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The role of ($\alpha\beta$) protomer interaction in determining functional chararacteristics of red cell Na,K-ATPase

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Abstract

We have examined the possibility that interaction of ($\alpha\beta$) protomers within a diprotomer is responsible for some anomalous characteristics of red cell Na,K-ATPase by examining their response to two inhibitors, FITC and H_2DIDS, which bind covalently, and to ouabain, which debinds slowly from red cell pumps. The phenomena we examined were: (1) the biphasic curve relating Na,K-ATPase activity to ATP concentration, and (2) protection of Na pumps against vanadate inhibition by external Na. If interaction of ($\alpha\beta$) protomers within a diprotomer were responsible for these phenomena, random inactivation of ($\alpha\beta$) protomers should have resulted in a high proportion of ($\alpha\beta$) protomers with an inhibited protomer as a partner, and therefore should have significantly altered the consequences of subunit interaction. With each inhibitor, 60–70% inhibition of ATPase activity did not alter the functional characteristics of the residual activity. We conclude that interaction of functional ($\alpha\beta$) protomers does not explain the phenomena which we investigated. This is consistent with our previous observation that Na,K pumps of red cell membranes exist as monomeric ($\alpha\beta$) protomers (Martin, D.W. and Sachs, V.R. (1992) J. Biol. Chem. 267, 23922–23929).

Key words: ATPase, Na^+/K^+-; Vanadate; Sodium pump; Sodium/potassium pump; Cation transport; Protomer interaction

1. Introduction

The reaction mechanism of the Na,K-ATPase, the membrane-bound enzyme which is present in almost all animal cells and which is responsible for the asymmetric distribution of Na and K across cell membranes, has been the subject of controversy for decades [1]. The enzyme consists of an α chain (M_r 112000 [2,3]) which contains all the substrate binding sites, and a β chain (protein M_r 35000 [4]); the two chains form an ($\alpha\beta$) heterodimer. In most purified preparations, the ($\alpha\beta$) protomer is organized in $(\alpha\beta)_2$ dimers or higher oligomers. Early studies found that Na promotes phosphorylation of the enzyme from ATP and K dephosphorylates the enzyme, and the results of transport experiments indicated that the movement of Na and K occurs consecutively. Therefore it seemed likely that the mechanism of the enzyme is ping-pong with respect to Na and K. However, it was soon found that in most enzyme preparations less than maximal expected phosphorylation from ATP could be achieved, and transport experiments were reported which seemed to show that Na and K movement might not occur consecutively, but simultaneously in a single step. These anomalous findings led to the proposal of a reaction mechanism in which the two halves of a diprotomer $(\alpha\beta)_2$ each perform the steps of a transport cycle out-of-phase, with interaction between the two protomers during the course of the cycle [5,6]. Such mechanisms accounted for less than maximal (half-of-sites) phosphorylation from ATP and for the apparent simultaneous movement of Na and K. It is now clear that, as first proposed, transport of Na and K is consecutive rather than simultaneous [7–9] and it has been shown

Abbreviations: Na,K-ATPase, Na + K-dependent adenosine triphosphatase; Ca-ATPase, Ca-dependent adenosine triphosphatase; FITC, fluorescein 5′-isothiocyanate; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid; EGTA, [ethylenebis (oxyethylenenitrilo)]tetracetic acid; DMF, *N,N*-dimethylformamide; PCMBS, *p*-chloromercuriphenylsulfonic acid; DTT, DL-dithiothreitol.

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that solubilized enzyme preparations, which consist almost exclusively of $(\alpha\beta)$ protomers, display Na,K-dependent ATPase activity [10,11] and are able to carry out all the partial reactions of a transport cycle [12]. Flip-flop models for the Na,K-ATPase reaction mechanism are no longer popular, and it is now widely believed that the mechanism is essentially that of the Albers-Post model [13] carried out by independent $(\alpha\beta)$ protomers.

Although the original bases for the proposal of protomer interactions in the Na,K-ATPase reaction mechanism no longer seem relevant, the concept has had continued popularity as a means of explaining functional properties of Na,K-ATPase preparations which escape ready explanation by the Albers-Post mechanism carried out by independent protomers. Unfortunately, since purified Na,K-ATPase preparations exist predominantly as diprotomers or higher oligomers [14,15], it is difficult to devise experiments to test these explanations. Preparations of soluble monomeric $(\alpha\beta)$ protomers seem ideal for such tests, but the preparations are unstable, and some of the anomalous phenomena might be difficult to demonstrate. Moreover, the results are subject to the criticism that whatever is done to measure the phenomenon may alter the aggregation state of the preparation. Recently, solubilized enzyme has been used to decide whether the biphasic curve relating ATPase activity to ATP concentration persists when monomeric preparations are used [11,16]; the results of the two studies conflict.

We have recently presented evidence which makes it most unlikely that $(\alpha\beta)$ protomers of red cell membranes exist to any significant extent as diprotomers [17]. This is probably in part due to the paucity of pumps in red cell membranes. If the red cell pump exists primarily as an $(\alpha\beta)$ protomer, then any phenomenon which can be demonstrated with these membranes cannot be ascribed to protomer interaction. We have used red cell membranes for the studies reported here.

Although it is unlikely that significant numbers of red cell pumps are organized as diprotomers, we further examined the possibility of protomer interaction by determining the effect of partial inhibition of ATPase activity on several phenomena which suggest such interaction. To examine the role of protomer interaction in these phenomena, we made use of a technique previously employed to evaluate the biphasic ATPase curve of sarcoplasmic reticulum Ca-ATPase [19] and of red cell Ca-ATPase [20] and to decide whether diprotomeric interaction accounts for the ordered release of occluded Rb in Na,K-ATPase preparations [21]. The rationale is simple. If an inhibitor reacts randomly with 50% of the $(\alpha\beta)$ protomers in a diprotomeric preparation and inhibits their function, then, of the remaining ATPase activity, 50% will be contributed by functioning protomers associated in diprotomers and 50% by functioning protomers associated with inhibited, nonfunctioning protomers. With higher levels of inhibition, the fraction of activity contributed by functioning protomers associated in diprotomers will be less: at 70% inhibition, only 30% of the residual activity will come from such functional diprotomers. If protomer interaction is responsible for an observed phenomenon, the characteristics of the observed phenomenon must be markedly altered at high levels of inhibition. We used three inhibitors which produce stable inhibition of Na,K-ATPase activity: FITC, H_2DIDS, and ouabain. The characteristics of the inhibitors and the assumptions underlying the method will be discussed below.

We have examined two phenomena which could be attributed to protomer interaction and which have been demonstrated with red cell preparations. The first of these anomalous phenomena is the finding that the curve relating ATPase activity to ATP concentration is biphasic [22–25]. This finding led to the suggestion that a functional enzyme unit contains two ATP sites, a regulatory site and a catalytic site, and that the functional unit is an $(\alpha\beta)_2$ diprotomer. An alternative explanation for biphasic ATPase curves has been advanced; it proposes that the biphasic curve results from the addition of ATP at two points in a transport cycle [26,27]. Modeling of the ATPase reaction using experimentally determined rate constants reproduces the biphasic ATPase curve.

The second phenomenon is not readily accounted for by an independent protomeric model with a single set of high affinity sites for Na and K. External Na protects against vanadate inhibition of Na,K-ATPase activity. Protection results from the interaction of Na with a high affinity external site; K competes with Na at this site, also with relatively high affinity, and promotes inhibition. In the absence of external Na, vanadate inhibition increases monotonically with external K concentration, but when Na is added to the external solution, the relation between pump activity and external K concentration is biphasic in the presence of vanadate. At K concentrations which saturate the transport sites, there is still significant protection by external Na against vanadate inhibition, and increasing external K beyond the point at which the transport sites are saturated continues to increase vanadate inhibition. It has been shown that the biphasic curve does not occur because Na and K each occupy one of the two K transport sites. The results suggest that there are regulatory sites with high affinity for external Na and K which coexist with the transport sites [28,29]. Such sites could exist on separate protomers interacting in a diprotomer, or both could coexist on the same protomer. A third possibility is that the phenomenon can be explained by some unusual kinetic mechanism, but we have not been able to find one.

2. Methods

Venous blood was collected from normal volunteers and anticoagulated with citrate-phosphate-dextrose solution. Broken membranes were prepared by osmotic lysis. Red cells were washed three times with an isosmotic (160 mM) NaCl solution, and the packed cells rapidly squirted with stirring into 10 volumes of an ice-cold solution containing 16 mM NaCl and 2.5 mM Tris Cl, pH 7.4 at 0°C. The membranes were then collected and washed five times with the lysing solution and twice with a solution containing Tris Cl 1.0 mM and Tris EDTA 0.5 mM, pH 7.4 at 37°C. The washed ghosts were resuspended in a solution which contained Tris Cl 2.0 mM and Tris EDTA 0.01 mM, pH 7.4 at 0°C, frozen and stored.

In order to react ghosts with FITC, ghost suspensions were thawed, and the ghosts washed twice in a solution which contained 16 mM choline chloride and 1 mM EGTA, then once with a 50 mM Tris Cl solution, pH 9.0 at room temperature. Ghosts were then suspended in a solution containing Tris Cl 30 mM, NaCl 100 mM, EDTA 0.5 mM, pH 9.0 at room temperature, and appropriate concentrations of FITC which was added as a DMF solution just before addition of the ghosts. The suspension was incubated at 22°C for 30 min, or for the times indicated in the legends. The reaction was ended by placing the suspension in an ice bath, and the suspension was centrifuged and the supernatant removed. The ghosts were then suspended in a solution which contained 5 mM DTT, 1 mg/ml albumin, 7 mM ATP, and 16 mM choline chloride, pH 7.4 at room temperature. After 5 min at room temperature, the suspension was centrifuged and the supernatant removed. The ghosts were washed three times in a solution which contained 16 mM choline chloride and 1 mM EGTA, and used for measurement of ATPase activity.

A similar procedure was used for reacting membranes with H_2DIDS. Ghosts were washed twice in a solution made up of 16 mM choline chloride and 1 mM EGTA, and once in a 50 mM Tris Cl solution, pH 9.0 at room temperature. Ghosts were suspended in a solution which contained Tris Cl 30 mM, choline chloride 100 mM, and EDTA 0.5 mM, pH 9.0 at room temperature. Appropriate amounts of H_2DIDS dissolved in DMF were added just before addition of the ghosts. The suspension was incubated at 22°C for 30 min, or for the times indicated in the legends. Binding was stopped by placing the suspension in an ice bath, and the suspension was centrifuged and the supernatant removed. The ghosts were resuspended in a solution which contained 5 mM DTT, 1 mg/ml albumin, and 16 mM choline chloride and the suspension was incubated for 5 min at 22°C. The suspension was centrifuged, the supernatant removed, and the ghosts washed three times with a 16 mM choline chloride solution containing 1 mM EGTA. The ghosts were used for measurement of ATPase activity.

Binding of ouabain was performed using ghosts which were washed three times in a solution made up of 16 mM choline chloride and 1 mM EGTA. The ghosts were resuspended in a solution which contained Tris Cl 30 mM, NaCl 16 mM, $MgCl_2$ 3 mM, and ATP 2 mM, pH 7.4 at 37°C, and the appropriate concentration of ouabain. The suspension was incubated at 37°C for 1 h or for the times indicated in the legends. The suspension was then transferred to an ice bath, centrifuged, and the supernatant removed. The ghosts were washed four times with a solution containing choline chloride 16 mM and EGTA 1 mM, and used for the measurement of ATPase activity.

In experiments in which ouabain-sensitive ATPase activity was estimated at micromolar concentrations of ATP, a modification of a previous described method [30] using [γ-^{32}P]ATP was employed. 20 μl of an appropriate dilution of red cell membranes was added to 180 μl of assay solution in microfuge tubes, and the assay was begun by transferring the tubes to a 37°C water bath. The assay solution usually contained Tris Cl 32 mM, NaCl 96 mM, KCl 16 mM, EDTA 0.5 mM, EGTA 0.5 mM, $MgCl_2$ 0.5 mM greater than the sum of concentrations of EDTA and ATP, and the appropriate concentration of ATP containing [γ-^{32}P]ATP, pH 7.4 at 37°C. Measurements were made with and without $2.5 \cdot 10^{-4}$ M ouabain. In some experiments, KCl was replaced by RbCl or LiCl as indicated in the figure legends. After 0.5 h incubation, the reaction was stopped by returning the tubes to an ice bath and adding 20 μl 10% SDS solution to each. 0.1 ml of a solution containing 2 M H_2SO_4, 4% NH_4molybdate, and 20 mM silicotungstic acid was added and the suspensions mixed. Phosphomolybdate was extracted into 0.8 ml isobutanol, the suspensions centrifuged, and 0.5 ml of the isobutanol layer removed and counted. Samples of the assay solutions were also counted, and ATPase activity was calculated [30]. Values obtained in the presence of $2.5 \cdot 10^{-4}$ M ouabain were subtracted from those obtained in ouabain-free solutions.

In experiments in which ouabain-sensitive ATPase activity was estimated at high concentrations of ATP, the measurement was made by an assay in which the phosphorylation of ADP is coupled to the oxidation of NADH [31]. The assay solution contained Tris Cl 32 mM, NaCl 96 mM, KCl 24 mM, EDTA 0.5 mM, ATP 2 mM, and $MgCl_2$ 3.5 mM; pH was adjusted to 7.4 at 37°C. Measurements were made with and without $2.5 \cdot 10^{-4}$ M ouabain, and the values obtained in the presence of ouabain were subtracted from the values obtained in its absence.

Intracellular cation concentrations of intact cells were altered by incubating washed cells for 36 h at 4°C

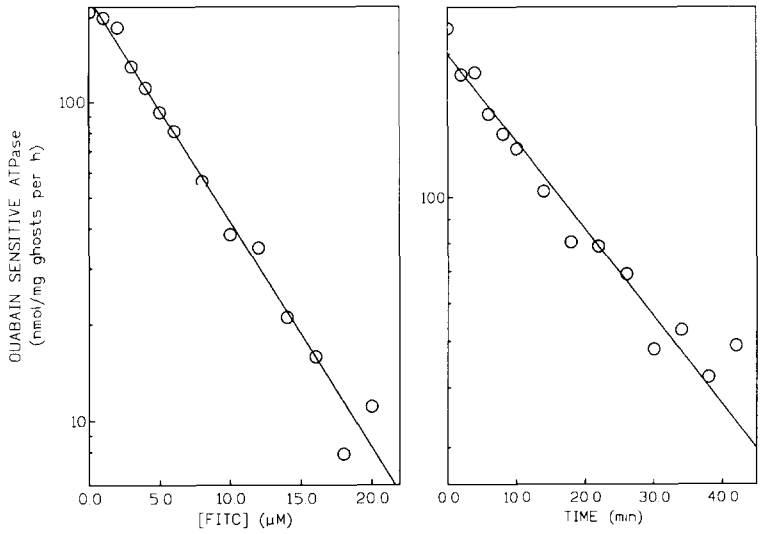


Fig. 1. Ouabain-sensitive ATPase activity of ghosts (1) incubated for 0.5 h at the indicated concentrations of FITC (left) and (2) incubated for the times indicated in 20 μM FITC (right). The data from which the left hand panel was drawn were fitted to the equation $v = v_0 e^{-k[FITC]}$ where v_0 = 212 nmol/mg ghosts per h and $k = 0.162/\mu M$. The data from which the right hand panel was drawn were fitted to the equation $v = v_0 e^{-kt}$ where v_0 = 200 nmol/mg ghosts per h and $k = 0.472$/min.

in buffered solutions of suitable composition containing PCMBS. During the incubation, the cells became permeable to cations and the intracellular cation concentrations approached the extracellular concentrations. The cells were then resealed by separating them from the PCMBS solution and incubating them for 1 h at 37°C in a solution which contained 1 mM DTT. The method has been described in detail [28].

Measurement of unidirectional K influx into intact cells was performed as previously described [32]. Cells were incubated at 37°C with solutions which contained ^{42}K. Uptake was calculated from the amount of ^{42}K accumulated by the cells and the specific activity of the solution.

Determinations were made in quadruplicate, and in the figures and table each point is the mean of four determinations and, except in Figs. 1, 2 and 3, the S.E. value is given unless, in the figures, it is smaller than the symbol. Curves were fitted to the data by a nonlinear least-squares method; the points were weighted by their variances.

3. Results

Since Na,K pumps are scarce in red cell membranes, it is difficult to determine the characteristics of inhibition using these membranes and, for the most part, we

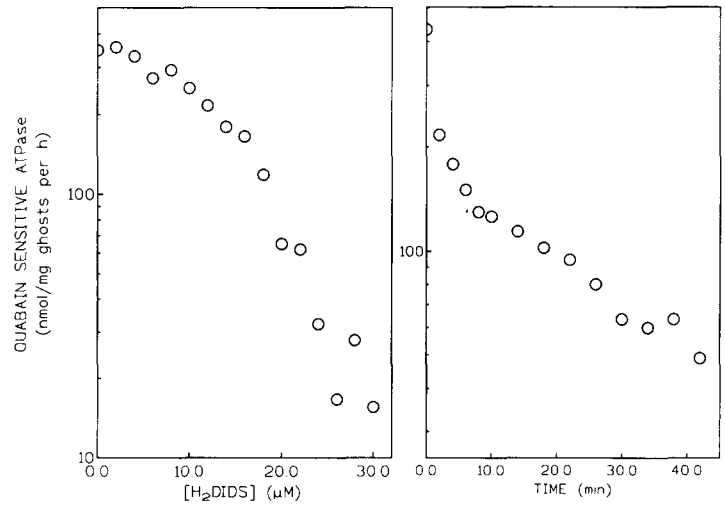


Fig. 2. Ouabain-sensitive ATPase activity of ghosts (1) incubated for 0.5 h at the indicated concentrations of H_2DIDS (left) and (2) incubated for the times indicated in 30 μM H_2DIDS (right).

rely on results obtained with other preparations to evaluate whether the inhibitors are sutiable for determining the role of protomer interaction within a diprotomeric functional unit. Nevertheless, we measured inhibition of red cell ATPase activity by the three agents as a function of inhibitor concentration during exposure of the membranes for a fixed time, and as a function of time of exposure of membranes to a fixed inhibitor concentration. Fig. 1 shows the results of the experiment with FITC. Inhibition increased as an exponential function of the FITC concentration to which membranes were exposed for a fixed time, and as an exponential function of time when membranes were exposed to a fixed concentration of FITC. FITC inhibition of red cell ATPase is pseudo first order, as it is with other ATPase preparations.

The situation with H_2DIDS is much less clear as shown in Fig. 2. Inhibition is not exponential with concentration especially at low concentrations of H_2DIDS and it is exponential with time only late in the time course. The situation is complicated by the fact that, under conditions in which it is convenient to inhibit red cell membrane ATPase, the anion transporter, band 3, which also binds H_2DIDS with high affinity, is present at a concentration of about 6 μM, within the range of H_2DIDS concentrations used in the inhibition incubation. The deviation of the curves in Fig. 2 from strict first order behavior is probably attributable to this complication. In purified ATPase preparations, inhibition by H_2DIDS under the conditions we used is clearly first order [33]. It is unlikely that the mechanism is much different with red cell ATPase than it is with ATPase from other sources.

Ouabain bound under the conditions we used is known to dissociate slowly. Fig. 3 shows that, when allowance is made for the partial reversal of inhibition during the influx measurement, ouabain inhibition is first order. Elution of ouabain bound to Na,K pumps in intact red cells is very slow, and in intact red cells, ouabain binding has been shown to be first order [18]. Slow elution of ouabain complicates the evaluation of the results of the experiment shown in Fig. 3, but ought to have little effect on the experiments reported below.

3.1. Effect of partial enzyme inhibition on the biphasic ATPase curve

To evaluate the experiments, it was convenient to fit the data to an expression relating ATPase activity to the ATP concentration over the entire range of ATP concentrations. The exact form of such a relation depends, of course, on the reaction mechanism chosen. Since we were primarily interested in describing the data, we utilized an ad hoc relation which assumes that the ATPase activity is the sum of the contribution of two processes, a high-affinity process with $V_{max} = V_1$ and $K_{1/2} = K_1$, and a low-affinity process with $V_{max} = V_2$ and $K_{1/2} = K_2$ [34]:

$$v = \frac{V_1[\mathrm{ATP}]}{K_1 + [\mathrm{ATP}]} + \frac{V_2[\mathrm{ATP}]}{K_2 + [\mathrm{ATP}]} \quad (1)$$

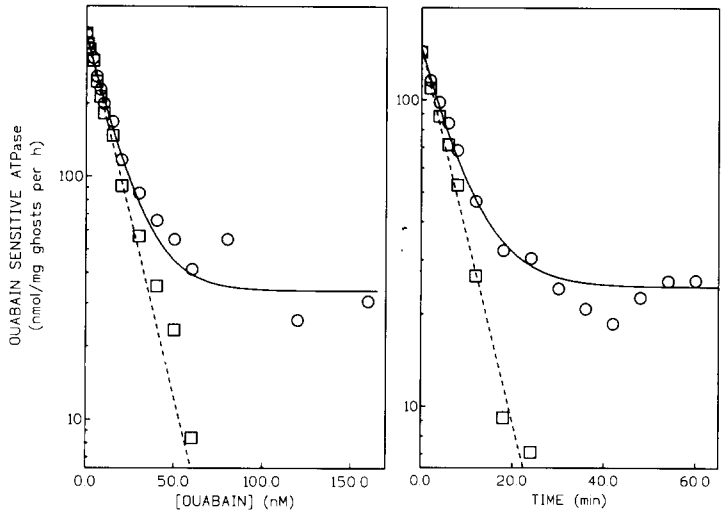


Fig. 3. Ouabain-sensitive ATPase activity of ghosts (1) incubated for 0.5 h at the indicated concentrations of ouabain (left) and (2) incubated for the times indicated in 10^{-7} M ouabain (right). The circles are the experimentally determined values and the solid curves are drawn to the equation $v = v_0 e^{-kx} + fv_0(1 - e^{-kx})$ where, for the left panel, x is [ouabain] (nM), and, for the right panel, x is time (min). The equation is derived from the assumption that ouabain binding is a first order process, but during the measurement of ATPase activity, ouabain slowly elutes from the enzyme. For the left hand panel, $v_0 = 379$ nmol/mg ghosts per h, $k = 0.0686$ nM^{-1}, and f (the fraction of the experimentally determined ATPase activity which is attributable to enzyme which has recovered from inhibition) is 0.089. For the right hand panel, $v_0 = 147$ nmol/mg ghosts per h, $k = 0.141$ min^{-1}, and $f = 0.166$. From these values, the ATPase activity which would have been measured if no elution had taken place was calculated, and the resulting values are plotted as squares in each panel. The dashed lines are $v = v_0 e^{-kx}$ with the values of v_0 and k given above.

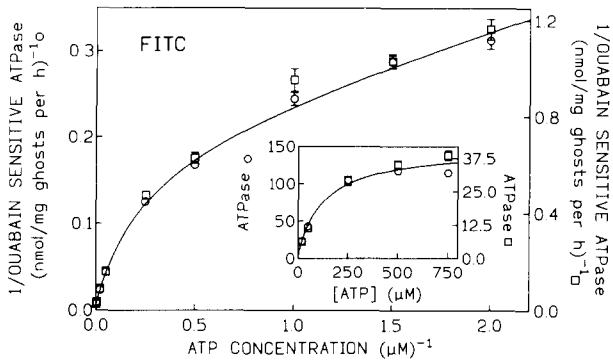


Fig. 4. Reciprocal of ouabain-sensitive ATPase activity vs. reciprocal of ATP concentration of control ghosts (○, left ordinate) and of ghosts which were exposed to 7 μM FITC for 30 min (□, right ordinate). The scale of the right ordinate is 1/0.28 the scale of the left ordinate; ATPase activity of ghosts exposed to FITC was 0.28 the activity of the corresponding control ghosts. The curve, drawn to the left ordinate, is the reciprocal of Eq. (1) with $V_1 = 4.17$ nmol/mg ghosts per h, $K_1 = 0.316$ μM, $V_2 = 145$ nmol/mg ghosts per h, and $K_2 = 131$ μM. The values of the parameters were obtained by fitting the results with the control ghosts to the reciprocal of Eq. (1). The inset is a direct plot of ATPase activity vs. ATP concentration. The results obtained at high ATP concentrations for control ghosts (○, left ordinate) and for FITC treated ghosts (□, right ordinate) are plotted. The scale of the right ordinate is 0.28 the scale of the left ordinate. The curve, drawn to the left axis, is Eq. (1) with the parameter values given above.

We fit the reciprocal of the data from the ATPase experiments to the reciprocal of Eq. (1) and extracted estimates of the four parameters.

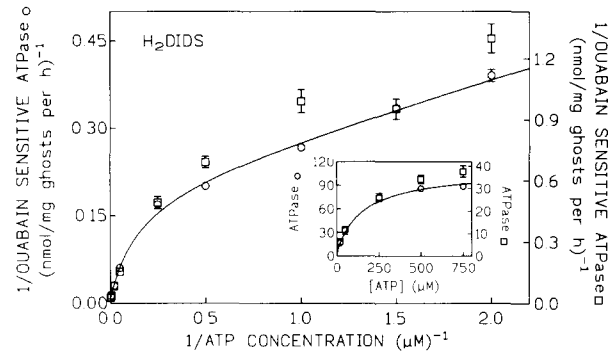


Fig. 5. Reciprocal of ouabain-sensitive ATPase activity vs. reciprocal of ATP concentration of control ghosts (○, left ordinate) and of ghosts which were exposed to 12 μM H_2DIDS for 30 min (□, right ordinate). Since ATPase activity of the ghosts exposed to H_2DIDS was 0.35 of the activity of the control ghosts, the scale of the right ordinate is 1/0.35 of the scale of the left ordinate. The curve is drawn to the left ordinate, and is the reciprocal of Eq. (1) with $V_1 = 4.27$ nmol/mg ghosts per h, $K_1 = 0.44$ μM, $V_2 = 105.2$ nmol/mg ghosts per h and $K_2 = 153$ μM. The values of the parameters were obtained by fitting the results with the control ghosts to the reciprocal of Eq. (1). The inset is a direct plot of ATPase activity vs. ATP concentration. The results obtained at high ATP concentration for control ghosts (○, left ordinate) and for H_2DIDS-treated ghosts (□, right ordinate) are plotted. The scale of the right ordinate is 0.35 the scale of the left ordinate. The curve, drawn to the left axis, is Eq. (1) with the parameters given above.

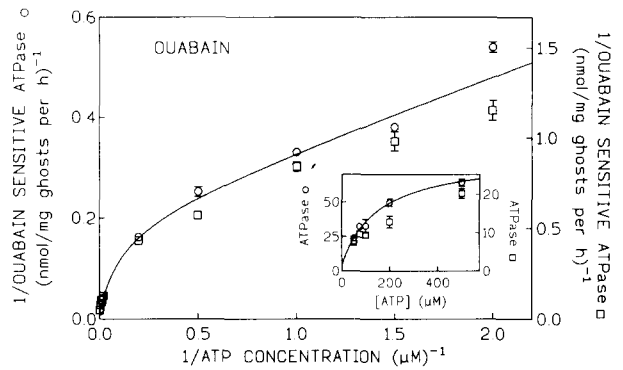


Fig. 6. Reciprocal of ouabain-sensitive ATPase activity vs. reciprocal of ATP concentration of control ghosts (○, left ordinate) and of ghosts which were exposed to $1.5 \cdot 10^{-8}$ M ouabain for 1 h (□, right ordinate). Since ATPase activity of the ghosts exposed to ouabain was 0.36 of the activity of the control ghosts, the scale of the right ordinate is 1/0.36 of the scale of the left ordinate. The curve is drawn to the left ordinate, and is the reciprocal of Eq. (1) with $V_1 = 4.21$ nmol/mg ghosts per h, $K_1 = 0.66$ μM, $V_2 = 79.1$ nmol/mg ghosts per h, and $K_2 = 151$ μM. The values of the parameters were obtained by fitting the results with the control ghosts to the reciprocal of Eq. (1). The inset is a direct plot of ATPase activity vs. ATP concentration. The results obtained at high ATP concentration for control ghosts (○, left ordinate) and for ouabain-treated ghosts (□, right ordinate) are plotted. The scale of the right ordinate is 0.36 the scale of the left ordinate. The curve, drawn to the left axis, is Eq. (1) with the parameters given above.

Fig. 4 shows the results of an experiment in which ATPase activity was partially inhibited by exposure of the membranes to FITC. In this experiment, ATPase activity in the FITC-treated membranes was 0.28 that in the control membranes so that, if the functional unit is a diprotomer, 72% of the residual activity must be attributed to active protomers associated with inhibited protomers. (0.28 of the ($\alpha\beta$) protomers are active; $0.28 \times 0.28 = 0.078$ of the diprotomers pair active ($\alpha\beta$) protomers, and $0.28 \times 0.72 + 0.72 \times 0.28 = 0.403$ pair an active ($\alpha\beta$) protomer with an inactive one. Total relative activity contributed by the active-active diprotomers is 2×0.078 (both protomers contribute), and by the active-inactive diprotomers, 0.403 (only the active protomers contribute). Fractional activity contributed by the active-inactive pairs is $0.403/(0.156 + 0.403) = 0.72$). Nevertheless, aside from the difference in scale, the curve for the control preparation adequately describes results obtained with the inhibited preparation.

Fig. 5 shows the results of a similar experiment in which membranes were exposed to H_2DIDS. Residual activity in the H_2DIDS-treated membranes was 0.35 that in the control membranes so that 65% of the residual activity results from functional protomers associated with inhibited protomers if the preparation is diprotomeric. There is no qualitative difference in the biphasic ATPase curve between the values obtained with the H_2DIDS-treated membranes and the values obtained with the control membranes.

Finally, Fig. 6 shows the results of the same experiment using membranes which were exposed to ouabain. The activity of the ouabain-treated membranes was 0.36 that of the control membranes so that 64% of the residual activity arises from functional protomers associated with inhibited protomers if the pumps are diprotomers. The shape of the curve obtained with the control membranes is the same as the shape of the curve obtained with the inhibited membranes except for the difference in scale.

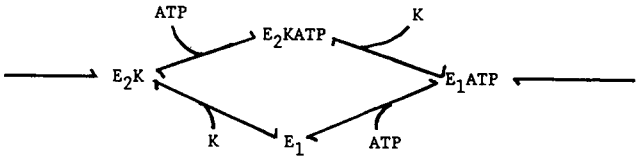
Since inhibition of two thirds or more of the ATPase activity with resulting reduction in the possibility for protomer interaction has no effect on the shape of the biphasic ATPase curve, it is unlikely that subunit interaction accounts for the biphasic curve.

3.2. Effect of Rb and Li on the biphasic ATPase curve

An alternative explanation for the biphasic ATPase curve has been advanced. In the overall transport cycle, the rate-limiting step at low ATP is the release of K at the inside surface. ATP, acting with low affinity, increases the rate of K release [35]. These findings led to the suggestion that the biphasic ATPase curve results from the presence within the ATPase reaction mechanism of a branched chain sequence in which ATP adds at two points [26,27] (Scheme 1).

In the upper pathway, ATP adds with low affinity to E_2K leading to the release of K and the formation of E_1ATP. In the lower pathway, K is released slowly, and then ATP adds with high affinity leading to the formation of E_1ATP. When K is the activating cation, flow through the upper pathway is much greater than flow through the lower pathway so that V_{max} of the high affinity component is much less than V_{max} of the low affinity component. Rb and Li can replace K in stimulating ATPase activity. Rb is released from E_2 less rapidly than K so that with Rb, the contribution of the high-affinity component to the overall ATPase activity should be less than with K; Li on the other hand is released much more rapidly than K so that when Li replaces K the contribution of the high-affinity component should increase [35,36].

Fig. 7 shows the results of an experiment in which we measured ATPase activity as a function of ATP concentration with Rb replacing K and with Li replacing K. The ATPase curve when Rb replaced K was biphasic. In three experiments in which Rb replaced K,



Scheme 1.

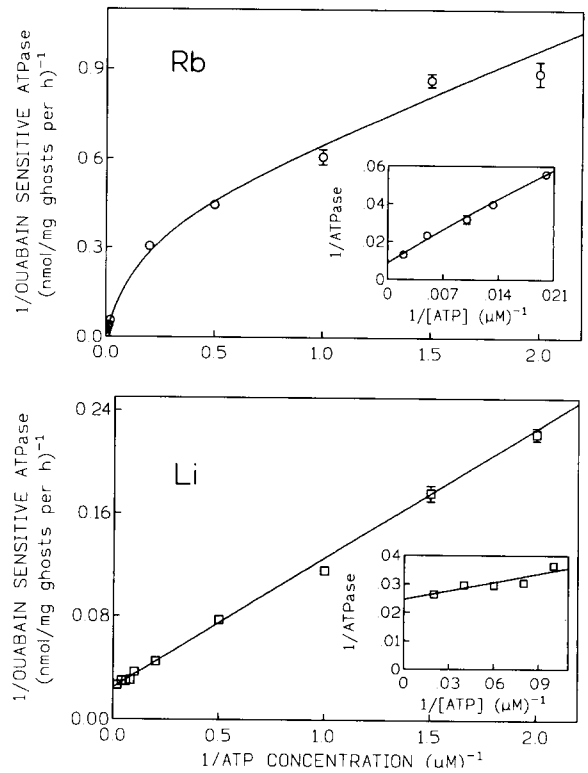


Fig. 7. Reciprocal of ouabain-sensitive ATPase activity measured in solutions in which K was replaced with 24 mM Rb (○) or 80 mM Li (□). For the Rb experiment, the curve is the reciprocal of Eq. (1) with $V_1 = 1.90$ nmol/mg ghosts per h, $K_1 = 0.62$ μM, $V_2 = 112$ nmol/mg ghosts per h, and $K_2 = 303$ μM. The values of the parameters were obtained by fitting the experimental values to the reciprocal of Eq. (1). The inset is an expanded plot of the results at the highest ATP concentrations, and the curve is the reciprocal of Eq. (1) with the parameter values given. For the Li experiment, the line is the reciprocal of $v = V[\text{ATP}]/(K + [\text{ATP}])$ with $V = 40.7$ nmol/mg ghosts per h, and $K = 4.11$ μM, obtained by fitting the experimental values to the equation. The inset is an expanded plot of the results at the highest ATP concentrations and the curve is the reciprocal of the equation given above with the same parameter values.

mean V_1 (V_{max} of the high-affinity component) was 1.85 nmol/mg ghosts per h, and for the three experiments shown in Figs. 4–6 with K as the activating cation mean V_1 was 4.22 nmol/mg ghosts per h. (V_2 varies considerably more from individual to individual than does V_1, so that the rate V_2/V_1 for each condition varies more than does V_1). The curve describing the experiment in which Li replaced K is a straight line and the affinity for ATP is high; there is no sign of the contribution of a low affinity component to the overall ATPase activity. The results are consistent with the branched chain mechanism as the explanation for the biphasic ATPase curve.

3.3. Na protection against vanadate inhibition

A simple explanation for the coexistence of high affinity sites at which Na and K modulate vanadate inhibition and transport sites which demonstrate rela-

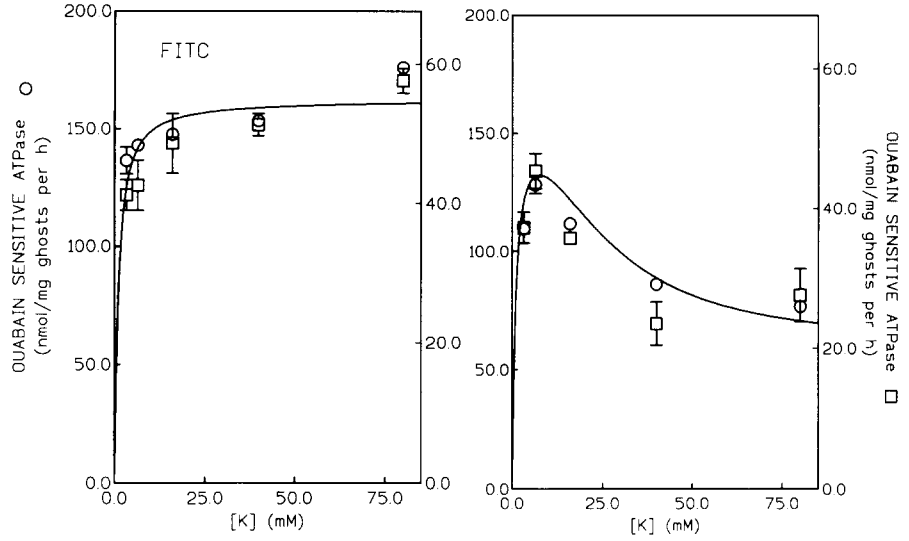


Fig. 8. Ouabain-sensitive ATPase activity vs. K concentration in the presence (right panel) and absence (left panel) of 1 μM vanadate. The ghosts were preincubated in the presence (□, right ordinate) and absence (○, left ordinate) of 7 μM FITC. ATPase activity of the FITC treated ghosts was 0.34 the activity of the control ghosts, and the scale of the right ordinates are 0.34 the scale of the left ordinates.

tively high affinity for K is that the two sets of sites exist on different protomers within a diprotomer, and that interaction of the protomers is responsible for the modulation of inhibition.

In the experiment shown in Fig. 8, membranes were exposed to FITC before the characteristics of vanadate inhibition were examined; activity in FITC-treated membranes was 0.34 that in control membranes. Activation of ATPase activity by K in vanadate-free solutions is shown in the left panel, and activation in the presence of vanadate in the right panel. In the presence of vanadate, activation is clearly biphasic. When pump activity is measured in the absence of external Na, the corresponding K-activation curve is monotonic and inhibition is nearly complete at all concentrations of K [8]. The biphasic K activation curve in the right hand panel occurs because Na protects against vanadate inhibition, and K competes with Na to reverse this inhibition [28,29]. Aside from the difference in scale, the curves in the right panel are superimposable; 66% inhibition of ATPase activity by FITC did not alter the properties of vanadate inhibition. If modulation of vanadate inhibition by external Na were due to protomer interaction, random inactivation of protomers by FITC would be expected to be equivalent to the removal of external Na; i.e., vanadate inhibition should

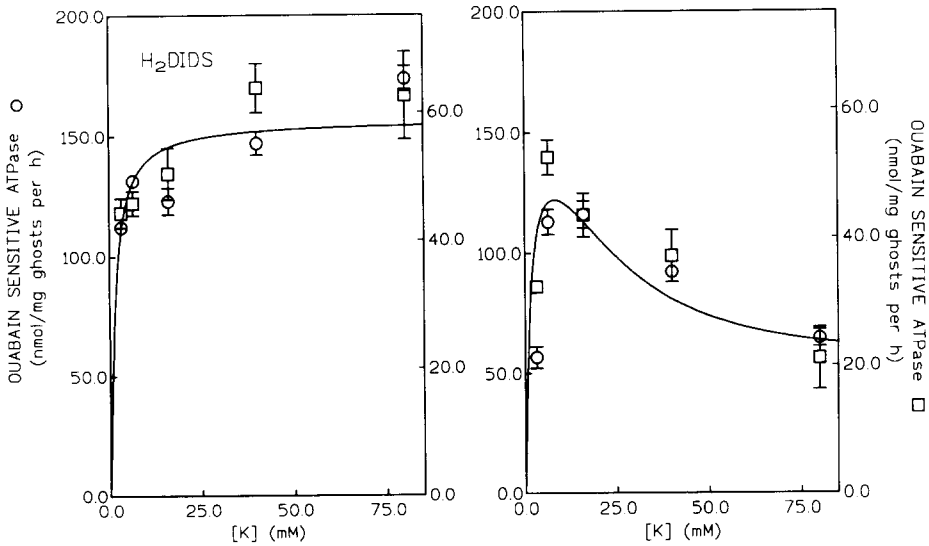


Fig. 9. Ouabain-sensitive ATPase activity vs. K concentration in the presence (right panel) and absence (left panel) of 1 μM vanadate. The ghosts were preincubated in the presence (□, right ordinate) and absence (○, left ordinate) of 12 μM H_2DIDS. ATPase activity of the H_2DIDS-treated ghosts was 0.38 the activity of the control ghosts, and the scale of the right ordinates were 0.38 the scale of the left ordinates.

Table 1
Inhibition of ouabain-sensitive K influx by vanadate in red cells preexposed to ouabain

Ouabain pre-exposure	K influx ± S.E.		Fraction uninhibited by ouabain	Vanadate 20 μM		Fraction uninhibited by vanadate	
	K 2.2 mM	K 22.6 mM		K influx ± S.E.			
				K 2.2 mM	K 22.6 mM	K 2.2 mM	K 22.6 mM
0	2.72 ± 0.03	3.74 ± 0.08	–	1.94 ± 0.01	0.54 ± 0.06	0.71	0.14
$0.5 \cdot 10^{-8}$ M	1.90 ± 0.07	2.83 ± 0.08	0.73	1.41 ± 0.01	0.50 ± 0.06	0.74	0.18
$1.0 \cdot 10^{-8}$ M	1.57 ± 0.01	2.22 ± 0.03	0.59	1.16 ± 0.02	0.42 ± 0.06	0.74	0.19
$4.0 \cdot 10^{-8}$ M	0.62 ± 0.01	0.90 ± 0.01	0.23	0.46 ± 0.04	0.18 ± 0.03	0.74	0.20

Red cells were prepared so that their intracellular Na concentration was 31.5 mmol/l cells and their intracellular K concentration was 4.74 mmol/l cells. The cells were exposed to ouabain at the indicated concentrations for 1 h at 37°C in a solution which contain 144 mM choline chloride and enough Mg-glycylglycine buffer pH 7.4 at 37°C to bring the final osmolality to 295 mosmol/kg H_2O. The cells were then washed three times in 160 mM choline chloride solution. K influx was measured in solutions which contained 10% by volume of a 295 mosmol/kg H_2O solution of Mg-glycylglycine pH 7.4 at 37°C, 88 mM NaCl, the indicated concentrations of KCl, choline chloride 53 mM in the low K solutions and 30 mM in the high K solutions, and either no vanadate or 20 μM vanadate. Measurements were made in the presence and absence of 10^{-4} M ouabain. The units for K influx are mmol/liter cells per h. Fraction uninhibited by vanadate was obtained by dividing a value from the columns listing values obtained in the presence of vanadate by the corresponding value obtained in the absence of vanadate.

have been greater in the FITC-treated membranes especially at low K concentrations, and the K-activation curve should have been less biphasic.

Fig. 9 shows the results of a similar experiment in which membranes were exposed to H_2DIDS instead of FITC. Activity in the H_2DIDS-treated membranes was 0.38 that in the control membranes. In both the presence and absence of vanadate, the K-activation curves in the H_2DIDS-treated membranes is superimposable on the curves in the untreated membranes, aside from the difference in scale.

To determine how inhibition by ouabain affects the interaction between external Na and vanadate, we measured K influx in intact cells rather than ATPase activity in membranes since inhibition caused by ouabain binding to intact red cells is nearly irreversible [18]. The results of the experiment are shown in Table 1. Cells were incubated with ouabain at several concentrations, washed, and K influx measured. Residual K influx in the ouabain treated cells at saturating external K ranged from 0.73 to 0.23 that of the control cells. K influx was measured in high Na solutions at two K concentrations with and without vanadate. If protomer interactions responsible for protection by external Na against vanadate inhibition were disrupted by ouabain, then one would predict that vanadate inhibition at low external K would be much greater in the ouabain-treated cells than in the control cells [9]. No difference was seen, and the results do not support the diprotomer hypothesis.

4. Discussion

To be useful in experiments like those reported here, an inhibitor must display several characteristics. The inhibitor must bind randomly to functional α chains, and binding of a single inhibitor molecule to a functional α chain must inhibit its function so that inhibition is strictly proportional to the number of inhibitor molecules bound. Inhibition must demonstrate pseudo-first order kinetics to be sure that inhibitor binding is random and to eliminate the possibility that binding of an inhibitor to one α chain in a diprotomer changes the affinity of a second site for the inhibitor. Finally, inhibition must be stable. It has been shown that these are characteristics of the inhibitors used in these studies: FITC [37–40], H_2DIDS [33], and ouabain [18,41]. In each case, inhibition is a linear function of the number of inhibitor molecules bound, and in each case the number of inhibitor molecules bound per mg protein at 100% inhibition equals the maximum number of phosphorylation sites per mg protein obtained with the same preparation.

The method also requires some characteristics of the Na,K pump. First it is necessary that each $(\alpha\beta)$ protomer exhibits Na,K-ATPase activity whether or not it is associated with another $(\alpha\beta)$ protomer in a diprotomer. It has repeatedly been demonstrated that $(\alpha\beta)$ protomers exhibit Na,K-ATPase activity in the monomeric state [10,12], and all doubts should now be dispelled by the recent demonstration of ATPase activity by $(\alpha\beta)$ monomers during active enzyme centrifugation [11]. However, the question of whether Na,K-ATPase preparations display half-of-the-sites reactivity persists [42,43]. Most ATPase preparations have less than the theoretically predicted phosphorylation capacity (6.8 nmol/mg protein based on a molecular weight of 145 000) and specific activity (68 μmol phosphate/mg protein per min based on a turnover of 10 000/min), and most often much less (~ 25%). This has led to a persistent debate about the nature of the inactive protein; whether it is impurity, denatured enzyme, or $\alpha\beta$ protomer participating in a half-of-the-sites reaction mechanism. Since even preparations with only ~ 25% of maximal activity are reported to consist only of α

and β chains, impurities seem to be an unlikely explanation for the discrepancy. The presence of significant amounts of denatured or partially denatured enzyme is a far more likely cause of the low specific activities. In those preparations which consist only of α and β chains and which have considerably less than 50% of expected activity, it is clear that significant amounts of the enzyme must be denatured even in the context of the half-of-the-sites mechanism; SDS, used in enzyme purification, is known to be capable of denaturing the enzyme [44,45]. It is hard to believe that when the activity of a preparation is 25% of the expected activity significant denatured enzyme is present, but when it is near 50% of expected activity, there is no denatured enzyme. At any rate, some laboratories report routine preparations with specific activity greater than 50% of the expected maximum [46], and nearly 100% of maximum phosphorylation and specific activity can be achieved [12,47–49], although in some cases uncertainty persists about the methods used for estimation of protein concentration. We have developed a relatively simple procedure for preparing Na,K-ATPase of specific activity 60 μmol P_i/mg protein per min or greater (protein determined by a modified Lowery method) (Martin, D.W. and Sachs, J.R., unpublished observations). If dimers exist, $(\alpha\beta)$ protomers associated in diprotomers must each demonstrate activity and be capable of phosphorylation. The demonstration that solubilized enzyme exhibits the same specific ATPase activity, almost equivalent to that of the nonsolubilized enzyme which is its source, whether the solubilized enzyme is a protomer or a diprotomer [46] provides conclusive evidence that the condition required for the validity of the inhibition experiments reported here, namely, that each $(\alpha\beta)$ protomer exhibits Na,K-ATPase activity whether or not it is associated with another $(\alpha\beta)$ protomer, is satisfied.

The situation is complicated by the possibility that denatured enzyme may be partially active. When conformationally dependent alterations in the pattern of trypsin digestion of α chains [50] are examined using enzyme preparations of low specific activity which contain only α and β chains, the digestion patterns indicate that all the enzyme undergoes Na and K dependent conformational changes even though much less than all the enzyme hydrolyzes ATP [51]. Another manifestation of partially denatured enzyme can be found in a recent report that transient phosphorylation (superphosphorylation) considerably greater than the stable phosphorylation seen at low ATP concentrations can be demonstrated if the ATP concentration is raised to high levels [52]. In that study, the level of superphosphorylation varied inversely with enzyme specific activity; when phosphorylation at low ATP concentrations was low, the amount of superphosphorylation was high, and in preparations which displayed approximately 50% of maximal phosphorylation at low ATP, the amount of superphosphorylation was relatively low. The results suggest that superphosphorylation occurs on partially denatured enzyme molecules. Moreover, in the presence of imidazole, superphosphorylation produced phosphorylation levels of nearly 6 nmol/mg protein, close to maximal.

In the experiments reported here, we used red cell membranes which are not exposed to detergents during preparation and therefore do not contain SDS-denatured enzyme.

Even though a protomer is inhibited, it is possible that it is still capable of modifying the behavior of its neighbor in a diprotomer. To reduce the likelihood of such an occurrence yielding misleading results, in these experiments we utilized three different inhibitors which produce modified enzyme with different properties. FITC inhibition results in pumps which do not interact with ATP, but which are capable of undergoing conformational changes between E_1 and E_2 in response to changes in the concentration of Na and K [37]. H_2DIDS also produces protomers which do not interact with ATP, but these protomers are frozen in an E_2 conformation and do not change conformation in response to changes in cation concentration [33]. Ouabain-inhibited pumps are also prevented from undergoing conformational transitions, but they are capable of interaction with ATP and cations, and such interaction alters the rate at which ouabain is released from the enzyme-ouabain complex [53]. Despite the markedly different mechanisms of inhibition and the different properties of the inhibited molecules, none of the inhibitors gave evidence of interfering with postulated protomer interactions.

Finally, the inhibition method we used here would not be valid if an uninhibited protomer in a diprotomer is able to dissociate from the inhibited protomer and form a new diprotomer with another uninhibited protomer. The inhibitors we used should not increase the likelihood of dissociation. In soluble preparations, dimerization is more likely when protomers are in the E_2 conformation; since ouabain selects E_2 conformations, it decreases the likelihood that diprotomers will dissociate [54]. H_2DIDS also promotes the E_2 conformation so that it too should inhibit dissociation [33]. FITC favors neither the E_1 nor the E_2 conformation [37] so that it should neither increase nor decrease the likelihood of dissociation. Even if all the uninhibited protomers were to dissociate from their inhibited partners, the chances that any significant numbers of new functional $(\alpha\beta)_2$ diprotomers could form are slim. Although the diffusion rate of $(\alpha\beta)$ protomers in red cell membranes is not known, comparison with the known diffusion properties of band III molecules seems appropriate. Since band III binds with high affinity with the cytoskeletal protein ankyrin, lateral diffusion is

very slow; at 37°C and about 50 mM total salt concentration, about 60% of band III molecules are totally immobilized, and the diffusion coefficient of the remaining molecules is about 10^{-11} cm^2/s [55,56]. Since red cell ankyrin also displays a high affinity binding site for Na,K-ATPase α chains [57], it is reasonable to believe that the same diffusion parameters can be used to evaluate the characteristics of $(\alpha\beta)$ chain diffusion. Using these parameters, one can calculate [58] that, if 60% of $(\alpha\beta)$ protomers are inhibited, if all the functional protomers dissociate from their inhibited partners (120 unpaired functional protomers/cell), and if 60% of the functional $(\alpha\beta)$ protomers are immobilized, 0.7 collisions between functional $(\alpha\beta)$ protomers would be expected over the 30 min. course of the ATPase assay. Alternatively, if all the functional $(\alpha\beta)$ protomers are mobile, 1.7 such collisions are expected. Recombination of functional $(\alpha\beta)$ protomers is not a serious concern.

Using the inhibition technique, we have examined two phenomena which could be explained by protomer interaction. One of these, the biphasic ATPase curve, has been examined with soluble enzyme preparations. Solubilization of renal Na,K-ATPase to $(\alpha\beta)$ monomers has been shown to be without effect on the biphasic ATPase curve [11], as expected if protomer interaction is not responsible, but the same procedure applied to duck nasal gland enzyme converted the biphasic curve to a rectangular hyperbola [16]. In this study, we found that inhibition of 60–70% of the ouabain-sensitive ATPase of red cell membranes does not change the characteristics of the biphasic curve, and so there is no evidence for protomer interaction.

A branched chain mechanism in which ATP adds at two steps in the reaction sequence has been suggested for the Na,K-ATPase as a logical consequence of the effect of ATP on the rate of release of K and its congeners from the enzyme [35]. The mechanism produces a biphasic ATPase curve using measured values of the rate constants of intermediate steps between E_2K and E_1K ATP in the reaction pathway [26,27]. The branched chain mechanism also accounts for the effect of ATP on the K–K exchange [59] and predicts the stimulation of ATPase activity by nonhydrolyzable ATP analogues at low concentrations of ATP [60]. Since Li is readily released from the enzyme before ATP adds [35], and therefore effectively converts the branched chain mechanism to a straight chain mechanism, replacement of K with Li should convert the biphasic ATPase curve to a hyperbolic curve. We have verified that prediction here. (The ATPase curve in the presence of Na alone is also hyperbolic, but the maximum Na ATPase is much less than the maximum Na,Li-ATPase). Despite the success of the branched chain mechanism, support persists for a reaction mechanism which assigns both a regulatory and catalytic role for ATP [61]. No such mechanism has ever been explicitly stated, analyzed and shown to be consistent with published observations. Support for a regulatory role for ATP is primarily derived from evidence that ATP 'analogues' bind to the enzyme either in the presence of ATP or of other ATP 'analogues' [62]. Evidence that such 'analogues' really interact with ATP sites is sometimes derived from the observation of competitive inhibition [27], which is a necessary but not sufficient condition, and from the observation that the 'analogues', like ATP, increase the rate of the $E_2K \rightarrow E_1$ transition [63]. Reynolds et al. [64] have shown, however, that in order for a ligand to speed the transition, all that is necessary is that the ligand have a higher affinity for E_1 than for E_2K at any site; the observation cannot be used to infer that the ligand binds at an ATP site.

The second phenomenon we investigated, protection by external Na against vanadate inhibition of pump activity, does not have such a ready explanation. From the characteristics of Na protection it is clear that high-affinity regulatory sites at which Na modulates vanadate inhibition must exist coincidentally with high-affinity sites which transport K [28,29]. In view of the results of the present study, the convenient explanation that regulatory sites exist on one protomer of an interacting diprotomer and the transport sites exist on the other protomer is untenable. Two possible explanations remain. It is possible that high-affinity regulatory sites and transport sites exist simultaneously on a single protomer, but this contradicts the general belief that, on each protomer, there is a single set of high-affinity cation binding sites which change from high Na affinity to high K affinity over the course of a transport cycle. It is also possible that some unusual kinetic mechanism may be responsible for the protective effect of external Na, but we have been unable to find such a mechanism.

Although dimerization is not required for Na,K-ATPase activity, there are many observations which are difficult to explain by the function of a single protomer, and these phenomena are often explained by protomer interaction within a diprotomer [61]. When such phenomena are observed in purified preparations in which it is clear that Na pumps are densely distributed and protomer interaction might occur, such explanations seem possible, but independent evidence for protomer interaction is required. For instance, protomer interaction has been advanced as an explanation for the finding that, in purified preparations, Scatchard plots of ADP binding in the presence of K are nonlinear [65], but similar experiments with solubilized preparations yield linear Scatchard plots [66]. Such independent evidence is, however, rarely provided. Red cell membranes seem to provide a convenient tool for obtaining such evidence. If a phenomenon can be

demonstrated with red cell membranes, it is very unlikely that it can be attributed to protomer interaction since it is very unlikely that diprotomers exist in red cell membranes [17]. If one remains unconvinced, the inhibition method used here with red cell membranes can be employed to reduce further the likelihood that diprotomer interaction is involved. In the absence of positive evidence for protomer interaction, other explanations for anomalous behavior should be sought.

Acknowledgment

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