Binding of 2(3′)-O-(2,4,6-Trinitrophenyl)ADP to Soluble αβ Protomers of Na,K-ATPase Modified with Fluorescein Isothiocyanate

EVIDENCE FOR TWO DISTINCT NUCLEOTIDE SITES*

(Received for publication, January 16, 1996, and in revised form, March 18, 1996)

Douglas G. Ward and José D. Cavieres†
From the Department of Cell Physiology and Pharmacology, Leicester University, Leicester LE1 9HN, United Kingdom

The overall reaction of well-defined solubilized protomers of Na,K-ATPase (one α plus one β subunit) retains the dual ATP dependence observed with the membrane-bound enzyme, with distinctive ATP effects in the submicromolar and submillimolar ranges (Ward, D. G., and Cavieres, J. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5323-5336). We have now found that the K⁺-phosphatase activity of the αβ protomers is still inhibited by 2(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP). What is most significant is that the TNP-ADP effect can be observed clearly with protomeric enzyme whose high affinity ATP site has been blocked covalently with fluorescein isothiocyanate. We conclude that nucleotides can bind at two discrete sites in each protomeric unit of Na,K-ATPase.

The sodium pump or Na,K-ATPase¹ uses the energy of ATP hydrolysis to power the active transport of Na⁺ and K⁺ ions across the plasma membrane (1). This integral membrane enzyme consists of α and β subunits. The α chain presents an ATP-binding site, a phosphorylation site, and a ouabain-binding site. The β subunit is a glycoprotein of unclear function, found in equimolar proportions with the α chain. We recently reported (2) that solubilized αβ protomers of highly purified sodium pump responded to ATP with high and low affinity effects, and also that the complex behavior did not result from protomer association to form soluble (αβ)₂ dimers. Those experiments demonstrated that a complex dependence on nucleotides was intrinsic to the αβ protomer. They could not decide, however, whether this arose from the presence of more than one ATP site per αβ protomer or from a single site whose function and affinity changed round the catalytic cycle. That is the question addressed in this paper.

It has long been recognized that ATP has two types of activatory effect during the sodium pump cycle (3). Na,K-ATPase becomes phosphorylated by ATP during the course of the reaction, and the small high affinity activation (K₀.5(high) < 1 μM) correlates well with the ATP requirement for phosphorylation of the E₁ form of the enzyme (4). The considerable low affinity stimulation of the overall cycle (K₀.5(low) approximately 150-300 μM) seems to result from accelerating a rate-limiting step in the E₂ form of the dephosphoenzyme (4, 5). ADP (6), non-phosphorylating ATP analogues (7), and acyl coenzymes A (8) can replace ATP in this low affinity effect. For these reasons, this is regarded as a regulatory nucleotide effect, in contrast with the "catalytic" (high affinity) ATP action that results in enzyme phosphorylation in the presence of Na⁺ ions. Affinity probes have so far returned what seems to amount to a single, high affinity ATP site on the α chain. The purine ring subsite has been mapped with 2-azido-ATP and 8-azido-ATP (9, 10), which label peptides identifying Gly⁵⁰² and Lys⁴₈₀ respectively, as the anchoring points. This is a region which had earlier been found to be labeled by FITC, after binding covalently to Lys⁴₂⁰ (11, 12). Probes for the 5′-triphosphate moiety of ATP, on the other hand, have identified a sequence between Asp⁷₁⁵ and Lys⁷₁⁷ on the α chain (13, 14).

The structural findings have polarized the rationalization of the two ATP actions toward two basic models: (i) a unique ATP-binding site whose affinity and function change round the reaction cycle and (ii) a membrane (αβ)₂ dimer, with one ATP site per subunit, where the interaction of αβ units leads to a degree of half-of-the-sites reactivity (15) during turnover. Evidence for the first arises from studies with the vanadate-inhibited enzyme (16), from experiments with TNP-ATP, a high-affinity ATP analogue (17), and, at least superficially, from the enzymatic competence of the αβ protomer in solution (2). There are a number of observations, however, that cannot be explained by a single site (18), and it now seems increasingly likely that TNP-ADP can access more than one class of binding sites (19).

A third possibility, i.e. the idea of two distinct nucleotide sites per αβ unit, has not had much currency because of the lack of supporting structural data, the controversy above, and a certain sense of economy. This was, however, a viable alternative hypothesis in the case of the soluble αβ protomer (2). We have approached the question by examining the behavior of soluble protomers whose high affinity ATP site has been irreversibly blocked by FITC. The covalently bound fluorescein suppresses phosphorylation by ATP and ablates the overall pump cycle, but does not affect backwards phosphorylation by inorganic phosphate, or the K⁺-phosphatase activity of the enzyme (19-21). A realistic setting was that the K⁺-phosphatase substrate binds at a place other than the FITC-blocked site.

The K⁺-phosphatase activity is the ability of the sodium pump to hydrolyze substrates like pNPP, carbamyl phosphate, and 3-OMFP, hydroxylases which do not support cation fluxes. It is an E₂-type of activity on account of not only its K⁺ require-
Two Nucleotide Sites in Soluble Protomeric Na,K-ATPase

Fig. 1. Inactivation of membrane-bound sodium pump by FITC: two experiments. The enzyme (2.4 mg of protein/ml) was incubated with 20 μM FITC for the times shown. Open symbols, ATP protection. Na,K-ATPase activity remaining after FITC treatment in the absence (open squares) and the presence (open circles) of 3 mM ATP. Solid symbols, an experiment, under similar inactivation conditions, to measure the K+-phosphatase activity (solid circles) as well as ATP hydrolysis (solid squares). The substrate concentrations were 1 mM [γ-32P]ATP for Na,K-ATPase assays and 40 mM pNPP (Tris) for the K+-phosphatase assays.

Fig. 2. Block of individual sodium pump protomers by FITC. K+-activated phosphatase activity (Vmax), Na,K-ATPase activity, and protein concentration were measured in FITC-treated and control sodium pump, in their original membrane-bound state (MB, stippled bars) and in the αβ protomers arising after solubilizing with C12E8 (αβ, solid bars). The specific activities of the FITC-treated enzyme are presented as percent of the specific activities of the respective control enzyme. Open bar, rescue of Na,K-ATPase specific activity to be expected after solubilization, had FITC inactivated only one-half of a functional (αβ)2 membrane dimer. At 20°C, the control K+-phosphatase specific activities (μmol-min⁻¹-mg⁻¹) of the membrane-bound and solubilized enzymes were 3.41 ± 0.08 and 1.28 ± 0.02, and the control Na,K-ATPase specific activities, 2.68 ± 0.11 and 1.54 ± 0.02, respectively. The initial treatment was done for 3 h, at 2.4 mg of protein/ml, and with or without 30 μM FITC, as detailed under “Experimental Procedures.”

Experimental Procedures

Enzyme Purification—Sodium pump was purified from pig kidney outer medulla with the zonal-rotor procedure of Jørgensen (25). The specific Na,K-ATPase activity ranged from 20 to 35 μmol-min⁻¹-mg⁻¹ at 37°C.

FITC Treatment—A sample of purified membrane-bound sodium pump was incubated at 20°C, in the dark, either (i) at 2.4 mg of protein/ml and with 20 μM FITC for periods up to 3 h, or (ii) at 1 mg of protein/ml and with 50 μM FITC for 30 min. This was done in a medium containing 100 mM NaCl, 50 mM TrisCl (pH 9.2), 5 mM EDTA and 0.5% dimethyl sulfoxide. A parallel control sample was handled similarly, omitting the FITC. The membranes were chilled and spun out at 350,000 × g for 15 min and at 4°C in a Beckman TL-100 ultracentrifuge. They were washed, and resuspended, with a solution (“2 S medium”) containing 300 mM KCl, 20 mM TES/K1 (pH 7.5), 2 mM EDTA, and 2 mM dithiothreitol. Ultracentrifugation and wash were omitted in the experiments of Fig. 1 (see below, under Enzyme Activities).

Solubilization to αβ Protomers—This was carried out essentially as described (2, 26), by mixing equal volumes of FITC-treated (or control) membranes in 2×5 medium and aqueous C12E8 at a detergent:protein mass ratio of 2.9:1. Insoluble material was removed by ultracentrifugation for 15 min at 350,000 × g. When assaying the enzymatic activities of soluble protomers, C12E8 was added to all media (2) at a concentration of 50 μM (critical micelle concentration).

Enzyme Activities—These were measured in washed membrane-bound enzyme, or in soluble protomers, as linear 6-point time courses at 20°C. Na,K-ATPase activity was determined (2) over a 2.5-min period, in 50 μl and at 1 mM [γ-32P]ATP, 2 mM MgCl2, 1 mM EDTA, 118 mM NaCl, 32 mM KCl, 10 mM TES/Na1 (pH 7.5), and 1 mM dithiothreitol. The reactions were stopped by freezing on a dry ice/ethanol bath. K+-phosphatase activity was routinely measured from six time points over 2.5 min in 50 μl of 1×5 medium (pH 7.5) supplemented with 6 mM MgCl2 plus pNPP (Tris salt). The release of p-nitrophenol was measured spectrophotometrically at 410 nm, after stopping the reactions with 0.3 ml of 1 M NaOH. In experiments in the presence of TNP-ADP or TNP-ATP, p-nitrophenol calibration curves were constructed for each of the TNP-nucleotide concentrations. All enzyme activities and errors were obtained from least squares linear fitting of the time courses. Most K+-phosphatase assays were done at several pNPP concentrations, ranging from 1.5 to 20 μM, in order to estimate Vmax by nonlinear regression (Sigmastat package, Corte Madera, CA). In the experiments in Fig. 1, the incubation with (or without) FITC was arrested at the times indicated by diluting (i) a 10-μl sample into 990 μl of chilled medium containing (mM) NaCl 130, KCl 20; histidine (pH 7.2) 25, [γ-32P]ATP 1, and MgCl2 1 (Fig. 1A) or (ii) 10 μl each into 700 μl of chilled assay medium suitable for K+-phosphatase or Na,K-ATPase activity determinations. Similar reaction media were used when 3-OMPP replaced pNPP as K+-phosphatase substrate; product release was then measured against 3-O-methylfluorescein calibration curves, from the
Two Nucleotide Sites in Soluble Protomeric Na,K-ATPase

Table I

| Experiment | Na,K-ATPase activity left in FITC-enzyme | Protein concentration | Control enzyme | FITT-treated enzyme | Control enzyme | FITT-treated enzyme |
|------------|----------------------------------------|-----------------------|----------------|-------------------|----------------|-------------------|
|            | % control enzyme                       | mg/ml                 |                |                   |                |                   |
| 1          | 40.4 ± 3.6                             | 0.16                  | 0.15           | 68 ± 0.1          | 68 ± 0.1       |
| 2          | 39.4 ± 4.4                             | 0.24                  | 0.24           | 69 ± 0.1          | 69 ± 0.1       |
| 3          | 3.2 ± 0.4                              | 0.30                  | 0.29           | 71 ± 0.1          | 71 ± 0.1       |
| Mean       | 3.2 ± 0.4                              | 0.30                  | 0.29           | 69 ± 0.1          | 69 ± 0.1       |

Table II

| Enzyme sample | Na,K-ATPase activity | K⁺-phosphatase activity (μmol · min⁻¹ · mg⁻¹) |
|---------------|----------------------|---------------------------------------------|
|               |                      | With nPMP With 3-OMFP                        |
| Membranes     |                      |                                             |
| Control       | 2.78 ± 0.13          | 2.95 ± 0.10 3.05 ± 0.12                      |
| + FITC        | 0.05 ± 0.01          | 0.22 ± 0.12 0.25 ± 0.06                      |
| αβ protomers  | 2.06 ± 0.17          | 2.51 ± 0.08 2.88 ± 0.07                      |
| + FITC        | 0.05 ± 0.01          | 1.77 ± 0.04 1.85 ± 0.03                      |

Results

Our results for the inactivation of the Na,K-ATPase activity of the sodium pump by FITC, the protective effect of ATP (20, 29), and the survival of the K⁺-phosphatase activity (20, 21) are shown in Fig. 1, for illustration purposes. We could also confirm that the Kₐ₅ for nPMP increases (approximately 4-fold in our hands) following irreversible FITC binding. The maximal K⁺-phosphatase rate remained high, at about 80% of the control activity (see Table II and legend to Fig. 4). In the experiments of Figs. 2 and 3, therefore, the K⁺-phosphatase activities used in the calculations were the fitted Vₘₐₓ values (estimated from determinations at 6 or more nPMP concentrations).

In order to test for independent high affinity and low affinity ATP sites, we wished to find out whether the K⁺-phosphatase activity of well-defined αβ protomers was inhibited by nuclease when the high-affinity ATP site had been blocked by the modified fluorescein. The limited thermal stability of the solubilized protomers, even at 20 °C (30), thwarted attempts at treating the enzyme with FITC at alkaline pH after detergent solubilization; lower pH values would only prolong the incubation period. The alternative was to solubilize the FITC-treated membrane enzyme. It has been reported that C₁₂E₈ solubilization of the FITC-modified sodium pump releases particles (30) whose sedimentation coefficient (s₂₀,₅₀) is 6.9 S, i.e. as expected for the αβ protomer (2, 26). The results presented in Table I average to an identical s₂₀,₅₀ value and confirm that, in our hands also, solubilization of the FITC-treated enzyme leads to αβ protomeric particles.

Before opting for the approach of solubilizing after FITC treatment, however, certainty was needed that the high affinity ATP site of every αβ protomeric unit was occupied by FITC when complete inactivation of the overall sodium pump reaction had been achieved. The degree of confidence was not as high as desirable for the present purpose (see "Discussion"), and the endurance of the K⁺-phosphatase activity in the FITC-inactivated membrane enzyme seemed open, therefore, to two alternative interpretations. One possible explanation was that, in each αβ protomeric unit, there was a site that bound the phosphatase substrate that was different from the high affinity ATP site (which was blocked by FITC). In this case, solubilization of the membrane-bound, FITC-modified sodium pump with C₁₂E₈ would release αβ protomers whose Na,K-ATPase specific activity (relative to the FITC-free control enzyme) was no more, and no less, than the (relative) Na,K-ATPase specific activity of the parent membrane-bound enzyme. The alternative possibility was that the high affinity ATP site and the K⁺-phosphatase substrate were different from each other. ATP hydrolysis, which requires cyclical E₁-E₂ transitions, would be prevented just as in the FITC-blocked protomer. As solubilization with C₁₂E₈ in our conditions, dissociates any (αβ)₂ dimers (2, 26), the good protomer should now be higher than that of the par-
after solubilization, and the specific activities (in \( \mu \text{mol-min}^{-1} \cdot \text{mg}^{-1} \)) calculated. The data represent 100 times the specific activity of the FITC-modified enzyme, divided by the relevant (ATPase or phosphatase) specific activity of the appropriate (membrane-bound or solubilized) control enzyme. In agreement with previous observations, and in spite of the very low level of Na,K-ATPase activity left over, the \( K^+ \) -phosphatase activity remains at a high 80% in both the membrane-bound and solubilized enzymes. The crucial feature, however, is that the percent Na,K-ATPase activity left in the soluble protomers does not differ from the percent Na,K-ATPase activity left in the parent membrane-bound enzyme. In the case of the “one-site-plus-dimer” hypothesis, one should have expected that the Na,K-ATPase increase to 51.6 ± 6.5% of the controls, after solubilization (clear plus filled bars on right). The experiment was repeated at several levels of inactivation of the Na,K-ATPase activity, and the result is shown in Fig. 3. It is apparent that, despite some dispersion in the data, the results are firmly anchored on the 1:1 correlation line expected for the “two-sites-per-protomer” hypothesis.

Table II shows that the protomeric FITC-enzyme can efficiently utilize the bulky 3-OMFP as a \( K^+ \) for the “two-sites-per-protomer” hypothesis. The experiment was repeated at several levels of inactivation of the Na,K-ATPase activity, and the result is shown in Fig. 3. It is apparent that, despite some dispersion in the data, the results are firmly anchored on the 1:1 correlation line expected for the “two-sites-per-protomer” hypothesis.

Two Nucleotide Sites in Soluble Protomeric Na,K-ATPase

**Fig. 4. Inhibition of the \( K^+ \)-PNPase activity of soluble protomeric Na,K-ATPase by TNP-ADP.** Data in the form of Dixon plots. A, control enzyme; B, FITC-treated enzyme. In A, the \( K^+ \)-phosphatase activity was assayed at the TNP-ADPNa-[\( S \)] concentrations shown and 2 (solid circles), 4 (open circles), and 6 \( \mu \text{m} \) pNPP (squares) and in B, at 3 (solid circles), 5 (open circles), and 9 \( \mu \text{m} \) pNPP (squares). The total sodium concentration was kept constant. After solubilization, the FITC-treated enzyme presented 88.6 ± 3.6% of the control \( K^+ \)-phosphatase specific activity and 10.7 ± 1.6% of the control Na,K-ATPase specific activity. The kinetic parameters of the \( K^+ \)-phosphatase reaction of the \( \alpha \beta \) protomers were (K<sub>19</sub> [\( S \)], unstable, and V<sub>max</sub>, \( \mu \text{mol-min}^{-1} \cdot \text{mg}^{-1} \)): 0.99 ± 0.16 and 2.12 ± 0.06, for the control enzyme, and 3.84 ± 0.12 and 1.88 ± 0.05, for the FITC-modified enzyme. The arrows in B indicate, for each pNPP concentration, the maximal level of 1/\( v \) expected had only the native component of the \( K^+ \)-phosphatase activity been inhibited by TNP-ADP.

It being clear that FITC can block an ATP site in every protomeric unit of the membrane-bound enzyme, we tested the effect of nucleotides on the \( K^+ \)-phosphatase activity of the soluble protomers. Experiments using ATP (Tris salt) to 5 \( \mu \text{m} \) (not shown) produced clear effects with the control membrane-bound and solubilized enzymes, but its effect on the FITC-modified enzyme seemed to be obscured by a much reduced affinity (cf. Refs. 19 and 21). Lower pNPP or higher ATP concentrations imposed unacceptably high spectrophotometric errors or ionic strength compensations, respectively, and we resorted to using TNP-ADP, which has proved useful in experiments with the membrane-bound FITC-modified enzyme (19). The result of one of three similar experiments is presented in Fig. 4, as Dixon plots, for the soluble protomers. Here, again, the affinity is very much reduced in the FITC-treated protomers. K<sub>i</sub> works out as 65 ± 8 \( \mu \text{M} \), compared with the native protomers at 0.084 ± 0.003 \( \mu \text{M} \), but it is evident that TNP-ADP inhibits the \( K^+ \)-phosphatase activity competitively well beyond what could be expected if it merely suppressed the 10% of the sodium pump that escaped FITC modification (arrows). Dixon plots of the \( K^+ \)-phosphatase activity of the parent membrane-bound FITC-modified enzyme (not shown) also reflected the decreased TNP-ADP affinity, as has been observed earlier (19), with K<sub>i</sub> values of 42 ± 9 \( \mu \text{M} \) and 0.52 ± 0.05 \( \mu \text{M} \) for the FITC-treated and control enzymes, respectively. TNP-ADP was also effective with the FITC-modified membrane enzyme, but the estimated inhibition constant was 4-fold that for TNP-ADP, and it was thereby not used with the soluble protomers. Picrate (2,4,6-trinitrophenolate) at 500 \( \mu \text{M} \) inhibited the \( K^+ \)-phosphatase activity of the FITC-modified enzyme by 13% in conditions that 250 \( \mu \text{M} \) TNP-ADP caused a decrease of over 70%. It seems safe to conclude, therefore, that the bulk of the TNP-ADP inhibition does not represent nonspecific effects of the trinitrophenyl group.

**DISCUSSION**

Our test for a discrete low affinity nucleotide site in Na,K-ATPase is based on searching for a known E<sub>2</sub> effect of nucleotides on \( \alpha \beta \) protomers with a blocked high affinity ATP site. We chose FITC as the blocking agent and the \( \alpha \beta \)-ATPase activity as the E<sub>2</sub> function. The covalent FITC binding to Lys<sup>551</sup> suppresses phosphorylation by ATP and the overall pump reaction, but does not prevent backwards phosphorylation by inorganic phosphate or abolish the \( K^+ \)-phosphatase activity of the enzyme. Obviously, the possibility had to be excluded that any observable low affinity nucleotide effects could arise from binding at a unique nucleotide site that was left vacant in a neighboring and interacting subunit.

It has been reported that, at 100% inactivation of the Na,K-ATPase activity, the protein mass that can incorporate 1 mol of FITC can otherwise bind 1 mol of ouabain (32) or form 1 mol of acid-stable phosphoenzyme (33). Our difficulty with this approach was that, with the best preparations of native enzyme, it had not been possible to achieve acid-stable phosphorylation or ouabain binding levels of 1 mol/mol protomeric unit (6.9 nmol/mg of protein) using the same standard phosphorylation or ouabain binding assays, and that this did not seem just a matter of protein assay method (34). For instance, the standard ouabain/phosphorylation ratio is subject to some uncertainty (35, 36), and it is now apparent that, depending on the experimental conditions, measurements of acid-stable phosphorylation can grossly underestimate the number of active sodium pump units (37, 38). In addition, it has been reported that FITC
can also bind to Lys, on the chain, which is not necessarily within the high affinity ATP-binding pocket. Any one, or a combination, of these complications could cause an overestimate of the number of functional high affinity ATP sites that are occupied by FITC. This possibility seems more plausible when considering the evidence that the membrane-bound Na,K-ATPase behaved, at least in some conditions, as a functional dimer of protomers (7, 18, 40–43).

The concerns above prompted the experiments shown in Figs. 2 and 3, which exclude the possibility that only one-half of a putative membrane dimer could bind FITC. If all active protomers in the membrane-bound enzyme are potential targets for the binding, the corollary would be that the K+-phosphatase substrate site should be different from the blocked high affinity ATP site. It would follow also that the competitive TNP-ADP inhibition of the K+-phosphatase activity of FITC-modified soluble protomers (Fig. 4) should result from TNP-ADP binding at a location different from the blocked site, probably at the pnpp-binding site. We have confirmed that, in the membrane-bound enzyme, FITC treatment decreases the affinities of PnPP, ATP, and TNP-ADP for their E, effects (19, 21) and find that this effect persists after solubilization to protomers. In the control enzyme, this decreases K, for TNP-ADP from 0.52 μM (cf. Ref. 17) to 0.084 μM, which agrees with the 8-fold decrease of K, observed for the low affinity ATP activation of the Na,K-ATPase activity upon solubilization (2). This downward shift did not occur with the FITC-modified enzyme, as K, changed from 42 μM in the membrane-bound enzyme (compare with 35 μM in Ref. 19) to 65 μM after solubilization. The possibility should be considered, therefore, that all of the FITC does is greatly lowering the affinity of two states of a single ATP site. If that were so, the FITC block of the Na,K-ATPase activity should be relieved by increasing the ATP concentration. This did not occur at 20 mM ATP when using the coupled enzyme spectrophotometric assay (19), and we now find that it does not happen at 20 mM [γ-32P]ATP either. Had FITC lowered both high and low affinities by, say, a thousand-fold (so that K, ~ 200 mM), we would have easily noticed an increase from 1% (at 2 mM) to 9% (at 20 mM) of the control Na,K-ATPase activity. By far, the most likely explanation is that ATP is utterly prevented from accessing the catalytic pocket by the bound fluorescein which also induces a distortion in the low-affinity nucleotide site. The block of the catalytic ATP site probably results from the restricted rotational motion of the bound fluorescein, as observed even through E, conformational changes (44).

It is conceivable that the catalytic ATP site and the phosphatase site could share binding groups, perhaps their phosphate subsites. At any rate, such overlap must be such that access be still allowed to the fluorescein moiety of 3-OMFP when another fluorescein blocks the catalytic ATP site of the FITC-enzyme (Table II). On the other hand, it seems probable that the second nucleotide site be the same as the phosphate site, a question that can be explored in more elaborate experiments. Fully competitive inhibition (same site) of the K+-phosphatase by nucleotides should lead to linear Dixon plots whereas partially competitive (allosteric) inhibition should show as hyperbolae (45). The plots in Fig. 4 look convincingly linear, but higher inhibitor and lower substrate concentrations might lead to a curvature (46). Downward-bent Dixon plots do appear when using ATP as the inhibitor with the membrane-bound sodium pump (21), and we have confirmed this, also with the protomers. Caution is needed in their interpretation, though, as in a partially modified enzyme the difference in kinetic parameters between FITC-inactivated and outlasting enzyme populations could lead to an artifactual downward curvature. For instance, had the FITC-modified enzyme been impervious to TNP-ADP inhibition, the plots in Fig. 4 would have bent down and become parallel to the abscissa, at maximal ordinate levels given by the arrows in panel B (and at much lower values of the abscissa, compare with panel A).

The test of Figs. 2 and 3 does not contradict the possibility that the membrane-bound sodium pump behave, at least in some conditions, as a dimer of protomers. What proves it is the absence of half-of-the-sites reactivity toward FITC. However, some of the evidence for a dimeric Na,K-ATPase may need reinterpreting in consideration of a second nucleotide site per protomeric unit, and the added element of complexity should be taken into account. The functional relationships between the two nucleotide sites, and their behavior during the reaction cycle, remain to be explored.

Acknowledgments—We thank Tim Walton for his help with the preparation of the enzyme and Purnima Mistry (National Centre for Macromolecular Hydrodynamics, Leicester University) for the analytical ultracentrifugation runs.

REFERENCES

1. Glynn, I. M. (1985) in The Enzymes of Biological Membranes (Martonosi, A. N., ed) Vol. 3, pp. 35–114, Plenum Press, New York.
2. Ward, D. G., and Cavieres, J. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5332–5336.
3. Kanazawa, T., Salto, M., and Tomonura, Y. (1967) J. Biochem. (Tokyo) 61, 555–566.
4. Peretz, R., Hegvary, C., and Kurne, S. (1972) J. Biol. Chem. 247, 6530–6540.
5. Glynn, I. M., and Richards, D. E. (1982) J. Biol. Chem. (Lond.) 257, 17–43.
6. Kaplan, J. H., and Kenney, L. J. (1982) Ann. N. Y. Acad. Sci. 402, 292–295.
7. Cavieres, J. D. (1987) FEBS Lett. 225, 145–150.
8. Kakar, S. S., Huang, W. H., and Askari, A. (1987) J. Biol. Chem. 262, 42–45.
9. Tran, C. M., Huston, E. E., and Farley, R. A. (1994) J. Biol. Chem. 269, 6558–6565.
10. Tran, C. M., Scheiner-Bobis, G., Schoner, W., and Farley, R. A. (1994) Biochemistry 33, 4140–4147.
11. Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., and Shively, J. E. (1984) J. Biol. Chem. 259, 5932–5935.
12. Kirley, T. L., Wallick, E. T., and Lane, L. K. (1984) Biochemistry. Res. Commun. 125, 767–773.
13. Ohita, T., Nagano, K., and Yoshida, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2071–2075.
14. Ovchinnikov, Y. A., Dzhandzhugazyan, K. N., Lutsenko, S. V., Mustayev, A. A., and Mudayyan, N. N. (1987) FEBS Lett. 217, 111–116.
15. Lazdunski, M. (1976) Prog. Biophys. Chem. 3, 82–140.
16. Smith, R. L., Zinn, K., and Cantley, L. C. (1980) J. Biol. Chem. 255, 9852–9859.
17. Moczydlowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 2346–2356.
18. Askari, A. (1987) Biochemistry 26, 359–361.
19. Scheiner-Bobis, G., Antonioli, J., and Farley, R. A. (1993) Biochemistry 32, 9592–9599.
20. Karish, S. J. D. (1980) J. Bioenerg. Biomembr. 12, 111–136.
21. Davis, R. L., and Robinson, J. D. (1989) Biochem. Biophys. Acta 953, 26–36.
22. Nagai, K., and Yoshida, H. (1966) Biochim. Biophys. Acta 128, 410–412.
23. Robinson, J. D. (1969) Biochemistry 8, 3348–3355.
24. Ward, D. G., and Cavieres, J. D. (1994) J. Biol. Chem. (Lond.) 482, 42–43P.
25. Jergensen, P. L. (1974) Biochem. Biophys. Acta 356, 36–52.
26. Broderius, J. R., Jacobsen, L., and Jergensen, P. L. (1983) Biochemistry. J. Bioenerg. Biomembr. 15, 382–101.
27. Schachman, H. K. (1959) Ultracentrifugation in Biochemistry, Academic Press, New York.
28. Tran, C. M., and Farley, R. A. (1986) Biochem. Biophys. Acta 850, 9–14.
29. Askari, A., and Modyanov, N. N. (1987) Biochemistry 26, 3610–3612.
30. Forbush, B. (1988) J. Biol. Chem. 263, 7961–7969.
31. Cavieres, J. D., and Ellory, J. C. (1975) in The Enzymes (Martonosi, A. N., ed) Vol. 3, pp. 5764–5772, Plenum Press, New York.
32. Koepsell, H., Hulla, F. W., and Fritzsch, G. (1982) J. Biol. Chem. 257, 10373–10374.
33. Ottolenghi, P., and Jensen, J. (1983) Biochem. Biophys. Acta 727, 89–100.
34. Ashari, A., and Ball, W. J. (1982) Biochemistry 21, 407–413.
35. Cavieres, J. D., and Ellory, J. C. (1975) Nature 255, 338–340.
Binding of 2′(3′)-O-(2,4,6-Trinitrophenyl)ADP to Soluble αβ Protomers of Na,K-ATPase Modified with Fluorescein Isothiocyanate: EVIDENCE FOR TWO DISTINCT NUCLEOTIDE SITES
Douglas G. Ward and José D. Cavieres

J. Biol. Chem. 1996, 271:12317-12321.
doi: 10.1074/jbc.271.21.12317

Access the most updated version of this article at http://www.jbc.org/content/271/21/12317

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 11 of which can be accessed free at http://www.jbc.org/content/271/21/12317.full.html#ref-list-1