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THE KINETIC PROPERTIES OF ADENYLATE DEAMINASE FROM HUMAN ERYTHROCYTES

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SUMMARY

Adenylate deaminase (AMP aminohydrolase, EC 3.5.4.6) was purified 500-fold from the human erythrocyte by DEAE-cellulose adsorption and chromatography on hydroxylapatite. Its isoelectric point is 5.0 and the pH of optimal activity is near 7.0. Although the enzyme is markedly activated by ATP and by monovalent cations, it is able to deaminate AMP in the absence of activators. At pH 7 the order of effectiveness for activation by monovalent cations is $K^+ > Rb^+ > Li^+ > Na^+ > Cs^+$. The presence of ATP or changes in pH somewhat alter their relative activities but not the order. dATP activates the enzyme as effectively as ATP. ADP activates to a lesser degree and GTP is without effect.

The enzyme displays a cooperative effect with substrate and also with its activators, monovalent cations and ATP. The activators thus increase the apparent affinity of the eznyme for AMP without affecting the maximum reaction velocity. When both cation and ATP are added, the substrate–velocity curve is changed from a pronounced sigmoidal curve to a rectangular hyperbola. The apparent affinity for AMP increases as the concentration of cation is raised. Conversely, the affinity for cation increases when the concentration of AMP is elevated or ATP is added.

Halide anions are non-competitive inhibitors and decrease in their effectiveness as follows: $F^- > I^- > Br^- > Cl^-$. P_i and 2,3-diphosphoglyceric acid inhibit the enzyme competitively and ATP counteracts that inhibition. In the presence of 1 mM ATP and 100 mM KCl the K_i values for P_i and 2,3-diphosphoglyceric acid are between 4 and 11 mM.

INTRODUCTION

The erythrocyte of man contains a considerable quantity of the enzyme adenylate deaminase (AMP aminohydrolase, EC 3.5.4.6) which catalyzes the deamination of 5′-AMP to form IMP [1, 2]. Since the mature human erythrocyte is unable to synthesize purine nucleosides or their phosphate esters de novo from purine ring precursors or to convert IMP into AMP [3,4], the action of this enzyme results in the irretrievable loss of the adenine moiety to the cell. This enzyme may therefore play an important regulatory role in the adenine nucleotide metabolism of the red blood cell [5].

Previous studies on AMP deaminase from human erythrocytes have been carried out on crude hemolysates only [5–11]. Askari and co-worker [7, 8] described the allosteric nature of the enzyme and its activation by either K^+ or NH_4^+ alone or by Na^+, Li^+ and Rb^+ in the presence of ATP, properties that are somewhat different from the deaminase obtained from other sources [12–19]. They also demonstrated its inhibition by P_i and 2,3-diphosphoglyceric acid [5, 8], a compound present in uniquely high amounts in most mammalian erythrocytes, including those of man [20].

In an attempt more fully to understand the regulation of its activity within the human erythrocyte we have partially purified AMP deaminase from this source and have carried out detailed studies on its kinetic properties. In this paper we present the results of this work with particular emphasis upon the activation of the enzyme by monovalent cations and by ATP and its inhibition by phosphates and halides.

MATERIALS AND METHODS

Purine bases, nucleosides, nucleotides, AMP monohydrate and Tris–ATP were purchased from Sigma Chemical Co. and CalBiochem. Tris–AMP was prepared either by passage of the sodium salt of AMP over columns of Dowex-50 in the Tris form or by neutralization of the acid monohydrate with Tris base (Sigma). The various Tris–anion buffers were prepared by direct titration of Tris base with the reagent grade acid, e.g. H_2SO_4 or HNO_3. The Tris salt of 2,3-diphosphoglycerate was prepared by passing the cyclohexylammonium salt (CalBiochem) through Dowex-50 in the Tris form. Other chemicals and materials were obtained as follows: DEAE-cellulose (coarse mesh, 1 mequiv/g capacity), dithioerythritol and Tricine base from Sigma Chemical Co.; hydroxylapatite (BioGel HT) from BioRad Laboratories; ampholyte pH 3–6 (LKB8142) from LKB Inst.; Carbowax (polyethylene glycol 20 000) from Union Carbide Corp.; Nessler's reagent from Hartman–Leddon Co.; crystalline bovine serum albumin from Armour Lab. and β-mercaptoethanol from Eastman Organic Chemicals. Dipyridamole was a gift from Geigy Pharmaceutical Co. All other chemicals were of reagent grade. Quartz-distilled deionized water was used in preparing all reagents and during enzyme purification. DEAE-cellulose was washed successively with base, acid, and base before use [21].

Either freshly-drawn heparinized blood from normal persons or blood anticoagulated with acid–citrate–dextrose solution (NIH Formula I) obtained by phlebotomy from patients with secondary polycythemia was employed in these studies. The plasma and buffy coat were removed by aspiration after centrifugation, and the erythrocytes were washed three times with several volumes of 0.154 M NaCl at 4 °C.

Assay of enzyme activity

The enzyme was assayed by measuring the amount of ammonia formed as described by Setlow and Lowenstein [12]. For maximal activation of the enzyme, the reaction mixtures contained 2.5 mM AMP, 2.5 mM ATP, 75 mM KCl and 50 mM Tris–Cl^- buffer, pH 7.0, in a volume of 2.0 ml. The diffusion bottles containing the reaction mixtures were warmed to 37 °C and the reaction was initiated by the addition of enzyme. The reaction was terminated by the addition of 0.2 ml saturated K_2CO_3, and the diffusion bottles were immediately sealed with rubber stoppers fitted with glass rods which had been dipped in 0.25 M H_2SO_4. After mixing for 1 h at room

temperature in a multi-purpose rotator (Model 150 V, Scientific Ind., New York, N.Y.), the diffused ammonia was reacted with Nessler's reagent and the color read at either 490 or 430 nm. Ammonia standards and reagent blanks were included with each experiment. The colorimetric assay was linear between 0.05 and 1.5 μmoles of ammonia. The enzymatic liberation of ammonia was proportional to the time of incubation up to 60 min. In the kinetic studies velocity represents micromoles NH_3 produced in 15 min under the conditions described. All experiments were designed so that not more than 15% of the substrate was consumed. In studies of cation activation, product concentration was kept under 20% of the K^+ content and 10% of the Na^+ content used in the reaction in order to minimize the activation by NH_4^+. This required the use of larger incubation volumes in some cases. For the kinetic studies, just prior to use the enzyme was dialyzed for 24 h against two changes of 10 mM Tris–Cl^- buffer, pH 7.0, which contained 1 mM β-mercaptoethanol.

One unit of enzyme is defined as that amount which produces one micromole NH_3 in one minute under conditions of maximal activation. Specific activity is expressed as units per milligram protein. Protein was assayed by the method of Lowry et al. [22] using bovine serum albumin as standard. Na^+ and K^+ were measured in an Eppendorf flame photometer.

Isoelectric focusing

Isoelectric focusing was performed according to the method of Vesterburg and Svensson [23].

Purification of adenylate deaminase from human red blood cells

Washed erythrocytes were suspended in 3 vol. of 3 mM sodium phosphate buffer, pH 7.0, containing 1 mM β-mercaptoethanol, and lysed by freezing and thawing twice. It was not necessary to remove the stroma before proceeding. The hemolysate usually possessed from 3 to 4 units of enzyme activity per ml of cells. All subsequent steps were carried out at 0–4 °C.

Batch adsorption on and elution from DEAE-cellulose

Following the method of Hennessey et al. [24] 100 ml of DEAE-cellulose (about 10 g dry weight) was added to 400 ml of hemolysate and the mixture mechanically stirred for 30 min. The unadsorbed material in the supernatant fraction, which contained most of the hemoglobin, was discarded after centrifugation for 15 min at 2000 $\times$ *g*. The adsorbent was washed with successive aliquots of the 3 mM phosphate buffer (totalling approx. 1200 ml) and filtered. It was further washed with the same phosphate buffer containing 50 mM KCl until the filtrate was colorless (approx. 600 ml). The adsorbed protein was then eluted from the DEAE-cellulose by stirring with 200 ml 3 mM sodium phosphate buffer, pH 7.0, containing 350 mM KCl. The eluate was collected after centrifugation and this step was repeated. The combined eluates were centrifuged for 15 min at 16 000 $\times$ *g* to remove any remaining adsorbent or stroma.

Chromatography on hydroxylapatite

Hydroxylapatite was suspended in 5 mM potassium phosphate buffer, pH 7.0, and packed in a 1 cm $\times$ 25 cm column. The undialyzed DEAE-cellulose eluate was applied to the column at a speed of 0.5 ml/min and the column was washed with

250 ml of 0.15 M phosphate buffer, pH 7, containing 1 mM β-mercaptoethanol. The enzyme was eluted with a linear gradient composed of 250 ml of 0.15 M phosphate buffer, pH 7, in the mixing chamber and 250 ml of 0.5 M phosphate buffer, pH 7, in the reservoir, both containing 1 mM β-mercaptoethanol. The fractions containing deaminase activity were pooled, placed in a dialysis bag, and concentrated with Carbowax to 1–2 units of enzyme activity per ml. 1 mM dithioerythritol was added and the enzyme was stored at −20 °C. These two steps produced a 300–800-fold purification of the enzyme in different preparations. The results of a typical purification are given in Table I. Because of the instability of the partially purified enzyme, attempts at further purification were unsuccessful.

TABLE I

PURIFICATION OF AMP DEAMINASE FROM HUMAN ERYTHROCYTES

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Hemolysate	33 440	349	0.01	100	—
DEAE-cellulose eluate	439	330	0.75	95	75
Hydroxylapatite chromatography	31.4	179	5.70	51	570

RESULTS

Stability

The enzyme was unstable and lost most of its activity in seven days both in low and high concentrations of Tris–Cl^- and KCl at 4 °C. High concentration of inorganic phosphate, i.e. 0.25–0.5 M, between pH 6 and 8, was found to protect the enzyme and further protection was afforded by the addition of 1 mM dithioerythritol and 10 mM ATP. Under these conditions the enzyme was also more stable to heating at 50 °C; more than 90% of the activity was retained after storage at −20 °C for up to four months. AMP, glycine, sucrose and glycerol had little effect upon enzyme stability.

Isoelectric point

The isoelectric point of the purified AMP deaminase is 5.0.

Effect of pH

The pH of optimal activity using Tris–Cl^- or Tricine–Cl^- buffer is between 6.5 and 7.2. When 1 mM ATP is added, the plateau of the pH optimum is shifted to a somewhat higher pH (6.7 to 7.5).

Substrate specificity and activators

The enzyme is highly specific for 5′-AMP. Even under conditions of maximal activation with K^+ and ATP, the following are not deaminated; adenine, adenosine, 5′-dAMP, 3′-AMP, ADP, ATP, guanosine, 5′-GMP, DPN and TPN.

The purified enzyme does not have an absolute requirement for either ATP or cations; 5'-AMP without activators is deaminated. Addition of monovalent cation (K^+, Rb^+, Na^+ or Li^+), ATP, dATP or ADP increases the rate of the reaction without affecting the maximum reaction velocity, whereas increased concentration of Tris ion does not have any effect. When both cation and ATP are present, the rate of the reaction is markedly enhanced at low substrate levels beyond that obtained with either cation or ATP alone and the substrate–velocity curve is changed from the typical "allosteric" or sigmoidal shape to a rectangular hyperbola. The effect of activation by cation and ATP appears to be synergistic. At very high substrate levels (30 mM AMP) the activity without activators approaches the maximal activity of the fully activated enzyme. Hill plots [25] as shown in Fig. 1 give a value of 2.7 for n with respect to

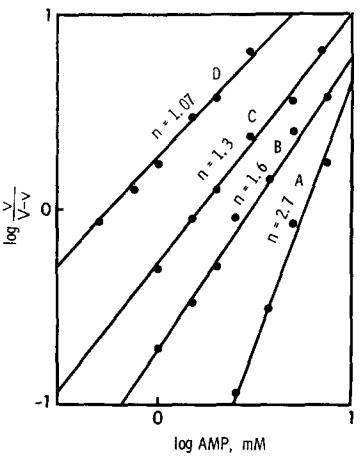

Fig. 1. Hill plot of adenylate deaminase activity in the presence and absence of activators. The reaction mixtures contained 50 mM Tris–Cl^- buffer, pH 7.0, variable concentrations of Tris–AMP and: (A) no addition, (B) 100 mM NaCl, (C) 100 mM KCl and 1 mM KCl, and (D) 100 mM KCl and 1 mM ATP. V represents the velocity in the presence of 100 mM KCl, 2.5 mM ATP, and 5 mM AMP and v represents velocity under experimental conditions described above.

AMP in the absence of activators, whereas with maximal activation, n approaches 1.

In what follows we shall use the term "apparent affinity constant" (K_{app}) after Setlow and Lowenstein [12], for the concentration of substrate which yields one-half of the maximum reaction velocity even under conditions when the enzyme does not follow Michaelis–Menten kinetics. The K_{app} for AMP in the presence of 100 mM KCl without ATP is 1.2 mM; when 1 mM ATP is added, the K_{app} is lowered to 0.2–0.8 mM.

Effects of monovalent cations

In contrast to studies with crude hemolysates [7], alkali metals other than K^+ activate the purified enzyme in the absence of ATP (Table II). At pH 7, Rb^+ is as effective as K^+, whereas Li^+ and Na^+ produce less of an effect, and Cs^+ has almost none. Addition of ATP increases the activation by Li^+ somewhat more than that by Na^+. Changes of pH do not affect the order of activation by monovalent cations but

TABLE II

THE EFFECT OF MONOVALENT CATIONS UPON ADENYLATE DEAMINASE

Reaction mixtures contained 100 mM Tris-Cl^- buffer at the indicated pH, 100 mM cation as the chloride salt, and 2.5 mM AMP. Activity is expressed as percentage of the activity observed with K^+ at the respective pH.

	pH 8.2 no ATP (%)	pH 7.0 no ATP (%)	pH 5.3 no ATP (%)	pH 7.0 2.5 mM ATP (%)
Li^+	76	83	76	94
Na^+	42	82	72	87
K^+	100	100	100	100
Rb^+	70	94	99	99
Cs^+	7	2	2	11
none	2	3	2	8

do slightly change their relative activity. The relative activity of Rb^+ increases as the pH is lowered; Na^+ and Li^+ have higher activities at pH 7.0.

Apparent affinities for AMP and alkali cations are interdependent and increase to almost the same degree upon the addition of ATP. As the concentration of substrate is raised, the apparent affinity for K^+ or Na^+ increases proportionally. Conversely, when the concentration of cation is raised, the apparent affinity for AMP also increases proportionally. For instance, at 2 mM AMP without ATP, the K_a (in this paper we use K_a to refer to the concentration of activator to achieve half-maximal stimulation) for K^+ is between 5 and 10 mM; at 2 mM AMP and 1 mM ATP, it is 0.8 mM; at 4 mM AMP and 1 mM ATP, it is 0.4 mM (Fig. 2).

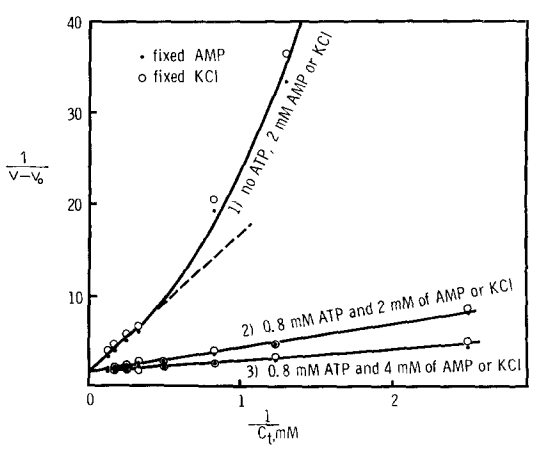

Fig. 2. Equivalence of apparent affinities of adenylate deaminase for monovalent cations and AMP. These studies were carried out at low concentrations of Tris–AMP containing 50 mM Tris-Cl^- buffer, pH 7. C_t denotes the total concentrations of either monovalent cation or AMP. v represents the velocity in the presence of cations and v_0 represents the small velocity with AMP alone in the absence of cations. Additions as noted in the figures were: (1) 2 mM AMP with variable concentrations of KCl or 2 mM KCl with variable concentrations of AMP without ATP; (2) the same as (1) but with 0.8 mM ATP; (3) 0.8 mM ATP and 4 mM AMP with variable concentrations of KCl or 4 mM KCl with variable concentrations of AMP.

Effects of anions

In the presence of various anions and 100 mM K^+ or Na^+ at pH 7, with 2.5 mM or no ATP, the AMP deaminase activity decreased in the following order: acetate > SO_4^{2-}, citrate, Cl^- > Br^- > NO_3^- > I^- > PO_4^{3-} > F^-. The relative activity of potassium and sodium salts of different anions is almost the same. Therefore, the effect of anions seems to be independent of cations. The velocity–concentration relationship of SO_4^{2-}, Cl^-, and NO_3^- were further studied as shown in Fig. 3.

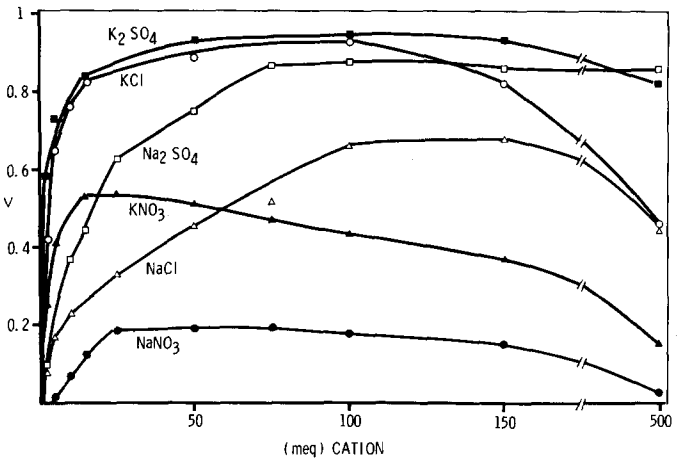

Fig. 3. The effect of various concentrations of sodium and potassium salts upon AMP deaminase activity. The reaction mixtures contained 50 mM Tris–anion, pH 7.0, 2.5 mM AMP and the concentrations of sodium and potassium salts indicated.

These data demonstrate that: (a) There is marked inhibition at the higher concentrations of all six salts, presumably related to the anion. Enzyme activity in the presence of NO_3^-, either as the potassium or sodium salt, decreases at concentrations above 150 mM. With SO_4^{2-} the activity remains high until more than 500 mequiv of salt is added. (b) In the absence of ATP, double reciprocal plots of these data result in lines which curve upward as $1/c$ increases, suggesting that the cation as well as AMP has a cooperative effect upon the enzyme. (c) The rates of ascent of the curves are related to cation concentration, the rise with potassium salts being steeper than that with sodium salts. (d) The maximal activity at 2.5 mM AMP is not only related to the species of cation present, but is also dependent upon the particular anion. The velocities observed are therefore undoubtedly a result of both cation activation and anion inhibition.

Inhibition by monovalent anions

F^- has been reported to inhibit rabbit skeletal muscle adenylate deaminase [26]. As shown in Fig. 4, F^- also inhibits the enzyme from the erythrocyte. This inhibition is non-competitive with substrate and is not significantly influenced by the addition of ATP. In the absence of ATP, the K_i for F^- is 2.4 mM whereas with 1 mM ATP the K_i is 1.75 mM. I^- also inhibits the enzyme non-competitively. The K_i for I^- in the presence of 1 mM ATP is 75 mM.

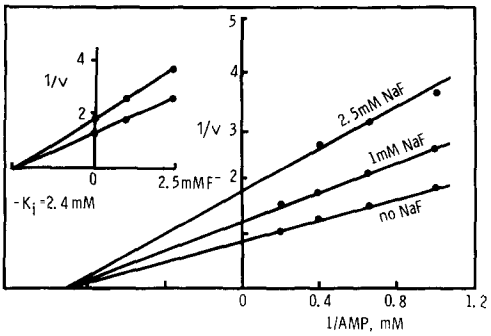

Fig. 4. Inhibition of adenylate deaminase by F^-. The reaction mixtures contained 50 mM Tris–Cl^- buffer, pH 7.0, 100 mM KCl, 1 mM ATP and various concentrations of AMP and NaF.

Inhibition of adenylate deaminase by phosphates

P_i, PP_i, and 2,3-diphosphoglycerate all inhibit the activity of adenylate deaminase, but the sensitivity of the enzyme to this inhibition varied in different enzyme preparations (Table III). The enzyme regularly lost much of its sensitivity to phosphate inhibition after hydroxylapatite chromatography, freezing or prolonged dialysis. Therefore, enzyme purified only through the DEAE-cellulose step was used for studies on the inhibition by these compounds. Besides 2,3-diphosphoglycerate, intracellular organic phosphates have little or no effect. Addition of ATP diminishes the inhibition by 2,3-diphosphoglycerate (Fig. 5A). The inhibition by 2,3-diphosphoglycerate is competitive with respect to AMP in the presence (Fig. 5B) and absence (not shown) of ATP. Inhibition of AMP deaminase by P_i is similar to that by 2,3-diphoshpoglycerate. In the presence of 1 mM ATP and 100 mM KCl, the K_i for both 2,3-diphosphoglycerate and P_i is between 4 and 11 mM.

TABLE III

THE EFFECT OF P_i, PP_i AND 2,3-DIPHOSPHOGLYCERATE UPON THE ACTIVITY OF ADENYLATE DEAMINASE AT VARIOUS STAGES OF PURIFICATION

The reaction mixtures contained 50 mM Tris–Cl^- buffer, pH 7, 75 mM KCl, 2.5 mM AMP, and 5 mM 2,3-diphosphoglycerate, 2.5 mM potassium phosphate or 2.5 mM potassium pyrophosphate in the presence or absence of 2.5 mM ATP. The activity is expressed as percentage of the velocity without inhibitors.

	2,3-Diphosphoglycerate		P_i		PP_i	
	No ATP (%)	2.5 mM ATP (%)	No ATP (%)	2.5 mM ATP (%)	No ATP (%)	2.5 mM ATP (%)
Hemolysate	37	91	39	78	53	96
DEAE eluate	52	95	39	80	27	93
Hydroxylapatite chromatography	69	93	93	100	86	103

Effect of divalent cations

None of the heavy metals tested stimulated enzyme activity. Mg^{2+}, Ca^{2+} and Ba^{2+} had little effect on adenylate deaminase, while Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+} and Ni^{2+} inhibited the enzyme.

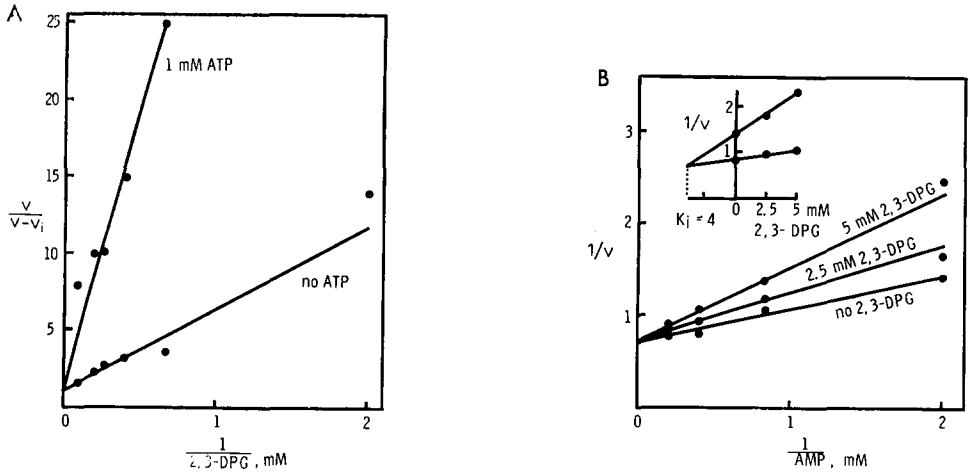

Fig. 5. Inhibition of adenylate deaminase by 2,3-diphosphoglycerate (2,3-DPG). (A) The reaction mixtures contained 50 mM Tris–Cl^- buffer, pH 7.0, 100 mM KCl, 2.5 mM AMP and various concentrations of 2,3-diphosphoglycerate with 1 mM or no ATP. (B) The reaction mixtures contained 50 mM Tris–Cl^- buffer, pH 7.0, 100 mM KCl, 1 mM ATP and various concentrations of AMP with 0, 2.5 or 5 mM 2,3-diphosphoglycerate. v_i represents velocity in the presence of inhibitor and v the velocity in its absence.

Activation of nucleoside polyphosphates

The effects of 1 mM nucleoside polyphosphates on the activity of adenylate deaminase were tested in the presence of 75 mM KCl. The enzyme is equally activated by ATP and dATP whereas CTP, GTP, UTP and ITP as well as DNP and TPN are without effect both in the presence and absence of ATP. ADP stimulates the deaminase approximately half as much as ATP or dATP, although presence of adenylate kinase in the partially purified enzyme was not excluded. The reciprocals of the increment increase in velocity resulting from added ATP plotted against the reciprocal of the ATP concentration at 1, 2.5 and 5 mM AMP in the absence of cations (Fig. 6) reveals:

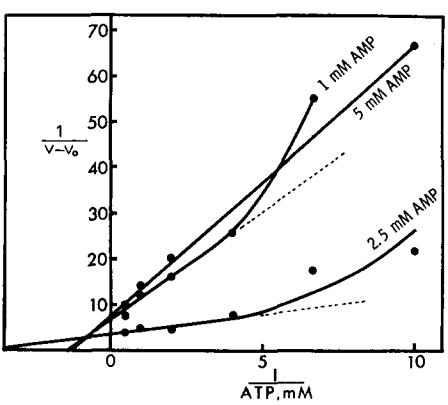

Fig. 6. The effect of AMP upon the activity of adenylate deaminase as a function of ATP concentration. Tris salts of AMP and ATP were used and each reaction mixture contained 100 mM KCl and 50 mM Tris–Cl^- buffer, pH 7.0. The velocity v represents NH_3 production in the presence of ATP and v_0 is the velocity with no ATP added.

(1) The K_a for ATP varies with AMP concentration: 0.19 mM at 1 mM AMP, 0.17 mM at 2.5 mM AMP and 2 mM at 5 mM AMP; (2) ATP has a cooperative effect on AMP deaminase as is indicated by the upward curvature at the right of the graph which is intensified at lower substrate levels. When 100 mM KCl is added, the double reciprocal plot of the increased velocity due to ATP and the ATP concentration becomes a straight line (Fig. 7).

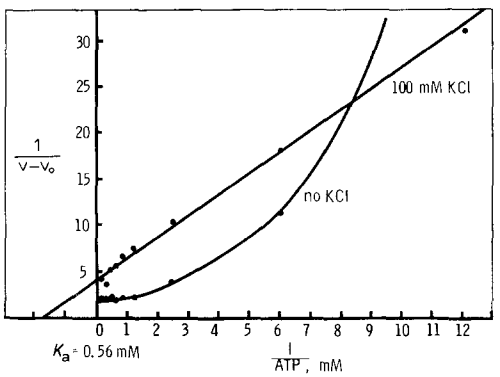

Fig. 7. The effect of KCl upon adenylate deaminase activity as a function of ATP concentration. The reaction mixtures contained 50 mM Tris–Cl^- buffer, pH 7.0, 2.1 mM AMP, and variable concentrations of ATP, with and without KCl. v and v_0 represent the velocity with and without ATP, respectively.

Inhibition by nucleoside monophosphates

In the absence of ATP, the enzyme is slightly inhibited by 2.5 mM of 5′-IMP, 2′-AMP and 3′-AMP. When 2.5 mM ATP is added, the inhibition by 5′-IMP and 2′-AMP is eliminated but not that of 3′-AMP. Double reciprocal plots of velocity against 5′-AMP concentration at several concentrations of 3′-AMP demonstrated that the inhibition is competitive with substrate. The K_i for 3′-AMP is 3.2 mM in the absence of ATP, and 1.3 mM in the presence of 2.5 mM ATP. Experiments with 1 to 2.5 mM AMP and various concentrations of dAMP (up to 10 mM) were carried out and no effect of dAMP on this enzyme was noted. Other nucleoside monophosphates such as 5′-GMP and 2′, 3′-cyclic AMP also had no effect on enzyme activity.

Lack of inhibition by nucleosides, purines and dipyridamole

Adenine, adenosine, inosine, guanine, and dipyridamole have been used as additives to help maintain the adenine pool in stored blood. These compounds were examined to ascertain whether or not their beneficial effect might result from inhibition of adenylate deaminase. Both in the presence and absence of ATP at concentrations up to twice substrate concentration, adenosine, inosine, guanine and adenine had no effect upon enzyme activity. There was also no demonstrable effect by 0.25 mM dipyridamole.

DISCUSSION

Adenylate deaminases from rabbit muscle [15], calf brain [12], rat liver [18] and crude hemolysates of human erythrocytes [8, 10] have been demonstrated to be allo-

steric enzymes with a cooperative effect of substrate upon enzyme activity. In this paper we have demonstrated that, when purified, the deaminase from erythrocytes not only displays this cooperative effect between the enzyme and substrate but also with respect to its activators. This indicates that the enzyme contains two or more allosteric sites in addition to catalytic sites [27–29].

In contrast to the results reported by Askari and Franklin [7] but in agreement with Razin and Mager [10], we found that AMP is deaminated in the absence of activators. The velocity–substrate concentration curve shows a high degree of sigmoidicity. This cooperative effect could be achieved either by the binding of substrate at the catalytic site with subsequent conformational change in the enzyme to facilitate binding of the next substrate molecule at other catalytic sites, or by the binding at the allosteric site where ATP is bound. The latter possibility appears attractive since ADP, like ATP, can activate the enzyme, and in view of their similar structures it is reasonable to postulate that AMP might also activate the enzyme at a common allosteric site. There is, however, some evidence to suggest that this is not the case since dAMP is not a substrate, inhibitor or activator for the enzyme whereas dATP is equally as effective as ATP as an activator.

Askari reported that activation of the deaminase in red cell hemolysates required the presence of a monovalent cation [8]. In contrast we have found that the partially purified enzyme is activated by ATP in the absence of cations. This is in agreement with the observation by Razin and Mager [10]. Although the Lineweaver–Burk plot shows a "competitive type" activation by ATP, the binding sites of ATP and AMP are definitely different. The presence of a specific allosteric site for ATP is also evidenced by the observation that ATP protects the enzyme against heat denaturation and prolongs the stability of the enzyme at 4 °C whereas AMP does not. The competitive inhibitory effect of GTP upon activation by ATP described for adenylate deaminases of some other tissues was not observed by us [13, 18, 30, 31].

Our findings on the kinetic behavior of adenylate deaminase suggest certain similarities to those of creatine phosphokinase as described by Askonas [32] and Kuby et al. [33]. In that case, the apparent affinity for substrate is dependent upon the concentration of divalent cation (Mg^{2+} or Mn^{2+}) and the affinity for the cation is dependent upon the concentration of substrate. Combination of substrate and cation is apparently obligatory before interaction with enzyme. With red cell adenylate deaminase, apparent affinities for both cation and AMP are lowered to the same degree upon addition of ATP. The apparent affinity for cation (or AMP) is proportional to the concentration of substrate (or cation) present in the reaction mixture whereas the V remains unchanged. It is therefore probable that the activation of adenylate deaminase by monovalent cations is achieved through the formation of an AMP–cation complex. The interaction between the AMP–cation complex and the enzyme is not an exclusive one since at higher concentrations AMP alone will bind to the enzyme.

Melchior [34] studied the sodium- and potassium-coordinated complexes of ATP and ADP and reported that Na^+ is completely buried in the molecular complex but, in contrast, K^+ is almost completely exposed. Kennard et al. [35] analyzed the X-ray crystallographic structure of Na-ATP and found that two sodium atoms are each bound to five phosphate oxygen atoms and the nitrogen atom in position 7 of the adenine ring. AMP may bind these cations in a somewhat similar manner. This binding to the ring nitrogen would pull electrons away from the carbon atom in position 6

facilitating hydroxylation with subsequent deamination. AMP possesses some degree of monovalent cation-binding specificity [36]. The monophosphate nucleotide complex with different monovalent cations might, like the ATP–cation complex [37], have different charge-steric shape relationships which further influence its ease of binding to the enzyme. The anomalous sequence of activation by monovalent cations observed here may result from a combination of cation selectivity by AMP and the affinity of the enzyme for the complex.

As pointed out by Stadtman [28], the "competitive type" kinetics in allosteric enzymes do not necessarily reflect the capacity of the inhibitor to compete with the substrate for a catalytically active site on the enzyme. Since orthophosphate, pyrophosphate and 2,3-diphosphoglycerate contain phosphate groups as do both substrate (AMP) and activator (ATP), it is hard to predict whether phosphates compete directly with AMP at a catalytic site or indirectly by binding to the allosteric site, changing the conformation of the protein to a form with a lowered affinity for the substrate. Another possibility is that phosphate esters and P_i may compete with AMP for Na^+ or K^+; Smith and Alberty [36] have shown, for instance, that P_i binds cations more tightly than AMP. The inhibition by phosphates is antagonized by ATP, suggesting that phosphates may compete with ATP at the allosteric site. However, the sensitivity of the enzyme to phosphate inhibition is diminished by prolonged dialysis or passage over a hydroxylapatite column whereas the affinities for ATP and AMP remain unchanged. Furthermore, phosphates do not protect the enzyme against heat denaturation as does ATP. It seems probable, therefore, that the binding site for phosphate to adenylate deaminase is different from those for ATP and for AMP. Therefore, the antagonistic action of phosphates and ATP may be a consequence of counteracting effects on enzyme conformation that are induced by the binding of activator and inhibitor at separate allosteric sites. Such an interpretation implies that adenylate deaminase from human erythrocytes has at least four different binding sites: at least one each for AMP or AMP–cation complex, ATP, phosphate or 2,3-diphosphoglycerate, and monovalent anions.

Conway and Cooke [1] suggested that in the red blood cell adenylate deaminase is reversibly bound to some inhibitory substance. Askari and Rao [5] reported its inhibition by 2,3-diphosphoglycerate and we have confirmed this observation. At the concentrations of K^+, ATP, AMP and 2,3-diphosphoglycerate prevailing within the normal erythrocyte we estimate that adenylate deaminase is inhibited between 25 and 50%. Even at 50% activity there is sufficient adenylate deaminase in human red blood cells to convert 1.5 μmoles of AMP into IMP per hour per milliliter of cells. This is far in excess of the in vivo rate of turnover of ATP measured by Lowy et al. [38] and Mager et al. [39].

There is evidence from the clinical literature which suggests that 2,3-diphosphoglycerate may not be an important in vivo regulator of adenylate deaminase. Pathologic increase or decrease of 2,3-diphosphoglycerate in erythrocytes is not necessarily accompanied by elevation or reduction of ATP [40–43]. One must therefore conclude that either there are other or additional factors which regulate the activity of adenylate deaminase or that 2,3-diphosphoglycerate, by its effects on this enzyme actually plays a minor role in regulation of adenine nucleotide metabolism in the red blood cell.

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