicles should produce no resonance shift for the 3 phosphate resonance and about —0.3 Hz for the 2 phosphate resonance in reasonable agreement with our observation with the right side out vesicles.

side of the right side out vesicles. These experiments show that the shift of the 2,3-diphosphoglycerate resonance observed in whole human red cells can be reproduced in a system containing a sided preparation of $(Na^+ + K^+)$-ATPase and the two enzymes, phosphoglycerate kinase and monophosphoglycerate mutase.

Inside out vesicles may also be used to demonstrate that the effect is mediated by phosphoglycerate kinase. When 350 μl of inside out vesicles are added to the monophosphoglycerate/2,3-diphosphoglycerate complex, the resonance shift is −0.3 Hz for the 3 phosphate (−0.2 Hz for the 2 phosphate); it rises to its normal value of −1.1 Hz (Fig. 4; −2.0 Hz for the 2 phosphate) when phosphoglycerate kinase is added. This shift is different in sign from that observed when the two enzymes are brought together in free solution (Table II, line 4) indicating that the inside out vesicles play a role in the complex. These data show that phosphoglycerate kinase is a necessary link between the $(Na^+ + K^+)$-ATPase and the monophosphoglycerate mutase. The results of these experiments with inside out and right side out vesicles provide very strong support for the linkage between metabolism and ion transport shown in Fig. 2.

Role of glyceraldehyde 3-phosphate dehydrogenase

We were intrigued by the observation of Kant and Steck [30] that either adenosine triphosphate (1 mM) or 2,3-diphosphoglycerate (1 mM) is an effective inhibitor of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) binding to the inside surface of human red cell membranes. We have shown that the ouabain-induced effects on 2,3 diphosphoglycerate ^{31}P resonances do not take place when all the components of red cell glycolysis are operative. It is possible * that changes in the availability of adenosine triphosphate consequent to 2 hour glucose depletion could be the reason. This would fit an inadvertent and otherwise unexplained observation we have made: that 2 mM adenosine triphosphate prevents the normal resonance shift observed when inside out vesicles are added to the enzyme/2,3-diphosphoglycerate complex.

Possibly these adenosine triphosphate effects could be mediated through regulation of glyceraldehyde-3-phosphate dehydrogenase binding. There is a well defined high affinity binding site for glyceraldehyde-3-phosphate dehydrogenase on the inside face of the cell [31] while there is as yet no evidence for a binding site for phosphoglycerate kinase. Hence, the dehydrogenase could serve as the link through which the kinase is attached to the membrane.

We carried out two preliminary experiments to see whether such an explanation was tenable. 0.2 mg/ml rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was added in solution to the two enzyme/2,3-diphosphoglycerate complex. The results are given in the bottom two lines of Table II. Though the addition of glyceraldehyde-3-phosphate dehydrogenase has little effect on the 2-phosphate resonance, it shifts the 3-phosphate resonance by 1.2 Hz, indicating that all three enzymes interact in solution to form a complex that affects the binding of 2,3-diphosphoglycerate.

We next studied the interaction of glyceraldehyde-3-phosphate dehydrogenase

* Possibly changes in the reduced nicotinamide adenosine dinucleotide content may be involved, since this compound inhibits glyceraldehyde-3-phosphate dehydrogenase binding to the membrane. This binding would be enhanced in depleted cells since they are not producing reduced nicotinamide adenosine dinucleotide.

with inside out vesicles, as measured by the ^{31}P resonance of glyceraldehyde 3-phosphate. As the bottom section of Table III shows, the inside out vesicles cause a 1.2 Hz ^{31}P resonance shift, which is reduced to 0.3 Hz when the inside out vesicles contain 10^{-6} M ouabain and 0.4 Hz when 300 μl right side out vesicles are used instead of 100 μl inside out vesicles. This experiment indicates that the ouabain-induced configurational change in the $(Na^+ + K^+)$-ATPase is transmitted to glyceraldehyde 3-phosphate. Since glyceraldehyde 3-phosphate is the substrate for glyceraldehyde 3-phosphate dehydrogenase, this finding further implicates glyceraldehyde-3-phosphate dehydrogenase as one of the links between glucose metabolism and ion transport. Additional experiments will be necessary to show how this link fits in the chain.

Acknowledgements

We are particularly grateful to Mr. Michael Toon whose skilled and devoted help was essential to these experiments. We should also like to thank Dr. Jeremy Knowles and Dr. Gary Ackers for their helpful suggestions. One of us (E.T.F.) has been supported by a fellowship from the Arthritis Foundation. This research was supported in part by grants number 5 RO1 GM15692-08 and HL 14820-05 from the National Institutes of Health.

References

1 Huestis, W.H. and Raftery, M.A. (1972) Biochem. Biophys. Res. Commun. 49, 428—433
2 Moon, R.B. and Richards, J.H. (1973) J. Biol. Chem. 248, 7276—7278
3 Henderson, T.O., Costello, A.J.R. and Omachi, A. (1974) Proc. Nat. Acad. Sci. U.S. 71, 2487—2490
4 Fossel, E.T. and Solomon, A.K. (1976) Biochim. Biophys. Acta 436, 505—511
5 Parker, J.C. and Hoffman, J.F. (1965) Fed. Proc. 24, 589
6 Hoffman, J.F. (1966) Am. J. Med. 41, 666—680
7 Romualdez, A., Sha'afi, R.I., Lange, Y. and Solomon, A.K. (1972) J. Gen. Physiol. 60, 46—57
8 Elford, B.C. and Solomon, A.K. (1974) Biochim. Biophys. Acta 373, 253—264
9 Deuticke, B., Duhm, J. and Dierkesmann, R. (1971) Pflügers Arch. 326, 15—34
10 Poznansky, M. and Solomon, A.K. (1972) J. Membrane Biol. 10, 259—266
11 Steck, T.L. and Kant, J.A. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. 31, pp. 172—180, Academic Press, New York, San Francisco, London
12 Feig, S.A., Segel, G.B., Shohet, S.B. and Nathan, D.G. (1972) J. Clin. Invest. 51, 1547—1554
13 Kyte, J. (1975) J. Biol. Chem. 250, 7443—7449
14 Rosa, R., Gaillardon, J. and Rosa, J. (1973) Biochem. Biophys. Res. Commun. 51, 536—542
15 Sasaki, R., Ikura, K., Sugimoto, E. and Chiba, H. (1975) Eur. J. Biochem. 50, 581—593
16 Kappel, W.K. and Hass, L.F. (1976) Biochemistry 15, 290—295
17 Solomon, A.K. (1952) J. Gen. Physiol. 36, 57—110
18 Lepke, S. and Passow, H. (1971) J. Membrane Biol. 6, 158—182
19 Duhm, J., Deuticke, B. und Gerlach, E. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1008—1016
20 Hoffman, J.F. (1962) Circulation 26, 1201—1213
21 Parker, J.C. and Hoffman, J.F. (1967) J. Gen. Physiol. 50, 893—916
22 Proverbio, F. and Hoffman, J.F. (1974) Ann. N.Y. Acad. Sci. 242, 459—460
23 Segel, G.B., Feig, S.A., Glader, B.E., Müller, A., Dutcher, P. and Nathan, D.G. (1975) Blood 46, 271—278
24 Schrier, S.L., Ben-Bassat, I., Junga, I., Seeger, M. and Grumet, F.C. (1975) J. Lab. Clin. Med. 85, 797—810
25 Hass, L.F., Caban, C.E., Kappel, W.K., Ottinger, W.E., White, E.L., Kranz, K.R. and Fritz, P.J. (1974) Comp. Biochem. Physiol. 47B, 879—893
26 Yoshida, A. and Watanabe, S. (1972) J. Biol. Chem. 247, 440—445
27 Rose, Z.B., Hamasaki, N. and Dube, S. (1975) J. Biol. Chem. 250, 7939—7942
28 Harkness, D.R., Thompson, W., Roth, S. and Grayson, V. (1970) Arch. Biochem. Biophys. 138, 208—219
29 Hoffman, J.F. (1969) J. Gen. Physiol. 54, 343s—350s
30 Kant, J.A. and Steck, T.L. (1973) J. Biol. Chem. 248, 8457—8464
31 McDaniel, C.F., Kirtley, M.E. and Tanner, M.J.A. (1974) J. Biol. Chem. 249, 6478—6485