Effect of Thiols on the Adenosine Triphosphatase Activity

of Cells of Various Origin

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Cysteamine and some other investigated thiols stimulate the ability of certain cell homogenates to hydrolyze ATP.**

In view of the finding by Wollemann and Feuer [4] that the SH group of coenzyme A functions as a phosphate group acceptor in the enzymic hydrolysis of ATP by a brain enzyme, it has been investigated whether the stimulatory effect of thiols on ATP-ases could be explained by the intermediary formation of S-phosphorylated thiols. Methods have been designed for the demonstration and determination of such intermediates. Thiol stimulated ATP-ases in human red blood cells, mouse liver, mouse liver mitochondria, ox brain and certain vegetables were studied. In no case could evidence be found for the intermediary formation of free S-phosphorylated thiols.

Enzymes hydrolyzing the $S-P$ bond in cysteamine S-phosphate appear to be common in biological material.

One often finds that the "ATP-ase activity" (as defined here: catalysts hydrolyzing one or more $P-O$ bonds in ATP) is stimulated by the presence of certain compounds containing a thiol group. There are numerous articles in the literature bearing on this fact.[2] For instance, Morales *et al.*[1] showed that the ATP-ase activity of myosin B is strongly stimulated by cysteine ethyl ester and by 2-guanidino ethanethiol. The isomeric compounds containing OH substituted for SH are usually not active in the systems.

The stimulating effect of thiols on these catalysts, which usually are enzymic in nature, could be explained in several ways, *e.g.* (*a*) by reduction of $S-S$ bridges oxidatively formed during preparation of the homogenate, (*b*) by preventing SH groups needed for full activity of the enzyme to become autooxidized during the hydrolysis of ATP, (*c*) by functioning as inhibitor removing

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** Abbreviations: [ ] = concentration

CASH	= cysteamine, 2-aminoethanethiol	P_i	= orthophosphate
CASP	= cysteamine S-phosphate	ADP	= adenosine 5'-diphosphate
RBC	= human red blood cells	ATP	= adenosine 5'-triphosphate

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chelators, (d) by causing new SH groups to form from S—S groups on the enzyme surface. The last mentioned reaction could also increase the catalytic activity of the protein. Perényi[3] has namely shown a synthetic polymer containing SH-groups to possess catalytic effect on the hydrolysis of p-nitrophenyl phosphate.

Another explanation of the observed effect of thiols on the ATP-ase activity is the possibility that the added thiols do not function merely as reducing agents but form S-phosphorylated thiols as intermediates:

$$\text{RSH} + \text{ATP} \xrightarrow{\text{enzyme}} \text{ADP} + \text{RSPO}_3^{2-} \xrightarrow{\text{enzyme}} \text{ADP} + \text{P}_i + \text{RSH} \quad (1)$$
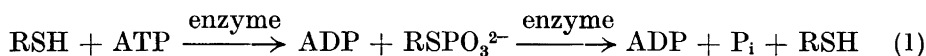
Such a reaction, catalyzed by an enzyme system from bovine brain, was observed by Wollemann and Feuer[4]. The thiol taking part in the reaction was coenzyme A. Hydrolytic enzymes capable of attacking the S—P bond in S-phosphorylated thiols are known[4-7].

Since some of the phosphorylated thiol intermediates are now synthetically available and since analytical methods for their determination exist[8,9] it seemed of interest to investigate whether or not S-phosphorylated thiols can be demonstrated as intermediates in the SH-group stimulated P_i formation from ATP.

METHODS

Since the investigations were planned to include studies on cells from a number of different sources, purification of individual ATP-ase activities was not attempted, but whole homogenates of cells were used.

Under such circumstances the "ATP-ase activity" will most likely include enzymes belonging to the category of unspecific phosphatases[2].

Minor amounts of P_i may also be liberated by enzymic reaction between a substrate present in the homogenate and ATP. When small amounts of homogenisate are used the last mentioned type of reactions can usually be neglected in comparison to the amount of P_i formed from ATP by hydrolytic enzymes.

The different kinds of enzymes that liberate P_i from ATP and which are stimulated by thiols could all form S-phosphorylated thiols as intermediates according to scheme (1). A study was consequently within the scope of the present investigation.

Cysteamine was chosen as the thiol most thoroughly investigated since the chemical properies of its S-phosphorylated derivate has been studied in detail[6,10].

In several cases the effect of cysteine, homocysteine and glutathione was also investigated. The phosphorylated derivatives corresponding to these thiols have not been synthesized *, but it was presumed that they could be analysed for by the same methods as have been worked out for CASP. The fact that the analytical procedure applied to CASP also can be utilized in the analysis of a large number of other synthesized compounds containing the $—SPO_3^{2-}$ group[8,9] justifies such an assumption.

Homogenates that had been incubated with ATP and CASH were tested for CASP (after deproteinization with TCA at 0°) by the following methods:

(a) P_i was determined colorimetrically in or without the presence of catalytic amounts of Hg^{2+} ions. In the presence of Hg^{2+} both P_i from enzymically hydrolyzed ATP and the amount of P_i esterified in CASP are measured[14]. In the absence of Hg^{2+} only P_i from ATP is determined (and a certain amount of P_i liberated from CASP by hydrolysis by the phosphate reagents; this amount was corrected for by blank determinations on CASP).

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* Cysteine S-phosphate is reported to have been synthesized by Binkley[7] from cysteine and $POCl_3$, but its properties differ so markedly from those of known S-phosphorylated thiols, that a confirmation of the structure of the synthesized compound is warranted.

The amount of P_i formed from ATP by hydrolysis by the phosphate reagents was small (of the order of 0.02 μmoles P_i per μmole of ATP);

(b) Bromine water [8] was used in place of Hg^{2+};

(c) Residual CASH was determined. Formation of CASP is accompanied by a decrease of free SH groups, which were determined colorimetrically [17];

(d) Paper chromatographic analysis according to Ref.[10]

Recovery experiments with CASP added to cell homogenates showed that about 0.1 μmole of CASP per ml could be detected and determined by methods (a) and (b).

It had to be established that the investigated cell homogenates did not contain catalysts hydrolyzing CASP at such a high rate that the detection of enzymically formed CASP would be prevented. It was indeed found that all investigated cell homogenates did contain catalysts hydrolyzing CASP to P_i and CASH. The rate of hydrolysis of CASP is exemplified in Tables 2 and 3. During a 30 min interval between $10-30$ % of added CASP was hydrolyzed. Rather high concentrations of CASP had to be used in these experiments. However, no evidence was found for inhibition of the hydrolyzing catalysts by these substrate concentrations. Nor were the catalysts saturated with substrate in these experiments. At least when the amount of thiol stimulation was large it should therefore be possible to demonstrate an intermediary formation of S-phosphorylated thiols.

RESULTS

Human red blood cells. Hemolyzed RBC, but not isolated stromata, contain a heat labile ATP-ase activity (*cf.* Clarkson and Maizels [11]) which was found to be stimulated by CASH or by cysteine, but not by glutathione or 2-mercaptoethanol (Table 1). (The corresponding ATP-ase activity of $3-4$ days old RBC was slightly stimulated also by glutathione). Cystamine was inhibitory. The increase in activity of the ATP-ase observed in the presence of CASH was further enhanced by Mg^{2+} and to some extent by Mn^{2+}, but not by Ca^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+} in similar concentrations. The optimal conditions for this ATP-ase activity were as follows: [ATP] = 3 mM; $[Mg^{2+}]$ = 3 mM; [CASH] about 10 mM; pH optimum = 7.0. The stimulatory effect of CASH increased linearly with [RBC] (Fig. 1).
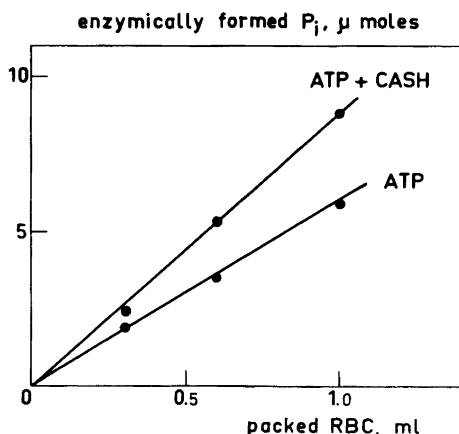

Fig. 1. Effect of RBC concentration on the formation of P_i from ATP. System: 10.0 μmoles of ATP + 15 μmoles of $MgCl_2$ ($\pm$ 10.0 μmoles of CASH) + RBC corresponding to *a* ml of packed cells + (2.0−*a*) ml of 0.25 M tris pH 7.2. Incubation: 30 min at 37°.

Table 1. Effect of various sulfur containing compounds on the ATP-ase activity of hemolyzed RBC and of liver homogenate.

Systems: RBC (corresponding to 25 % of packed cells) + ATP (5 mM) + $MgCl_2$ (5 mM) + tris pH 7.5 (0.13 M). The amount of P_i released at 100 % activity was 2.14 μmoles/ml. Liver (2 %) + ATP (5.0 mM) + tris pH 7.2 (0.15 M). The amount of P_i released at 100 % activity was 6.06 μmoles/ml. Incubation in both cases: 30 min at 37°.

Addition (5.0 μmoles/ml of each)	Relative P_i formation, %	
	Hemolyzed RBC	Liver homogenate
None	100	100
CASH	127	146
Cysteine	142	149
Homocysteine	—	131
Glutathione	100	118
2-Mercaptoethanol	100	—
Cystamine	89	—

It was demonstrated earlier [6] that CASP is only slowly hydrolyzed by RBC at pH 7.

CASP or cysteine S-phosphate could not be demonstrated as intermediates in the reaction.

It may be mentioned in this connection that the pyrophosphatase activity of RBC is stimulated some 40 % by 5 mM CASH at pH 7.2. CASP was not formed in detectable amounts during the reaction.

Mouse liver and mouse liver mitochondria. The ATP-ase activity of homogenized mouse liver slices is strongly stimulated by CASH, cysteine or homocysteine even in the presence of EDTA (Tables 1 and 2). Glutathione is

Table 2. Effect of CASH on the ATP-ase activity of various tissues and the catalytic hydrolysis of CASP by liver and brain homogenates. Incubation: 30 min at 37°.

System (figures within parentheses give concentrations in μmoles/ml)	pH	μmoles of P_i or CASH(*)formed per ml
Liver (20 mg/ml) + ATP (5.0) + tris (160)	7.2	6.06
above + CASH (10.0)	7.2	8.84
Liver (20 mg/ml) + ATP (5.0) + EDTA (25) + tris (160)	7.2	2.31
above + CASH (10.0)	7.2	6.02
Mitochondria (from 100 mg liver/ml) + ATP (5.0) + tris (160)	7.2	5.05
above + CASH (4.0)	7.2	7.34
Ox brain (20 mg/ml) + ATP (10.0) + tris (160)	8.0	1.51
above + CASH (10.0)	8.0	1.89
Liver (90 mg/ml) + CASP (10.0) + tris (160)	7.2	3.40 *
Ox brain (40 mg/ml) + CASP (10.0) + tris (160)	7.2	0.57 *
above + $MgCl_2$ (5.0)	7.2	1.01 *

Table 3. Effect of CASH on the ATP-ase activity of various fresh vegetables. Controls consisted of: cell homogenate (90 mg of fresh vegetable) + ATP (10.2 μmoles) + tris (200 μmoles) in a total volume of 1.0 ml. Samples contained in addition to the controls CASH (5 μmoles). The rate of hydrolysis of CASP was determined with 90 mg of cell homogenate + 6.0 μmoles of CASP in 1.0 ml buffer (200 μmoles of tris). It was corrected for the spontaneous hydrolysis of CASP. (The pH 6.0 buffers had been adjusted with acetic acid). Incubation: 30 min at 37°.

| Vegetables * | μmoles P_i formed | | | | | | μmoles CASP hydrolyzed at pH 7.0 |
| | pH 6.0 | | pH 7.0 | | pH 8.0 | | |
	control	CASH	control	CASH	control	CASH	
Cabbage	3.50	3.81	2.56	2.95	1.54	1.78	1.1
Red beets	5.64	5.64	3.92	3.72	2.48	1.92	1.5
Red beets tops	10.2	8.70	7.70	5.78	3.26	2.70	2.0
Green pepper	1.78	1.88	1.41	1.54	0.85	0.85	2.0
Cucumber	1.25	1.30	0.88	1.50	0.53	1.30	1.7
Potato	10.2	9.74	10.2	8.95	10.1	9.26	1.7
Asparagus	7.81	7.96	8.12	7.12	5.20	6.11	1.5
Carrot	2.02	1.91	1.32	1.38	0.71	0.85	1.0
Tomato	0.85	0.85	0.77	1.08	0.60	0.60	0.6

* The parts for household use were investigated (unless otherwise stated).

only weakly or not at all stimulatory. (Some of the thiol compounds were found to react with ATP or a degradation product thereof at the thiol end of the molecule. This observation is under further study).

The ATP-ase activity of mitochondria is stimulated to some extent by CASH, cysteine and homocysteine at pH 7.2 (Table 2).

Neither with homogenized liver slices nor with liver mitochondria could a S-phosphorylated thiol be detected as an intermediate in the thiol stimulated hydrolysis of ATP.

Mouse heart and mouse muscle. The ATP-ase activity of these tissues was not effected by 10 mM CASH in the pH range 6—9.

Ox brain. The ATP-ase activity was only slightly stimulated by CASH at pH 7—8. Cysteine and glutathione in similar concentrations had about the same effect (Table 2). The intermediary formation of an S-phosphorylated thiol could not be demonstrated.

Vegetables. In Table 2 the effect of CASH on the ATP-ase activity of various vegetables is reported. A marked stimulatory effect on the ATP-ase activity was only observed in the case of cucumber. In several cases CASH was inhibitory. In no case could the formation of CASP be demonstrated.

DISCUSSION

The fact that S-phosphorylated thiols of a small molecular weight can not be demonstrated as intermediates in the thiol stimulated ATP-ase activity of various cells does not exclude the intermediary formation of either protein bound S-phosphates or S-phosphorylated proteins, similar to the ones suggested, *e.g.*, by Brahms and Kakol [13].

The present investigation has shown that the stimulatory effect of the investigated thiols on the ATP-ase activity is not due to a formation of free S-phosphates according to scheme (1) but to some other factor, perhaps one or several of those mentioned earlier in this paper.

EXPERIMENTAL

CASP was prepared as described previously [14].

RBC were obtained from fresh blood and were washed several times with 0.9 % NaCl and stored at —20°. They were used within two days. The RBC were hemolyzed by thawing and subsequent addition of an equal volume of distilled water immediately before use.

Stromata were prepared at 0° according to Herbert [15] and were suspended in 0.9 % NaCl before use.

Mitochondria were prepared at 0—4° according to Schneider [12].

Tissues were homogenized at 0° in a Potter-Elvehjem homogenizer.

Determination of CASP. The incubated sample (1.0 ml) was deproteinized at 0° by the addition of 1.0 ml of 10 % TCA. After rapid centrifugation at 0° the sample (a ml, maximally containing 1 μmole of P_i + CASP) was added to (3.4—a) ml of water, 0.1 ml of Hg^{2+} [14], 0.5 of elon [16] and 1.0 ml of molybdate [16]. In the control 0.1 ml of Hg^{2+} was substituted for 0.1 ml of water. The tubes were set aside for 1 h and then read at 660 mμ. The amount of CASP present was calculated as described under Methods above. The presence of EDTA did not interfere. When the bromine method [8] was used 0.1 ml of saturated bromine water was substituted for the Hg^{2+} solution. The values were corrected for the amount of P_i present in the homogenates and for the small amount of P_i liberated from ATP by the phosphate reagents.

Determination of residual thiols was performed after deproteinization with 10 % TCA by the method of Grunert and Phillips [71]. Proper blanks were used to estimate the disappearance of thiols in the absence of ATP.

The rate of hydrolysis of CASP (Table 2) was determined as described previously [6].

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