Na^+,K^+-ATPase: LIGAND-INDUCED ALTERATIONS IN SUBUNIT CROSSLINKING

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1. Introduction

($Na^+ + K^+$)-dependent adenosinetriphosphatase (Na^+,K^+-ATPase EC 3.6.1.3) is the enzyme that performs the energy-dependent transports of Na^+ and K^+ across the plasma membrane [1]. The purified enzyme contains two polypeptides in association with the lipids of the membrane. The α-subunit with mol. wt ~100 000 is the catalytic unit of the enzyme, and the β-subunit with mol. wt ~40 000 is a glycoprotein of uncertain role. To explore the quaternary structure of the enzyme, several studies on the chemical crosslinking of the enzyme subunits have been attempted, and the results have been interpreted in support of an $\alpha_2\beta_2$ structure of the enzyme [2–6]. Here we report that the formation of a crosslinked α,α-dimer in the presence of Cu^{2+}-phenanthroline is influenced by certain physiological ligands of the enzyme. The results provide little new information concerning the quaternary structure of the enzyme. They do clearly show, however, the usefulness of crosslinking reagents as novel conformational probes of Na^+,K^+-ATPase.

2. Methods

Procedures for the preparation of dog kidney enzyme, chemical crosslinking, and SDS–polyacrylamide gel electrophoresis have been described [6]. Crosslinking was done at 24°C in a mixture containing 0.1 mg enzyme protein, 0.25 mM $CuSO_4$, 0.5 mM phenanthroline, 50 mM Tris–HCl (pH 7.4) and the indicated concentrations of ligands, in 0.2 ml. After a 10 min incubation period, EDTA was added to 30 mM final conc. and the reaction was then terminated with SDS [6].

3. Results

As we have shown [6], when the native enzyme is reacted with Cu^{2+}-phenanthroline, the major crosslinked products are an α,β-dimer and an α,α-dimer. Our preliminary experiments showed that the enzyme's physiological ligands affect the formation of the α,α-dimer but not that of the α,β-dimer. Under the standard conditions of crosslinking in section 2 both dimers are formed. To facilitate the visualization and the measurement of changes in the intensity of the α,α-dimer, we have eliminated the α,β-dimer band from the gels by the addition of EDTA after the completion of the crosslinking reaction (see section 2). As we have shown [6], the α,β-dimer formed under these conditions is not a covalently crosslinked product, and is easily dissociated in the presence of EDTA.

The effects of Na^+,K^+ and ATP on α,α-dimer formation are shown in fig.1. It is evident that:

(i) The dimer is formed in the absence of ligands, in the presence of ATP, and in the presence of Na^+ + ATP;
(ii) The dimer is not formed in the presence of K^+ + ATP.

In experiments whose results are not presented, it was found that if ATP is not added to the reaction mixture the crosslinking patterns in the presence of Na^+ and K^+ are not different from that observed in the absence of ligands. Apparently, the simultaneous presence of K^+ and ATP is required for the prevention of α,α-dimer formation.

The effects of varying concentrations of Na^+,K^+ and ATP on the crosslinking reaction were determined by measuring changes in the intensities of α,α-dimer and α-monomer bands on the gels. Figure 2 is an

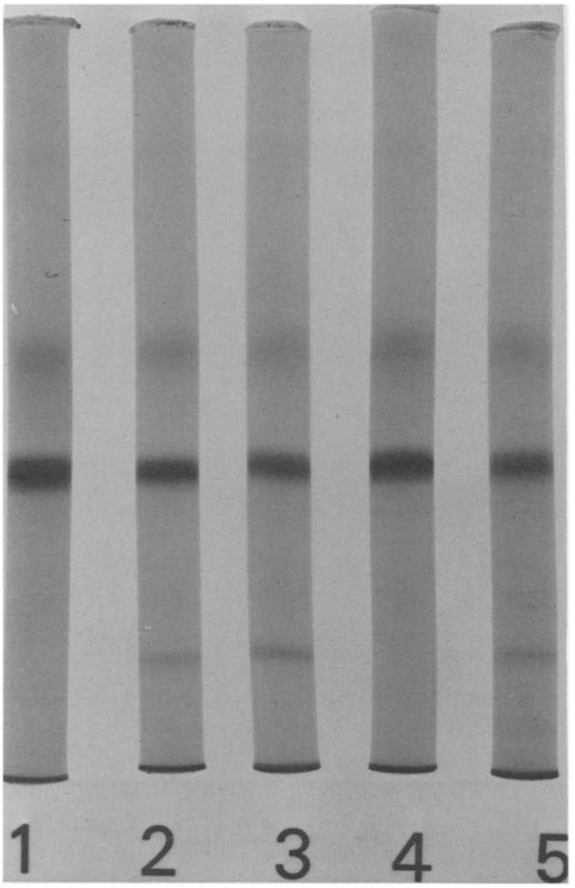

Fig.1. Effects of Na^+,K^+ and ATP on the crosslinking of Na^+,K^+-ATPase in the presence of Cu^{2+}-phenanthroline. After completion of the crosslinking reactions, enzyme samples were subjected to SDS–polyacrylamide gel electrophoresis. Migration was from bottom to top. The three prominent bands, from top to bottom, are: β-monomer, α-monomer and α,α-dimer. Gel 1, control enzyme without crosslinking; gel 2, crosslinked in the absence of ligands; gel 3, crosslinked in the presence of 1 mM ATP; gel 4, crosslinked in the presence of 1 mM ATP + 20 mM K^+; gel 5, crosslinked in the presence of 1 mM ATP + 80 mM Na^+.

example of such an experiment showing variations in the amount of α,α-dimer formed as a function of ATP concentration at 20 mM K^+. If the area under each α,α-dimer peak is determined, and the results plotted, it becomes evident that half-maximal effect of ATP is obtained at ~0.2 mM, and that 1 mM ATP is required for maximal effect.

As shown by the data of fig.3, the inhibitory

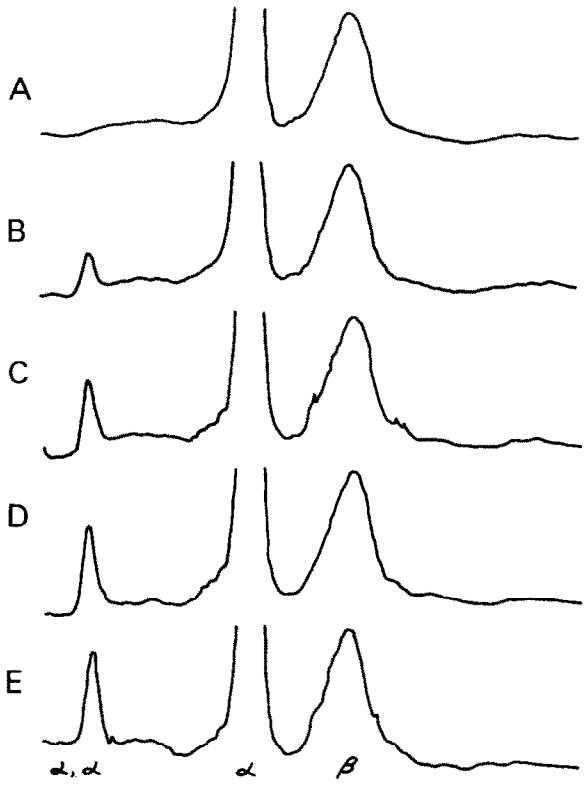

Fig.2. Effects of varying concentrations of ATP on the formation of crosslinked α,α-dimer in the presence of Cu^{2+}-phenanthroline and 20 mM K^+. Photometric scans of the gels were made after the completion of crosslinking reactions and subsequent electrophoresis of the samples. (A) 1 mM ATP; (B) 0.2 mM ATP; (C) 0.15 mM ATP; (D) 0.1 mM ATP; (E) no ATP.

effect of K^+ on α,α-dimer formation can be overcome by Na^+; and Na^+ seems to be competitive with K^+.

In the presence of 20 mM K^+, the effects of P_i and several nucleotides (at 1 mM) were compared with that of ATP. Inhibition of dimer formation was observed with ADP and CTP, but not with inorganic phosphate, AMP and UTP. Under the conditions of crosslinking experiments no significant hydrolysis of any of the nucleotides was detected. We have not yet investigated the possibility of the phosphorylation of the enzyme by ATP under these conditions. If it occurs, however, it does not seem to be relevant to the observations reported here, because ADP is also effective.

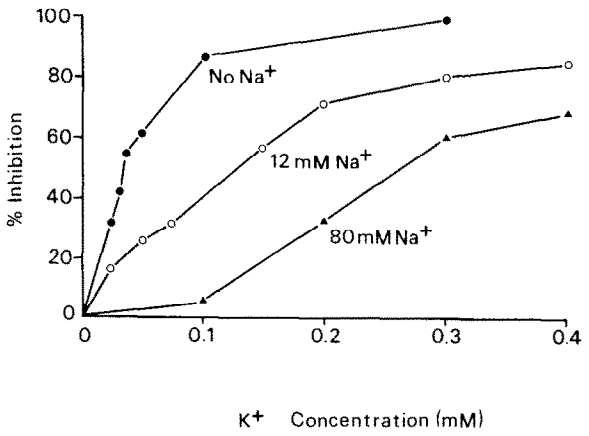

Fig.3. Inhibitory effects of varying K^+ concentrations on the formation of crosslinked α,α-dimer in the absence of Na^+ and in the presence of 12 mM Na^+ and 80 mM Na^+. ATP was present at 1 mM in all reaction mixtures. Photometric scans were made as in fig.2, and area under each α,α-dimer peak was determined. % inhibition of dimer formation was calcaluated in comparison with the control value obtained in the absence of K^+.

4. Discussion

It has been suggested [2,3] that the formation of crosslinked α,α-dimer in the presence of Cu^{2+}-phenanthroline indicates the existence of a stable non-covalent α,α-dimer in the native state of the enzyme. This is a reasonable suggestion, especially since the assumption of the existence of a native α-oligomer is consistent with the estimated molecular weight of the enzyme, and provides a convenient explanation for a variety of observations on the biochemical and transport properties of the enzyme [1]. As we have pointed out [6], however, because of uncertainties concerning the nature of the reactions of Cu^{2+}-phenanthroline with the enzyme, and considering the usual difficulties associated with the interpretation of all crosslinking studies in membrane systems [7], the mere formation of an α,α-dimer can not be taken as unambiguous establishment of a stable α-oligomer in the native enzyme.

If no assumptions on the quaternary structure of the enzyme are made, the crosslinked dimer may simply be taken as a product of a reaction between two α-subunits and crosslinking reagents. Because the combination of K^+ and ATP inhibits the formation of this product, and that of Na^+ and ATP does not, it is clear that the rate of product formation depends on the nature of the alkali cation used. Comparison of the data of fig.3 with the kinetic parameters for the antagonistic effects of K^+ and Na^+ on a variety of partial reactions of Na^+,K^+-ATPase [1] clearly suggests that Na^+,K^+-effects on the crosslinking reaction must be due to the selective interactions of these ions with the enzyme. The existence of distinct conformational states of the enzyme, one with bound Na^+ and the other with bound K^+, has been indicated by experiments on nucleotide binding to the enzyme [8], by different reactive states of the enzyme as revealed by phosphorylation studies [9] and by experiments on the effects of ligands on the tryptic digestion of the enzyme [10]. Therefore, it is reasonable to assume that the different cation-induced conformational states have different reactivities in the crosslinking reaction.

The role of ATP in the crosslinking reaction is not clear. The similar effects of ATP and ADP on the crosslinking reaction are in agreement with the similar effects of the two nucleotides on the interconversion of Na^+-form and K^+-form of the enzyme as detected by other conformational probes [8,10]. The limited experiments of fig.2 show that the app. K_m value of ATP (0.2 mM) is about the same as the app. K_m of ATP for the low-affinity binding site of the K^+-form of the enzyme as determined by experiments of different nature [10]. Therefore, it is tempting to suggest that it is the binding of ATP to the K^+-form of the enzyme that is responsible for the inhibition of crosslinking reaction. The indicated similarity between the K_m values may be fortuitous, however, because ATP is capable of interacting not only with the enzyme, but also with the other reactants of the crosslinking reaction. Complex formation between Cu^{2+} and ATP certainly occurs, and the formation ATP–Cu^{2+}-phenanthroline complex can not be ruled out. The clarification of the precise role of ATP must await the results of more extensive experiments that are in progress. In spite of this uncertainty, the utility of the crosslinking reactions for the study of ligand-induced conformational changes in Na^+,K^+-ATPase is clearly indicated by the data.

Acknowledgements

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