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ORGANIC PHOSPHATES MODULATE ANION SELF-EXCHANGE ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

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Anion transport across the red cell membrane has been measured as sulfate self-exchange flux ($\vec{J}_a$) in fresh and metabolically depleted human red cells. Depletion of metabolic stores by a starvation of the cells decreases $\vec{J}_a$ by 50%. A similar effect was observed when ATP was acutely and selectively depleted by iodoacetamide. This inhibition was independent of the presence of calcium and reversible after metabolic rejuvenation of the cells. Ghosts prepared from fresh red cells exhibited the same value of $\vec{J}_a$ as fresh red cells. By contrast, ghosts prepared from depleted red cells exhibited a decrease in $\vec{J}_a$ which was reverted by a physiological concentration of ATP. The effect of ATP was dependent on its concentration ($K_m \approx 40$ μM) and on the duration of the metabolic depletion: complete restoration of $\vec{J}_a$ was obtained only in ghosts prepared from red cells acutely depleted of ATP by a 2 h incubation with iodoacetamide. After a 20 h starvation, $\vec{J}_a$ restoration was never more than 80%. We postulate that ATP acts primarily through the phosphorylation of band 3 protein, the anion exchanger; it acts also through the stabilization of the normal organization of the membrane. This latter effect may involve the phosphorylation of membrane components, but also a direct interaction, as shown by the influence of other organic phosphates (2,3-diphosphoglycerate and inositol hexaphosphate) on $\vec{J}_a$ in the absence of ATP.

The exchange of bicarbonate and chloride anions across the erythrocyte membrane plays a key role in CO_2 transport in vivo. This exchange takes place during each respiratory cycle and is mediated by protein band 3 [1]. This protein is made of two distinct parts: (i) an integral domain bearing the anion-binding site(s), (ii) a cytoplasmic segment which is the locus of multiple interactions between the integral domain of the protein, the cytoskeleton and cytosolic proteins [2–5]. Structural and functional independence of the two domains have been claimed after differential scanning calorimetry [6] or anion-exchange measurement after proteolytic cleavage of the cytoplasmic segment [7].

Anion transport through band 3 is known as an energy-independent process and its kinetics were shown to be governed by pH and temperature [1]. Low [8] showed that increased internal calcium inhibited the exchange reaction. Motais et al. [9] assigned this effect to ATP depletion and protein lattice rearrangement as evidenced by the shape changes of the red cells.

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Supplementary data to this article are deposited with, and can be obtained from, Elsevier Science Publishers B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/296/72305/777(1984) 253. The supplementary information includes: Three tables containing the parameters necessary to compute $\vec{J}_a$.

Abbreviations: EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

The role of the metabolic status of the red cell on the activity of several membrane proteins and on the organization of the membrane has been recently highlighted [10,11]. This led us to investigate its influence on the function of band 3 protein.

Studying the anion-exchange reaction in bank blood stored for several weeks, we showed a progressive inhibition of anion transport which was reversible with metabolic rejuvenation of the cells [12]. The present report demonstrates that the anion-transport kinetics are inhibited after prolonged depletion (24 h) of the ATP stores of the cells and restored after cell metabolic rejuvenation or ATP addition to red cell ghosts. This constitutes a new example of the influence of the metabolic status of the red cells on a non-energy-consuming transport [13,14].

Methods and Materials

Studies on red blood cells. Fresh blood was obtained from healthy adult donors at the local blood bank. It was drawn on heparin and immediately processed. Plasma and white blood cells were discarded and the red blood cells were washed three times with the buffer used for measuring sulfate self-exchange (medium A). This medium was the following: 48.5 mM Na_2SO_4, 1.5 mM $MgSO_4$, 0.1 mM EGTA-Tris, 25 mM Hepes, 87–90 mM KCl to adjust osmolarity at 300 mosmol/l. pH was adjusted with 0.5 M NaOH to the desired value from 6.3 to 7.3.

The red blood cells were then resuspended in medium A at 10% hematocrit and incubated at 37 °C for 3 h in the presence of $Na_2^{35}SO_4$ as a tracer (0.5 μCi/ml) and glucose (10 mM). Isotope distribution was measured at the end of the incubation period. The loaded red blood cells were washed once at 4 °C before the measurement of sulfate self-exchange.

Metabolic depletion and restoration. Metabolic depletion as estimated from the disappearance of ATP and diphosphoglycerate was obtained after 20 h incubation at 37 °C of the washed red cells in medium A in the absence of glucose. Chloramphenicol (25 μg/ml) was added to prevent bacterial growth.

To replenish the metabolic stores, starved red blood cells were incubated for 3 h at 37 °C with a metabolic cocktail: 5 mM pyruvate, 5 mM inosine, 5 mM glucose, 5 mM phosphate, 0.5 mM adenine (medium B). A selective depletion of ATP was achieved by incubating the fresh red cells with 5 mM recrystallized iodoacetamide and 5 mM inosine for 90 min at 37 °C. Diphosphoglycerate concentration was constant in these conditions. Diphosphoglycerate could also be selectively depleted at constant ATP level by incubation of the red cells in the presence of 1 mM sodium vanadate ($NaVO_3$) [15,16].

It is worth mentioning that equilibrium exchange of SO_4^{2-} in starved red cells was much longer to obtain than in fresh red cells. The cells were thus incubated in the presence of 50 mM SO_4^{2-} during all the metabolic depletion period.

Studies on resealed ghosts. Resealed ghosts were prepared according to the method of Bodemann and Passow [17] with minor modifications. The red blood cells were washed three times with a buffer containing 150 mM NaCl and 25 mM Hepes (pH 7.4). They were resuspended in the same buffer at 50% hematocrit and hemolyzed at 2 °C with 10 vol. of a hemolyzing solution made up with 4 mM $MgSO_4$, 1.7 mM acetic acid, 0.1 mM EGTA-Tris and the compounds to be incorporated, i.e., $Na_2^{35}SO_4$ (1 μCi/ml) and when necessary, organic phosphates; pH was adjusted to 6.0. After 5 min stirring on ice, pH was readjusted to 7.0 by adding 1 M Tris, and isotonicity was restored by the addition of an hypertonic solution of KCl and K_2SO_4 such as to yield 50 mM sulfate final concentration. Then the red cell ghosts were allowed to reseal by incubating at 37 °C for 45 min. The ghosts were spun down at 35000 × g for 10 min at 2 °C and were washed twice at 2 °C in medium A.

Measurement of ^{35}S distribution (r_{SO_4}). Due to the variation of the Donnan equilibrium and sulfate distribution with pH at constant temperature, it was important to measure the ^{35}S distribution and to check that equilibrium was attained in any experimental condition. In fresh red blood cells, the equilibrium was attained in about 2 h.

^{35}S distribution across the red cell membrane was determined at different pH as:

$$r_{SO_4} = \frac{[SO_4]_{in}}{[SO_4]_{out}} = \frac{\text{dpm } ^{35}\text{S/ml } c_{H_2O}}{\text{dpm } ^{35}\text{S/ml supernatant}} \quad (1)$$

where c_{H_2O} is the cellular water content measured by drying to constant weight an aliquot of packed red cells. $[SO_4]_{in}$ was measured on 100 mg packed red cells which were hemolyzed with 0.9 ml H_2O and deproteinized with the same volume of 15% trichloroacetic acid. 0.5 ml of the clear extract were dissolved in 3 ml of the scintillator ACS (Amersham).

Sulfate self-exchange flux. This was determined as the efflux of ^{35}S from loaded red cells or ghosts at sulfate distribution equilibrium. 0.2–0.3 ml of loaded washed cells were mixed at 37°C with 8 ml medium A at the pH value of the loading medium. The appearance of the radioactivity in the extracellular water phase was followed at suitable time intervals and related to the overall suspension radioactivity.

Determination of the SO_4 efflux rate constant (k, min^{-1}) was obtained from the slope of the linear regression line of $\ln(1 - S_t/S_{max})$ versus time (t), where S_t is the radioactivity in the extracellular compartment at time t and S_{max} the radioactivity at isotopic equilibrium:

$$S_{max} = \frac{R}{1 - H\left[1 - (r_{SO_4} \cdot c_{H_2O})\right]} \quad (2)$$

where R is the overall suspension radioactivity, H the hematocrit. The unidirectional sulfate exchange flux ($\vec{J}_a$) was calculated according to Ref. 18 from the relation:

$$\vec{J}_a(\text{mmol} \cdot \text{cm}^{-2} \cdot \text{min}^{-1}) = k \cdot \frac{V}{A} \cdot C \quad (3)$$

where V is the solvent volume inside the red cells or ghosts, i.e., mean cell volume $\times c_{H_2O}$, A is the mean surface area ($1.42 \cdot 10^{-6}$ $cm^{-2} \cdot cell^{-1}$ [18]) and C is the intracellular sulfate concentration in $mmol \cdot cm^{-3}$ cell water. Mean cell volume was measured by Coulter S counter analyses.

Miscellaneous. pH was measured at 37°C with the Radiometer BMS microassembly. Both supernatant pH (pH_e) and intraerythrocytic pH (pH_i) were analyzed after separation of the cells. pH_i was determined in the hemolyzate obtained after two episodes of freezing and thawing.

Intraerythrocytic concentrations of ATP and diphosphoglycerate were determined according to current enzymatic methods [19,20].

Electrophoresis of membrane proteins was achieved in white erythrocyte ghosts prepared by hypotonic lysis in 5 mM sodium phosphate (pH 7.4) as described by Dodge [21]. Antiproteinases were added in all the buffers as 0.1 mM phenylmethylsulfonyl fluoride. The cell membranes were solubilized in a 10% glycerol/1% SDS/1% 2-mercaptoethanol/50 mM Tris (pH 8.6) solution for 2 min at 100°C. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was performed on 5–15% acrylamide gradient slabs with a 3.5% acrylamide stacking gel [22].

Chemicals. $Na_2^{35}SO_4$ and the scintillator, ACS, were from Amersham; ATP disodium salt, creatine phosphate, creatine kinase and Hepes were from Boehringer; diphosphoglycerate penta sodium salt, inositol hexaphosphate, EGTA and EDTA were from Sigma; iodoacetamide and sodium vanadate were from Merck; electrophoresis compounds were from Bio-Rad. All the chemicals used were of analytical grade.

Results

Anion transport in intact erythrocytes

Table I shows the effect of time on anion self-exchange in red blood cells stored at 4°C in citrate/ phosphate/ dextrose medium. Sulfate transport was preserved close to normal initial values (day of collection) up to 5 weeks of storage. Anion transport began to slow down after 6 weeks. As shown on Fig. 1, the impairment of anion transport occurs as ATP content decreases and appears independent of diphosphoglycerate content. SDS-polyacrylamide gel electrophoresis of membrane proteins of red cells stored in these conditions did not show any abnormality, in particular no irreducible high molecular weight compound was noticed. The proportion of the different proteins was within normal limits up to 7 weeks. Rejuvenating the red cells on day 52 by incubation in medium B led to restoration of anion transport and of ATP and diphosphoglycerate content of the erythrocytes.

Following this observation, we studied the effect of metabolic depletion on anion transport in fresh red cells. Starvation of fresh red cells by incubation at 37°C for 20 h in the absence of glucose led to a large decrease in anion transport

TABLE I

RELEVANT PARAMETERS OF ANION TRANSPORT MEASUREMENT DURING IN VITRO AGEING OF RED BLOOD CELLS STORED IN CITRATE/PHOSPHATE/DEXTROSE/ADENINE MEDIUM

pH_e = extracellular pH, pH_i = intraerythrocytic pH, $r_{H^+} = [H^+]_{out}/[H^+]_{in}$, $r_{SO_4} = [SO_4]_{in}/[SO_4]_{out}$, c_{H_2O} = intraerythrocytic water, MCV = mean cell volume (in 10^{-15} l), k = rate constant of anion self-exchange (s^{-1}), $\vec{J}_a$ = anion self-exchange flux (mmol·cm^{-2}·min^{-1}) ($\times 10^6$).

	pH_e	pH_i	r_{H^+}	r_{SO_4}	c_{H_2O}	MCV	k	$\vec{J}_a$
J_0	6.70	6.61	0.813	0.872	0.66	90	0.082	1.50
J_{15}	6.71	6.62	0.813	0.881	0.665	95	0.079	1.55
J_{22}	6.72	6.62	0.794	0.91	0.67	93	0.074	1.48
J_{28}	6.70	6.60	0.794	0.94	0.691	97	0.066	1.45
J_{40}	6.70	6.61	0.813	0.922	0.722	101	0.055	1.30
J_{45}	6.71	6.63	0.832	0.954	0.710	100	0.046	1.10
J_{52}	6.70	6.62	0.832	0.933	0.719	103	0.041	1.00
After metabolic restoration:								
J_{52}	6.70	6.58	0.759	0.831	0.730	104	0.075	1.65

as well as almost complete disappearance of ATP and diphosphoglycerate (Fig. 2A and Table II). Rejuvenation of the metabolic stores after 3 h incubation of the starved red cells in medium B restored anion transport to normal (Table III). Fig. 2B shows that these effects were independent of the presence of calcium or of bovine serum albumin when they were added to the incubation medium during the starvation period. These results rule out the possibility that the inhibition of anion transport might be due to the accumulation of Ca^{2+} inside the cells or to the presence of a metabolic derivative of membrane lipids [23].

It is worth noting that in expressing the present results, we did take into account the well-known

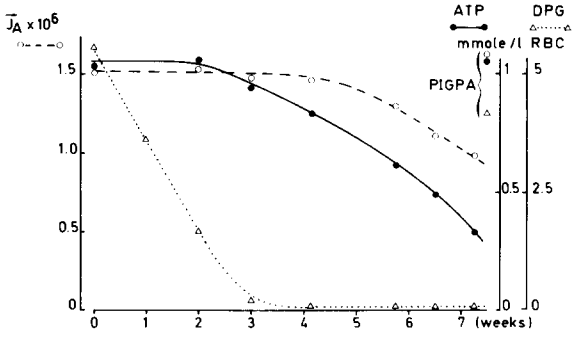

Fig. 1. Time-course of development of metabolic depletion and sulfate self-exchange flux ($\vec{J}_a$) inhibition in bank blood stored on citrate/phosphate/dextrose/adenine medium. The [^{35}S]sulfate efflux in a 3% (v/v) red cell suspension was measured under self-exchange conditions (Donnan equilibrium) at 37 °C and is expressed in mmol·min^{-1}·cm^{-2}. On day 52, the washed red cells were rejuvenated by a 3 h incubation at 37 °C in the presence of pyruvate, inosine, glucose, phosphate and adenine (PIGPA), RBC, red blood cells; DPG, diphosphoglycerate.

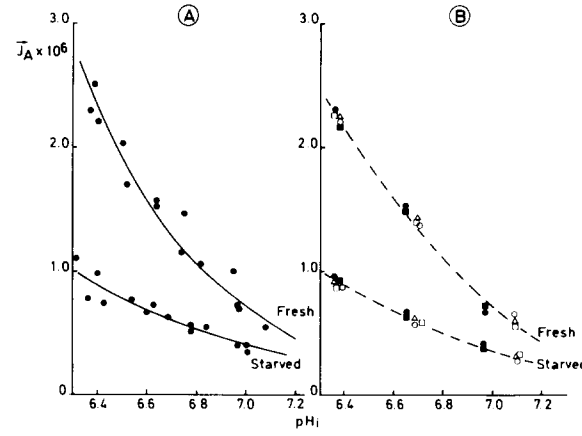

Fig. 2. Variation of sulfate self-exchange flux ($\vec{J}_a$) with intraerythrocytic pH (pH_i) in fresh red cells and red cells depleted by a 20 h incubation at 37 °C in the absence of glucose. $\vec{J}_a$ was measured as the [^{35}S]sulfate efflux in a 3% (v/v) red cell suspension under self-exchange conditions (Donnan equilibrium) at 37 °C and is expressed in mmol·min^{-1}·cm^{-2}. (A) The incubation medium was 50 mM SO_4^{2-}, 1.5 mM Mg^{2+}, 25 mM Hepes (pH 6.3–7.3), Na^+, K^+, Cl^-, up to 300 mosmol/l. (B) To this incubation medium were added 2 mM $CaCl_2$ (△), 3 g/l bovine serum albumin (■), 30 g/l bovine serum albumin (□), EDTA (○) or EGTA (●).

TABLE II

EFFECT OF METABOLIC DEPLETION OF RED BLOOD CELLS ON SULFATE SELF-EXCHANGE KINETICS

Sulfate self-exchange flux ($\vec{J}_a$) was measured at Donnan concentration equilibrium as the efflux of ^{35}S from cells previously loaded in a medium containing 50 mM Na_2SO_4 and 0.5 μCi/ml ^{35}S. The pH-dependence of $\vec{J}_a$ (d log $\vec{J}_a$/d pH) was calculated in the pH range 6.3–7.2. Selective depletion of ATP was obtained by a 90 min incubation of the red cells (RBC) in the presence of 5 mM iodoacetamide and 5 mM inosine. Selective depletion of diphosphoglycerate (2,3-DPG) was obtained by a 90 min incubation of the red cells in the presence of 1 mM $NaVO_3$. Metabolic restoration of starved red cells was achieved after 3 h incubation with medium B (phosphate/inosine/glucose/pyruvate/adenine). All manipulations were performed at 37 °C. Data are mean ± S.D.

	$\vec{J}_a$ (pH 6.7) (mmol·cm^{-2}·min^{-1}) ($\times 10^6$)	d log $\vec{J}_a$/d pH$_i$	ATP (mmol·l^{-1} RBC)	2,3-DPG (mmol·l^{-1} RBC)
Control	1.30	−0.84	1.10 ± 0.12	5.3 ± 0.3
Iodoacetamide	0.90	−0.66	0.01 ± 0.01	4.7 ± 0.3
$NaVO_3$	1.09	−0.77	0.81 ± 0.05	0.05 ± 0.01
Iodoacetamide + $NaVO_3$	0.68	−0.56	0	0.04 ± 0.01
Starved (20 h)	0.62	−0.54	0.02 ± 0.02	0
Restoration medium B	1.25	−0.78	1.05 ± 0.25	3.8 ± 1.0

TABLE III

RELEVANT PARAMETERS OF ANION TRANSPORT MEASUREMENT IN RED CELLS, FRESH, STARVED FOR 20 h AND AFTER METABOLIC RESTORATION

For details see legend of Table I.

Red cell type	pH$_e$	pH$_i$	r_{H^+}	r_{SO_4}	c_{H_2O}	MCV	k (min^{-1})	$\vec{J}_a$ (mmol·cm^{-2}·min^{-1}) ($\times 10^6$)
Fresh	6.36	6.31	0.89	0.96	0.683	94	0.121	2.61 ± 0.45
	6.68	6.59	0.80	0.86	0.677	90	0.079	1.47 ± 0.09
	6.93	6.79	0.72	0.73	0.667	90	0.071	1.10 ± 0.05
	7.17	6.99	0.65	0.64	0.663	93	0.044	0.61 ± 0.13
Starved	6.33	6.30	0.93	1.08	0.720	101	0.037	1.00 ± 0.09
	6.63	6.60	0.84	0.96	0.713	103	0.028	0.69 ± 0.03
	6.88	6.80	0.83	0.87	0.709	92	0.028	0.56 ± 0.03
	7.11	6.98	0.74	0.74	0.698	102	0.021	0.41 ± 0.05
Rejuvenated	6.34	6.30	0.92	1.00	0.718	106	0.098	2.62 ± 0.20
	6.66	6.59	0.84	0.87	0.715	106	0.067	1.54 ± 0.10
	6.93	6.79	0.72	0.76	0.711	103	0.053	1.03 ± 0.07
	7.18	7.02	0.70	0.67	0.692	100	0.043	0.70 ± 0.03

variations of pH$_i$ with pH$_e$ in starved red cells, due to the disappearance of highly concentrated nondiffusible anions such as ATP and diphosphoglycerate (5–6 mM). Therefore, anion transport values were all calculated at the measured pH$_i$ in each sample. Similarly, r_{SO_4} and c_{H_2O} were measured in each condition and their variation with pH$_i$ is shown in Fig. 3.

The pH-dependence of sulfate transport across the red cell membrane has been reporeted by Schnell [24] and ascribed to change in the pK of amino-acid side chains in the band 3 protein [25,26]. The values calculated from our results between pH 6.3 and 7.2 was d log $\vec{J}_a$/d pH$_i$ = −0.84 in fresh red cells and −0.54 in starved red cells or a 30% inhibition (Table II). This was restored to normal values after rejuvenation of the metabolic stores of the cells.

Selective depletion of the ATP and diphosphoglycerate content

The relative role of ATP and diphosphogly-

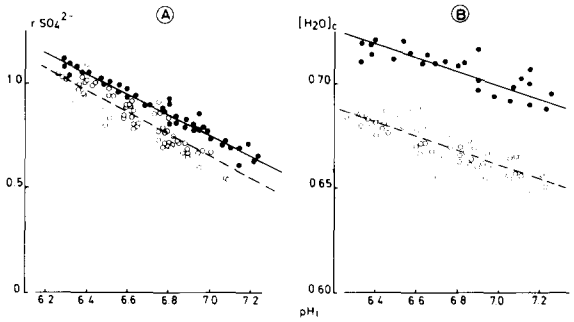

Fig. 3. (A) Variation with intracellular pH (pH_i) of the sulfate anion distribution ratio, r_{SO_4}, across the red cell membrane in fresh (○) and depleted (●) red cells (20 h incubation at 37 °C without glucose), (B) Variation with pH_i of the intracellular water ratio c_{H_2O} (g H_2O/g red cells) in fresh (○) and depleted (●) red cells.

cerate on anion transport was studied by selective depletion of each of these organic phosphates. Selective depletion of ATP was carried out (at constant diphosphoglycerate content) in the presence of iodoacetamide and inosine. Depletion of diphosphoglycerate (at constant ATP content) was obtained by addition of $NaVO_3$ to the incubating medium. Table II gives the results. Depletion of ATP at normal diphosphoglycerate content led to a 33% inhibition of sulfate transport. This inhibition was about halved when diphosphoglycerate was absent in the presence of ATP. The combined effect (incubation in the presence of iodoacetamide and inosine, then $NaVO_3$) resulted in approx. 50% inhibition of sulfate transport, i.e., the addition of the two effects. Similarly, the pH dependence of sulfate transport was lowered in both conditions (Table II).

Sulfate transport in red cell ghosts

The effects of organic phosphates on anion transport were studied by incorporating these compounds in hemolyzed, resealed cells (ghosts). Preliminary experiments showed that the distribution of sulfate (r_{SO_4}) was equal to 0.95 ± 0.03 when ghosts were resealed on medium A containing various organic phosphate compounds: 3 mM ATP and 10 mM creatine phosphate, 5 mM diphosphoglycerate or 2 mM inositol hexaphosphate. r_{SO_4} was therefore considered constant, equal to 0.95. Similarly, water content measured in these conditions was found constant within experimental error, approx. 0.95.

Control studies with ghosts prepared from fresh red cells, without the addition of ATP, showed that sulfate transport at pH 6.5 was identical to that measured in fresh red cells. When ghosts prepared from fresh red cells were resealed on 1.5 mM ATP and creatine phosphate and creatine kinase, the same normal value of $\vec{J}_a$ was observed. This demonstrated that ATP per se is not necessary to achieve a normal anion flux across fresh red cell membrane.

At alkaline pH values, $\vec{J}_a$ exhibited a higher value in ghosts compared to red cells. Consequently, the pH dependence of $\vec{J}_a$ was slightly lower in ghosts than in intact red cells: −0.72 and −0.84, respectively. This difference is related to the constancy of r_{SO_4} in ghosts, whereas it decreases from 1 to 0.5 in red cells between pH 6.4 and 7.2. When ghosts were prepared from starved red blood cells $\vec{J}_a$ was similar to the value observed in intact starved red cells, that is, inhibited by 60% at pH 6.5. Resealing ghosts on ATP prepared from starved red blood cells restored $\vec{J}_a$ to near normal values (up to 80% restoration). Experiments performed at varying concentrations of ATP showed that half the effect was obtained for ATP concentration varying from 35 to 64 μM at pH 6.3 and 7.3, respectively (Fig. 4). Ghosts prepared from red cells whose ATP content had been selectively depleted with iodoacetamide for 2 h evidenced a similar inhibition of $\vec{J}_a$ as the iodoa-

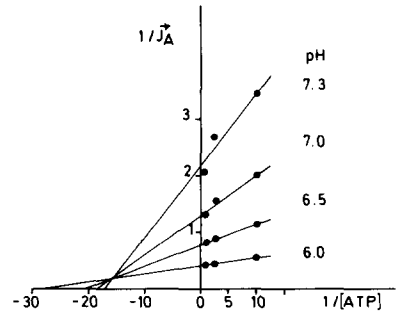

Fig. 4. Lineweaver-Burk plot from a study of the dependence of sulfate self-exchange flux ($\vec{J}_a$) on ATP concentration. Red cells were incubated at 37 °C for 20 h then hemolyzed and resealed on ATP (0.1–1 mM), 10 mM creatine phosphate and 25 U/ml creatine kinase. $\vec{J}_a$ is expressed in (mmol·min^{-1}·cm^{-2}) ($\times 10^6$) and ATP in mmol·l^{-1}.

cetamide-treated intact cells. This inhibition was completely reverted by resealing these ghosts on 1.5 mM ATP.

These results suggest two mechanisms for the effect of ATP: (i) the phosphorylation of specific residues of the protein and (ii) a direct binding effect. This latter hypothesis was checked by resealing ghosts on 5 mM diphosphoglycerate, the physiological concentration of diphosphoglycerate inside human fresh red cells. This led to an increase of $\vec{J}_a$ of 30% at pH 6.7. In these experiments, the diphosphoglycerate concentration remained constant. Similarly, ghosts were resealed on 2 mM inositol hexaphosphate, a non-natural, nonhydrolyzable organic phosphate [27]. The increase of $\vec{J}_a$ was comparable to that observed after the addition of diphosphoglycerate.

Discussion

The present study demonstrates that sulfate exchange, a model of anion transport through protein band 3, is highly dependent upon the metabolic status of the red cell. Metabolic depletion inhibited $\vec{J}_a$ and its pH dependence; they were reverted to normal values after metabolic rejuvenation. This phenomenon was studied in long-term energy stores-depleted red cells as well as after acute depletion. Our results may be compared with those reported by Motais and his group [9] who observed an inhibition of chloride transport (also mediated by protein band 3) after ATP depletion in intact red cells at 0 °C. However, after rejuvenation of the energy stores of the cells, these authors observed the recovery of a normal shape of the red blood cell, but still a partly inhibited Cl^- transport. In the light of our study, and as will be discussed below, this discrepancy is probably due to the incomplete restoration of the ATP concentration which appears to be a prerequisite for the full expression of the anion self-exchange system in long-term-depleted red cells. Our results are well in keeping with other reports, demonstrating that several other so-called non-energy-dependent transport systems across the cell membrane are inhibited by the alteration of their metabolic status, essentially by the disappearance or lowering of the intracellular ATP concentration. Among various examples which have been recently published are urate transport [13], glucose transport [14], sodium-dependent passive potassium transport [28], sodium-calcium exchange in the squid axons [29] and other [30,31]. Results obtained with red cell ghosts suggest that the mechanism of the dependence of anion transport on ATP concentration is not unique. One hypothesis is that ATP acts as a phosphoryl donor, by phosphorylating specific sites on protein band 3 [32,33]. We observed that ghosts prepared rapidly from fresh cells and resealed on a medium which does not contain ATP exhibit a normal $\vec{J}_a$ value. This agrees with results reported by Funder and Wieth [18] who demonstrated identical Cl^- transport values in fresh red cells and in ghosts prepared therefrom. This observation could indicate that phosphorylation of band 3 is preserved in fresh red cell ghosts, either because the phosphatase which specifically hydrolyzes band 3 phosphoryl groups is cytosolic and is eliminated during the preparation of the ghosts or because its kinetics are slow in the present experimental conditions.

After metabolic depletion, half the value of the maximal effect of ATP was obtained at micromolar concentration of ATP (Fig. 4) which favors the role of ATP-mediated phosphorylations of protein residues [32,33]. Specific characteristics of phosphorylation of band 3 protein are not yet well documented. Several sites of phosphorylation of band 3 have been reported on the cytoplasmic segment, none on the integral segment which is responsible for anion transport. As the relationship between these two segments is still ill-defined, the present results do not allow any speculation on the site(s) of band 3 which could modulate the anion-transport function. Diphosphoglycerate and ATP have been shown to bind to the cytoskeleton, ensuring, through this binding, an increased mobility of its components [34]. It has also been shown that after metabolic depletion, the lateral mobility of the integral domain of the transport protein was lowered [35,36]. An alteration of anion self exchange kinetics is reasonably expected in these circumstances. Arguments for this interpretation are found in the partial restoration of the $\vec{J}_a$ value in ghosts resealed on 5 mM diphosphoglycerate or on 2 mM inosinol hexaphosphate which are not considered as phosphoryl donors.

Full restoration of $\vec{J}_a$ after ATP depletion was

observed in intact starved red cells after restoration of their metabolic stores by a 3 h incubation in medium B. This was observed also in ghosts prepared from acutely ATP-depleted red cells resealed on 1.5 mM ATP. By contrast, ghosts prepared from 20-h starved red cells exhibited only 80% restoration of $\vec{J}_a$ at the maximum when resealed on 1.5 mM ATP and up to 10 mM (results not shown). This indicates that the duration of the metabolic depletion is an important factor in the mechanism of inhibition of the anion-transport system, acting either through the progressive inhibition of the protein kinase responsible for band 3 phosphorylation or through a disordering of the membrane components. This has already been shown for phospholipid asymmetry [37] and for spectrin physical state and arrangement in the membrane [38].

In conclusion, the mediated anion transport across the red cell membrane is dependent upon the metabolic state of the red cell through phosphorylation of membrane proteins and possibly through its influence on the architecture of membrane components.

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