Substrate Specificity and Aspects of Deamination Catalyzed by Rabbit Muscle 5'-Adenylic Acid Aminohydrolase*

(Received for publication, October 28, 1969)

CAROL L. ZIELKE† and C. H. SUELTER§
From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

SUMMARY

A total of 24 nucleotides were examined as substrates for 5'-AMP aminohydrolase (EC 3.5.4.6). The following nucleotides were deaminated at the indicated maximum velocity relative to AMP (100): adenosine 5'-phosphoramidate (73), N°-methyl AMP (20), dAMP (18), adenosine 5'-monosulfate (13), adenosine (1), and ADP (1). Other substrates for which maximum velocities were not determined included N°-ethyl-AMP, formycin 5'-monophosphate, 2'-O-methyl-ADP, and α,β-methylene-ADP. 3'-AMP, 3',5'-AMP, and 3,5-d-ribofuranosyladenine 5'-phosphate were not deaminated but were effective inhibitors. The pH optimum for ADP deamination was more acidic (pH 5.0 to 5.5) than the optimum for AMP or adenosine (pH 6.3 to 7.0). Heat inactivation data and the concurrent elution of the activity for AMP and ADP from cellulose phosphate are consistent with a single enzyme being responsible for the multiple activities. Aspects of the mechanism are discussed.

MATERIALS AND METHODS

Rabbit muscle 5'-adenylic acid aminohydrolase was prepared as described by Smiley et al. (1). Unless otherwise indicated, activity was determined in 0.15 M KCl, 0.05 M Tris-cacodylate, pH 6.3, and 50 μM Tris-AMP (Assay 1) or 0.10 M (CH₃)₂NCl, 0.05 M Tris-cacodylate, pH 6.3, and 50 μM Tris-AMP (Assay 2). Specific activities in micromoles per min per mg were calculated from the change in absorbance per unit time per mg of protein by the following relationship: micromoles per min per mg = ΔA per min per mg per F where F, the change in molar absorbance, is equal to 8.86 × 10⁶ m⁻¹ cm⁻¹ at 265 nm, 0.30 × 10⁵ m⁻¹ cm⁻¹ at 285 nm, and 0.12 × 10⁵ m⁻¹ cm⁻¹ at 290 nm.

All chemicals were reagent grade or purified as previously described (1). Analogues of 5'-AMP were purchased from Sigma or P-L Biochemicals unless otherwise designated. Tubercidin monophosphate (4-amino-7-p-ribosanonyl 5'-monophosphate-7H-pyrrolo[2,3-d](2N)pyrimidine) and tubercidin monophosphate methyl ester were obtained from Dr. William J. Wechter of Upjohn Company, Kalamazoo, Michigan. 3,5-d-Ribofuranosyladenine 5'-phosphate was kindly donated by Dr. Nelson Leonard at the University of Illinois, Urbana, Illinois. Dr. R. J. Suhadolnik of the Albert Einstein Medical Center, Philadelphia, Pennsylvania, donated toyoacamin monophosphate (4-amino-5-εamino-7-p-ribosanonyl 5'-monophosphate-7H-pyrrolo[2,3-d](2N)pyrimidine). Formycin monophosphate (4-amino-7-p-ribosanonyl 5'-monophosphate pyrazolo[3,4d](2H)pyrimidine) was a gift of Dr. S. Nishimura of the National Cancer Center Research Institute, Tokyo, Japan. The 2'-O-methyl-ADP was supplied by Dr. F. Rottman, Michigan State University, East Lansing, Michigan; the N°-ethyl-AMP and the mixture of 6-amino-9-psicofuranyl 1'-phosphate and psicofuranyl 6'-phosphate were donated by Drs. K. W. Rabinowitz and W. A. Wood of the same institution.

N°-Methyl-AMP was synthesized from AMP at pH 4.5 by the method of Griffin and Reese (7). The purified product exhibited only one spot in Solvent A (see Legend, Table III), and the RF value of 0.75 agrees well with the RF of 0.76 previously reported (7).

N°-Methyl-AMP was synthesized from N°-methyl AMP by the procedure of Brookes and Lawley (8). The absorption spectrum at pH 6.7 was identical within experimental error to that previously reported for N°-methyl adenosine in water, λmax = 265 nm and λmin = 229 nm (9). Paper chromatography of N°-methyl-AMP in solvent Systems A and in 1% ammonium...
**TABLE I**

| Substrate Analogue | \(K_m\) (mM) | \(V_{max}\) (pmoles/min/mg) | pH (pH optimum) |
|--------------------|-------------|--------------------------|----------------|
| AMP                | 0.40        | 100                      | 6.3 (6.3-7.0)  |
| Adenosine          | 7.4         | 35                       | 6.3            |
| N^4-methyl AMP     | 1.8         | 20.4                     | 6.3 (6.4-6.6)  |
| dAMP               | 2.3         | 15.5                     | 6.3            |
| Adenosine monosulfate | 3.2       | 10-16                    | 6.3            |
| Adenosine          | 20          | 1.1                      | 6.5 (6.5-6.8)  |
| ADP                | 0.8         | 0.20                     | 6.3 (6.0-5.6)  |
| 6-Chloropurine 5'-ribonucleotide | 9.5 | 0.94                     | 5.2            |
| Adenosine 5'-phosphothioate | 1.6 | 0.4                     |                |
| \(\alpha,\beta\)-methylene-ADP | +         | +                        |                |
| 2'-O-methyl-ADP    | +           | +                        |                |
| \(N^4\)-ethyl-AMP  | +           | +                        |                |
| Formycin 5'-monophosphate | +    | +                        |                |

- The following compounds were not deaminated under the conditions described under "Materials and Methods": ATP, 2'-AMP, 3'-AMP, 3',\(\beta\)-cytic AMP, 6-amino-\(\beta\)-psicofuranyl 1'-phosphate and \(\beta\)-psicofuranyl 6'-phosphate mixture, N^4-methyl-AMP, 6-mercaptourine 5'-ribonucleotide, GMP, GDP, GTP, CMP, 3'-\(\beta\)-ribofuranosyladenine 5'-phosphate, tubercidin 5'-monophosphate methyl ester, and toyo-camycin 5'-monophosphate.

- Kinetic parameters were obtained from Lineweaver-Burk plots of initial velocities (Assay 1) at substrate concentrations from 10^{-5} M to 10^{-3} M (except in the case of adenosine 5'-monosulfate and N^4-methyl-AMP where the maximum substrate concentration was 2 x 10^{-4} M) at the pH indicated in Column 4. \(V_{max}\) is reported as percentage \(V_{max}\) for AMP (1240 pmoles deaminated per min per mg).

- Parentheses indicate the pH optimum. For AMP a broad optimum was observed at pH 6.3 to 7.0 with 1.9 mM Tris-AMP (Assay 2), while a rather sharp optimum was observed at pH 0.3 with either 0.05 mM Tris-AMP, 0.042 mM Tris-ADP (Assay 2), or 0.05 mM Tris-AMP, 0.1 mM KCl, and 0.05 mM Tris-cacodylate all at 30°C. Conditions of the pH studies for N^4-methyl AMP, adenosine, and ADP were as follows: 0.05 mM Tris-N^4-methyl-AMP, 0.15 mM KCl, and 0.05 mM Tris-cacodylate; 8.2 mM Tris-ADP, 0.1 mM KCl, 0.1 mM Tris-cacodylate (pH 5 to 7.8) or 0.1 mM sodium acetate (pH 3.5 to 5.5).

- From reference (10) for rat muscle AMP aminohydrolase.

- The "±" denotes that determination was observed. Kinetic parameters were not determined because of an insufficient amount of material.

- From reference (34: 20: 45) on Whatman No. 1 paper washed with 1% ammonium sulfate indicated approximately 3% contamination by AMP and adenosine methylphosphate.

- Sodium ADP and barium adenosine 5'-monosulfate and 6-mercaptopurine 5'-ribonucleotide were converted to Tris salts by passage over Dowex 50W-X8 (Tris) at room temperature. The less than 5% AMP contamination of ADP was not removed since the high protein concentration required for the study of ADP deamination deaminated all AMP during the mixing phase. The acid forms of all other analogues were titrated to the desired pH with Tris base.

**TABLE II**

| Compound | Solvent A | Solvent B | Solvent C | Solvent D |
|----------|-----------|-----------|-----------|-----------|
|          | Minus enzyme | Plus enzyme | Minus enzyme | Plus enzyme |
| AMP      | 0.25       | 0.52      | 0.56      | 0.30      |
| N^4-methyl-AMP | 0.25       | 0.52      | 0.60      | 0.30      |
| IMP      | 0.31       | 0.82      | 0.82      | 0.43      |
| ADP      | 0.11       | 0.22      | 0.22      | 0.42      |
| IDP      | 0.57       | 0.19      | 0.21      | 0.42      |
| Adenosine | 0.11      | 0.35      | 0.35      | 0.43      |
| Inosine  | 0.36       | 0.42      | 0.42      | 0.42      |
| Adenosine phophoramide | 0.19     | 0.19      | 0.19      | 0.19      |

- Solvent systems: A, saturated ammonium sulfate-0.1 M potassium phosphate buffer, pH 7.2-isopropanol (79:20:45); B, isobutyric acid-1 N ammonia-0.1 M sodium EDTA (100:60:1.6); C, isopropanol-concentrated ammonium hydroxide-H_2O (7:1:3); D, concentrated ammonium hydroxide-H_2O-isobutyric acid (10:32.9:66.1).

- The spectrum of this material after elution from the chromatogram with 0.3 M KCl was characteristic of the inosine moiety (\(\lambda_{max}\) pH 7.0 = 248.5 nm).

**Fig. 1.** 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 as previously described (3). Samples of approximately 4.0 ml were collected and assayed for AMP deaminase in 0.1 M KCl containing 1 mM mercaptoethanol at pH 7.0 and 30°C. The unusual small protein peak preceding the main peak was not examined.
TABLE III

Heat denaturation of adenylic acid aminohydrolase

| Substrate     | First order rate constant for inactivation |
|---------------|-------------------------------------------|
|               | (CH₃)₄NCl system x 10⁻¹² | KCl system³ |
| AMP           | 5.1 | 0.168 |
| ADP           | 3.5 | 0.161 |
| Adenosine     | 3.2 | Not determined |

* Conditions of incubation in the (CH₃)₄NCl system. Protein which was passed over a Sephadex G-25 column equilibrated with 0.10 m (CH₃)₄NCl, 0.05 m (CH₃)₄N-cacodylate, pH 7.2, and 2 mM mercaptoethanol was incubated at 1.3 mg per ml 40° in a closed test tube from which aliquots were removed for assay. Protein concentration per ml of assay were 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 mM Tris-cacodylate, pH 6.4; 1 mM Tris-ADP and 0.05 mM Tris-cacodylate, pH 6.4; and 1 x 10⁻⁴ moles adenosine, 0.1 m KCl, 0.05 mM Tris-cacodylate, pH 6.4.

³ Conditions of incubation in the KCl system. Deaminase (0.48 mg per ml) was incubated at 0.2° 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 (CH₃)₄NCl, and 0.05 mM Tris-cacodylate, pH 6.3. The AMP assay contained 50 µM Tris-AMP and 0.10 mM potassium succinate, pH 6.5. The protein concentration in the ADP and AMP assays was 4.8 µg per ml and 0.24 µg per ml, respectively.

DB spectrophotometer equipped with a Sargent SRL recorder. Under these conditions analogues which were not hydrolyzed at a rate greater than 0.05 optical density per min per mg were not considered substrates.

Methylamine from N⁶-methyl-AMP deamination was detected with an F and M 402 gas chromatograph equipped with a flame detector. The column, 3 mm × 6 feet, packed with Chromosorb 103, 100 to 120 mesh (Anspec Company, Ann Arbor, Michigan), was conditioned overnight at 250° with N₂ gas flow. The samples and methylamine standard (1.0 µg per µl) were run at 105° with N₂ as the carrier gas.

RESULTS

This preparation of AMP-aminohydrolase catalyzed the deamination of several of the 24 analogues of AMP examined (Table I). Whether or not AMP-aminohydrolase was solely responsible for the observed deaminations was examined by three approaches. First, the products of adenosine, ADP, and adenosine phosphoramidate deamination chromatographed with the same Rf values as inosine, IDP, and inosine phosphoramide when examined in three solvent systems (Table II). Second, the ratio of specific activities for AMP and ADP deamination was constant within experimental error throughout a single activity peak eluted from a cellulose phosphate column during enzyme preparation (Fig. 1). Third, the first order rate constant for inactivation of the enzyme as measured by the loss of AMP, ADP, and adenosine deaminating activities were essentially identical under two different conditions of heat inactivation (Table III).

Examination of Table I shows that in addition to the 6-amino group, the 6-methylamino, 6-ethylamino, and 6-chloro substituent (10) of the purine ribonucleotide were susceptible to hydrolysis. AMP, however, was the preferred substrate, having the largest V₅₀ and lowest Kₗ values. Displacement of the sulphydryl residue from 6-mercaptopurine 5'-ribonucleotide was not detected. On the other hand, in the absence of K⁺ (Assay 2) 0.165 mM 6-mercaptopurine 5'-ribonucleotide activated AMP deamination 32-fold.

With respect to the 5'-substituent, a free 5'-hydroxyl (adenosine) or second phosphoryl group (ADP) reduced the maximum rate of deamination to 1% or less of that observed for AMP at pH 6.3 to 6.5; only adenosine showed a significant change in Kₗ, being 50 times greater the than AMP. Interestingly, substitution of a methylene group for the oxygen of the phosphodiester linkage of ADP did not prevent deamination although it did prevent its role as an activator, i.e. α,β-methylene-ADP could not substitute for ADP as an activator even at 1 x 10⁻⁴ m at which ADP activation is essentially maximal (11). ATP with three phosphoryl groups was not a substrate.

Alterations at the position 2' either substantially reduced or abolished activity. AMP deamination was reduced to 0.18 V₅₀ (AMP) with a concomitant increase in Kₗ. Although 2'-O-methyl-AMP was slowly deaminated, 2'-AMP was neither.

1 C. Zielke and C. H. Suelter, unpublished observation.
N6-methyl-AMP was mixed with 12 µg of protein and incubated for 30 to 60 min after which an aliquot containing an equivalent of 0.214 nmoles of substrate was spotted on Whatman No. 1 paper for 30 to 60 min after which an aliquot containing an equivalent and developed with Solvent System C. The product was quantitatively eluted with 0.3 M Tris-2-[N-morpholino]ethane sulfonate, and the absorption at 248.5 nm was determined. A total of 0.210 nmoles of IMP was recovered from the chromatogram.

Analysis of purine ribonucleotide product from N6-methyl-AMP deamination

| Criteria | Spectral characteristics* |
|----------|--------------------------|
| Product | N6-Isoxinosine | N6-Methylinosine |
| pH 7     | 1.70                  | 1.66            | 1.78          |
| pH 12    | 1.08                  | 1.08            | 1.67          |
| Vmax     | 248.5                 | 248.5           | 250           |
| pH 7     | 253                   | 254             | 250           |
| pH 12    | 253                   | 254             | 250           |

* Spectral characteristics for each substance were determined at pH 7 (0.5 M Tris-2-[N-morpholino]ethane sulfonate) and pH 12 (40 mM KOH) after elution of each spot from the chromatogram with 0.3 M KCl.

The broad substrate specificity observed for this preparation as noted in Table I might be interpreted in terms of contaminating enzymes such as (a) a second purine aminohydrolase or (b) an enzyme such as a kinase or phosphatase catalyzing the conversion of substrate under study to an alternate compound susceptible to attack by the AMP aminohydrolase. However, neither of these interpretations are convincing in light of the heat inactivation data or constant ratio of specific activities for AMP and ADP deamination throughout the activity peak obtained from the cellulose phosphate column during enzyme preparation. Furthermore, prior conversion to an alternate compound was not observed for adenosine, AMP, and adenosine phosphoromimidate deamination since the corresponding isosine derivative was the only observed product in each case (Table II). The different pH optima for adenosine and ADP deamination (Table I), which may reflect the total charge of the substrate, have previously been observed with a nonspecific adenine nucleotide aminohydrolase from Porphyra crista mode, a red marine alga (10). As with the nonspecific algal enzyme, the pH optimum was lowest for ADP, intermediate for AMP, and highest for adenosine.

Comparison of the maximum rates of deamination, the Km values and the pH optima for adenosine, AMP, ADP, adenine monosulfate, and adenosine phosphoromimidate (Table I) and also the lack of substrate activity with ATP would seem to emphasize the critical nature of the total negative charge of the substrate, or more specifically, at the position 5'. Wollenden, Sharpless, and Allan (17) have observed with the nonspecific Taka-diestase adenosine deaminase that nucleotides bearing monosubstituted phosphates appeared to bind only in the form in which the phosphate residue bears a single negative charge. With the muscle enzyme, a more rigorous analysis of variations in Km and Vmax values for these substrates as a function of pH is necessary before the nature of the influence of the negative charge on deamination can be evaluated. Steric factors, of course, cannot be entirely disregarded.

In summary, alterations in the purine, ribose, and phosphate moieties of AMP affect catalysis: analogues with substituents at the 1', 2', 7', and 3'-position of AMP were not substrates for the muscle enzyme whereas AMP with substitution or substituents at positions 6, 5', and occasionally 2' was deaminated. The suggestion by a reviewer that the effective inhibitor, 3B-d-ribosyluridyladenine 5'-phosphate, be considered a transition state analogue (21) is worthy of consideration.
Although the number of AMP analogues tested for substrate activity might be considered relatively small, the data are consistent with a requirement for somewhat rigid stereochemical and electronic configuration. Furthermore, this preparation of AMP-aminohydrolase, although less specific than previous preparations from animal sources (2, 15–20), has a more restrictive substrate specificity than that exhibited by the calf duodenal and Taka-diastase adenosine aminohydrolases. In the latter two cases, both N1-methyl- and 3-isoadenosine (23–25) as well as numerous other analogues were deaminated. The specificities of these enzymes have recently been reviewed (26).

Aspects of Mechanism—The Dimroth rearrangement of N'-methyladenosine or N'-methyladenylic acid resulting in the formation of the N6 methyl derivative, recently examined in some detail by Macun and Wolfenden (27), is presumed to occur through a ring opening reaction by analogy with rearrangements of N1-methyl pyrimidines (28). In light of the feasibility of this rearrangement, it seemed possible that the enzymatic deamination of AMP could likewise involve a ring opening at the N1-position followed first by the release of ammonia and subsequently by the incorporation of the N6 of adenylic acid into the ring as the N1 of inosinic acid. However, the quantitative deamination of N6-methyl-AMP to IMP and methylamine eliminates the ring opening mechanism in favor of a more direct hydrolysis. Furthermore, the dechlorination of 6-chloropurineribonucleotide by the rat muscle enzyme (10) is not consistent with the ring opening mechanism or the prior formation of a Schiff base. The formation of a purinyl enzyme intermediate or a nucleophilic attack with formation of a tetrahedral intermediate previously suggested for the calf and Taka-diastase adenosine aminohydrolase (23, 29) cannot be excluded with the present data. It should be noted, however, that the adenosine aminohydrolase contains no reported cofactors, whereas the AMP aminohydrolase is a zinc metallo enzyme (31). This essential cation would presumably be involved in the catalytic event and as such assist in a nucleophilic attack by H2O at the position 6.

Acknowledgments—We are grateful to Mr. Joseph Abbate for assisting in the gas chromatographic analysis.

Inhibition studies utilizing stable transition state analogues recently reported by Evans and Wolfenden (30) lend further support to a nucleophilic substitution reaction by water in reactions catalyzed by the calf duodenal and Aspergillus oryzae adenosine deaminases.

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*J. Biol. Chem.* 1971, 246:1313-1317.

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