EVIDENCE FOR A PHOSPHORYLATED INTERMEDIATE OF RED-CELL MEMBRANE ADENOSINE TRIPHOSPHATASE

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A phosphorylated intermediate of the Mg-dependent and Na- and K-activated ATPase has been identified in several tissues (Post et al., 1965; Albers et al., 1963; Skou, 1960). Although Na and K transport is related directly to ATPase activity in the red cell, a phosphorylated intermediate of red-cell ATPase could not be demonstrated by Heinz and Hoffman (1965). We have confirmed their observations that such an intermediate could be obscured by the relatively large amount of stable ^{32}P-labeled material formed during incubation of the membranes at 37° with γ-^{32}P-ATP (Blostein and Cooper, 1966). This material failed to undergo turnover, as was reflected in no loss of radioactivity upon addition of excess nonradioactive ATP. Also, in contrast to observations made with other tissues (Post et al., 1965), the addition of K ions after pre-labeling with Mg and Na ions alone failed to dissociate bound ^{32}P from membrane material.

Evidence is presented that during short-period incubation of red-cell membranes with γ-^{32}P-ATP at 0° there is rapid uptake and turnover of ^{32}P consistent with the existence of an intermediate of Mg-dependent ATPase activity. It is probable that at this temperature this active fraction can be demonstrated only because the rate of formation of stable ^{32}P-labeled material is greatly reduced.

Materials and Methods

Erythrocyte membranes were prepared by osmotic lysis of fresh saline-washed human erythrocytes. After centrifugation, the post-hemolytic residue was washed four times with 1 mM Tris HCl containing 1 mM EDTA, pH 7.5, and then once with 2 mM Tris HCl; the residue was made up to one-half the original packed cell volume of the washed cells. The preparation obtained was equivalent to approximately 15 X 10^9 cells/ml and 5 mg protein/ml. ATPase activity was 12 mμmoles ADP or P_i liberated/min/mg membrane protein at 37°, of which 40% was ouabain-sensitive. The nucleoside diphosphokinase of the erythrocyte (Mourad and Parks, 1965) was absent in the washed membrane preparation. The preparation was frozen and thawed twice before use, to ensure complete permeability of substrates. γ-^{32}P-ATP was prepared according to the method of Pfleiderer (1961) and purified on Dowex-1(Cl^-) according to the procedure of Glynn and Chappell (1964). Other reagents were purchased from Sigma Chemical Co.

Results and Discussion

The results depicted in Fig. 1 demonstrate the kinetics of labeling and $^{32}P_i$ release at 0° during short-period incubation of red-cell membranes with γ-^{32}P-ATP. At this temperature only 1% of the ATP was hydrolyzed during

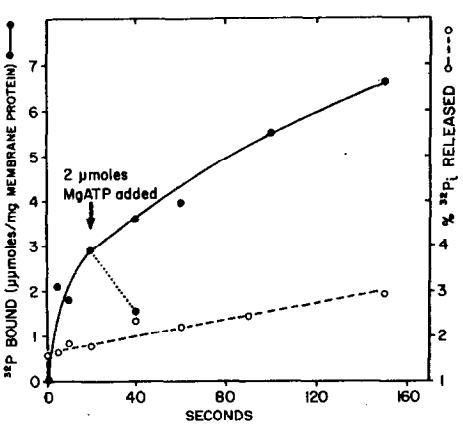

FIG. 1. FORMATION AND TURNOVER OF ^{32}P-LABELED MATERIAL IN RED-CELL MEMBRANES. 0.24 ml membranes (1.1 mg protein) was added to the reaction medium, which consisted of 0.12 mM $MgCl_2$, 50 mM NaCl, 10 mM KCl, 0.02 mM Tris ATP; final vol 0.4 ml, containing γ-^{32}P-ATP, 8.7 X 10^5 cpm. The reaction was terminated with 9 vol 5% trichloroacetic acid containing 5 mM P_i and 2.5 mM ATP. The precipitate obtained after centrifugation was washed 5 times with 4 ml of the same buffer, dissolved in 0.1 N NaOH. Aliquots were removed for determination of protein and radioactivity. Incubation and all maniputations were carried out at 0°.

the 2-min period. Uptake of ^{32}P by the membranes was rapid initially (2 μμmoles P/mg protein within 5 sec) but then slowed to an almost linear rate of 0.03 μμmoles/mg/sec. The striking reduction in radioactivity, equivalent to 1.4 μμmoles bound P/mg, induced by a 'chase' of excess nonradioactive Mg ATP, indicates rapid turnover of the material that was labeled in the early stages. Extraction of phospholipids from the labeled material failed to remove bound radioactivity. Uptake was abolished by omission of Mg ions or addition of EDTA. However, the addition of ouabain (0.2 mM) failed to affect significantly either ATPase activity, as determined by $^{32}P_i$ release, or the amount of bound ^{32}P at 0°.

The turnover of bound ^{32}P has been considered to be due to (1) Mg-dependent ATPase activity (according to equations 1A and 1B) and/or (2) unrelated ATP–ADP exchange(s), as in equation 2 :

Equation 1A: $ATP + X \rightleftharpoons ADP + XP$

Equation 1B: $XP \longrightarrow X + P_i$

Equation 2: $ATP + Y \rightleftharpoons ADP + YP$

where the solid arrows designate routes of breakdown of labeled material.

The presence of (^{14}C)ADP–ATP exchange activity at 0° is indicated in Fig. 2, from which data the rate of exchange of labeled ADP into ATP was calculated (Expt I, Table 1).

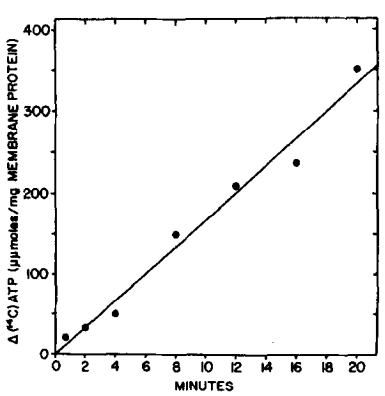

FIG. 2. (^{14}C)ADP–ATP EXCHANGE REACTION OF RED-CELL MEMBRANES AT 0°. Conditions were as described in the text and in Fig.1; final membrane protein concentration was 1.2 mg/ml. The reaction was terminated with an equal volume of 5% trichloroacetic acid containing 2 mM each of ATP, ADP, and AMP. The nucleotides were separated according to the method of Wadkins and Lehninger (1958) and their radioactivity determined. The results were corrected for low adenylate kinase activity by subtracting cpm in AMP from cpm in ATP. (^{14}C)ADP, 4.6×10^5 cpm (8.7 nμmoles) per ml.

TABLE 1. COMPARISON OF THE MINIMAL RATE OF BREAKDOWN OF ^{32}P-LABELED MATERIAL WITH THE RATE OF (^{14}C)ADP–ATP EXCHANGE

Experiment	(^{14}C)ADP–ATP exchange	Loss of bound ^{32}P
	μμmoles/mg/sec	μμmoles/mg/sec
I	0.3	$\geq$ 0.5
II	0.3	$\geq$ 0.8

Conditions were as described in the text and in Fig. 1 and 2. Final membrane protein concentration was 0.9 mg/ml in Expt I and 1.2 mg/ml in Expt II.

The exchange data are expressed in absolute amounts based on initial concentration and specific radioactivity of labeled ADP. This was possible since the reaction rate was linear and the change in ATP and ADP concentrations due to ATPase activity at 0° was $\leq$ 10% in 20 min. The rate of exchange thus obtained was compared with the minimal rate of loss of bound ^{32}P. This determination was carried out simultaneously with and under conditions identical to those for the exchange experiment, by labeling the membranes with ^{32}P as described in Fig. 1. A 'chase' of excess Mg ATP was added at 30 sec and the reaction was terminated at 2, 5, and 8 sec thereafter. Since it was found that the amount of decrease in ^{32}P following the chase was maximal in 2 sec, the loss of bound ^{32}P calculated on the basis of the 2-sec value is a minimal value. It is evident from the data summarized in Table 1 that the minimal rate of loss of bound ^{32}P is approximately twice the net rate of transphosphorylation as measured by the (^{14}C)ADP–ATP exchange. The difference between these values (0.2 and 0.5 μμmoles in Expt I and II, respectively) probably is due to hydrolysis of the bound ^{32}P, as depicted in Equation 1B.

It is possible that the (^{14}C)ADP–ATP exchange does not measure the true rate of transphosphorylation, i.e., that the rate of interchange of ADP with non-covalently-bound ADP, formed from ATP, is slow compared with the rate of transphosphorylation between a phosphorylated intermediate and bound

ADP, as pointed out by Boyer (1960). However, the observed rates of formation (Fig. 1) and breakdown (Table 1) of the intermediate are of an order of magnitude similar to the rate of $^{32}P_i$ release under similar conditions, as illustrated in Fig. 1 (0.5 μmoles/mg/sec). Therefore, it is probable that the observed turnover of bound ^{32}P is related to a significant extent to the reactions which constitute ATPase activity and which are outlined in Equations 1A and 1B.

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