Temperature Effects on Sodium Pump Phosphoenzyme Distribution in Human Red Blood Cells

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ABSTRACT Phosphorylation of red cell membranes at ambient temperatures with micromolar [^{32}P]ATP in the presence of Na ions produced phosphoenzyme that was dephosphorylated rapidly upon the addition of ADP or K ions. However, as first observed by Blostein (1968, *J. Biol. Chem.*, 243:1957), the phosphoenzyme formed at 0°C under otherwise identical conditions was insensitive to the addition of K ions but was dephosphorylated rapidly by ADP. This suggested that the conformational transition from ADP-sensitive, K-insensitive Na pump phosphoenzyme ($E_1 \sim P$) to K-sensitive, ADP-insensitive phosphoenzyme ($E_2 P$) is blocked at 0°C. Since the ATP:ADP exchange reaction is a partial reaction of the overall enzyme cycle dependent upon the steady state level of $E_1 \sim P$ that is regulated by [Na], we examined the effects of temperature on the curve relating [Na] to ouabain-sensitive ATP:ADP exchange. The characteristic triphasic curve seen at higher temperatures when [Na] was between 0.5 and 100 mM was not obtained at 0°C. Simple saturation was observed instead with a $K_{0.5}$ for Na of ~1 mM. The effect of increasing temperature on the ATP:ADP exchange at fixed (150 mM) Na was compared with the effect of increasing temperature on (Na + K)-ATPase activity of the same membrane preparation. It was observed that (*a*) at 0°C, there was significant ouabain-sensitive ATP:ADP exchange activity, (*b*) at 0°C, ouabain-sensitive (Na + K)-ATPase activity was virtually absent, and (*c*) in the temperature range 5–37°C, there was an ~300-fold increase in (Na + K)-ATPase activity with only a 9-fold increase in the ATP:ADP exchange. These observations are in keeping with the suggestion that the $E_1 \sim P \rightarrow E_2 P$ transition of the Na pump in human red cell membranes is blocked at 0°C. Previous work has shown that the inhibitory effect of Na ions and the low-affinity stimulation by Na of the rate of ATP:ADP exchange occur at the extracellular surface of the Na pump. The absence of both of these effects at 0°C, where $E_1 \sim P$ is maximal, supports the idea that external Na acts through sites on the $E_2 P$ form of the phosphoenzyme.

INTRODUCTION

Characterization of the transport processes mediated by the Na pump has relied extensively on information obtained from studies on human red blood cells. The

availability of resealed ghost preparations (Hoffman, 1962; Schwoch and Passow, 1973; Wood and Passow, 1981) has enabled the investigator to independently vary cation concentrations in the cytoplasmic and extracellular compartments and to characterize the cation activation of transport. The accessibility of the cytoplasmic compartment at hemolysis has also allowed the nucleotide requirements of the various transport modes of the Na pump to be characterized (Glynn and Hoffman, 1971; Glynn et al., 1971; Glynn and Karlish, 1976; Kaplan and Kenney, 1982). Recent work using resealed ghosts or inside-out vesicles (Blostein, 1983) has attempted to relate the biochemical partial reactions of (Na + K)-ATPase to the various transport modes of the Na pump in human red blood cells.

The sidedness of the cation-activating effects of the ouabain-sensitive ATP:ADP exchange reaction has been recently characterized (Kaplan and Hollis, 1980; Kaplan, 1982). This reaction involves phosphorylation of the pump protein by ATP followed by the phosphorylation of ADP by the Na pump phosphoenzyme and it is probably the biochemical transformation accompanying the Na:Na exchange mode of the Na pump (Garrahan and Glynn, 1967; De Weer, 1970, 1983; Glynn and Hoffman, 1971; Kaplan, 1982, 1983). The activation of ATP:ADP exchange by Na ions shows three distinct phases in both kidney enzyme preparations (Beaugé and Glynn, 1979; Kaplan et al., 1981) and porous red cell membranes (Kaplan and Hollis, 1980). These consist of a high-affinity activation in the range of 0–5 mM Na, a high-affinity inhibitory phase in the range of 5–15 mM Na, and a low-affinity stimulation that does not saturate in the physiological range and progressively increases between ~20 and 200 mM Na. The low-affinity stimulation by Na ions has been accounted for in terms of the steady state distribution of Na pump phosphoenzyme between the $E_1\sim P$ (ADP-sensitive, K-insensitive) and E_2P (ADP-insensitive, K-sensitive) forms as originally described in Na pump models by Post et al. (1969) and Albers et al. (1968). As [Na] is increased, the distribution of phosphoenzyme between the $E_1\sim P$ and E_2P forms shifts in favor of the $E_1\sim P$ form.

The effects of Na ions on the $E_1\sim P:E_2P$ distribution have not been characterized for the red cell Na pump, although many studies have been carried out using (Na + K)-ATPase enzyme from a variety of other sources with higher specific activities (Yoda and Yoda, 1982; Kuriki and Racker, 1976; Hara and Nakao, 1981). In order to directly relate the results of the ATP:ADP exchange reaction in resealed ghosts to red cell Na pump phosphoenzyme forms, we initiated studies to characterize the phosphoenzyme distribution in red cell membranes. Initial experiments were carried out at 0°C, and an observation previously made by Blostein (1968) was confirmed. At 0°C, the red cell Na pump phosphoenzyme is insensitive to the addition of K ions (Blostein, 1968) and is rapidly dephosphorylated by ADP; at higher temperatures, it shows sensitivity to both K and ADP. This response to changes in temperature is not shown by the kidney enzyme (Post et al., 1969; White and Blostein, 1982; Hara and Nakao, 1981) or brain enzyme (Nørby et al., 1983), where the phosphoenzyme at 0°C is composed of both the $E_1\sim P$ and E_2P forms. We have investigated the consequences of the temperature response of the red cell Na pump phosphoenzyme

to partial pump reactions that depend upon either $E_1 \sim P$ levels (the ATP:ADP exchange) or E_2P levels [the (Na + K)-ATPase activity]. A brief description of some of these results has appeared previously (Kaplan and Kenney, 1983).

MATERIALS AND METHODS

Preparation of Red Cell Membranes

Membranes were prepared from freshly drawn blood according to the method of Wood and Passow (1981) with some modifications (Kaplan, 1982). Following hemolysis on the gel filtration column, the fractions containing membranes were pooled and centrifuged at 20,000 g for 5 min at 0°C, the supernatants were removed, and the membranes were washed by repeated centrifugation and resuspension in a phosphate buffer (5 mM Na_2HPO_4, pH 8.0) until white. The membranes were then frozen, thawed, and washed twice in 10 mM Hepes-Tris, 5 mM EDTA-Tris, pH 7.5, followed by two washes and a final resuspension in 20 mM Tris, pH 7.5 (with HCl). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The membranes were frozen and stored at −20°C.

Measurement of ATP:ADP Exchange Activity

The ATP:ADP exchange activity was measured in a suspension containing 50 μl of buffer and salts and 50 μl of membrane suspension. The assay was initiated by the addition of membranes. The final concentrations of the components of the assay were: 10 μM $MgCl_2$, 25 μM ATP, 25 μM [³H]ADP, 10 mM Hepes (pH 7.5 with Tris), and NaCl plus choline Cl, so that their sum in most experiments was 150 mM. At each Na concentration, samples of membranes and diadenosine pentaphosphate (300 μM) were preincubated at 22°C with ouabain (2 × 10⁻⁴ M) for 10 min before the assay to ensure maximal inhibition by ouabain. In the experiments shown in Fig. 3, NaCl plus choline Cl was 450 mM. At the high membrane concentrations employed, allowance was made for the dilution of the salts by the membrane suspension. This dilution was determined by measuring the [ATP] before and after the addition of membranes to the salt solution. [ATP] was measured by the method of Kimmich et al. (1975) and was found to be diluted 1.67-fold after addition of membranes (rather than 2-fold, if the membranes occupied negligible volume). The concentrations of buffers, salts, and nucleotides in the solution (50 μl) to which the membrane suspension was added were 1.67-fold higher than in the final assay. Reaction times were chosen so that ATP hydrolysis during the assay was low (<7%). The ATP:ADP exchange assay was terminated by mixing a 20-μl sample with 10 μl of 10% trichloroacetic acid (TCA) at 60 (0°C), 15 (22°C), or 2 min (37°C), when the rates were linear. The samples were frozen before processing and the thawed samples were processed according to the method of Kaplan and Hollis (1980). Exchange activity was expressed as nanomoles P_i transferred to ADP per milligram membrane protein per hour. The difference between the exchange activity in the absence and presence of ouabain (10⁻⁴ M) was taken as the Na pump-mediated component of the exchange activity.

Phosphorylation of Red Cell Membranes

Approximately 300 μg of membranes was suspended in a total volume of 400 μl containing 20 mM NaCl, 0.1 mM $MgCl_2$, and 25 μM EGTA. Blanks contained 20 mM KCl instead of NaCl. The reaction was initiated by the addition of a 2-μM solution of ATP (40 μl) with 0.4 μCi [³²P]ATP (final [ATP] = 0.2 μM). After 15 (0°C), 10 (22°C), or 3 s (37°C), the reaction was terminated by adding 10 ml of a 10% TCA solution containing 1 mM

ATP and 10 mM phosphate (at 0°C) and poured onto a Millipore glass fiber filter (24 mm) in a Millipore filtration manifold and filtered under vacuum pump aspiration. The filter was washed five times with 15 ml of the TCA solution, and the Cerenkov radiation of the ^{32}P remaining on the filter was counted in an LS 7500 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). In dephosphorylation experiments, instead of stopping the reaction at the times specified above, a 1-ml solution of 25 mM CDTA (1,2-cyclohexanediaminetetracetic acid) plus 20 mM NaCl was added, and the reaction was terminated 10 or 20 s later as described above. In experiments where the sensitivity of phosphoenzyme to ADP or K ions was examined, the CDTA solution contained 25 mM ADP or 25 mM KCl, respectively, in addition to NaCl.

Measurement of (Na + K)-ATPase Activity

The (Na + K)-ATPase activity of red cell membranes was assayed using a modification of the method of Brotherus et al. (1981) in a total volume of 0.6 ml containing the following: 125 mM NaCl, 25 mM KCl, 4 mM $MgCl_2$, 4 mM Na ATP, 60 mM Tris-HCl, 0.75 mM EDTA, pH 7.5. The assay was initiated by the addition of the membrane suspension (100 μl), which had been preincubated for 10 min at 22°C in the presence or absence of ouabain (10^{-4} M). The amount of protein used varied with the temperature of incubation, and was ~1.0 mg for 0°C and 0.5 mg for 5, 10, 22, and 37°C. The incubation time also varied with the assay temperature and was 24 h for 0, 5, and 10°C, 90 min for 22°C, and 20 min for 37°C. The assay was terminated by the addition (1 ml) of a solution that contained 1% ammonium molybdate, 0.5 M HCl, 1.5% sodium dodecyl sulfate, and 170 mM ascorbic acid, and the samples were then incubated at 0°C for 10 min. This was followed by the addition of a 1.5-ml solution of 70 mM Na citrate, 155 mM Na arsenite plus glacial acetic acid (20%), and incubation at 37°C for 5 min. After 20 min at 22°C, the optical density of the samples at 850 nm was determined on a Beckman UV 24 spectrophotometer, and compared with the optical density of Na_2HPO_4 standards prepared in the appropriate range.

The ATPase activity is expressed as nanomoles P_i liberated per milligram protein per hour, and the difference between the activity measured in the absence and presence of ouabain (10^{-4} M) was taken as the Na pump-mediated component of the activity. All activities were corrected for nonenzymatic hydrolysis of ATP by the inclusion of blanks that did not contain membranes.

The two Na pump activities measured and compared in this work were assayed under different conditions. The Na,K-ATPase was measured under optimal conditions for this activity. The ATP:ADP exchange activity was measured in the absence of K ions, at low nucleotide levels, and with low [Mg]. The low nucleotide levels were employed to make this work compatible with our previous studies on red cell membranes where the Na activation properties were characterized at 22°C, and where K ions and elevated Mg levels were shown to inhibit the ATP:ADP exchange activity (Kaplan, 1982; Kaplan and Hollis, 1980).

Materials

[^{3}H]ADP and [γ^{32}P]ATP were obtained from Amersham Corp., Arlington Heights, IL. Salts, buffers, ADP, ATP (grade 1, prepared from phosphorylation of adenosine), Luciferin-Luciferase (type L-0633), TCA, and phosphoenolpyruvate were obtained from Sigma Chemical Co., St. Louis, MO. Pyruvate kinase and diadenosine pentaphosphate were from Boehringer Mannheim Biochemicals, Indianapolis, IN. The polyethyleneimine thin-layer chromatography plates used for nucleotide separation were obtained from

Brinkmann Instruments, Inc., Des Plaines, IL. The membranes were prepared on a K100/45 gel filtration column from Pharmacia Fine Chemicals, Piscataway, NJ. The beads used in the column were Bio-Gel A-50m from Bio-Rad Laboratories, Richmond, CA. The screen mesh for the column was 44-μm mesh from Small Parts, Inc., Miami, FL. Millipore filters were type AP40 microfiber glass disks (without binder resin) obtained from Millipore Corp., Bedford, MA.

RESULTS

Phosphorylation of Red Cell Membranes

When red cell membranes were incubated in 20 mM NaCl plus 0.1 mM MgCl$_2$ and phosphorylation was initiated by the addition of 0.2 μM [^{32}P]ATP, an acid-stable phosphoenzyme was formed (see Materials and Methods). In Table I are

TABLE I

Sensitivity of Na Pump Phosphoenzyme (EP) to Ligands

Temperature	Fraction of EP remaining		
	CDTA	CDTA + ADP	CDTA + K
°C			
0	0.81	0.33	0.89
22	0.42	0.24	0.23
37	0.37	0.51	0.10

Phosphorylation and dephosphorylation were performed as described in Materials and Methods. The fraction of acid-stable EP remaining after phosphorylation had been halted by the addition of the ligands shown in the table was determined. The added ligands were either Na (20 mM) + CDTA (25 mM, column 2), or Na + CDTA + ADP (25 mM, column 3), or Na + CDTA + K (25 mM, column 4). The ligands were present for 15 (0°C), 10 (22°C), or 3 s (37°C) before dephosphorylation was halted by the addition of TCA (see Materials and Methods). The values of the fraction of EP remaining varied from different membrane preparations, which reflects the difficulty in measuring EP levels in such a low specific activity preparation. However, at 37°C, the presence of K ions always gave a substantially lower level of EP remaining than did ADP, and at 0°C, this situation was always reversed. The level of Na-dependent phosphorylation of red cell membranes varied between 0.2 and 0.5 pmol/mg protein in different membrane preparations from different donors in the temperature range 0–37°C. The experimental results shown are representative of at least three additional experiments of the same type. Each determination was carried out in duplicate.

shown the effects of the addition of K ions and of ADP on the amount of phosphoenzyme when phosphorylation was halted by the addition of CDTA (with 20 mM Na to keep [Na] constant). At elevated temperatures (>0°C), the addition of K ions caused a more rapid breakdown of phosphoenzyme. At 0°C, however, the addition of K ions had little effect, while ADP caused a rapid dephosphorylation. These observations are similar to those previously reported by Blostein (1968). The lack of sensitivity of phosphoenzyme to the addition of K ions at 0°C is independent of the [Na] present during phosphorylation (not shown).

Effect of Temperature on Na Activation of ATP:ADP Exchange

The ATP:ADP exchange rate was measured using porous red cell membranes (see Materials and Methods) and the effect of [Na] on the ouabain-sensitive component of the activity was examined. As previously described (Kaplan and Hollis, 1980), a triphasic curve is observed at 22°C (Fig. 1). The curve is
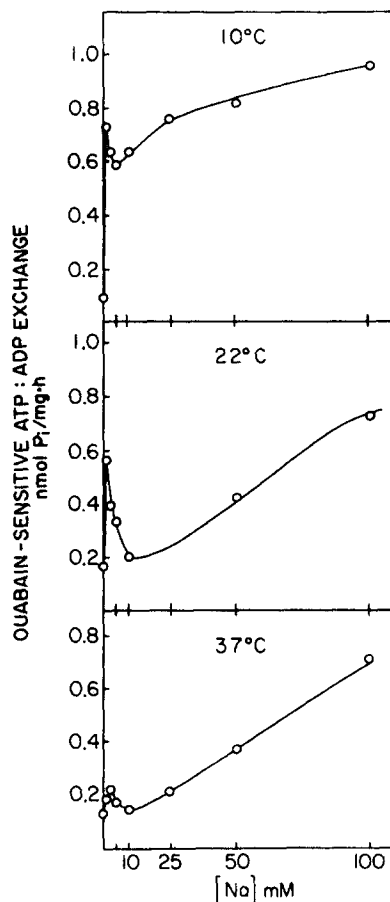

FIGURE 1. Activation of ouabain-sensitive ATP:ADP exchange in red blood cell membranes by Na ions. The measurements of ATP:ADP exchange were carried out as described in Materials and Methods at 37, 22, and 10°C. The points shown are the means of the duplicate determinations; the range of each pair is within the size of the points in the figure.

composed of an activating limb (0–1 mM Na), followed by inhibition in the range 3–10 mM Na, and finally a low-affinity stimulation is seen that does not saturate in the concentration range tested. At 37, 22, and 10°C, the overall pattern is the same (Fig. 1). Although the shapes of the curves differ in detail, they are all composed of both activating phases (low and high affinity) and an inhibitory phase. At 5°C and below, the Na activation of ATP:ADP exchange is altered

(Fig. 2). At this temperature, only high-affinity activation is observed, and the curve relating [Na] and ouabain-sensitive ATP:ADP exchange activity shows simple saturation kinetics with a $K_{0.5}$ for Na at ~1 mM.

Since saturation was not observed at temperatures >5°C and at [Na] within the physiological range, we examined the effects of higher (150–450 mM) [Na] on exchange at three different temperatures. The results are shown in Fig. 3. Increasing [Na] had no effect on the ATP:ADP exchange at 0°C, as saturation had already occurred at a much lower [Na] (see Fig. 2). However, at 22 and 37°C, the exchange rate was stimulated by increasing [Na].
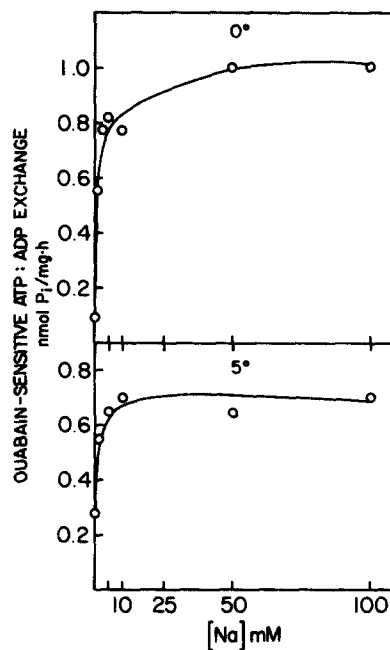

FIGURE 2. Activation of ouabain-sensitive ATP:ADP exchange in red cell membranes by Na ions. Measurements of ATP:ADP exchange were carried out as described in Materials and Methods at 0 and 5°C.

Temperature Dependence of (Na + K)-ATPase Activity

Having characterized the temperature dependence of the ATP:ADP exchange, a reaction dependent upon $E_1\sim P$, we then examined the effects of temperature on an enzyme reaction that involves E_2P levels, the (Na + K)-ATPase activity. In Fig. 4, the results of such studies are shown. At 37°C, there is significant ouabain-sensitive (Na + K)-ATPase activity (80% of total ATPase activity), but as the temperature is decreased, the ouabain-sensitive component of total ATPase is progressively reduced. For example, at 5°C, there is a small but significant ouabain-sensitive (Na + K)-ATPase activity; at 0°C, this activity is absent. The lack of ouabain sensitivity at 0°C is not due to a lack of sensitivity of the assay under these conditions. At 0°C, greatly extended (24 h) assay times and higher
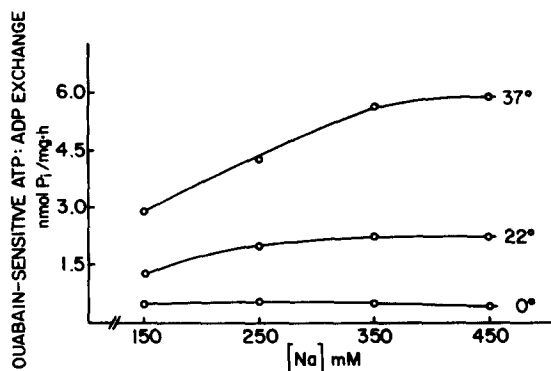
FIGURE 3.   The effects of elevated |Na] on ouabain-sensitive ATP:ADP exchange in red cell membranes. Measurements of ATP:ADP exchange were carried out as described in Materials and Methods at 0, 22, and 37°C. The sum of NaCl plus choline Cl was held constant at 450 mM.

protein concentrations (10 mg/ml) were used in order to obtain adequate hydrolysis of ATP, and no ouabain sensitivity of this hydrolysis was observed. The activity measured at 37°C in the present study (~300 nmol P_i/mg protein · h) agrees well with previous estimates made under similar experimental conditions (~400 nmol P_i/mg protein·h; Bond and Hudgins, 1981).

Since we had observed appreciable variation in the ATP:ADP exchange activities of different membrane preparations, we examined the effect of temperature on both (Na + K)-ATPase activity and ATP:ADP exchange (in the presence of 150 mM Na) in the same membrane preparation. The results of these experiments are shown in Fig. 5, where the ouabain-sensitive components of the measured activities are presented. Several points emerge from these data. At 0°C, there is significant ATP:ADP exchange activity, but no (Na + K)-ATPase activity; in the temperature range 5–37°C, there is a 300-fold increase
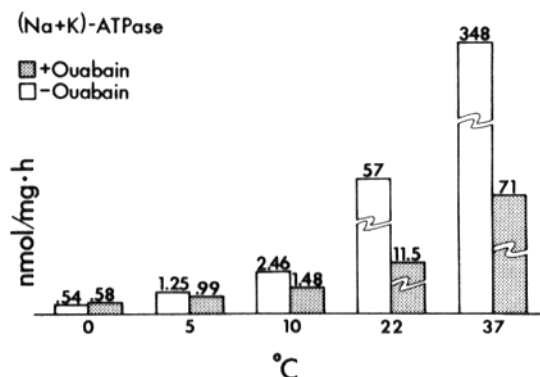

FIGURE 4.   The effect of increasing temperature on (Na + K)-ATPase activity of red cell membranes. The (Na + K)-ATPase activity of red cell membranes was determined as described in Materials and Methods over the range of temperatures indicated in the figure.

FIGURE 5.   A comparison of the effects of increasing temperature on the ouabain-sensitive ATP:ADP exchange and ouabain-sensitive (Na + K)-ATPase activity of a single preparation of red cell membranes. The (Na + K)-ATPase activities and the ATP:ADP exchange activities of the same preparation of red cell membranes were determined as described in Materials and Methods over the temperature range indicated in the figure.

in (Na + K)-ATPase activity and only a 9-fold increase in the ATP:ADP exchange activity.

DISCUSSION

The involvement of phosphorylated enzyme intermediates in the reaction cycle of the Na pump was first proposed by Post and Albers and co-workers (Post et al., 1969; Albers et al., 1968) and remains the basis of the major current working hypothesis of the Na pump. The Post-Albers scheme can be schematized as follows:

FIGURE 6.   A simplified scheme for the reaction cycle of the Na pump.

The conformational transition between the two major phosphoenzymes, $E_1 \sim P$ (ADP-sensitive, K-insensitive) and E_2P (ADP-insensitive, K-sensitive), is directly involved in the Na translocation step of the transport cycle. Studies on Na pump enzyme from a variety of sources have demonstrated the simultaneous presence of the $E_1 \sim P$ and E_2P forms of the phosphoenzyme. The findings described in the present paper confirm earlier observations by Blostein (1968), which indicated that in the red cell, the Na pump shows a striking temperature dependence,

and at $0°C$, only the $E_1 \sim P$ form is produced following Na-dependent phosphorylation by ATP (see Table I). This suggests that at $0°C$, the conformational transition $E_1 \sim P \rightarrow E_2P$ is blocked and is in contrast to the results of studies on kidney enzyme (White and Blostein, 1982; Post et al., 1969; Hara and Nakao, 1981), brain (Plesner et al., 1981), and electroplax (Kuriki and Racker, 1976), where at $0°C$, the phosphoenzyme is composed of both the $E_1 \sim P$ and E_2P forms, which are interconvertible. In kidney (Hara and Nakao, 1981), high concentrations of Na ions are needed to generate phosphoenzyme predominantly in the $E_1 \sim P$ form at $0°C$, and high concentrations of Na ions are required to drive the $E_2P \rightarrow E_1 \sim P$ transition (Taniguchi and Post, 1975). It has recently been shown (Nørby et al., 1983) that Tris-Cl can influence the conformation of the enzyme in favor of E_1 forms. It is possible that Tris-Cl might also influence the $E_1 \sim P \rightarrow E_2P$ transition and the temperature response of this equilibrium.

The partial reaction of the (Na + K)-ATPase, which depends upon the steady state levels of $E_1 \sim P$, is the ATP:ADP exchange reaction. This was first characterized by Fahn et al. (1966a, b) in partially purified enzyme preparations from electric eel. The reaction consists of the phosphorylation of Na pump enzyme by ATP and the subsequent phosphorylation of ADP by the phosphoenzyme $E_1 \sim P$. The Na activation of this reaction has been characterized in kidney (Beaugé and Glynn, 1979; Kaplan et al., 1981) and in porous red cell membranes (Kaplan and Hollis, 1980, and this work). The Na activation curve is composed of three phases: (a) a high-affinity stimulation (0.5–3 mM Na), (b) a high-affinity inhibitory phase (5–15 mM), and (c) a low-affinity stimulation (25–150 mM Na) that does not saturate in the physiological range. Recent work using caged ATP in resealed ghosts has enabled the localization of these effects to cytoplasmic and extracellular aspects of the Na pump protein (Kaplan and Hollis, 1980; Kaplan, 1982). The high-affinity stimulation is due to internal Na, and presumably reflects the Na requirement for phosphorylation. The inhibitory and low-affinity stimulatory sites are at the extracellular surface of the membrane.

In the present work, the characteristic triphasic curve is seen at all temperatures studied between 10 and $37°C$ (Fig. 1). The low-affinity stimulation of ATP:ADP exchange by Na ions has been interpreted as occurring via the reversal of $E_1 \sim P \rightarrow E_2P$ (Beaugé and Glynn, 1979) at extracellular sites for Na (Kaplan and Hollis, 1980; Kaplan, 1982), leading to a higher steady state level of $E_1 \sim P$ and an enhanced rate of ATP:ADP exchange. Na_o binding takes place at what are external Na unloading sites in Na:K exchange or loading sites for Na:Na exchange. At $0°C$, both the high-affinity inhibition and low-affinity stimulation of ATP:ADP exchange are absent. The simple saturation curve seen at $0°C$ when the activation by Na ions of ATP:ADP exchange is examined (Fig. 2) is similar to that seen at higher temperatures with kidney enzyme after treatment with N-ethylmaleimide (NEM) (Beaugé and Glynn, 1979). The $K_{0.5}$ for Na activation of ATP:ADP exchange in NEM-treated kidney enzyme is ~ 7 mM (Beaugé and Glynn, 1979), while in the red cell membrane Na pump at $0°C$, the $K_{0.5}$ is ~ 1 mM (this work). Treatment of the enzyme with NEM has been shown to block the $E_1 \sim P \rightarrow E_2P$ transition (Fahn et al., 1966a). The absence of low-affinity stimulation by Na ions at $0°C$ is in keeping with the results of the

phosphorylation studies. At $0°C$, all of the phosphoenzyme is in the $E_1\sim P$ form (Blostein, 1968; this work); thus, the steady state level of $E_1\sim P$ is maximal and (external) Na ions that stimulate the exchange at higher temperatures by increasing the $E_1\sim P:E_2P$ ratio are without effect.

The inhibition of the rate of ATP:ADP exchange by low concentrations of Na ions at the external surface of the pump has been interpreted as the effect of Na ions on the rate of phosphoenzyme hydrolysis. Beaugé and Glynn (1979) showed that at $0°C$, low [Na] inhibits the rate of breakdown of EP (presumably E_2P). This would effectively result in making a portion of the enzyme unavailable for ATP:ADP exchange until it is dephosphorylated and rephosphorylated again back to $E_1\sim P$. In the present work, the inhibitory effect of Na ions at low concentration is clearly present at elevated temperatures and is absent at $0°C$ (see Figs. 1 and 2). Since we know that the inhibition occurs at external Na sites (Kaplan and Hollis, 1980; Kaplan, 1982) and is absent at $0°C$ when E_2P is absent, we conclude that the inhibition occurs through Na ions binding to E_2P phosphoenzyme and that E_2P has sites exposed to Na ions at the external surface of the membrane. That E_2P is the form of the phosphoenzyme having sites for Na at the external membrane surface is a point of distinction between the model of the Na pump reaction mechanism of Post et al. (1978) and the later, modified form (Karlish et al., 1978). The latter authors explicitly assumed that all external effects of Na ions occur on the E_2P form of the phosphoenzyme. The present work lends strong experimental support to that assumption. However, the same arguments cannot be applied to the absence of Na stimulation of ATP:ADP exchange. It is tempting to conclude that the present studies support models that have Na unloading from E_2P sites at the external surface after the conformational transition $E_1\sim P \rightarrow E_2P$, as proposed by Karlish et al. (1978), rather than from $E_1\sim P$ (Post et al., 1978; De Weer, 1983). The absence of stimulation by Na_o of ATP:ADP exchange at $0°C$, where E_2P is absent, would be expected in either case. Since $E_1\sim P$ is already maximal at $0°C$, no low-affinity stimulation by Na ions would occur whether Na acts on $E_1\sim P$ or E_2P at the external surface.

A consequence of blocking the $E_1\sim P \rightarrow E_2P$ transition at $0°C$ can be seen when the temperature dependence of (Na + K)-ATPase and ATP:ADP exchange activities are compared (Figs. 4 and 5). The (Na + K)-ATPase activity, which requires the complete cycle including the $E_1\sim P \rightarrow E_2P$ transition, is not detectable at $0°C$, whereas the ATP:ADP exchange proceeds at a significant rate. This supports the conclusion that E_2P is absent at $0°C$. A comparison of the effects of increasing temperature on these reaction rates reveals profound differences. The absolute rates differ by about three orders of magnitude and both increase with increasing temperature. However, between 5 and $37°C$, the (Na + K)-ATPase activity increases by ~300-fold, whereas the ATP:ADP exchange increases by only 9-fold (Fig. 5). This lower sensitivity of the exchange reaction rate to increasing temperature reflects an increase in temperature-sensitive rate constants and the concomitant fall in the proportion of phosphoenzyme that is $E_1\sim P$ as the temperature rises. At a fixed [Na], the ratio $E_1\sim P:E_1\sim P + E_2P$ is greatest at $0°C$ (where it is ~1), and at higher temperatures it is <1.

If unloading of Na ions at the external surface occurs from E_2P as has been

suggested, the present results suggest that ouabain-sensitive Na transport in red blood cells will cease at 0°C. This prediction is difficult to test since the low pump density in red blood cells leads to very small ouabain-sensitive fluxes as the temperature is lowered. Previous studies by Willis et al. (1980) on the effects of temperature on ouabain-sensitive K influx in red blood cells from a variety of species have included data on humans. These authors report that between 5 and 37°C, there is a 400-fold increase in ouabain-sensitive K uptake rates in a situation where the contribution from K:K exchange was likely to be small (Na-loaded cells). This effect is in good agreement with the 300-fold increase in (Na + K)-ATPase activity in red cell membranes over the same range of temperatures reported here (Fig. 5).

It has been suggested that the phosphorylated enzymes seen in Na pump studies in the absence of K ions are not intermediates in the normal working of the pump, i.e., when Na and K ions are both present (Skou, 1975). A recent model for the (Na + K)-ATPase reaction mechanism explicitly models Na-dependent reactions (in the absence of K ions) in terms of the usual phosphoenzyme intermediates and rejects their competency as intermediates in the (Na + K)-ATPase and (by inference) in the coupled active Na and K transport (Plesner et al., 1981). The present study cannot explicitly reject or support such models. However, it is of interest that in red cell membranes, when K-sensitive phosphoenzymes are not detected (at 0°C), ouabain-sensitive (Na + K)-ATPase activity is similarly absent.

To what can we attribute the difference in response to temperature of the red cell Na pump and other Na pump preparations? Either the protein itself is different in the red cell system or the temperature effects reflect differences in the various membrane environments. White and Blostein (1982) have suggested that these differences may be explained simply on the basis of the different lipid environments of the pump proteins. Studies on the effects of the phosphoenzyme characteristics of the kidney enzyme preparation after altering lipid composition have not yet been reported. A systematic investigation of the effects of drastically altering red cell membrane composition (cholesterol levels, phospholipid composition, etc.) on the $E_1\sim P:E_2P$ ratio is hampered by the low number of copies of the Na pump per human red cell. A comparison of the human red cell Na pump to other blood cell Na pumps that have a higher pump density and different membrane composition may help to resolve the source of the striking temperature effects reported in the present work.

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