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MINI-REVIEW

$(Na^+ + K^+)$-ATPase: On the Number of the ATP Sites of the Functional Unit

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Abstract

Questions concerning the number of the ATP sites of the functional unit of $(Na^+ + K^+)$-ATPase (i.e., the sodium pump) have been at the center of the controversies on the mechanisms of the catalytic and transport functions of the enzyme. When the available data pertaining to the number of these sites are examined without any assumptions regarding the reaction mechanism, it is evident that although some relevant observations may be explained either by a single site or by multiple ATP sites, the remaining data dictate the existence of multiple sites on the functional unit. Also, while from much of the data it is clear that the multiple sites of the unit enzyme represent the interacting catalytic sites of an oligomer, it is not possible to rule out the existence of a distinct regulatory site for ATP in addition to the interacting catalytic sites. Regardless of the ultimate fate of the regulatory site, any realistic approach to the resolution of the kinetic mechanism of the sodium pump should include the consideration of the established site–site interactions of the oligomer.

Key Words: $(Na^+ + K^+)$-ATPase; sodium pump; sodium ion; ATP; regulatory site; catalytic site; potassium ion; oligomeric structure; site–site interactions.

Introduction

A strange feature of the controversies surrounding the ATP sites of $(Na^+ + K^+)$-ATPase is that it is not always clear what the controversies are about. The confusion is partly due to the fact that disagreements on the number of ATP sites overlap with those on several other interesting issues (e.g., the reaction

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mechanism of the enzyme), and often sufficient care is not taken to clarify what is the central subject of a debate. It is necessary, therefore, that we begin with a brief history of matters pertaining to the ATP sites of the enzyme, and identify the issues that will and will not be addressed here.

In early studies on crude enzyme preparations it was noted (Czerwinski *et al.*, 1967; Neufeld and Levy, 1969) that the initial velocity of the $Na^+ + K^+$-dependent ATPase activity as a function of ATP concentration exhibited negative cooperativity,[2] and this was explained by suggesting the presence of two distinct enzymes in such preparations: One a Na^+-activated ATPase with a high affinity for ATP, and the other a $Na^+ + K^+$-activated enzyme with lower affinity for the substrate. Subsequently, it was pointed out (Post *et al.*, 1972) that the assumption of two enzymes was not necessary and that the phenomenon could be explained by the existence of a low-affinity allosteric site for ATP in addition to the high-affinity catalytic site. With the development of what is now known as the Albers–Post scheme for the reaction mechanism of the enzyme (Glynn, 1985), the low-affinity effect of ATP was suggested to be the enhancement of the slow conversion of the enzyme species from which P_i had just departed (E_2K^+) to the species (E_1Na^+) that could accept ATP at the high-affinity catalytic site (Hegyvary and Post, 1971; Post *et al.*, 1972).

In 1973, soon after the suggestion of a regulatory role of the low-affinity ATP site, the possibility of the functional significance of the dimeric structure of the enzyme was proposed by two groups (Repke and Schon, 1973; Stein *et al.*, 1973). These reaction schemes and their subsequent modifications by others (Cantley *et al.*, 1978; Froehlich *et al.*, 1976; Robinson and Flashner, 1979) were all variations of the flip-flop or alternating site mechanisms first proposed for malic dehydrogenase (Harada and Wolfe, 1968) and alkaline phosphatase (Lazdunski, 1970). Such mechanisms were applied to $(Na^+ + K^+)$-ATPase not only because they were fashionable at the time, but also because they explained certain unusual properties of the enzyme, e.g., the fact that in some preparations it seemed that only half of the enzyme could be phosphorylated by ATP (Glynn and Karlish, 1975). A further achievement of an alternating site model was that it eliminated the need for distinct catalytic and regulatory ATP sites, since the catalytic site of one protomer could act as the regulatory site of the other.

Such was the state of affairs until 1980–1981 when several reports appeared (Craig and Kyte, 1980; Moczydlowski and Fortes, 1981; Peters *et al.*, 1981; Smith *et al.*, 1980) containing data which seemed to be inconsistent with the alternating site mechanism. By a twist of logic, the apparent

[2] The term cooperativity is used in the phenomenological sense to indicate the nonlinear nature of the double-reciprocal plot or the Scatchard plot.

discredit of this model was then turned by some into the advocacy of the notion that the functional unit of the enzyme is a monomer (i.e., the α,β-protomer) with a single ATP site. This idea has since been picked up by others, and considerable effort has been spent to rationalize all catalytic and transport properties of the enzyme in its context. On previous occasions we have pointed out how little solid evidence exists in support of the single-site monomeric enzyme (Askari, 1982; Askari and Huang, 1985). Here, with consideration of more recent findings, I shall make the same point, and show that while some relevant observations may be explained either by a single site or by multiple ATP sites, the remaining data make it necessary to assume the existence of multiple sites on the functional unit of the enzyme. I shall also point out that although from much of the data it is clear that the multiple sites represent the interacting catalytic sites of an oligomer, it is not possible to rule out the existence of a distinct regulatory site for ATP in addition to the catalytic site. The conclusion that there are multiple ATP sites is independent of whether or not an alternating site model applies to this enzyme. In fact, it should be emphasized that the complexities of the reaction mechanism of the enzyme and the associated controversies (e.g., whether or not Na^+ and K^+ may bind to the enzyme simultaneously) are not the primary topics of consideration here, though at the end I shall briefly discuss the implications of multiple ATP sites for the reaction mechanism.

In the following sections each line of evidence that is pertinent to the number of ATP sites of the enzyme will be examined separately. Many of these lines are unnecessarily intertwined in the original literature.

Half-Site Reactivity of the Enzyme

As indicated already, the early observations that the enzyme may exhibit half-site reactivity (e.g., in respect to phosphoenzyme formation) was one rationale behind the suggestion that the enzyme is an oligomer, and for the proposal of an alternating site mechanism. The subsequent findings of three laboratories (Craig and Kyte, 1980; Moczydlowski and Fortes, 1981; Peters *et al.*, 1981) indicating that the molecular weight of the minimum asymmetric unit of the enzyme (i.e., the α,β-protomer) had been underestimated, and that the protein concentration of enzyme preparations had been overestimated through the use of the Lowry method, led to the conclusion that the enzyme was not half-site reactive. In turn, this was used as one of the arguments against the alternating site model, and in favor of the monomeric single-site enzyme (Cantley, 1981; Kyte, 1981). Three points concerning these findings and conclusions should be noted: (1) The initial impression (Craig and Kyte, 1980) that quantitative amino acid analysis was a more accurate way of

determining the true protein content of the enzyme than the Lowry method was followed by the actual comparison of the two methods in several laboratories. However, the reported overestimation of the protein content by the Lowry method in the hands of various groups ranged from 10 to 90% (Koepsell *et al.*, 1982; Moczydlowski and Fortes, 1981; Peters *et al.*, 1981), raising doubts about the reliability of the quantitative amino acid analysis as applied to this enzyme. The most recent report (Chetverin, 1986) in which detailed data on the comparison of amino acid analysis, the Lowry method, and total nitrogen determination by the Kjeldahl method were presented showed that quantitative amino acid analysis was in fact less reliable than the Lowry method, and that it underestimated the true protein content of the enzyme! Clearly, until the dust settles down, arguments against the enzyme's half-site reactivity which are based on "true" protein content can not be considered valid. (2) While the demonstration of half-site reacitivity is excellent evidence for the oligomeric structure of an enyzme (or for two interacting sites of the same ligand on a monomer), the demonstration of all-site reactivity provides no information on the number of ligand sites on the functional unit. (3) Demonstration of half-site reactivity need not rely on the knowledge of the "true" protein content. Ligand-induced doubling or halving of the maximal capacity of the enzyme for binding a ligand, or for covalent modification by a ligand, is good evidence for half-site reactivity. Such evidence, in fact, has been obtained (a) in experiments on the covalent reaction of one α-subunit either with another α-subunit or with a β-subunit (Askari and Huang, 1980; Periyasamy *et al.*, 1983); (b) in studies on enzyme phosphorylation by P_i (Askari and Huang, 1981; Askari *et al.*, 1983); and (c) in experiments on rubidium ion that is "occluded" by the enzyme (Glynn *et al.*, 1985). Also of interest are the experiments of Chetverin (1986) suggesting the posttranslational blockade of the N-termini of half of the α-subunits. On the whole, observations of half-site reactivity of the enzyme continue to provide strong support for the existence of negative site–site interactions in the oligomeric membrane-bound enzyme.

Cooperativities of the Initial Velocity and Equilibrium Binding Plots

One of the major items of evidence used in support of the monomeric single-site enzyme has been the argument that the well-known negative cooperativity[2] of the substrate–velocity of the enzyme can be explained by the slow isomerization of two states of the monomeric enzyme with low and high affinities for ATP at the same site; and that it is not necessary to assume

[2]See footnote 2.

site–site interactions of an oligomer, or the existence of catalytic and regulatory sites, to explain the shape of this plot (Moczydlowski and Fortes, 1981; Smith *et al.*, 1980). That cooperativity of kinetic origin may arise from a variety of mechanisms that do not involve multiple substrate sites has been known for a long time (Askari and Huang, 1985; Neet, 1981). It is unfortunate that recognition of possible applicability of such mechanisms to $(Na^+ + K^+)$-ATPase was not accompanied by that of another equally well-established fact: A monomeric enzyme with a single substrate site that exhibits kinetic cooperativity due to slow transitions would not show cooperativity in equilibrium binding plots (Neet, 1981). The negative cooperativity of plots of ATP binding to the enzyme in the presence of K^+ was demonstrated many years ago (Norby and Jensen, 1974), and the phenomenon has been confirmed repeatedly (Jensen and Ottolenghi, 1976; Jensen *et al.*, 1984; Ottolenghi and Jensen, 1983; Schoner *et al.*, 1977). Ligand-induced cooperativities in the plots of phosphoenzyme formation from P_i under equilibrium conditions (Askari and Huang, 1984) and of the equilibrium binding of ouabain to the enzyme (Hansen, 1984; Ottolenghi and Jensen, 1983) have also been clearly demonstrated. These data, considered together with the half-site reactivity experiments, require the existence of two equal populations of sites in the functional unit. In respect to ATP sites, therefore, the unit must be a dimer, or a higher oligomer, capable of functional symmetry. Whether the asymmetry is induced or preexisting cannot be established easily, though certain data favor the former (Ottolenghi and Jensen, 1983).

The original literature advocating the notion that the slow transitions of the single-site monomer are responsible for the cooperativity of the substrate–velocity curve also contain calculations showing the reasonable agreement of the proposed kinetic model with experimental data (Moczydlowski and Fortes, 1981; Smith *et al.*, 1980). It is important to note that any measure of credibility of the single-site model that is implied by such "quantitative" agreements is also not deserved, since the equations derived for the model are independent of the assumptions concerning the physical relationship between the ATP sites of the two states of this model. It is also important to emphasize that while the cooperative plots under equilibrium conditions establish the existence of multiple sites, they do not rule out the significance of slow transitions to the enzyme function. Slow transitions and intersubunit site–site interactions may occur in the same enzyme (Askari and Huang, 1984; Neet, 1981).

Ratio of ATP Binding Sites to Phosphorylation or Ouabain Binding Sites

There is general agreement in the field that in purified membrane-bound preparations of the enzyme the maximal number of high-affinity ATP binding

sites is equal to the maximal phosphorylation capacity or the ouabain binding capacity of the enzyme. Many years ago experiments of Hegyvary and Post (1971) on the equilibrium binding of ATP to a crude preparation of enzyme suggested that there may be some low-affinity binding of ATP in excess of the high-affinity binding to the catalytic site. This has been confirmed by subsequent experiments of several laboratories with highly purified preparations and improved techniques (Ball, 1986; Koepsell, 1978; Schuurmans-Stekhoven *et al.*, 1981, 1983; Yamaguchi and Tonomura, 1980). Because of the well-known limitations of the radioligand binding studies on ligands of low affinity, and continued skepticism of the field about the validity of the results of the above studies, recently we used a different technique for the detection of the ATP sites. Examination of the effects of varying concentrations of ATP and other nucleotides on the unidirectional dissociation of the ouabain–enzyme complex demonstrated that the complex contained two sites with different affinities for ATP and with different nucleotide specificities (Kakar *et al.*, 1985). There is of course no easy way of relating the properties of the ligand sites of ouabain-complex enzyme to those of the sites on the native enzyme, but the technique does show unambiguously that the membrane-bound enzyme contains two ATP sites for each ouabain binding or phosphorylation site. Using the same technique, we have also demonstrated (unpublished observations) the existence of two sites for eosin (tetrabromofluorescein) or TNP-ATP, two inhibitors of the enzyme that are thought to bind to the ATP site. Two eosin sites per phosphorylation site have also been demonstrated by Skou and Esmann (1983); but only one of the TNP-ATP sites has been detected by other techniques (Moczydlowski and Fortes, 1981). We should also consider briefly the studies with ATP analogs that inhibit the enzyme through covalent modification. The results of some of these studies are difficult or impossible to explain without the assumption of multiple ATP sites (Dzhandzhugazyan and Modyanov, 1985; Patzelt-Wenczler and Schoner, 1981; Patzelt-Wenczler and Mertens, 1981; Koepsell *et al.*, 1982). Experiments with fluorescein isothiocyanate have been interpreted in favor of monomeric single-site enzyme primarily because inhibition seemed to require the reaction of one mole of the probe per one mole of α-subunit (Fortes and Han, 1985). This stoichiometry, however, is subject to the same uncertainties about protein assays that were discussed in the section on half-site reactivity.

Since it is now clear that there is an ATP site in excess of the high-affinity ATP site involved in phosphoenzyme formation, we may ask how the two sites are related. The uncertainties discussed in a previous section on whether all or only half of the α,β-protomers of the membrane-bound enzyme are phosphorylated by ATP, also create uncertainties on the nature of the extra ATP site. If we assume, as it seemed to be certain a few years ago, that all

protomers of the membrane-bound enzyme are phosphorylated, we must then conclude one of two alternatives: Either each protomer of the oligomeric enzyme (with site–site interactions among the catalytic sites) also constains a distinct regulatory site, or that each protomer contains two catalytic sites with negative interactions, as has been suggested for other enzymes (Fersht, 1975). On the other hand, since the more recent data indicate again that only half of the membrane-bound protomers is phosphorylated (Chetverin, 1986; Ottolenghi and Jensen, 1985), the extra ATP site could be on the half of the protomers that is not ordinarily phosphorylated due to negative site–site interactions. If this is the case, the ligand-induced cooperativities of the equilibrium binding data that were discussed in a previous section would indicate that the half of the protomers that is phosphorylated is also an oligomer, meaning that the functional unit is a tretramer of α,β-protomers. It will be noted below that several other lines of evidence also suggest a tetrameric state.

ATP Binding to the Phosphoenzyme and the Vanadate-Complexed Enzyme

Years ago it was recognized that some variations of the alternating site model of the enzyme required the binding of ATP to the phosphoenzyme (Glynn and Karlish, 1975). With the discovery that vanadate inhibits the enzyme through tight binding to the P_i release site, and the assumption of an alternating site model, Cantley *et al.* (1978) concluded that ATP should also bind to the vandate-complexed enzyme. Failure of the same group to detect such binding was the basis for the later conclusion that the alternating site model was not operative, and for one of the first considerations of the monomeric single-site model (Smith *et al.*, 1980). Subsequently, however, the binding of ATP and other nucleotides to the phosphoenzyme and the vanadate-complexed enzyme was demonstrated unambiguously by our laboratory (Askari and Huang, 1982, 1984; Huang and Askari, 1981) and by others (Fukushima *et al.*, 1984; Hobbs *et al.*, 1985; Shuurmans-Stekhoven *et al.*, 1983). Simultaneous bindings of ATP and P_i to the enzyme have also been indicated by studies on the K^+,K^+-exchange that is carried out by enzyme (Sachs, 1981; Karlish and Stein, 1982). Needless to say, these findings do not prove the alternating site model, but they certainly pose problems to the monomeric single-site model. To avoid these, it has been pointed out that the single-site model can be made compatible with the simultaneous bindings of ATP and P_i if we assume that after the binding of ATP to the catalytic site and enzyme phosphorylation, the phosphoenzyme is so distorted that the low-affinity ATP binding occurs to the site from which ADP has departed (e.g., Karlish and Stein, 1982). With the assumption of such a conformational

change that separates the P_i site from the adenine binding site, Robinson *et al.* (1986) have proposed an ingenious modification of the original kinetic scheme of the single-site enzyme that is compatible with the data on the effects of ATP, P_i, and K^+ on enzyme velocity. The scheme has the shortcoming that it does not permit the binding of ATP to the phosphoenzyme in the absence of K^+, a phenomenon for which there is ample evidence (Fukushima *et al.*, 1984; Hobbs *et al.*, 1985). More importantly, the validity of this kinetic scheme, as that of its simpler predecessors, is of course independent of assumptions on the structural relationships between the ATP sites. Considering that the simultaneous bindings of two ATP molecules have been demonstrated (the previous section), the simultaneous bindings of ATP and P_i, or ATP and vanadate, must also be considered as strong evidence in favor of the existence of multiple ATP sites on the unit enzyme.

Chemical Cross-Linking of Enzyme Subunits

The results of studies on the reaction of the enzyme with chemical cross-linking reagents are among the strongest evidence in favor of the oligomeric structure of the enzyme and the existence of intersubunit site–site interactions. Some of the cross-linking studies, however, are also among the most widely quoted evidence in support of the monomeric single-site enzyme. To see how this confusing state of affairs has come about, it is necessary that we examine these studies in some detail.

Early experiments with the membrane-bound enzyme (Kyte, 1972, 1975) showing the formation of a covalently cross-linked α,β-dimer upon exposure to one type of reagent, and the formation of a cross-linked α,α-dimer upon reaction with another type of reagent, were interpreted to indicate the minimum oligomeric structure of $(\alpha,\beta)_2$. Similar results with the membrane-bound enzyme were obtained later in several laboratories (Askari *et al.*, 1980; Giotta, 1976; Liang and Winter, 1977; Sweadner, 1977). Experiments of three laboratories with detergent-solubilized enzymes, however, complicated matters. In spite of the use of different detergents and conditions, the findings of these studies were essentially the same: In the presence of a detergent, formation of cross-linked α,β-dimer could be demonstrated, but not that of the cross-linked α,α-dimer. Two of the laboratories (Huang and Askari, 1979; Liang and Winter, 1977) recognized the possibility that detergent treatment of the native enzyme may have abolished or altered its α,α-contact, but the third (Craig and Kyte, 1980) emphasized the alternative that the formation of cross-linked α,α-dimer observed only in the absence of detergents may have been due to the random collisions of α,β-protomers that were highly concentrated, rather than associated, within the membrane phase. It is

important to note that while these studies created doubts about the existence of α,α-associations within the membrane, they firmly established the existence of α,β-associations; and they showed that the ratio of α to β in the unit enzyme was indeed 1 (Craig and Kyte 1980; Liang and Winter 1977).

To resolve the uncertanties concerning the existence of α,α-interactions within the membrane, a series of studies on the membrane-bound enzyme were conducted in our laboratory (Askari *et al.*, 1980; Askari and Huang, 1980; Huang and Askari, 1981; Periyasamy *et al.*, 1983). The most pertinent aspects of the findings were as follows: First, the formation of both cross-linked α,β-dimer and α,α-dimer in the presence of a variety of cross-linking reagents could be induced or modified specifically by physiological ligands of the enzyme under conditions where the conformational transitions induced by these ligands had been demonstrated by experiments other than cross-linking. Second, it was shown that several specific ligand conditions that induced or reduced α,β-dimer formation in the presence of one cross-linking reagent had the same effects on the formation of α,α-dimer in the presence of another reagent. Since the existence of α,β-association in the native enzyme had already been established, the inevitable conclusion of the above findings was that the same ligand-induced conformational transitions that affected the α,β-domain also affected the α,α-domain of the oligomer, thus establishing $(\alpha,\beta)_2$ as the minimum size of the structural unit of the membrane-bound enzyme. That cross-linked α,α-dimer formation was not due to random collisions of protomers was also established by showing the same ligand-induced cross-linking patterns in different membranes in which the density of the enzyme differed at least by a factor of 10^4 (Periyasamy *et al.*, 1983). A further finding of these studies was that when cross-linking depended on the prior phosphorylation of the enzyme, the maximal level of α,α-dimer formed was half of the total α-content of the enzyme, suggesting that the oligomer was in fact a tetramer rather than a dimer of α,β-protomer.

While the results of the cross-linking studies with the membrane-bound enzyme can now be clearly interpreted, the same cannot be said about the results of such studies with the detergent-solubilized enzyme. After the inconclusive studies already mentioned, two additional reports on the cross-linking properties of the detergent-solubilized enzyme have appeared. The results are conflicting. Craig (1982a,b) reported that an apparently monomeric $C_{12}E_8$-solubilized enzyme that exhibited activity when added to a reaction mixture yielded only a cross-linked α,β-dimer when it was exposed to glutaraldehyde under assay conditions. Based on these findings, and the assumptions that glutaraldehyde is capable of detecting all modes of subunit associations, he concluded that the α,β-protomer had complete enzymatic and transport function. The questionable validity of the assumption of

glutaraldehyde as a universal cross-linker aside, in our laboratory the same solubilized preparation used by Craig underwent spontaneous cross-linking; and the characteristics of the cross-linked products, with or without glutaraldehyde, indicated that the preparation was at least a dimer of α,β-protomer (Periyasamy *et al.*, 1983). In view of these discrepancies, it is clearly not justified to quote the cross-linking studies with $C_{12}E_8$-solubilized enzyme in support of the monomeric single-site enzyme. The other aspects of studies on the $C_{12}E_8$-solubilized enzyme are addressed below.

The Detergent-Solubilized Enzyme

The first active detergent-solubilized preparations of the enzyme were shown to be oligomers of the α,β-protomer (Esmann *et al.*, 1979, 1980; Hastings and Reynolds, 1979). Subsequent studies with a $C_{12}E_8$-solubilized enzyme that exhibited activity for a short while when added to a reaction mixture suggested that the predominant species of the preparation prior to the addition to the reaction mixture was the α,β-protomer (Brotherus *et al.*, 1981). This observation was hastily interpreted in favor of the already proposed notion of the monomeric single-site enzyme. In the previous section I have pointed out the problems associated with the only reported study that has attempted to assess the association state of $C_{12}E_8$-enzyme under reaction conditions through cross-linking experiments. To my knowledge, there is no other report in which the issue of the association state of the solubilized enzyme under reaction conditions is addressed experimentally, though the literature is full of statements assigning "complete function" to the α,β-protomer. Meanwhile, studies of several laboratories on various preparations of $C_{12}E_8$-enzyme have clearly indicated the interconversions of the protomeric and oligomeric species in the solubilized state, and have suggested the involvement of these association–dissociation phenomena in the well-known instability of the solubilized preparations (Esmann, 1984, 1986; Jorgensen and Andersen, 1986), and in the ligand-induced conformational transitions and function of the enzyme (Hayashi *et al.*, 1985, 1986; Nakao *et al.*, 1985). It has also become clear by now that the early beliefs (Craig, 1982b) on the identity of the properties of the membrane-bound and $C_{12}E_8$-solubilized preparations were not correct, and that the properties of the two differ in many important respects (Esmann, 1985; Esmann and Skou, 1984; Hayashi *et al.*, 1985, 1986; Jensen and Ottolenghi, 1983; Jorgensen and Andersen, 1986; Ottolenghi *et al.*, 1986). Even if we make the unjustified assumption that the observed properties of a $C_{12}E_8$-solubilized enzyme are the properties of a free-standing α,β-protomer, it is not clear at all how the many changes induced by solubilization are reflected in the transport function of the

enzyme.[3] To complicate matters further, it has become evident that $C_{12}E_8$ and other amphiphiles have profound effects on the catalytic activities of the enzyme that have nothing to do with enzyme solubilization but seem to be due to amphiphile binding to hydrophobic regulatory sites that are on the extramembraneous domains of the enzyme subunits (Huang *et al.*, 1985, 1986; Kakar *et al.*, 1987). On the whole, while it is evident that detergent-solubilized and detergent-altered preparations will continue to be valuable research tools, it is equally clear that we should stop referring to the early studies on these preparations as evidence favoring the single-site monomeric enzyme.

Electron Microscopic Studies

Such studies on the membrane-bound enzyme, either before or after crystallization, have shown repeatedly that enzyme forms corresponding to the α,β-protomer and its oligomers are observed within the membrane (Haase and Koepsell, 1979; Herbert *et al.*, 1985; Mohraz *et al.*, 1985, 1986, 1987; Ovchinnikov *et al.*, 1985; Vogel *et al.*, 1977; Zampighi *et al.*, 1986). That the observed protomers may be the result of the disruption of oligomeric structure during sample preparation has been suggested (Haase and Koepsell, 1979). In a more recent report on enzyme crystals (Zampighi *et al.*, 1986), while it has been concluded that the enzyme exhibits strong tendency to form stable dimers, it has also been stated that dimer formation is the result of a process that occurs during the purification of the enzyme and is independent of function. Neither the report nor the cited references, however, provide evidence for the artifactual nature of the oligomerization. It should be obvious that without independent evidence electron microscopic studies cannot establish the association state of the functional unit of the enzyme. Mohraz *et al.* (1986) and Ovchinnikov *et al.* (1985) are correct, however, in concluding that taken in conjunction with all other evidence, the electron microscopic studies indicate the oligomeric nature of the functional membrane-bound enzyme.

Radiation Inactivation Experiments

The results of radiation inactivation experiments were among the earliest data to suggest the oligomeric nature of the membrane-bound enzyme

[3] It is often said that a turnover of an enzyme that exhibits $Na^+ + K^+$-dependent ATPase activity is equivalent to the active transports of Na^+ and K^+ even if transport cannot be measured in the preparation (e.g., Craig, 1982b). That this need not be true should be abundantly clear on general theoretical grounds. That it is not true is strongly suggested by the most interesting findings of Harvey and Blostein (1986).

(Kepner and Macey, 1968). This and subsequent studies (Ottolenghi and Ellory, 1983) reaching similar conclusions dealt with the inactivations of the catalytic activities of the enzyme. Studies involving the radiation inactivations of the transport functions of a purified enzyme incorporated into phospholipid vesicles were interpreted in favor of the α,β-protomer being the functional unit (Karlish and Kempner, 1984). More recent radiation inactivation studies on the target size of the ouabain-sensitive fluxes in intact red cells, however, suggest again that the *in situ* association state of the enzyme corresponds to that of a dimer or a tetramer of the α,β-protomer (Hah *et al.*, 1985). Glynn (1985) has summarized the problems associated with the interpretation of the radiation inactivation data. When considered along with all other evidence, the great majority of the radiation inactivation data clearly support the oligomeric structure of the unit enzyme.

Simplicity and Complexity

An argument often used in favor of the monomeric single-site enzyme is that it is simpler than an oligomeric enzyme, or one with catalytic and allosteric sites for ATP. This may be true. The task, however, is not to create an article of primitive art, but to understand the workings of a sodium pump that is given to us by nature. This does seem to be quite complex, either because it is, or because we have not characterized it sufficiently. Insisting on the validity of a simple model that fits only some of the known facts will only delay the ultimate understanding of the system. Besides, who said that in biology simplicity is a virtue?

Implications for the Reaction Mechanism of the Enzyme

The Albers–Post scheme involving the enzyme's Na^+-dependent phosphorylation, its K^+-dependent dephosphorylation, and two major conformational transitions of the phospho- and the dephosphoenzyme has dominated the field for almost two decades. While there have always been some nagging doubts about its validity, the uncertainties have become more serious during the past few years. There are now solid data challenging the proposition that the phosphoenzyme formed in the presence of Na^+ is an intermediate of ATP hydrolysis in the presence of Na^+ and K^+ (Norby, 1985; Plesner *et al.*, 1981; Skou, 1985). We may safely assume that it will be a while before these controversies are settled. How the questions on the reaction mechanism are eventually answered, however, depends a great deal on our ability to resolve the remaining issues on the nature of multiple ATP sites

(e.g., is there an ATP regulatory site in addition to the multiple interacting sites of the oligomer?), and on how we fit the site–site interactions into the reaction mechanism. Concerning the latter point, it may seem surprising that for more than a decade following the suggestion of the existence of negative interactions between the protomers of the oligomeric enzyme, the only explicit recognition given to these interactions has been the statement that both protomers of the dimer may undergo the reactions of the Albers–Post scheme, but alternatively or out of phase. Such gross neglect of the subunit interactions, however, is not limited to this field. The classical models of sequential interaction and concerted symmetry of oligomeric enzymes (Koshland *et al.*, 1966; Monod *et al.*, 1965) deal with the effects of subunit interactions on equilibrium ligand binding to the enzyme, but not with the role of these interactions in the steady-state reaction kinetics. Although the theoretical aspects of the steady-state properties of the interacting oligomers have slowly developed (e.g., Hill, 1977; Hill and Levitzki, 1980; Ricard *et al.*, 1974; Ricard and Noat, 1984), their applications to real systems have been few. It is in this context that the special significance of the recent work of Plesner (1987) may be appreciated. In an elegant application of the formalism of Hill (1977) to the case of dimeric (Na^+ + K^+)-ATPase with negative interactions, Plesner shows that the predicted kinetic mechanism may be reduced to one in agreement with the bicyclic mechanism that had already been proposed as an alternative to the Albers–Post scheme (Plesner *et al.*, 1981). Needless to say, the ultimate fate of these and other alternative kinetic mechanisms for the enzyme will be decided in the laboratory. Regardless of how the scales are tipped by future experiments, however, it is clear that approaches similar to that of Plesner (1987), in which the subunit interactions are treated with due respect, will be necessary for the proper analysis and interpretation of the data.

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