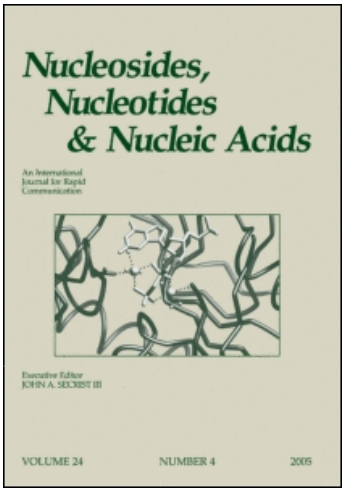

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Synthesis of New Fluorescent Nucleoside Analogues and Application to the Study of Human Deoxycytidine Kinase

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SYNTHESIS OF NEW FLUORESCENT NUCLEOSIDE ANALOGUES AND APPLICATION TO THE STUDY OF HUMAN DEOXYCYTIDINE KINASE

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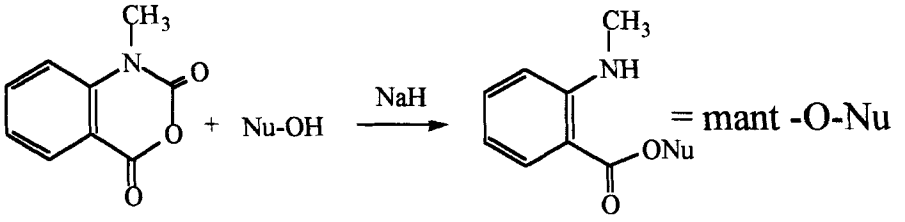
ABSTRACT: We have determined the affinity of human deoxycytidine kinase with respect to new fluorescent N-methylanthraniloyl cytidine derivatives or non fluorescent enantiomeric cytidine analogues. New results regarding the enantioselectivity and the mechanism of the enzyme are presented.

Deoxycytidine kinase (dCK) is an important enzyme in the salvage reaction of nucleoside synthesis. The enzyme has a broad substrate specificity and is therefore it plays an important role in the *in vivo* activation of many nucleoside analogues used in antiviral chemotherapy. The enzymatic mechanism is complex with the occurrence of bimodal kinetic curves, negative cooperativity and the apparent existence of two binding sites or states[1,2]. Furthermore, dCK has a markedly low enantioselectivity and is able to catalyze the phosphorylation of both enantiomers of cytidine[3] or adenosine [4] analogues. Consequently, dCK has been suggested to be a key enzyme in the activation of L-nucleoside analogues to the corresponding L-5'-triphosphates which are the active entities in antiviral chemotherapy. We are currently studying the enantioselectivity of dCK with respect to a broad series of substrates. In the present work, we determined the affinity of the enzyme to newly prepared fluorescent cytidine derivatives and to non fluorescent enantiomeric nucleoside or nucleotide analogues using fluorescence spectroscopy.

MATERIALS AND METHODS

Enzyme : Recombinant human dCK was produced and purified from the human dCK cDNA sequence[5]. It had a histidine tag sequence and was more than 90% pure with very similar properties compared to the non tagged enzyme.

Synthesis of N-methylanthraniloyl (mant) nucleosides :



5'-*O*-Mant-cytidine, **1**, 2'(3')-*O*-mant-cytidine, **2**, 5'-*O*-mant-2'-deoxycytidine, **3**, 3'-*O*-mant-2'-deoxycytidine, **4**, where prepared through selective protection of the adequate hydroxyl and amino groups of cytidine and deoxycytidine with monomethoxytrityl chloride or *t*-butyldimethylsilyl chloride. The desired compounds were obtained after treatment with sodium hydride and N-methyl isatoic anhydride, followed by acidic deprotection. Combustion analyses and spectroscopic properties (^{1}H NMR, FAB-MS) were consistent with the proposed structures. All compounds exhibited a characteristic maximum of fluorescence emission at 440 nm upon excitation at 320-350 nm[6].

Non fluorescent ligands: The ß-D- or L-cytidine analogues have been prepared as previously reported[3].

Fluorescence experiments: The tryptophan intrinsic fluorescence was excited at 290 nm and the emission measured at 332 nm to titrate a 0.5 µM solution of the enzyme with the ligands. The fluorescence of mant-derivatives was excited at 350 nm and the emission observed at 440 nm in the titration of a 0.1 µM solution of ligand with the enzyme up to a 1.5 µM concentration. The values of Kd parameters were determined as previously described[7].

RESULTS AND DISCUSSION

In the study of nucleoside and nucleotide transforming enzymes, the efficiency of the N-methylanthraniloyl fluorophore is probably due to its high quantum yield and its

modest steric hindrance resulting in relatively slight modifications of the binding compared to the parent compounds[6]. To characterize the binding sites and the enantioselectivity of human dCK we have prepared several mant derivatives of cytidine (**1-4**) not accessible by the direct synthetic method involving unprotected precursors [6]. The binding of these compounds and of a series of unlabelled enantiomeric cytidine derivatives was studied using the variations of either the intrinsic tryptophan fluorescence of the enzyme or the fluorescence of the mant derivatives.

Using tryptophan fluorescence, the titration curves of dCK by the mant compounds **1-4** and the enantiomeric pairs (D or L) ß-dC, ß-ddC, ß-araC, ß-riboC, ß-dA and ß-ATP were biphasic, except for ATP. These results demonstrate the existence of two binding sites (or conformational enzymatic states) for nucleosides and their analogues, and one site (or state) for ATP. The first binding (Kd_1: 0.6-4.3 μM) was much more stronger than the second (Kd_2: 45-300 μM). The enzyme was only slightly enantioselective in binding the ligands, with a preference for D-enantiomers (Kd_1: 0.6-1.5 μM) compared to L-enantiomers (Kd_1: 1.3-2.7 μM). The study of the titration of compounds **1-4** with dCK using the fluorescence of the mant group showed an initial non specific binding to the enzyme and suggested that ATP,$MgCl_2$ is the first substrate to bind to the enzyme in the reaction mechanism.

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