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Ouabain-Induced Changes in the Tertiary And the Quaternary Conformations of (Na^+ + K^+)-Activated Adenosine Triphosphatase

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SUMMARY

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Purified preparations of (Na^+ + K^+)-activated adenosine triphosphatase (EC 3.6.1.3) contain two major polypeptides: the catalytic subunit (α-subunit), and a glycoprotein (β-subunit) of unknown function. Effects of ouabain on the chemical crosslinking of the subunits in the presence of *o*-phenanthroline and Cu^{2+} were studied, and the following results were obtained: (1) When the native enzyme was exposed to 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline, a crosslinked α,α-dimer was obtained in the presence of Na^+ + ATP, but not in the presence of either Na^+, or K^+, or K^+ + ATP. Exposure of the preformed ouabain–enzyme complex to the same reagent resulted in the formation of the α,α-dimer. The level of this dimer was lower than that obtained from the native enzyme in the presence of Na^+ + ATP, and was not affected by Na^+, ATP, and K^+. (2) When the native enzyme was preincubated with Na^+ + Mg^{2+} + ATP and then exposed to 0.25 mM Cu^{2+} and 1.25 mM *o*-phenanthroline, the α,α-dimer was obtained. Addition of K^+ to the preincubated enzyme prior to the addition of the crosslinking reagent prevented dimer formation. Addition of ouabain to the preincubated enzyme, did not affect dimer formation but did block the K^+ effect on dimer formation. (3) When the native enzyme was exposed to 0.25 mM Cu^{2+} and 0.5 mM *o*-phenanthroline, an α,α-dimer was obtained under all ligand conditions except when K^+ + ATP was present. With the preformed ouabain–enzyme complex, the dimer was obtained, and its formation was not affected by K^+ + ATP. (4) The formation of an α,β-dimer in the presence of Cu^{2+} and *o*-phenanthroline was not affected by ouabain. These findings indicate that the minimum quaternary structure of $\alpha_2\beta_2$ exists in both the native enzyme and the ouabain–enzyme complex, but that ouabain binding induces changes in α-subunit conformation and in α-subunit interactions within the oligomeric structure of the enzyme.

INTRODUCTION

Cardiac glycosides inhibit the membrane-bound Na^+, K^+-ATPase[1] and the ion translocations that are carried out by the enzyme (1). The interactions of cardiac glycosides with the myocardial enzyme are also responsible for part, if not all, of the pharmacologic effects of these drugs on the heart (1, 2). Numerous experiments on the effects of cardiac glycosides on the reactions catalyzed by the enzyme and studies on the effects of the enzyme's physiological ligands on the kinetics of the drug–enzyme interaction have suggested that significant conformational transitions result from the binding of the drug to the enzyme (1, 3). Occurrence of such transitions and their transmission to areas of the cell membrane adjacent to the enzyme, have also been suggested as the basis of the positive inotropic effects of cardiac glycosides (4). Direct demonstration and measurement of the drug-induced structural transitions of the enzyme, however, have been more difficult to achieve. Successful attempts include studies on the effects of ouabain on the reactivity of enzyme sulfhydryl groups (5) and on the temperature sensitivity of the enzyme (6). Recently, we have demonstrated that several conformational transitions of Na^+, K^+-ATPase and alterations in its subunit interactions that are induced by the enzyme's physiological ligands are detectable through crosslinking experiments (7, 8). The primary purpose of this report is to show that ouabain-induced structural changes in Na^+, K^+-ATPase may also be studied through the novel and technically

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[1] Abbreviations used: Na^+, K^+-ATPase, (Na^+ + K^+)-activated adenosine triphosphatase; SDS, sodium dodecyl sulfate.

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simple experiments on the crosslinking of the enzyme subunits.

Highly purified enzyme from dog kidney outer medulla with the specific activity of 1000–1500 μmol *P*/mg/hr was prepared by the "rapid" version of the procedure of Jorgensen (9). Methods and procedures for crosslinking experiments, SDS–polyacrylamide gel electrophoresis of the samples, and identification and measurement of the enzyme subunits and crosslinked products have been described in detail (7, 8, 10). Briefly, 50 μg of enzyme protein was mixed with a solution containing the indicated concentrations of ligands and crosslinking reagents, and 50 mM Tris–HCl (pH 7.4), in a final volume of 0.1 ml. Crosslinking reagent was either 0.25 mM $CuSO_4$ and 0.5 mM, *o*-phenanthroline, or 0.25 mM $CuSO_4$ and 1.25 mM *o*-phenanthroline. After incubation at 24°C for the indicated periods, reactions were terminated by one of the following two ways: When the detection of both α,α-dimer and α,β-dimer was desired, SDS was added to obtain a final detergent concentration of 5%. When the detection of α,α-dimer, but not that of α,β-dimer, was desired (experiments of Fig. 1 and 2, and Table 1) EDTA was added to a final concentration of 30 mM, followed immediately by the addition of SDS as indicated above. Aliquots were then subjected to SDS–polyacrylamide gel electrophoresis. Stained gels were either photographed or scanned photometrically. In some of the experiments, preformed ouabain–enzyme complexes were used. These were obtained by incubating the enzyme with 10^{-4} M ouabain, 1 mM EDTA, and 50 mM Tris–HCl (pH 7.4) at 37°C in the presence of (a) 100 mM NaCl, 3 mM $MgCl_2$, and 2 mM ATP for 10 min; (b) 3 mM $MgCl_2$ and 3 mM inorganic phosphate for 10 min; and (c) 3 mM $MgCl_2$ for 45 min. The enzyme–ouabain complexes were washed thoroughly with 50 mM Tris–HCl (pH 7.4) at 4°C, assayed for Na^+, K^+-ATPase activity to assure that complete inhibition was obtained, and then used in the crosslinking experiments. In other experiments, ouabain effects on crosslinking were determined by adding the drug to the reaction mixtures as indicated in the text. Enzyme assay and measurement of ^{32}P incorporation from ATP were done as described before (8). All gel patterns presented here are representative of at least three experiments with identical results. Ouabain, *o*-phenanthroline, and ATP were obtained from Sigma Chemical Company (St. Louis, Mo.).

When the purified native enzyme, or the ouabain–enzyme complex obtained from it, is subjected to SDS–polyacrylmide gel electrophoresis under conditions used here, two prominent bands are obtained (Fig. 1, gels 1 and 6). It is well established (3) that the larger has a molecular weight of about 100,000 and is the catalytic subunit (α-subunit), and that the other (β-subunit) is a glycoprotein of uncertain role with a molecular weight of about 40,000. It is also known ((10), and references therein) that when the native enzyme is exposed to Cu^{2+} and *o*-phenanthroline, a variety of crosslinked products of the two subunits are obtained, and that in experiments of short duration the major products are an α,α-dimer and an α,β-dimer. In recent reports (7, 8) we demonstrated that (a) the formation of the α,α-dimer, but not that of the α,β-dimer, is influenced by Na^+, K^+, and ATP;

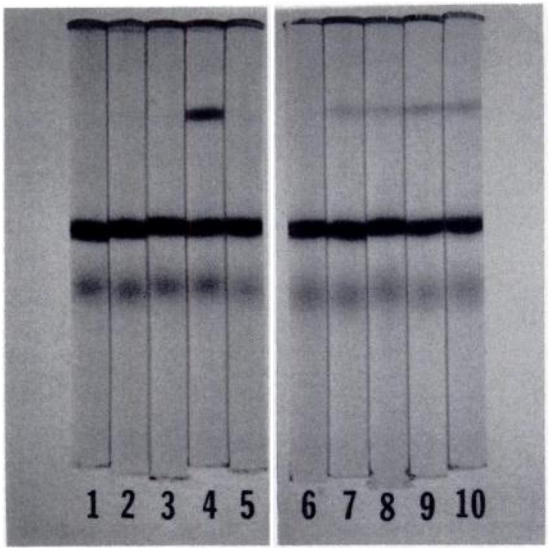

FIG. 1. *Effects of Na^+, K^+, and ATP on the formation of crosslinked α,α-dimer from the native enzyme (gels 1–5) and the ouabain–enzyme complex (gels 6–10) in the presence of 0.25 mM Cu^{2+} and 1.25 mM 0-phenanthroline*

Crosslinking experiments were done as described in the text for the duration of 2 min. Identical aliquots from the various samples were then subjected to SDS–polyacrylamide electrophoresis. Migration was from top to bottom. The three prominent bands, from top to bottom, are α,α-dimer, α-monomer, and β-monomer. Gels 1 and 6, control samples without crosslinking; gels 2 and 7, 100 mM Na^+; gels 3 and 8, 25 mM K^+; gels 4 and 9, 100 mM Na^+ + 1 mM ATP; gels 5 and 10, 25 mM K^+ + 1 mM ATP. The ouabain–enzyme complex was formed in the presence of Na^+, Mg^{2+}, and ATP, and used after thorough washing as indicated in the text. Experiments were also done with ouabain–enzyme complexes obtained in the presence of Mg^{2+} and P_i + Mg^{2+}. The gel patterns obtained from these were identical to those of gels 6–10 and are not presented.

and (b) two distinct patterns of ligand effects on the formation of the α,α-dimer are obtained depending on the molar ratio of Cu^{2+} and *o*-phenanthroline. The following experiments were done to determine the effects of ouabain on these two crosslinking patterns.

Crosslinking in the presence of 0.25 mM Cu^{2+} and 1.25 mM o-phenanthroline. The effects of Na^+, K^+, and ATP on the formation of the α,α-dimer from the native enzyme and from the ouabain–enzyme complex are shown in Fig. 1. The results obtained with the native enzyme are similar to those described before (8) and are presented here for direct comparison with the results obtained with the ouabain–enzyme complex. When the native enzyme is used, it is apparent that the α,α-dimer is not formed in the presence of either Na^+ or K^+ or K^+ + ATP, but that the dimer is formed in the presence of Na^+ + ATP. The experiments with the ouabain–enzyme complex (regardless of whether this is formed in the presence of Na^+ + Mg^{2+} + ATP, or in the presence of Mg^{2+} + *P*i, or in the the presence of Mg^{2+}) show that (a) the α,α-dimer is formed under all tested ligand conditions; (b) there is neither stimulation by Na^+ and ATP, nor inhibition by K^+, of this dimer formation; and (c) the level of the crosslinked dimer obtained from the ouabain–enzyme complex is considerably less than the maximal level obtained from the native enzyme in the presence of Na^+

and ATP. In experiments the results of which are not shown, it was found that the level of α,α-dimer obtained from the ouabain–enzyme complex did not increase when the duration of the experiment was extended beyond that of the experiments of Fig. 1. The fact that a crosslinked α,α-dimer is formed at all from the ouabain–enzyme complex is strong indication of the existence of a noncovalent α-oligomer within this complex (11). The lack of effects of Na^+, K^+, and ATP on this dimer formation is easily explained. We know already (8) that the Na^+, ATP-stimulated dimer formation from the native enzyme is dependent on the formation of the phosphoenzyme. It is also known (1) that when the ouabain–enzyme complex is formed first, and then exposed to Na^+, ATP, and Mg^{2+}, the phosphoenzyme does not form. In experiments similar to those done with the native enzyme (8), the ouabain–enzyme complex was exposed to Na^+, [γ-^{32}P]-ATP, Cu^{2+}, and *o*-phenanthroline under the conditions of crosslinking experiments. As expected, no Na^+-dependent phosphoenzyme formation could be detected (results not shown). This is consistent with the lack of stimulatory effects of Na^+ and ATP, and the absence of inhibitory effect of K^+, on dimer formation from the ouabain–enzyme complex. What can be concluded from the observation that in the absence of enzyme phosphorylation, the α,α-dimer does form from the ouabain–enzyme complex, but not from the native enzyme? Recalling that the absence of crosslinking cannot be taken as indication of the absence of an oligomeric structure (11), the most reasonable explanation is that an α-oligomer exists in both the native enzyme and the ouabain–enzyme complex, but the interactions among the α-subunits differ in the two enzyme species. This indicates the different conformational states of the α-subunits in the native enzyme and the ouabain–enzyme complex.

As indicated above, and reported before (8), when the crosslinking reagent is 0.25 mM Cu^{2+} + 1.25 mM *o*-phenanthroline, the phosphoenzyme that is formed as the result of the reaction of the native enzyme with ATP participates in the formation of the crosslinked α,α-dimer. Extensive past studies (1) have shown that ouabain can bind to both unphosphorylated and phosphorylated forms of the enzyme. The question arose, therefore, as to whether ouabain binding to the phosphoenzyme alters its reactivity in the crosslinking reaction. The following series of experiments were done to answer this question.

The results of the experiments presented in Table 1 show that when the enzyme is preincubated under conditions that are optimal for the phosphorylation of the enzyme by ATP, namely, in the presence of Na^+ + Mg^{2+} + ATP, and then exposed to the crosslinking reagent, the α,α-dimer forms. When preincubation is done in the same way and ouabain is added prior to the addition of the crosslinking reagent, the amount of α,α-dimer formed is the same as it is without the addition of ouabain. This indicates that the crosslinking reaction involving the phosphoenzyme proceeds regardless of whether ouabain is bound to the enzyme or not. The data of Table 1 also show that when K^+ is added to the reaction mixture prior to crosslinking, the level of α,α-dimer is significantly reduced. This is in accord with the expected reduction in the steady-state level of the phosphoenzyme upon K^+ addition (3). It is apparent from the data, however, that if K^+ is added after the addition of ouabain no reduction in the amount of α,α-dimer is observed. This is consistent with the well-known phenomenon that ouabain prevents the stimulatory effect of K^+ on the discharge of phosphoenzyme (1, 3).

TABLE 1

Effects of ouabain on the ATP-induced formation of the crosslinked α,α-dimer

Preincubation of the enzyme with indicated ligands, but without crosslinking reagent, was done under conditions described in the text. After the indicated period, crosslinking reagent (0.25 mM Cu^{2+} + 1.25 mM *o*-phenanthroline) was added. One minute later, the reaction was terminated, the sample subjected to gel electrophoresis, and the level of α,α-dimer measured as indicated in the text. Ligand concentrations were: 100 mM Na^+, 25 mM K^+, 2 mM Mg^{2+}, 2 mM ATP, and 0.1 mM ouabain. Dimer levels obtained in various samples are expressed as percentage of the level obtained in sample 3.

Enzyme sample	Preincubation condition	Crosslinked α,α-dimer (% of maximal amount obtained)
1	60 s with Mg^{2+}	0
2	60 s with Mg^{2+}+Na^+	0
3	30 s with Mg^{2+}+Na^++ATP	100
4	70 s with Mg^{2+}+Na^++ATP	96
5	Same as sample 3, followed by 30 s with ouabain	100
6	Same as sample 3, followed by 10 s with K^+	8
7	Same as sample 5, followed by 10 s with K^+	92

Crosslinking in the presence of 0.25 mM Cu^{2+} and 0.5 mM 0-phenanthroline. Comparison of the effects of Na^+, K^+, and ATP on the native enzyme and the ouabain–enzyme complex is shown in Fig. 2. In agreement with previous observations (7, 8), when the native enzyme is used, the α,α-dimer is formed under all conditions except with K^+ and ATP are present simultaneously. With the ouabain–enzyme complex, however, this prevention of dimer formation by K^+ + ATP is not observed.[2] As discussed before (7, 8), the results with the native enzyme indicate the existence of a stable conformational state of the enzyme with bound K^+ and bound ATP at a low-affinity site. Obviously, the formation of this conformational state is prevented when ouabain binds to the enzyme. The interference of ouabain with K^+ binding to the enzyme (12) is sufficient to explain these results.

Lack of effect of ouabain on the formation of the crosslinked α,β-dimer. Reaction of the enzyme with Cu^{2+} and *o*-phenanthroline also leads to the formation of an α,β-dimer (10) and this process is not affected by Na^+, K^+, and ATP (7). To investigate the possible effects of ouabain on α,β-dimer formation, experiments similar to those of Figs. 1 and 2 were done. Reactions were terminated, however, by the addition of SDS instead of EDTA and SDS. As indicated elsewhere (7, 8, 10), the omission of EDTA at this stage permits the detection of the α,β-

[2] In gels of Fig. 2 faint bands of products whose molecular weights are integral multiples of that of the α-monomer may be apparent. Close examination of the gels may suggest the existence of ligand effects on the formation of these products. We have not made a systematic study of these products, because it is not certain that the formation of a crosslinked product other than a dimer provides any additional information concerning the quaternary structure of a protein (11).

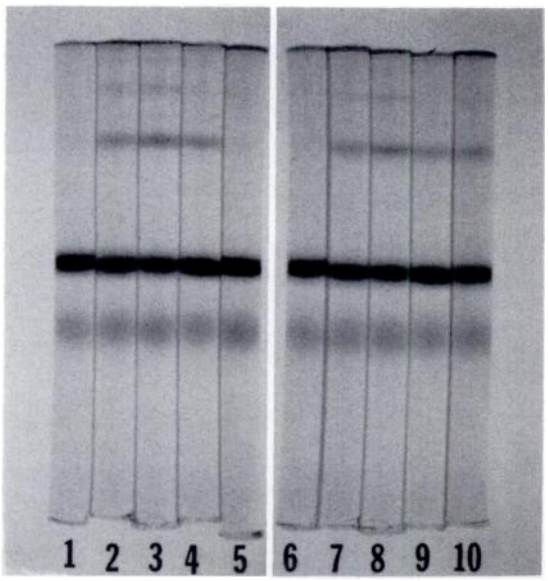

FIG. 2. *Effects of Na^+, K^+, and ATP on the formation of crosslinked α,α-dimer from the native enzyme (gels 1–5) and the ouabain–enzyme complex (gels 6–10) in the presence of 0.25 mM Cu^{2+} and 0.5 mM o-phenanthroline*

Experiments were done as described in the legend to Fig. 1. Gels 1 and 6, control samples without crosslinking; gels 2 and 7, 100 mM Na^+; gels 3 and 8, 25 mM K^+; gels 4 and 9, 100 mM Na^+ + 1 mM ATP; gels 5 and 10, 25 mM K^+ + 1 mM ATP. The ouabain–enzyme complex was formed in the presence on Na^+, Mg^{2+}, and ATP, and used after thorough washing as indicated in the text. Experiments were also done with ouabain–enzyme complexes obtained in the presence of Mg^{2+} and P_i + Mg^{2+}. The gel patterns obtained from these were identical to those of gels 6–10 and are not presented.

band on SDS-gels. These experiments did not reveal any influence of ouabain of α,β-dimer formation (results not shown).

In spite of the difficulties associated with the interpretation of all crosslinking studies of membrane proteins (11), the cumulative evidence pertaining to Na^+, K^+-ATPase strongly suggests, as discussed elsewhere (13), that the formations of the crosslinked α,α-dimer and α,β-dimer are indicative of the minimum quaternary structure of $\alpha_2\beta_2$. The studies presented here suggest, therefore, that this quaternary structure is retained in the ouabain–enzyme complex. If the formation of the crosslinked α,α-dimer indicates the existence of the non-covalent association of α-monomers in the native state, then ligand-induced alterations in the crosslinking must reflect ligand-induced alterations in the nature and the strength of subunit interactions. In turn, such changes in subunit interactions must be caused by ligand-induced changes in the tertiary structures of the subunits. On this basis, our recent studies concerning the effects of Na^+, K^+, and ATP on the formation of the crosslinked α,α-dimer (8) have been interpreted to show the existence of at least three distinct conformational states of the α-subunit: a phosphorylated form of the enzyme, the dephosphoenzyme containing bound K^+ and ATP at a low-affinity site, and the dephosphoenzyme liganded with anything but the combination of K^+ and ATP. The studies presented here show that upon ouabain interaction with the enzyme, while no change in the minimum quaternary structure of the enzyme is observed, alterations in α-subumit conformation and in the α-subunit interactions within the $\alpha_2\beta_2$ structure are detected.

Another interesting point revealed by the present data is that the crosslinking experiments do not distinguish between the previously suggested conformational states of the ouabain–enzyme complex that are dependent on the reaction conditions used to obtain the complex (1, 5). This simply indicates that a conformational state suggested by one probe may not be detectable by another, and hence the need for the utilization of different probes in order to achieve the complete elucidation of all conformational transitions of the enzyme.

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