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Enzymic Hydrolysis of Cysteamine S-Phosphate by Human Erythrocytes

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Human erythrocytes are shown to contain an enzyme that hydrolyzes cysteamine S-phosphate into cysteamine and *ortho*-phosphate. The enzyme activity, which is absent from serum and practically absent from isolated stromata, is optimal at pH 5.7 (5.5—6.0). Some kinetic data are presented.

In addition to the wellknown biological significance of several compounds containing phosphorylated amino and hydroxyl groups, some recently published reports support the possibility that also compounds containing phosphorylated thiol groups may take part in cellular processes.

The first demonstration of an enzymic S-phosphorylation of a biologically important thiol seems to have been performed by Feuer and Wollemann [1–5] in a study of the formation of "active acetate". Using an enzyme system of brain these authors could show that a phosphate group from adenosine-5'-triphosphate is enzymically transferred to the thiol group of coenzyme A forming coenzyme A S-phosphate *. Smith *et al.* have recently obtained evidence for the formation of this compound in *E. coli* [7,8].

An earlier suggestion by Lynen *et al.*[9,10] and by Lipmann and collaborators [11] that coenzyme A S-phosphate or coenzyme A S-diphosphate should be formed as an intermediary step in the formation of "active acetate" in yeast, was later abandoned [12] (*cf.* Refs. [13–15]).

Reports by other workers indicated that coenzyme A might possibly be S-phosphorylated by enzymes in rabbit liver [16] and spinach [17].

Recent studies by Brahms and Kakol [18] give support to the view that thiol groups of myosin are phosphorylated during the action of this protein on adenosine-5'-triphosphate (*cf.* Ref.[19]).

A study of the properties and possible biological significance of S-phosphorylated thiols of a small molecular size is subject to investigations by the present author. As a part of this program a method was recently developed for

* Regarding the nomenclature applied to these compounds see footnote in Ref.[6]

the preparation of various substances belonging to this category [6,20]. A sodium salt of a compound with some structural relationship to coenzyme A S-phosphate, cysteamine S-phosphoric acid (S-(2-aminoethyl) phosphorothioic acid), was thus prepared [6], and the present paper deals with the enzymic hydrolysis of this compound.

Enzymes capable of hydrolyzing cysteine S-phosphate (prepared from cysteine and $POCl_3$) and the phosphorothioate ion have been demonstrated to be present in rat kidney extracts [21,22]. Forrest *et al.*[23] have desribed a "thionophosphatase", present in *Aspergillus oryzae*, which is capable of hydrolyzing certain aromatic esters of phosphorothioic acid. Feuer and Wollemann [4] have demonstrated the enzymic hydrolysis of coenzyme A S-phosphate by a brain extract.

RESULTS AND DISCUSSION

Preliminary investigations showed that when cysteamine S-phosphate is incubated with RBC * at pH 5—6 it is rapidly converted into *ortho*-phosphate and a nitroprussiate positive substance, which was eventually identified as cysteamine (2-aminoethanethiol).

The factor in RBC responsible for the observed hydrolysis was heat sensitive. Complete inactivation occurred in 5 min at 50°C, pH 5.6, or in 5 min 60°C, pH 6.3. About 50% of the activity remained after exposure to 50°C for 5 min at pH 6.3. (The heating took place in the presence of EDTA (25 mM) and remaining activities were determined at the pH's indicated).

Two obvious possibilities present themselves for a quantitative study of the hydrolysis of cysteamine S-phosphate: a) determination of the thiol released, or b) determination of the *ortho*-phosphate formed. Pilot experiments indicated that the thiol group of cysteamine is rapidly oxidized by RBC (even under nitrogen). The reaction could therefore not be followed quantitatively by the appearence of the thiol without special precautions beeing taken.

On the other hand, the acid lability of the remaining cysteamine S-phosphate (*cf.* Fig. 2) made a determination of liberated *ortho*-phosphate impractical.

It was found, however, that added cysteamine could be quantitatively recovered after incubation with RBC if the incubation took place in the presence of EDTA and the pH was simultaneously kept below *ca.* 8. The enzyme responsible for hydrolysis of cysteamine S-phosphate was not inhibited by EDTA in the concentrations necessary for quantitative recovery of cysteamine. This fact made a determination of the liberated thiol convenient for a quantitative study of the reaction, and this method was therefore adopted.

An investigation of the effect of pH (Fig. 1) showed activity maximum at about pH 5.7 (5.5—6.0). Consequently all subsequent experiments were performed at this pH. It is evident from Fig. 1 that the enzyme is only moderately active at physiological pH. Although recovery of liberated cysteamine is not quite quantitative after incubation at pH's above *ca.* 8, it could be concluded from the experiments performed, that only negligible activity remains between pH 8 and 9.

* Abbreviations used: RBC = human erythrocytes; EDTA = (ethylenedinitrilo)tetraacetic acid.

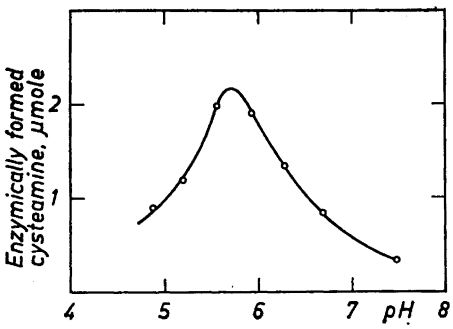

Fig. 1. Effect of pH on the enzymic hydrolysis of cysteamine S-phosphate. Test system (2 ml final volume): cysteamine S-phosphate 9.0 µmole, EDTA 100 µmole, citrate buffer 170 µmole, hemolyzed RBC corresponding to 0.25 ml of packed cells. pH of the complete system as indicated. Blanks contained water instead of RBC. Incubation: 30 min at 37°C. The presented curve constitutes of the average result from 6 different blood samples (individual variations were found to be small).

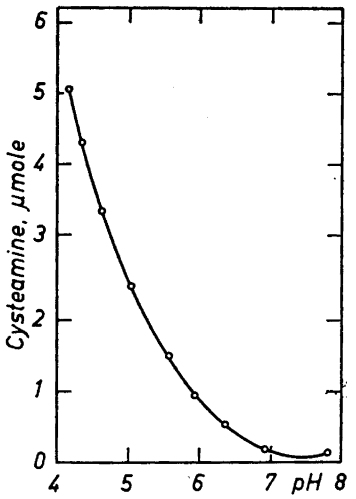

Fig. 2. The spontaneous hydrolysis of cysteamine S-phosphate at 37°C and 30 min as a function of pH. Test system (2 ml final volume): cysteamine S-phosphate 9.0 µmole, EDTA 100 µmole and citrate buffer 170 µmole. pH of the complete system as indicated.

For comparison, the spontaneous hydrolysis of cysteamine S-phosphate in the pH range in question is represented in Fig. 2. As was demonstrated earlier [6] acid hydrolysis of the compound yields the same products as the enzymic hydrolysis. This is in contrast to the behaviour of cysteine S-phosphate as prepared by Binkley [21]. Acid hydrolysis of this compound was reported to give phosphorothioate, ammonia and (presumably) pyruvate. Hydrolysis by rat kidney extract, however, gave cysteine and *ortho*-phosphate. On the other hand, acid hydrolysis of coenzyme A S-phosphate yields coenzyme A and *ortho*-phosphate [4,8].

The enzymic hydrolysis of cysteamine S-phosphate by erythrocytes follows zero order kinetics during the time of the experiments described. The enzyme activity, which seems to be primarily present in the interior of the cells *, is completely absent in serum (Table 1). Substrate saturation of the enzyme is reached at a concentration close to 5 mM (Fig. 3). Higher substrate concentrations are slightly inhibitory.

The effect of enzyme concentration is described in Fig. 4.

* The small activity found in stromata can possibly be attributed to coprecipitation by proteins from the interior of the cells during the preparation of stromata.

Table 1. Enzyme activity of various blood fractions. Test system (2 ml final volume): cysteamine S-phosphate 9.0 μmole, EDTA 100 μmole, citrate buffer 170 μmole. Furthermore the respective solutions contained: hemolyzed RBC corresponding to 0.25 ml of packed cells; stromata corresponding to 0.25 ml of packed cells and serum 0.25 ml. Blanks contained water instead of blood fraction. Final pH 5.6. Incubation: 30 min at 37°C.

Blood fraction	Enzymically formed cysteamine, μmole
Hemolyzed RBC	2.0
Stromata	0.1
Serum	0.0

A phosphomonoesterase having the same optimal pH range as the enzyme described in this paper is known to be present in human erythrocytes (see, *e.g.* Ref.[24]). The previously studied enzyme is only slightly inhibited by fluoride (6—10 % inhibition observed in 10 mM fluoride as determined in RBC hemolysate [25,26]; nor is the purified enzyme markedly inhibited by fluoride [27]), and appears not to be inhibited by small concentrations of EDTA [28].

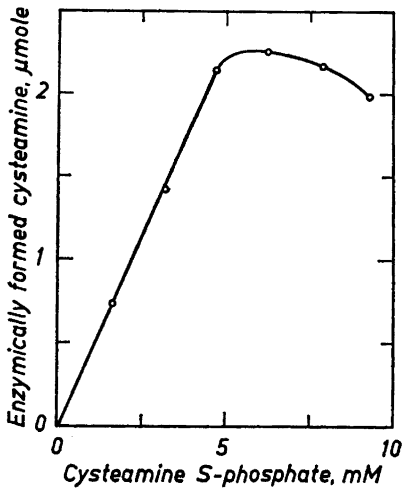

Fig. 3. Effect of cysteamine S-phosphate concentration. Test system (2 ml final volume): EDTA 100 μmole, citrate buffer 170 μmole, cysteamine S-phosphate as indicated and hemolyzed RBC corresponding to 0.25 ml of packed cells. Final pH 5.6. Blanks contained water instead of RBC. Incubation: 30 min at 37°C.

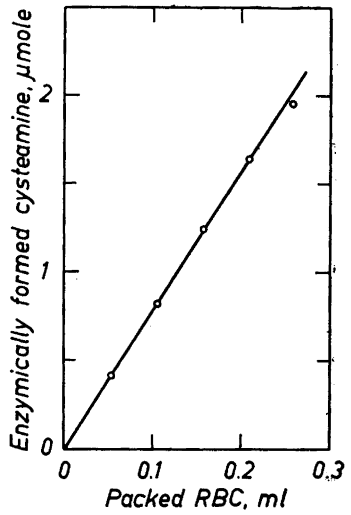

Fig. 4. Effect of enzyme concentration. Test system (2 ml final volume): cysteamine S-phosphate 9.0 μmole, EDTA 100 μmole, citrate buffer 170 μmole and hemolyzed RBC as indicated. Final pH 5.6. Incubation: 30 min at 37°C.

The enzyme hydrolyzing cysteamine S-phosphate shows similar properties, namely: a) the effect of fluoride is small (5 % inhibited by 10 mM NaF and 34 % inhibited by 30 mM NaF); b) it is not inhibited by EDTA (tested up to 75 mM).

Further comparative studies have not been carried out.

EXPERIMENTAL

Materials. Cysteamine S-phosphate was prepared as previously described [6]. RBC were washed several times with 0.9 % NaCl and stored at −20°C. They were used within seven 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°C according to Herbert [29] and were suspended in 0.9 % NaCl before use.

Recovery experiments with cysteamine incubated for 30 min at 37°C with RBC showed that the thiol was quantitatively recovered when the incubation took place in the presence of 50 mM EDTA at pH's between 3 and *ca.* 8 (pH's below 3 were not tested). At pH 3−6 25 mM EDTA was generally sufficient for quantitative recovery, but since the enzyme subject to study, as mentioned, was practically unaffected by the EDTA concentration, 50 mM EDTA was used throughout the experiments.

Determination of cysteamine in the presence of cysteamine S-phosphate. To the incubation tube, containing up to 0.25 ml of hemolyzed RBC in 50 mM EDTA in a total volume of 2.0 ml, was added 1.5 ml of 10 % HPO_3. The mixture was rapidly stirred for 10 sec and 4.0 ml of a 1 M phosphate buffer of pH 7.0 was immediately added. (In order to prevent excessive hydrolysis of cysteamine S-phosphate present, the time allowed for protein precipitation by HPO_3 was kept as short as possible). After stirring the mixture was centrifuged. In the resulting solution of pH *ca.* 6, remaining cysteamine S-phosphate was found to be hydrolyzed at a rate of less than 0.05 % per min at 25°C. The over-all hydrolysis of cysteamine S-phosphate during this procedure was less than 2 %.

Cysteamine was finally determined colorimetrically at 520 mμ with nitroprussiate according to Grunert and Phillips [30], and the results were corrected for the nitroprussiate positive substances already present in the RBC. (The recently developed method [31] employing 2-chloromercuri-6-nitrophenol * as a thiol reagent could not be utilized since this reagent catalyzes the hydrolysis of cysteamine S-phosphate [6].)

Usually the deproteinized solution was slightly colored (owing to the solubilization of some proteins by the change in pH by the addition of the buffer of pH 7.0). Thus a correction was made for the absorption of the test solution at 520 mμ without added nitroprussiate.

In each experiment, where enzyme activity was determined, a correction was made for the spontaneous hydrolysis of cysteamine S-phosphate at the pH in question.

Standardization of the colorimetric method was carried out by the use of: a) commercially available cysteamine hydrochloride, and b) cysteamine solutions obtained by acid hydrolysis of cysteamine S-phosphate. Because of the highly hygroscopic properties of commercial cysteamine and cysteamine hydrochloride, method b) gave more consistent results. However, the two methods agreed within ± 1 %.

Identification of cysteamine as a product of the enzymic hydrolysis of cysteamine S-phosphate. 50 μmole of cysteamine S-phosphate, 1 ml of hemolyzed RBC and 5 ml of 0.15 M citrate buffer, pH 5.6, were incubated at 37°C under nitrogen. A blank sample, carried through the same procedures as the test sample, contained only RBC and buffer. The reaction was stopped after 3 h by the addition of 3.5 ml 10 % trichloroacetic acid. Hydrogen sulfide or ammonia were not detected as products of the hydrolysis. After centrifugation the supernatant solution was divided into two equal parts.

* This numbering follows more closely the system adopted by *Chemical Abstracts*, than the one used previously [31].

One part was treated with iodine in methanol until no more iodine was reduced. Paper chromatography * of the resulting solution was carried out in 1-butanol:acetic acid:water, 4:1:1, and in ethanol:pyridine:water, 2:1:1. Subsequent spraying with ninhydrin and nitroprussiate-cyanide gave, as the only difference between the test and the blank, spots with positions identical with those of cystamine (2,2'-dithiobisethylamine). The spots from the blank sample were faint and did not coincide with the R_F's of cystamine. (Iodine oxidation of the samples was carried out since chromatography of cysteamine in the above solvents gives less satisfactory results than chromatography of cystamine).

The second part of the supernatant solution was made alkaline with KOH and 25 μmole of 2-chloromercuri-6-nitrophenol in 1 ml of 0.1 M NaOH were added. The resulting solution was chromatographed according to Ref.[31] using solvents No. 1 and 3. Only one thiol spot, identical in position with that of MNP-cysteamine [31], was found in this way. (Since EDTA forms a complex with 2-cloromercuri-6-nitrophenol at neutral or alkaline pH's[31], EDTA was omitted from the incubation mixture in this experiment.)

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* Before chromatography most of the trichloroacetic acid was removed by drying the spotted papers in a stream of hot air.

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