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Product activation of human erythrocyte AMP deaminase

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Abstract IMP was found to activate AMP deaminase in crude glucose-depleted human erythrocyte lysates. Activation of the enzyme by IMP is due to prevention of the inhibitory effect of inorganic phosphate. At 1 mM AMP and 2–3 mM phosphate the addition of 2–5 mM IMP accelerates the AMP deamination two to three times.

Key words: Erythrocyte; AMP deaminase; Adenylate metabolism; Enzyme activation

1. Introduction

In human erythrocytes, AMP deaminase (EC 3.5.4.6) irreversibly hydrolyses AMP to IMP. The enzyme is extremely active (V_{max} is about 200 mmol/h/l cells) [1–4]; thus, it appears that the effective regulation of AMP deamination in the cell is important for the control of normal levels of adenylates, especially ATP. Despite extensive studies of the enzyme, its specific role in the cell and the mechanisms responsible for its regulation are still unclear. Moreover, kinetic data obtained on isolated AMP deaminase conflict with results obtained in vivo; the rate of AMP deamination extrapolated from kinetic parameters determined for the isolated enzyme at actual intracellular concentrations of AMP and in the presence of effectors [1,3,5–9] is significantly greater than that found in native erythrocytes [10–15]. This discrepancy may result either from alteration of enzyme properties during purification or from the lack of some effectors (which are present in vivo) in the reaction mixtures used to study the isolated AMP deaminase.

The aim of this work was to study the AMP deaminase of erythrocytes under conditions closely simulating those which exist intracellularly. Specifically, the kinetics of AMP degradation in crude lysates of erythrocytes was studied.

2. Materials and methods

2.1. Preparation of haemolysates

Healthy donor blood anticoagulated with CPD (citrate phosphate dextrose solution) was centrifuged at 1500×*g* for 10 min. The plasma and the upper layer of cells containing platelets and leukocytes were removed. The erythrocytes were washed three times by centrifugation at 1500×*g* for 10 min in four volumes of a solution containing 150 mM NaCl, 30 mM KCl, and 5 mg/l gentamicin (solution A). The washed erythrocytes were resuspended in solution A to a haematocrit of 40–50% and incubated at 37°C for 24 h. After incubation, the erythrocytes were washed three times with solution A, then two times with solution B containing 150 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, and 3 mM β-mercaptoethanol (pH 7.4), and resuspended in solution B to a haematocrit of 50%. To lyse cells, dry saponin was added to the suspension (final concentration 5 mM) and the mixture was incubated for 15 min. Cellular membranes were removed by centrifugation at 3500×*g* of 1 h.

2.2. Dialysis of haemolysate

For dialysis, a membrane with a molecular weight cut-off of 12 kDa (Sigma, USA) was used. 20 ml of haemolysate was dialysed against 1 l of solution B at 4°C for 12 h with permanent stirring.

2.3. Assay for AMP deaminase

Before the experiments, haemolysates were diluted with solution B to a haemoglobin concentration of approximately 80 g/l. All incubations were carried out in teflon cells thermostatted at 37°C with permanent stirring. AMP, inorganic phosphate, 2,3-diphosphoglycerate, IMP, inosine and hypoxanthine were prepared as 10–100 mM stock solutions in solution B.

The reaction was started by the addition of AMP (0.5–10 mM) to 2 ml of the haemolysate. The reaction was terminated by the addition of two volumes of 0.5 M $HClO_4$. The mixture was vigorously stirred and centrifuged at 1500×*g* for 10 min. The pH of the supernatant was adjusted to 7.0 with a saturated solution of K_2CO_3. Three aliquots were withdrawn from the haemolysate: 0, 2, and 4 min after the addition of AMP. The rate of AMP degradation was determined as the difference in AMP concentrations between the zero time and subsequent samples and was expressed as mmol AMP hydrolysed per h per litre of cells. We assumed that 1 l of erythrocytes contains 300 g of haemoglobin [16].

2.4. Determinations of metabolite concentrations

The perchloric acid extracts were analysed by HPLC [17]. The concentration of haemoglobin was determined spectrophotometrically at 416 nm. The inorganic phosphate was determined using a phosphorus test kit from Kone (Finland).

3. Results

Fig. 1 shows the dependence of the initial rate of AMP degradation on the concentration of AMP (V_{AMP}) in crude haemolysates. Analysis of the Lineweaver-Burk and Hill plots revealed a sigmoidal curve with $n = 2.3$, $V_{max} = 100$ mmol/h/l cells, and $S_{0.5} = 4$ mM (data of four experiments). IMP was a major product of AMP degradation, suggesting that AMP deamination was largely responsible for the shape of V_{AMP}. Fig. 1 shows the initial rates of AMP degradation (open circles) and those after 2 min of preincubation with AMP (solid circles). After 2 min of preincubation, AMP degradation products (IMP, inosine and hypoxanthine) were formed, and their stimulatory effect on AMP deamination became evident.

The substrate rate dependences for AMP degradation in the presence of IMP, inosine or hypoxanthine shown in Fig. 2 suggest that IMP is an activator of AMP deaminase.

Fig. 3 shows the effects of IMP and inorganic phosphate added to a dialysed haemolysate on the V_{AMP}. Without the effectors, V_{AMP} appears as a simple hyperbolic function, with K_m and V_{max} of about 6 mM and 200 mmol/h/l cells, respectively. These results are consistent with those obtained for isolated enzyme under the same conditions [6,8,9]. IMP was ineffective when added to the dialysed haemolysate. This ob-

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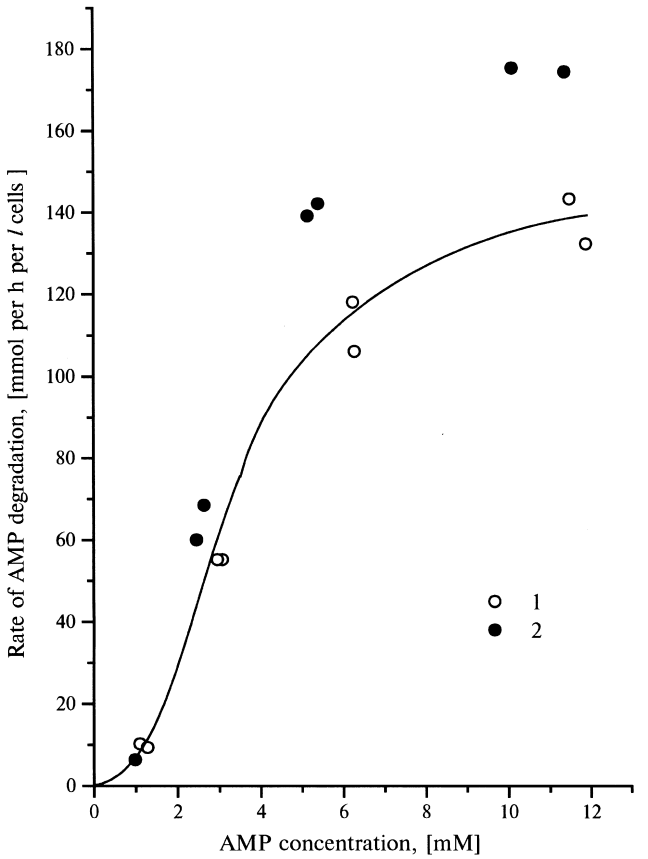

Fig. 1. Rate of AMP degradation in crude erythrocyte lysates plotted agains AMP concentration: (1) the initial rate and (2) the rate determined 2 min after addition of AMP.

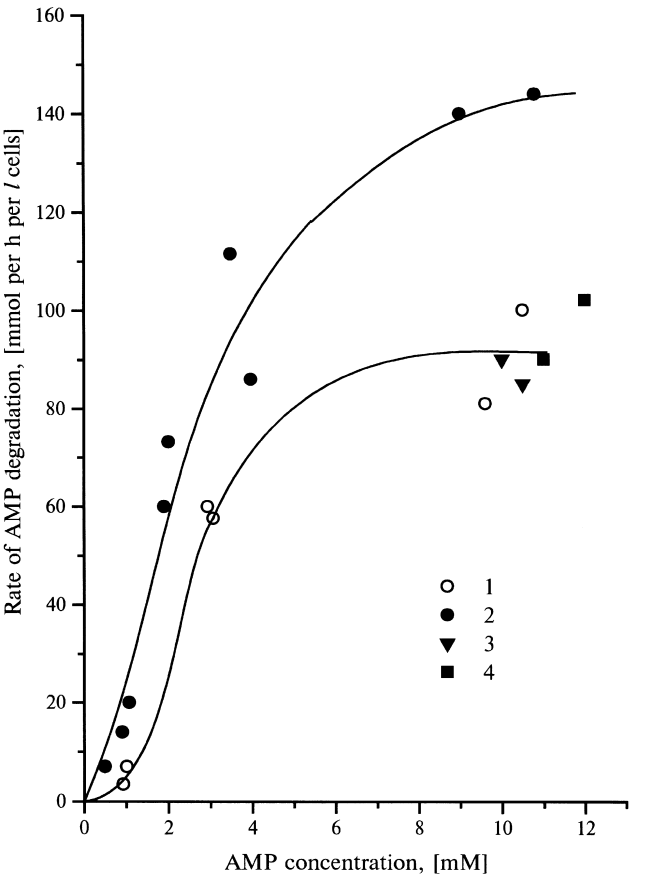

Fig. 2. Initial rate of AMP degradation in crude erythrocyte lysates as a function of AMP concentration: (1) control, (2) 5 mM IMP, (3) 2 mM inosine or (4) 4 mM hypoxanthine. The effectors were added simultaneously with AMP.

servation suggested that crude haemolysates contained some low-molecular-weight inhibitor of AMP deaminase whose effect was abolished by added IMP. Thus, the effect of IMP on AMP deaminase inhibited by 2,3-diphosphoglycerate or inorganic phosphate was studied (Table 1). IMP activated the enzyme in dialysed haemolysate containing inorganic phosphate and showed no effect in the presence of 2,3-diphosphoglycerate.

4. Discussion

Lushchak and Storey have shown that IMP inhibits purified AMP deaminase from the salmon white muscle [18]. In contrast, we found that IMP stimulates the enzyme in crude erythrocyte lysates, but does not affect AMP deaminase in dialysed haemolysates. This observation suggests that a low-molecular-weight inhibitor of AMP deaminase exists in crude haemolysates, and IMP acts by diminishing its effect. Our data on the combined effect of IMP and inorganic phosphate on AMP deaminase suggest that inorganic phosphate is likely to be such an inhibitor. Crude haemolysates contain 1.5–2.5 mM orthophosphate; at a concentration of 1 mM, AMP was deaminated at a rate of about 5 mmol/h/l cells (Figs. 1 and 2). The same rate was observed in the dialysed haemolysates containing 2–3 mM KH_2PO_4 (Table 1). In both cases, 5 mM IMP accelerated AMP deamination two to three times. We believe that IMP and inorganic phosphate compete for the common binding site on the AMP deaminase molecule. IMP had no effect on the inhibition of AMP deaminase by 2,3-diphosphoglycerate. This observation needs further studying because inorganic phosphate and 2,3-diphosphoglycerate bind to the same site on the AMP deaminase [7,19,20].

Table 1
Effect of IMP on the AMP degradation rate in the presence of 2,3-diphosphoglycerate or inorganic phosphate in dialysed haemolysates

Inhibitor	IMP concentration	Rate of AMP degradation (mmol/h/l cells)
–	–	15.1 ± 1.0 (2)
–	5 mM	15.3 ± 2.0 (3)
KH_2PO_4 2 mM	–	5.7 ± 0.9 (2)
–	5 mM	9.8 ± 1.0 (2)
2,3-Diphosphoglycerate 3 mM	–	2 ± 0.1 (2)
–	5 mM	1.9 ± 0.2 (2)

The initial AMP concentration was 1 mM. The data shown are representative of three experiments. Means ± S.D.; numbers of measurements are indicated in parentheses.

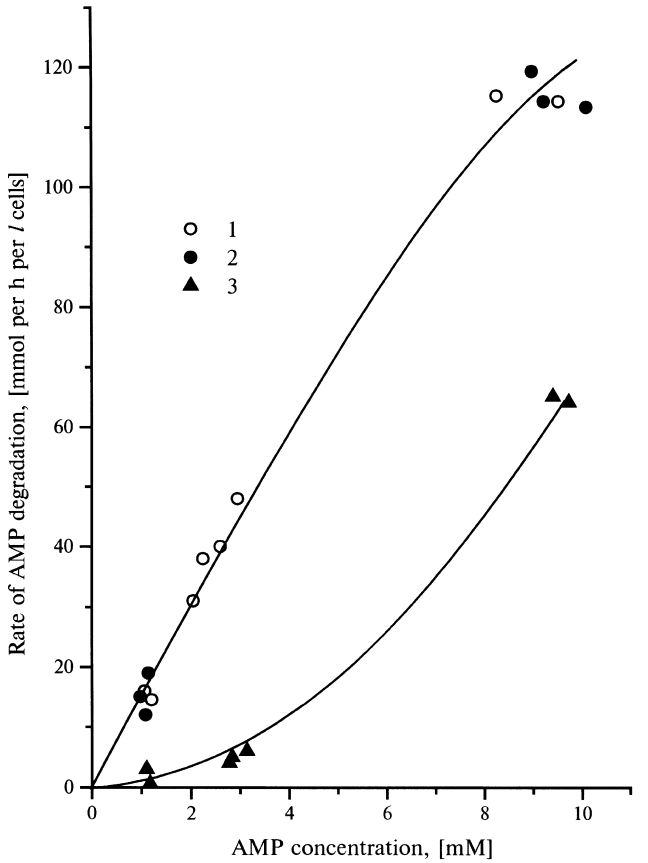

Fig. 3. Substrate rate dependence of AMP degradation in dialysed erythrocyte lysates: (1) control, (2) 5 mM IMP or (3) 5 mM KH_2PO_4. Inorganic phosphate was added 30 min before the addition of AMP; IMP was added simultaneously with AMP.

Our results emphasise that it is difficult to understand how AMP deaminase is regulated within the cells by studying the isolated enzyme: many intracellular effectors exist and the net effect on AMP deaminase activity depends on their interplay.

Activation of AMP deaminase by IMP results in a positive feedback in the AMP degradation pathway. If the intracellular AMP level increases (e.g. when cell membrane damage significantly activates transporting ATPases), this positive feedback would result in an irreversible, self-accelerated adenylate pool loss. Eventually, this will lead to cell death. Under normal physiological conditions, when the rate of AMP degradation is low (40–60 mmol/h/l cells [10–15]) and the IMP concentration is about 0.01 mM [21,22], the product activation of AMP deaminase has no effect on erythrocyte metabolism. We believe that the product activation of AMP deaminase may serve as the mechanism for elimination of severely damaged cells.

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