

Cleavage of Modified Adenosine Nucleotides by Two Phosphatases with Differing pH Optima

BARTH H. RAGATZ and GINA MODRAK

Northwest Center for Medical Education

Indiana University School of Medicine, Gary, Indiana 46408

Introduction

Various ADP analogs have been investigated for effectiveness as inducers of platelet aggregation *in vitro* (7, 12). This information is used to assess requirements of the platelet ADP receptor for certain nucleotide structural features (4). Since platelet rich plasma may contain some phosphatase activities (2), the short-range susceptibility of the ADP analogs to phosphatase cleavage must be investigated initially to rule out possible secondary effects with the platelet suspension system from nucleotide hydrolysis products. Some novel information found about the substrate specificities of the two model phosphatases used for this purpose is reported in this present investigation.

Materials and Methods

The ADP analogs were obtained from Sigma Chemical Company, St. Louis, Missouri, or from P-L Biochemicals, Inc., Milwaukee, Wisconsin as chromatographically homogeneous crystalline powders. The two enzymes were purchased from Sigma Chemical Company as acid phosphatase (P-3752) and as alkaline phosphatase (P-3877). The nucleotide analogs were dissolved in the buffer appropriate for the incubation with a given enzyme present at the concentration of 1.0 mg/ml. Alkaline phosphatase was dissolved in 0.1M glycine—NaOH buffer, pH 10.0 at 1.0 mg/ml concentration while acid phosphatase was dissolved in 0.1M disodium citrate, pH 5.0 at the same concentration. Aliquots of enzyme (0.1 ml) were added to 1.0 ml of substrate in the appropriate buffer, mixed and incubated at 25°C. Aliquots (30 μ l) of reaction mixture were spotted at one hour intervals on cellulose MN-300 (Sigma Chemical Company) thin layer plates (15 g powder slurried in 90 ml of distilled water, coated on glass plates in 0.25 mm. layers, and air dried overnight) and developed immediately in a solvent mixture consisting of saturated ammonium sulfate (80 ml): 1.0M sodium acetate (18 ml): isopropanol (2 ml) (9). Compounds were visualized by ultraviolet illumination or by exposure to iodine vapor and were identified by R_f comparison with standards chromatographed on the same plate.

Results

All parent compounds were well resolved from possible hydrolysis products in this chromatography solvent system and easily detected at indicated concentrations. The use of cellulose thin layer plates for the superior resolution of nucleoside, nucleotide, or carbohydrate mixtures has been documented previously (11). The four compounds mentioned in this report were shown to be

stable in the pH 3 - pH 10 range at room temperature (25°C) for periods up to four hours; this suggests that all were suitable from this stand point to be used in platelet aggregation studies. Upon prolonged incubation in 0.155M NaCl, pH 6.8, at 25°C, the following relative stabilities (in decreasing order) were demonstrated by thin layer chromatographic analysis: meth ADP >> Me ADP, ϵ ADP, ϵ AMP > AMP > ADP.

TABLE I *Relative Susceptibility of Adenosine Nucleotides and Analogs to Potato Acid Phosphatase (3.1.3.2)*

AMP > ϵ AMP
ADP > ϵ ADP
MeADP: No change in 5 hours
MethADP: No change in 5 hours

The relative susceptibilities of the modified adenosine nucleotides to acid phosphatase attack are shown in comparison to the corresponding naturally occurring adenosine nucleotides in Table I. It can be seen that the additional bulky ring present in the etheno compounds (ϵ AMP and ϵ ADP) causes some impedence of enzyme mediated phosphate cleavage. These etheno compounds may very well be the largest purine-like substrates used with acid phosphatase so far. Both MeADP and methADP are resistant to acid phosphatase attack, showing that this degree of pentose substitution is not tolerated by the enzyme and that this P-C-P linkage is resistant to enzymatic cleavage as is expected.

TABLE II *Relative Susceptibility of Adenosine Nucleotides and Analogs to Calf Intestine Alkaline Phosphatase (3.1.3.1)*

AMP; ϵ AMP: Similar Susceptibilities
MeADP > ϵ ADP
2ADP $\leftrightarrow$ AMP + ATP
MethADP: No change in 5 hours

Table II shows the comparative susceptibility of these adenosine derivatives to alkaline phosphatase hydrolysis. As with acid phosphatase, the bulky etheno compounds are tolerated, and the P-C-P linkage is not cleaved by this enzyme. The pentose derivative, MeADP, is hydrolyzed by alkaline phosphatase, indicating that some steric accomodations are available in the active site of this enzyme, or that other binding regions are of greater importance in determining enzyme-analog interaction. Although AMP is observed more rapidly in the reaction mixture when ADP is used as principal substrate, ATP is also ultimately detected, suggesting transphosphorylation has occured: 2 ADP $\leftrightarrow$ AMP + ATP.

Discussion

It is not too surprising that ϵ AMP and ϵ ADP serve as substrates for both of the present phosphatases since Secrist *et. al.* have shown that ϵ ATP could be used as a phosphate, pyrophosphate or adenylate donor, or as an allosteric

effector in selected ATP requiring enzyme systems (10). The additional bulky ring is well tolerated by the present enzymes and it is seen that there is no requirement for a free amino group on the purine ring for hydrogen bonding interactions with either of the active sites of the two enzymes. Changes in location of cationic charge and pK are also not problematic in present enzyme—analog interactions; other regions in these etheno nucleotide molecules may be of greater importance in this interaction. Concerning bulky substrate analogs, Ikawa *et. al.* (5) hydrolyzed several phenyl substrates with acid phosphatase and Cathala *et. al.* (1) reported that even bulky naphthyl substrates are well tolerated by alkaline phosphatase.

This report states that MeADP is cleaved by alkaline phosphatase, but not by acid phosphatase. This compound, and one other pentose variant, adenine arabinofuranoside-5'-diphosphate, were shown to be poor substitutes for ADP at the platelet ADP receptor in platelet aggregation studies by Ragatz *et. al.* (8). Lesser substrate specificity for variations in sugar moieties of other analogs with alkaline phosphatase has been previously reported (1, 3).

The P-C-P linkage found in methADP is not cleaved by either phosphatase. This is to be expected from other enzymatic studies reported by Myers *et. al.* (6). Acidic or basic hydrolysis of this compound is only accomplished upon prolonged treatment at 100°C (6). Transphosphorylation does not occur with any of the analogs in the presence of alkaline phosphatase, but does occur with ADP as substrate, as reported previously by Georgatsos for the placental enzyme (3).

These three kinds of analogs have been shown to be stable over a broad pH range and in the presence of two phosphatases analogous to enzymatic activities which may be encountered in human plasma. Thus, the present compounds are deemed appropriately stable for platelet aggregation studies. The present investigation has also shown that an impressively bulky ring system can be accommodated by the active site regions of two unique phosphatases, that pentose conformation is important in limiting the interactions of some analogs with nucleotide-utilizing enzymes, and that P-C-P bonds remain stable under diverse hydrolytic circumstances.

Acknowledgments

The authors wish to acknowledge the encouragement of Dr. P. G. Iatridis and Dr. William Baldwin in this project. Assistance provided by Mr. Robert Hansen in the early stages of this project has also been appreciated.

Literature Cited

1. CATHALA, G., C. BRUNEL, D. CHAPPELET-TORDO and M. LAZDUNSKI. 1975. Bovine Kidney Alkaline Phosphatase: Purification, Subunit Structure, and Metalloenzyme Properties. *J. Biol. Chem.* **250**:6040-6045.
2. DIEM, K. and C. LENTNER. 1970. *Documenta Geigy Scientific Tables*, Seventh Edition. J. R. Geigy, Publisher. 596-597.
3. GEORGATSOS, J. G. 1967. Specificity and Phosphotransferase Activity of Purified Placental Alkaline Phosphatase. *Arch. Biochem. Biophys.* **121**:619-624.
4. GOUGH, G., M. H. MAGUIRE and F. PENGLIS. 1972. Analogues of Adenosine 5'-Diphosphate-New Platelet Aggregators. *Molec. Pharmacol.* **8**:170-177.

5. IKAWA, T., K. NISIZAWA and T. MIWA. 1964. Specificities of Several Acid Phosphatases from Plant Sources. *Nature*. **203**:939-940.
6. MYERS, T. C., K. NAKAMURA and J. W. FLESHER. 1963. Phosphonic Acid Analogs of Nucleoside Phosphates. I. The Synthesis of 5' Adenylyl Methylendiphosphonate, a Phosphonic Acid Analog of ATP. *J. Amer. Chem. Soc.* **85**:3292-3295.
7. PEARCE, P. H. and M. C. SCRUTTON. 1977. Interaction of Human Blood Platelets with the 2',3'-Dialdehyde Derivatives of Adenosine Diphosphate and Adenosine Triphosphate. *Biochem. Soc. Transact.* **5**:138-139.
8. RAGATZ, B. H., P. G. IATRIDIS and S. G. IATRIDIS. 1976. Presentation, 92nd Annual Meeting, Indiana Academy of Science. Valparaiso, Indiana.
9. RANDERATH, K. 1961/62. A Comparison Between Thin-Layer Chromatography of Nucleic Acid Derivatives. *Biochem. Biophys. Research Commun.* **6**:452-457.
10. SECRIST, J. A., J. R. BARRIO, N. J. LEONARD and G. WEBER. 1972. Fluorescent Modification of Adenosine—Containing Coenzymes. Biological Activities and Spectroscopic Properties. *Biochem.* **11**:3499-3506.
11. STAHL, E. 1965. *Thin-Layer Chromatography: A Laboratory Handbook*. Springer-Verlag. 447-451.
12. STONE, J. V., R. V. SINGH, H. HORAK and P. G. BARTON. 1976. Sulfhydryl Analogues of Adenosine Diphosphate: Chemical Synthesis and Activity as Platelet-Aggregating Agents. *Can. J. Biochem.* **54**:529-533.