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ATP SYNTHESIS IN HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

Human erythrocyte membranes were prepared so that they contained only membrane bound enzymes and retained many of the permeability characteristics of intact erythrocytes. Specific substrates were then sealed into these membrane by the technique of hemolysis reversal. When NAD^+, ADP and triose phosphate were added to membrane interiors and $^{32}P_i$ was added to the external medium, there was a net synthesis of ATP which appeared predominantly within or bound to membranes. The synthesis of ATP was mediated by membrane bound glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase which seem to be linked so that there is transfer of substrates between enzymes.

INTRODUCTION

Mammalian erythrocytes require ATP both for the maintenance of their shape[1] and their intracellular sodium and potassium content[2,3]. The pathway by which ATP controls cell shape is unknown, but sodium–potassium transport requires a membrane localized ATPase (ATP phosphohydrolase, EC 3.6.1.3) (ref. 4). The ATP necessary for these functions has been assumed to come from the intracellular pool. We have shown that erythrocyte membranes contain a specific complement of enzymes, including glyceraldehydephosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) and phosphoglycerate kinase (ATP: D-3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3) (ref. 5). These two enzymes are organized in membranes[6] and could catalyze the synthesis of ATP if they were linked so that substrates could pass smoothly from one enzyme to the next. Prior studies were performed on membranes which were completely permeable to the substrates used[6]; therefore, it was impossible to determine if these enzymes could act in concert to synthesize ATP at the membrane. The object of this study was to determine whether an erythrocyte membrane preparation which had restricted permeability for phosphorylated intermediates could mediate the synthesis of ATP. Membrane synthesized ATP would be the preferred substrate for ATP requiring steps simply because of its proximity. Using the technique of

hemolysis reversal, known amounts of specific substrates and enzymes were 'sealed' into these human erythrocyte membranes which otherwise contain only membrane bound enzymes, carriers and binding sites. Membranes whose intracellular contents were known and circumscribed were incubated with extracellular ^{32}P-labeled P_i. The net synthesis of intracellular ATP by these membranes is reported*.

MATERIALS AND METHODS

Materials

Radioactive $^{32}P_i$ was obtained from Volk and New England Nuclear Companies and consisted of highly labeled H_3PO_4 with no added carrier phosphate. Other materials were obtained from sources previously described[5,6]. Enzymes used in substrate assays were obtained from Calbiochem.

Preparation of erythrocyte membranes

Instead of prolonged dialysis[6], the red cells were swirled for 5 min at 4° in the following empirically derived solutions: (a) 0.05 M NaCl (pH 7.1); (b) 0.03 M NaCl (pH 6.9), 0.5 mg bovine serum albumin per ml; (c) 0.019 M NaCl (pH 6.9), 0.25 mg bovine serum albumin per ml; (d) 0.009 M NaCl (pH 6.9), 0.25 mg bovine serum albumin per ml; (e) 0.154 M NaCl (pH 6.8), 0.25 mg bovine serum albumin per ml. The pH was adjusted by the use of Tris–HCl buffers at a final concn. of approx. 0.1 mM. The resulting membrane suspension contained 10^{10} erythrocyte membranes per ml.

Hemolysis reversal[8,9]

A 126-ml solution containing 900 μmoles of NAD^+, 750 μmoles of ADP, 1700 μmoles of DL-glyceraldehyde 3-phosphate, 70 μmoles ouabain, 800 μmoles of 2-mercaptoethanol, 120 mg of bovine serum albumin, and in some cases 10 mg of hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) (capable of converting 1500 μmoles glucose per min) at pH 6.9 was added with stirring at 4° over 10 min to 14 ml of membrane suspension. Isotonicity was restored by addition of 6.2 ml of 3.08 M KCl, 0.36 ml of 0.2 M calcium acetate and 0.6 ml of 0.5 M $MgCl_2$, followed by incubation for 32 min at 37°. The glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase reaction was limited during this period because the P_i resulting from triose phosphate hydrolysis did not exceed 2 mM. The ATP produced accounted for the unlabeled membrane ATP found in both the controls and experimental tubes. The suspension was centrifuged for 10 min, 4° at 12 000 $\times$ g, and the membranes were washed twice in 100 ml of a solution comprised of 145 mM KCl, 0.5 mM calcium acetate, 5 mM $MgCl_2$ and 1.25 mM 2-mercaptoethanol at a pH of 6.6. A recent report states that stepwise removal of hemoglobin results in membranes which have increasing permeability[10]. Our results indicate that pH control and 37° incubation are crucial, and that membrane integrity can be restored so as to provide membrane/suspending medium ratios of 20–40 for ADP, NAD^+ and triose phosphate.

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* A preliminary report of these studies has appeared[7].

Reactions

Membranes were incubated at 37° in a final vol. of 15.0 ml with isotonic solutions of $^{32}P_i$-labeled (80 μC to 120 μC per reaction) phosphate buffer (pH 7.4), along with 6.5 mM 2-mercaptoethanol and isotonic KCl or NaCl. To obtain the levels of intramembrane substrates and P_i at the beginning of the reactions, identically constituted tubes were kept at 4° and were then separated and assayed along with the experimental tubes. To measure the amount of substrate leak or depletion during incubation, isotonic Tris buffer (pH 7.4) was substituted for the $^{32}P_i$ buffer in otherwise identical reactions, which were incubated at 37° and treated in parallel with the experimental tubes. These Tris-substituted flasks also controlled any synthesis of ATP that might have occurred *via* reactions other than the glyceraldehydephosphate dehydrogenase, phosphoglycerate kinase coupled system; *i.e.* adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3). The values recorded in the tables are the differences between the experimental tubes and the Tris-substituted controls. The reactions were stopped by the addition of 30 ml of cold wash solution and immediate centrifugation. The clear supernatant was saved for chromatographic analysis and determination of P_i content[11] and isotopic activity. Radioactivity was measured by planchetting duplicate samples, drying them to constant weight and counting in a Nuclear Chicago thin window gas-flow counter Model D-47. The membrane sediment was washed once by resuspending to 45 ml, and the membranes were extracted twice with 10 ml of 10% trichloroacetic acid at 4°.

Assay of trichloroacetic acid filtrates

Anion exchange chromatography of phosphorylated substrates[12,13] was performed on columns calibrated with authentic compounds, whose identity and position was confirmed by determining the ratios of base to pentose to total and labile phosphate[14]. The amount of NAD^+, ADP and ATP present was estimated by measurements of the absorption at 260 mμ of the indicated fractions. ATP synthesis was calculated from the incorporation of $^{32}P_i$ of known specific activity into the designated ATP containing NH_4Cl fractions which absorbed at 260 mμ. It was assumed that both the β and γ phosphates of ATP were labeled since adenylate kinase is present in membranes[15]. Significant radioactivity was found only in fractions containing P_i or ATP.

The substrate content of membranes was also determined by enzyme assays on neutralized filtrates in which the change of reduced pyridine nucleotide co-enzymes was recorded at 340 mμ, and a millimolar extinction coefficient of 6.22 was used. Glucose 6-phosphate was assayed with glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49). NAD^+ was assayed with alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1). ATP and glucose were measured with the coupled hexokinase, glucose-6-phosphate dehydrogenase reaction. Triose phosphates were measured with triosephosphate isomerase (D-glyceraldehyde-3-phosphate keto-isomerase, EC 5.3.1.1) coupled to α-glycerolphosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8). Fructose diphosphate was estimated by adding aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7) to triosephosphate isomerase and α-glycerolphosphate dehydrogenase. Adenosine diphosphate was estimated with pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) coupled to lactate dehydrogenase (L-lactate: NAD

oxidoreductase, EC 1.1.1.27). Authentic substrates were added at values equivalent to 0.01 μmole/ml original material and were detected by the assay systems used.

Extracts of membranes, prior to hemolysis reversal, were analyzed by chromatographic and enzymic methods, and less than 0.01 μmole/ml glucose, ADP, ATP, triose phosphate, fructose diphosphate, glucose 6-phosphate or NAD^+ was found. Following hemolysis reversal, membranes contained as the mean of three experiments 1.41 μmoles/ml triose phosphate, 1.33 μmoles/ml ADP and 1.23 μmoles/ml NAD^+.

RESULTS

The substrate requirements for ATP synthesis were determined as well as the site of ATP synthesis (Table I). All three substrates were required for optimum $^{32}P_i$ incorporation into ATP, and when expressed as the concentration, 10 times as much ATP appeared in membranes as in the supernatant medium. Small amounts of [^{32}P]ATP appeared in membranes containing only NAD^+ and triosephosphate. These membranes contained 0.53 μmole of adenine in the chromatographic fractions

TABLE I

SUBSTRATE REQUIREMENTS FOR ATP SYNTHESIS IN ERYTHROCYTE MEMBRANES

Membranes from a single donor were prepared so that they contained the four variations in substrate content listed. 15-ml reactions containing $2 \cdot 10^{10}$ membranes in a volume of 2 ml, 0.2 mM ouabain and 40 mM $^{32}P_i$ with a specific activity of 350 000 counts/min per μmole P_i were incubated for 15 min at 37°. Membranes and supernatant fraction in each case were separated, extracted and chromatographed, and the radioactivity recovered in the ATP containing eluates is recorded below. For the calculation of ATP synthesis it is assumed that both β and γ phosphates of ATP were labeled. A single experiment is described; however, a similar experiment gave comparable results.

Substrate content of membranes	*Membranes*			*Supernatant fraction*		
	^{32}P in ATP-containing eluates (counts/min)	*ATP*		*^{32}P in ATP-containing eluates (counts/min)*	*ATP*	
		μmole	*μmole/ml*		*μmole*	*μmole/ml*
ADP + NAD^+ + triose-*P**	23 600	0.034	0.017	15 100	0.022	0.0017
ADP + NAD^+	0			0		
ADP + triose-*P**	0			0		
NAD^+ + triose-*P**	3 600	0.005	0.0025	0		

* Triose phosphate.

which ordinarily contain ADP. NAD nucleosidase (NAD glycohydrolase, EC 3.2.2.5) is present in erythrocyte membranes[16], and a consequence of its attack on NAD^+ would be adenosine 5′-pyrophosphoryl-5-ribose (ADP ribose). Further degradation of ADP ribose to ADP could account for the apparent recovery of material in the ADP fraction and for the subsequent appearance of small amounts of [^{32}P]ATP.

DEVERDIER has cautioned against complete reliance on $^{32}P_i$ incorporation into ATP as a measure of ATP synthesis since a $^{32}P_i$–ATP exchange occurs in human hemolysates in the presence of triose phosphate and NAD^+ (ref. 17). It was important,

therefore, to confirm the isotopic data relating to membrane mediated ATP synthesis by conventional chemical means. However, the non-ouabain inhibitable ATPase which accounts for 20–60% of the total erythrocyte membrane ATPase[18] destroyed 67% of ATP added to membranes in preliminary experiments in 10 min at 37° even in the presence of 2.5 mM ouabain. Therefore, hexokinase was sealed into some membrane preparations, and 30 mM glucose was added to the external medium in order to convert both added and newly synthesized ATP into glucose 6-phosphate, hopefully reducing the amount of ATP susceptible to ATPase attack. The glucose 6-phosphate formed was not further degraded by membranes since no $NADP^+$ had been added, and no glucose-6-phosphate dehydrogenase was present[5]. The results of 3 experiments are summarized in Table II. The sum of ATP, measured chromatographically, and glucose 6-phosphate measured enzymatically, was taken to represent the amount of ATP initially present (see experimental procedures) *plus* ATP which had been newly formed. The increase in this quantity with time over comparable values of the controls is recorded as the ΔATP + glucose 6-phosphate. While the numbers are small, there was a consistently demonstrable net increase in ATP

TABLE II

NET SYNTHESIS OF ATP IN MEMBRANES DEMONSTRATED BY STANDARD CHEMICAL METHODS

Approx. $2 \cdot 10^{10}$ membranes containing hexokinase as well as triose phosphate, ADP and NAD^+ were incubated with 20 mM $^{32}P_i$ and 0.1 mM ouabain for the designated times at 37°. 30 mM glucose was added to all tubes except those reported in the last column. Results are given as μmoles per $2 \cdot 10^{10}$ membranes. The term ΔATP + Glc-6-*P* indicates the increase of that sum over the control value. In the controls, Tris buffer was substituted for $^{32}P_i$. [^{32}P]ATP is calculated as described in Table I. Three experiments, A, B, and C, are described.

Expt.	*Control*	*Time of incubation*		
		15 min	*30 min*	*30 min**
ATP				
A	0.52	0.35	0.38	0.70
B	0.43	0.28	0.33	0.49
C	0.45	0.25	0.30	—
Glc-6-*P*				
A	0.04	0.31	0.33	0
B	0	0.20	0.26	0
C	0.05	0.33	0.38	—
ATP + Glc-6-*P*				
A	0.56	0.66	0.71	0.70
B	0.43	0.48	0.59	0.49
C	0.50	0.58	0.68	—
ΔATP + Glc-6-*P*				
A	—	0.10	0.15	0.14
B	—	0.05	0.16	0.06
C	—	0.08	0.18	—
[^{32}P]ATP				
A	—	0.05	0.09	0.13
B	—	0.08	0.12	0.11
C	—	0.10	0.14	—

Abbreviation: Glc-6-*P*, glucose 6-phosphate.
* No glucose added.

which was in the same order of magnitude as the isotopic data presented in Table II as [^{32}P]ATP. There was no advantage in the use of the glucose 6-phosphate trap.

Effects of Na^+ and K^+ on ATP synthesis

External K^+ and internal Na^+ are known to stimulate at least one of the erythrocytic membrane ATPases[18,19] and hence ATP breakdown. To determine whether these vectorial ionic considerations affected the measurement of ATP synthesis by membranes, reactions were run in which the internal and external cationic compositions were independently varied. Membranes were prepared so that they contained either K^+ or Na^+ as the major internal cation. These membranes were then incubated with $^{32}P_i$ in solutions which contained either K^+ or Na^+ as the major external cation. Where the internal and external cations differed, a parallel reaction was run containing ouabain. The chloride salts were used throughout. As seen in Table III, the preservation of ATP was slightly improved in the presence of ouabain; however, there was no consistent effect of cationic variation on our measurement of ATP synthesis by either of the two methods used.

TABLE III

ATP SYNTHESIS WITH VARYING INTRACELLULAR AND EXTRACELLULAR CONTENT OF Na^+ AND K^+

All reactions contained 30 mM glucose and were incubated for 30 min at 37° .The $^{32}P_i$ composition of the medium was 30 mM in Expt. A and 40 mM in Expt. B. When ouabain was used, the concentration was 0.1 mM in Expt. A and 0.4 mM in Expt. B. The cation content of K^+ loaded membranes was 140 mM K^+ and 0.3 mM Na^+ in Expt. A and 156 mM K^+ and 0.6 mM Na^+ in Expt. B. The cation content of Na^+ loaded membranes was 159 mM Na^+ and 0.6 mM K^+ in Expt. A and 152 mM Na^+ and 0.6 mM K^+ in Expt. B. The K^+ external medium contained 140 mM K^+ and 1.6 mM Na^+, while the Na^+ medium contained 141 mM Na^+ and 0.1 mM K^+. Values in the table are given as μmoles per $2 \cdot 10^{10}$ membranes. The terms Δ ATP + Glc-6-*P* and [^{32}P]ATP are as described in prior tables and the text. Two Expts., A and B, are described.

Cation	*ΔATP + Glc-6-P*		*[^{32}P]ATP*	
	A	*B*	*A*	*B*
Intramembrane K^+ extracellular				
K^+	0.10	0.24	0.08	0.13
Na^+	0.08	0.29	0.10	0.12
Na^+ and ouabain	0.25	0.35	0.10	0.12
Intramembrane Na^+ extracellular				
K^+	0.14	0.24	0.10	0.12
Na^+	0.23	0.27	0.12	0.07
K^+ and ouabain	0.24	0.24	0.12	0.13

DISCUSSION

The synthesis of ATP by the human erythrocyte membrane was studied in a system comprised of the membrane's own permeability characteristics, its bound enzymes and carriers, and containing within it only designated substrates and cofactors. External $^{32}P_i$ traversed the membrane, and then essentially only in the presence of ADP, NAD^+ and triose phosphate was partially converted into intracellular ATP. The synthesis of ATP seemed to be time dependent and occurred *via* the membrane bound glyceraldehydephosphate dehydrogenase and phosphoglycerate

kinase system since all three substrates *plus* P_i were required and $^{32}P_i$ was incorporated into ATP. Of the alternative possible routes of ATP synthesis, adenylate kinase would have required only ADP and would not have resulted in $^{32}P_i$ incorporation into ATP. It is possible that some of the [^{32}P]ATP formed could have resulted from a $^{32}P_i$–ATP exchange which has been described in hemolysates supplied with NAD^+ and triose phosphate[17]. However, the net synthesis of ATP was demonstrated both by isotopic and standard chemical means, and both methods yielded comparable results.

In prior studies[6,20] erythrocyte membranes or membrane fragments which were freely permeable to the substrates used did synthesize ATP from NAD^+, ADP and triose phosphate added to the medium. This finding simply confirmed the presence of glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase in the membranes, but said nothing about organization or linkage of enzymes, since the free passage of substrates through the membranes allowed abundant interchange of intermediates between enzymes. The current report emphasized organizational aspects of ATP synthesis and utilized a preparation of erythrocyte membranes which approximated the permeability characteristics of intact erythrocytes, including their relative impermeability to phosphorylated intermediates. The results indicated that membrane bound glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase are positioned or linked so that intracellular substrates can be attacked or transferred between enzymes with the resultant synthesis of ATP. Since the intramembrane ATP concentration is 10 times as high as the ATP concentration in the medium, it is proposed that the ATP synthesized stays within or on membranes.

It was anticipated that a requirement for active cation transport would have reduced the amount of intramembrane ATP (refs. 3, 18) and that this decrease in ATP would in turn have been reversed by ouabain. In fact, this was not observed, and we can only offer the following alternative explanations: (1) the method was not sensitive enough to detect the changes, (2) the additional Na^+–K^+-stimulated ATPase activity[3,18] was not significant when compared to that already functioning, or (3) increased utilization of ATP served as a further stimulus for ATP synthesis.

It has been demonstrated that membrane bound enzymes are organized and linked so that they can use intracellular glycolytic substrates and either intracellular or extracellular P_i to synthesize intramembrane ATP. This newly formed ATP is adjacent to the membrane, and its possible role as preferred substrate for shape maintenance or membrane ATPase in Na^+–K^+ transfer remains to be determined.

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