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Na^+,K^+-ATPase: HALF-OF-THE-SUBUNITS CROSS-LINKING REACTIVITY SUGGESTS AN OLIGOMERIC STRUCTURE CONTAINING A MINIMUM OF FOUR CATALYTIC SUBUNITS

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Summary: When Na^+,K^+-ATPase was reacted with Cu^{2+} and o-phenanthroline under conditions where the formation of a cross-linked dimer of the catalytic subunit (α,α-dimer) is dependent on the prior phosphorylation of the enzyme by ATP, it was found that (a) only half of the α-subunit content is phosphorylated, and only half is cross-linked; and (b) a phosphorylated α-subunit is cross-linked to an unphosphorylated α-subunit. It is suggested that the functional unit of the membrane-bound enzyme contains at least four α-subunits, and that ligand-induced half-of-the-sites reactivity may be exerted across two different intersubunit domains of the tetramer.

Introduction: Highly purified preparations of Na^++K^+-activated adenosinetriphosphatase (Na^+,K^+-ATPase, EC 3.6.1.3) contain two major polypeptides in association with membrane lipids (1). One is the catalytic subunit (α-subunit) with molecular weight of about 100,000, and the other (β-subunit) a glycoprotein of unknown role with molecular weight of about 40,000. Although it is established that the enzyme is an oligomer consisting of several of each subunit (1,2), little is known about the exact composition and structure of the functional oligomer within the membrane. The studies presented here provide preliminary information on the number of α-subunits, their geometric arrangement, and the nature of their interactions in a membrane-bound enzyme preparation.

Methods: The highly purified, but still membrane-bound, enzyme from dog kidney outer medulla with specific activity of 1000-1500 μmoles Pi/mg/h was prepared according to Jorgensen (3). Methods and procedures for enzyme assay, measurement of ^{32}P-incorporation into the enzyme, cross-linking experiments, SDS-polyacrylamide gel electrophoresis of samples, and identification and measurement of enzyme subunits and cross-linked products have been described before (2,4,5).

Results: In recent studies (2) we showed that when the enzyme was exposed to 0.25 mM Cu^{2+} and 1.25 mM o-phenanthroline, cross-linking of the α-subunits

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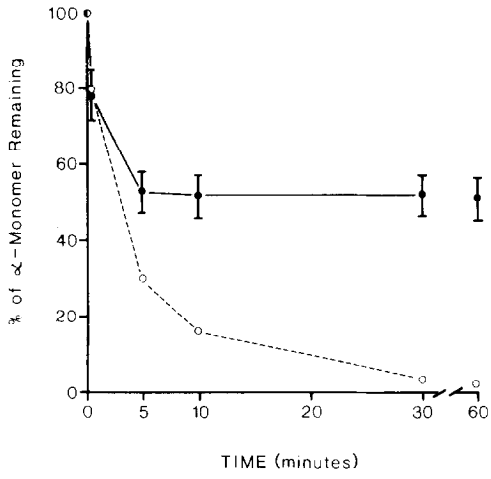

Fig. 1. Time-course of disappearance of the α-subunit upon exposure of the enzyme to cross-linking reagents. The enzyme was reacted at 24° C either with 0.25 mM Cu^{2+} and 0.5 mM o-phenanthroline (o), or with 0.25 mM Cu^{2+}, 1.25 mM o-phenanthroline, 1 mM ATP, and 100 mM Na^{+} (•). Under the latter conditions cross-linking does not occur unless ATP is present (2). Reactions were terminated by the addition of SDS and EDTA, samples (5 μg of enzyme protein) were subjected to SDS-gel electrophoresis, and the levels of stained and scanned α-monomer bands were determined by the measurement of area under each peak (2). The indicated values for the experiments done in the presence of ATP (•) are averages of the results obtained with four different enzyme preparations. Accurate measurements of the accumulated cross-linked products could not be made. In such experiments of long duration, the products consist of α,α-dimer and several α-aggregates of higher molecular weights. In experiments with the uncross-linked control enzyme it was shown that area under the α-monomer peak was linear with protein concentration up to 7 μg of enzyme protein applied to the gels.

did not occur unless ATP was present, and that cross-linking was optimal under conditions that were optimal for the formation of the phosphoenzyme; namely, in the presence of Na^{+} and ATP. Because the steady state level of phosphoenzyme was attained within 10 seconds, while the level of cross-linked α,α-dimer continued to rise for several minutes; and since the terminal phosphate of ATP was incorporated into both α-monomer and α,α-dimer, it appeared that the phosphorylated α-subunit participates in the formation of the cross-linked α,α-dimer (2). From these findings the following question arose: If, as indicated by past studies (1), no more than half of α-subunits can be phosphorylated by ATP, what fraction of α-subunits is cross-linked under optimal conditions for phosphorylation? Experiments of Fig. 1 showed that the answer is half. Also included in Fig. 1 are data indicating that when the enzyme was exposed to Cu^{2+} and o-phenanthroline under conditions where

Table 1. Relation of enzyme phosphorylation to α-subunit content. The enzyme was reacted at 24° C with 50 μM [^{32}P]ATP, 2 mM Mg^{2+}, and 100 mM Na^+ for 15 seconds; the reaction was terminated by the addition of $HClO_4$; and ^{32}P-incorporation was determined (2). A similar experiment in which Na^+ was replaced with K^+ was also done. From the difference between the results, the level of Na^+-dependent ^{32}P-incorporation was calculated. Enzyme samples (5 μg of protein) were subjected to SDS-polyacryamide gel electrophoresis, and amount of α-subunit as per cent of the total protein applied to the gel was determined (1,7). α-Subunit content was calculated on the basis of molecular weight of 96,000. The indicated values are averages of three determinations. Note that the maximal level of ^{32}P-incorporation obtained in the presence of ATP, Mg^{2+}, and Na^+ is the same as that obtained in the presence of ATP, Cu^{2+}, o-phenanthroline, and Na^+ (reference 2 and Table 2).

a. Phosphorylation (nmoles ^{32}P/mg of enzyme protein)	b. α-Subunit Content (nmoles/mg of enzyme protein)	a/b
4.72 ± 0.05	8.13 ± 0.32	0.58

cross-linking is not dependent on prior phosphorylation of the enzyme (2), all of α-subunits were cross-linked.

Experiments of Table 1 confirmed the findings of others (1), and showed that under optimal conditions for enzyme phosphorylation in the presence of Na^+, ATP, and Mg^{2+}, about half of the α-subunits was phosphorylated.

We knew already that cross-linking did not occur between two unphosphorylated subunits (2). It remained to be determined, therefore, whether cross-linking occurred between two phosphorylated subunits, or between a phosphorylated and an unphosphorylated subunit. In experiments of Table 2, the enzyme was cross-linked in the presence of [^{32}P]ATP. The reaction time was short (1 min) to avoid the formation of cross-linked products other than α,α-dimer. After termination of the reaction, the enzyme was subjected to gel electrophoresis and the specific activities of α-monomer and α,α-dimer were determined. These were about the same as the specific activity of α-monomer obtained from a control enzyme that was phosphorylated in the absence of cross-linking. The data of Table 2, in conjunction with those of Fig. 1 and Table 1, indicate that cross-linking occurs between a phosphorylated α-subunit and an unphosphorylated α-subunit.

Discussion: That the functional unit of Na^+,K^+-ATPase contains at least a dimer of the α-subunit has been established (2 and references therein). Half-

Table 2. Comparison of the specific activity of phosphorylated α-monomer obtained from the uncross-linked enzyme with those of phosphorylated α-monomer and phosphorylated α,α-dimer obtained from the cross-linked enzyme. A portion of the enzyme was reacted at 24° C with 50 μM [^{32}P]ATP, 2 mM Mg^{2+}, and 100 mM Na^{+} for 15 seconds. Another was reacted with 50 μM [^{32}P]ATP, 0.25 mM Cu^{2+}, 1.25 mM o-phenanthroline, and 100 mM Na^{+} for 60 seconds. In the latter case less than maximal cross-linking of α-subunits occurred. Note, however, that in each case the reaction time was sufficient to obtain maximal steady state level of phosphorylation (2). Similar experiments in which Na^{+} was replaced with K^{+} were also done. Reactions were terminated by the addition of $HClO_4$, and levels of total Na^{+}-dependent ^{32}P-incorporations were determined. These were 4.24 ± 0.05 nmoles/mg for the control enzyme reacted with ATP, Mg^{2+}, and Na^{+}; and 4.43 ± 0.06 nmoles/mg for the enzyme reacted with ATP, Cu^{2+}, o-phenanthroline, and Na^{+}. The precipitates obtained from the reactions containing Na^{+} were solubilized in SDS, and aliquots (5 μg of protein) were subjected to SDS-gel electrophoresis at pH 2.4. Gels were either sliced and counted, or stained and scanned photometrically. For each peak, specific activity is expressed as total cpm per area (in arbitrary units) under the peak. The indicated values are averages of three determinations. In separate experiments with the control enzyme it was determined that area under the α-monomer peak was linear with protein concentrations up to 7 μg of enzyme protein applied to the gels.

Enzyme	α-Monomer peak a.cpm	b.area	a/b	α,α-Dimer peak a.cpm	b.area	a/b
Phosphorylated but not cross-linked	5100±100	174±15	29.3			
Phosphorylated and cross-linked	3475±25	118±6	29.4	1850±50	54±7	34.2

of-the-sites reactivity of the enzyme has been suggested (6) on the basis of observations that about half of the α-subunit content of the enzyme is phosphorylated (1,7), and that under certain conditions there seems to be one high-affinity binding site for each of several ligands (i.e., ATP, ouabain, and vanadate) per phosphorylation site (8 and references therein). The data of Fig. 1 are the most direct demonstration of functional dissimilarity between two halves of the α-subunits. It is important to note, however, that neither the phosphorylation experiments, nor the cross-linking data of Fig. 1, are sufficient to indicate the existence of genuine half-of-the-sites reactivity. Because the homogeneity of the enzyme preparation is far from being established, the apparent negative cooperativity may be explained by assuming the existence of different populations of α-dimers within the preparation. This explanation, however, becomes implausible when our findings of Table 2 are considered. Upon reflection it becomes evident that the

simplest way of explaining the functional dissimilarities between two halves of the α-subunits in respect to both phosphorylation (Table 1) and cross-linking (Fig. 1), and the apparent cross-linking of a phosphorylated subunit to an unphosphorylated subunit (Table 2), and the fact that phosphorylation preceeds cross-linking (2), is to assume the following: 1. The functional unit of the enzyme contains four (or an integral multiple of four) α-subunits. 2. Genuine half-of-the-sites reactivity in respect to phosphorylation is exerted across one of three potential intersubunit domains within the α-tetramer. 3. Half-of-the-sites reactivity in respect to cross-linking is exerted at the one intersubunit domain of a dimer of two half-phosphorylated α-dimers.

Several important issues related to the above conclusions should be considered:

1. Granted that there must be an α-tetramer, the question arises whether the genuine half-of-the-sites reactivities that are observed are due to pre-existing asymmetry within the tetramer, or due to ligand-induced alterations in the tetramer with initial symmetry (9,10). Needless to say, the question can not be answered with certainty; and most of the approaches used to resolve similar questions in cases of purified soluble proteins (9, 10) are not applicable to this membrane-bound enzyme. However, the occurrence of ligand-induced conformational changes in the α-subunit has been well-established through several different approaches (2,11,12); and this fact alone would favor the proposal that the observed half-of-the-sites reactivities are induced by ligand binding (9).

2. Two recent studies (13,14) have demonstrated that active and detergent-solubilized preparations of the enzyme contain a dimer of the α-subunit. These findings are not inconsistent with the existence of a tetramer in the membrane-bound preparation used in our studies. Indeed, the fact that the membrane-bound tetramer can be converted to a stable dimer, may be taken as support for the existence of 2:2:2 point group symmetry of the tetramer (10).

3. The membrane-bound preparation used in the present studies was treated with SDS in the course of purification. In a recent report (8) it was shown that the apparent number of certain ligand sites of the enzyme was altered upon SDS-treatment; and it was suggested that subunit interactions of SDS-treated enzyme may be different from those of native enzyme of the membrane. Therefore, whether the conclusions reached by our studies can be extrapolated to the native enzyme must await the outcome of cross-linking experiments on the enzyme of less purified or intact membranes. Such studies are in progress.

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