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ARGININE MODIFICATION WITH BUTANEDIONE INHIBITS THE POTASSIUM ATPase OF *Streptococcus faecalis*

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The K^+-ATPase of *Streptococcus faecalis* is inhibited by incubation with the arginine-modifying reagent 2,3-butanedione. The inactivation proceeds by pseudo - first order kinetics and a double-logarithmic plot of the pseudo - first order rate constants versus reagent concentrations yields a reaction order of 1.14 with respect to butanedione. Partially inactivated ATPase exhibits a decreased maximal velocity but the same affinity for ATP, as compared to the native enzyme. Butanedione modification is inhibited by adenine nucleotides. These results indicate the involvement of most likely one crucial arginyl residue in adenine nucleotide binding by the ATPase.

We previously described the purification to homogeneity of a K^+-ATPase from the Gram-positive organism *Streptococcus faecalis* (1). The enzyme consists of a single polypeptide component of relative M_r = 78,000. When reconstituted into proteoliposomes, the ATPase electrogenically translocates potassium and thus appears to serve in the accumulation of this cation by the bacterial cell (2). The recent cloning of the gene encoding the *S. faecalis* ATPase (Solioz, Mathews, and Fürst, submitted) has made this enzyme accessible to protein engineering through manipulation of the cloned DNA, a technique that has already successfully been applied to various enzymes, including transport proteins (3-5). To efficiently employ protein engineering requires, however, that some prior knowledge exists of amino acids or regions of the polypeptide that are crucially involved in catalysis. To this end, we here demonstrate the participation of most likely one essential arginyl residue in nucleotide binding by the *S. faecalis* ATPase.

MATERIALS AND METHODS

The K^+-ATPase of *S. faecalis* (ATCC9790) was purified to homogeneity according to Hugentobler *et al.* (1) and protein concentrations estimated as

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described (6). ATPase activity was measured at 37°C by recording the rate of NADH oxidation, coupled to the hydrolysis of ATP, at 366-550 nm with a Shimadzu dual wavelength spectrophotometer. Aliquots (0.05 ml) of ATPase were added to 0.95 ml of 10 mM Na-Hepes, 50 mM Na-borate pH 7.5, 5% glycerol, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.5 mg/ml soybean phospholipids (Asolectin, Associated Concentrates, Woodside, NY), 0.5 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, and 1 U/ml of each, lactate dehydrogenase and pyruvate kinase (enzymes from Boehringer Mannheim). Calibration of the assay was accomplished by adding defined amounts of ADP.

For chemical modification, aliquots of 1 ml containing 0.125 mg of ATPase in the buffer used for purification were supplemented with 0.1 ml 0.5 M Na^+-borate and the pH was adjusted to the desired value with NaOH. The modification reactions were conducted at 30°C and initiated by adding 2,3-butanedione. To terminate the reactions, aliquots were diluted 20-fold by adding them to the assay mixture for the determination of the ATPase activity. Residual 2,3-butanedione did not interfer with this assay.

RESULTS

Incubation of the K^+-ATPase with 2,3-butanedione in borate buffer at pH 7.5 caused time-dependent inactivation of the enzyme. Fig. 1 depicts the inactivation kinetics with 1 mM butanedione in a semi-logarithmic plot of activity versus time. Inactivation could be taken to completion and followed pseudo - first order kinetics down to less than 8% residual activity. The reaction was dependent on the presence of borate, and was reversible. These latter two properties appear to be characteristic for the butanedione-arginyl interaction (7-11). In the absence of butanedione, no loss of activity was observed (not shown). The apparent first order rate constant,

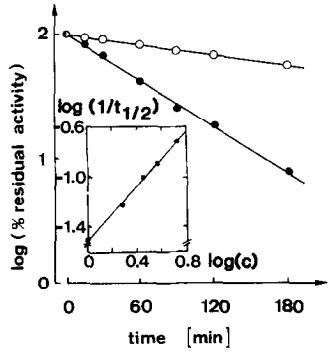


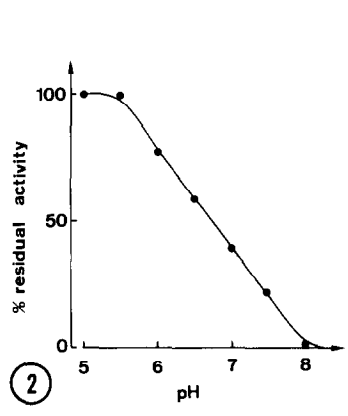
Fig. 1. Kinetics of inactivation of the ATPase by 2,3-butanedione. The ATPase was incubated at pH 7.5 in the presence of 50 mM borate with 1 mM 2,3-butanedione for different times. Residual activity was defined as the difference between parallel incubations with and without butanedione. (●), control in the absence of MgADP; (○), in the presence of 1 mM MgADP. Inset, half-times of inactivation in min, $t_{1/2}$, at various butanedione concentrations in mM, c, plotted according to: $\log(1/t_{1/2}) = n\log(c) - \log k_2$, where n is the reaction order in respect to butanedione and k_2 the second order rate constant.

k_1, defined as ln(activity) = $-k_1 t + C$, where t is the time and C a constant, varied proportionally with the butanedione concentration. This is shown in a double-logarithmic plot in the inset of Fig. 1. Under the conditions of the experiment, the second-order rate constant, k_2, amounts to 23 $min^{-1}M^{-1}$ and the slope of the curve, n, is 1.14. This kinetic behavior suggests that the reaction of a single arginyl residue per ATPase molecule leads to inactivation of the enzyme (12). However, a more complicated model where the reaction of one of several essential arginyl residues with equal reactivity toward butanedione inactivates the ATPase, cannot be discounted.

Protection of the ATPase from butanedione inhibition was offered by MgADP. Under the experimental conditions of Fig. 1, 1 mM MgADP increased the half-time of inactivation by 1 mM butanedione from 50 to 208 min (Fig. 1). To observe the protection exerted by MgATP, the non-hydrolyzable analog β,γ -methylene adenosine 5'-triphosphate was employed. This nucleotide also protected against inactivation by butanedione, albeit to a somewhat lower degree than MgADP (half-time of inactivation at 1 mM = 125 min, not shown). Pseudo - first order rates of inactivation were maintained in the presence of both of these nucleotides. The protection from butanedione inactivation by adenine nucleotides points to a central role of at least one arginyl residue in the binding of these substrates.

The pH of the incubation medium had a dramatic effect on the inactivation (Fig. 2). Below pH 5.5, no inactivation took place upon incubation with 1 mM butanedione for 60 min, whereas complete inhibition was apparent following treatment under the same conditions at pH 8.0. Although the cause of this pH-dependency is not clear, it has generally been observed for the inactivation by butanedione (8-11).

In Fig. 3, the kinetic properties of the partially inactivated ATPase were investigated. Modification of the enzyme with butanedione left the K_m for MgATP unchanged at 0.1 mM, while V_{max} was reduced from 2 μmol/min/mg to 0.95 μmol/min/mg. Thus, the residual activity is probably due to unmodified ATPase still present rather than to modified ATPase with altered proper-



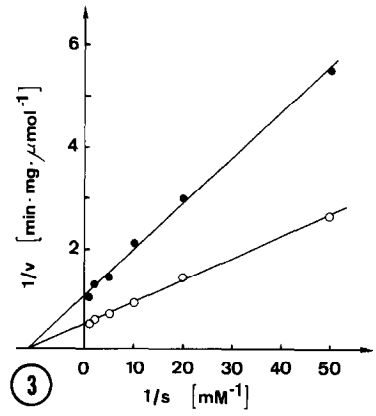


Fig. 2. pH-profile of the inactivation of the ATPase by butanedione. The ATPase was incubated with 1 mM 2,3-butanedione for 60 min in 50 mM borate in media adjusted to different pH-values. The residual activity was measured at pH 7.5.

Fig. 3. Lineweaver-Burk plot for modified and native ATPase. The ATPase was incubated at pH 7.5 with (●) and without (○) 1 mM 2,3-butanedione for 60 min. The activity of the modified and the native enzyme was measured in the assay medium described under "Materials and Methods", but containing the respective concentration of ATP.

ties, in support of the concept that arginyl residues at active rather than regulatory sites are the target for butanedione inactivation. Proof of this proposal of course requires additional experimentation, as discussed below.

DISCUSSION

The data presented here strongly suggest, but do not prove, that modification of a single arginyl residue crucially involved in catalysis by the K^+-ATPase of S. faecalis leads to inactivation of the enzyme. The same proposal has been put forth for, among other enzymes, several closely related ion-motive ATPases, namely the plasma membrane ATPases of Schizosaccharomyces pombe (11) and Neurospora crassa (8), the Ca^{2+}-ATPases of sarcoplasmic reticulum (13) and the erythrocyte membrane (14), the K^+H^+-ATPase of gastric mucosa (10), and the Na^+K^+-ATPase (9). For the latter, however, a later report favors two or more essential arginyl residues that react with butanedione (15).

Recently, molecular cloning has allowed to derive the protein sequences for several of the enzymes mentioned above, namely for the Ca^{2+}-ATPase of sarcoplasmic reticulum (16), the yeast plasma membrane ATPase (17), the

Na^+K^+-ATPase (18,19), as well as the K^+-ATPase of S. faecalis (Solioz, Mathews, and Fürst, submitted). A number of amino acid residues and short sequence stretches have been conserved in all these enzymes, underlining their evolutionary relationship and the concept, that they probably operate by the same underlying mechanism. In addition, the availability of these primary structures has, for the first time, allowed limited speculation on structure/function relationships in these proteins (20). This has augmented interest in the identification of residues essential for catalysis.

In this light, future work on arginine modification has to be aimed at pinpointing the essential residues, and the question of the presence of one or several such residues in a protein becomes of secondary importance. Two routes appear feasible to identify arginyl residues responsible for enzyme inactivation. Irreversible arginine modification with phenylglyoxal (11,16) could be employed to generate labeled peptides for sequencing. Proof of the functional involvement of reactive arginyl residues thus revealed will, however, require additional tests. An alternative and more direct method is furnished by modern molecuar biology: arginyl residues suspected to be essential for enzyme function are exchanged against other amino acids at the level of the cloned gene, and the resulting in vitro mutated gene product functionally analyzed. This method appears to be the method of choice for the K^+-ATPase of S. faecalis.

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