RABBIT SKELETAL MUSCLE 5'-AMP AMINOHYDROLASE: SOME PHYSIOCHEMICAL PROPERTIES AND CHARACTERIZATION AS A ZINC METALLOENZYME

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ABSTRACT

RABBIT SKELETAL MUSCLE 5'-AMP AMINOHYDROLASE: SOME PHYSIOCHEMICAL PROPERTIES AND CHARACTERIZATION AS A ZINC METALLOENZYME

By Carol Louise Zielke

Rabbit skeletal muscle AMP aminohydrolase [EC 3.5.4.6], purified to homogeneity by adsorption of the enzyme from a curde extract to cellulose phosphate and elution with 1 M KCl, was characterized with respect to a) kinetic parameters for substrates, activators, and inhibitors, b) physical properties, and c) metal cofactor requirement.

The enzyme deaminated not only AMP and dAMP but also N^6-methyl AMP, adenosine 5'-phosphoramidate, adenosine 5'-monosulfate, ADP, adenosine, formycin 5'-monophosphate, N^6-ethyl AMP, AmDP and α,β-methylene ADP. The kinetic parameters were obtained for all but the last four. The pH optima for ADP, adenosine, and N^6-methyl AMP deamination were 5.0-5.5, 6.5-6.75 and 6.4-6.6, respectively. Fourteen additional analogs were not deaminated. Data are presented which are consistent with one enzyme being responsible for deamination of adenosine, AMP, and ADP. 3'-AMP, 3',5'-cyclic AMP, and 3-iso AMP, although not deaminated, were inhibitors of AMP aminohydrolase. Inhibition by 3-iso AMP

was linear competitive for AMP (K_i = 32 μM); inhibition of dAMP deamination was complicated by nonlinear kinetics. IDP and 6-mercaptopurine 5'-ribonucleotide activated the enzyme; the concentration of IDP required for 50% activation was 100 times that required for activation by ADP.

The absorbance coefficient for AMP aminohydrolase at 280 nm was 9.15 OD/mg protein on a dry weight basis. A molecular weight of 278,000 for the native enzyme was determined by sedimentation equilibrium centrifugation. From a subunit molecular weight of 69,000 obtained upon denaturation of the enzyme in sodium dodecyl sulfate and mercaptoethanol followed by electrophoresis on sodium dodecyl sulfate impregnated polyacrylamide gels, it was concluded that AMP aminohydrolase consists of 4 subunits of identical molecular weight.

Enzyme assayed in the absence of activators or in the presence of the activator ADP was strongly inhibited by di- and tricarboxylic acids; the K^+-activated enzyme was less sensitive to such inhibition. Limited kinetic analysis was consistent with a competitive interaction between citrate (K_i = 0.11 mM) and ADP. Metal binding agents inhibited the enzyme as a function of time. The data suggest that AMP aminohydrolase contained a bound metal cofactor.

A total of 2.58 gram atoms zinc per 278,000 grams protein was detected upon atomic absorption analysis of the purified enzyme: magnesium, iron, calcium, and cobalt (neutron activation analysis) were not observed in stoichiometrically significant quantities. Zinc was required for enzymatic activity as demonstrated by the loss of enzymatic activity concurrent with the formation of Zn [8-hydroxyquinoline 5-sulfonate]$_3$ following titration with the chelator. The removal of 3 gram atoms zinc per mole enzyme decreased activity by greater than 90%. Apoenzyme, obtained by incubation in 8-hydroxyquinoline 5-sulfonate followed by chromatography on Sephadex G-25, contained less than 0.5 gram atom zinc as shown by atomic absorption analysis. The addition of zinc, cobalt, iron, and manganese sulfate but not nickel, copper, cadmium, or magnesium sulfate reactivated the enzyme. High concentrations of zinc (the very ion required for activity), copper, cadmium, and nickel in the assay inhibited enzymatic activity.

A preliminary investigation of the kinetic parameters of the zinc, cobalt, and manganese reconstituted enzyme was made: both K_m and V_{max} for AMP were affected. Although enzyme containing 2.58 gram atoms zinc and 4 gram atoms zinc exhibited similar K_m and V_{max} values for AMP in KCl, the relative velocity of the ADP activated enzyme containing less than 4.0 gram atoms of zinc per mole enzyme was less than that observed for enzyme reconstituted with 4.0 gram atoms zinc per mole enzyme. The data suggest that there are two different classes of zinc in AMP aminohydrolase.

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By

Carol Louise Zielke

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Dedicated to my husband and my parents

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ABBREVIATIONS

AMP	adenosine 5'-monophosphate
3'-AMP	adenosine 3'-monophosphate
2'-AMP	adenosine 2'-monophosphate
ADP	adenosine 5'-diphosphate
α,β-methylene ADP	α,β-methylene adenosine 5'-diphosphonate
AmDP	2'-O-methyladenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
AMP-NH_2	adenosine 5'-phosphoramidate
dAMP	2'-deoxyadenosine 5'-monophosphate
GMP	guanosine 5'-monophosphate
GDP, GTP	guanosine 5'-di, and triphosphate
CMP	cytidine 5'-monophosphate
3-iso AMP	3-β-D-ribofuranosyladenine-5'-phosphate
TMACl	Tetramethylammonium chloride
Tris	Tris(Hydroxymethyl)aminomethane
8-OHQ5SA	8-hydroxyquinoline-5-sulfonic acid
EDTA	Ethylenediaminetetraacetic acid
Cacodylic	Dimethylarsinic acid
Mes	2(N-Morpholino)ethane sulfonic acid
DTE	dithioerythritol
TCA	trichloroacetic acid
OP	ortho-phenanthroline or 1,10 phenanthroline
SDS	sodium dodecyl sulfate

OD	optical density or absorbance
K_m	the concentration of substrate at which the reaction rate is one half V_{max}
V_{max}	the rate of catalysis at saturating substrate
n_H	Hill slope
K_i	the dissociation constant for the enzyme inhibitor complex
$K_{a_{app}}$	the concentration of activator required for 50% activation
K_f	formation constant for metal ligand complex defined by the relationship: $K_f = [ML]/[M][L]$.

INTRODUCTION

The substrate specificity, activation and inhibition, physical properties, and requirement for a metal cofactor have been reinvestigated for rabbit skeletal muscle AMP aminohydrolase. This study was prompted by the observation that the enzyme purified by the method of Smiley _et al_ (1) exhibited sigmoidal substrate saturation curves in the absence of K^+, was activated by K^+, ADP, and ATP, and was inhibited by GDP and GTP. None of these phenomena were observed with the enzyme as purified by Lee (2) although Lyubimova and Matlina (3) observed activation of the skeletal muscle enzyme by ADP and ATP as early as 1954. This thesis points out additional differences between the two preparations of rabbit muscle AMP aminohydrolase.

LITERATURE REVIEW[1]

Distribution

5'AMP aminohydrolase [EC 3.5.4.6] catalyzes the deamination of adenylic acid to inosinic acid and ammonia. In 1928 Schmidt demonstrated that two different enzymes were responsible for deamination of AMP and adenosine in muscle extracts (4). Furthermore the enzyme responsible for the presence of IMP in muscle specifically deaminated muscle adenylic acid (5'-AMP) and not yeast adenylic acid (3'-AMP). This was the first indication that muscle and yeast adenylic acid were chemically different (5).

Although skeletal muscle remains the single best source of AMP aminohydrolase, the enzyme occurs widely throughout nature. In addition to those sources enumerated by Lee in his review (6), AMP aminohydrolase activity has been observed in the following organisms and tissues: reptiles (7), erythrocytes (8), snail (9), unfertilized fish eggs (10), invertebrates (11), and the particulate fraction of pea seeds (12). The skeletal muscle enzyme occurs in both the sarcoplasm (13) and the microsomes (6);

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[1] The Literature Review presented here will appear as part of a Chapter on "Purine, Purine Nucleoside, and Purine Nucleotide Aminohydrolases" by C. L. Zielke and C. H. Suelter in The Enzymes, 3rd edition, Vol. 2, P. D. Boyer, Editor, New York: Academic Press, (1970) in press.

frog muscle enzyme is located within or just beneath the sarcolemma of the muscle bundle (14). In heart tissue activity occurs in the cytoplasm (15), in microsomes, heavy mitochondria and myofibrils (16). AMP aminohydrolase activity has been found in most areas of the rat brain (17); the ATP activated enzyme occurred in both structural as well as soluble fractions of the cell (18). At least 15% of the erythrocyte enzyme is bound to the red cell membrane (19). However in the kidneys and gills of fresh water fish, AMP aminohydrolase activity occurred only in the cytoplasmic fraction (20).

Molecular Properties

Purification and Homogeneity

Several preparations for 5'-AMP aminohydrolase have been described which yield homogeneous enzyme (1, 21, 22). Of these the method utilizing the direct adsorption of AMP aminohydrolase from a crude rabbit muscle extract with cellulose phosphate provides an elegant one-step purification of enzyme in high yields; the eluted enzyme was homogeneous by ultracentrifuge and electrophoretic criteria (1). This method has been adapted with slight modifications for preparations from rat (23), chicken breast (22), carp (24) and elasmobranch fish (*Raia clavata*) muscle (25). While the enzyme from chicken breast muscle was homogeneous by electrophoretic, ultracentrifugal, and immunoelectrophoretic criteria, no data were given for the rat, carp, and elasmobranch fish muscle preparations. A previous preparation of the rat muscle enzyme (26) and the enzyme purified from rat liver (27) were not homogeneous. No criteria were published for the 200-fold purified calf brain enzyme (28).

Chemical and Physical Properties

Whereas the rabbit muscle (1) and calf brain preparations (28) required mercaptoethanol and KCl or LiCl for stability over extended time periods, the preparation described by Lee was not affected by reducing or oxidizing agents (2). Multivalent anions such as tripolyphosphate, 3-iso AMP, ATP, and GTP but not substrate stabilized the calf brain enzyme against heat inactivation (28, 29).

The molecular weight of 270,000 obtained by Wolfenden et al (30) for the muscle enzyme from sedimentation-diffusion data ($S_{20,w} = 11.1$ S, $D_{20,\ w} = 3.75 \times 10^{-7}$ $cm^2\ sec^{-1}$, and $\bar{v} = 0.731$ calculated from the amino acid content) is in reasonable agreement with the 320,000 reported by Lee ($\bar{v} = 0.75$) (31). The rabbit muscle enzyme has a normal amino acid content; that is, no unusually low or large amounts of amino acids were found. Of the 32 cysteine/half cystine residues per molecule based on a molecular weight of 270,000, 6.2 were rapidly titrated with p-mercuribenzoate (30). Typical protein absorption spectra were reported for elasmobranch fish (25), carp (24), rat (26), and rabbit muscle enzyme (1). An $E^{1\%}_{280\,nm\; 1\,cm} = 9.13$ OD has been reported for the latter (32). The atypical absorption spectrum with a maximum at 275 to 276 nm observed by Lee (31) is indicative of contaminating bound nucleotides.

Rabbit antisera for the chicken breast enzyme strongly inhibited the AMP aminohydrolase from that tissue but it had no effect on the chicken brain, heart or erythrocyte enzyme; furthermore the brain and breast muscle enzyme exhibited differences in substrate specificity. The data are consistent with at least two types of chicken 5'-AMP aminohydrolase (22). Isoenzymic patterns, while perhaps implied by differences in certain kinetic parameters, have not been delineated for other preparations.

Catalytic Properties

Specificity

In general AMP aminohydrolase specificities have not been thoroughly defined perhaps because of difficulties until recently in obtaining pure enzyme. In addition to deamination of AMP and dAMP, the muscle enzyme catalyzes the deamination of N^6-methyl AMP, N^6-ethyl AMP, formycin-5'-monophosphate, adenosine 5'-monosulfate, adenosine 5'-phosphoramidate, adenosine, ADP (32), adenosine 5'-phosphorothioate and 6-chloropurine 5'-ribonucleotide (23). ATP, GMP, CMP, 2'-AMP, 3'-AMP, 3',5'-cyclic AMP, 3-iso AMP, N^1-methyl AMP, toyocamycin 5'-monophosphate, tubercidin-

5'-monophosphate, and 6-mercaptopurine 5'-ribonucleotide are not deaminated (32). The elasmobranch fish muscle, carp muscle, and chicken brain enzymes appear to be specific for AMP and dAMP (25, 24, 22). Extracts from pea seed and erthrocytes and the purified calf brain enzyme are specific for AMP (12, 33, 29).

Kinetics

The kinetic parameters of various muscle AMP aminohydrolases, presented in Table 1, are similar except for the lower specific activities exhibited by the fish enzymes for which no criteria of homogeneity are presently available. Specific activities reported for the brain enzymes (not shown in Table 1) are for calf, 15 μmoles/min per mg (34) and for chicken, 30 μmoles/min per mg (22). The apparent K_m (AMP) values for the calf enzyme varied from 2.7 mM to 8.4 mM depending upon the pH and the activator present (28). Although the pH optimum for AMP deamination varies depending upon the source, it normally occurs in a range from pH 5.9 to 7.1 (2, 12, 24, 25, 28, 35-37).

Activation

Most preparations of AMP aminohydrolase are activated by monovalent cations and nucleoside di- or triphosphates (see Table 2). Potassium is generally the most effective monovalent cation activator except for the calf brain

Table 1. Kinetic Constants for AMP Deamination by Several Preparations of AMP Aminohydrolase

Source (Ref.)	K_m mM	V_{max} umoles/min-mg[a]	Conditions
Rabbit Muscle			
(21)	1.4	1660	0.1 $\underline{M}$ Na^+-succinate, pH 6.4, 30°
(1, 32)	0.4	1200[b]	0.05 $\underline{M}$ $Tris^+$-cacodylate, pH 6.3, 0.15 $\underline{M}$ KCl, 30°
Rat Muscle			
(26)	1.4	1140	0.1 $\underline{M}$ succinate, pH 6.4, 0.5 $\underline{M}$ KCl, 30°
(23)	0.95	1330[b]	0.02 $\underline{M}$ K^+-cacodylate, pH 6.5, 1 mM mercaptoethanol, 30°
Fish - Elasmobranch			
(25)	1.52	350	0.1 $\underline{M}$ K^+-succinate, pH 6.6, 30°
Carp Muscle			
(24)	0.4	180	0.5 $\underline{M}$ K^+-succinate, pH 6.4, 30°

[a] V_{max} was calculated from the Michaelis-Menten equation where K_m is the value reported in this table and v the specific activity of the purest fraction at the concentration of AMP used for the standard assay.

[b] V_{max} values were obtained from double reciprocal plots.

Table 2. Monovalent Cation and Nucleotide Activators of AMP Aminohydrolase

Source	Activator	Comments	Ref.
Brain			
Calf	$Li^+ > Na^+ > K^+ > Rb^+$ $> Cs^+ > NH_4^+ = (CH_3)_4N^+$		(28)
	$ATP^{-3} >$ dATP > ITP > CTP = UTP		(29)
	$Li^+ = Na^+$ K^+, Rb^+, Cs^+ inhibited NH_4^+ no effect	Activator ATP present	(28)
	$Li^+ > Na^+ > NH_4^+$ K^+, Rb^+, Cs^+ no effect	Inhibitor GTP present	(34)
Chicken	ATP		(22)
Dog	ATP		(38)
Rabbit	ATP		(39)

	ATP + Na^+	Synergistic effect	(39)
Particulate	Na^+, K^+	required	(18)
Soluble	Na^+, K^+	Activate but not required	(18)
Rat	ATP		(40)
Erythrocytes			
Human	K^+, NH_4^+	Required	(37)
Soluble	ATP $\gg$ ITP, GTP, UTP	No effect in absence of K^+ or Na^+	
	Na^+, Li^+, Rb^+	Occurred only in presence of ATP	
Membrane	K^+	Required	(19)
Bound	ATP no effect		
Cat and Dog	ATP	Monovalent cations had no effect	(37)
Rabbit	ATP = ADP $>$ GTP	Assay contained K^+	(41)
Liver			
Rat	Na^+, K^+, Li^+		
	ATP, ADP	Assay contained Na^+	(27)

Table 2. (Continued)

Source	Activator	Comments	Ref.
<u>Muscle</u>			
Carp	ATP		(42)
	$K^+ > Cs^+ > Na^+ > NH_4^+ > Rb^+$		(24)
Elasmobranch Fish	$K^+ > Rb^+ > NH_4^+ \sim Li^+ > Na^{++} \gg (CH_3)_4N^+$	Cs^+, no effect	(25)
	ATP > 2'-AMP	In KCl	(25)
Rabbit	$K^+ = Na^+ > Li^+ > Rb^+ \sim NH_4^+ \gg Cs$		(1)
	ATP, ADP		(43, 3)
Rat	K^+, ADP		(44)
Chicken	ATP		(22)

Ehrlich Ascites Tumor Cells	ATP	In KCl	(45)
Pea Seed	$NH_4 > Li^+ > Na^+$		(12)

enzyme where lithium is more effective. Although Li^+, Na^+, Rb^+, Cs^+, and NH_4^+ can often substitute for K^+, the relative order of effectiveness is not consistent when enzymes from different sources are compared. Monovalent cations are not required for the brain, muscle, and rat liver enzymes since the same V_{max} was observed at high concentrations of AMP in either the presence or absence of cations. However, the enzyme associated with a brain particulate fraction (18), Ehrlich Ascites Tumor cells (45), and the human erythrocyte membrane (19) is reported to absolutely require K^+ for activity. The soluble human erythrocyte enzyme (which constitutes 85% of the total AMP aminohydrolase content of this cell) was activated only by K^+ and NH_4^+ (37); activation by Na^+, Li^+, and Rb^+ required the presence of ATP. ATP alone did not activate the soluble enzyme but did lower the effective concentration for K^+ activation. In contrast the cat and dog erythrocyte enzyme were activated by ATP but not by monovalent cations either in the presence or absence of ATP (37).

In the absence of activators, AMP aminohydrolase from brain (46), erythrocytes (41, 47), muscle (43), and liver (27) give sigmoid curves for velocity versus AMP concentration which become hyperbolic after the addition of monovalent cations, adenine nucleotides, or a combination of monovalent cations and adenine nucleotides. That is, for the rabbit muscle enzyme (43), addition of K^+, ADP, or ATP give normal hyperbolic saturation curves for AMP as represented by a change in the Hill slope, n_H, from 2.2 to 1.1; V_{max} remained the same. On the other hand the soluble erythrocyte enzyme (47) and the calf brain enzyme (28) required the presence of both monovalent cations and ATP before saturation curves became hyperbolic. In contrast, the bound human erythrocyte membrane enzyme did not exhibit sigmoid saturation curves and K^+ activation was not affected by ATP (19).

Inhibition

A variety of anions such as inorganic phosphate (22, 25, 2, 41, 48-50), sulfate (50), nitrate (50), pyrophosphate (2, 29), tripolyphosphate (29), carboxylic acids (32, 44), 2,3-diphosphoglyceric acid (47), fluoride (12, 25, 2, 36, 48, 49), 3'-AMP (25, 29, 32), GTP (27, 40, 43), GDP (43), and 3-iso AMP (29) have been shown to inhibit AMP aminohydrolase. GTP inhibits the ATP activation of the enzyme from rat brain, heart and liver, calf brain, and rabbit muscle but had no effect on the elasmobranch fish muscle enzyme (25). In the case of the calf brain enzyme GTP inhibition appears to be competitive for ATP [$K_{i_{app}}$ = 10 μM]. 3-Iso AMP was not a substrate but was an effective competitive inhibitor for substrate in the brain [$K_{i_{app}}$ = 60 μM] and muscle enzymes. Inhibition of the erythrocyte enzyme by physiological concentrations of 2,3-diphosphoglycerate may prevent early depletion of the adenine nucleotide pool, an important physiological factor since mature erythrocytes lack enzymes for synthesis of the adenine ring and AMP (47).

Carboxylic acids have been reported either to have no effect (2, 28), to activate (35), or to inhibit (32) AMP aminohydrolase. While the activation of the rabbit muscle enzyme was not thoroughly examined (35), the reported inhibition of this enzyme by citrate, succinate, and maleate was most effective in the absence of activators or in the presence of ADP (32). Limited kinetic data were consistent with a competitive interaction between citrate and ADP. The enzymatic activity in intact myofibrills was activated by ATP, ADP, and ITP in succinate buffer but not in citrate buffer (51). With the rat enzyme citrate, succinate, cacodylate, acetate, and lactate decreased $K_{m_{app}}$ for AMP and increased the Hill slope; V_{max} decreased only slightly (44). At physiological concentrations of lactate, nucleoside triphosphates actually inhibited; ADP and GDP reversed these inhibitions.

The inhibition by carboxylic acids, o-phenanthroline, and dithioerythritol led to the discovery of 2.8 gram atoms Zn^{2+} per 300,000 grams of rabbit muscle AMP aminohydrolase (32). The reported inhibitions by Zn^{2+} (2, 36), Cu^{2+} (2, 28, 52), Fe^{3+} (2), Ag^{+} (2, 52), Cd^{2+} and Ni^{2+} (2, 28), and Hg^{2+} (35, 36) might be explained in terms of an interaction with a sulfhydral group(s) necessary for catalysis or by a displacement of the presumably required Zn^{2+}.

Iodoacetate had no effect on the rabbit muscle enzyme (2, 35) but did inhibit the carp muscle and pea seed enzyme (12, 36). Organic mercurials are also reported to inhibit the enzyme from several sources (2, 12, 24, 25, 36). Except for the preliminary report by Wolfenden et al (30) that mercurials desensitized the rabbit muscle enzyme to allosteric inhibition by GTP, the role of sulfhydral residues in AMP aminohydrolase is not understood.

Mechanism

Except for the fact that deamination of AMP by the rabbit muscle enzyme was irreversible between pH 6.0 and 9.0 (2) a detailed discussion of the mechanism for 5'-AMP deamination is at present premature. The sigmoidal relationship for substrate saturation and activation by monovalent cations and adenine nucleotides is consistent with mechanisms involving active site-effector site interaction. The activation brought about by this site-site interaction is a relatively slow 1st order process independent of protein concentration (53) and is comparable to observations reported for yeast glyceraldehyde-3-phosphate dehydrogenase (54) and homoserine dehydrogenase (55). The activation was discussed in terms of a simple scheme similar to those proposed by Rabin (56) and Weber (57) which provides a plausible explanation for the sigmoid curve for initial velocities versus substrate concentration without involving additional phenomena such as cooperative interactions between catalytic sites.

The hydrolytic deamination catalyzed by rabbit muscle AMP aminohydrolase may be facilitated by Zn^{2+} in contrast to the mediation of a common purinyl enzyme intermediate for adenosine aminohydrolase catalysis (58, 59).

Considerations of Physiological Function

The physiological role of AMP aminohydrolase is not clearly understood. Enzymatic activity in muscle is markedly reduced in the dystrophic mouse (60, 61), in humans suffering from Duchanne type muscular dystrophy (62), in hypokaliemic periodic paralysis (63) and upon denervation of normal and dystrophic mouse gastronemii (64). Activity is reported to increase in both transplanted and primary hepatomas (48) and in precancerous livers prior to the onset of neoplasia induced by feeding or by intra-abdominal injections of the potent carcinogen 3'-methyl-4-dimethylaminoazobenzene (65). The weak carcinogen 4'-methyl-4-dimethylaminoazobenzene was not effective (65). Increases in enzyme activity concomitant with altered nuclear-nucleolar morphology, nuclear RNA content, and nuclear RNA biosynthesis were also observed after injections of thioacetamide, a hepatocarcinogen (66, 67).

AMP aminohydrolase activity was low but distinguishable in the leg, diaphragm and heart muscle of a 20-24 day old rabbit fetus (68). The activity in the heart remained low in both neonatal and adult life, whereas a rapid increase occurred in the activity of the enzyme in the diaphragm during the 4 or 5 days before parturition reaching a maximum activity immediately after birth. In contrast the enzymic activity of the mixed leg muscles remained relatively constant until 8-9 days after birth when it began to rise steadily reaching an adult value of 7-8 times that of the fetal muscle within 14 days. This increase occurred when the animal began independent movement. Similar but qualitatively different effects were observed with guinea pig and rat leg muscle and chicken leg and pectoral muscle. Increases in aldolase, myokinase and creatine phosphokinase activity were roughly parallel to increases in AMP aminohydrolase activity.

Although it has been reported that increased AMP aminohydrolase activity occurred during prolonged stimulation of muscle bundles (69-71), the participation of this enzyme in the contractile process seems unlikely in light of the lack of significant changes in the levels of AMP and IMP during a single contraction of frog abdominal muscle (72). This is corroborated by the absence of AMP aminohydrolase activity in muscle of some invertebrates (73-75) and in human uterine muscle (76).

It is tempting to consider regulation of the concentration of AMP, a known effector of several glycolytic enzymes (77), by the antagonistic action of adenine and guanine nucleotides on AMP deamination as a control factor in glycolysis and gluconeogenesis (77). Setlow et al (40) suggested the participation of AMP aminohydrolase in a self regulating system for purine nucleotide interconversion as presented in Figure 1.

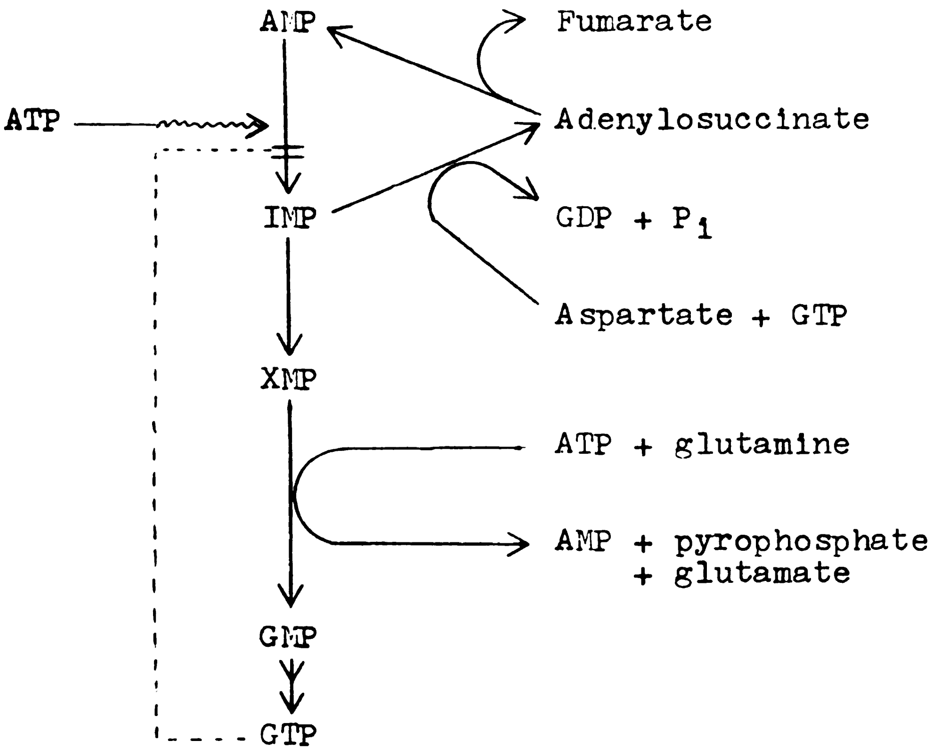

Figure 1. Purine Nucleotide Interconversions

As the GTP concentration decreases, AMP aminohydrolase inhibition is released with a concommitant increase in IMP and GTP which completes the self-regulating system by inhibiting the AMP aminohydrolase. In the case of the rat and calf brain enzymes, the ATP activation and GTP inhibition were observed at the overall *in vivo* concentrations of these nucleotides and AMP (28, 29). However such a control mechanism based upon kinetically observed changes with an _in vitro_ system is subject to presently undefined effects by other factors _in vivo_. (For example, although AMP could be postulated as a control factor of glycogenolysis in muscle _via_ its activation of phosphorylase _b_, Helmreich and Cori could not detect a sufficient increase in AMP concentration _in vivo_ during electrical stimulation of muscle to explain the rapid increase in phosphorylase activity (78).) At present only preliminary data exist as to (A) the effects of divalent metals such as Mg^{2+} and Ca^{2+} upon the activation and inhibition by nucleotides (34, 44) and (B) the effects of anions other than nucleotides (32, 44). An additional factor, the availability of substrate, activators, and inhibitors to the enzyme in the cell has not been investigated. Consequently, the control and function of AMP aminohydrolase remain interesting questions.

MATERIALS AND METHODS

Enzyme Preparation

AMP aminohydrolase from rabbit muscle was prepared by the method of Smiley, et al. (1). Since the crystalline enzyme was less stable than enzyme obtained directly from cellulose phosphate and since it showed evidence of heterogeneity upon ultracentrifuge analysis, the eluted enzyme in 1 M KCl from the cellulose phosphate column was used directly for all experiments. Although previously reported yields for this preparation were 36%, essentially 100% recovery of enzymatic activity from the column can be achieved if the amount of 0.45 M KCl wash solution is kept to 500 ml or less per pound of muscle.

The enzyme was stored as previously described (1). For those experiments where it was desirable to remove the activator, K^+, (43) the enzyme was chromatographed on Sephadex G-25 equilibrated with tetramethylammonium chloride (TMACl) at either pH 6.3 or 7.1 and 1 mM in mercaptoethanol.

Protein Determination

Protein concentrations were determined by measurement of absorbance at 260 nm and 280 nm according to the method of Warburg and Christian (79) or from the absorbtion coefficient of 9.15 OD for a 1% solution at 280 nm in a 1 cm light path (see page 29 for a description of this determination).

Kinetic Assays

Kinetic assays were run on a Beckman DU spectrophotometer equipped with a Gilford optical density convertor with offset and a Sargent SRL recorder. Temperature was maintained at 30^o with a circulating water bath. For AMP concentrations less than 0.2 mM the decrease in absorbance at 265 nm was measured according to the method of Kalckar (80); at AMP concentrations greater than 0.2 mM and 5 mM the increase in OD was determined at 285 nm and 290 nm, respectively. The change in absorbance per minute was converted to μmoles substrate deaminated per minute by the relationship:

$$\mu\text{moles/minute} = \Delta\,\text{OD per minute}/F$$

where F is equal to -8.86 at 265 nm, +0.30 at 285 nm, and +0.12 at 290 nm (53). Specific activity is expressed as μmoles substrate deaminated per min/mg protein.

Three basic assay systems were used: Assay 1, the KCl activated system, contained 0.15 M KCl, 0.05 M Tris-cacodylate, pH 6.3, and 50 μM Tris AMP unless otherwise indicated; Assay 2, the ADP activated system, contained 0.10 M TMACl, 0.05 M Tris cacodylate, pH 6.3, 100 uM Tris ADP, and 50 μM Tris AMP unless otherwise indicated;

Assay 3, the method used to determine activity in the absence of activators, consisted of 0.10 M TMACl, 0.05 M Tris cacodylate, pH 6.3, and Tris AMP at the concentrations indicated.

The enzyme was diluted into 0.5 M KCl, or 0.5 M TMACl, 0.05 M Tris cacodylate, and 1 mM mercaptoethanol before assay unless stated otherwise.

Reagents

All reagents were ACS Reagent grade or the best grade available. Spectropure sulfate salts (Johnson, Matthey and Co., Ltd.) of zinc, cobalt, ferrous, magnesium, manganese, nickel, and cadmium were obtained from Jarrell-Ash Co. of Waltham, Massachusetts. Stock solutions of 1000 ppm metal were prepared with analytically weighed samples. Harleco atomic absorption standards (1000 ppm) for $CuSO_4$ and $CaCl_2$ were supplied by Scientific Products. All solutions were prepared using double distilled water, the second distillation being done in an all glass apparatus.

Trizma Base (Tris), cacodylic acid, mercaptoethanol, dithioerythritol (DTE), o-phenanthroline (OP), ethylenediaminetetraacetic acid (EDTA), cellulose phosphate, and Sephadex G-25 coarse were obtained from Sigma. The TMACl, 8-hydroxyquinoline-5-sulfonic acid (8-OHQ5SA), and m-phenanthroline (Alfred Bader Chemicals) were from

Aldrich Chemical Co. Tetramethylammonium hydroxide was supplied by Mallinkrodt, methylamine by Eastman Organic Chemicals, Chelex 100 (Na^+) 50-100 mesh by Bio Rad, and dimethylsulfate by Matheson, Coleman, and Bell.

The TMACl was recrystallized twice from hot absolute ethanol and then passed over Chelex 100 ($Tris^+$) to remove divalent metal ions. Carboxylic acids were recrystallized from water or organic solvents, treated with Chelex 100, and adjusted to pH 6.4 with Tris base. A Sargent Model SL pH meter equipped with a 30070-10 combination electrode was used for all pH measurements. Zinc, magnesium, and calcium were not detected in the carboxylic acid solutions by atomic absorption analysis. Chelex 100 (Na^+) was washed in a sintered glass funnel with three cycles of 1 N HCl and water and then titrated to pH 7.0 or 7.5 with Tris base. Visking Corporation dialysis tubing was boiled twice in $NaHCO_3$, three times in sodium EDTA, and rinsed three times in water and stored at 4^o in water.

Substrate Analog Study

Source of Analogs

Analogs of the substrate AMP were obtained from the following sources: Sigma (AMP, adenosine, ADP, ATP, adenosine monosulfate, 2'-AMP, 3'-AMP, 3',5'-cyclic AMP, dAMP, GMP, GDP, GTP, and CMP); P-L Biochemicals (adenosine phosphoramidate and 6-mercaptopurine 5'-ribonucleotide); Miles Laboratories (α,β-methylene Adenosine diphosphonate); Tubercidine monophosphate and tubercidine monophosphate methyl ester were obtained from Dr. William J. Wechter of UpJohn Co., Kalamazoo, Michigan; 3-iso AMP was kindly donated by Dr. Nelson Leonard of the University of Illinois, Urbana, Illinois; Dr. R. J. Suhadolnik of the Albert Einstein Medical Center, Philadelphia, Pennsylvania, donated toyocamycin monophosphate (7-deaza, 7-cyano AMP); Formycin monophosphate (8-aza, 9-deaza AMP) was a gift of Dr. S. Nishimura of the National Cancer Center Research Institute, Tokyo, Japan; Dr. F. Rottman of Michigan State University, East Lansing, Michigan kindly supplied the 2'-O-methyl ADP (AmDP) and K. W. Rabinowitz of the same institution donated N^6-ethyl AMP and the mixture of 6-amino-9-D-psicofuranosylpurine 1'-phosphate and 6'-phosphate.

The sodium salt of ADP and the barium salts of 5'-adenosine monosulfate and 6-mercaptopurine 5'-ribonucleotide were converted to the Tris salts by passage over Dowex 50 WX8 ($Tris^+$) at room temperature. The acid forms of all other analogs were titrated to the desired pH with Tris base. No attempt was made to remove the less than 5% AMP contaminant in ADP analog studies since the high concentration of enzyme used deaminated the AMP almost as fast as the sample could be mixed.

Synthesis of N^1-Methyl AMP

N^1-Methyl AMP was synthesized from AMP and dimethylsulfate at pH 4.5 by the method of Griffin and Reese (81). The purified product exhibited only one spot in Solvent A (see page 28) and the R_f value of 0.75 agrees well with the R_f = 0.76 previously reported (81). In 0.1 N HCl λ_{max} was 258 mμ and λ_{min} was 232 mμ.

Synthesis of N^6-Methyl AMP

N^6-Methyl AMP was synthesized from N^1-methyl AMP by the procedure of Brooks and Lawly (82). The absorption spectrum at pH 6.7 was identical within experimental error to that previously reported for N^6-methyl adenosine in water, λ_{max} = 265 mμ and λ_{min} = 229 mμ (83). Paper chromatography of N^6-methyl AMP in solvent system B (see page 28) indicated that the sample contained less than 1.7% AMP and 2.1% N^6-methyl AMP methylester. The R_f values for AMP and N^6-methyl AMP in system B were 0.23 and 0.41 respectively.

The change in absorbance for the enzymatic deamination of N^6-methyl AMP was determined using a Cary Model 15 spectrophotometer. The spectrum of a known amount of N^6-methyl AMP, $\epsilon_{265\ nm} = 16.3 \times 10^3$ OD/mM (83), was determined from 340 nm to 220 nm before and after the addition of 16 μg of AMP aminohydrolase to sample and reference cells. The absorbance change resulting from deamination of N^6-methyl AMP (at pH 6.3) was calculated in ΔOD/mM at several wavelengths: 265 nm, -10.65; 270 nm, -11.23; 275 nm, -10.10; 285 nm, -5.45; 290 nm, -3.24; and 295 nm, -1.23.

Deamination of Analogs

The deamination of analogs of AMP was examined by recording the absorption spectrum of each before and after the addition of 10-40 μg of enzyme to both sample and reference cells of a Beckman DB spectrophotometer equipped with a Sargent SRL recorder. Those analogs deaminated showed changes in their absorption spectrum within 15 minutes. Assay 1 was used except approximately 60 μM analog was substituted for the AMP.

Kinetic parameters were obtained from Lineweaver-Burk plots of initial velocities (in Assay 1) at substrate concentrations from 10^{-5}M to 10^{-2}M except in the case of adenosine monosulfate and N^6-methyl AMP where the maximum concentration of analog was 2.0 x 10^{-4}M. The Hill slope (n_H) was obtained from a plot of Log $[(V_{max}/v) - 1]$ versus Log [S].

Purine Product Characterization

Substrate analogs and protein (5-80 μg) were mixed on a watch glass and incubated 10-70 minutes before the products were chromatographed on Whatman #1 paper or polyethylenimine impregnated cellulose MN 300 thin layer plastic sheets (Brinkman Instruments, Inc.). Impurities were removed from the latter prior to use by ascending irrigation with double distilled water according to method 1 of Randerath and Randerath (84). Chromatograms were developed with the following solvent systems: (A) saturated ammonium sulfate:0.1 M potassium phosphate buffer, pH 7.2:isopropanol (79:19:2); (B) 1% ammonium sulfate:acetic acid:isopropanol (34:20:45) chromatographed on Whatman #1 paper washed with 1% ammonium sulfate; (C) isobutyric acid:1 N ammonia:0.1 M sodium EDTA (100:60:1.6); (D) 10 ml concentrated ammonium hydroxide added to 32.9 ml water and mixed with 66.1 ml of isobutyric acid; (E) 1 M acetic acid-ammonium acetate buffer, pH 3.8:95% ethanol (30:70); (F) 0.5 M sodium formate buffer pH 3.3. Descending chromatography was used for all systems except for solvents B and F which were used for ascending chromatography. Solvent F was used to develop chromatograms on the polyethylenimine cellulose thin layer sheets.

Detection of Methylamine

Methylamine from N^6-methyl AMP deamination was detected with an F & M 402 gas chromatograph equipped with a flame detector. The column, 3 mm x 6 feet, packed with Chromosorb 103, 100/120 mesh (Aspec Co., Ann Arbor, Michigan), was conditioned overnight at 250° with N_2 gas flow. The samples were run at 105° with N_2 as the carrier gas. Solutions of methylamine at 1.0 $\mu g/\mu l$ and NH_3 at 9 $\mu g/\mu l$ (ammonium chloride in KOH) were prepared as standards.

Physical Properties

Extinction Coefficient of AMP Aminohydrolase

The extinction coefficient of AMP aminohydrolase was determined by the method of Hoch and Vallee (85) using trichloroacetic acid precipitation of protein followed by drying to constant weight. After concentrating the enzyme to approximately 8 mg protein/ml by placing it in a dialysis bag around which dry Sephadex G-200 (fine) was packed to absorb solvent, AMP aminohydrolase was chromatographed on Sephadex G-25 equilibrated with 0.10 M KCl at pH 7.0 with potassium phosphate buffer. The optical density of the eluted protein was determined at 260 and 280 nm by dilution into 0.8 M KCl, 0.05 M Tris cacodylate, pH 6.3, and 1 mM mercaptoethanol. Three ml samples containing a total of 7 to 7.5 OD units (at 280 nm) of protein were placed in conical glass test tubes (previously dried to constant weight), precipitated with an equal volume of 20-30% TCA, allowed to stand at room temperature for 15 minutes, and then centrifuged 15 minutes at top speed in a clinical centrifuge. The supernatant was carefully removed with a Pasteur pipet. The precipitate was washed once with 2 ml 20% TCA, centrifuged, and the supernatant removed. After a second washing with 2 ml 10% TCA, the precipitate was dried to constant weight at 105° and weighed to ±0.1 mg on a Mettler balance.

Molecular Weight of AMP Aminohydrolase

The molecular weight of AMP aminohydrolase was determined by the Yphantis Sedimentation Equilibrium method for dilute protein solutions using the Spinco Model E Ultracentrifuge equipped with Rayleigh interference optics (86). Enzyme was chromatographed on Sephadex G-25 equilibrated with 0.20 M KCl, 0.05 M Tris Mes, pH 7.2, and 1 mM mercaptoethanol. The protein sample (0.575 mg protein/ml) and a blank of the 0.20 M KCl buffer were placed in a double sector 12 mm Epon filled cell and centrifuged at 293.0°K and 12,590 RPM. The Rayleigh patterns were recorded on Kodak II-G photographic plates. The fringe displacements of the photograph focused at 2/3 plane were measured at 0.1 mm intervals on a Bausch and Lomb Microcomparator. The data were treated according to the procedure given by Yphantis.

Subunit Molecular Weight

Enzyme was chromatographed on Sephadex G-25 equilibrated with 0.1 M TMACl at pH 7.0 with Tris phosphate buffer and 1 mM mercaptoethanol to remove KCl which interferes with denaturation by sodium dodecyl sulfate. The enzyme (96 ug/ml) and standards (bovine serum albumin, RNase, and DNase) were incubated 3 hours at 37° in 1% SDS, 0.1 M sodium phosphate buffer, pH 7.1, and 1% mercaptoethanol. SDS polyacrylamide gels and gel buffer were prepared according to a modification of the procedure of

Weber and Osborn (87). The gels were pre-electrophoresed to remove ammonium persulfate before application of the protein samples. A total of 20-80 μl of 1:1 mixtures of the enzyme solutions and 50% glycerol was layered on separate gels followed by electrophoresis at room temperature for 2.5 hours at 7.5 ma/gel. The gels were removed from the glass gel tubes, stained 4 hours with Coomaasie Blue solution (2.5 ml 1% Coomaasie Brilliant Blue, 5 ml absolute methanol, and 10% TCA to 100 ml), and then destained over a 48 hour period with several changes of 10% TCA. Migration distances (mobility) of each standard and sample were measured from the origin.

Characterization of AMP Aminohydrolase as a Zinc Metalloenzyme

Preparation of Enzyme for Trace Metal Analyses

To insure that the enzyme was not contaminated with divalent metal ions during preparation all glassware, pipets, and nalgeneware were carefully washed and rinsed, soaked in 2 N HCl for six hours or more, and then rinsed with double distilled water. A pyrex Waring blender was substituted for the metal meat grinder and stainless steel Waring blender used in the initial stages of the enzyme preparation. All reagents except mercaptoethanol and Tris base were passed over Chelex 100 ($Tris^+$) to remove contaminating metal ions and were then stored in polyethylene bottles. The cellulose phosphate was treated as previously described (1) and then soaked in 0.01 M Tris EDTA, pH 7.5, for 1 week, and washed exhaustively with double distilled water before equilibration with extraction buffer.

A total of 100-200 mg protein from the crude extract (10-20 ml of the supernatant obtained after centrifugation of the extraction mixture) was dialyzed against three changes (300 ml each) of 0.1 M TMACl, pH 7.0 with 50 μM Tris phosphate, and 1 mm mercaptoethanol (Buffer A) over a 72 hour period to reduce potassium and phosphate to levels that would not interfere with the metal analysis. The sample was transferred to a 100 ml pyrex Berzalius beaker with pyrex cover glass and evaporated to moist dryness before wet ashing. Two blanks containing an amount of Buffer A equivalent to that in the sample were similarly treated.

Purified AMP aminohydrolase (25 ml at 4 mg protein per ml) was dialyzed against 3 changes (250 ml each) of 0.97 M KCl, 0.01 M Tris Mes, pH 7.1, and 1 mM mercaptoethanol for 72 hours at 4°. In order to remove potassium 10 ml of the dialyzed enzyme was chromatographed on a Sephadex G-25 column (2.5 cm x 22 cm) equilibrated with Buffer A. Before equilibration with buffer all Sephadex columns were washed with 2 to 3 column volumes of 0.01 M Tris EDTA, pH 7.5, to remove metal ions followed by exhaustive rinsing with double distilled water. The enzyme was collected in Nalgene test tubes and the more concentrated fractions were combined (a total of 25 mg protein) and evaporated to moist dryness in Berzalius beakers. Two blanks containing Buffer A collected from the column before protein was applied were similarly treated.

After all samples had cooled, 10 ml of concentrated nitric acid (Baker Analyzed ACS Reagent grade) was added to the samples and blanks. The samples were kept at room temperature for 15 minutes and then heated slowly and allowed to reflux until clear and colorless insuring complete oxidation of all organic material. Finally all samples were evaporated to moist dryness, cooled, the beaker sides and cover rinsed with 5 ml water, heated and then cooled before quantitative transfer to 25 ml (25 mg samples) or 50 ml (50 mg samples) volumetric flasks. Concentrated HCl (Baker Analyzed ACS Reagent grade) at 0.125 ml/25 ml was used to insure solubilization of all inorganic material.

Metal analyses were accomplished with the Perkin Elmer 303 Atomic Absorption Spectrophotometer at the following wavelengths: Zn, 2138 A; Ca, 4227 A; Mg, 2852 A; Fe, 2483 A, and Cu, 3247 A. Samples were aspirated into an air-acetylene flame and the percent absorption of three readings was averaged and converted to OD. Standards for each metal were completed before each analysis using standard solutions made by dissolving analytically weighed spectropure metals or metal oxides (Johnson, Matthey and Co., Ltd.) in concentrated HCl. The standard matrix contained water and 0.5 ml concentrated HCl/100 ml. The ppm of metal were calculated from a standard curve or by proportion after subtraction of the blank readings from the sample readings.

Ten ml of the samples and blanks for the purified enzyme were sent to Dr. H. Nass of the Union Carbide Corp. (Tuxedo, New York) for Neutron Activation Analysis of Co, Mn, and Cu.

Preparation of Apo Enzyme[2]

ApoAMP aminohydrolase (enzyme from which the bound metal has been removed) was prepared by incubating enzyme at 3-4 mg protein per ml in 15 to 19 mM Tris 8-OHQ5SA at pH 7.1 in 1 M KCl for 12 to 15 hours until the activity of the enzyme was less than 10% of the original activity. The chelator (8-OHQ5SA) was removed by chromatography on Sephadex G-25: for metal analysis enzyme was eluted with Buffer A and for reconstitution studies Buffer B (0.5 M KCl, 0.02 M Tris Mes, pH 7.1, and 1 mM mercaptoethanol) was used since the enzyme was generally more stable in this buffer.

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[2] The methods and conditions by which apoenzyme was obtained and by which reconstitution of the apoenzyme was achieved were those that worked but are not necessarily the most optimum. Except for preliminary studies which indicated that reactivation did not occur as readily in TMACl as it did in KCl, the methods and conditions described were the only ones tried.

Reconstitution of Apo Enzyme

Apoenzyme in Buffer B was reconstituted by incubating samples containing 0.10 mg protein/ml in the presence of 0-10 gram atoms of metal (as the spectropure metal sulfate) per mole of enzyme. The activity of enzyme at each level of metal was determined with Assay 1 containing 10 mM Tris AMP. The assay mixture was freed of metal ions before use by treatment with Chelex 100 ($Tris^+$).

To determine the kinetic constants of reconstituted enzyme, apoenzyme in Buffer B was incubated with 3.7 to 4.0 gram atoms of zinc, cobalt, or manganese sulfate overnight. The enzyme (1.5 to 2.0 mg protein/ml) was chromatographed on Sephadex G-25 equilibrated with Buffer A to remove excess metal ion and KCl and aliquots were diluted in either 0.5 M KCl or 0.5 M TMACl containing 0.02 M Tris Mes, pH 7.1 and 1 mM mercaptoethanol for assay.

Apoenzyme (16 mg) reconstituted with 3.7 gram atoms zinc/mole enzyme was chromatographed on Sephadex G-25 as previously described; the recovered enzyme (9 mg) was wet washed and analyzed for zinc.

Stability Towards Metal Binding Agents

Inhibition of AMP aminohydrolase by a number of metal binding agents in the presence or absence of KCl was studied by exposing the enzyme at 0.1 mg protein/ml and 30° to these inhibitors. The loss in activity was followed over a 6 hour period using Assay 1 or 2. The inhibition data are expressed as Vi/Vo x 100% where Vi is the activity after exposure to the chelating agent and Vo is the activity before exposure. The small amount of chelating agent transferred from the incubation mixture to the assay mixture did not affect the assay.

Correlation of the Removal of Zinc and Loss of Enzymatic Activity

Zinc was removed from the enzyme by a modification of the method of Simpson and Vallee (88) using 8-OHQ5SA. Enzyme (3.59×10^{-6}M) in 0.4 M KCl or TMACl, 0.1 M Tris Mes, pH 7.1, and 1 mM mercaptoethanol was titrated with 8-OHQ5SA and the OD recorded at 370 nm against a blank containing all reagents except protein. The amount of zinc $[8\text{-OHQ5SA}]_3$ complex formed, calculated from standard curves of $OD_{370\ nm}$ versus zinc concentration at each concentration of 8-OHQ5SA used, was correlated with enzymatic activity remaining. Activity was determined immediately after appropriate dilution of aliquots (25 μl) removed from the sample cuvette. The immediate determination of activity after dilution was necessary since a time dependent reactivation, presumably due to readsorption of zinc by apoenzyme, was frequently observed. Aliquots (25 μl) were also withdrawn from the blank cuvette to maintain an equal volume in each cuvette. All calculations were corrected for dilution of the protein during the titration.

RESULTS AND DISCUSSION

Kinetic Properties of AMP Aminohydrolase

Substrate Specificity

The AMP aminohydrolase purified by Lee (21) was specific for AMP; dAMP, the only other substrate, was deaminated at 1% the rate of AMP deamination (2). Enzyme prepared by the method of Smiley et al (1) deaminated not only 5'-AMP and dAMP but also adenosine, ADP, N^6-methyl AMP, N^6-ethyl AMP, adenosine 5'-monosulfate, AMP-NH_2, AmDP, α,β-methylene ADP, and formycin 5'-monophosphate. The kinetic parameters (K_m and relative V_{max}) for these compounds are presented in Table 3. No data for N^6-ethyl AMP, AmDP, α,β-methylene ADP, and formycin 5'-monophosphate was obtained due to lack of sufficient material. The following compounds were not deaminated under the conditions described in Materials and Methods: ATP, 2'-AMP, 3'-AMP, 3',5'-cyclic AMP, 6-amino-9-D-psicofuranosylpurine 1'-, and 6'-phosphate mixture, N^1-methyl AMP, 6-mercaptopurine 5'-ribonucleotide, GMP, GDP, GTP, CMP, 3-iso AMP, tubercidine 5'-monophosphate, and tubercidin 5'-monophosphate methyl ester.

pH Optima

The pH optima for deamination of ADP, adenosine,

Table 3. Kinetic Parameters for Deamination of AMP Analogs Catalyzed by AMP Aminohydrolase

Substrate Analog	K_m (mM)	Relative V_{max}[a]	n_H
AMP	0.40	100	1.0
AMP-NH_2	49	73.4	1.0
N^6-Methyl AMP	1.8	20.4	
dAMP	2.3	18.5	1.1
Adenosine monosulfate	3.2	10 to 16	1.1
Adenosine[b]	20.0	1.1	
ADP	0.8	0.20	1.3

[a] Reported as % V_{max} for AMP (1240 μmoles deaminated/min per mg) in 0.15 M KCl and 0.05 M Tris cacodylate buffer, pH 6.3.

[b] The kinetic parameters for adenosine were obtained at the pH optimum, 6.5, for adenosine deamination.

and N^6-methyl AMP determined in either Tris cacodylate or sodium acetate buffer are given in Figure 2. The optimum for ADP deamination occurred between pH 5.0 and 5.5, a marked shift to acid pH from the optimum (pH 6.3-7.0) observed for AMP deamination.[3] Since the pK_a for the terminal phosphate of ADP is 6.44 (89) ADP exists as ADP^{2-} between pH 5.25 to 5.50 whereas at pH 6.44 it exists as a 1:1 mixture of ADP^{2-} and ADP^{3-}. The observed shift may indicate that ADP^{3-} is less readily deaminated than ADP^{2-}. The sharp optimum for adenosine (pH 6.5 to 6.75) and for N^6-methyl AMP (pH 6.4 to 6.6) deamination at non-saturating substrate concentrations shows a slight shift to more alkaline pH from the observed optimum (pH 6.3) for AMP deamination under similar conditions.

Activation and Inhibition of AMP Aminohydrolase by Purine Nucleotides

Activation

Both ADP and ATP are allosteric activators for AMP deamination in the absence of the monovalent cation activator K^+ (43, 90). Data for activation by other purine nucleotides are presented in Table 4. Of the analogs tested only 6-mercaptopurine 5'-ribonucleotide and IDP activated the enzyme. Although according to the data in Table 2, 6-mercaptopurine 5'-ribonucleotide is a better

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[3] Unpublished observation, K. L. Smiley, Jr.

Figure 2. The Effect of pH on Deamination of ADP, Adenosine, and N^6-Methyl AMP

The assay mixture for ADP deamination contained 0.10 M KCl, 0.10 M buffer at variable pH, and 8.2 mM Tris ADP. The reaction was initiated by adding 15 μg of enzyme to 1 ml of assay mixture: □, sodium acetate; ■, Tris cacodylate.

The assay mixture for adenosine deamination contained 0.10 M KCl, 0.10 M Tris cacodylate buffer, 3 mM adenosine and 30 μg enzyme/ml, ●.

For N^6-methyl AMP deamination, the assay mixture contained 0.15 M KCl, 0.05 M Tris cacodylate, 50 μM N^6-methyl AMP, and 0.8 μg enzyme/ml, △.

The pH of each assay was read before and after the reaction.

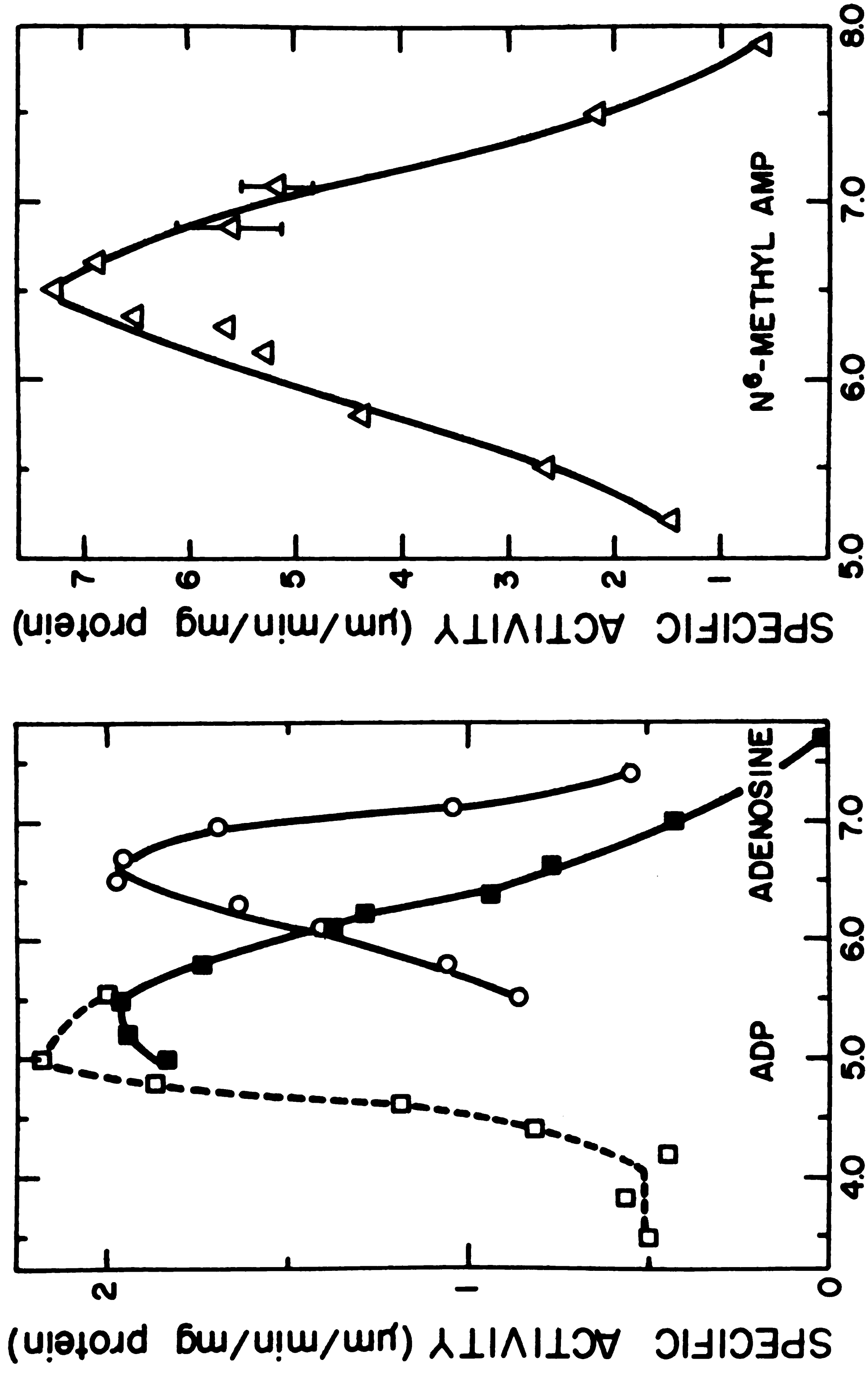
SPECIFIC ACTIVITY (μm/min/mg protein)
7
6
5
4
3
2
1
5.0
6.0
7.0
8.0
pH
N6-METHYL AMP
SPECIFIC ACTIVITY (μm/min/mg protein)
2
1
0
4.0
5.0
6.0
7.0
pH
ADP
ADENOSINE

Figure 2.

Table 4. Activation of AMP Aminohydrolase by Purine Ribonucleotides

Purine Nucleotide	[A] μM	Specific Activity
		μm/min per mg Protein
Control	0	0.72
6-Mercaptopurine 5'-ribonucleotide	82	14.1
	165	26.0
Inosine diphosphate	70	2.9
	150	7.6
Tubercidin monophosphate	36	0.66
	72	0.84
Toyocamycin monophosphate	47	1.4
	94	2.4
CMP	95	0.64

Activity was determined with Assay 3 containing 50 μM Tris-AMP. The protein concentration in the assay varied from 0.2 to 0.5 μg/ml.

activator than IDP, activation by this compound was not pursued further due to a lack of material. The Hill plot for IDP activation is presented in Figure 3 along with the data for ADP and ATP activation obtained by K. L. Smiley. (Due to the high absorbance of IDP at 265 nm, the data presented was obtained at suboptimum concentrations of this activator.) The $K_{a_{app}}$ value for IDP is 1.2 mM compared to 10 μM and 50 μM observed with ADP and ATP, respectively.[4] However the observed maximum velocity at 50 μM AMP for IDP activation was 75% of that observed with ADP. Hence the 6-amino group of ADP, which substantially reduces the concentration of activator required for half maximum activation, is not required for the activation *per se*.

Inhibition

In addition to inhibition by GDP and GTP (43, 90), the enzyme is subject to inhibition by 3',5'-cyclic AMP, 3'-AMP, α,β-methylene ADP, and 3-iso AMP (see Table 5). At concentrations of less than 0.3 mM 2'-AMP had no effect on activity. N^1-methyl AMP did not inhibit significantly at 6 times the concentration of AMP in the assay.

3-Iso AMP was a potent inhibitor of AMP, dAMP, N^6-methyl AMP, ADP, AMP-NH_2, and adenosine monosulfate deamination (data not shown). The Dixon plot (Figure 4) and double reciprocal plot (not shown) for 3-iso AMP

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[4] Unpublished observation, K. L. Smiley, Jr.

Figure 3. Hill Plots for IDP, ADP, and ATP Activation of AMP Aminohydrolase

Initial velocities, v, were obtained using Assay 2 and varying the concentration of activator. V_m was determined from a Lineweaver-Burk plot of 1/v versus 1/[A]. The data for IDP activation was obtained using enzyme chromatographed on Sephadex G-25 equilibrated with 0.5 M TMACl, 0.05 M Tris cacodylate, pH 6.3, and 1 mM mercaptoethanol. The concentration of activator required for 50% activation, $K_{a_{app}}$, was estimated from the intercept of the Hill plot (Log $[v/(V_m-v)]$ versus Log [A]M) where Log $[v/(V_m - v)] = 0$.

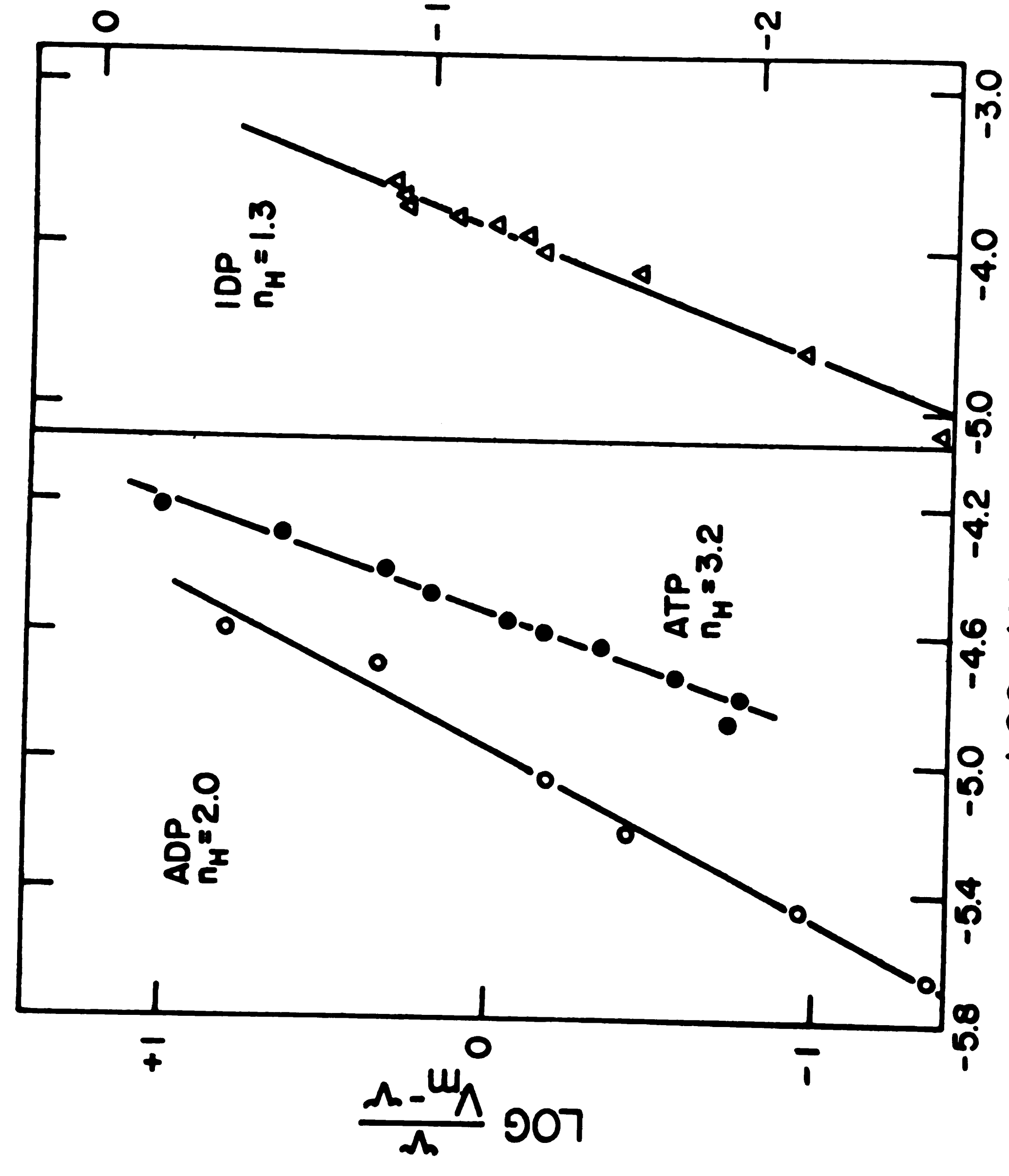

IDP
$n_H = 1.3$
ATP
$n_H = 3.2$
ADP
$n_H = 2.0$
LOG (ACTIVATOR) M
-3.0
-4.0
-5.0
-4.2
-4.6
-5.0
-5.4
-5.8
0
-1
-2
+1
0
-1
LOG $\frac{v}{V_m - v}$

Figure 3.

Table 5. Inhibition of AMP Deamination by Purine Nucleotides

Inhibitor	[I] mM	% Inhibition[a]
3-Iso AMP	0.01	33
	0.10	73
3'-AMP	0.10	14
	0.17	41
3',5'-cyclic AMP	0.10	14
	0.14	23
	0.17	46
2'-AMP	0.30	5[b]
α,β-Methylene ADP	0.10	21
	0.13	41
N^1-Methyl AMP	0.18	15[c]

[a] Activity was determined in Assay 1 containing 50 µM Tris AMP. In order to obtain data at total nucleotide concentrations greater than 0.2 mM where the absorption is greater than 2.5 OD ($\epsilon_{265\ nm}^{AMP} = 14.1 \times 10^3$), the cuvette was blanked against 50 µM AMP. The reaction was initiated by the addition of 0.2 µg protein/ml to the assay containing the indicated amount of inhibitor. Percent inhibition is reported with respect to activity in the absence of inhibitor.

[b] Due to the high optical density the assay was run in cuvettes with a path length of 5 mm instead of the usual 10 mm.

[c] The assay contained 32 µM Tris AMP/ml.

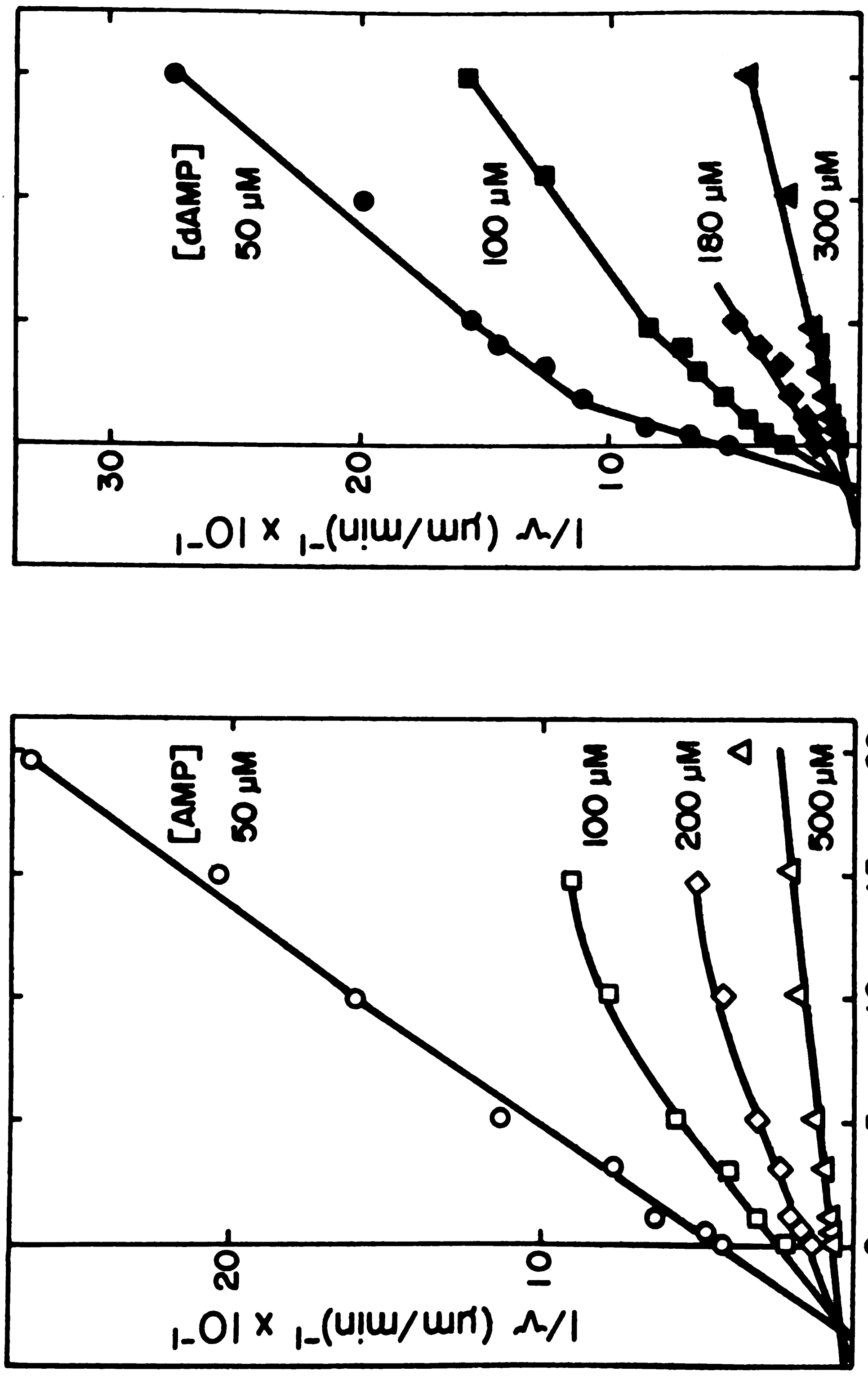
Figure 4. 3-Iso AMP Inhibition of AMP and dAMP Deamination

Activity was measured using Assay 1 at the following concentrations of substrate:

AMP ○, 50 μM; □, 100 uM; ◇, 190 μM; △, 515 μM

dAMP ●, 50 uM; ■, 100 μM; ◆, 180 μM; ▲, 300 uM.

The AMP and dAMP reaction mixtures contained 0.24 ug protein per ml and 7.9 ug protein per ml, respectively.



[dAMP]
50 μM
100 μM
180 μM
300 μM
1/v (μm/min)⁻¹ x 10⁻¹
10
20
30
0
5
10
15
[3-ISO-AMP] x 10⁺⁵ M
[AMP]
50 μM
100 μM
200 μM
Δ
500 μM
1/v (μm/min)⁻¹ x 10⁻¹
10
20
0
5
10
15
20
[3-ISO-AMP] x 10⁺⁵ M

Figure 4.

inhibition of AMP deamination is characteristic of competitive inhibition. The K_i value for 3-iso AMP obtained from the Dixon plot was 37 μM. A replot of the slopes from the Lineweaver Burk plots versus inhibitor concentration was linear (see Figure 5): the K_i of 32 μM determined from the intercept on the horizontal axis in Figure 5 is in good agreement with the K_i determined from the Dixon plot. The data are consistent with 3-iso AMP exhibiting linear competitive inhibition for AMP deamination according to the terminology of Cleland (91). Neither AMP nor 3-iso AMP caused substantial changes in the Hill slopes for 3-iso AMP inhibition or for AMP deamination, respectively (see Tables 6 and 7).

The inhibition of dAMP deamination by 3-iso AMP is not clearly defined. Both the Dixon (Figure 4) and Lineweaver Burk plots were markedly nonlinear. In the Dixon plot the curvature is especially noticeable at low substrate concentration where at increasing concentrations of 3-iso AMP less inhibition is observed than would be predicted. This could occur by four different mechanisms: (A) iso-AMP is deaminated; (B) iso-AMP is activating the reaction; (C) the ability of iso-AMP to bind to the enzyme decreases upon increasing saturation with the inhibitor; or (D) iso-AMP binds to more than one type of site each with a different binding constant. Possibility (A) seems unlikely in that significant deamination of iso-AMP did not

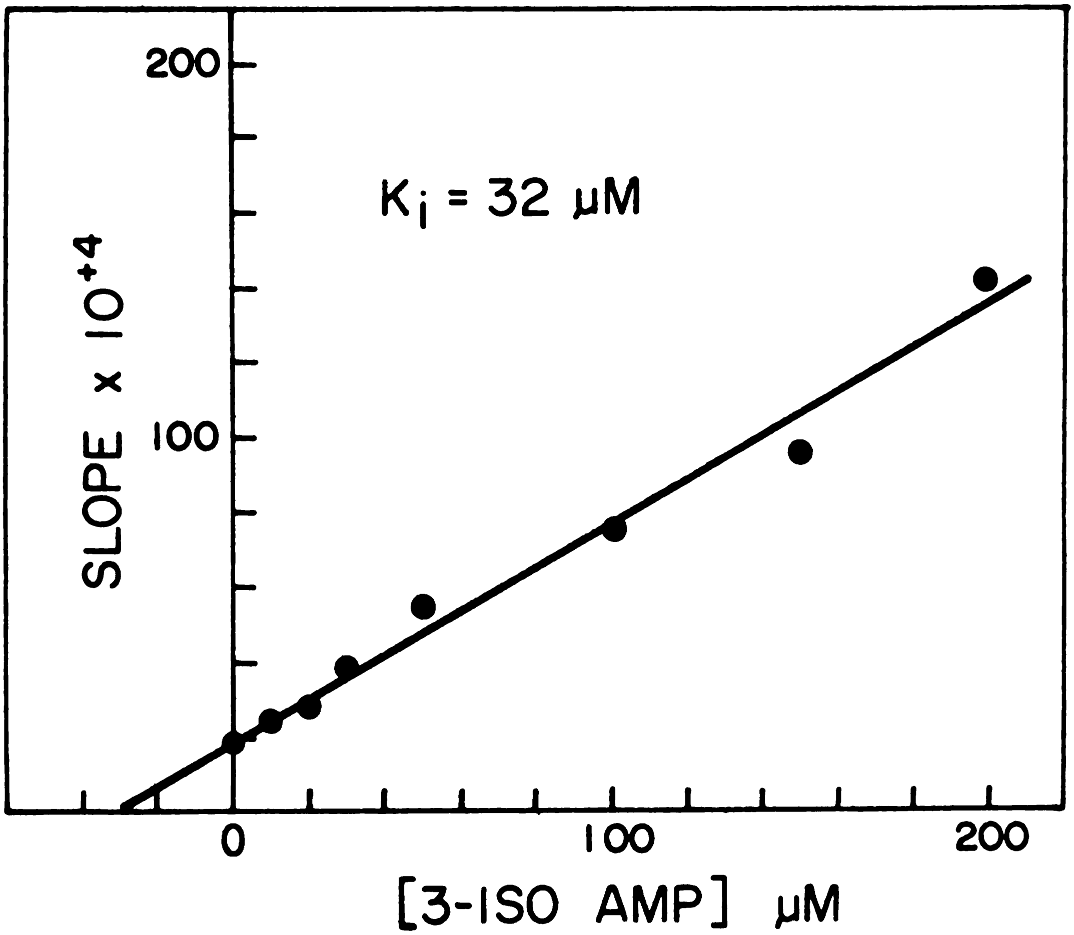


Figure 5. Replot of Slopes from Lineweaver Burk Plot of 3-Iso AMP Inhibition of AMP Deamination Versus the Concentration of 3-Iso AMP

Table 6. The Effect of AMP and dAMP on the Hill Slope for 3-Iso AMP Inhibition

[AMP] mM	$-n_H$ (3-Iso AMP)	[dAMP] mM	$-n_H$ (3-Iso AMP)
0.05	0.95	0.05	0.70
0.10	0.87	0.10	0.79
0.19	0.87	0.18	1.05
0.52	1.10	0.30	1.04
0.80	1.0	0.60	0.97

The data from Figure 3 were plotted according to the Hill equation (see Figure 1) where V is the velocity of substrate deamination in the absence of inhibitor and $v = v_i$, the activity of the inhibited reaction.

Table 7. The Effect of 3-Iso AMP on the Hill Slope for AMP and dAMP

3-Iso AMP μM	n_H (AMP)	n_H (dAMP)
0	1.00	1.3
10	1.07	1.2
50	1.04	1.3
150	1.00	1.1

The data from Figure 3 were plotted according to the Hill equation where V_m is the maximum rate of AMP or dAMP deamination and v is the activity of the reaction as a function of substrate concentration at constant concentrations of inhibitor.

occur over a 45 minute period at twice the protein concentration used in the dAMP study. Mechanisms B, C, and D cannot be distinguished with the data available. Unlike the data for AMP, 3-iso AMP effects the Hill slope for dAMP and vice versa (see Tables 6 and 7). The Hill slopes of < 1 for iso-AMP suggest that the enzyme is exhibiting negative cooperativity with respect to the inhibitor.[5] The Monod, Wymen, and Changeux theory (93) for allosteric enzymes cannot account for $n_H < 1.0$ (94) although Levitzki and Koshland have pointed out that enzymes with Hill slopes of less than 1 for some effectors and greater than 1 for others are common phenomena (95). For example aspartate transcarbamylase, which displays positive cooperativity ($n_H > 1$) for aspartate (96), exhibits negative cooperativity for the inhibitors GTP and CTP (95): from the theory of negative cooperativity the incomplete inhibition by nucleotide triphosphates at concentrations which should have completely inhibited the reaction was rationalized. AMP aminohydrolase may be yet another example of both positive and negative cooperativity, the former exhibited by substrate and nucleotide activators and the latter by the

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[5] Negative cooperativity is characterized by decreasing affinity of the enzyme for ligand upon saturation and by $n_H < 1$. It may arise from ligand-induced conformational changes in a single protein species or from a mixture of two or more proteins or subunits (isoenzymes) with different intrinsic binding constants. For instance, glyceraldehyde-3-phosphate dehydrogenase exhibits negative cooperativity for NAD$^+$ binding as a result of conformational changes upon binding of successive molecules of NAD$^+$ (92).

inhibitor 3-iso AMP, at least with respect to inhibition of dAMP deamination.

One Enzyme Responsible for Deamination of all Substrates

The less specific behavior of AMP aminohydrolase towards purine nucleotide analogs could be due to contamination by other aminohydrolases. In addition, deamination of ADP might be effected by transphosphorylation of ADP to ATP and AMP with myokinase followed by deamination of AMP by the specific AMP aminohydrolase. However, deamination of various substrates by a single enzyme, AMP aminohydrolase, is consistent with the results obtained from studies of enzyme homogeneity, heat inactivation, elution from cellulose phosphate, and product characterization.

Homogeneity

AMP aminohydrolase was homogeneous by ultracentrifugal and electrophoretic criteria (1).

Heat Inactivation

The rates of heat inactivation of ADP, AMP and adenosine deaminating activity were identical within experimental error when examined under 2 conditions, 62° in KCl and 40° in TMACl (Table 8). In the latter system the slightly greater rate of heat inactivation for AMP than for ADP and adenosine activity may be the consequence

Table 8. Heat Denaturation of Adenylic Acid Aminohydrolase

	Rate of Heat Inactivation k (min^{-1})	
Substrate	$(CH_3)_4NCl$ System[a]	KCl System[b]
AMP	2.2×10^{-3}	7.3×10^{-2}
ADP	1.5×10^{-3}	7.0×10^{-2}
Adenosine	1.5×10^{-3}	ND[c]

[a] Conditions of incubation in the $(CH_3)_4NCl$ (TMACl) system: Protein which was passed over a Sephadex G-25 column equilibrated with 0.10 M TMACl, 0.05 M $(CH_3)_4N^+$-cacodylate, pH 7.2, and 2 mM mercaptoethanol was incubated at 1.3 mg/ml, 40°C in a closed test tube from which aliquots were removed for assay. Protein concentration per ml of assay was 0.26, 13, and 32.5 μg in the AMP, ADP and adenosine systems, respectively. Assay solutions contained 1 mM Tris AMP and 0.05 M Tris cacodylate, pH 6.4; 1 mM Tris ADP and 0.05 M Tris cacodylate, pH 6.4; and 0.1 mM adenosine, 0.10 M KCl, 0.05 M Tris cacodylate, pH 6.4.

[b] Conditions of incubation in the KCl system: Deaminase (0.48 mg/ml) was incubated at 62°C in 1 M KCl, 1 mM mercaptoethanol, and 0.10 mM potassium phosphate, pH 7.0. The ADP assay contained 0.1 mM Tris ADP, 0.10 M TMACl, and 0.05 M Tris cacodylate, pH 6.3. The AMP assay contained 50 uM Tris AMP and 0.10 M potassium succinate, pH 6.5. The protein concentration in the ADP and AMP assays was 4.8 μg/ml and 0.24 μg/ml, respectively.

[c] Not determined.

of an additional dilution required before AMP aminohydrolase could be assayed which could introduce an additional inactivation.

Elution of AMP and ADP Aminohydrolase Activity from Cellulose Phosphate

The ratio of specific activities for AMP and ADP deamination remained constant within the protein peak eluted from a cellulose phosphate column with a linear gradient from 0.45 M KCl to 1.0 M KCl (Figure 6). The recovery of ADP aminohydrolase activity with respect to the crude extract was not determined due to the presence of interfering enzymes, i.e. myokinase, in the extract.

Product Characterization

The purine ribonucleotide products of adenosine, AMP, and ADP deamination were characterized by paper chromatography (Table 9). The product of ADP deamination chromatographed with the same R_f value as commercial IDP in solvent systems A and D. The product for adenosine deamination exhibited the same R_f value as inosine in systems A, D, and E. No IMP was detected in either of the ADP or adenosine reaction mixtures. Since the relative roles of deamination of ADP and adenosine are only 0.2 to 1% that observed for AMP deamination, any AMP formed by reactions involving contaminating enzymes such as myokinase would be immediately deaminated to IMP at the concentrations of protein used. Since this was not observed, the

Figure 6. Elution Profile of AMP and ADP Aminohydrolase Activities From Cellulose Phosphate

Enzyme was eluted with a 400 ml linear gradient from 0.45 to 1.0 M KCl containing 1 mM mercaptoethanol at pH 7.0. Samples of approximately 4.0 ml were collected and assayed for AMP aminohydrolase activity in 0.10 M potassium succinate at pH 6.4 and 50 μM AMP. ADP deamination was examined at 0.10 mM Tris ADP in 0.10 M TMACl and 0.05 M Tris cacodylate, pH 6.3. The small protein peak preceding the main peak was not assayed.

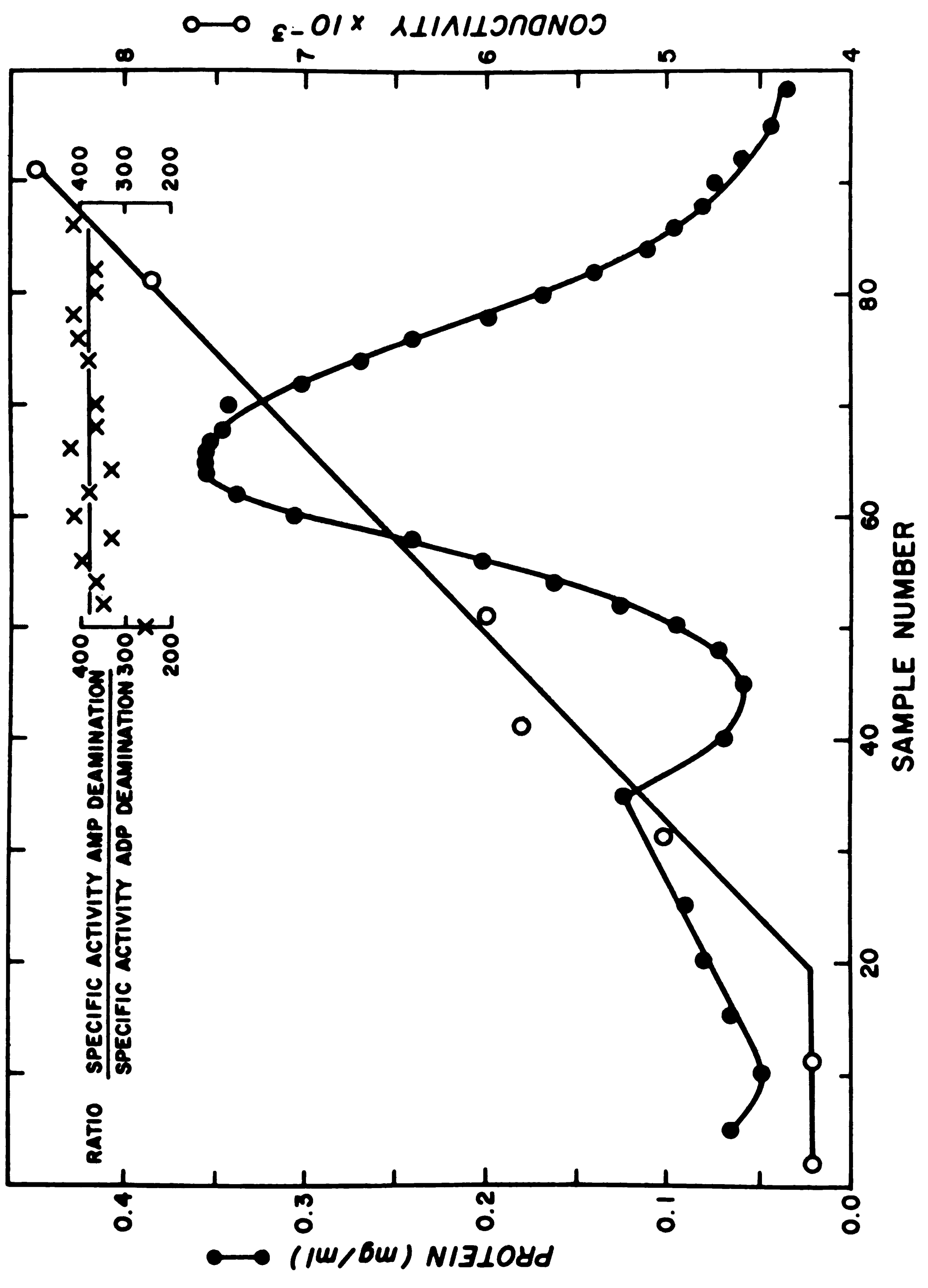
CONDUCTIVITY x 10^-3
8
7
6
5
4
RATIO
SPECIFIC ACTIVITY AMP DEAMINATION
SPECIFIC ACTIVITY ADP DEAMINATION
400
300
200
400
300
200
PROTEIN (mg/ml)
0.4
0.3
0.2
0.1
0.0
20
40
60
80
SAMPLE NUMBER

Figure 6.

Table 9. Chromatographic Characterization of the Products of Adenosine, ADP, and AMP Deamination

	R_f							
	Solvent A		Solvent C		Solvent D		Solvent E	
Compound	Minus Enzyme	Plus Enzyme	Minus Enzyme	Plus Enzyme	Minus Enzyme	Plus Enzyme	Minus Enzyme	Plus Enzyme
AMP	0.25	0.52	0.56	0.30				
IMP	0.51	0.52		0.30			0.38	
ADP	0.30	0.58	0.42	0.22				
IDP	0.57		0.21					
Adenosine	0.11	0.33			0.82	0.43	0.56	0.59
Inosine	0.36	0.32	0.55		0.42		0.58	

data are consistent with the direct deamination of ADP and adenosine to IDP and inosine, respectively.

General Discussion

The differences between the preparations of Lee and Smiley *et al.* for the rabbit muscle AMP aminohydrolase may be related to the higher K_m (1.4 mM) for AMP obtained by Lee (2) than observed by Smiley and Suelter (0.4 mM) (43). Since the enzyme prepared by the method of Smiley *et al.* exhibited still greater K_m values for adenosine and ADP and very low relative rates of deamination, their deamination by Lee's preparation was no doubt too slow to be readily observed. The high $K_{m_{app}}$ and low V_{max} observed for the purified calf brain enzyme (28) may also explain the inability of Setlow and Lowenstein to observe deamination of several analogs (29) readily deaminated by the muscle enzyme.

Deamination of ADP by myofibrills (97), actomyosin gels (98), and skeletal muscle extracts (99, 100) has been reported. Data obtained with crude extracts suggested that both AMP and ADP but not ATP were deaminated by an enzyme extracted with water. Deamination of ADP by these extracts was much slower than AMP deamination which is consistent with the suggestion that AMP aminohydrolase is responsible for both activities.

Although the AMP aminohydrolase purified by the method developed by Smiley et al. appears to be less specific than enzyme prepared by other methods, the limited kinetic data indicates that this enzyme still exhibits relatively narrow specificity with respect to the stereochemical and electronic configuration of the substrate. N^6-methyl AMP was deaminated with a relative velocity of 20% V_{AMP}; the K_m increased 4.5 fold. Ethylation of the 6-amino group further decreased the rate of deamination.[6] Hydrolysis of the thio group from 6-mercaptopurine 5'-ribonucleotide was not detected although Murray and Atkinson (23) have reported the dechlorination of 6-chloropurine 5'-ribonucleotide by rat muscle AMP aminohydrolase isolated by a modification of the rabbit muscle preparation. The 6-thio analog did, however, activate the rabbit muscle enzyme.

Substitutions at other than the 6 position of the purine ring frequently proved detrimental. N^1-methyl AMP was neither a substrate nor an inhibitor. GMP was not deaminated although GDP and GTP inhibited AMP deamination in both the K^+- and ADP-activated systems (43). Tubercidin monophosphate and toyocamycin monophosphate, in which the N-7 of the purine ring is replaced by a carbon and a carbon plus cyano group, respectively, were neither substrates, activators, nor inhibitors of adenylic acid aminohydrolase.

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[6] Unpublished observation, C. Zielke.

Formycin monophosphate, in which C-8 is replaced by nitrogen and N-9 by carbon, was deaminated.

3-Iso AMP which exhibits differences in both aromaticity of the purine ring and stereochemistry (101) was an effective competitive inhibitor for AMP; 3-iso AMP was not significantly deaminated even at 70 times the enzyme levels used for AMP deamination. Inhibition of the rabbit muscle enzyme by 3-iso AMP was slightly greater than that observed for the calf brain AMP aminohydrolase as indicated by the lower K_i value of 32 μM for the muscle enzyme versus 60 μM for the brain enzyme (29). In the case of 3-iso AMP inhibition of dAMP deamination interpretation is complicated by the nonlinearity of the data. The enzyme appeared to exhibit negative cooperatively for 3-iso AMP in this case.

Substitutions in the ribose moiety also have marked effects on deamination. Replacement of the 2'-hydroxy group of AMP by hydrogen in dAMP decreased the relative V_{max} to 18% that observed for AMP; the K_m increased from 0.4 mM to 2.3 mM. The psicose analog showed no substrate activity.

Deamination of both adenosine and ADP proceeds at 1.1 and 0.2% V_{max} (AMP), respectively. Although the K_m value for ADP is not much greater than that observed for AMP at pH 6.3, the value for adenosine increased two orders of magnitude indicating that the phosphate moiety may be important for binding of substrate. ATP was not deaminated under the conditions studied. Although the emphasis on adenosine and ADP as substrates may seem greater than warranted, the deamination of ADP is sufficiently rapid to eliminate use of this compound for binding studies with what is a very effective allosteric activator. Replacement of an oxygen in the 5'-phosphate by an amino group did not change V_{max} substantially, but the K_m for adenosine phosphoramidate was even greater than observed with adenosine or ADP. Both kinetic parameters are affected by substitution of a sulfate group for the 5'-phosphate in adenosine monosulfate. 2'-AMP and 3'-AMP are interesting in that neither is deaminated but 3'-AMP is an inhibitor of AMP deamination whereas 2'-AMP showed no inhibition.

In summary alterations in the purine, ribose, and phosphate moieties of AMP affected catalysis: analogs with substituents at the 1,2,7,2'- and 3'- positions of AMP (dAMP and AmDP were exceptions) were not substrates of the muscle enzyme while analogs with substitutions at the 6 and 5' position were generally deaminated although at slower rates.

Mechanism of AMP Deamination

More than one mechanism for the enzymatic deamination of AMP seems likely. The first and most obvious is the direct hydrolysis of the 6-amino group with the formation of IMP and ammonia. The second mechanism involves a rearrangement of the N-1 and N-6 nitrogens of the purine ring similar to the Dimroth rearrangement proposed by Macon and Wolfenden (102) for the conversion of N^1-methyl adenosine to N^6-methyl adenosine at neutral and alkaline pH. Such rearrangements are analogous to those observed with N^1-methyl pyrimidines (103). For the deamination of AMP this mechanism might proceed by a ring opening at the N-1 position followed first by the release of ammonia from the N-1 position and then by incorporation of the N-6 of adenylic acid into the ring as the N-1 of inosinic acid.

The direct hydrolysis and ring rearrangement mechanisms cannot be distinguished for AMP deamination since ammonia is released in both cases. However the deamination of N^6-methyl AMP could differentiate between the two possibilities since hydrolysis would involve release of IMP and methylamine while rearrangement would involve the release of N^1-methyl IMP and ammonia.

The products of N^6-methyl AMP deamination were therefore examined by paper and gas chromatography. The purine nucleotide product chromatographed with the same R_f value as IMP in 3 solvent systems (see Table 10). The characteristics of the absorption spectrum of this purine product (Table 11) were identical within experimental error to that of commercial IMP in 0.1 M HCl, in 0.1 M potassium phosphate buffer at pH 7.2, and in 0.1 M KOH. The spectra of IMP and the isolated product differ markedly

Table 10. Chromatographic Characterization of the Purine Product of N^6-Methyl AMP Deamination

Compound	R_f					
	Solvent A		Solvent C		Solvent F	
	Minus Enzyme	Plus Enzyme	Minus Enzyme	Plus Enzyme	Minus Enzyme	Plus Enzyme
AMP	0.25	0.52	0.56	0.30	0.69	0.40
N^6-MeAMP	0.25	0.52	0.60	0.30	0.69	0.39
IMP	0.51	0.52		0.30		0.37

Table 11. Spectral Characteristics of the Purine Nucleotide Product of N^6-Methyl AMP Deamination

	Solvent		
Characteristic	0.1 M HCl	0.1 M Potassium Phosphate Buffer pH 7.2	0.1 M KOH
		Product of N^6-methyl AMP deamination[a]	
250:260	1.41	1.66	1.05
270:260	0.49	0.55	0.58
280:260	0.16	0.24	0.19
λ_{max} nm	249	249	254
		Commercial IMP[b]	
250:260	1.38	1.64	1.05
270:260	0.51	0.57	0.59
280:260	0.19	0.27	0.19
λ_{max} nm	249	248.5	254
		N^1-methyl inosine (104)	
	pH 1	pH 6	pH 12
250:260	1.42	1.51	1.60
270:260	0.57	0.68	0.67
280:260	0.23	0.37	0.35
λ_{max} nm	250	251	249

[a] The deaminated product of N^6-methyl AMP was adsorbed onto Dowex 1x2 (Cl^-) and eluted with 0.01 N HCl. The peak tubes were combined and concentrated under vacuum. The absorption spectrum was determined with a Beckman DU spectrophotometer.

[b] The sample cuvette contained 0.134 mM NaIMP obtained from Sigma.

from N^1-methyl inosine at alkaline pH (the 250/260 ratio for IMP is 1.05 versus 1.60 for N^1-methyl inosine). The accuracy of this method should detect 10% contamination with the N^1-methyl derivative; the results obtained are consistent with at least 90% of the substrate being converted to IMP.

The gas chromatogram on Chromosorb 103 of the amine product from N^6-methyl AMP deamination (Figure 7B) revealed a peak with a retention time of 1.37 minutes which was identical with that observed for the standard, methylamine (1.35 minutes). The methylamine released from N^6-methyl AMP deamination was estimated to be 0.48 μg/μl compared to a theoretical maximum of 0.50 μg/μl indicating greater than 95% conversion of substrate to the expected product. The gas chromatogram of the product from AMP deamination (Figure 7A) shows that extraneous amines do not arise during the analysis. The technical inability to detect NH_3 on the gas chromatogram[7] does not completely eliminate a possibility that deamination proceeds through a ring opening mechanism with the intermediate leaving the enzyme surface before ammonia or methylamine is released. This intermediate might then collapse through a rate controlled equilibrium process with the loss of both ammonia

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[7] Since a flame detector was used, products such as NH_3 which do not contain carbon are not detectable. According to Figure 3 of the Technical Bulletin FF-181 (Johns-Manville Co., Celite Division) ammonia emerges before methylamine when chromatographed on Chromosorb 103.

Figure 7. Gas Chromatography of the Amine Products from AMP and N^6-Methyl AMP Deamination

A total of 0.975 μmoles Tris AMP (A) and 1.073 μmoles Tris N^6-methyl AMP (B) were incubated under cover in 0.2 M KCl and 0.05 M Tris cacodylate buffer, pH 6.3, containing 5.8 μg AMP aminohydrolase. The total volume of the reaction mixture was 52 μl. After thirty minutes the reaction was stopped by addition of 10 μl of 4 N KOH; 1 μl samples were injected into the gas chromatograph with a Hamilton syringe and the resultant spectrum recorded. Samples A and B contained a maximum of 0.26 μg NH_3/μl and 0.50 μg methylamine/μl, respectively, assuming complete deamination of AMP and N^6-methyl AMP. The standard, Sample C, contained 1 μg methylamine/μl. The amount of methylamine released upon deamination of N^6-methyl AMP was estimated by proportion from the peak areas of B (the unknown) and C (the standard). Peak areas were obtained by weight. The area for C was multiplied by 2 to correct for the difference in attenuation at which the samples were run.

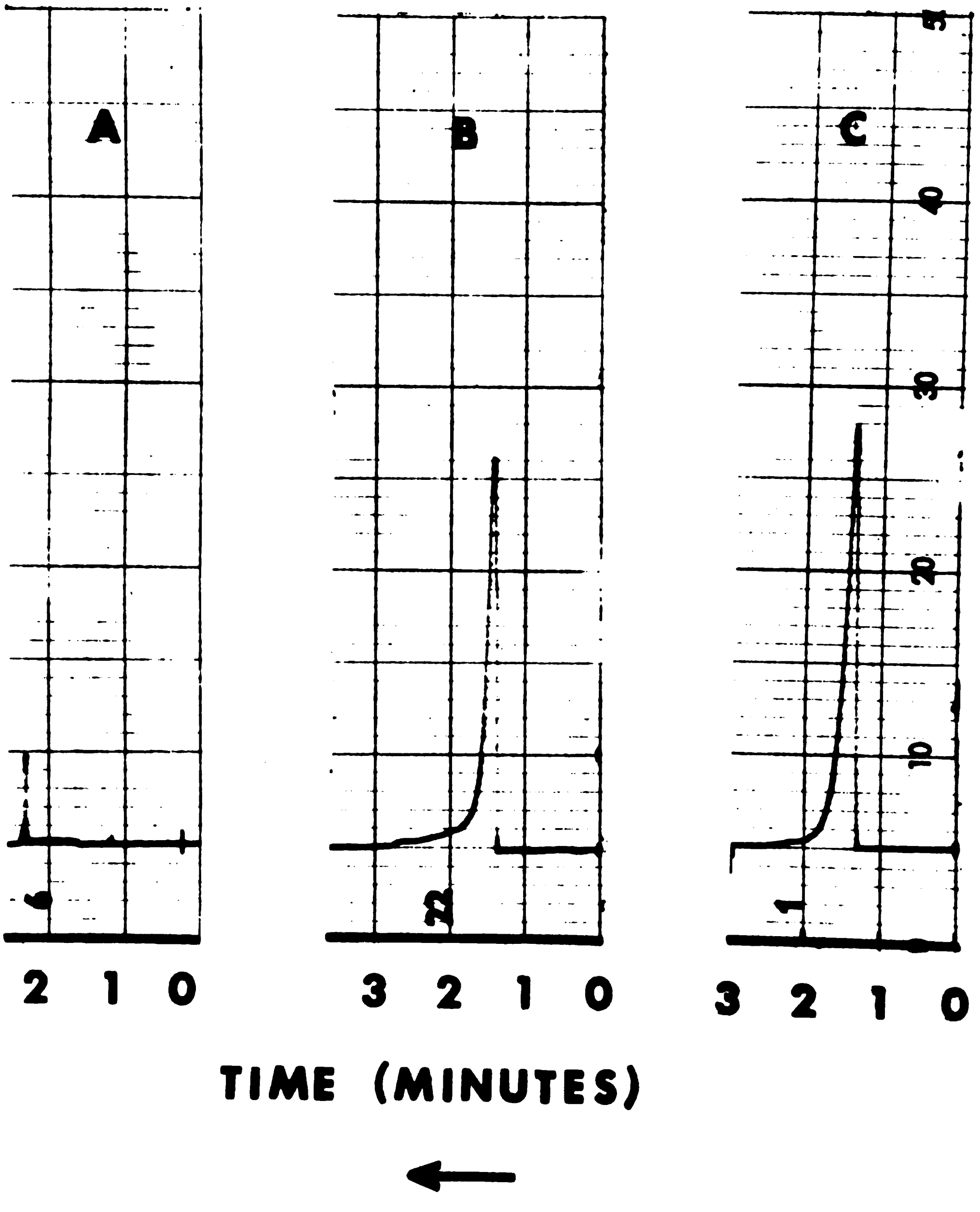

Figure 7.

and methylamine. Furthermore the quantity of N^1-methyl IMP and IMP formed would depend upon which pathway of ring reformation is favored. However since only one purine product which exhibited R_f values and spectral characteristics corresponding to those obtained for IMP was observed, this latter mechanism seems unlikely. The data are consistent with a direct hydrolysis of AMP or N^6-methyl AMP by AMP aminohydrolase. An additional argument in favor of the direct hydrolysis mechanism is the observed dechlorination of 6-Cl purine 5'-ribonucleotide by the rat muscle enzyme (23). Rearrangement of this compound is not possible. Although the chloro analog has not been checked for substrate activity with the rabbit enzyme, it seems unlikely that these enzymes would exhibit different mechanisms in light of the many similarities observed with these enzymes.

Physical Properties of AMP Aminohydrolase

Absorption Coefficient

The absorption coefficient ($A^{1\%}_{280\ nm}$) for a 1 cm light path of AMP aminohydrolase was determined gravimetrically by TCA precipitation as described in Materials and Methods. According to Hoch and Vallee (85) TCA precipitation is quantitative for proteins of molecular weight greater than 12,000. The values for $OD_{280\ nm}$/mg protein obtained from three trials are summarized in

Table 12. The calculated absorption coefficient was (9.15 ± 0.34) OD/10 mg protein dry weight: the 4% error is within the range expected for sample sizes of 5-10 mg protein (85).

Table 12. The Absorption Coefficient for AMP Aminohydrolase Measured at 280 nm

Trial	$OD_{280\ nm}$/10 mg Protein	Standard Deviation
1	9.29	± 0.2 OD/10 mg
2	8.76	± 0.2
3	9.40	± 0.1
Average of 3	9.15	

Molecular Weight

The weight average molecular weight of AMP aminohydrolase was determined by the Yphantis Sedimentation Equilibrium method for dilute protein solutions (86). The data are plotted as the Log (ΔY) (or Log (Fringe Displacement)) versus the square of the distance (in cm^2) from the center of rotation (see Figure 8). The molecular weight was calculated according to the following equation:

$$\bar{M}_w = \frac{\sigma_w \cdot 2.303 \cdot R \cdot T}{\omega^2\ (1-\bar{v}\rho)}$$

Figure 8. Sedimentation Equilibrium Centrifugation of AMP Aminohydrolase

The observed fringe displacements for the Rayleigh interference patterns obtained with the camera focused at 2/3 plane are presented as a plot of Log (ΔY) versus the square of the radius (cm^2) from the center of rotation. The data were obtained using an initial concentration of 0.06% AMP aminohydrolase at pH 7.2 in 0.2 M KCl, 0.05 M Tris Mes, and 1 mM mercaptoethanol in an Epon filled double sector cell 12 mM thick. The (I) designates the standard deviation of the readings at that position in the cell.

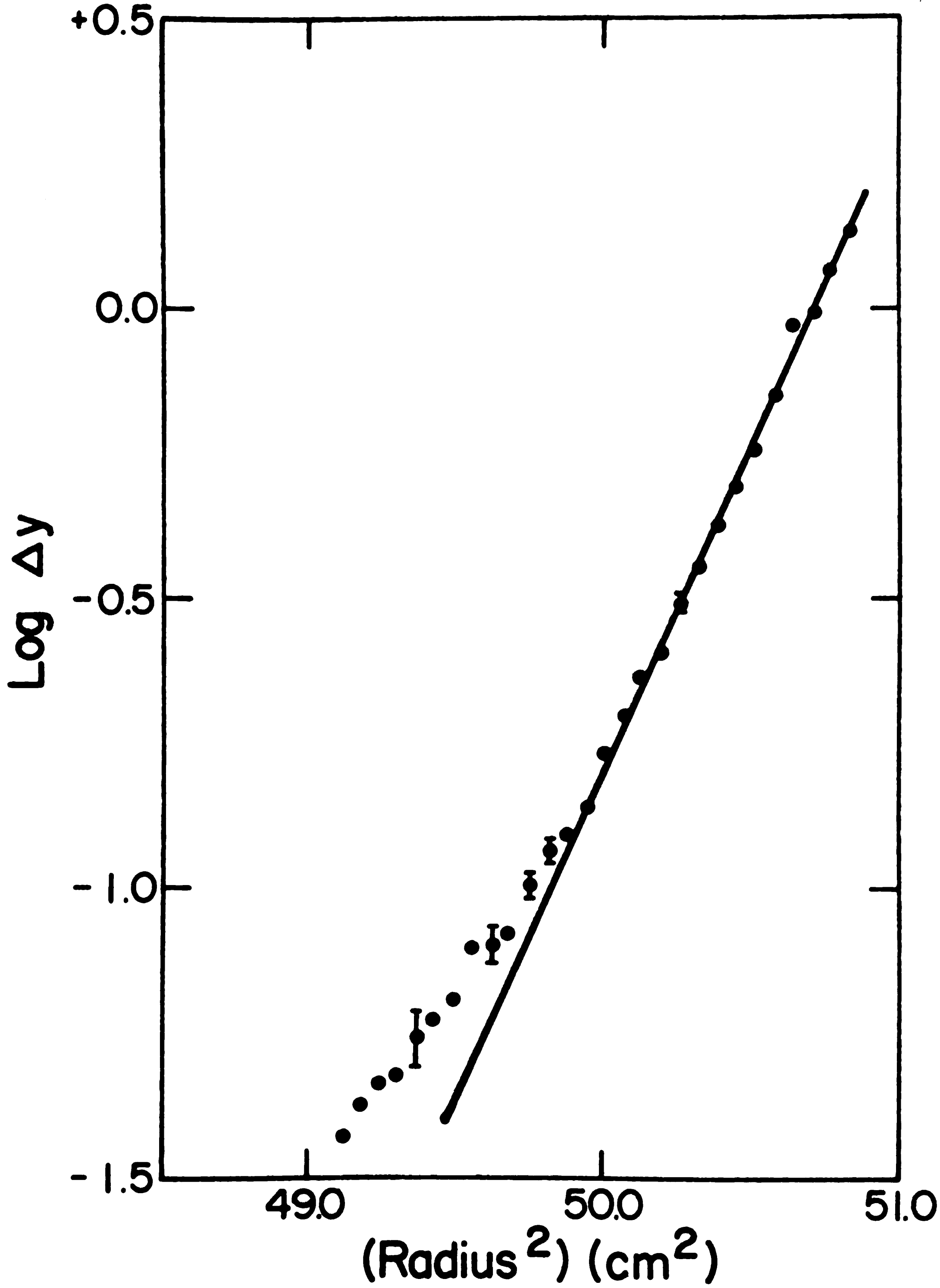

Figure 8.

where

σ_W = 2·slope of the Log (ΔY) versus R^2 plot or 2.266

R = gas constant, 8.314×10^7 erg/degree

ω = 2 RPM/60 π sec min^{-1} or $1.048 \cdot 10^{-1}$ (RPM)$(sec/min)^{-1}$

RPM = 12,590

ρ = density of solvent, 1.0088 g/ml

$\bar{\bar{v}}$ = partial specific volume of solute (0.731 cc/g) determined from amino acid composition (30).

The calculated molecular weight, 278,000, is in good agreement with the molecular weight of 272,000 obtained by Wolfenden *et al*.(30) from $S_{20,W,D}$ data but in poor agreement with the higher molecular weight of 320,000 obtained by Lee (31).

No high molecular weight components attributable to aggregation were present from visual inspection of Figure 8; i.e., there was no deviation from linearity upon approach to the bottom of the cell. However the decreased slope of the experimental points observed at fringe displacements of less than 120 μ indicate the presence of a low molecular weight species. Whether or not dissociation of the enzyme has occurred cannot be established from this data; rigorous analyses at several protein concentrations are required before such a mechanism can be proposed. The effect which ionic strength or K^+ may have upon such a dissociation would be of interest since the enzyme is known to be unstable at ionic strengths of less than 0.5 or in the absence of K^+ (1).

Subunit Molecular Weight

The allosteric kinetics obtained with AMP and several effectors and the large molecular weight suggest the presence of more than one polypeptide chain. The simplest, most rapid method of determining the subunit molecular weight and concurrently the total number of polypeptide chains in a protein is the sodium dodecyl sulfate polyacrylamide disc gel electrophoresis method developed by Shapiro et al.(105). According to Weber and Osborn (87) the accuracy of this method is better than 10% in the molecular weight range of 15,000 to 100,000 and resolution of polypeptide chains differing in molecular weight by 10% can be achieved. Furthermore the electrophoretic mobility of polypeptide chains on SDS gels is independent of the isoelectric point and amino acid composition of the polypeptide chain and dependent only upon the molecular weight.

The electrophoretic patterns obtained for AMP aminohydrolase and three standards on the SDS gels are presented in Figure 9. AMP aminohydrolase (D-F) migrated as one band with the same mobility (2.2 ± 0.1 cm from the origin) as bovine serum albumin (C), a standard with a known molecular weight of 69,000 (106). The subunit molecular weight of

Figure 9. Electrophoretic Patterns for SDS Denatured AMP Aminohydrolase, RNase, DNase, and Bovine Serum Albumin on SDS Polyacrylamide Gels

The electrophoretic patterns observed for three standards and AMP aminohydrolase on SDS polyacrylamide gels after 2.5 hours electrophoresis at 7.5 ma per tube are presented as follows: (A) 20 μg RNase, MW 13,500 (87); (B) 20 μg DNase, MW 31,500 for fast moving band[8]; (C) 20 μg Bovine serum albumin, MW 69,000 (106); (D) 68 μg AMP aminohydrolase; (E) 25 μg AMP aminohydrolase; (F) 52 μg AMP aminohydrolase denatured in SDS buffer plus urea. The direction of electrophoresis is from bottom to top.

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[8] The slow moving band in DNase is a contaminant in the Sigma sample (unpublished data, Dr. J. A. Boezi).

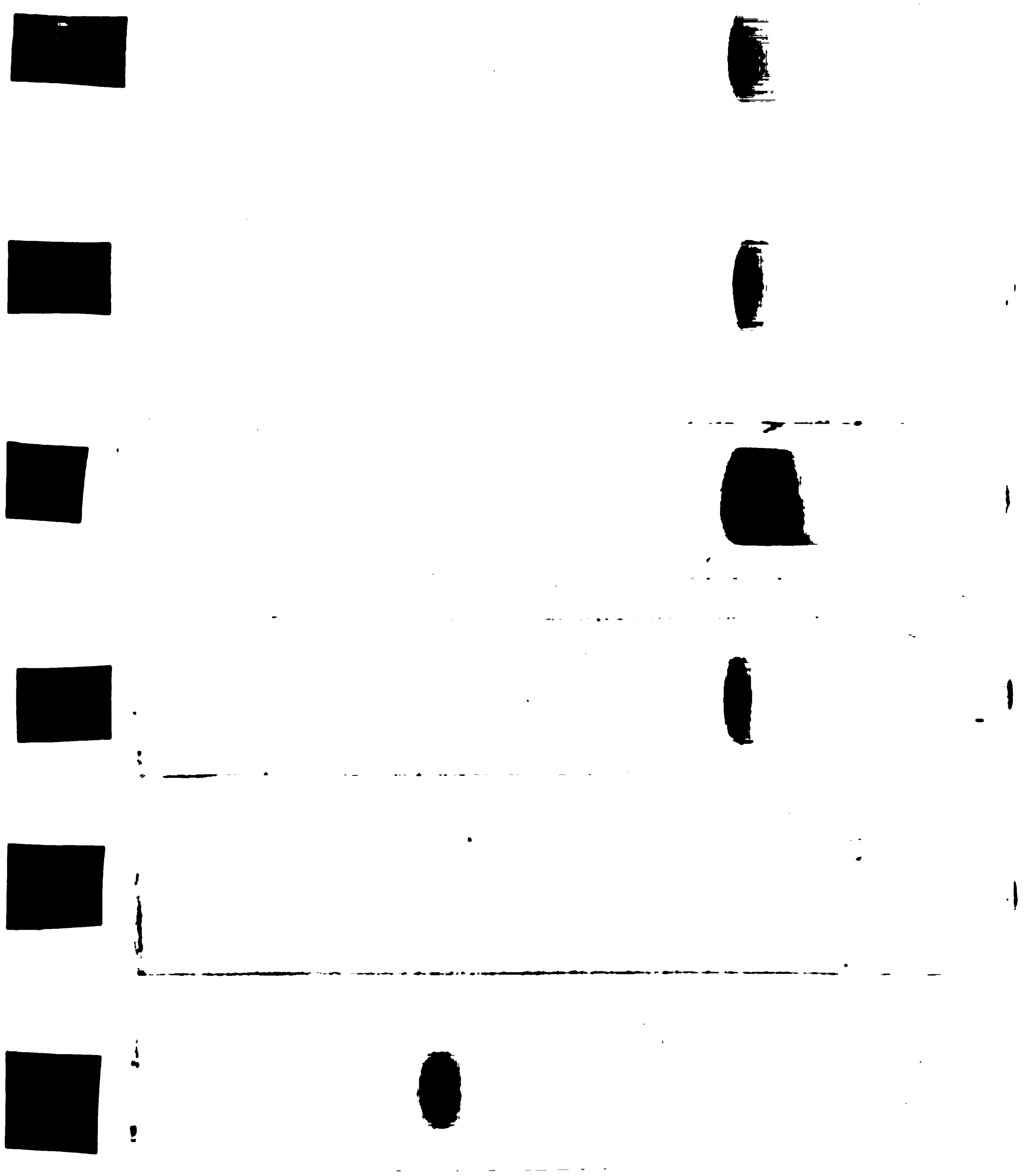

Figure 9.

AMP aminohydrolase is estimated as 69,000. Enzyme incubated in both urea and SDS buffer to insure complete denaturation (F) still moved with the same mobility as enzyme incubated in SDS buffer (D, E). Even at high protein concentrations (D) only one band could be detected for the aminohydrolase. The data are consistent with AMP aminohydrolase being a tetramer of like chains, at least with respect to molecular weight. The molecular weight calculated for the tetramer is 276,000 which is within 2% of the 278,000 obtained by equilibrium centrifugation.

The Divalent Metal Component of AMP Aminohydrolase

The Effect of Carboxylic Acids Upon Activity

Since the ADP activation of AMP aminohydrolase was not observed in the presence of Tris succinate buffer, the effect of several di- and tri-carboxylic acids on activity was investigated under three different conditions (Table 13). Enzyme assayed at 0.52 mM AMP in the absence of activators was especially sensitive to inhibition by succinate, maleate, and citrate while the ADP-activated enzyme was inhibited by citrate, maleate, fumarate, and malonate. The K^+ activated enzyme was less sensitive to carboxylic acid inhibitions: only citrate inhibited more than 20% at 0.01 molar. Acetate, a monocarboxylic acid, did not inhibit.

Table 13. Inhibition of AMP Aminohydrolase by Carboxylic Acids

The enzyme was diluted into 0.5 M salt [$(CH_3)_4NCl$ for the high AMP assay and the ADP activated system and KCl for the potassium activated system], 0.05 M Tris cacodylate pH 6.3, and 2 mM dithioerythritol. The protein concentration in the assay was 0.26 µg/ml.

Carboxylic Acid (Tris salt)	Conc. (mM)	Percent Inhibition		
		High AMP[a]	K^+-Activated[b]	ADP-Activated[c]
Oxalic	1	6	10	
	10	36	21	26
Fumaric	1		5	13
	2.5		12	61
	10	28		
Maleic	1	24	4	17
	7.5		15	78
	10	58		
Citric	1	3	19	43
	10	44	32	82

Succinic	1	13		6
	5		12	
	10	75	21	34
Aspartic	5	15	0	0
	10	29	0	15
	15		14	
Acetic	10	5		6
	15		6	
Malic	1	0		12
	10	52	18	32
Malonic	1			10
	10	24	0	41
	20		18	

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[a] Each assay contained 0.52 mM Tris AMP, 0.10 M TMACl and 0.05 M Tris cacodylate, pH 6.3, in 1 ml volume. Reactions were followed at 285 nm.

[b] Conditions for assay were 50 µM Tris AMP, 0.15 M KCl, and 0.05 M Tris cacodylate, pH 6.3. Reactions were followed at 265 nm.

[c] The ADP-activated system contained 50 µM Tris AMP, 100 uM Tris ADP, 0.1 M TMACl, and 0.05 M Tris cacodylate, pH 6.3. Assays were followed at 265 nm.

The inhibition of this enzyme by di- and tricarboxylate anions contradicts previously reported results obtained in Na^+ or K^+ succinate buffers. According to Nikiforuk and Colowick (35) citrate, acetate, and lactate activated AMP aminohydrolase assayed at pH 5.9; Lee could not reproduce their data (2). Their failure to observe inhibition may be attributed to the presence of Na^+ or K^+ in the assay since in this case the K^+ activated enzyme was also relatively insensitive to carboxylate anions. Furthermore, the failure to observe activation by ATP or ADP in the earlier studies may have been due to the presence of succinate (2), which at 0.1 M completely inhibits the ADP activation. The insensitivity of the calf brain AMP aminohydrolase to such anions (28) may reflect species or tissue differences.

The effect of citrate on ADP activation is presented as a Woolf plot of $[ADP]/v_i$ versus $[ADP]$ at constant levels of citrate and a suboptimal substrate concentration of 50 μM (Figure 10). While interpretation of such data for an allosteric enzyme is difficult, citrate does not change the observed maximum velocity of the ADP activated AMP deamination as indicated by the identical slopes obtained in the Woolf plot. Furthermore the lines are linear except for slight curvature in the absence of citrate. Thus the data are consistent with a competitive interaction between citrate and ADP. From the intercept

Figure 10. The Effect of Citrate on ADP Activation of AMP Aminohydrolase

The data are presented in a Woolf plot, [ADP]/v_i versus [ADP] at constant levels of citrate where v_i represents the initial velocity of the inhibited reaction. The reaction was followed at 265 nm in 0.15 M TMACl, 0.05 M Tris cacodylate, pH 6.3, 50 μM Tris AMP and variable amounts of ADP. Tris citrate concentrations were as follows: ○ - no citrate; ● - 0.1 mM; □ - 0.175 mM; ■ - 0.25 mM; and △ - 0.50 mM. Each point represents the average of 2 or more assays at 0.15 μg enzyme per ml. The insert represents a plot of the [ADP]/v_i intercepts versus citrate concentration. The K_i for citrate was determined from the intercept on the citrate axis.

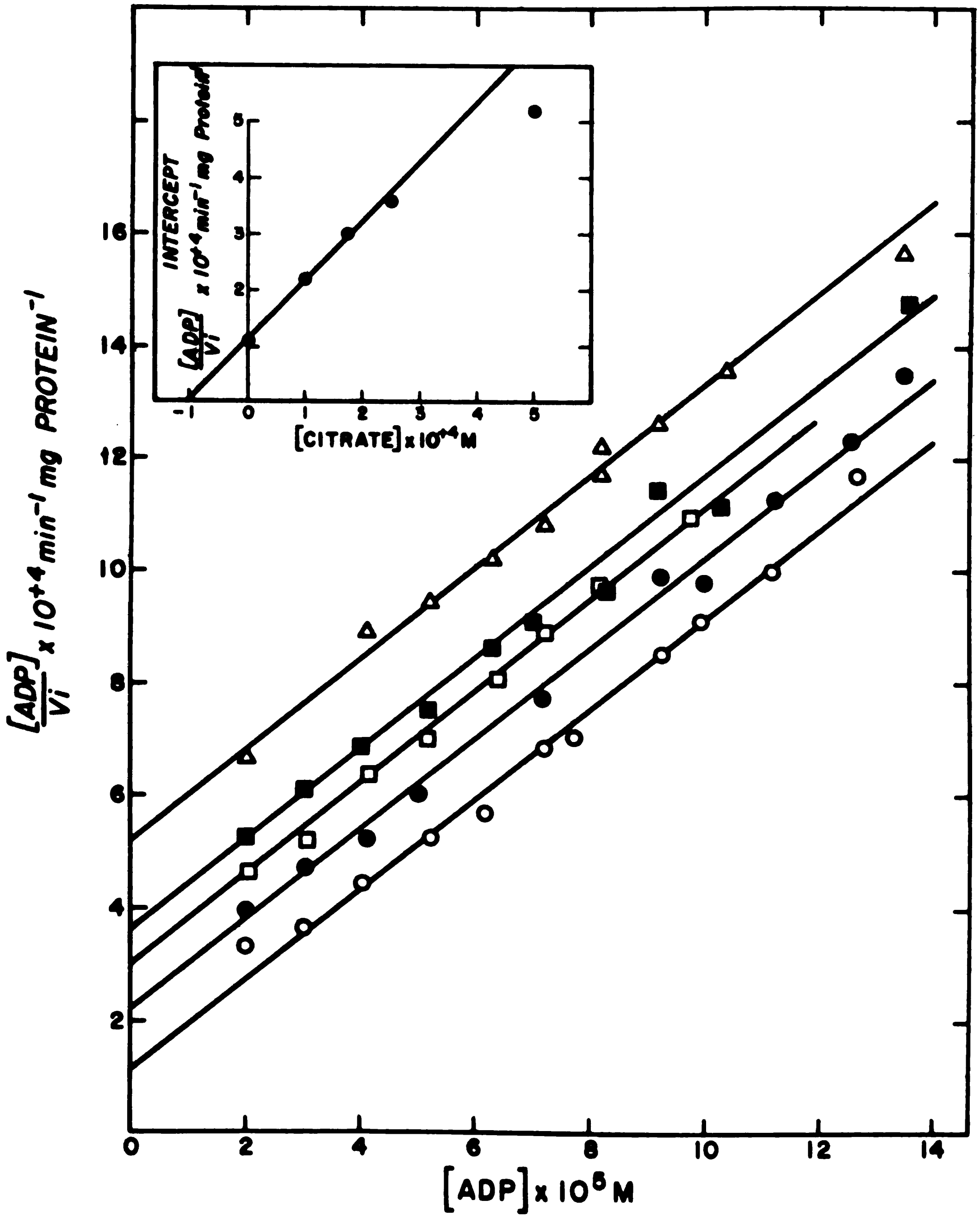
INTERCEPT
[ADP]/Vi x 10+4 min-1 mg Protein-1
5
4
3
2
1
-1
0
1
2
3
4
5
[CITRATE] x 10+4 M
[ADP]/Vi x 10+4 min-1 mg PROTEIN-1
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
[ADP] x 10^5 M

Figure 10.

on the [citrate] axis of the line drawn through a plot of the [ADP]/v_i intercepts versus citrate concentration, a K_i for citrate inhibition of 0.11 mM was determined.

The Hill slope for ADP activation in the presence of increasing citrate concentrations decreased from 1.7 to 1.1 (Figure 11) while the $K_{a_{app}}$ for ADP calculated from the Hill plot increased: $K_{a_{app}}$ for ADP was 22 µM, 30 µM, 36 µM, 42 µM, and 63 µM at 0, 0.10 mM, 0.175 mM, 0.25 mM, and 0.50 mM citrate, respectively. Perhaps citrate is binding at the ADP site eliminating the cooperativity observed for ADP activation.

Citrate and ADP, although dissimilar in structure, do possess common metal binding properties; consequently citrate may inhibit ADP activation by interaction with an enzyme bound cation necessary for activator binding. Although other mechanisms of inhibition could be proposed such as dissociation of subunits or conformational changes, the presence of a metal ion component seemed, intuitively at least, a good possibility.

Inhibition[9] of AMP Aminohydrolase by Metal Binding Agents

AMP aminohydrolase, in either KCl or TMACl at pH 7.1 was inhibited by several metal binding agents including

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[9] In this thesis inhibition is defined as any loss of activity caused by metal binding agents. Theoretically there are two mechanisms by which an inhibition can be realized: (A) binding of the inhibitor *in situ* or (B) removal of the metal from the protein. In either case

Figure 11. Hill Plot for Citrate Inhibition of ADP Activation

The data from Figure 8 for ADP activation at various levels of the inhibitor, citrate, are plotted according to the Hill equation: Log $[(V_{max}/v_i) - 1]$ versus Log [ADP] M, where V_{max} is the observed maximum velocity of the reaction at 50 μM Tris AMP and v_i is the velocity of the inhibited reaction.

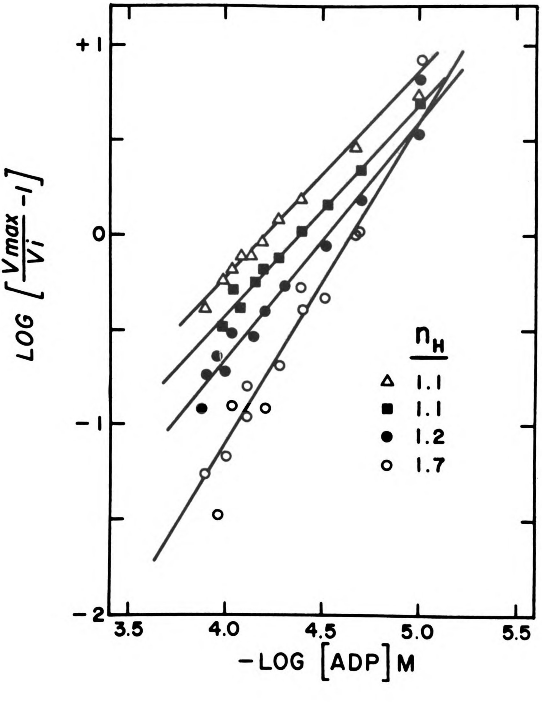
LOG $\left[\frac{V\,max}{V_i}-1\right]$
+1
0
−1
−2
3.5
4.0
4.5
5.0
5.5
−LOG [ADP] M
n_H
△ 1.1
■ 1.1
● 1.2
○ 1.7

Figure 11.

o-phenanthroline, ethylenediaminetetraacetate, citrate, 8-hydroxyquinoline-5-sulfonate, dithioerythritol, and mercaptoethanol (see Figures 12-15). In all cases enzyme in the absence of K^+ was more sensitive to inhibition by the metal binding agents. For example, 0.18 mM OP inhibited the enzyme in TMACl to the same extent as 1.0 mM OP in KCl; in 0.67 mM 8-OHQ5SA the inhibition in TMACl was 78% versus 37% in KCl during the same time span. Although 50 mM citrate inhibited the enzyme in TMACl, this concentration of citrate did not inhibit the enzyme in KCl (Figure 13). This greater sensitivity to metal binding agents in the absence of K^+ may reflect a weaker binding constant of the enzyme for its metal component or greater accessibility of the chelating agent to the metal as a result of a conformational change. A second possibility is that a significant amount of the inhibitor(s) may be complexed with K^+ thereby reducing the available concentration of inhibitor(s). No stability constants for potassium complexes with the metal binding agents used in this study were available; however, if one assumes a K_f for $KCitrate^{2-}$ similar to $KADP^{2-}$ ($\sim 5\ M^{-1}L$) (107) the free

---

inhibition may be reversible or irreversible. The loss of enzyme activity may be restored upon exposure to environmental conditions similar to those under which the enzyme was exposed to the inhibitor. In mechanism (B) the reversibility is dependent upon the ability of the apoprotein to reassociate with the metal once dissociation occurs. Irreversible inhibition (sometimes referred to as <u>inactivation</u>) results from an irreversible loss of activity by denaturation or by covalent binding at the active site. For further discussion see Reference 119.

Figure 12. Semilogrithmic Plot of o-Phenanthroline Inhibition of AMP Aminohydrolase as a Function of Time

AMP aminohydrolase (0.10 mg protein/ml) was incubated in 0.5 M salt (KCl or TMACl), 0.10 M Tris Mes, pH 7.1, and 1 mM mercaptoethanol at 30°. Aliquots were removed at the intervals indicated for assay in Assay 1 containing 50 µM Tris AMP (A) or Assay 2 containing 50 µM Tris AMP and 100 µM Tris ADP (B). The concentrations of metal binding agents in the KCl incubation mixtures (A) were ○ - control, X - 0.6 mM m-phenanthroline, △ - 1 mM OP, and □ - 2 mM OP. For the TMACl incubation mixtures (B) the concentrations were ● - control, + - 0.18 mM m-phenanthroline, ▲ - 0.05 mM OP, and ■ - 0.18 mM OP. The degree of reactivation during the assay is represented by the broken lines: --- 1 mM OP and -•- 2 mM OP in A.

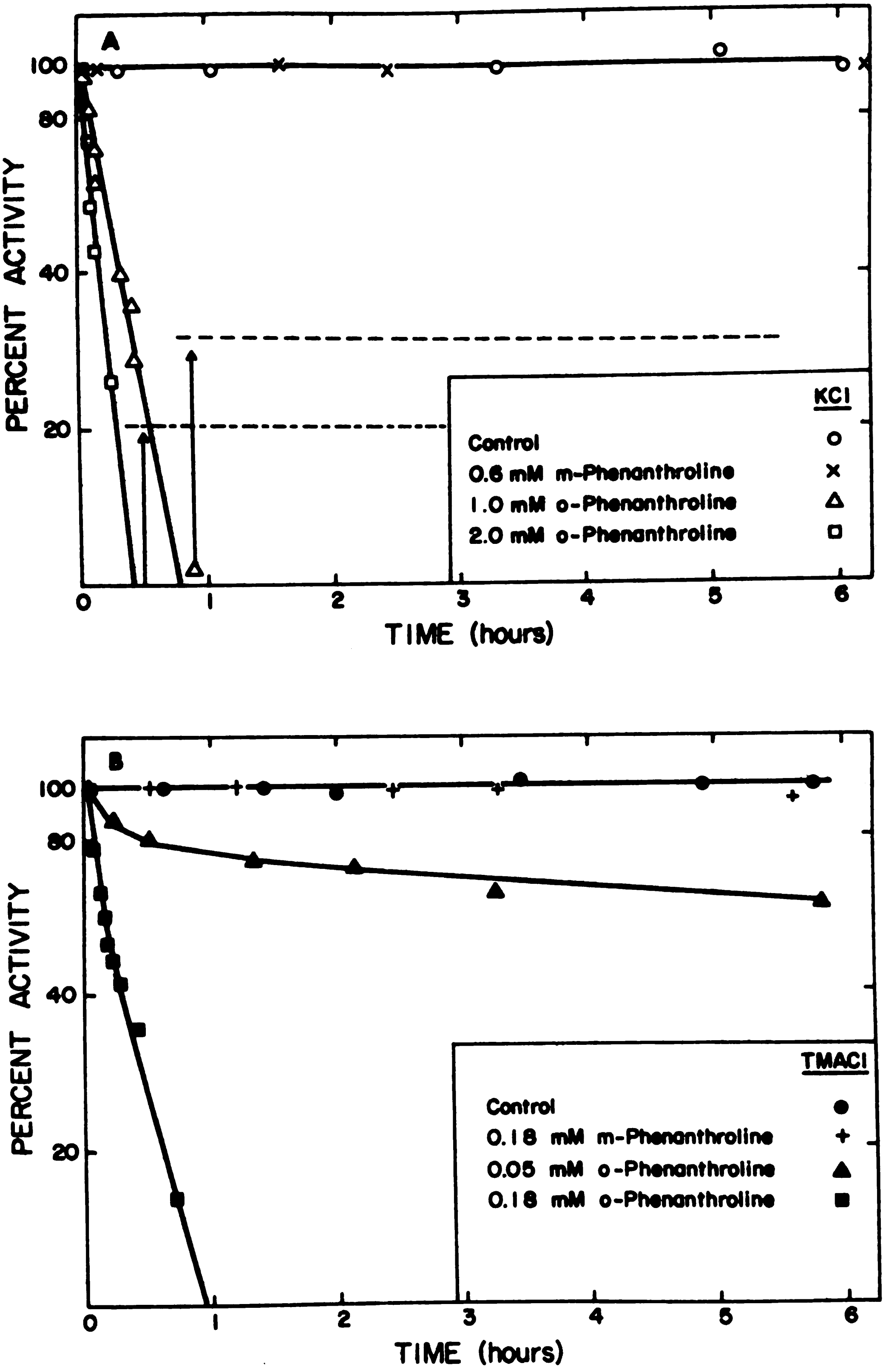
A
PERCENT ACTIVITY
100
80
40
20
0
1
2
3
4
5
6
TIME (hours)
KCl
Control ○
0.6 mM m-Phenanthroline ×
1.0 mM o-Phenanthroline △
2.0 mM o-Phenanthroline □
B
PERCENT ACTIVITY
100
80
40
20
0
1
2
3
4
5
6
TIME (hours)
TMACl
Control ●
0.18 mM m-Phenanthroline +
0.05 mM o-Phenanthroline ▲
0.18 mM o-Phenanthroline ■

Figure 12.

Figure 13. **Semilogarithmic Plot of Citrate and EDTA Inhibition of AMP Aminohydrolase as a Function of Time**

Conditions are described in Figure 12. Concentrations of metal binding agent in the incubation mixture were as follows: ○,● - control; □,■ - 50 mM Tris Citrate; △,▲ - 100 mM and 60 mM Tris EDTA, respectively. The open symbols represent the KCl system and the closed symbols the TMACl system.

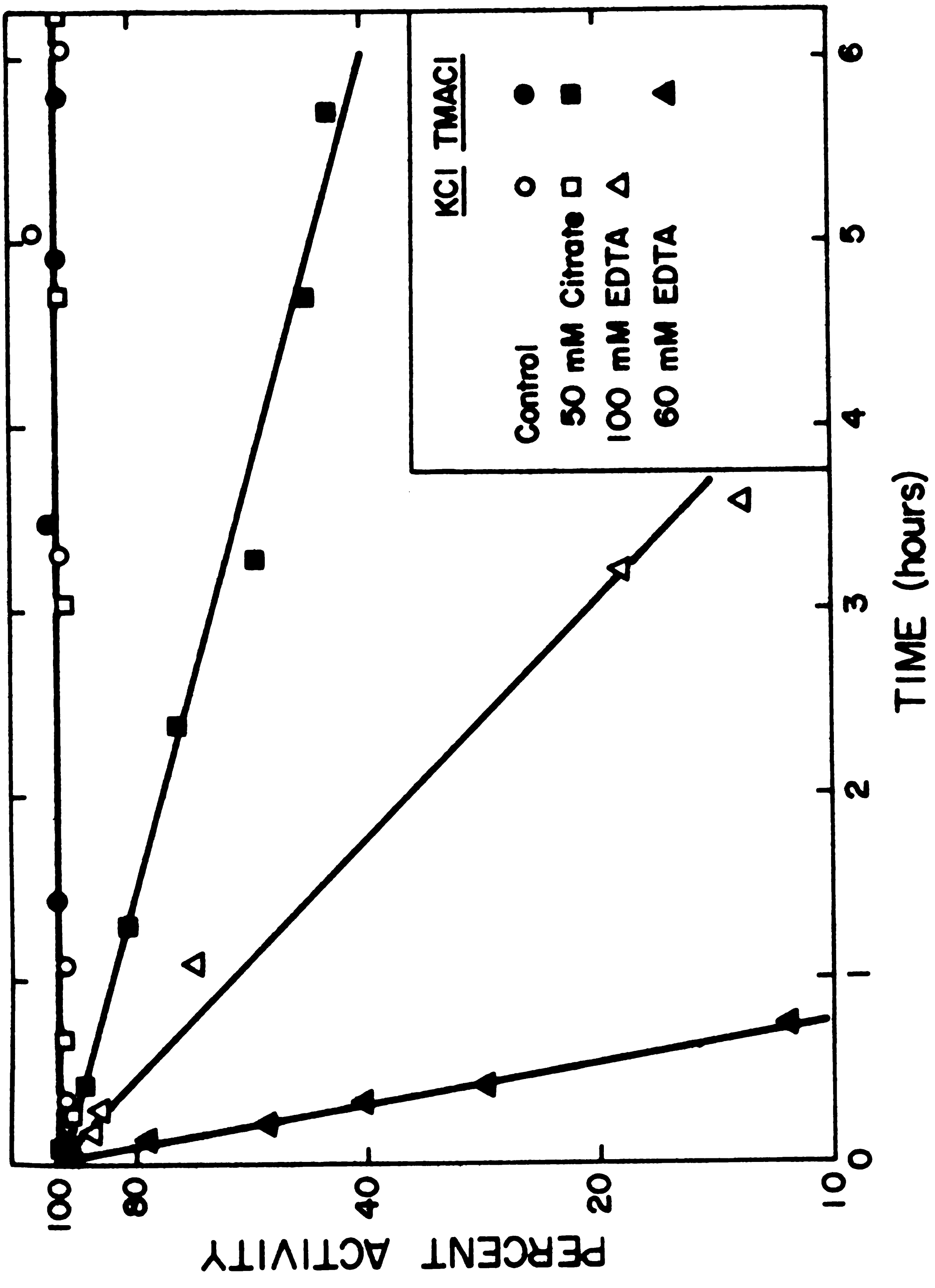
PERCENT ACTIVITY
100
80
40
20
10
TIME (hours)
0
1
2
3
4
5
6
KCl TMACl
Control ○ ●
50 mM Citrate □ ■
100 mM EDTA △
60 mM EDTA ▲

Figure 13.

Figure 14. Semilogarithmic Plot of 8-OHQ5SA Inhibition of AMP Aminohydrolase as a Function of Time

Enzyme was treated and assayed as described in Figure 12. The concentrations of inhibitor in the incubation mixture were as follows: ○ , ● - control; ▲ - 0.2 mM 8-OHQ5SA; and □ , ■ - 0.67 mM 8-OHQ5SA. The open symbols represent the KCl system and the closed symbols represent the TMACl system.

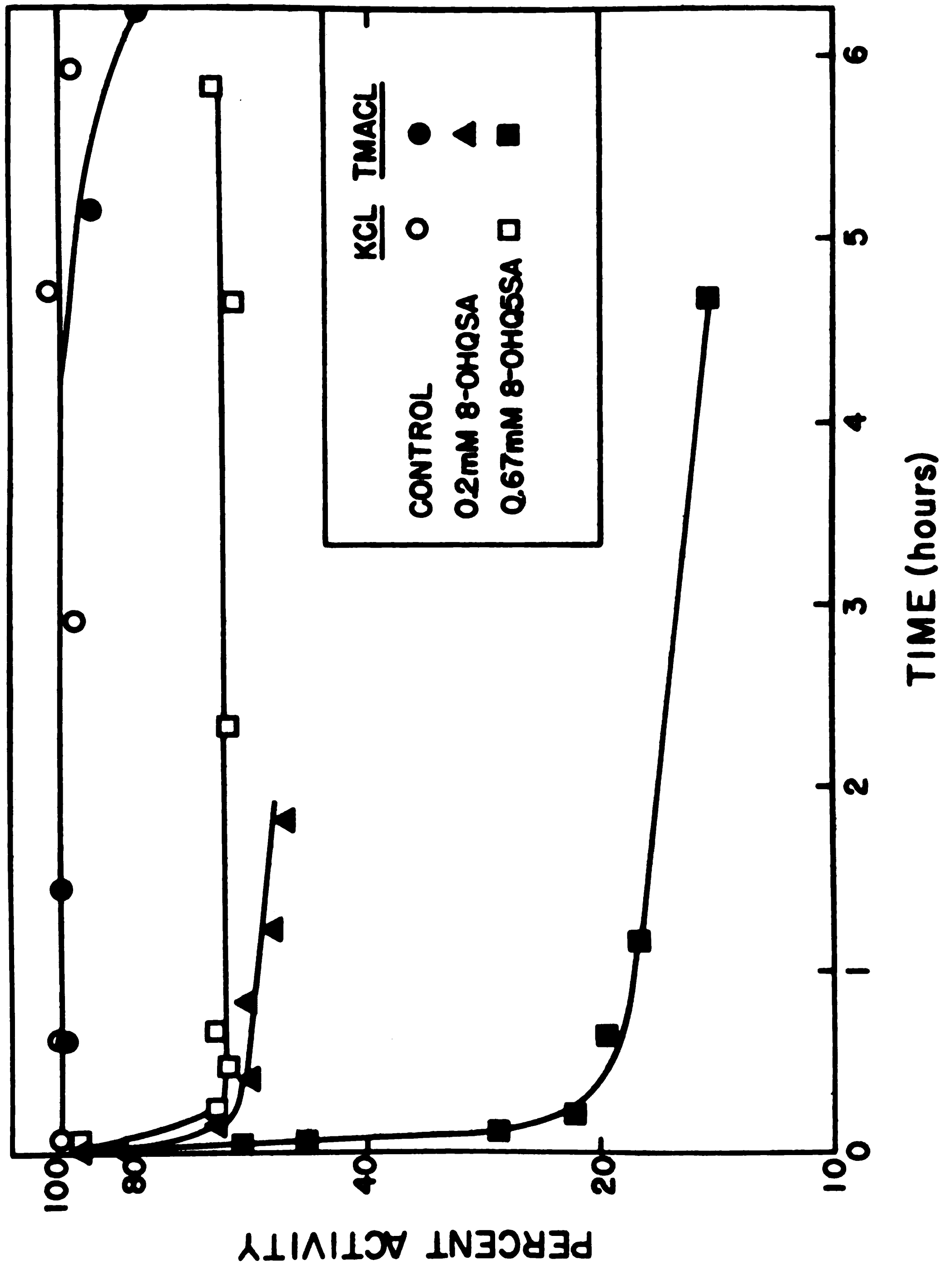
KCL
TMACL
CONTROL ○ ●
0.2mM 8-OHQSA ▲
0.67mM 8-OHQ5SA □ ■
PERCENT ACTIVITY
100
80
40
20
10
TIME (hours)
0
1
2
3
4
5
6

Figure 14.

Figure 15. Semilogarithmic Plot of the Inhibition of AMP Aminohydrolase by Thiol Compounds as a Function of Time

Conditions for incubation are described in Figure 12. The following concentrations of thiol compounds were used: ○, ● - 1 mM mercaptoethanol (control); ▼- 40 mM mercaptoethanol; □, ■ - 1 mM DTE; and △, ▲ - 10 mM DTE.

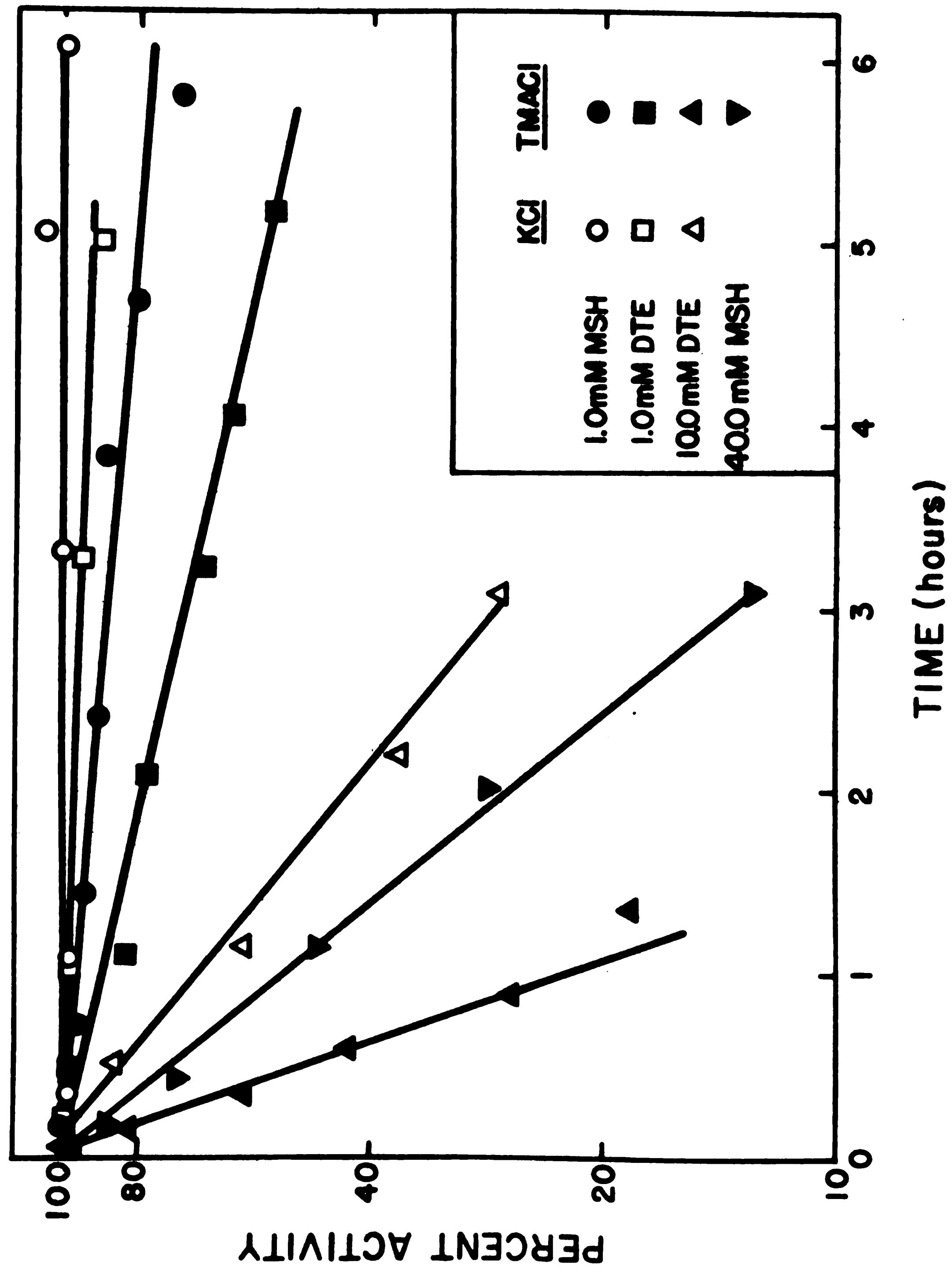
PERCENT ACTIVITY
100
80
40
20
10
0
1
2
3
4
5
6
TIME (hours)
KCl
TMACl
1.0mM MSH ○ ●
1.0mM DTE □ ■
10.0mM DTE △ ▲
40.0mM MSH ▼

Figure 15.

citrate concentration at 50 mM total citrate and 0.5 M KCl is reduced to 15 mM. Such a reduction in free citrate concentration might account for the decreased sensitivity of the enzyme to citrate inhibition in KCl.

In the study of OP inhibition in KCl the enzyme was reactivated upon dilution in the assay mixture; the extent of reactivation depended upon the concentration of OP in the incubation mixture. Although this reactivation phenomenon was not explored further, two explanations can be proposed: either the inhibition by OP is truly reversible upon dilution or the presence of a trace metal(s) in the assay mixture may have removed some of the OP inhibition through competition with the metal-enzyme for OP or displacement of a metal$[OP]_x$ complex from the enzyme (119).

Meta-phenanthroline did not inactivate AMP-aminohydrolase, thus binding of the phenanthroline ring system to the enzyme is not responsible for the observed inhibition by OP. The inhibition by OP via sulfhydral oxidation, as was shown to be the case with rabbit muscle aldolase (108), seems unlikely since mercaptoethanol which protects against such inhibition was always present in the protein incubation mixtures.

Although AMP aminohydrolase absolutely requires thiols such as mercaptoethanol for stability (1), an excess of either DTE or mercaptoethanol caused a time dependent inhibition (Figure 15). The contradictory effects of thiols can be explained by (A) a sensitive sulfhydral protected from oxidation by low concentrations of thiols and (B) the relative ability of thiols and dithiols to bind metals (109, 110). An additional possibility that high concentrations of thiols reduce disulfide bonds cannot be eliminated with the present data. Mercaptoethanol, a monothiol, was a less effective inhibitor than DTE, a dithiol. For example, after one hour in the TMACl incubation mixture, 10 mM DTE inhibited the enzyme 78% whereas 40 mM mercaptoethanol inhibited only 50%. The data are consistent with the ability of dithiols to form stable bidentate chelates with divalent metals (109).

The inhibition of AMP aminohydrolase by metal binding agents possessing widely different ligands is consistent with the enzyme requiring a metal for activity. Since many known zinc metalloenzymes are subject to inhibition by thiols such as DTE, mercaptoethanol, cysteine, and 2,3-dimercaptoethanol (111-115), zinc is a good possibility for the metal component of AMP aminohydrolase. Direct evidence that the enzyme contained zinc or another metal was obtained by quantitative metal analysis.

Quantitative Metal Analyses of Metal Content of AMP Aminohydrolase

Purified AMP aminohydrolase and an aliquot of the crude extract were analyzed for the presence of seven transition metals by atomic absorption spectroscopy and neutron activation analysis. The average of the analyses for two preparations are presented in Table 14. In order to eliminate metal ion contamination, the enzyme was purified using the precautions outlined in Materials and Methods.

Zinc increased from 0.7 gram atom per mole enzyme in the crude extract to 2.58 gram atoms per mole after purification based on a molecular weight of 278,000 for the native enzyme. Extrinsic metals such as calcium, magnesium, and iron, although present in significant quantities in the crude extract, were not found in stoichiometrically significant amounts in the purified enzyme. Neither were significant amounts of metals found in the TMACl solutions used for dialysis and Sephadex chromatography.

The neutron activation analyses for cobalt, manganese, and copper were reported as minimum detection limits for an instrumental analysis without radiochemical separations. In each case the detection limit for the blank was higher than that for the sample: cobalt, <35 ppb blank and <20 ppb sample; copper, <250 ppb blank and <100 ppb sample; and manganese, <350 ppb blank and <300 ppb sample. The detection limits for copper and manganese were too high to rule out the presence of 0.4 gram atom copper or 1.5 gram atoms of manganese in the samples. However, for cobalt the detection limit was low enough to eliminate the

Table 14. Metal Content of the Crude Extract and Purified AMP Aminohydrolase[a]

Fraction	Crude Extract[b]		Purified AMP Aminohydrolase[c]	
Metal	Micro Grams per Gram Protein	Gram Atoms per Mole Enzyme	Micro Grams per Gram Protein	Gram Atoms per Mole Enzyme
Zn	166 ± 13	0.705	605 ± 10	2.58
Ca	96 ± 14	0.67	<10	<0.07
Mg	46 ± 8	0.53	< 5	<0.06
Fe	78 ± 2	0.39	≤50[d]	≤0.25[d]
Cu	5	0.03	[e]	
Co	ND[f]		[e]	
Mn	ND		[e]	

[a] Reported as the average of the determinations for two enzyme preparations based on a molecular weight of 278,000 for the native enzyme.

[b] An aliquot of the Supernatant after centrifugation of the crude muscle extract was treated as described in Materials and Methods before analysis.

[c] The pooled fractions from the cellulose phosphate column were dialyzed against KCl buffer and chromatographed on Sephadex G-25 as described in Materials and Methods.

[d] The instrumental detection limit for Fe was 0.05 ppm. Since Fe was not detected at 1 mg protein/ml, the Fe content is reported as less than or equal to this minimum.

[e] Samples were analyzed by Neutron Activation. The values obtained for the blank solutions were greater than those obtained for the samples. See text for further discussion.

[f] ND, not determined.

presence of greater than 0.1 gram atom cobalt per mole enzyme.

The enzyme containing 2.58 gram atoms zinc per mole enzyme was fully active with respect to previous preparations of the enzyme: the K_m for AMP and V_{max} were 0.4 mM and 1345 µm/min/mg protein, respectively (Assay 1). ADP activation was not as great since the specific activity in Assay 2 was only 70 µm/min/mg protein versus the specific activity of 110-130 µm/min/mg protein usually observed. Considering the non integral amount of zinc found upon metal analysis, it is conceivable that some zinc was lost during the purification procedure with a resultant decrease in ADP activation. Loss of ADP activation without a concurrant loss in maximum activity for the K^+-activated enzyme is consistent with two different types of zinc.

Divalent Metal Ion Requirement for Enzymatic Activity

Removal of Zinc from AMP Aminohydrolase and the Concurrent Loss of Enzymatic Activity

The trace metal analyses and inhibition by metal binding agents suggest that AMP aminohydrolase contains strongly bound zinc. However the data does not establish that zinc is required for activity. Therefore it is necessary to establish that the enzyme loses activity as a function of the loss of zinc.

In the presence of excess 8-OHQ5SA, zinc and 8-OHQ5SA form a 1:3 complex which exhibits a distinct absorption maximum at 370 nm (see Figure 16) with an extinction coefficient of 11.1 x 10^3 OD/mm and a stability constant, Log β_3, of 20.[10] The absorption obeys Beer's law; i.e., at excess 8-OHQ5SA the absorption of the complex is a linear function of zinc concentration (data not shown). Since AMP aminohydrolase was rapidly inhibited by 8-OHQ5SA, the method described by Simpson and Vallee (88) was adapted to correlate the loss of enzymatic activity with formation of a Zn[8-OHQ5SA]$_3$ complex.

AMP aminohydrolase (3.59 x 10^{-6}M) was titrated with Tris 8-OHQ5SA as described in Materials and Methods. Addition of this chelator resulted in a rapid inhibition the extent of which was dependent on the amount added (see Figure 14). The extent of inhibition is presented as a plot of Percent Activity Remaining versus Moles of Zn[8-OHQ5SA]$_3$ formed (Figure 17). Ninety percent of the activity was lost after formation of 3 moles of Zn[8-OHQ5SA]$_3$ per mole enzyme. After the loss of 3.5 moles of zinc turbidity prevented further measurements. There is some disagreement between the 3 to 3.5 gram atoms of zinc titrated by 8-OHQ5SA and the 2.58 gram atoms of zinc per mole of native enzyme as previously determined. However, the theoretical absorbance for the Zn [8-OHQ5SA]$_3$

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[10] Private communication, B. L. Vallee.

Figure 16. The Visible and UV Spectrum of 8-OHQ5SA Before and After the Addition of Zinc Sulfate

The spectra were recorded from 500 nm to 220 nm with a Beckman DB Spectrophotometer equipped with a Sargent Recorder. The sample and reference cells contained 0.4 M TMACl and 0.1 M Tris Mes, pH 7.2. The ——— represents the spectrum of 0.18 mM 8-OHQ5SA and the ----- represents the spectrum of 0.18 mM 8-OHQ5SA plus 0.02 mM zinc sulfate.

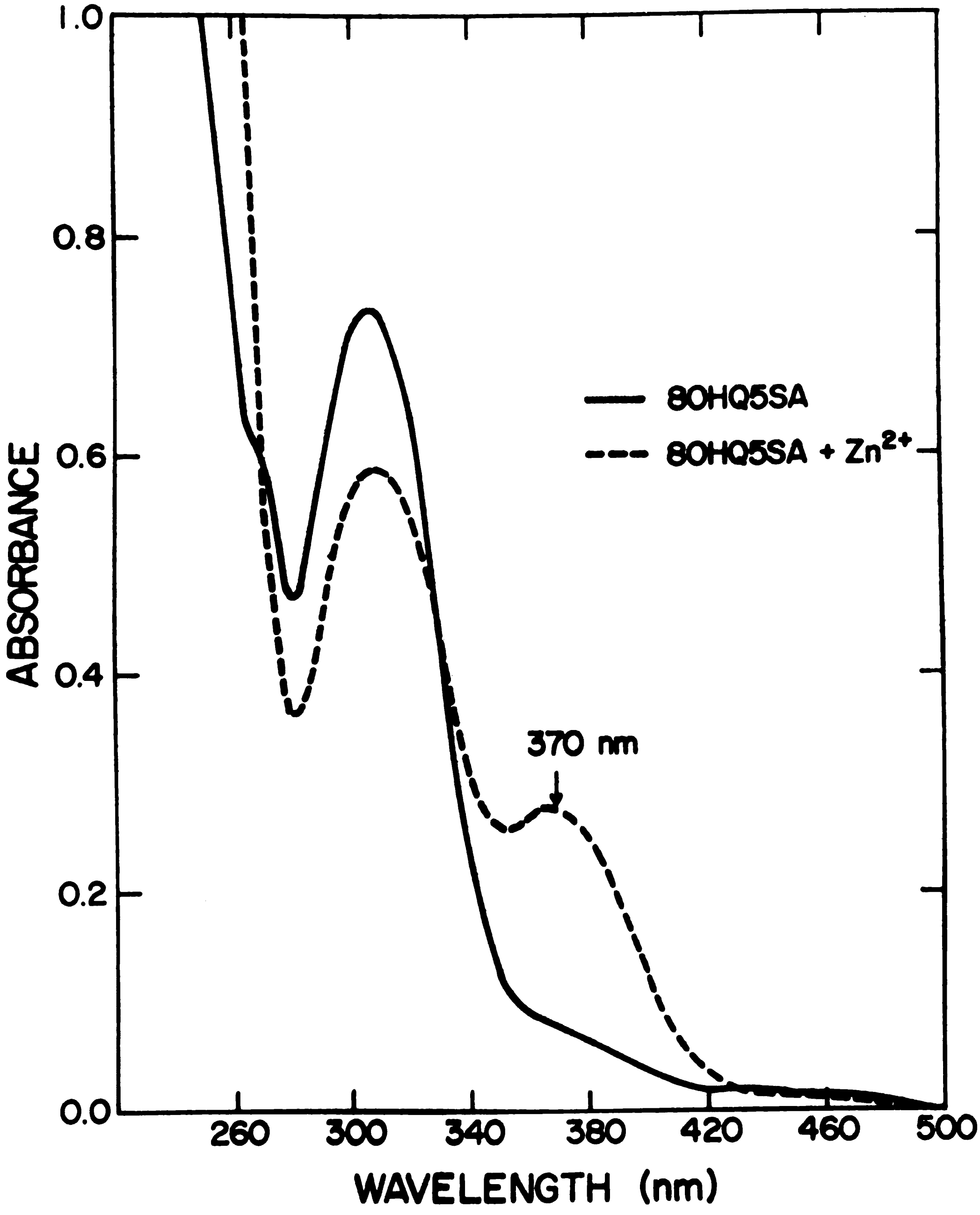
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
260
300
340
380
420
460
500
WAVELENGTH (nm)
8OHQ5SA
8OHQ5SA + Zn²⁺
370 nm

Figure 16.

Figure 17. Correspondence Between Activity of AMP Aminohydrolase and the Formation of the Zn[8-OHQ5SA]$_3$ Complex

The percent enzymatic activity remaining as a function of the Zn[8-OHQ5SA]$_3$ complex formed is presented. Enzyme was chromatographed on Sephadex G-25 equilibrated with 0.5 M salt (KCl or TMACl), 0.10 M Tris Mes, pH 7.1, and 1 mM mercaptoethanol. Increments of 8-OHQ5SA were added to 3.6×10^{-6}M enzyme under conditions described in Materials and Methods and the absorption at 370 nm was recorded; aliquots were withdrawn and diluted for assay after each addition. The activity of the enzyme incubated in KCl (--○--) before the addition of inhibitor was 108 μm AMP deaminated/min per mg protein at 50 μM AMP; the specific activity of enzyme incubated in TMACl (--●--) was 65 μm/min per mg protein in the same assay. The assay mixture was passed over Chelex 100 (Tris$^+$) prior to use to remove contaminating metal ions.

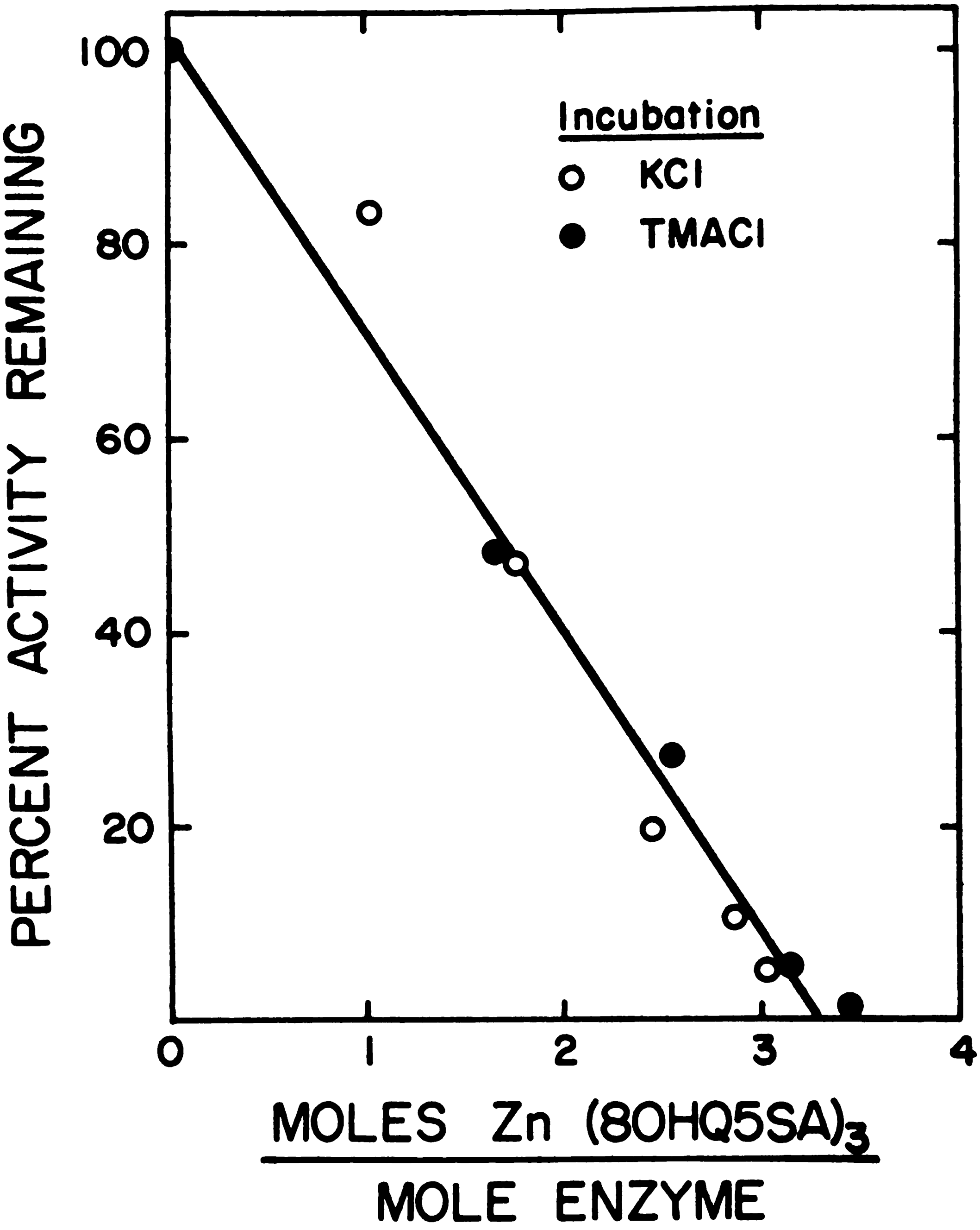
PERCENT ACTIVITY REMAINING
100
80
60
40
20
0
1
2
3
4
MOLES Zn (8OHQ5SA)$_3$ / MOLE ENZYME
Incubation
○ KCl
● TMACl

Figure 17.

complex, assuming 4 gram atoms zinc per mole enzyme (at 3.59×10^{-6} mm enzyme per ml) is only 0.17 OD units. With such a low total absorbance some error can be expected. The data are consistent with zinc being required for enzymatic activity and that loss of activity is due to complexation by 8-OHQ5SA.

To establish that zinc is indeed removed from the enzyme in contrast to formation of an inactive ternary complex, purified enzyme incubated in 8-OHQ5SA was chromatographed on Sephadex G-25 to separate the enzyme from excess 8-OHQ5SA and the $Zn[8\text{-}OHQ5SA]_3$ complex. The recovered enzyme generally exhibited ~15% of the activity of the native enzyme at 10 mM AMP and contained 0.45 gram atoms of zinc per mole enzyme as determined by atomic absorption analysis. The 15% residual activity closely corresponded to the 17% residual zinc based upon the 2.58 gram atoms zinc per mole native enzyme; thus the lower activity is consistent with the loss of zinc from the enzyme rather than formation of an inactive ternary complex.

Reactivation of Apo-AMP Aminohydrolase by Transition Metal Ions

Since AMP aminohydrolase could be obtained relatively free of zinc, reconstitution of the enzyme by the addition of zinc and other transition metals was attempted.

The apoenzyme was titrated with spectropure sulfate salts of zinc, cobalt, manganese, iron, nickel, magnesium, copper, and cadmium; activity at each concentration of metal was determined at 10 mM AMP. The gram atoms of metal added per mole of enzyme varied from 0 to 4 or 10. Zinc reactivation (Figure 18) approached 70-80% that observed for native enzyme. Cobalt, iron, and manganese, in order of decreasing effectiveness, also reactivated the apoenzyme (Figure 19). Nickel, magnesium, copper, and cadmium were ineffective in restoring activity to the apoenzyme.

Kinetic Properties of the Zinc, Cobalt, and Manganese AMP Aminohydrolase

Since the replacement of zinc by other transition metals alters the kinetics and specificity of other zinc metallo enzymes (116, 117), the kinetic parameters for AMP deamination in KCl and for ADP activation in TMACl were investigated for the zinc, cobalt, and manganese reconstituted enzyme and compared with those of the native and apoenzyme (see Table 15). The ferrous enzyme was too unstable to obtain reliable data.

An aliquot of apoenzyme reconstituted by addition of 3.7 gram atoms metal sulfate per 278,000 grams of enzyme was chromatographed on Sephadex G-25 to remove excess divalent cations and potassium chloride. The zinc and cobalt enzymes were 20 to 30% more active after chromatography, however only 60-70% of the protein applied to the column was recovered. These observations are consistent

Figure 18. Reactivation of Apo-AMP Aminohydrolase by Titration with Spectropure Zinc Sulfate

The apoenzyme was reactivated at pH 7.1 as described in Materials and Methods. Atomic absorption analysis indicated that the apoenzyme contained 0.45 gram atoms zinc per mole enzyme, therefore the titration curve is corrected for this residual amount of zinc. Enzyme activity was measured with assay 1 containing 10 mM Tris AMP. All reagents including the assay mixture had been treated with Chelex 100 ($Tris^+$) to remove contaminating metal ions.

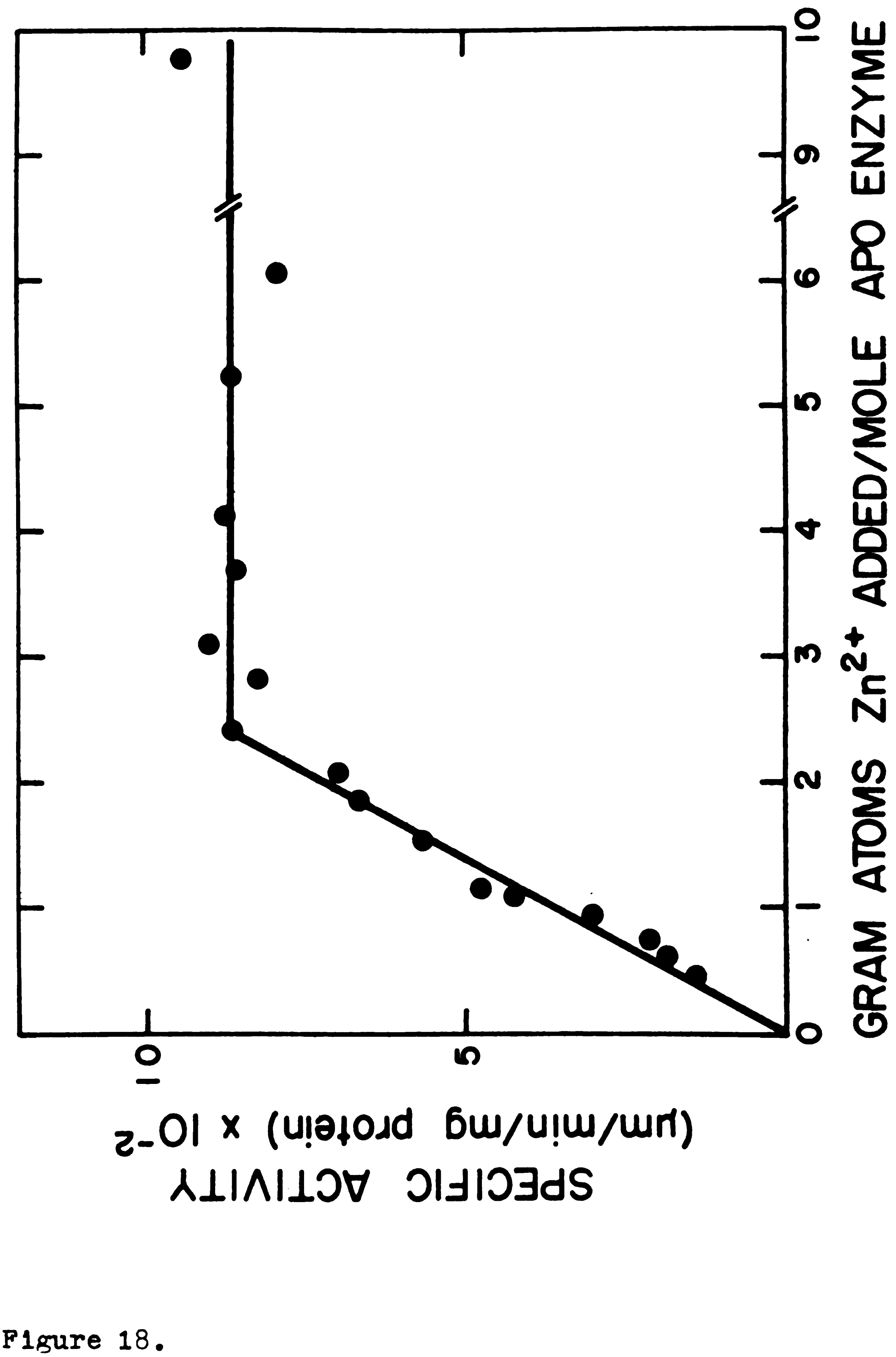
GRAM ATOMS Zn^{2+} ADDED/MOLE APO ENZYME
0
1
2
3
4
5
6
9
10
SPECIFIC ACTIVITY
(μm/min/mg protein) x 10^{-2}
5
10

Figure 18.

Figure 19. Reactivation of Apo-AMP Aminohydrolase by Titration with Spectropure Transition Metal Sulfates

Conditions of reactivation are described in Figure 18 and Materials and Methods. Since the apoenzyme used in this study was not analyzed for the presence of zinc, the data were not corrected for residual zinc and thus the plots do not pass through zero activity.

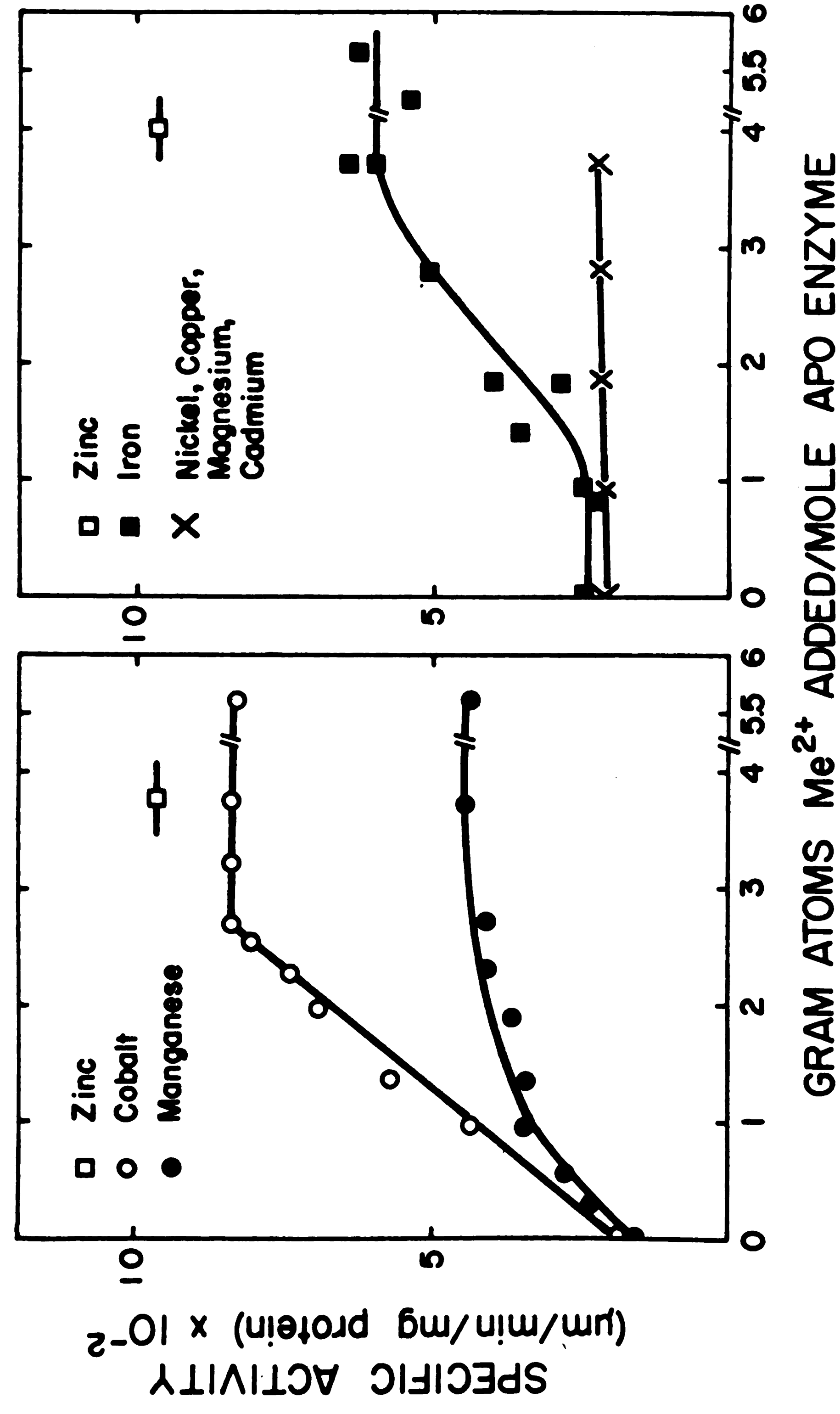
SPECIFIC ACTIVITY
(µm/min/mg protein) x 10^{-2}
10
5
10
5
□ Zinc
○ Cobalt
● Manganese
□ Zinc
■ Iron
× Nickel, Copper, Magnesium, Cadmium
0
1
2
3
4
5.5
6
0
1
2
3
4
5.5
6
GRAM ATOMS Me^{2+} ADDED/MOLE APO ENZYME

Figure 19.

with selective binding of the denatured enzyme (which perhaps was incapable of being reactivated by zinc) to the Sephadex resulting in recovery of enzyme with a higher specific activity. The activity of the manganese enzyme reverted to that of the apoenzyme after chromatography indicating a weaker binding of manganese with AMP aminohydrolase.

The observed V_{max} for the zinc reconstituted enzyme (4 gram atoms zinc per mole enzyme) was 1550 µm/min per mg protein or 13% greater than that observed with the native enzyme (Table 15). Both enzymes exhibited the same K_m for AMP. The data suggest that the 4th atom of zinc is not necessary for maximum enzymatic activity in the K^+-activated system. Enzyme which contained 4 gram atoms zinc per mole enzyme had a specific activity of 150 µm/min per mg protein when assayed at saturating ADP and 50 µM AMP; however enzymes with an estimated 1.85 gram atoms of zinc, although still activated by ADP, exhibited a considerable reduction in observed maximum velocity at 50 µM AMP. It was previously pointed out (page 101) that the native enzyme which contained 2.58 gram atoms of zinc (by atomic absorption analysis) also exhibited a lower relative activity in the ADP-activated system. Although the kinetic results are only preliminary, they suggest that zinc functions in more than one capacity; i.e., zinc may be necessary for both enzymatic activity and for ADP activation.

Table 15. Summary of Metal Content and Kinetic Parameters for Native AMP Aminohydrolase, Apo-AMP Aminohydrolase, and the Zinc, Cobalt, and Manganese Reconstituted Enzyme

		Kinetic Parameters				
		AMP Deamination[a]		ADP Activation		
Metal	Gram Atoms Metal per Mole Enzyme	K_m (mM)	V_{max}	$K_{a_{app}}$ (μM)[b]	V^c	n_H (ADP)
Zn	2.58 (d)	0.40	1345			
	0.45 (e)	0.35	300			
	1.85 (f)			15	88	2.1
	4.0 (g)	0.36	1550	24	150	1.9
Co	3.7 (h)	0.63	1200	30	80	2.4
Mn	3.7 (h)	0.93	560			

[a] The kinetic parameters for AMP deamination (Assay 1) were determined from Lineweaver-Burk plots of 1/v versus 1/ AMP . V_{max} is expressed as µM substrate deaminated/min per mg protein.

[b] The $K_{a_{app}}$ for ADP was determined from the Hill plot for ADP activation (Assay 2 with 50 uM Tris AMP) as described in Figure 3.

[c] V is the observed maximum velocity at 50 µM Tris AMP when activated by ADP. The value obtained (in µM AMP deaminated/min per mg protein) was estimated from a modified Lineweaver-Burk plot of 1/v versus 1/ ADP ^{n} where n is the Hill slope for ADP activation.

[d] Native enzyme--zinc content determined by atomic absorption analysis (see Table 11).

[e] Apoenzyme--zinc content (105.8 µg Zn^{2+}/g protein) determined by atomic absorption analysis.

[f] Reconstituted enzyme--obtained by the addition of 1.4 gram atoms of zinc to apoenzyme (e) followed by chromatography on Sephadex G-25 in Buffer A.

[g] Reconstituted enzyme--obtained by the addition of 3.7 gram atoms of zinc to apoenzyme (e) before chromatography on Sephadex G-25. The enzyme contained 944 µg Zn^{2+}/g protein by atomic absorption analysis (average of 2 analyses).

[h] Gram atoms of metal added to the apoenzyme before Sephadex G-25 chromatography.

The first is consistent with the low activity found in the apoenzyme versus the high activity of the native and reactivated enzyme: the second is consistent with citrate inhibition of ADP activation. Since the enzyme has 4 subunits of identical molecular weight, the binding of 4 atoms of zinc to the apoenzyme is consistent with one zinc per subunit. However very careful binding studies are necessary before the actual relationship between the total number of zinc atoms bound and enzymatic activity can be established.

The reconstitution of the apoenzyme with divalent cations other than zinc affects both the maximum rate of reaction and to some extent the K_m for AMP in KCl. The cobalt reactivated enzyme showed an increase in K_m for AMP from 0.36 mM to 0.63 mM and a decrease in maximum velocity relative to enzyme containing 4 gram atoms of zinc. Furthermore the cobalt enzyme was not as effectively activated by ADP: the observed maximum velocity at 50 μM AMP was 0.53 $V_{(Zn)}$. A greater increase in K_m from 0.36 to 0.93 occurred with the manganese enzyme; the relative maximum velocity in KCl was 0.36 $V_{(Zn)}$. ADP activation of the manganese enzyme was not studied since the enzyme lost activity when chromatographed on Sephadex G-25 equilibrated with TMACl.

Since atomic absorption analyses of the cobalt and manganese reactivated enzymes were not done, the stoichiometry of metal to enzyme used for determination of K_m and V_{max} is not known, especially with respect to the replacement of the residual zinc (<0.5 gram atom per mole enzyme) in the apoenzyme. Therefore the kinetic parameters obtained for the cobalt and manganese enzymes are only approximate values.

Until the present no AMP aminohydrolase has been shown to be a metalloenzyme. Lee (2) and Nikiforuk and Colowick (35) observed no dependence of activity upon divalent cations. However their judgement was based upon (A) the observed inhibition rather than activation of the enzyme by Cu^{2+}, Zn^{2+}, Fe^{2+}, and Ag^{+} and (B) the failure of known metal binding agents such as cyanide, EDTA, and cysteine to inhibit the enzyme. Trace metal analysis was not attempted by these investigators. Indeed the rabbit muscle enzyme prepared according to the method of Smiley <u>et al</u> (1) is also inhibited by divalent metals added to the assay. At 0.1 mM, Cu^{2+} and Zn^{2+} inhibited 98% and 87%, respectively, while 0.5 mM Ni^{2+} or Cd^{2+} inhibited 23%. Inhibition by Co^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+} was less than 10% at 1.0 mM. Similarly yeast alcohol dehydrogenase and beef liver glutamate dehydrogenase are actually inhibited by Zn^{2+} the very metal essential for catalytic activity (118, 111). The ions Cu^{2+}, Fe^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+} also inhibited (118). Hence inhibition of zinc metalloenzymes by transition metals is no criteria for eliminating a metal cofactor requirement. Furthermore failure to observe inhibition by EDTA, a strong metal chelator, is not justifiable evidence that a metal ion is absent. The high association constants for metal EDTA complexes apply only for the tetradentate complex and their association constants are altered if some of the coordination sites of the metal are already occupied by protein ligands. Therefore inhibition may not be observed (119). The failure of EDTA to inhibit yeast alcohol dehydrogenase is attributed to such steric hinderance (118). The preparation of AMP aminohydrolase used here required higher concentrations for inhibition than would be expected indicating steric factors may be interfering with binding of EDTA to the metal ion. Setlow and Lowenstein observed slight activation of the purified calf brain AMP aminohydrolase by Mg^{2+} and Mn^{2+}; however Zn^{2+} did not activate (28). The nonspecific adenine nucleotide aminohydrolase from Red Marine Algae is reported to be activated by divalent metals: Ca^{2+} was the most effective activator of this enzyme (120). No metal analysis for this enzyme was reported.

The data presented in this thesis partially fulfill the postulates first enumerated by Vallee (121) for identification of a metalloenzyme: (A) The enzyme is homogeneous by physiochemical techniques (1); (B) Metal analysis performed on the purified enzyme show an increase of zinc, "intrinsic metal," to protein ratio with respect to the crude extract and a decrease in "extrinsic metal" to protein ratio; (C) Iron, calcium, magnesium, and cobalt were present in stoichiometrically insignificant amounts (the analyses for copper and manganese were ambiguous due to insensitivity of the analytical method); (D) The zinc content of the enzyme correlated with the specific activity; and (E) Enzyme activity was inhibited by a variety of metal binding agents. Three additional postulates have not yet been fully satisfied. The stoichiometry of zinc to enzyme has not been absolutely established. The ratio of gram atoms of zinc per mole of enzyme, although a small number, 2.58, was not an integral number. It seems possible that the pure enzyme is not isolated with its full zinc complement especially in light of the fact that the enzyme consists of four subunits of the same molecular weight. Although the prevention or reversal of the inhibition by metal binding agents was not investigated, enzyme from which zinc had been removed by incubation in 8-OHQ5SA followed by chromatography on Sephadex G-25 was found to be readily reconstituted not only by Zn^{2+} but also by Co^{2+}, Mn^{2+}, and Fe^{2+}. Reconstitution by metals other than zinc resulting in enzymatically functional proteins is a common phenomenon among zinc metalloenzymes (88, 116, 117). The replacement of zinc, a diamagnetic ion, by paramagnetic Co^{2+} or Mn^{2+} makes further investigation of the role of the divalent metal in the enzyme possible through spectral and nuclear magnetic resonance studies. Investigations along these lines are of special interest in light of the kinetic data which indicate (but do not prove) that the zinc atom(s) perform two roles: (A) The zinc atom(s) is absolutely necessary for enzymatic activity although its function remains unknown (for example zinc may participate by binding AMP, by orientation of either substrate, by polarization of the bond to be broken, or by maintaining the enzyme in an active conformation); (B) Zinc may be required at the activator site. Precedence for two classes of tightly bound metal atoms has been established in alkaline phosphatase, a zinc metalloenzyme where 2 of the 4 zinc atoms are essential for activity (88). Horse liver alcohol dehydrogenase also possesses two classes of zinc (122): two of the four zinc atoms are essential for activity while the remaining zinc atoms stabilize the quaternary structure of the enzyme.

Although the data are not sufficient to propose a molecular model for the enzyme and the mechanism of action of zinc, it is tempting to suggest that zinc is directly involved in the hydrolysis of the 6-amino group from AMP in a manner analogous to the hydrolysis of peptide bonds by carboxypeptidase A, a known zinc metalloenzyme. In the latter enzyme recent x-ray crystallographic studies indicate that zinc may participate in polarization of the carbonyl of the peptide linkage to be hydrolyzed (123-125).

SUMMARY

1. Rabbit skeletal muscle AMP aminohydrolase deaminated the following analogs in order of decreasing V_{max}: AMP, AMP-NH_2, N^6-methyl AMP, dAMP, adenosine monosulfate, adenosine and ADP. The enzyme also deaminated N^6-ethyl AMP, formycin 5'-monophosphate, AmDP, and α,β-methylene ADP.
2. 6-Mercaptopurine 5'-ribonucleotide and IDP ($K_{a_{app}}$ = 1.3 mM) activated the enzyme.
3. 3'-AMP, 3',5'-cyclic AMP, and 3-iso AMP inhibited AMP deamination. 3-Iso AMP was a linear competitive inhibitor for AMP (K_i = 32 μM). With dAMP as the substrate the enzyme exhibited negative cooperativity with respect to this inhibitor.
4. Deamination of adenosine, AMP, and ADP by a single enzyme was consistent with the results obtained from studies of enzyme homogeneity, heat inactivation, elution from cellulose phosphate, and product characterization.
5. Analysis of the products of N^6-methyl AMP by paper and gas chromatography indicated that only IMP and methylamine were formed. The data favor the direct hydrolysis of substrate rather than a Dimroth-like rearrangement.

6. The $A^{1\%}_{280\ nm}$ (1 cm light path) for AMP aminohydrolase was 9.15 OD on a dry weight basis.
7. A molecular weight of 278,000 for the native enzyme was obtained from sedimentation equilibrium centrifugation in 0.2 M KCl and 1 mM mercaptoethanol at pH 7.2.
8. AMP aminohydrolase consists of 4 subunits of identical molecular weight (69,000) by SDS polyacrylamide electrophoresis.
9. Citrate, succinate, maleate, fumarate, malate, and malonate strongly inhibited the activity of enzyme assayed in the absence of K^+. Citrate was a competitive inhibitor (K_i = 0.11 mM) for ADP activation and eliminated the cooperative kinetics observed for ADP by decreasing the n_H for ADP from 1.7 to 1.1.
10. The enzyme was strongly inhibited by metal binding agents such as o-phenanthroline, 8-hydroxyquinoline-5-sulfonate, ethylenediaminetetraacetate, citrate, mercaptoethanol, and dithioerythritol.
11. Atomic absorption analysis demonstrated that the native enzyme contained 2.58 gram atoms Zn^{2+}/278,000 grams protein. Only zinc showed an increase in metal to protein ratios during purification. Magnesium, calcium, iron and cobalt were present in stoichiometrically insignificant amounts in the pure enzyme.
12. Zinc was essential for enzymatic activity as shown by the correspondence between loss of activity and the

formation of the $Zn[8\text{-}OHQ5SA]_3$ complex after addition of the chelator 8-OHQ5SA. After treatment with this chelator, enzyme chromatographed on Sephadex G-25 and analyzed by atomic absorption spectroscopy contained 0.45 gram atoms zinc per mole enzyme; enzymatic activity showed a corresponding decrease in activity.

13. Apoenzyme was reconstituted by titration with the spectropure sulfates of zinc, cobalt, iron, and manganese. Nickel, cadmium, copper, and magnesium did not reactivate the apoenzyme.
14. Enzyme reconstituted with zinc, cobalt, and manganese showed differences in both K_m and V_{max} for AMP deamination.

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