

THE CONSEQUENCES OF ALTERING ATP

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Abstract - The general purpose of the research is to assess the size of the space available to the adenine moiety of adenosine triphosphate (ATP) in its enzyme binding sites and to ascertain the dimensional limits for the expression of enzyme-coenzyme activity. The fluorescence of certain of the synthetic "stretched-out" analogues of ATP, e.g. linear-benzo-ATP, enables them to be used for the characterization of adenine nucleotide binding sites of representative enzymes. New synthetic methodology had to be devised to obtain intermediates in the linear-naphthoadenine and linear-benzocyclobutadienoadenine series, and unusual rearrangements were also encountered.

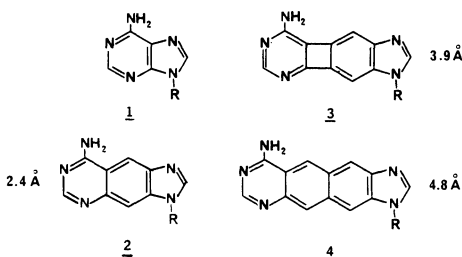
INTRODUCTION

Almost every sequence of metabolic processes involves one or more reactions with molecules of adenosine 5'-triphosphate. ATP is central to energy processing in all cells and is more-over the final energy transfer molecule in almost all cellular processes (Ref. 1). What is known concerning the binding of ATP to any cohort enzyme? The best information provided by X-ray analysis for an enzyme-ATP complex is a composite structure for hexokinase (Ref. 2 & 3), in which "the adenine is situated in a shallow depression on the surface of the large lobe of the enzyme at the entrance to its deep cleft" (Ref. 2). Binding of the substrate glucose to the bottom of the cleft causes a 12° rotation of one lobe of hexokinase relative to the other, closing the cleft (Ref. 4 & 5). Since the distance between the γ -phosphate of the ATP and the 6-hydroxy of glucose is nearly 6 Å (note: at 3-Å resolution), transfer of the γ -phosphate to glucose (Ref. 6) requires that the Mg^{2+} -ATP and the glucose be brought closer together. To determine the extent of further conformational change and to establish the active-site geometry, a crystal structure of the ternary glucose-enzyme Mg^{2+} -ATP complex would be required (Ref. 7). We seek an answer to binding to representative enzymes in dimensionally quantitative terms with specific sets of enzymes and cofactors. We are encouraged in this enterprise by the demonstrated (Ref. 8) conformational fluctuation and flexibility of proteins (Ref. 9), as opposed to mechanical rigidity, and by a dynamic description of enzyme action (Ref. 10). The importance of ATP is further illustrated by the calculation (Ref. 11) that a person uses and resynthesizes his own body weight of ATP daily. One-sixth of all known enzymes require ATP or a related adenine-containing cofactor (1) such as AMP, ADP, cyclic AMP, NAD^+ , NADPH, FAD, or coenzyme A; yet for none of these is the binding site of the adenine moiety described more fully than as a "pocket", "slot", or "surface".

DIMENSIONAL PROBES OF ENZYME-COENZYME BINDING SITES

We decided to assess the size of the space available for adenine with dimensional probes of the enzyme-coenzyme binding sites, which are related to the natural coenzymes by defined dimensional changes in the molecules. While natural nucleoside triphosphates other than ATP have been found, not surprisingly, to be mainly inactive with ATP-requiring enzymes and while many analogues of adenine nucleotides have been made and tested for enzyme activation and inhibition, we have been concentrating on stretched-out analogues that are unique in retaining the terminal pyrimidine and imidazole rings. Thereby, the normal hydrogen-bonding sites, e.g., N1, N⁶, and N7, of the adenine moiety are retained, while a spacer is formally inserted between the terminal rings. The spacer has dimensions known from X-ray structure determinations of corresponding hydrocarbon systems. Thus, the formal insertion of a benzene ring into the center of the adenine ring system (2) stretches the original (1) linearly by 2.4 Å, insertion of a benzocyclobutadiene unit (3) stretches it by 3.9 Å, and insertion of a naphthalene unit (4) stretches it by 4.8 Å. Although the heteroaromatic ring systems in 2-4 differ electronically from adenine, which will be reflected in their basicity, nucleophilicity, and π -binding characteristics, at least such differences are related to one major structural feature. The degree of interaction of compounds in this series with

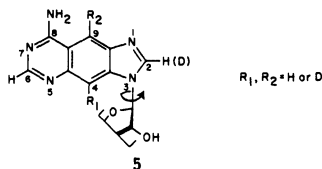
DIMENSIONAL PROBES OF ADENINE BINDING SITES



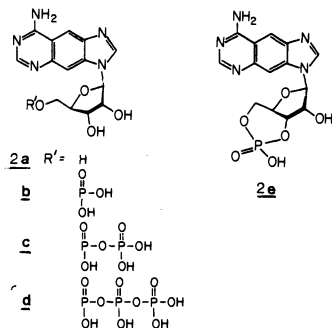
appropriate enzymes reveals the effect of incrementally greater spacing between N7 and 6-NH₂, and N9 and N3 of the original adenine ring.

With the synthetic dimensional probes in the lin-benzoadenine series (Ref. 12), it has been possible to improve descriptions of binding and therefore to define more accurately the spatial basis of activity and inhibition in comparing the biochemical behavior of the synthetic analogues with the natural coenzymes. The steps involved in assessing stretched-out versions of adenosine 5'-triphosphate as dimensional probes of the binding sites on specific enzymes have been the following: (a) synthesis of the necessary heterocyclic nuclei, (b) N-ribosidation at the site requisite for direct analogy, (c) mono-, di-, and triphosphorylation, (d) determination of enzyme binding and kinetics relative to the natural substrate or cofactor, and (e) fluorescence spectroscopy for detecting the mode of enzyme binding.

During the synthesis of the heteroaromatic ring of lin-benzoadenine nucleotides, each of the C protons could be replaced specifically by deuterium to a different extent (5), and the relative intensities allowed unequivocal assignment of the individual proton resonances



throughout the series 2a-e and in p¹,p²-di-lin-benzoadenosine 5'-pyrophosphate. This technique greatly reduced the number of experiments and variables involved and prevented misassignments of the signals upon changing the multiplicity of phosphate groups and solution concentration. As infinite dilution is approached, the 6-H becomes a "monitor" of charge (protonation takes place on the pyrimidine ring), the 4-H is sensitive to phosphate ionization and is therefore indicative particularly of the *syn* conformation that predominates under acidic conditions (pD ~ 4.0) (There is no analogy to the 4-H in the adenine nucleotide series.), and the 2-H is responsive to the *anti* conformation (pD ~ 8.5) of 2b-d. When the base is unprotected, lin-benzoadenine nucleotides are stacked in aqueous solution, with



association constants of a least one order of magnitude greater than those of the corresponding adenine nucleotides. Head to tail orientations of stacked lin-benzoadenine nucleotides in aqueous solution were indicated by the deuterium substitution effect on relaxation times (DESERT). The intramolecular stacking pattern of the N,N-dimethyl derivative of lin-benzoadenine [8-(dimethylamino)imidazo[4,5-g]quinazoline] in the crystal was shown to be a head to tail alternate stack by X-ray analysis.

The fluorescence of the active, stretched-out derivatives of the nucleic acid bases, nucleosides, and nucleotides has increased their utility (Ref. 12). For example, the fluorescence quantum yield of lin-benzo-AMP (2b) is 0.44 and its fluorescence lifetime is 3.7 nsec at pH 8.5 in 50 mM Tris-HCl buffer, and the nucleotides show sensitivity of the fluorophore to environmental conditions, such as divalent metal ions and stacking.

Adenosine cyclic 3',5'-monophosphate-dependent protein kinase

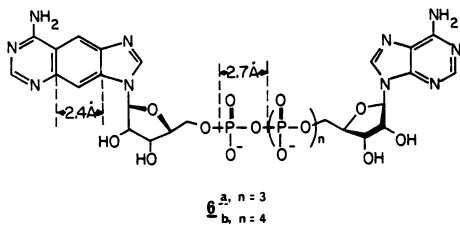
The interaction of lin-benzoadenosine di- and triphosphates with the catalytic subunit and type II holoenzymes of adenosine cyclic 3',5'-monophosphate (cAMP) dependent protein kinase has been investigated by steady-state kinetics and fluorescence spectroscopy (Ref. 13). lin-Benzo-ADP (2c) is a competitive inhibitor of the catalytic subunit with respect to ATP with a K_i (8.0 μM) similar to the K_i for ADP (9.0 μM). This value agrees well with the K_d (9.0 μM) determined by fluorescence polarization titration. Type II holoenzymes from bovine brain and skeletal muscle have K_d values for lin-benzo-ADP of 3.4 μM and 3.5 μM , respectively, and each binds approximately 2 mol/mol of R_2C_2 tetramer. Furthermore, fluorescence polarization studies indicate that both the catalytic subunit and type II holoenzyme bind lin-benzo-ADP rigidly, so that there is little or no rotation of the lin-benzoadenine portion of the molecule within the nucleotide binding site. lin-Benzo-ATP (2d) is a substrate for the phosphotransferase activities of protein kinase with peptides, water, or type II regulatory subunit as phosphoryl acceptors. lin-Benzo-ATP is the best nucleotide substrate (besides ATP) reported for the catalytic subunit (Leu-Arg-Arg-Ala-Ser-Leu-Gly as the phosphoryl acceptor).

Fluorescence displacement titration technique

Displacement of lin-benzo-ADP (2c) has provided the basis of a new fluorescence displacement titration technique for characterizing the nucleotide binding site on the catalytic subunit of adenosine 3',5'-cyclic monophosphate-dependent protein kinase in Prof. Robert Roskoski's laboratory at the Louisiana State University Medical Center, New Orleans. By calculation of the dissociation constants, K_d , of a series of nucleotides for the bovine skeletal muscle type II catalytic subunit from the decreases in the fluorescence polarization of lin-benzo-ADP which accompany their displacement from the catalytic subunit, it is possible to map the exact requirements for the most tenacious binding of the nucleotide moiety (Ref. 14). By using the same fluorescence method, one can determine which metal ions promote binding and support phosphotransferase activity and which metal ions do not.

Adenylate kinase inhibition

In the design of inhibitors of adenylate kinase, the 1000-fold greater inhibition of $\text{A}(5')\text{P}_5(5')\text{A}$ compared with $\text{A}(5')\text{P}_4(5')\text{A}$ suggested that the additional phosphate of the former, which lengthens the phosphate chain by about 2.7 Å, is crucial for strong inhibition. The question was asked as to whether there would be a similar inhibition-enhancing effect of stretching one of the terminal adenines by 2.4 Å (Ref. 15). Accordingly, we synthesized P^1 -(lin-benzo-5'-adenosyl)- P^4 -(5'-adenosyl)tetraphosphate (6a) and P^1 -(lin-benzo-5'-adenosyl)- P^5 -(5'-adenosyl)pentaphosphate (6b) from lin-benzoadenosine 5'-monophosphoromorpholidate with adenosine 5'-triphosphate and with adenosine 5'-tetraphosphate. These mixed dinucleoside oligophosphates are competitive with respect to ATP and AMP and are potent inhibitors of porcine muscle adenylate kinase, with association constants of $2 \times 10^6 \text{ M}^{-1}$ for the tetraphosphate and $2 \times 10^5 \text{ M}^{-1}$ for the pentaphosphate, respectively, as determined by kinetics and fluorescence experiments. These compounds (6a,b) are intramolecularly stacked when free in aqueous solution, as judged by their low fluorescence



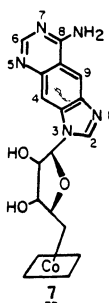
quantum yield and short lifetime compared with lin-benzo-AMP (2b). When adenylate kinase is present, the fluorescence quantum yields and lifetimes of 6a and 6b are increased. The reversal of quenching signifies that the intramolecular stacking has been broken and that these inhibitors are therefore bound to the enzyme in an "open" or "extended" form of the oligophosphate chain.

lin-Benzo-ATP and firefly luciferase

lin-Benzo-ATP (2d) has been shown to be an acceptable substrate for light production in the firefly luciferase system (Ref. 16). This ATP analogue displays strong enzyme binding and a reduced rate of enzyme catalysis compared with ATP. Variations in the color or the bioluminescence emission suggest that a lateral extension in the purine base induces an incremental change in the conformation of luciferase in the vicinity of the excited light emitter.

Profluorescent analogue of coenzyme B₁₂, lin-benzoadenosylcobalamin

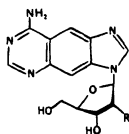
The fluorescence of the lin-benzoadenosine system became of special advantage when we synthesized the profluorescent analogue of coenzyme B₁₂, lin-benzoadenosylcobalamin (7), which has the laterally extended nucleoside in the upper axial position (Ref. 17). This compound is nonfluorescent in solution but, on homolytic (light) or heterolytic (acid, cyanide) cleavage of the carbon-cobalt bond, forms fluorescent products. In addition, fluorescence is detectable on binding of the coenzyme analogue to ribonucleotide reductase,



and the observed fluorescence polarization of the lin-benzoadenosyl moiety indicates that it is bound loosely to the enzyme when the coenzyme is partially dissociated. The stabilized radical pair, also detectable by EPR in the case of adenosylcobalamin, is considered to be the first intermediate in the ribonucleotide reductase reaction and is prerequisite for catalysis but does not guarantee that ribonucleotide reduction will be completed. Compound 7 was found to be an effective competitive inhibitor of ribonucleotide reductase from Lactobacillus leichmanii.

2'-Deoxy-lin-benzoadenosine 5'-triphosphate

In order to accomplish the synthesis of the 2'-deoxy-lin-benzoadenosine phosphates, it was necessary to develop a new general synthesis of 2'-deoxyribonucleotides from the parent ribonucleosides (Ref. 18).



8a R=OH lin-benzoadenosine

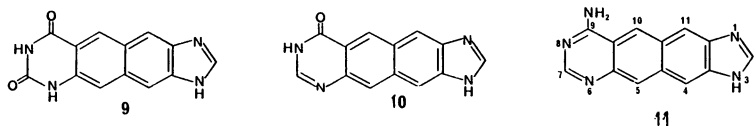
8b R=H 2'-deoxy-lin-benzoadenosine

2'-Deoxy-lin-benzoadenosine (8b) was synthesized via reductive deoxygenation of 2-(β-D-ribofuranosyl)-8-methylthioimidazo[4,5-g]quinazoline. The 5'-mono-, di-, and triphosphates were prepared by chemical and/or enzymatic methods (Ref. 19). The 5'-diphosphate was found to be a substrate for phosphorylation by pyruvate kinase and was compared with various natural and extended substrates in kinetic assays. When 2'-deoxy-lin-benzoadenosine 5'-triphosphate was tested in a nick-translation experiment with E. coli DNA polymerase I, a very low level of ³²P incorporation from [α-³²P]TTP into poly d(AT) was observed. Nearest neighbor analysis indicated that the analogue was not significantly incorporated into internal positions in the polymer. In DNA-sequencing reactions, the analogue caused chain

termination at adenine residues, although termination was less uniform and less efficient than with 2',3'-dideoxy-ATP. These experiments show that lin-benzoadenine can form a widened Watson-Crick base pair with thymine. They strongly suggest, though they do not prove, that the enzyme is able to attach the analogue to DNA.

lin-Naphthoadenine and lin-Naphthoadenosine

For the lin-naphtho series of analogues (4) it was necessary to develop regioselective syntheses that would result in appropriate tetra- β -substituted naphthalene intermediates. The first of these depended upon an application of the trimerization of acetylenes with cobalt catalysis, together with preferential electrophilic desilylation at two of the eventual four β positions of the naphthalene ring without competitive electrophilic displacement of α -hydrogens (Ref. 20). lin-Naphthoxanthine (9) and lin-naphthohypoxanthine (10), which were the first compounds to be prepared in the lin-naphtho series, exhibit

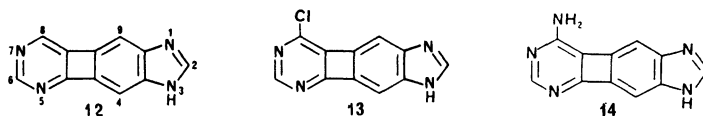


intense fluorescence. lin-Naphthoxanthine was not oxidized to lin-naphthouric acid by xanthine oxidase but functioned as a noncompetitive inhibitor. However, lin-naphthohypoxanthine was readily converted to lin-naphthoxanthine by xanthine oxidase. In this reaction, lin-naphthohypoxanthine functioned as a competitive inhibitor of xanthine oxidase. The enzymatic results for the naphtho analogues when compared with the benzo analogues demonstrate, in part, a useful application of defined dimensional probes for determining the limiting spatial restrictions of the binding region for xanthine oxidase. Thus, whereas hypoxanthine and lin-benzohypoxanthine are oxidized first in the pyrimidine ring and then in the imidazole ring, lin-naphthohypoxanthine is oxidized to lin-naphthoxanthine, i.e., in the pyrimidine ring, but no further. The K_m values for lin-benzohypoxanthine and lin-naphthohypoxanthine are similar. The limiting lateral extension of the substrate for xanthine oxidase to be able to act on the imidazole ring appears to lie between 2.4 and 4.8 Å. Further refinement of the limit should be possible as lin-bcb-hypoxanthine becomes available (series represented by 3). The fluorescence properties of the lin-naphthopurines, coupled with potential biological activity, complement those of the benzopurines which have demonstrated both favorable spectroscopic properties and enzyme activity.

lin-Naphthoadenine (11) and lin-naphthoadenosine (4, R = ribosyl) have now been synthesized for the first time, utilizing the availability of the intermediate linear-naphthohypoxanthine from a shortened, efficient synthesis (Ref. 21). 5,6-Dimethylbenzimidazole, protected by a bulky group on N, was subjected to selective benzylic bromination. The tetrabromo product, when treated with sodium iodide under Finkelstein conditions, generated a dibromo-*o*-xylylene intermediate that could be trapped by maleic anhydride or *N*-hydroxymaleimide, with aromatization by loss of 2 HBr. This Diels-Alder cycloaddition approach to the otherwise difficultly available tetra- β -substituted naphthalenes was followed by stepwise conversions of the terminal anhydride or *N*-hydroxyimide ring to a suitably substituted pyrimidine ring. lin-Naphthoadenine (11) and lin-naphthoadenosine are brilliantly fluorescent, exhibiting high fluorescent yields ($\phi = 0.57, 0.64$) and long lifetimes ($\tau = 20.5, 22.4$ nsec in ethanol purged of oxygen). Neither is a substrate for adenosine deaminase, showing that a lateral extension of 4.8 Å is too great for a satisfactory fit at the enzyme active site, whereas a 2.4 Å-extension (both lin-benzoadenine and lin-benzoadenosine) is tolerated.

The synthesis of the lin-benzocyclobutadienoadenine derivatives (3) was delayed by the observation of an unexpected transience of the intended 1,3-diazabiphenylene precursors (Ref. 22 & 23). Discovery of a rearrangement reaction may be regarded as an unusual "consequence" of trying to alter the ATP molecule! Two independent syntheses of the 1,3-diazabiphenylene ring system have been developed. The first of these involves thermal extrusion of nitrogen from a condensed pyridazine precursor, and the second utilizes a diethynylpyrimidine in a cobalt-catalyzed co-oligomerization reaction. The 1,3-diazabiphenylenes so synthesized undergo facile transformations to isoquinolines upon treatment with either acid or base. The intermediacy of 1,3-benzodiazocines between a pair of electrocyclic reactions, followed by small-molecule extrusion, best explains the results obtained.

We have now returned from this diverting rearrangement to concentration on the compound that has survived (12), and the compounds that we expect to survive (13, 14), thermolysis of the corresponding cinnoline derivatives with extrusion of N_2 (Ref. 24).



Ribosidations, with structural discrimination between the isomers, displacement of Cl in the case of 13, amination in the case of 12, and subsequent phosphorylation stages with the correct (3-substituted) ribosyl derivatives would follow in order to be able to compare directly the ATP analogues with 2.4, 3.9, and 4.8 Å separation between the terminal rings. The lin-bcb-hypoxanthine, obtainable from 13 and/or 14, is desired especially for oxidation in the presence of xanthine oxidase, for fine-tuning the width of substrate acceptable to that enzyme.

CONCLUSION

While there was no a priori reason to expect the synthetic lin-benzoadenine nucleotides (as in 2), including lin-benzo-ATP, to be active in a certain enzyme system, they have indeed shown selective binding and either activity or inhibition. In defining enzyme-coenzyme binding sites, aided by their fluorescence properties, they have found multiple use. The challenge of synthesis of the other stretched-out versions (3, 4) of the natural bases, nucleosides, and nucleotides has resulted in the development of new synthetic methodology and the discovery of unexpected rearrangements. Initial enzyme experiments have been carried out at the base and riboside levels for the highly fluorescent lin-naphtho series. In this and in the lin-benzocyclobutadieno series, enzyme experiments at the ribotide level are intended that should reveal further details of the dimensional requirements for enzyme-coenzyme or enzyme-substrate binding and activity.

Acknowledgment - This work was supported by a research grant from the National Institutes of Health (GM 05829). The research collaboration of Professor Robert Roskoski, Jr., Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana, is gratefully acknowledged.

REFERENCES

1. H. Morowitz, Science **82** 3, No. 4, 20-22 (1982).
2. M. Shoham and T.A. Steitz, J. Mol. Biol. **140**, 1-14 (1980).
3. E.A. Meritt, M. Sundaralingam, R.D. Cornelius, and W.W. Cleland, Biochemistry **17**, 3274-3278 (1978).
4. W.S. Bennett, Jr. and T.A. Steitz, Proc. Natl. Acad. Sci. USA **75**, 4848-4852 (1978).
5. C.M. Anderson, F.H. Zucker, and T.A. Steitz, Science **204**, 375-380 (1979).
6. W.A. Blättler and J.R. Knowles, Biochemistry **18**, 3927-3933 (1979).
7. W.S. Bennett, Jr. and T.A. Steitz, J. Mol. Biol. **140**, 211-230 (1980).
8. G. Weber, Biochemistry **11**, 864-878 (1972).
9. F.R.N. Gurd and T.M. Rothgeb, Adv. Protein Chem. **33**, 73-165 (1979).
10. G. Careri, P. Fasella, and E. Gratton, CRC Critical Reviews in Biochemistry **3**, 141-164 (1975).
11. W.D. McElroy, Cell Physiology and Biochemistry, 3rd. ed., p. 59, Prentice-Hall, Englewood Cliffs, New Jersey.
12. N.J. Leonard, Acc. Chem. Res. **15**, 128-135 (1982) and references therein.
13. F.T. Hartl, R. Roskoski, Jr., M.S. Rosendahl, and N.J. Leonard, Biochemistry **22**, 2347-2352 (1983).
14. D. Bhatnagar, R. Roskoski, Jr., M.S. Rosendahl, and N.J. Leonard, Biochemistry **22**, 6310-6317 (1983).
15. P. VanDerLijn, J.R. Barrio, and N.J. Leonard, Biochemistry **18**, 5557-5561 (1979).
16. M.S. Rosendahl, N.J. Leonard, and M. DeLuca, Photochem. Photobiol. **35**, 857-861 (1982).
17. M.S. Rosendahl, G.M. Omann, and N.J. Leonard, Proc. Natl. Acad. Sci. USA **79**, 3480-3484 (1982).
18. R.A. Lessor and N.J. Leonard, J. Org. Chem. **46**, 4300-4301 (1981).
19. R.A. Lessor K.J. Gibson, and N.J. Leonard, Biochemistry, in press.
20. K.P. Moder and N.J. Leonard, J. Am. Chem. Soc. **104**, 2613-2624 (1982).
21. T.M. Stevenson and N.J. Leonard, J. Org. Chem., in press.
22. M. d'Alarcao and N.J. Leonard, J. Am. Chem. Soc. **105**, 5958-5960.
23. V. Bakthavachalam, M. d'Alarcao, and N.J. Leonard, J. Org. Chem. **49**, 289 (1984).
24. V. Bakthavachalam, Ph.D. Thesis, University of Illinois, 1983.