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Activation and inhibition of (Na^+ + K^+)-ATPase by *N*-ethylmaleimide

(Na^+ + K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is inhibited by $1 \cdot 10^{-3}$ M *N*-ethylmaleimide[1]. This is due to the prevention of a conformational change of the phosphorylated enzyme from a Na^+-sensitive form to a K^+-sensitive form by enhancing an ATP–ADP exchange reaction[2]. Hydroxylamine activates this enzyme in the absence of certain heavy divalent metals (Me^{2+}) and inhibits the enzyme in the presence of these cations[3]. *N*-Ethylmaleimide at about $3 \cdot 10^{-4}$ M activates the (Na^+ + K^+)-ATPase both in the presence and the absence of $2 \cdot 10^{-2}$ M hydroxylamine. Furthermore, *N*-ethylmaleimide prevents the inhibition of the enzyme by hydroxylamine and Me^{2+}, with half maximal activation at about $1 \cdot 10^{-4}$ M (ref. 4). The present study is an attempt to explore the mechanism by which *N*-ethylmaleimide inhibits and activates the (Na^+ + K^+)-ATPase and the mechanism by which *N*-ethylmaleimide prevents the inhibition of the hydroxylamine–Me^{2+}-treated enzyme.

(Na^+ + K^+)-ATPase was prepared from frozen rabbit kidney cortex according to the method of POST AND SEN[5]. The enzyme was preincubated with *N*-ethylmaleimide at a different pH for 20 min at 23°. The buffer solution contained $1 \cdot 10^{-2}$ M 2-(*N*-morpholino)ethanesulfonic acid (MES), $1 \cdot 10^{-2}$ M glycine and $1 \cdot 10^{-2}$ M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). The desired pH was obtained by adding diethylamine. After removing excess *N*-ethylmaleimide with β-mercaptoethanol and adjusting the pH to 7.5 with $4 \cdot 10^{-2}$ M Tris–TES, the activity of the (Na^+ + K^+)-ATPase was determined as described earlier[4].

The activity of the (Na^+ + K^+)-ATPase was not altered by preincubation between pH 6 and 9 (Fig. 1). Below and above these pH's the enzyme was inhibited. When the enzyme was preincubated in the presence of $3 \cdot 10^{-4}$ M *N*-ethylmaleimide, the activity of the enzyme increased between pH 6 and 8. This increase in activity

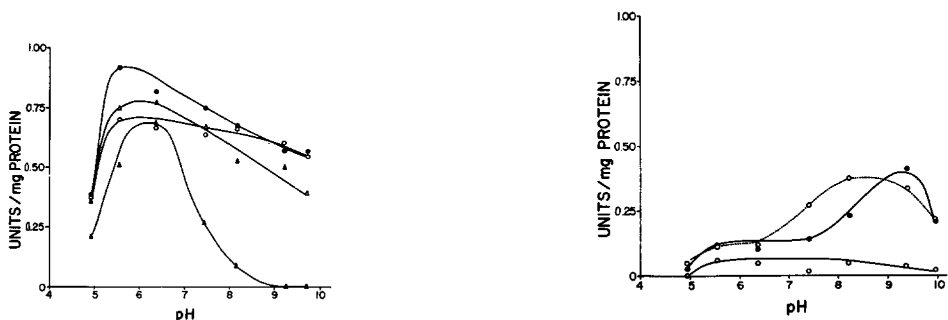

Fig. 1. Effect of *N*-ethylmaleimide preincubation (23°, 20 min) on (Na^+ + K^+)-ATPase at different pH. The activity was determined as described in the text. One unit is 1 μmole ATP hydrolyzed per min. ○—○, control; ●—●, $3 \cdot 10^{-4}$ M; △—△, $1 \cdot 10^{-3}$ M; ▲—▲, $1 \cdot 10^{-2}$ M *N*-ethylmaleimide.

Fig. 2. Effect of *N*-ethylmaleimide preincubation (23°, 20 min) on methylhydroxylamine + Cu^{2+} inhibition of (Na^+ + K^+)-ATPase. The enzyme was preincubated as in Fig. 1. The activity was determined in the presence of $2 \cdot 10^{-2}$ M methylhydroxylamine and $2 \cdot 10^{-5}$ M Cu^{2+} with a ratio of EDTA to Cu^{2+} of 2:1. ○—○, $2 \cdot 10^{-2}$ M methylhydroxylamino + $2 \cdot 10^{-5}$ M Cu^{2+}; ▲—▲, $3 \cdot 10^{-4}$ M *N*-ethylmaleimide + $2 \cdot 10^{-2}$ M methylhydroxylamine + $2 \cdot 10^{-5}$ M Cu^{2+}; ○ · · · ○, $1 \cdot 10^{-3}$ M *N*-ethylmaleimide + $2 \cdot 10^{-2}$ M methylhydroxylamine + $2 \cdot 10^{-5}$ M Cu^{2+}.

was larger at lower pH than at higher pH. When N-ethylmaleimide was increased to $1 \cdot 10^{-3}$ M this activation was again observed but was less pronounced, with inhibition occurring above pH 7. When N-ethylmaleimide was further increased to $1 \cdot 10^{-2}$ M no activation occurred at lower pH; whereas inhibition was observed at higher pH, with complete inhibition above pH 9. The ouabain-insensitive ATPase was not affected by N-ethylmaleimide under these conditions.

The ($Na^+ + K^+$)-ATPase was almost completely inhibited by $2 \cdot 10^{-2}$ M N-methylhydroxylamine and $2 \cdot 10^{-5}$ M Cu^{2+} together, independent of the pH during preincubation (Fig. 2). When the enzyme was preincubated with $3 \cdot 10^{-4}$ M N-ethylmaleimide this inhibition was prevented above pH 8. Increasing the concentration of N-ethylmaleimide to $1 \cdot 10^{-3}$ M prevented the hydroxylamine–Cu^{2+} inhibition of the enzyme above pH 7. N-Ethylmaleimide preincubation restored the activity of the hydroxylamine–Cu^{2+}-treated enzyme to 80–90% of the control value. In the presence of $2 \cdot 10^{-2}$ M N-methylhydroxylamine alone, N-ethylmaleimide activated and inhibited ($Na^+ + K^+$)-ATPase similarly, as shown in Fig. 1. The same results were observed when $2 \cdot 10^{-5}$ M Cu^{2+} was present alone.

The inhibition of the ($Na^+ + K^+$)-ATPase by higher N-ethylmaleimide concentrations above pH 7 is probably due to an alkylation of a SH group[1,2]. The activation of the ($Na^+ + K^+$)-ATPase by low N-ethylmaleimide concentrations occurs around pH 6. At such low pH alkylation of sulfhydryl groups by N-ethylmaleimide is very slow[6]. Therefore, it is unlikely that the activation of the ($Na^+ + K^+$)-ATPase by low N-ethylmaleimide concentrations is due to the alkylation of a SH group. A possibility is that a reaction occurs between N-ethylmaleimide and an imidazole group[7,8], which has a pK around 6 in protein. A similar reaction between N-ethylmaleimide and an imidazole group in the N-ethylmaleimide activation of α-glycerophosphate dehydrogenase was recently proposed by APITZ-CASTRO AND SUAREZ[9].

The rate of reaction of N-ethylmaleimide with SH groups inraceses rapidly above pH 7 [6]. The same is the case for the prevention of the hydroxylamine–Cu^{2+} inhibition of the ($Na^+ + K^+$)-ATPase by N-ethylmaleimide (Fig. 2). This strongly suggests the participation of a SH group in the inhibition of the enzyme by hydroxylamine and Cu^{2+}. This SH group is probably identical to the inhibitory site of the ($Na^+ + K^+$)-ATPase proposed earlier[4].

In summary, the results show that N-ethylmaleimide can react with three different sites which affect the activity of ($Na^+ + K^+$)-ATPase. One is the SH group by which N-ethylmaleimide prevents a conformational change of the enzyme. The second site is probably an imidazole group by which N-ethylmaleimide activates the enzyme. The third site is another SH group by which N-ethylmaleimide prevents the inhibition of ($Na^+ + K^+$)-ATPase by hydroxylamine and divalent cations. It has also been shown that there is a fourth site by which N-ethylmaleimide inhibits the Na^+-dependent phosphorylation of the enzyme[10].

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