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REVERSIBLE INACTIVATION OF ($Na^+ + K^+$)-ATPase BY USE OF A CLEAVABLE BIFUNCTIONAL REAGENT *

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Summary

1. Purified ($Na^+ + K^+$)-ATPase, prepared from rabbit kidney outer medulla, is incubated with the bifunctional NH_2-directed reagent dimethyl 3,3′-dithiobispropionimidate. This results in a cross-link between the subunits of the enzyme and a simultaneous reduction of the ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities.

2. The most abundant cross-link product is a dimer of the two different subunits of the enzyme.

3. Reduction of the disulfide cross-link by dithioerythritol results in partial recovery of the original subunit structure of the enzyme and of the ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities.

4. These results suggest that a free mobility of the subunits of the ($Na^+ + K^+$)-ATPase system relative to each other is essential for proper functioning of both enzyme activities.

Introduction

The enzyme ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is involved in the active transport of sodium and potassium ions across mammalian plasma membranes [1—5]. The protein part of the enzyme consists of two different subunits: a protein with a molecular weight of approx. 100 000, the catalytic subunit (α) and a glycoprotein (β) with a molecular weight of around 50 000 [3]. The catalytic subunit can be phosphorylated by ATP and appears to contain the active centre of the enzyme complex, whereas the function of the glycoprotein has not yet been established. The conformation of the enzyme

* Studies on ($Na^+ + K^+$) activated ATPase. Paper No. XLIII.

is influenced by the presence of Na^+, K^+ and Mg^{2+} ions resulting in a change of the tryptic digestion pattern [6], reactivity with the arginine reagent butanedione [7], and reactivity with sulfhydryl reagents [8]. This suggests that the enzyme complex is rather mobile. It has not yet been established whether the free mobility of both subunits is essential for the activity of this enzyme.

We have approached this problem by making use of the cleavable bifunctional reagent dimethyl 3,3'-dithiobispropionimidate. This reagent reacts with amino groups and can be split into two molecules of 3-thiopropionimidate by reducing agents like dithioerythritol [9,10]. By treating highly purified ($Na^+ + K^+$)-ATPase from rabbit kidney outer medulla with this reagent, we are able to link the two subunits of this enzyme in a reversible way. The ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities are also partially and reversibly inactivated, indicated that the free mobility of the subunits of the enzyme complex is essential for full activity of the enzyme.

Materials and Methods

Enzyme preparation

($Na^+ + K^+$)-ATPase was purified from rabbit kidney outer medulla, essentially as described by Jørgensen [11]. The preparation was washed to remove adhering ATP and ions as described before [12]. The enzyme was stored at −20° C in 0.25 M sucrose, 0.2 M triethanolamine (pH 10). The specific ($Na^+ + K^+$)-ATPase activity was 1000—2000 μmol P_i/mg protein · h, without any ouabain-insensitive ATPase activity. The K^+-stimulated *p*-nitrophenylphosphatase activity was 20—40% of the ($Na^+ + K^+$)-ATPase activity. According to sodium dodecyl sulfate gel electrophoresis, the enzyme was at least 95% pure.

Enzyme assays

($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities were determined as described before [12].

Incubation with dimethyl 3,3'-dithiobispropionimidate

0.5—2 mg/ml ($Na^+ + K^+$)-ATPase was incubated for 10 min at 25°C in 0.2 M triethanolamine (pH 10) containing various concentrations of dimethyl 3,3'-dithiobispropionimidate. The 100-μl reaction mixture was then placed on a Sephadex G-25 coarse column (100 × 5 mm), equilibrated in 0.2 M triethanolamine (pH 10). The enzyme was eluted free of contaminants and 50-μl samples were taken for protein determination. Then 450-μl samples were mixed with either 50 μl 100 mM dithioerythritol in 0.2 M triethanolamine (pH 10) or 50 μl triethanolamine alone. After 15-min incubation at 25°C, 20-μl samples were taken for determination of the ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities. The remaining eluate was centrifuged and used for sodium dodecyl sulfate gel electrophoresis.

Gel electrophoresis

Gel electrophoresis was performed according to Davies and Stark [13] in slab gels (1 × 16 × 16 mm) containing 3.5% acrylamide. The protein was dissolved in electrode buffer (0.1 M sodium acetate/0.1 M sodium tetraborate/

0.1% (w/v) sodium dodecyl sulfate, adjusted to pH 8.5 with acetic acid), containing in addition 10% sodium dodecyl sulfate and 10 mM *N*-ethylmaleimide. (*N*-ethylmaleimide was present as a functinal blocking agent for SH groups in order to prevent cross-linking due to disulfide formation by air oxidation). An additional advantage of the alkylation by *N*-ethylmaleimide is the fact that (for no known reason) the mobility of the catalytic subunit is decreased. This resulted in better separation from the glycoprotein, which in this type of gel had a relatively high apparent molecular weight. After allowing the protein to enter the gels slowly at 10 mA/gel, the current was set at 40 mA and electrophoresis was continued for 16 h.

Gels were fixed and stained in 2.5% (w/v) Coomassie blue-250 in H_2O/CH_3COOH/CH_3OH (5 : 1 : 4, v/v) at 60°C for 3 h. The gels were destained by diffusion of the unbound stain in H_2O/CH_3COOH/CH_3OH (8 : 1 : 1, v/v) with several changes of destaining medium.

A mixture of polymers of bovine serum albumin, prepared by reaction of albumin with dimethylsuberimidate in 0.2 M triethanolamine (pH 8.5)/dimethylsulfoxide (7 : 3, v/v), was used to calibrate the gels.

Miscellaneous techniques

Protein was determined according to Lowry et al. [14] with bovine serum albumin serving as a standard. The number of free amino groups in the (Na^+ + K^+)-ATPase preparation was determined fluorometrically with fluorescamine (Fluram) according to Böhlen et al. [5] with the exception that 1% sodium dodecyl sulfate was added to all samples before adding Fluram. Leucine was used as standard.

Materials

ATP was obtained from Boehringer (Mannheim, F.R.G.), *N*-ethylmaleimide and *p*-nitrophenylphosphate (disodium salt) from Merck (Darmstadt, F.R.G.). *p*-Nitrophenylphosphate was converted to the imidazole salt by ion-exchange chromatography over a Dowex 50 column (H^+ form) and subsequent neutralization with imidazole. Dimethyl 3,3′-dithiobispropionimidate was purchased from Pierce (Rockford, U.S.A.).

All other chemicals were of reagent grade.

Results

Effect of reagent concentration

Highly purified (Na^+ + K^+)-ATPase from rabbit kidney outer medulla was incubated for 30 min at 25°C with dimethyl 3,3′-dithiobispropionimidate in 0.2 M triethanolamine (pH 10). After incubation, the enzyme was freed from excess reagent by gel filtration and samples of the eluate were treated with and without dithioerythritol. Fig. 1 shows that both the (Na^+ + K^+)-ATPase and the K^+-stimulated *p*-nitrophenylphosphatase activities decreased in a concentration dependent fashion. This inactivation was partly reversed by the addition of dithioerythritol. The inhibitory effect on the (Na^+ + K^+)-ATPase activity was slightly higher than on the K^+-stimulated *p*-nitrophenylphosphatase activity.

Sodium dodecyl sulfate gel electrophoresis of (Na^+ + K^+)-ATPase prepara-

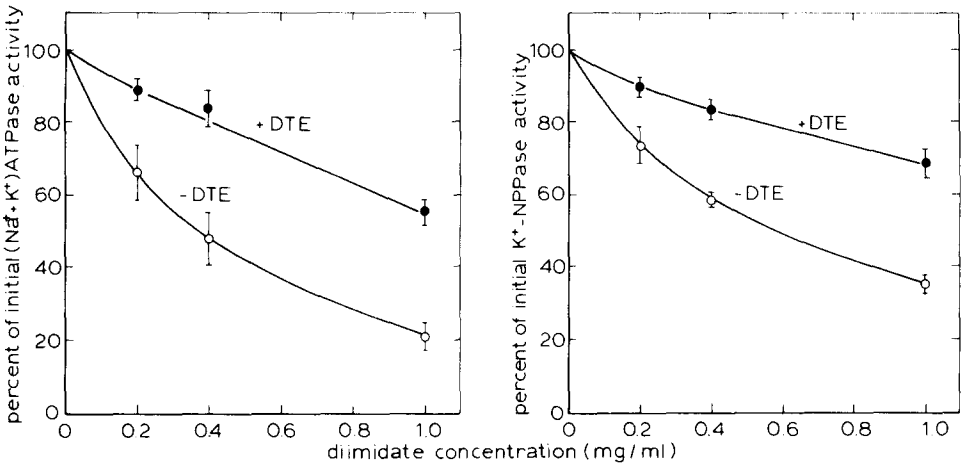
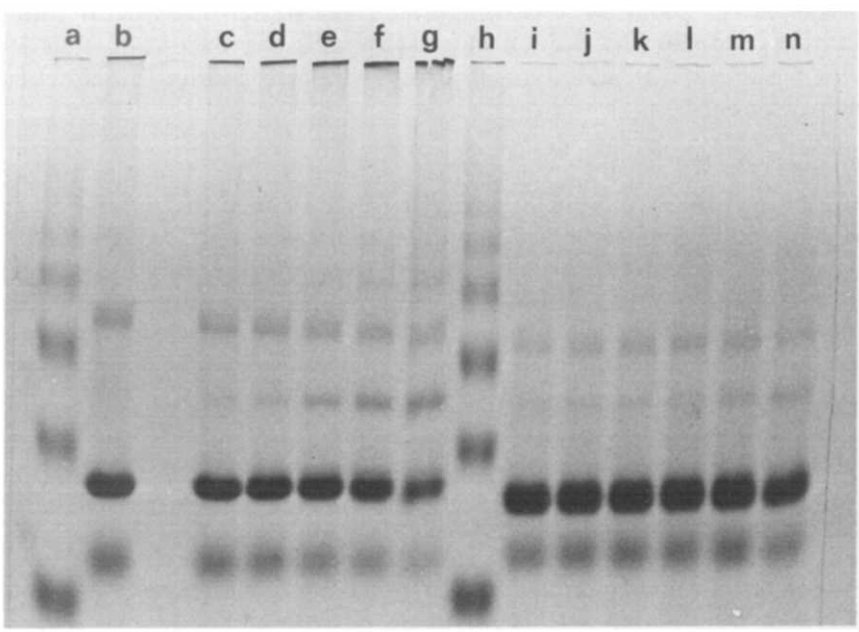


Fig. 1. Effect of treatment of $(Na^+ + K^+)$-ATPase with dimethyl 3,3′-dithiobispropionimidate on enzyme activities. $(Na^+ + K^+)$-ATPase (0.5 mg/ml) was incubated with increasing concentrations of dimethyl 3,3′-dithiobispropionimidate in 0.2 M triethanolamine (pH 10) for 10 min at 25°C. After gel filtration and incubation with (●———●) and without (○———○) dithioerythritol (DTE), activities of $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase were determined and expressed as percentage of the activities in the absence of the reagent. Values are given as means with standard errors of 4 experiments.



DTP(mg/ml)	0	.02	.07	.2	.4	1	0	.02	.07	.2	.4	1
DTE	−	−	−	−	−	−	+	+	+	+	+	+

Fig. 2. Gel electrophoretic patterns of $(Na^+ + K^+)$-ATPase treated with dimethyl 3,3′-dithiobispropionimidate (DTP). $(Na^+ + K^+)$-ATPase (0.5 mg/ml) was treated with increasing concentrations of DTP and incubated after gel filtration either with or without dithioerythritol (DTE). The concentration of DTP was 0.02 mg/ml (gels c and j), 0.07 mg/ml (gels d and k), 0.2 mg/ml (gels e and l), 0.4 mg/ml (gels f and m) and 1 mg/ml (gels g and n), respectively. On gels b and i, samples did not contain this reagent. Samples b—g were not treated with dithioerythritol; samples i—n were treated with this reagent. On gels a and h, albumin polymers were run as reference.

tions, treated with increasing concentrations of dimethyl 3,3'-dithiobispropionimidate, showed that in addition to the α and β bands (and a minor amount of an α_2 dimer) (Fig. 2, gel b) some new bands appeared (Fig. 2, gels c—g). The most prominent of these was a band with an apparent molecular weight of 160 000. The appearance of high molecular weight bands at higher reagent concentrations (>2 mg/ml, Fig. 2, gels e—g) was accompanied by a clearly diminished intensity of the α and β subunit bands (Fig. 2, gel g). After treatment with dithioerythritol, the original situation was reestablished, except that minor amounts of the 160 000 band were still present, when previously high concentrations of dimethyl 3,3'-dithiobispropionimidate were used (Fig. 2, gels i—n). The presence of dithioerythritol also led to the disappearance of some high molecular weight material which did not enter the gels in the absence of a reducing reagent.

Effect of pH

When the incubation with dimethyl 3,3'-dithiobispropionimidate was carried out at different pH values (Fig. 3), the degree of inhibition was higher at more alkaline pH values. After addition of dithioerythritol both the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities were partially restored, when the reaction with dimethyl 3,3'-dithiobispropionimidate had been carried out at pH 8 to 10. They were even more inhibited when the inactivation took place at pH 7 (Fig. 3). Fig. 3 suggests that the degree of inactivation, at least for the $(Na^+ + K^+)$-ATPase activity, is equal at pH 8, 9 and 10. But the differing degree of inactivation by dimethyl 3,3'-dithiobispropionimidate makes a comparison difficult. However, the same degree of inactivation as obtained with 1 mg dimethyl 3,3'-dithiobispropionimidate at pH 8 (residual activity 47 ± 3.8%, Fig. 3) was obtained with 0.4 mg of the reagent

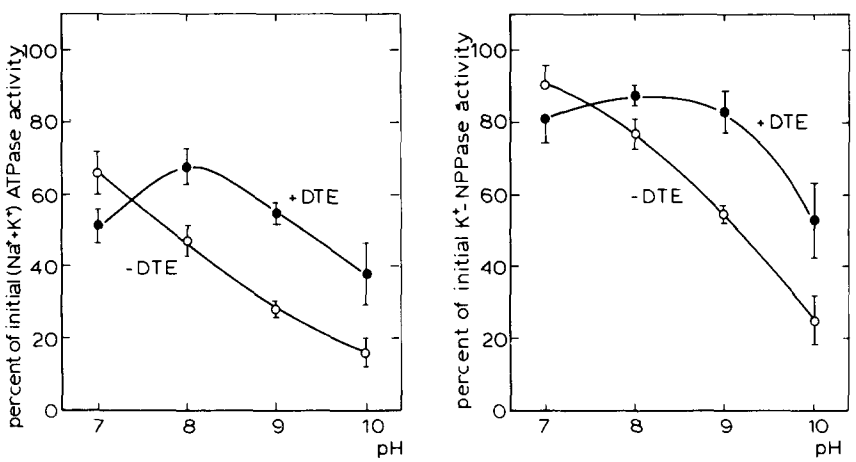


Fig. 3. Effect of treatment of $(Na^+ + K^+)$-ATPase with DTP on enzyme activities: pH dependence. $(Na^+ + K^+)$-ATPase (0.5 mg/ml) was incubated with 1 mg/ml DTP for 10 min at 25°C in 0.2 M triethanolamine at the indicate pH. After gel filtration and incubation with (●———●) and without (○———○) dithioerythritol (DTE), activities of $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase were determined and expressed as a percentage of the activities in the absence of the reagent. Values are given as means with standard errors of 4 experiments.

at pH 10 (residual activity 48 ± 7.2%, Fig. 1). However, in the latter case the recovery of the activity (up to 84 ± 4.6%, Fig. 1) is higher than after inactivation at pH 8 (68 ± 4.7%).

Gel electrophoresis of samples, treated with dimethyl 3,3′-dithiobispropionimidate at pH 8 instead of 10 showed formation of the same high molecular weight bands, but in this case treatment with dithioerythritol did apparently not result in restoration of the original situation.

Other conditions

When instead of a 0.2 M triethanolamine buffer a 0.2 M $Na_2CO_3/NaHCO_3$ buffer was used during incubation with the cross-linking reagent, there was also reversible inactivation of the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities. In this case, however, the samples without dithioerythritol did not enter the gel, suggesting that very extensive cross linkage occurred. After treatment with dithioerythritol the original bands with a minor amount of the 160 000 band were found.

The presence of 5 mM ATP in the preincubation medium has no effect on the degree of inactivation of the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities (Table I). Moreover the degree of reactivation by dithioerythritol and the electrophoretic pattern are unaffected by the presence of ATP (Fig. 4, cf. gels c and d with f and g; and gels j and k with m and n).

In most experiments dithioerythritol is added after the gel filtration step. An alternative approach is to mix dithioerythritol with dimethyl 3,3′-dithiobispropionimidate before incubation rather than after the gel filtration step, which cleaves the disulfide bond of the reagent reductively and thus yields double the molar amount of 3-thiopropionimidate. In this case the difference in inhibition of the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities is even larger than in the gel filtration procedure (cf. Fig. 5a and b with Fig. 1a and b). However, analysis of the number of modified amino groups (Fig. 5c) shows that without addition of dithioerythritol nearly double the number of amino groups is modified than after simultaneous addition of dithioerythritol and the reagent. This indicates that dimethyl 3,3′-di-

TABLE I

EFFECT OF ATP ON INHIBITION BY DIMETHYL 3,3′-DITHIOBISPROPIONIMIDATE

Purified $(Na^+ + K^+)$ATPase (1 mg/ml) was incubated with 1 mg/ml dimethyl 3,3′-dithiobispropionimidate in 0.2 M triethanolamine (pH 10) for 10 min at 25°C with and without 5 mM ATP present. After gel filtration the preparation is incubated with and without 10 mM dithioerythritol and after appropriate dilution the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities were determined. Values are quoted as activities expressed as percentages of those obtained with samples treated in the same way but without dimethyl 3,3′-dithiobispropionimidate present.

	$(Na^+ + K^+)$-ATPase activity		K^+-stimulated *p*-nitrophenylphosphatase activity	
	−ATP	+ATP	−ATP	+ATP
Without dithioerythritol	15	15	36	37
With dithioerythritol	46	48	65	68

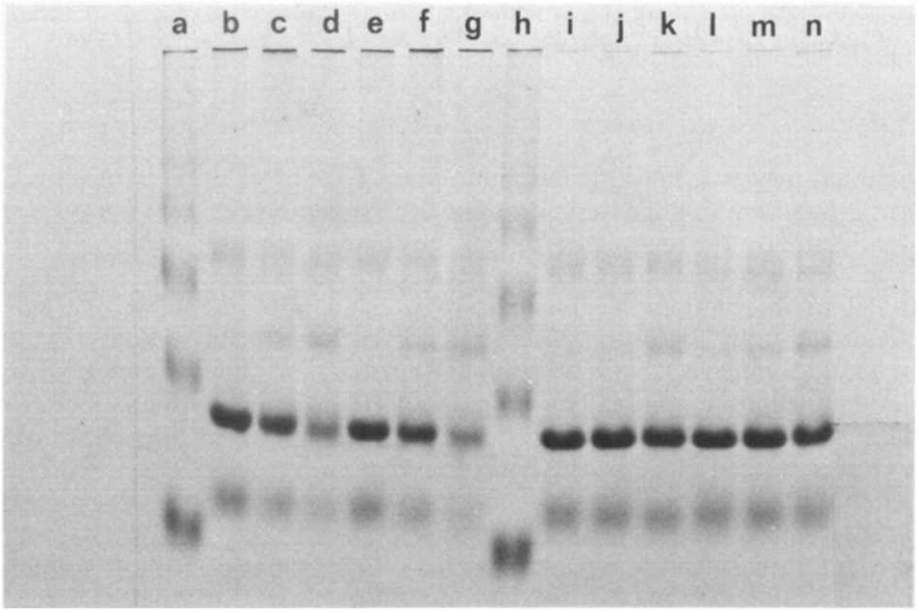


Fig. 4. Gel electrophoretic patterns of $(Na^+ + K^+)$-ATPase treated with dimethyl 3,3'-dithiobispropionimidate (DTP): effect of ATP and dithioerythritol (DTE). $(Na^+ + K^+)$-ATPase (0.5 mg/ml) was treated with DTP with or without 5 mM ATP present. After gel filtration the enzyme was incubated either with or without dithioerythritol. The concentrations of DTP are 0 (gels b, e, i and l), 0.2 (gels c, f, j and m) and 1 mg/ml (gels d, g, k and n), respectively. ATP is present in the experiments of slots e—g and l—n. Samples b—g are not treated with dithioerythritol; samples i—n are treated with this reagent. On gels a and h albumin polymers are run as reference.

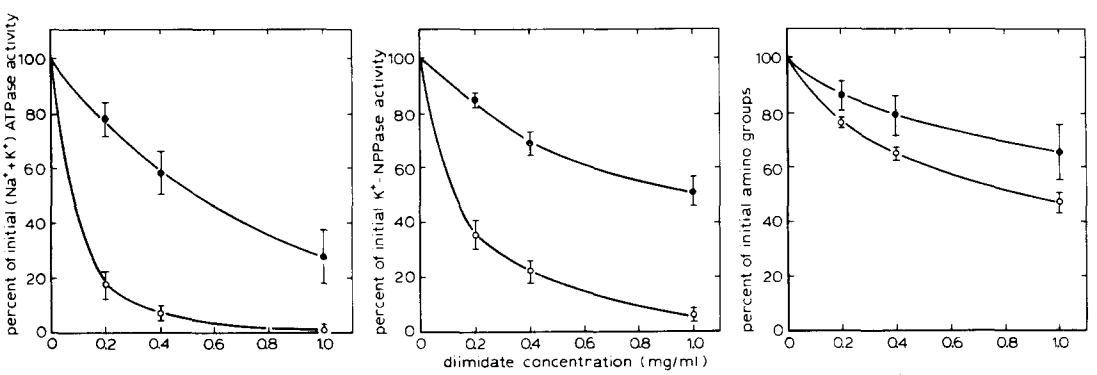


Fig. 5. Comparison of the effects of dimethyl 3,3'-dithiobispropionimidate (DTP) and 3-thiopropionimidate on the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities and on the number of residual amino groups. $(Na^+ + K^+)$-ATPase (2 mg/ml) was incubated at pH 10 for 30 min at 25° C with DTP, with (●———●) or without (○———○) pretreatment for 10 min with 10 mM dithioerythritol. The $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities and the number of unmodified amino groups were determined and expressed as percent of the values obtained without addition of the reagent. Values represent means and standard errors of three experiments.

thiobispropuinimidate reacts more effectively with amino groups than twice the molar amount of 3-thiopropionimidate.

Discussion

Bifunctional reagents, in particular imidoesters, have been used for the study of subunit interaction in oligomeric proteins and larger molecular aggregates (for a review see Ref. 16). However, the effect of this treatment on the biological function of the various systems has been investigated in only a few cases. The use of bifunctional imidoesters has one disadvantage, which has become apparent after a recent investigation by Browne and Kent [17]. These authors found that a monofunctional imidoester reacts with an amino group at pH 8 under formation of an *N*-alkylimidate as intermediate. This compound either reacts with ammonia to yield the expected amidine or with water to form a free amine. If an additional amino group is present close to the *N*-alkylimidate, a further reaction with the second amino group may occur, which results in bridge formation between two amino groups. In case the two reactive amino groups are located on different subunits a monofunctional imidoester can thus lead to a cross-link, which one would normally not expect from a monofunctional reagent. This explains why the reaction of $(Na^+ + K^+)$-ATPase with dimethyl 3,3′-dithiobispropionimidate at low pH values leads to cross-links which are not split by dithioerythritol. This also explains the formation of a cross-link upon reaction of $(Na^+ + K^+)$-ATPase with ethylacetimidate (unpublished data and Ref. 18) and the formation of cross-links in troponin with methylacetimidate [19] and in chromatin with ethylacetimidate [20]. The possibility of cross-linking apparently depends upon the orientation of the reactive amino groups, since no cross-link has been observed in other systems like mitochondrial inner membranes [21—22] and glyceraldehyde-3-phosphate dehydrogenase [23].

Browne and Kent [17] have also found that when the reaction with imidoesters is carried out at pH 10 there is much less formation of *N*-alkylimidates, which means that a cross-link with monofunctional reagents is less likely to occur. We have, therefore, carried out most experiments with dimethyl 3,3′-dithiobispropionimidate at pH 10, at which pH value the resulting cross-links can indeed be cleaved by reducing agents like dithioerythritol.

The formation of cross-links is accompanied by a reduction of the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities, which reduction is only partially reversible. The incomplete reversibility is probably due to the fact that modification of the amino groups as such has an inhibitory effect on both enzyme activities (24; de Pont et al., unpublished observations). Reduction of the disulfide bridge in the reagent cleaves the cross-link, but it has no effect on the modification of the amino groups. The fact that ATP does not decrease the inactivation is in agreement with our finding that ATP does not protect against modification of amino groups by imidoesters, in contrast to the modification of arginine [7], sulfhydryl [12,25] or tyrosine residues [26].

The parallelism between reversible inactivation of $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities and the reversible appearance of the $\alpha\beta$ cross-link, which is apparent after reaction with dimethyl 3,3′-dithiobispropionimidate at pH 10, does not occur at pH 8. At this pH value a minor

restoration of the ($Na^+ + K^+$)-ATPase activity is not accompanied by a clear change in the electrophoretic pattern. This may be due to the fact that small changes in the amount of the various bands are difficult to detect in electropherograms, Sweadner [17] also used dimethyl 3,3′-dithiobispropionimidate to study subunit interaction in ($Na^+ + K^+$)-ATPase, but only at pH 8.5. She found that the $\alpha\beta$ dimer is formed both with and without β-mercaptoethanol present. Addition of β-mercaptoethanol or dithiothreitol did not result in disappearance of the dimer. The lack of recovery in her study may be due not only to the lower pH used (8.5 vs. 10 in this study), but also to the different incubation conditions (longer incubation time, different buffer). Moreover, she did not measure the effect on enzyme activities, whereas in our study the effect of cross-linking on the subunit structure has only been studied when the activity was not completely abolished.

The main finding of this study is that reversible cross-linking of ($Na^+ + K^+$)-ATPase leads to a parallel, reversible inhibition of the ($Na^+ + K^+$)-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activites. The main product formed by cross-linking is a 160 000 molecular weight band, which can be split in an α and a β subunit after treatment with dithioerythritol. Kyte [27] has found that dimethylsuberimidate treatment also results in an $\alpha\beta$ cross-link, but he has not correlated cross-linking with inhibition of enzyme activity. The α subunits of ($Na^+ + K^+$)-ATPase can also be cross-linked by the Cu-phenanthroline catalysed oxidation of cysteine residues to a disulfide bridge (28—30; Schoot et al., unpublished observations). In the latter case, cross-linking is also accompanied by loss of the ($Na^+ + K^+$)-ATPase and K^+-stimulated activities [30,31], but no attempts to reverse this process have been reported.

It thus seems that the free mobility of the α and β subunits relative to each other is essential for the proper functioning of the ($Na^+ + K^+$)-ATPase activity as well as the K^+-stimulated *p*-nitrophenylphosphatase activity.

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