Regulation of platelet AMP deaminase activity *in situ*

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The regulation of platelet AMP deaminase activity by ATP, GTP and phosphate was studied in human platelets *in situ*, and *in vitro* after partial purification. In intact platelets, a similar 50 % decrease in cytosolic ATP was induced by either glucose starvation or treatment with H₂O₂. During starvation, AMP deaminase was in the inhibited state, as ATP consumption was mostly balanced by the accumulation of AMP. During H₂O₂ treatment, however, the enzyme was in the stimulated state, as the AMP formed was almost completely deaminated to IMP. Cytosolic GTP fell by 40–50 % in both starvation and H₂O₂ treatment. In contrast, intracellular phosphate was 4–5-fold higher in starved than in H₂O₂-treated cells. These data point to phosphate as the main regulator of AMP deaminase activity *in situ*. This conclusion was verified by kinetic analysis of partially purified AMP deaminase. At near-physiological concentrations of MgATP, MgGTP and phosphate, the *s*_{0.5} (substrate half-saturation constant) for AMP was 0.35 mM. Half-maximal stimulation by MgATP occurred at a concn. between 2 and 3 mM. This stimulation was antagonized by the inhibitory effects of phosphate (IC₅₀ = 2.0 mM) and MgGTP (IC₅₀ = 0.2–0.3 mM), which acted in synergism (IC₅₀ is the concentration causing 50 % inhibition). We conclude that the difference in adenylate catabolism between starved and H₂O₂-treated platelets is due to the distinct phosphate concentrations. During starvation, refeeding and H₂O₂ treatment, the values of the adenylate charge and the phosphorylation potential were kept closely co-ordinated, which may be effected by AMP deaminase.

INTRODUCTION

When cellular ATP utilization and regeneration are no longer balanced, the action of adenylate kinase results in an increase in the concentration of AMP. Further catabolism of AMP, either by deamination to IMP by AMP deaminase, or by dephosphorylation to adenosine by 5'-nucleotidase, leads to a depletion of the adenine nucleotide pool. The pathway of AMP degradation and its control has been studied extensively for a number of cells (Lowenstein, 1972; Van den Berghe *et al.*, 1977; Matsumoto *et al.*, 1979; Bontemps *et al.*, 1986; Whitlock & Terjung, 1987; Altschuld *et al.*, 1987). In platelets, the AMP dephosphorylation route appears to be absent (Harris & Crawford, 1973; Holmsen *et al.*, 1974). Upon platelet activation (Holmsen *et al.*, 1972; Fukami *et al.*, 1976) or when platelets are exposed to metabolic stress (Holmsen & Robkin, 1977, 1980; Murer *et al.*, 1981; Holmsen, 1985), AMP deaminase is stimulated so that hardly any AMP accumulates, and the decrease in cytosolic ATP and ADP is almost completely recovered in IMP and its degradation products, inosine and hypoxanthine. As a result the adenylate energy charge (AEC), defined as follows:

$$\text{AEC} = (\text{ATP} + \frac{1}{2}\text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$$

(Atkinson, 1977), is maintained at a high value. In contrast, when platelets are deprived of glucose, AMP deaminase is not stimulated, so there is a massive accumulation of AMP (Akkerman *et al.*, 1978, 1983). Here, the value of AEC falls in parallel with cytosolic ATP, and the platelets lose their responsivity.

Platelet AMP deaminase (EC 3.5.4.6) has been purified and its regulation by substrate AMP, activator ATP and inhibitors P_i and GTP extensively studied (Ashby & Holmsen, 1981, 1983). Kinetic analyses suggested that enzyme activity *in situ* is mainly regulated by substrate concentration (Ashby & Holmsen, 1983). However, these studies left unexplained why the enzyme is turned on during platelet activation and metabolic stress, when AMP concentrations are hardly increased, but shuts off during starvation despite massive accumulation of AMP. In the present study we have monitored changes in cytosolic ATP, GTP, P_i and AMP in platelets during starvation and metabolic stress. Using a double-labelling technique with [³²P]P_i and [³H]adenine, we were able to simultaneously monitor changes in P_i and GTP, and in cytosolic ATP, AMP, IMP, inosine and hypoxanthine. Exposure of platelets to low doses of H₂O₂ was used here as a metabolic stress model (Holmsen & Robkin, 1977; Verhoeven *et al.*, 1985). We report that intracellular P_i is increased in starved platelets but not in H₂O₂-treated platelets. Kinetic analysis of partially purified AMP deaminase confirmed that the enzyme activity is highly sensitive to changes in P_i at physiological concentrations of the other modulators.

MATERIALS AND METHODS

Studies on intact platelets

Platelet isolation and incubation. Human platelet-rich plasma was prepared as described previously (Tysnes *et al.*, 1985), and incubated for 60 min at 37 °C with 0.1

Abbreviations used: AEC, adenylate energy charge; *s*_{0.5}, substrate half-saturation constant; I₅₀ and A₅₀, half-maximal inhibition and activation constants respectively.

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mCi of [^{32}P]P_i/ml (carrier-free; Amersham International) and 3 μM -[2- ^3H]adenine (specific radioactivity 10 Ci/mmol; Amersham International). Subsequently, gel-filtered platelets were prepared in glucose-, phosphate- and Ca²⁺-free Tyrode's solution (pH 7.25; osmolarity 300 mosm/litre) containing 1 mM-KCN (Akkerman *et al.*, 1983; Holmsen *et al.*, 1983). The gel-filtration procedure lasted for 20–30 min. Platelet numbers were determined by cell counting and were standardized at 3.5×10^8 cells/ml by dilution in gel-filtration buffer. The gel-filtered platelets were warmed to 37 °C (5 min) and then shaken at 200 strokes/min in a shaking water bath. Platelet suspensions were divided into three portions. To one portion, glucose was added to a final concentration of 5 mM at the start of the incubation (refeeding). To a second portion, 5 mM-glucose was added and then 150 μM -H₂O₂ was added after 10 min. The third portion of gel-filtered platelets was incubated without further additions (referred to as starved platelets). It should be noted that starvation was already induced during gel-filtration and continued during cell counting (10 min) and prewarming. At the times indicated, samples were collected for subsequent analysis.

Analysis of metabolites. Samples of cell suspension were rapidly mixed with 2 vol. of EDTA/ethanol [10 mM-EDTA in 86% (v/v) ethanol, pH 7.4, 0 °C]. After centrifugation (10000 g, 4 °C, 2 min), the supernatants were analysed for ^3H - and ^{32}P -labelled adenylates and their derivatives, and for ^{32}P -labelled GTP, P_i and glycolytic intermediates after separation by two-dimensional paper chromatography (Holmsen *et al.*, 1983). Radiolabelled spots were identified by co-chromatography of a mixture of unlabelled standards, cut out, and the radioactivity was determined by liquid scintillation counting. The labelling procedure is essential to distinguish cytosolic ATP, ADP, GTP and phosphate from the large amounts of granule-stored compounds, which are not available for cellular energy metabolism (Holmsen, 1985). Previous studies have shown that in such [^{32}P]P_i- and [^3H]adenine-labelled platelets, all cytosolic metabolites investigated are labelled to the same specific radioactivity (Daniel *et al.*, 1980; Holmsen *et al.*, 1983). Changes in the labelling of each compound therefore accurately reflect changes in their cytosolic concentrations. The chromatography system used fails to separate GTP from UTP (Holmsen *et al.*, 1983). In untreated platelets, at least 60% of total ^{32}P -radioactivity in the GTP+UTP spot represents GTP (D'Souza & Glueck, 1979; Daniel *et al.*, 1980). No corrections were made for this contamination with UTP.

For the determination of intracellular [^{32}P]P_i, samples of cell suspension were directly layered on top of 100 μl of phthalate [dinonyl/dibutyl (2:3, v/v), 0 °C], and the platelets were immediately removed from the extracellular medium by centrifugation (7000 g, 1 min, 4 °C; Verhoeven *et al.*, 1987). The supernatants were rapidly mixed with 2 vol. of EDTA/ethanol, and ^{32}P -labelled extracellular P_i was determined after two-dimensional paper chromatography as described above. Total [^{32}P]P_i was determined in EDTA/ethanol extracts of the gel-filtered platelets without prior centrifugation through oil. Extracellular [^{32}P]P_i amounted to 20–40% of total [^{32}P]P_i, depending on the incubation conditions. Intracellular [^{32}P]P_i was calculated as the difference between total and extracellular [^{32}P]P_i. Of the radiolabelled adenylates, less

than 2% appeared in the supernatant after centrifuging the platelets through the oil layer, for which no corrections were made.

The AEC [(ATP + $\frac{1}{2}$ ADP)/(ATP + ADP + AMP)] was calculated from the ^3H radioactivity in ATP, ADP and AMP (Atkinson, 1977). The phosphorylation potential [ATP/(ADP $\times$ P_i)] was calculated from the ^{32}P radioactivity in ATP, ADP and intracellular P_i (Slater, 1979); the ^{32}P radioactivity in ATP was divided by 2 to allow for the presence of two labelled phosphate groups compared with only one in ADP (Holmsen *et al.*, 1983). No attempts were made to determine the specific ^{32}P radioactivity of intracellular P_i; hence the derived values are only relative measures of the phosphorylation potential.

Experiments with partially purified AMP deaminase

Starting material. AMP deaminase was purified from outdated human platelet concentrates (about 7 days old). The platelet suspensions were adjusted to 1 mM with respect to EDTA. As erythrocytes contain an AMP deaminase with different kinetic properties than the platelet form (Lian & Harkness, 1974; Ashby & Holmsen, 1981), care was taken to remove most of the residual erythrocytes by centrifugation (6 min, 300 g, room temperature). Thereafter, the platelets were collected by a further centrifugation (20 min, 8000 g, 10 °C). The platelets were resuspended in 30 mM-Tris/HCl (pH 7.4)/120 mM-NaCl/5 mM-EDTA, and sedimented again (5 min, 10000 g, 10 °C). After removing the supernatant, the platelet pellets were stored at –18 °C. Packed cells were used within 10 days of preparation.

Purification procedure. Packed cells from 7–10 batches of platelet concentrates were lysed by slow thawing in 9 vol. of lysis buffer (5 mM-Tris/HCl/3 mM-EGTA/2 mM-2-mercaptoethanol/0.1% Triton X-100, pH 7.0, at 4 °C). The lysate was gently stirred for 30 min (0 °C) and then centrifuged for 30 min at 20000 g and 4 °C. AMP deaminase was purified from the supernatants essentially as described by Spychala *et al.* (1986). In short, the enzyme was adsorbed batchwise on to phosphocellulose (either Whatman P-11 or Sigma) in 250 mM-KCl and 2 mM-2-mercaptoethanol (pH 7.0), which usually bound 70–95% of total enzyme activity. After packing the resin in a column, AMP deaminase was eluted with a double linear gradient from 0–100 mM-potassium phosphate and from 550–650 mM-KCl (containing 2 mM-2-mercaptoethanol, pH 7.0). Finally, the enzyme was concentrated by rechromatography on phosphocellulose and single-step elution with 2 M-KCl (containing 2 mM-2-mercaptoethanol, pH 7.0). AMP deaminase activity was assayed by the rate of ammonia production at 25 °C in a 50 mM-succinate buffer (pH 6.5) containing 0.05% albumin, 10 mM-AMP and a final concentration of 150 mM-KCl according to Chaney & Marbach (1962). The specific activity of the final preparation was 41–54 $\mu\text{mol/min per mg}$ of protein. The procedure used yielded 15–25% of initial AMP deaminase activity with a 360–500-fold purification. The final enzyme preparation was stored at 4 °C, and used within 3 days of purification.

Occasionally, AMP deaminase activity was also purified from fresh pig platelets. Pig blood, anti-coagulated in 10.5 mM-citric acid/12.8 mM-sodium citrate/16.6 mM-dextrose, was left at rest overnight, which allowed most of the erythrocytes to sediment. After removal of any residual erythrocytes by centrifugation, the platelets were

collected and packed cells were prepared as described above. No differences were found in purification behaviour and kinetics between AMP deaminase prepared from the outdated human platelets and the fresh pig platelets (results not shown). Therefore AMP deaminase isolated from the outdated platelets was assumed to be similar to that from fresh human platelets.

Kinetic analysis. The kinetics of AMP deaminase activity were studied at 25 °C in a 50 mM-cacodylate buffer (pH 7.2) containing 150 mM-KCl, 0.05 % bovine serum albumin and near-physiological concentrations of AMP (approx. 50 μ M; Holmsen & Robkin, 1980), ATP (approx. 3 mM; Ashby & Holmsen, 1983; Holmsen, 1985), GTP (approx. 0.4 mM; Holmsen, 1985) and P_i (approx. 4 mM; Fukami *et al.*, 1980). In order to simulate the situation in the starved and H_2O_2 -treated platelets, the kinetics were also studied at a lower concentration of ATP and at elevated concentrations of AMP and P_i , which were arbitrarily chosen at 1 mM, 250 μ M and 10 mM respectively. Mg^{2+} was added to an excess of 1 mM above the concentrations of ATP plus GTP; under these conditions, 90.1 % of ATP and GTP was complexed with Mg^{2+} (Spychala & Marszalek, 1987). Final Na^+ concentrations never exceeded 10 mM. Reactions were started by the addition of 0.6–3.0 μ g of enzyme protein/ml of assay mixture. At different times thereafter, samples were collected for determination of ammonia or IMP. When studied at 250 μ M-AMP (or higher concentrations), samples were added directly to the phenol/hypochlorite reagent and ammonia was determined (Chaney & Marbach, 1962). At lower concentrations of AMP, the enzyme activity was measured by the rate of [3H]IMP formation using [$2-^3H$]AMP (specific

radioactivity 10–20 Ci/mmol; Amersham International) as substrate. Aliquots from the incubation mixture were rapidly mixed with 1 vol. of EDTA/ethanol and then analysed for [3H]IMP after separation by one-dimensional paper chromatography on Whatman no. 1 paper using propanol/butanol/acetone/formic acid/30 % trichloroacetic acid in water/77 mM-EDTA (250:150:150:125:100:8, by vol.; Holmsen *et al.*, 1983). The IMP spot was identified by co-chromatography with unlabelled standard, cut out, and the radioactivity was determined by liquid scintillation counting. Formation of ammonia and [3H]IMP at 250 μ M- and 50 μ M-AMP respectively was linear both with time and with the amount of enzyme during consumption of up to 20 % of substrate (results not shown).

For determination of the kinetic parameters V_{max} (maximal velocity), $s_{0.5}$ (substrate half-saturation constant) and h (Hill coefficient), the non-linear regression method was used as described by Spychala *et al.* (1986) and Spychala & Marszalek (1987). The calculations were performed using a computer program written in BASIC. Half-maximal inhibition (I_{50}) and activation (A_{50}) constants were derived directly from the plots of initial velocities of ammonia or [3H]IMP formation versus concentration of effectors.

RESULTS

Effect of starvation, refeeding and H_2O_2 treatment

3H - and ^{32}P -labelled platelets were gel-filtered into a medium containing cyanide but no glucose. Incubation of such platelets (starvation) resulted in a gradual fall in [3H]ATP to approx. 50 % of the initial level at 30 min (Fig. 1a). Addition of glucose (5 mM; refeeding) pre-

Fig. 1. Effect of starvation, refeeding and H_2O_2 treatment on adenylate metabolism

[3H]Adenine- and [^{32}P] P_i -labelled platelets were gel-filtered into a glucose-free medium containing 1 mM-KCN, and subsequently incubated at 37 °C. One portion of the suspension was incubated in this medium without further additions (starvation; ○). To a second portion, 5 mM-glucose was added at the start of the experiment as indicated by the open arrowheads (refeeding; ●), and to a third portion 150 μ M- H_2O_2 was added at 10 min after glucose, as indicated by the filled arrows (■). At the times indicated, samples were withdrawn from the incubations for analysis of [3H]ATP (a), [3H]AMP and [3H]IMP (including its degradation products [3H]inosine and [3H]hypoxanthine) (b), [^{32}P]GTP (+ UTP; see the Materials and methods section) (c) and intracellular [^{32}P] P_i (d). Data are representative for three similar experiments.

Fig. 2. Relationship between AEC and phosphorylation potential

From the experiment presented in Fig. 1, the values of the AEC and phosphorylation potential were calculated for the different time points, and $\log(1 - \text{AEC})$ was plotted versus $\log(\text{phosphorylation potential})$ according to Reed (1976). AEC was calculated using the ^3H radioactivity in ATP, ADP and AMP, whereas the phosphorylation potential $[\text{ATP}/(\text{ADP} \times \text{P}_i)]$ was calculated using the ^{32}P radioactivity of ATP, ADP and intracellular P_i ; the ^{32}P radioactivity in ATP was divided by 2 to allow for two labelled phosphate groups versus one in ADP (Holmsen *et al.*, 1983). Symbols are as defined for Fig. 1 (○, starvation; ●, refeeding; ■, H_2O_2 -treated). The regression line was: $y = 3.47 - 0.74x$ ($n = 11$; $r = 0.9927$; $t = 24.7$; $P < 0.001$). Similar results were obtained for the two other experiments.

vented this decrease in ATP. When such glucose-repleted platelets were incubated with $150 \mu\text{M}$ H_2O_2 , cytosolic ATP decreased to approx. 50 % of control values within 5 min. Changes in ^{32}P ATP paralleled those in ^3H ATP (results not shown). In the starved platelets, the fall in ATP was accompanied by a marked accumulation of ^3H AMP, and approx. 20-fold elevated levels were reached at 30 min of incubation (Fig. 1b). Simultaneously, ^3H IMP (plus ^3H inosine and ^3H hypoxanthine) increased only slightly. In contrast, during H_2O_2 treatment of glucose-repleted platelets, ^3H AMP increased only slightly, whereas ^3H IMP (and its products) more than doubled within the first 5 min. In the starved platelets, the fall in ^3H ATP was almost completely recovered in AMP and ADP (results not shown), whereas in the H_2O_2 -treated platelets, the ^3H label was mainly recovered as IMP, inosine and hypoxanthine. No ^{32}P radioactivity could be detected in AMP or IMP, in agreement with previous reports (Holmsen *et al.*, 1983).

In the starved platelets, ^{32}P GTP (+ UTP) gradually fell to about 50 % of the initial level after 30 min of incubation (Fig. 1c). Addition of glucose prevented this fall, and ^{32}P GTP was maintained close to the initial level throughout incubation. H_2O_2 induced an abrupt fall in ^{32}P GTP to approx. 60 % of the initial level within the first 5 min.

After gel filtration of platelets into the glucose-free, KCN-containing medium, intracellular ^{32}P P_i was 2.7 ± 0.6 -fold higher than ^{32}P ATP (mean $\pm$ S.D.; $n = 3$).

Assuming that the ^{32}P label is homogeneously distributed among cytosolic ATP and P_i , and that the ^{32}P label in ATP is only present in the β - and γ -phosphoryl groups (Holmsen *et al.*, 1983), this figure indicates approx. 5-fold higher levels of P_i than ATP. As human platelets contain little glucose (Solomon & Gaut, 1970), starvation probably started during gel-filtration. Presumably, glycolytic intermediates and some ATP are consumed during the initial stage of starvation (Verhoeven *et al.*, 1985), thus explaining the initially high ^{32}P P_i level. Upon prolonged incubation, intracellular ^{32}P P_i increased further (Fig. 1d). Addition of glucose induced an abrupt fall in ^{32}P P_i to 20–25 % of the initial level. Concomitantly, the sum of ^{32}P radioactivities in sugar phosphates increased by several-fold (results not shown). H_2O_2 caused a transient increase in ^{32}P P_i (Fig. 1d), whereas the amount of ^{32}P -labelled fructose 1,6-bisphosphate and, notably, dihydroxyacetone phosphate was markedly and sustainedly increased (results not shown; Verhoeven *et al.*, 1985).

The data presented in Fig. 1 clearly show that AMP deaminase was stimulated during H_2O_2 -induced ATP catabolism, but inhibited during starvation, in agreement with previous reports (Holmsen & Robkin, 1977; Akkerman *et al.*, 1978, 1983). Of the metabolites monitored, only intracellular phosphate showed large variations, being 4–5-fold higher in the starved platelets (in which AMP deaminase was in the inhibited state) than in the refeed and H_2O_2 -treated platelets (which had a stimulated enzyme). This suggests a crucial role for intracellular P_i in the regulation of the activity of AMP deaminase *in situ*. In the glucose-repleted and the H_2O_2 -treated suspensions, the AEC value was maintained above 0.85, but in the starved platelets the AEC fell gradually from 0.87 at the start of the incubation to 0.55 at 30 min (results not shown). The phosphorylation potential, as calculated from the ^{32}P radioactivity in ATP, ADP and intracellular P_i , varied in parallel with the AEC. This is also illustrated in Fig. 2, in which $\log(1 - \text{AEC})$ is plotted versus $\log(\text{phosphorylation potential})$, according to Reed (1976). Apparently, these regulatory parameters are strongly correlated in human platelets, independent of the incubation conditions ($r = 0.993$, $n = 11$; $P < 0.001$).

Kinetic study of AMP deaminase

Partial purification of platelet AMP deaminase. To verify that platelet AMP deaminase is indeed under phosphate control *in situ*, kinetic analysis was performed on partially purified enzyme at near-physiological concentrations of AMP, ATP, P_i and GTP. The purification procedure used was designed to identify the possible existence of multiple forms of the enzyme which have been demonstrated in various human tissues (Ogasawara *et al.*, 1982), rat small intestine (Spychala *et al.*, 1986) and chicken kidney and liver (Spychala & Marszalek, 1987). However, platelet AMP deaminase was eluted from the phosphocellulose column in a single peak at about 50 mM-phosphate (0.6 M-KCl). Moreover, AMP deaminase that was eluted in the first and last part of the peak showed identical sigmoidal and regulatory kinetics (results not shown). Hence platelets probably contain only one form of AMP deaminase, corresponding to the human-liver-type isoenzyme (Ogasawara *et al.*, 1982) and form II from chicken liver (Spychala & Marszalek, 1987) and rat small intestine (Spychala *et al.*, 1986).

Table 1 gives the basal kinetic properties of our enzyme

Table 1. Basal kinetics of partially purified AMP deaminase

AMP deaminase activity was determined at various substrate concentrations by ammonia production in the absence or presence of 1 mM-MgATP, 4 mM- P_i or 0.4 mM-MgGTP as indicated. Assays were started with the addition of 2.64 μ g of enzyme protein/ml of incubation mixture. The kinetic parameters $V_{\max}$ (maximum velocity), $s_{0.5}$ (substrate half-saturation constant) and h (Hill coefficient) were calculated for one set of experimental data by non-linear regression analysis as described in the Materials and methods section. Confidence limits are indicated as s.d.s which were derived from the diagonal elements of the covariance matrix (Spychala *et al.*, 1986; Spychala & Marszalek, 1987). The data are representative for two to four independent experiments.

	$V_{\max}$ (nmol/min)	$s_{0.5}$ (mM)	h
Control	29.4 $\pm$ 0.8	5.0 $\pm$ 0.2	1.7 $\pm$ 0.1
+ MgATP	35.0 $\pm$ 2.0	0.36 $\pm$ 0.05	1.0 $\pm$ 0.2
+ P_i	26.9 $\pm$ 1.1	5.8 $\pm$ 0.4	1.9 $\pm$ 0.2
+ MgGTP	30.5 $\pm$ 0.4	8.4 $\pm$ 0.1	3.2 $\pm$ 0.1
+ P_i + MgGTP	29.1 $\pm$ 0.7	12.8 $\pm$ 0.2	3.6 $\pm$ 0.1

Fig. 3. ATP activation of platelet AMP deaminase

To a final volume of 100 μ l of assay mixture, 0.27 μ g of partially purified AMP deaminase was added and enzyme activity was determined by [3 H]IMP production at 50 μ M- $[^3$ H]AMP, 0.4 mM-MgGTP and 1 mM free Mg^{2+} , and either 4 mM- (●) or 10 mM- P_i (○). Data are representative for experiments with two different enzyme preparations.

preparation. Under the assay conditions used, the maximal velocity of AMP deamination was only slightly affected by the effectors studied. In contrast, the regulatory properties of the enzyme strongly depended on the presence of effectors. In their absence, the saturation curve for AMP was sigmoidal, with $s_{0.5} = 5.0$ mM and $h = 1.7$. Inclusion of 3 mM-MgATP in the assay mixture markedly increased the affinity for AMP ($s_{0.5} = 0.35$ mM) and changed the kinetics to hyperbolic ($h = 1.0$). Phosphate (4 mM) and MgGTP (0.4 mM) were only slightly inhibitory when added alone, but in combination the saturation curve for AMP was markedly shifted to higher substrate concentrations ($s_{0.5} = 12.8$ mM) and showed an increased degree of co-operativity ($h = 3.6$). Although GTP alone produced only moderate inhibition when complexed with Mg^{2+} , free GTP was highly inhibitory ($K_i = 10$ μ M). This property of platelet AMP deaminase distinguishes it from the erythrocyte form (Lian & Harkness, 1974). The kinetic properties of our enzyme

preparations were similar to those reported previously (Ashby & Holmsen, 1981, 1983).

Combined effect of MgATP, MgGTP and P_i . Next, the individual effect of each of the three modulators on enzyme activity was tested in the presence of near-physiological concentrations of the others. At all AMP concentrations tested, the inhibitory effect of P_i plus MgGTP (4 mM and 0.4 mM respectively) was abolished by the presence of 3 mM-MgATP (results not shown). Hence at this MgATP concentration, enzyme activity is mainly regulated by changes in substrate concentration (Ashby & Holmsen, 1983). Inhibition by P_i plus MgGTP became evident, however, when MgATP was lowered (Fig. 3). Enzyme activity decreased sigmoidically and was fully inhibited at 1 mM-MgATP. At 4 mM- P_i , half-maximal stimulation (A_{50}) was observed at 2 mM-MgATP. At higher phosphate concentrations, more MgATP was required to overcome inhibition by P_i plus MgGTP, and A_{50} was increased to approx. 3 mM-MgATP at 10 mM-phosphate (Fig. 3). Similar results were obtained at two substrate concentrations tested (50 μ M and 250 μ M; result not shown).

Subsequently, enzyme activity was studied as a function of MgGTP concentration either at 50 μ M-AMP and 3 mM-MgATP (Fig. 4a), or at 250 μ M-AMP and 1 mM-MgATP (Fig. 4b). Under either condition, enzyme activity was hardly affected by the inclusion of MgGTP up to 0.2 mM. A further increase to 0.4 mM-MgGTP resulted in only a moderate inhibition when tested at the high MgATP concentration (Fig. 4a). At 1 mM-MgATP, however, an abrupt fall in enzyme activity was observed when MgGTP was increased from 0.2 to 0.3 mM (Fig. 4b). Surprisingly, this MgGTP effect was independent of the P_i concentration in the assay mixture; in the absence of MgGTP, enzyme activity was already lower at 10 mM- P_i than at 4 mM- P_i , and this same difference in enzyme activity was found at all MgGTP concentrations tested (Figs. 4a and 4b). Full inhibition of enzyme activity by a physiological concentration of MgGTP (0.4 mM) was observed at 10 mM- P_i , 250 μ M-AMP and 1 mM-MgATP (Fig. 4b). Apparently MgGTP reinforced the inhibitory effect of P_i . This interpretation is strengthened by the data presented in Fig. 5, which shows that in the presence of 250 μ M-AMP and 1 mM-MgATP, addition of 0.4 mM-MgGTP led to a decrease in the I_{50} from approx. 15 mM-

Fig. 4. Inhibition of AMP deaminase activity by MgGTP

Enzyme activity was measured at either 4 mM- (●) or 10 mM-P_i (○), by [³H]IMP production at 50 μM-[³H]AMP and 3 mM-MgATP (a), or by ammonia production at 250 μM-AMP and 1 mM-MgATP (b). In (a), 0.3 μg of enzyme protein was tested in 100 μl of assay mixture, whereas in (b) 6.6 μg was tested in a final volume of 3 ml. Note the different scales for enzyme activity between panels (a) and (b). Data are representative for three independent experiments.

Fig. 5. Effect of MgGTP on P_i inhibition of AMP deaminase

Of the partially purified enzyme preparation, 1.9 μg of protein was added to 3 ml of assay mixture and enzyme activity was measured by ammonia production at 250 μM-AMP and 1 mM-MgATP at different concentrations of P_i in the absence (○) or presence (●) of 0.4 mM-MgGTP. In the absence of P_i, enzyme activity was 4.66 and 4.06 nmol/min in the absence and presence respectively of MgGTP. Data are representative for four similar experiments.

to 2 mM-phosphate. At 50 μM-AMP and 3 mM-MgATP, the effect of MgGTP was less pronounced, resulting in a half-optimal P_i concentration of approx. 8 mM (results not shown).

Comparison of kinetic data with studies on intact platelets

For platelets incubated in the presence of glucose, intracellular AMP and ATP concentrations are estimated at approx. 50 μM and 3 mM respectively (Holmsen & Robkin, 1980). At these concentrations, AMP deaminase

activity was not affected by either 4 mM-P_i or 0.4 mM-GTP alone, but with these effectors in combination the enzyme activity was reduced by one-third (Table 2). AMP formation, such as occurs in platelets upon challenge with H₂O₂, would therefore result in rapid deamination. The concurrent fall in GTP (Fig. 1c) would further enhance the rate of deamination (cf. Fig. 4). At 10 mM-P_i, which was arbitrarily chosen to reflect the situation in the starved platelets, AMP deaminase activity was reduced to approx. one-third of the control value (Table 2). As AMP increased, phosphate inhibition was lost. However, accumulation of AMP coincided with a decrease in cytosolic ATP. At 250 μM-AMP and 1 mM-MgATP, enzyme activity was again highly sensitive to physiological P_i concentrations, so that at 10 mM-P_i AMP deaminase activity was fully inhibited (cf. Fig. 4b). At this P_i concentration, the concurrent 40% fall in GTP (cf. Fig. 1c) would not result in marked reactivation of the enzyme (Fig. 4b).

DISCUSSION

In the present study, two novel observations have been made that may explain the differential regulation of AMP deaminase activity during starvation and H₂O₂ treatment of intact platelets. Firstly, intracellular P_i was 4–5-fold higher in the starved platelets than in either the refed or the H₂O₂-treated platelets (Fig. 1d). Deamination of AMP was slow when intracellular P_i was high, and rapid at low or normal P_i levels. Secondly, the sensitivity of partially purified platelet AMP deaminase to P_i inhibition was markedly increased by physiological concentrations of MgGTP (Figs. 4 and 5). In the absence of MgATP and P_i, the K_i for GTP was less than 10 μM (results not shown), which is in accordance with a previous report (Ashby & Holmsen, 1981). However, cytosolic GTP is probably complexed to Mg²⁺, but until now MgGTP has not been included in the kinetic analysis

Table 2. Combined effect of phosphate and MgGTP on AMP deaminase activity

Enzyme activity at 50 μ M- and 250 μ M-AMP was measured by [3 H]IMP and ammonia production respectively. Enzyme activity was expressed as a percentage of the activity measured with AMP and MgATP alone, which was 0.7 ± 0.3 nmol/min per ml of assay mixture at 50 μ M-AMP, 16 ± 6 nmol/min per ml at 250 μ M-AMP and 3 mM-MgATP, and 14 ± 3 nmol/min per ml at 250 μ M-AMP and 1 mM-MgATP. Data are means $\pm$ s.d. for the results obtained with four to six different enzyme preparations, except for 10 mM- P_i /0.4 mM-MgGTP where results from two separate experiments are given. *Significant differences with respect to the enzyme activity with AMP and MgATP alone, as determined by paired Student's *t* test ($P < 0.05$). Abbreviation: n.d., not detectable.

Additions	AMP... MgATP...	Enzyme activity (% of control)		
		50 μ M 3 mM	250 μ M 3 mM	250 μ M 1 mM
		(100)	(100)	(100)
4 mM- P_i		$96 \pm 2^*$	93 ± 8	$88 \pm 2^*$
0.4 mM-MgGTP		101 ± 2	96 ± 11	$76 \pm 5^*$
4 mM- P_i + 0.4 mM-MgGTP		$68 \pm 5^*$	91 ± 15	$23 \pm 8^*$
10 mM- P_i + 0.4 mM-MgGTP		29, 42	86, 92	n.d.

of platelet AMP deaminase. For our enzyme preparation an I_{50} of 0.1 mM was observed for MgGTP (results not shown). At 0.1–0.4 mM-MgGTP, enzyme activity was highly sensitive to phosphate inhibition at physiological P_i concentrations. When combined, the data obtained suggest that the inhibition of AMP deamination observed in starved platelets is mainly caused by the elevated P_i concentration.

We can now try to explain the differential effects of starvation and H_2O_2 treatment on adenylate metabolism in human platelets. In control platelets supplied with glucose, intracellular P_i is approx. 4 mM (Fukami *et al.*, 1980), and AMP deaminase is probably in the stimulated state (Table 2; Ashby & Holmsen, 1983). Nevertheless, the enzyme shows little activity as little IMP is formed (Fig. 1*b*). In these platelets, ATP consumption is balanced by rapid ATP regeneration, thus keeping the concentration of AMP low. In order to explain the complete absence of AMP deamination in this condition, one must assume that the intracellular AMP concentration is much lower than the presumed 50 μ M (Holmsen & Robkin, 1980). Alternatively, an as yet unidentified inhibitory mechanism might be involved. Upon starvation, AMP deaminase is shifted to the inhibited state owing to the fall in ATP and the increase in P_i (Fig. 1). As a consequence, very little IMP is formed despite massive accumulation of AMP. Nevertheless, because of this huge increase in substrate concentration, the enzyme shows more activity than in the control platelets, as illustrated by the higher rate of IMP formation (Fig. 1*b*). Refeeding restores ATP regeneration and AMP is reconverted to ATP through the adenylate kinase reaction (Akkerman & Gorter, 1980; Akkerman *et al.*, 1983). Simultaneously, intracellular P_i decreases sharply (Fig. 1*d*) due to its incorporation into glycolytic intermediates which accumulate (Verhoeven *et al.*, 1985). As a consequence, AMP deaminase returns to the stimulated state. Subsequent H_2O_2 addition induces the formation of AMP which is now rapidly deaminated (Fig. 1*b*).

During H_2O_2 treatment, ATP catabolism is not accompanied by P_i accumulation. Previously, H_2O_2 has been shown to induce a quantitative shift of phosphate from cytosolic ATP to glycolytic intermediates, notably fructose 1,6-bisphosphate and dihydroxyacetone phos-

phate (Verhoeven *et al.*, 1985). Depletion of the cytosolic adenylate pool through deamination of AMP was also observed upon treatment of platelets with platelet-activating agents (Akkerman *et al.*, 1984), cyanide (Akkerman *et al.*, 1984), fluoride (Murer *et al.*, 1981) and low doses of 2-deoxyglucose (Holmsen *et al.*, 1974; Holmsen & Robkin, 1980). In all of these conditions, ATP catabolism is accompanied by an unaltered or decreased rather than increased intracellular P_i concentration. These observations therefore support the hypothesis that the AMP deaminase activity is mainly determined by intracellular P_i .

Regulation of AMP deamination by P_i has also been described for a number of other cells, such as human erythrocytes (Bontemps *et al.*, 1986) and lymphoblasts (Matsumoto *et al.*, 1979), rat hepatocytes (Vincent *et al.*, 1982) and mouse ascites tumour cells (Sauer, 1978). In rat liver, fructose-induced ATP depletion was also accompanied by rapid deamination of AMP (Van den Berghe *et al.*, 1977). Activation of AMP deaminase was induced by a decrease in both P_i and GTP, with the largest contribution made by GTP. In rat heart myocytes, the flux through AMP deaminase was found to depend on the metabolic state of the cells just before ATP catabolism was initiated, with IMP formation favoured when respiring cells were de-energized (Altschuld *et al.*, 1987). In this case, AMP deamination coincided with a sharp reduction in cytosolic GTP, suggesting a crucial role for this nucleotide also in this cell type. Unfortunately, intracellular P_i was not measured (Altschuld *et al.*, 1987). In platelets, cytosolic GTP also fell during H_2O_2 treatment (Fig. 1*c*). However, this is not a sufficient explanation for the observed activation of AMP deaminase, since a similar decrease in GTP was also seen during starvation, when the enzyme was inactive (Fig. 1), and the inhibitory action of MgGTP on partially purified AMP deaminase was abolished in the presence of the other effectors (Table 2).

The role of AMP deamination in platelet energy metabolism remains unclear. When ATP utilization exceeds ATP regeneration, AMP is formed rapidly in the adenylate kinase reaction, the activity of which is high in platelets (Haslam & Mills, 1967). For some cell types, deamination of AMP is thought to enhance ATP re-

generation in glycolysis through ammonia activation of phosphofructokinase (Yoshino & Murakami, 1985). In platelets, AMP deamination parallels secretory responses, suggesting a role in energy metabolism coupled to secretion (Holmsen *et al.*, 1972). However, thrombin-induced platelet responses and activation of glycolytic flux were not disturbed when AMP deaminase was inhibited by coformycin (Ashby *et al.*, 1983). Deamination of AMP may also serve in the stabilization of the AEC, as has been shown for several tissues (Chapman *et al.*, 1976; Atkinson, 1977; Matsumoto *et al.*, 1979; Pradet & Raymond, 1983). AEC values around 0.9 appear crucial for normal platelet function (Holmsen & Robkin, 1977; Murer *et al.*, 1981; Akkerman *et al.*, 1983). Whereas aggregation and secretion responses remain unaffected by H_2O_2 treatment (Holmsen & Robkin, 1977; Verhoeven *et al.*, 1985), starvation leads to a gradual loss in platelet function in parallel with a lowering of AEC (Akkerman *et al.*, 1983).

Besides AEC, the phosphorylation potential $[ATP/(ADP \times P_i)]$ is an important determinant in the homeostatic regulation of cellular energy metabolism. Whereas AMP is the primary regulator in energy charge control, the role of P_i is emphasized by the phosphorylation potential. In some cell types AEC appears most important in coupling energy utilization and production (see above), whereas the phosphorylation potential appears more important in others (Erecinska & Wilson, 1978; Sussman *et al.*, 1980). Determination of the phosphorylation potential in platelets has been seriously hampered by the presence of intracellular P_i in metabolically active and inactive granule-stored pools (Fukami *et al.*, 1980). In the present study, this problem was bypassed by using radiolabelled P_i which is incorporated only into the metabolically active pool under the conditions used (Fukami *et al.*, 1980). Sauer (1978) suggested that in Ehrlich ascites tumour cells, both parameters may be linked by the dependence of AMP degradation on intracellular P_i concentration. In line with this, Reed (1976) reported on the close co-ordination of both regulatory parameters in rat liver and kidney. The present study reveals that this also holds for human platelets during starvation, refeeding and H_2O_2 treatment (Fig. 2). In platelets this close co-ordination may be effected by AMP deaminase. Until now, the AEC has been considered to be the most important factor of cellular homeostasis in platelets. In view of the present data, however, a possible role for the phosphorylation potential cannot be ruled out.

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REFERENCES

- Akkerman, J. W. N. & Gorter, G. (1980) *Biochim. Biophys. Acta* **590**, 107–116
- Akkerman, J. W. N., Gorter, G. & Sixma, J. J. (1978) *Biochim. Biophys. Acta* **541**, 241–250
- Akkerman, J. W. N., Gorter, G., Soons, H. & Holmsen, H. (1983) *Biochim. Biophys. Acta* **768**, 34–41
- Akkerman, J. W. N., Driver, H. A., Dangelmaier, C. A. & Holmsen, H. (1984) *Biochim. Biophys. Acta* **805**, 221–228
- Altschuld, R. A., Gamelin, L. M., Kelley, R. E., Lambert, M. R., Apel, L. E. & Brierley, G. P. (1987) *J. Biol. Chem.* **262**, 13527–13533
- Ashby, B. & Holmsen, H. (1981) *J. Biol. Chem.* **256**, 10519–10523
- Ashby, B. & Holmsen, H. (1983) *J. Biol. Chem.* **258**, 3668–3672
- Ashby, B., Wernick, E. & Holmsen, H. (1983) *J. Biol. Chem.* **258**, 321–325
- Atkinson, D. E. (1977) *Cellular Energy Metabolism and its Regulation*, Academic Press, New York
- Bontemps, F., Van den Berghe, G. & Hers, H.-G. (1986) *J. Clin. Invest.* **77**, 824–830
- Chaney, A. L. & Marbach, E. P. (1962) *Clin. Chem.* **8**, 130–132
- Chapman, A. G., Miller, A. L. & Atkinson, D. E. (1976) *Cancer Res.* **36**, 1144–1150
- Daniel, J. L., Molish, I. R. & Holmsen, H. (1980) *Biochim. Biophys. Acta* **632**, 444–453
- D'Souza, L. & Glueck, H. I. (1979) *Thromb. Haemostasis* **38**, 990–1001
- Erecinska, M. & Wilson, D. F. (1978) *Trends Biochem. Sci.* **3**, 219–223
- Fukami, M. H., Holmsen, H. & Salganicoff, L. (1976) *Biochim. Biophys. Acta* **444**, 633–643
- Fukami, M. H., Dangelmaier, C. A., Bauer, J. S. & Holmsen, H. (1980) *Biochem. J.* **192**, 99–105
- Harris, G. L. & Crawford, N. (1973) *Biochim. Biophys. Acta* **291**, 701–719
- Haslam, R. J. & Mills, D. C. B. (1967) *Biochem. J.* **103**, 773–784
- Holmsen, H. (1985) *Annu. Rev. Physiol.* **47**, 677–690
- Holmsen, H. & Robkin, L. (1977) *J. Biol. Chem.* **252**, 1752–1757
- Holmsen, H. & Robkin, L. (1980) *Thromb. Haemostasis* **42**, 1460–1472
- Holmsen, H., Day, H. J. & Setkowsky, C. A. (1972) *Biochem. J.* **129**, 67–82
- Holmsen, H., Setkowsky, C. A. & Day, H. J. (1974) *Biochem. J.* **144**, 385–396
- Holmsen, H., Dangelmaier, C. A. & Akkerman, J. W. N. (1983) *Anal. Biochem.* **131**, 266–272
- Lian, C.-Y. & Harkness, D. R. (1974) *Biochim. Biophys. Acta* **341**, 27–40
- Lowenstein, J. M. (1972) *Physiol. Rev.* **52**, 382–414
- Matsumoto, S. S., Raivio, K. O. & Seegmiller, J. E. (1979) *J. Biol. Chem.* **254**, 8956–8962
- Murer, E. H., Davenport, K., Siojo, E. & Day, H. J. (1981) *Biochem. J.* **194**, 187–192
- Ogasawara, N., Goto, H., Yamada, Y., Watanabe, T. & Asano, T. (1982) *Biochim. Biophys. Acta* **714**, 298–306
- Pradet, A. & Raymond, P. (1983) *Annu. Rev. Plant Physiol.* **34**, 199–224
- Reed, E. B. (1976) *Life Sci.* **19**, 1307–1322
- Sauer, L. A. (1978) *Cancer Res.* **38**, 1057–1063
- Slater, E. C. (1979) *Methods in Enzymol.* **55**, 235–245
- Solomon, H. M. & Gaut, Z. N. (1970) *Biochem. Pharmacol.* **19**, 2631–2638
- Spychala, J. & Marszalek, J. (1987) *Comp. Biochem. Physiol.* **B 88**, 1077–1082
- Spychala, J., Marszalek, J., & Kucharczyk, E. (1986) *Biochim. Biophys. Acta* **880**, 123–130
- Sussman, I., Erecinska, M. & Wilson, D. F. (1980) *Biochim. Biophys. Acta* **591**, 209–223
- Tysnes, O.-B., Verhoeven, A. J. M., Aarbakke, G. M. & Holmsen, H. (1985) *Thromb. Res.* **40**, 329–338
- Van den Berghe, G., Bronfman, M., Vanneste, R. & Hers, H.-G. (1977) *Biochem. J.* **162**, 601–609
- Verhoeven, A. J. M., Mommersteeg, M. E. & Akkerman, J. W. N. (1985) *J. Biol. Chem.* **260**, 2621–2624

Verhoeven, A. J. M., Tysnes, O. -B., Cook, C. A., Horvli, O. & Holmsen, H. (1987) *J. Biol. Chem.* **262**, 7047–7052
Vincent, M. F., Van den Berghe, G. & Hers, H.-G. (1982) *Biochem. J.* **202**, 117–123

Whitlock, D. M. & Terjung, R. L. (1987) *Am. J. Physiol.* **253**, C426–C432
Yoshino, M. & Murakami, K. (1985) *J. Biol. Chem.* **260**, 4729–4732

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