Biochimica et Biophysica Acta, 298 (1973) 291–296

BBA 76229

INTERRELATIONSHIPS BETWEEN HYDROXYLAMINE INHIBITION OF MICROSOMAL (Na^+, K^+)-ATPase AND PHYSICOCHEMICAL CHANGES IN MEMBRANE ORGANIZATION

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(Received September 20th, 1972)

SUMMARY

Hydroxylamine inhibits rat brain microsomal (Na^+, K^+)-ATPase. The inhibition is pH dependent and is reversed by the metal chelator EDTA. No effects of hydroxylamine and EDTA were detectable after treatment of the microsomal particles with the non-ionic detergent Lubrol PX. Hydroxylamine induces particle aggregation as observed by an increase in turbidity and this phenomenon may explain the inhibitory effect of hydroxylamine on the (Na^+, K^+)-ATPase in terms of a decreased access of substrate and activators to their respective sites.

INTRODUCTION

Studies of the mechanism of (Na^+, K^+)-ATPase have demonstrated a Na^+-dependent transfer of the terminal phosphate group of ATP to the enzyme with the formation of glutamyl-γ-phosphate[1].

(Na^+, K^+)-ATPase activities from various species are inhibited by hydroxylamine in concentrations between 20 mM and 1.0 M (refs. 2, 3). One possible explanation for this finding was that hydroxylamine dephosphorylates the acid-precipitable acyl phosphoenzyme, because labelled glutamyl γ-hydroxamate has been isolated[4]. However, Chignell and Titus[5] were unable to demonstrate the formation of the hydroxamate intermediate in the native enzyme. Furthermore, these authors[6] observed that the artificial substrates glutamyl γ-hydroxamate and acetyl hydroxamate were not hydrolysed by brain microsomes, emphasizing that (Na^+, K^+)-ATPase itself was not capable of removing the hydroxamate group blocking the active site. Bader *et al.*[2] found that hydroxylamine in the presence of Ca^{2+} prevents the Na^+-dependent phosphorylation of the enzyme rather than the splitting of the acylphosphoenzyme complex. They observed that a low concentration of hydroxylamine (*e.g.* 20 mM) activated the (Na^+, K^+)-ATPase activity in the absence of Ca^{2+}. Finally, Schoner *et al.*[7] found that hydroxylamine, up to concentrations of about 0.8 M, had no effect on ATP hydrolysis.

In an attempt to resolve these discrepancies, a microsome preparation from rat brain was isolated and used to reexamine the effects of hydroxylamine on the hydrolysis of ATP by native (Na^+, K^+)-ATPase. The results demonstrate a pH-

dependent inhibition of (Na^+, K^+)-ATPase by hydroxylamine, which could be prevented by the metal chelator EDTA. The same effect was observed with deoxycholate-treated microsomes. After treatment of microsomes with the non-ionic detergent Lubrol PX, (Na^+, K^+)-ATPase was neither activated nor inhibited by 40 mM hydroxylamine. Turbidity measurements of the microsomal particles demonstrated a hydroxylamine-induced particle aggregation which could be prevented by EDTA. The findings emphasize the limitations of the usefulness of hydroxylamine in elucidating the enzyme mechanism of native (Na^+, K^+)-ATPase.

EXPERIMENTAL

Preparation of microsomes

Fresh rat brains were homogenized at 0 °C in a solution containing 0.25 M sucrose, 30 mM histidine–HCl buffer, (pH 6.54) and 1.0 mM EDTA. The homogenate was centrifuged at $600 \times g$ for 10 min. The resulting supernatant was centrifuged at $10000 \times g$ for 20 min to sediment a pellet of heavy microsomes and mitochondria. The resulting supernatant was then centrifuged at $40000 \times g$ for 100 min to sediment microsomes which were washed and finally suspended in 30 mM histidine–HCl buffer (pH 6.54) to give about 0.9 mg membrane protein per ml. The membranes were stored at −35 °C until use.

Incubation of microsomes with detergents

For treatment with deoxycholate[8], thawed microsomes were mixed with 0.55 mg deoxycholate per ml at room temperature and diluted with 30 mM histidine–HCl buffer (pH 6.54) to give a final deoxycholate concentration of less than 0.08 mg/ml. Lubrol PX extraction was done as follows: a solution of 1.2% Lubrol PX, 30 mM histidine–HCl buffer (pH 6.53) 0.8 mM disodium ATP and 0.04% bovine serum albumin was added to an equal volume of the microsome solution. After cooling for 45 min in an ice bath the solution was centrifuged at $100000 \times g$ for 65 min. The supernatant was carefully removed and used.

Enzyme assay

The solution used for the assay of (Na^+, K^+)-ATPase activity contained 5.0 mM Tris–ATP, 100 mM NaCl, 20 mM KCl, 5.0 mM $MgCl_2$, 0.2 mg membrane protein in 30 mM histidine–HCl or 30 mM Tris–HCl buffers at pH values as indicated in the text. The total volume was 0.6 ml EDTA and hydroxylamine were dissolved in the incubation medium in concentrations as indicated. The enzyme reactions were allowed to run for 30 min at 30 °C and aliquots of 100 μl were then taken for phosphate determination[9]. The specific (Na^+, K^+)-ATPase activity (*e.g.* the difference between the total ATPase and the ouabain-insensitive (Mg^{2+})-ATPase activity) was 12–16 μmoles P_i/mg protein per h. Protein was determined by the method of Lowry *et al.*[10].

Turbidity measurements

Structural changes of the microsomal particles were observed at 20 °C by measuring changes in turbidity using a Zeiss monochromator with an absorbance indicator. The semiacceptance angle of the photomultiplier was below 1°. Thus the

detector views the primary beam and only little of the scattered light. Total volume in the cuvette was 3000 μl. Concentrated solutions of 20 μl, with a pH adjusted to that of the sample, were added as reagents during the course of assay. Turbidity is expressed in terms of Δ, the fractional adsorbance increment[11], defined as

$$\Delta = (A - A_0)/A_0 \tag{1}$$

where A_0 is the absorbance at a membrane protein concentration C μg/ml (here C is 200 μg/ml) under standard conditions, namely at 540 nm, 20 °C, atmospheric pressure and with 30 mM Tris–HCl (pH 8.14) or 30 mM histidine–HCl buffer (pH 6.72) as dispersion medium. A is the absorbance under altered conditions. The aggregation experiments were carried out at constant membrane protein concentration of 200 μg/ml, and

$$\Delta = \frac{(A/C) - (A_0/C)}{(A_0/C)} \tag{2}$$

is equivalent to Eqn 1.

To determine the maximum Δ attributable to aggregation of membrane particles we used the theories outlined by Wallach *et al.*[11]. We assumed that all aggregates, termed i-mers for aggregates containing i original particles, as well as the original particles are spherical and that the volume of the i-mer formed is equal to that of the particles of which it is composed. This implies that* (ref. 11)

$$\frac{A}{A_0} = \sum_{i}^{\max \cdot i\text{-mer}} (i)\,(w_i) \tag{3}$$

or, from Eqn 1,

$$\Delta + 1 = \sum_{i}^{\max \cdot i\text{-mer}} ((i)w_i) \tag{4}$$

where $w_i = i\,N_i/N_0$ is defined as the weight fraction of i-mers. N_0 is the number of the original monomer particles and N_i is the number concentration of i-mers.

RESULTS

Hydroxylamine inhibits the hydrolysis of ATP by native (Na^+, K^+)-ATPase (Fig. 1) and the inhibition is pH dependent. 40 mM hydroxylamine produced approximately 28% inhibition at pH $\approx$8. At lower pH values no inhibition occurred. We did not obtain significant activation of native (Na^+, K^+)-ATPase by hydroxylamine at any pH value, as opposed to by Bader *et al.*[2]. Fig. 1 also shows that increasing concentrations of the metal chelator EDTA when added to the incubation medium reactivated the inhibited (Na^+, K^+)-ATPase. This observation confirms other reports[2]. When microsomes were incubated with deoxycholate (Na^+, K^+)-ATPase was inhibited by approximately 30% by hydroxylamine, as demonstrated in Fig. 2. Furthermore, Fig. 2 shows that (Na^+, K^+)-ATPase in microsomal particles after

* This approach is not applicable to very large particles in which absorbance is not a simple function of radius[12,13].

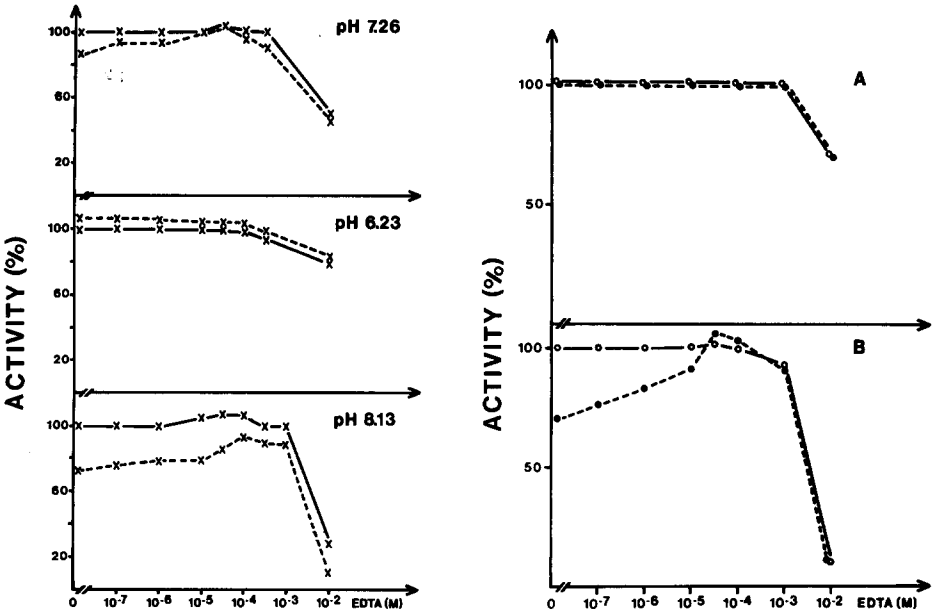

Fig. 1. Effects of EDTA and pH on the inhibition of (Na^+,K^+)-ATPase by hydroxylamine. The microsomes were incubated at various concentrations of EDTA at 30 °C in the presence of 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$ and 5 mM Tris–ATP in the following buffers: 30 mM histidine–HCl at pH 6.23 and 7.26. 30 mM Tris–HCl at pH 8.13. ×---×, in the presence of 40 mM hydroxylamine; ×—×, in the absence of hydroxylamine.

Fig. 2. Effect of EDTA on the inhibition of (Na^+, K^+)-ATPase by hydroxylamine. A. Lubrol PX-treated microsomes incubated as described in Fig. 1, in 30 mM Tris–HCl buffer (pH 8.13) in the presence of 40 mM hydroxylamine (×---×) and in the absence of hydroxylamine (×—×). B. Deoxycholate-treated microsomes incubated as described in Fig. 1, in 30 mM Tris–HCl buffer (pH 8.13) in the presence of 40 mM hydroxylamine (×---×) and in the absence of hydroxylamine (×—×).

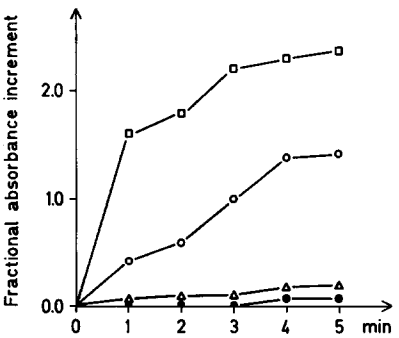

Fig. 3. Effects of EDTA, pH and hydroxylamine on the fractional absorbance increment of microsomal particles, incubated in the following solutions: □—□, 30 mM Tris–HCl buffer (pH 8.14) in the presence of 100 mM hydroxylamine; ○—○, 30 mM Tris–HCl buffer (pH 8.14 in the presence of 40 mM hydroxylamine; ●—●, 30 mM Tris–HCl buffer (pH 8.14) in the presence of $1 \cdot 10^{-4}$ M EDTA. After 3 min of incubation, hydroxylamine was added to give a final concentration of 40 mM. △—△, 30 mM histidine–HCl buffer (pH 6.27) in the presence of 40 mM hydroxylamine.

treatment with Lubrol PX was neither activated nor inhibited by hydroxylamine. Failure in inhibition might result from structural changes of the microsomal particles, due to Lubrol, as the particles have been shown to dissociate into small subunits averaging 60 Å in diameter after incubation with Lubrol[13].

Addition of hydroxylamine to suspensions of microsomal particles produces a fractional absorbance increment of $\Delta = 2.4$ and $\Delta = 1.4$ for measurements taken 5 min after addition of 100 mM and 40 mM, respectively, (Fig. 3). If we consider the simple case in which all the particles in the presence of 100 mM hydroxylamine aggregate to form a mixture of trimers ($w_3 = 0.44$) and tetramers ($w_4 = 0.56$ and $w_i = 0$ for $4 < i < 3$), then from Eqn 4 $(1 + \Delta) = 3.56$, and $\Delta = 2.56$, which fully accounts for the $\Delta = 2.4$ observed. A similar calculation reveals that a mixture of dimers and trimers would have a $\Delta = 1.60$, close to the $\Delta = 1.4$ observed upon addition of 40 mM hydroxylamine. In the presence of EDTA or at low pH (Fig. 3) a frictional absorbance increment of $\Delta = 0.1$ and $\Delta = 0.2$, respectively, was observed, which gives good reason to assume that no aggregation occurs.

DISCUSSION

The observation that hydroxylamine releases bound ^{32}P from trichloroacetic acid-denatured, phosphorylated brain microsomes has been taken to indicate that acylphosphate is an intermediate of (Na^+, K^+)-ATPase[14,15]. On the other hand, hydroxylamine has no effect on the trichloroacetic acid-denatured phosphorylated enzyme from *Electrophorus* electric organ[16]. However, with native misrosomal particles from *Electrophorus* electric organ the dephosphorylating effect of hydroxylamine and its inhibitory effect on (Na^+, K^+)-ATPase are in good agreement, 30% and 25%, respectively. Our results agree with others in that hydroxylamine inhibits ATP hydrolysis by native (Na^+, K^+)-ATPase. This inhibition was found to be pH dependent with a maximum effect of 28% at $pH \approx 8$. No inhibition was observed at pH values below 6.2. From our experiments we do not know the site of inhibition and especially whether hydroxylamine inhibits the native transport enzyme by hydroxylaminolysis of the glutamyl γ-phosphate which is synthesized in the presence of ATP and Na^+. Now, with various preparations, this transphosphorylation process has a pH optimum between 6.9 and 7.1 (ref. 18) and inhibition was observed at pH higher than 7.4. Therefore we find it reasonable to suppose that our observed inhibition of native (Na^+, K^+)-ATPase is not due to hydroxylaminolysis of the glutamyl γ-phosphate residue, since we report maximal inhibition by hydroxylamine at $pH \approx 8$.

Hydroxylamine is a nucleophilic reagent which can probably react with a variety of chemical groupings in the microsomal membrane and might thereby induce changes in their organization. This possibility was investigated using turbidimetry. Our measurements clearly show that hydroxylamine induces an aggregation of microsomal particles and this aggregation must result in an inhibition of membrane-bound (Na^+, K^+)-ATPase activity because the access of substrate and activators to their respective sites is decreased[8]. We have been able to correlate the degree of aggregation induced by hydroxylamine (measured by the change in fractional absorbance increment) with the degree of inhibition of enzyme activity produced by this reagent under a variety of conditions. To summarize, (i) the effect of pH

on the association of the microsomal particles in the presence of hydroxylamine parallels the degree of inhibition of ATP hydrolysis by native (Na^+, K^+)-ATPase; (ii) in the presence of EDTA only negligible changes in fractional absorbance increments were observed upon addition of hydroxylamine. Similarly, addition of hydroxylamine to a suspension containing EDTA produces a much smaller inhibition of native (Na^+, K^+)-ATPase; (iii) no changes occurred in ATP hydrolysis or fractional absorbance increment upon addition of hydroxylamine to membrane particles previously solubilized with the non-ionic detergent Lubrol PX.

We therefore believe that the turbidity increment produced by the addition of hydroxylamine to suspensions of microsomal particles represents primarily a physicochemical change in the organization of these membranes. However, it must be emphasized that the change in turbidity may also include a change in $m=n/n_0$, the ratio of refractive index of the particles to that of the medium. Unfortunately, we cannot, at present, evaluate changes in m. A second weakness of our work is that it seeks to describe a heterogeneous group of particles in terms of a few average parameters. We again wish to emphasize that the microsomal particle fraction is a population of particles which are supposedly similar in composition, overall organization and many physicochemical properties.

ACKNOWLEDGEMENT

This work was supported by The Danish Medical Research Council. Gratitude is expressed to Miss Nina Høffding for excellent technical assistance.

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