DIMENSIONAL PROBES OF ENZYME BINDING SITES: SYNTHESIS AND BIOLOGICAL ACTIVITY

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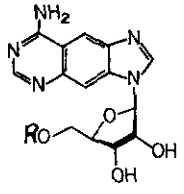
We have introduced the concept of testing the dimensional restrictions of enzyme-active sites by synthesizing cofactor analogues which are related to the natural cofactors by defined dimensional changes in the molecules (Leonard et al., 1976; Scopes et al., 1977). Linear-benzoadenine nucleotides (1), defined by the formal insertion of a benzene ring (actually four carbons) into the center of the purine ring system, have been synthesized and their chemical integrity and purity have been analyzed by HPLC, paper electrophoresis, and ^{31}P NMR spectroscopy. While many analogues of adenine nucleotides have been made and tested for enzyme activation and inhibition, the linear-benzoadenine nucleotides (1) are unique in retaining the terminal pyrimidine and imidazole rings of adenine intact, as well as the attached ribosyl phosphate(s).



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We have found that stretched-out versions of adenine nucleotides, which are 2.4 Ångstroms wider than normal due to the formal insertion of a benzene ring between the terminal rings, exhibit strong enzyme binding, act as cofactors in enzyme reactions, and have spectroscopic properties, including useful fluorescence, that help define their environments. There was no sure way to predict such findings a priori. We have recognized that the heteroaromatic ring system in 1 differs electronically from adenine, which is reflected, for example, in basicity, nucleophilicity, and π-bonding characteristics, but the similarities in peripheral rings, in ring locus of protonation, and in relative position and conformation of ribosyl phosphate attachment emboldens us to call the lin-benzoadenine nucleotides "dimensional probes". Their interactions with enzyme systems can tell us, inter alia, the effect of greater spacing between N7 and 6-NH_2, N9 and N3 of adenine.

The lin-benzoadenine nucleotides bind strongly and have generally somewhat slower enzymatic rates with a representative group of kinases, comprising pyruvate kinase, phosphofructokinase, phosphoglycerate kinase, hexokinase, and acetate kinase. Lin-benzo-AMP is a substrate for AMP deaminase. Lin-benzo-ADP acts as a substrate for primer independent polynucleotide phosphorylase (Micrococcus luteus) in the presence of Mn^{2+}. Lin-benzo-ATP is an activator of E. coli aspartate transcarbamylase equivalent to ATP. Lin-benzo-cAMP maximally activates protein kinase from brain or muscle. As for adenylate kinase, which catalyzes the

Table I: Adenylate Kinase[a]

$ATP \cdot Mg^{2+} + AMP \xrightleftharpoons{\text{Myokinase}} ADP \cdot Mg^{2+} + ADP$	
+	Lin-benzo-ATP + AMP
-	Lin-benzo-ATP + Lin-benzo-AMP
-	ATP + Lin-benzo-AMP
-	Lin-benzo-ADP
+	Lin-benzo-ADP + ADP

[a] + means reaction, - means no reaction took place.

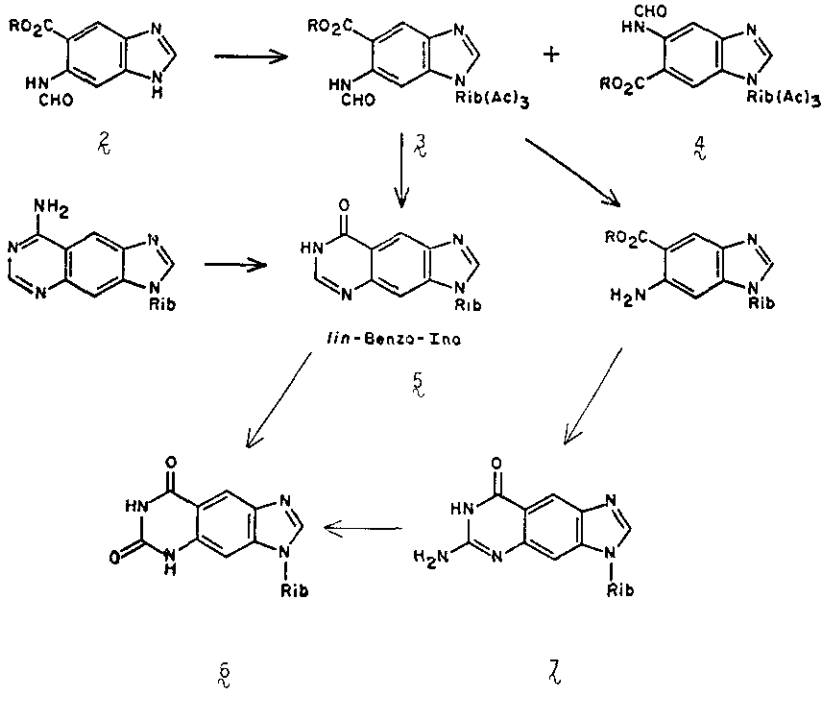
transfer of phosphate between MgATP and AMP and the reverse reaction between MgADP and ADP, our results (Table I) support the two-site model in which the first site is specific for AMP or ADP and the second, less demanding, is specific for ATP or ADP. The stretched-out analog, lin-benzo-ADP, with the enzyme alone remains unchanged, while the mixture of lin-benzo-ADP and ADP comes to equilibrium with five species present: AMP, ADP, ATP, lin-benzo-ADP, and lin-benzo-ATP. Thus, it is clear that one of the ADP binding sites is highly specific and corresponds to the AMP binding site. Neither lin-benzo-ADP nor lin-benzo-AMP can react at that site.

The failure of some enzymes to function with the stretched-out versions of the adenine nucleotides is to be expected. Even lack of activity will provide a positive indication of the discrimination of the particular enzyme with respect to the dimensional requirements, inter alia, of the cofactor or substrate binding site (Leonard et al., 1976). As an example, current experiments carried out on T7 phage DNA-directed RNA synthesis in vitro show, not too surprisingly, that lin-benzo-ATP does not replace ATP for RNA synthesis and does not inhibit ATP incorporation into RNA (D. Nakada, University of Pittsburgh). Also, lin-benzo-ATP does not get incorporated by primase (dnaG protein), a rifampicin-resistant RNA polymerase (Bouché et al., 1975), into a polynucleotide chain, the synthesis of which is template-directed (Rowen and Kornberg, 1978). These experiments indicate that normal Watson-Crick base pairing is exacting in template-directed polymerizations. The lin-benzoadenine nucleotides show useful fluorescence properties and sensitivity to environmental conditions, and it is possible to designate the cases of interaction of phosphate with the heteroaromatic moiety. Fluorescence spectroscopy was used to investigate the interaction of lin-benzoadenine nucleotides with Mg^{2+}, Mn^{2+}, and Co^{2+}. The association constants for the formation of such complexes were obtained from measurements of steady state fluorescence quenching. Phase and modulation measurements of the fluorescence lifetimes of lin-benzoadenine nucleotides vs. Co^{2+} concentration permitted determination of the static component of the quenching due to intramolecular complex formation. The association constants of the lin-

benzoadenine nucleotides with all of the divalent metal ions studied were greater than those observed for the corresponding adenine nucleotides and were in the order: lin-benzo-ATP > lin-benzo-ADP > lin-benzo-AMP. Fourier transform ^{1}H NMR of lin-benzo-ATP in the presence of Co^{2+} showed broadening of the aromatic proton signals, the 2-H signal (corresponding to the 8-H in ATP) being the most affected. Models are proposed to explain the phosphate-base interaction, the influence of metal ions on base protonation, and the intramolecular quenching observed in the complexes due to paramagnetic ion (Co^{2+}, Mn^{2+}) and base interaction.

In the synthesis of the lin-benzoinosine, -xanthosine, and -guanosine series, it has been preferable to incorporate the ribose unit earlier in the process than we did for the lin-benzoadenosine series (Leonard et al., 1976) in order to enhance the solubility characteristics of the intermediates. For example, lin-benzoinosine (5) was synthesized by ribosidation of the mercuri salt of 5-carbethoxy-6-formamidobenzimidazole (2) to give a mixture of 1- and 3-tri-O-acetyl-β-D-ribofuranosyl derivatives (3 and 4, respectively, Scheme I). After separation, the 1-substituted intermediate was ring-closed to 5 with concomitant deprotection of the sugar residue. Conversion of lin-benzo-Ino (5) to lin-benzo-IMP (5--5'-monophosphate) was accomplished chemically, while preparative-scale enzymatic oxidation of 5 gave lin-benzoxanthosine (6) in 86% yield. Xanthine oxidase was also surprisingly efficient in converting lin-benzo-IMP to lin-

Scheme I



benzo-XMP (6--5'-monophosphate). The enzyme reactions of the 5'-mono-, 5'-di-, and 5'-triphosphates of 5, 6, and of lin-benzoguanosine (7) are of particular interest because these are the first dimensional probes for substrates and cofactors in the -NH-CO- series of nucleotides.

This work is part of a long-range goal, which is to provide enough purine ring analogues with known dimensional alterations that the limiting size of an enzyme binding "pocket", "slot", or "surface" for the substrate or cofactor can be accurately defined.