BBA 75591

THE EFFECT OF HYDROXYLAMINE ON TRANSPORT ATPASE

G. SACHS, M. M. LONG, T. TSUJI AND B. I. HIRSCHOWITZ
Department of Medicine, University of Alabama Medical Center, Birmingham, Ala. (U.S.A.)
(Received November 2nd, 1970)

SUMMARY

When pig brain microsomes or NaI-treated microsomes are preincubated with ATP and NH_2OH, phosphorylation from [γ-^{32}P]ATP in the presence of Mg^{2+} *plus* Na^+ is inhibited by 80% or more, whereas hydrolysis is only slightly reduced. This shows that NH_2OH is capable of reacting with the native enzyme, and various explanations for these findings are discussed.

INTRODUCTION

When preparations from a variety of tissues of ($Na^+ + K^+$)-activated Mg^{2+}-dependent ATPase are incubated with [γ-^{32}P]ATP, a phosphoenzyme is formed[1,2]. Since it has been shown that NH_2OH dephosphorylates the acid-precipitated proteins, and that, using labelled *N*-propyl hydroxylamine, a γ-glutamyl hydroxamate can be isolated from the hydrolysate[3] it would appear that the phosphate is bound to a γ-glutamyl carboxyl group in the enzyme.

On this basis, NH_2OH might be expected to inhibit the enzyme, which is not what has been found[4,5]. One possible explanation for this finding is that NH_2OH does not react with the γ-glutamyl phosphate when the enzyme is in its native configuration. However, in the presence of certain metals[6] or when microsomes are treated with deoxycholate[7] or with electric organ ATPase[8] NH_2OH does inhibit transport ATPase.

In the case of pig brain microsomes, NH_2OH does not appear to inhibit the ATPase reaction. However, preincubation with ATP, cations and NH_2OH significantly lowers phosphorylation, but does not appreciably alter hydrolysis. These data have been presented in preliminary form[9].

METHODS

Pig brain microsomes were prepared by previously described methods[10] and treated with NaI by the method of NAKAO *et al.*[11]. NH_2OH was prepared from the hydrochloride by reaction with sodium methoxide in methanol followed by fractional distillation. [γ-^{32}P]ATP was prepared by a minor modification of the method of GLYNN AND CHAPPELL[12].

($Na^+ + K^+$)-ATPase activity was assayed in a solution contaning, in 1 ml, 1 mg enzyme protein, 20 mM Tris buffer, pH 7.4, 1 mM Tris-ATP, 1 mM $MgCl_2$, and, as necessary, 100 mM NaCl and 20 mM KCl. Following 20 min incubation at

37°, protein was precipitated with 10% trichloroacetic acid and the phosphate analyzed by the FISKE–SUBBAROW procedure[13]. Alternatively, for short incubation times, 1 mM [γ-^{32}P]ATP was used, and the phosphomolybdate samples extracted into isobutanol and counted. A standard curve was run to correct for non-extracted counts.

Preincubation was carried out at 37° for 30 min in the presence of 10 mM Na_2ATP, 10 mM $MgCl_2$ with or without 1 M NH_2OH and in the case of the NaI-treated microsomes with the addition of 20 mM KCl. The preparation was centrifuged and washed 3 times with the addition of 10 mM acetyl phosphate in the first wash to remove residual NH_2OH. Omission of the acetyl phosphate was found not to affect the results, and hence was omitted in later experiments.

The enzyme was then resuspended, and the same preparation assayed for ATPase activity as above, and for Na^+-dependent phosphorylation as detailed below.

The treated enzyme was incubated at 0° for 15 sec with 20 mM Tris–HCl, pH 7.4, 1 mM tris-[γ-^{32}P]ATP, 1 mM $MgCl_2$ with or without 100 mM NaCl. The protein was precipitated by 10% trichloroacetic acid containing 3 mM ATP and 10 mM inorganic phosphate, washed 3 times, suspended in hyamine hydroxide, and aliquots of the solution were counted in a liquid scintillation counter.

All reagents were reagent grade and dissolved in double glass distilled water.

RESULTS

The ATPase present in microsomal fractions from pig brain is stimulated by the presence of Na^+ *plus* K^+. In this particular preparation there was a 2-fold increase of activity when Na^+ and K^+ were present. The stimulation was increased by NaI treatment, when a 6-fold stimulation was seen with the addition of Na^+ *plus* K^+.

Addition of 1 M NH_2OH directly to the incubation mixture did not significantly inhibit the ($Na^+ + K^+$)-ATPase activity, and only slightly increased the activity when assayed in the presence of Mg^{2+} *plus* Na^+. Phosphorylation, however, was significantly reduced. These data are summarized in Table I for the NaI-treated microsomes.

When microsomes or NaI-treated microsomes were preincubated at 37° with ATP, NH_2OH or ATP *plus* NH_2OH, there was a non-selective reduction in the specific activity of the preparation and a slight reduction in the ($Mg^{2+} + Na^+ + K^+$)/Mg^{2+} ratio.

When untreated microsomes were used for the preincubation experiments

TABLE I

ATPase ACTIVITY AND PHOSPHORYLATION USING NaI-TREATED MICROSOMES

Additions	*Hydrolysis* (*μmoles P_i/mg protein*)	*Phosphorylation* (*pmoles P_i/mg protein*)
Mg^{2+}	0.74	182
Mg^{2+}, NH_2OH	0.80	104
Mg^{2+}, Na^+	0.91	1232
Mg^{2+}, Na^+, NH_2OH	1.03	228
Mg^{2+}, Na^+, K^+	4.10	206
Mg^{2+}, Na^+, K^+, NH_2OH	3.75	191

TABLE II

UNTREATED MICROSOMES

Assay	*Preincubation conditions*					
	ATP, Na+		*NH_2OH, Na+*		*ATP, NH_2OH, Na+*	
	*Hydrolysis**	*Phospho-rylation***	*Hydrolysis**	*Phospho-rylation***	*Hydrolysis**	*Phospho-rylation***
Mg^{2+}	0.64 ± 0.07	32 ± 6	0.41 ± 0.09	26 ± 10	0.90 ± 0.10	16 ± 8
Mg^{2+}, Na^+	0.70 ± 0.09	257 ± 17	0.88 ± 0.09	239 ± 22	0.76 ± 0.12	21 ± 15
Mg^{2+}, Na^+, K^+	1.33 ± 0.12	—	1.22 ± 0.05	—	1.26 ± 0.19	—

* Hydrolysis is expressed in μmoles P_i released/mg protein ± S.D. (n = 5).
** Phosphorylation is expressed as pmoles P_i incorporated/mg protein ± S.D. (n = 5).

TABLE III

NaI-TREATED MICROSOMES

Assay	*Preincubation conditions*					
	ATP, Na+, K+		*HN_2OH, Na+, K+*		*ATP, NH_2OH, Na+, K+*	
	*Hydrolysis**	*Phospho-rylation***	*Hydrolysis**	*Phospho-rylation***	*Hydrolysis**	*Phospho-rylation***
Mg^{2+}	0.50 ± 0.06	125 ± 19	0.60 ± 0.04	200 ± 20	0.4 ± 0.13	115 ± 39
Mg^{2+}, Na^+	0.61 ± 0.04	1500 ± 89	0.66 ± 0.03	1100 ± 114	0.052 ± 0.19	325 ± 74
Mg^{2+}, Na^+, K^+	2.14 ± 0.21	—	1.90 ± 0.30	—	1.93 ± 0.17	—

* Hydrolysis is expressed as μmoles P_i released/mg protein ± S.D. (n = 5).
** Phosphorylation is expressed as pmoles P_i incorporated/mg protein ± S.D. (n = 5).

in the presence of ATP and Na^+, in addition to NH_2OH there was significant inhibition of phosphorylation, with little effect on the hydrolysis rate relative to the ATP or NH_2OH preincubated enzyme (Table II).

In the case of the NaI-treated enzyme, preincubation affected neither hydrolysis nor phosphorylation. However, if K^+ was added to the preincubation, phosphorylation was significantly depressed, with little effect on hydrolysis (Table III). In the absence of K^+, the data obtained were not significantly different from those of column 1 in Table III.

With both these preparations, therefore, the phosphorylation reaction can apparently be dissociated from the overall hydrolysis rate provided both ATP and NH_2OH are present in the preincubation. Neither of these agents alone are effective, and in the case of NaI treatment there is an additional requirement for K^+ in the preincubation.

The hydrolysis rate tested in the presence of Mg^{2+} *plus* Na^+ was not significantly increased.

Using short incubation times for both hydrolysis and phosphorylation did not affect the pattern of the above data, and extending the time for phosphorylation studies to 1 min incubation did not substantially increase the amount of protein-bound phosphate detected.

DISCUSSION

The current view of ATP hydrolysis is that there are two intermediates formed in the reaction:

$$E + \text{ATP} \underset{\text{Na}^+}{\overset{\text{Mg}^{2+}}{\rightleftharpoons}} E_1\text{–P} + \text{ADP}$$

$$E_1\text{–P} \overset{\text{Mg}^{2+}}{\rightleftharpoons} E_2\text{–P}$$

$$E_2\text{–P} \underset{\text{K}^+}{\overset{\text{Mg}^{2+}}{\rightleftharpoons}} E_2 + \text{P}_i$$

Ouabain prevents the breakdown of E_2–P in the presence of K^+, whereas *N*-ethyl maleimide prevents the interconversion of the two forms of the intermediate[14,15].

Various explanations are possible for the findings reported, which fall into two general groups:

The effect is due to the presence of unreacted NH_2OH in the assay medium

1. NH_2OH dephosphorylates the denatured enzyme. This is unlikely since NH_2OH is not an effective nucleophile under these conditions of low pH.

2. NH_2OH accelerates the breakdown of E_2–P: If the breakdown of E_2–P is rate limiting, as would be suggested by the effect of K^+ on the overall reaction, and the quantity of E_2–P detected in the presence of K^+, then the reaction rate tested in the presence of Mg^{2+} *plus* Na^+ would increase. Since it does not, NH_2OH does not act by increasing the rate of breakdown of E_2–P, if this is rate-limiting.

3. NH_2OH reduces the formation rate of E_2–P: In the case of the enzyme reaction in the presence of Mg^{2+} *plus* Na^+, it is considered that ATP hydrolysis is catalyzed by an Mg^{2+}-ATPase different from the $(Na^+ + K^+)$-ATPase. Since, therefore, the overall rate is independent of E_2–P, the formation of E_2–P could be completely inhibited without an effect on hydrolysis. In the presence of K^+, however, where E_2–P is considered to be the intermediate of the $(Mg^{2+} + Na^+ + K^+)$-enzyme, slowing of formation of E_2–P would not affect the overall reaction rate, depending on the relative rates of formation and breakdown of E_2–P. If the rate of formation of E_2–P is rate limiting reduction of E_2–P by the presence of NH_2OH would therefore lead to inhibition of hydrolysis. Since this does not occur, breakdown of E_2–P must be rate limiting, to the extent that 80% inhibition of formation can occur without affecting the overall hydrolysis.

These above explanations depend on the continuing presence of an effective concentration of NH_2OH and do not account for the necessity of ATP in the reaction mixture.

The requirement for ATP in obtaining an effect of NH_2OH preincubation suggests that NH_2OH reacts with the enzyme, and that the effects observed are not due to contamination with unreacted NH_2OH, since ATP would not be expected to affect this.

An alternative view is that NH_2OH reacts with the enzyme:

NH_2OH effects are due to the presence of reacted NH_2OH

1. NH_2OH reacts with the γ-glutamyl phosphate forming the hydroxamate. In this case, if the hyroxamate breakdown is slow, even in the presence of K^+, hydrolysis should be inhibited, if the phosphate is an intermediate.

2. NH_2OH reacts with other groups in the enzyme protein or lipid component and alters the sensitivity of E_2–P to trichloroacetic acid or other denaturing agents.

BADER *et al.*[16] have recently suggested that the effects of NH_2OH may be accounted for by the presence of a variable amount of metal in the enzyme preparation. They used concentrations of 20 mM, whereas in this work 1 M NH_2OH was used. At lower concentrations, NH_2OH reacts slowly with carboxyl phosphate compounds, hence the effects of 20 mM NH_2OH are probably accounted for by some internal rearrangement of a metal from a storage to an inhibitory site. At 1 M, however, one would predict, as happened for the trichloroacetic acid-denatured protein, formation of the hydroxamate, with attendant inhibition of phosphorylation which, in part, occurs.

Consideration of the fast and slow labelling from ATP and ITP has also led SKOU[17] to suggest that the covalent phosphorylated intermediates in the ATPase reaction may be absent in the Na^+ *plus* K^+ hydrolysis.

Clearly, a variety of nucleophilic or carboxyl groups directed reagents should be used to clarify the complex action of NH_2OH on the enzyme and to establish the necessity of the γ-glutamyl phosphate intermediate in the presence of both Na^+ and K^+.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health TIAM Grant 2A-5286 and Grant AM08541 and National Science Foundation Grant GB-8351.

REFERENCES

1 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1964) 1437.
2 R. W. ALBERS, S. FAHN AND G. J. KOVAL, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 474.
3 A. KAHLENBERG, P. R. GALSWORTHY AND L. R. HOKIN, *Arch. Biochem. Biophys.*, 126 (1968) 34.
4 W. SCHONER, R. KRAMER AND W. SEUBERT, *Biochem. Biophys. Res. Commun.*, 23 (1966) 403.
5 C. CHIGNELL AND E. TITUS, *Federation Proc.*, 25 (1966) 655.
6 H. BADER AND A. BROOM, *Biochim. Biophys. Acta*, 139 (1967) 517.
7 C. F. CHIGNELL AND E. TITUS, *Biochim. Biophys. Acta*, 159 (1968) 345.
8 S. FAHN, G. J. LOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 243 (1968) 1993.
9 G. SACHS, M. M. LONG AND B. I. HIRSCHOWITZ, *The Physiologist*, 10 (1967) 296.
10 G. SACHS, J. D. ROSE AND B. I. HIRSCHOWITZ, *Arch. Biochem. Biophys.*, 119 (1967) 277.
11 T. NAKAO, Y. TASHIMA, K. NAGANO AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 755.
12 I. M. GLYNN AND J. B. CHAPPELL, *Biochem. J.*, 90 (1904) 147.
13 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
14 S. FAHN, M. R. HURLEY, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 241 (1966) 1890.
15 R. L. POST, S. KUME, T. TOBIN, B. ORCUTT AND A. K. SEN, *J. Gen. Physiol.*, 54 (1969) 306S.
16 H. BADER, A. B. WILKES AND D. H. LEAN, *Biochim. Biophys. Acta*, 198 (1970) 583.
17 J. C. SKOU AND C. HILBERG, *Biochim. Biophys. Acta*, 185 (1969) 198.