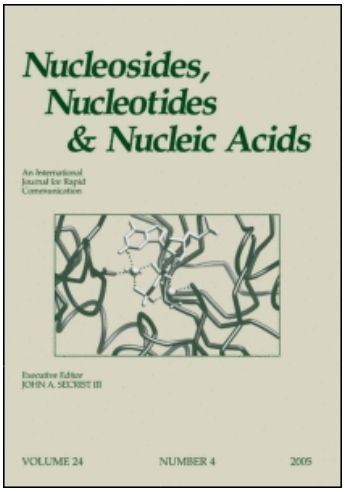

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KINETIC AND MOLECULAR MODELING OF NUCLEOSIDE AND NUCLEOTIDE INHIBITION OF MALATE DEHYDROGENASE

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ABSTRACT

We studied the inhibition of mitochondrial malate dehydrogenase (mMDH) by the nucleotides cAMP, AMP, ADP, ATP. The experimental kinetic studies showed that the nucleotides were competitive inhibitors and that cAMP was probably the most potent inhibitor. To explain these observations, we used molecular modeling to determine the location, orientation, and relative binding energy of the nucleotides to mMDH. The order of the calculated binding energies, from lowest (most favorable) to highest, was cAMP, AMP, ADP, and ATP, which corresponded somewhat to the order of the experimentally determined inhibition constants.

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INTRODUCTION

Malate dehydrogenase (MDH) is well characterized. It catalyzes the reversible reaction of the oxidation of malate by NAD^+ to produce NADH and oxaloacetate. Isozymes of MDH play crucial roles in the citric acid cycle, malate shuttle system, and gluconeogenic metabolic processes. The substrate binding sites in the x-ray crystal structures[1–3] of MDH from various sources are remarkably similar, despite differences in their overall tertiary structures. The structure of mitochondrial malate dehydrogenase (mMDH) from porcine heart is less similar to cytosolic (cMDH) from porcine heart than to the MDH structures from the prokaryotic enzyme of *Escherichia coli* (eMDH).[3] Furthermore, the monoclinic crystal of eMDH soaked in 10 mM NAD was isomorphous with the apo-enzyme crystals.[1] This indicates that the enzyme undergoes little structural change upon binding with NAD.

To better understand the structure and function of MDH, researchers have studied the binding of NAD and other ligands to MDH. For example, Labrou et al.[4–5] modeled the interactions between chimeric biomimetic dye ligands and bovine heart mMDH. These studies used the crystal structure of eMDH[1] because at that time of their publication, the crystal structure of mMDH was not available. Another computational study[6] also used the eMDH crystal structure to model the entire enzyme reaction mechanism of MDH.

Kinetic studies[7] showed that AMP, ADP, and ATP are competitive inhibitors of porcine heart mMDH, and therefore assumed to bind at the NADH binding site. Furthermore, Sulimovici and Lunenfeld[8] showed that cAMP inhibits mMDH, although the observed cAMP concentrations were significantly higher than occurs physiologically.

In our study, we repeated the kinetics studies of the inhibition of MDH by cAMP, AMP, ADP, and ATP to verify the previous experiments and to quantitatively compare the extent of inhibition. We then applied molecular modeling to understand the experimental results.

The goals of this study, therefore, are (a) to measure the K_i of cAMP, AMP, ADP, and ATP in the kinetics of MDH, (b) to model the interactions between various adenine compounds and mMDH, (c) to compute the relative binding energies of the various nucleotides to mMDH, and (d) compare the calculated binding energies to the experimental inhibition results from the enzyme kinetics. These goals will aid in providing a foundation for calculating binding energies between various nucleoside and nucleotide analogues and their receptor enzymes.

MATERIALS AND METHODS

Enzyme Kinetics Materials

The nucleotides, nucleoside, enzyme, and coenzyme were obtained from Sigma. Malate dehydrogenase (E.C. 1.1.1.37) came from porcine heart (mitochondrial) and was suspended in 2.8 M ammonium sulfate solution, pH 6.0. Oxaloacetate salt was also obtained from Sigma. Sodium phosphate buffer components were supplied by J. T. Baker. Assays were placed in a Bacharach Coleman® Model 35 Spectrophotometer and results were recorded by a Goerz Servogor 120 recorder. An ice-water slurry was used to maintain enzyme at 4°C.

Enzyme Kinetics Methods

Basic components of the assay solutions included oxaloacetate at a fixed 3.33 mM concentration, NADH at varied concentrations (0.025 to 0.100 mM), and nucleoside or nucleotide at a fixed 1.0 mM concentration. All solutions were made up in 0.1 mM sodium phosphate buffer (pH 7.4). A specific volume of enzyme (0.100 mL) was added to each assay to begin the reaction. Each enzyme preparation was diluted in buffer to provide a final enzyme activity that could be accurately measured. Dilutions were typically 500 fold from the original enzyme suspension. Oxidation of NADH was continuously recorded over a time interval of one minute with the spectrophotometer set at 340 nm, the characteristic absorbance peak of NADH. Activity was calculated from the initial linear portion of each recorded trace. To determine reaction rates, the data were then converted to μmoles NADH oxidized/2 min. Molar extinction coefficient for NADH = $6200\,M^{-1}\,cm^{-1}$. All data generated from individual inhibitor assays were normalized as a percent of the V_{max} for the appropriate control curve for that specific day. The resulting data were averaged to produce final Lineweaver-Burke plots.

Molecular Modeling Methods

We carried out all calculations using the AMBER force field of the Discover/Insight II program suite from Accelrys, Inc., on a Silicon Graphics O2 workstation.

We used the X-ray coordinates for the citrate:mMDH complex from the Protein Data Bank (PDB ID: 1MLD). To study the binding of the various inhibitors to the enzyme, we used a starting conformation and orientation of the coenzyme and various inhibitors in the catalytic site

corresponding to that of the NAD coenzyme found in the crystal structure of eMDH, which possesses 59% residue homology to mMDH. By superimposing all atoms of the critical active site residues of the eMDH structure onto the corresponding active site residue atoms of mMDH, we created a model of how the coenzyme and the inhibitors might appear in the coenzyme-binding site of mMDH.

The charges on all the atoms of the molecules were assigned automatically by Insight II based on the AMBER force field, with the following exception: we explicitly assigned the partial charges of the phosphate moiety of the inhibitors to give the correct total formal charge for AMP, ADP, and ATP. Although these values might not be exact, they are sufficiently accurate for the molecular modeling to represent the inhibitors in the correct protonated state at physiological conditions.

The total conformational energy of the enzyme-coenzyme and enzyme-inhibitor complexes was minimized using the conjugate gradient method without non-bonding interaction cutoff. The dielectric constant was set to 4.0, with distance dependence. The degree of convergence was set for an RMS value of less than 0.01 kcal/mol-Å. During minimization, all atoms in the system (including the enzyme, the coenzyme and inhibitors, and the solvent) within an 8 Å sphere around the inhibitor were allowed to vary.

To take into account the effect of desolvation of the various nucleotide inhibitors, with their differing anionic charge, we included explicit water molecules. The binding energy (ΔE_B) was thus defined as $\Delta E_B = E_{Inhibitor+Enzyme+Water} - (E_{Inhibior+Water} + E_{Enzyme+Water})$, where $E_{Inhibitor+Enzyme+Water}$ is the total energy of a minimized complex, $E_{Inhibitor+Water}$ is the energy of the minimized inhibitor water complex, and $E_{Enzyme+Water}$ is the energy of the minimized enzyme:water complex with no inhibitor in the coenzyme site. $E_{Inhibitor+Water}$ was obtained by utilizing a conformational searching procedure for nucleotides and nucleosides[10] that produces an ensemble of minima from which a Boltzmann average energy could be calculated. The nucleotide minimum that possesses a conformational energy closest to the calculated Boltzmann average energy was selected and solvated with a specified number of explicit water molecules and minimized to give $E_{Inhibitor+Water}$. For the calculation of binding energies, we made sure that the total number of waters in the complex of enzyme and inhibitor equaled the sum of number of waters with the inhibitor and with the enzyme.

Based on the above definition for the binding energy, a negative value of ΔE_B means that the binding is energetically favorable, whereas a positive value means that the binding is unfavorable.

The relative binding energy $\Delta\Delta E_B$ was defined as the difference between binding energies of any two complexes. For example, when we use $\Delta\Delta E_B$ to compare the binding energies of the cAMP complex with the AMP complex,

we subtracted the binding energy of the cAMP complex from the binding energy of the AMP complex. In other words, $\Delta\Delta E_B = \Delta E_{B,AMP} - \Delta E_{B,cAMP}$, where a positive value means that the binding of AMP to the enzyme is less energetically favorable than the binding of cAMP. The calculation of $\Delta\Delta E_B$ may lead to the cancellation of errors due to the approximation involved in force field simulations.[9]

RESULTS AND DISCUSSION

Kinetic Results

Figure 1 and Table 1 summarize the kinetic results. The data show that all the nucleotides competitively inhibit mMDH with respect to the coenzyme NADH in the following order: cAMP > AMP > ADP ≈ ATP. (All the experimental values of K_i, however, are within experimental error, so the molecular modeling helps delineate the relatively binding of the nucleotides to mMDH.) Due to the observed competitive inhibition of mMDH by the

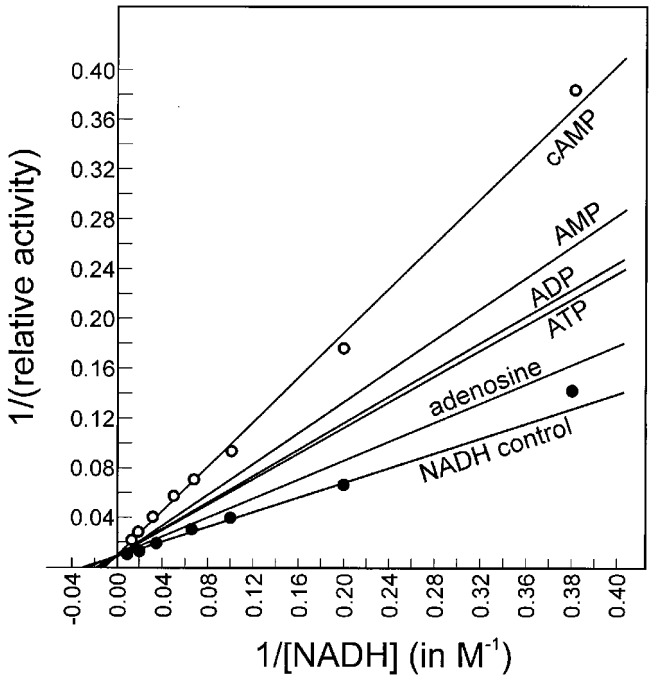

Figure 1. Double-reciprocal plot for the inhibition of malate dehydrogenase. Open circles are data points for cAMP; closed circles are data points for the NADH control. Other data points were omitted to avoid cluttering the diagram.

Table 1. Inhibition Constants (K_i), Binding Energies (ΔE_B), Relative Binding Energies ($\Delta\Delta E_B$), and Ligand Strain Energy (E_{strain}) for the Competitive Inhibition of mMDH by the Nucleotides cAMP, AMP, ADP, ATP

Inhibitor	K_i (mM)	ΔE_B(kcal/mol)	$\Delta\Delta E_B$(kcal/mol)[a]	E_{strain} (kcal/mol)
cAMP	0.56 ($\pm$0.1)[b]	$-$73.4	0.0	0.7
AMP	0.95 ($\pm$0.3)	$-$64.6	8.8	6.4
ADP	1.34 ($\pm$1.0)	$-$61.6	11.8	9.8
ATP	1.36 ($\pm$0.7)	$-$49.1	24.2	16.2

[a]Relative to cAMP.
[b]Error ranges in K_i were calculated from the standard errors for the K_m without inhibitor and the K_m with inhibitor.

various nucleotides, we concluded that the inhibitors were binding to the coenzyme-binding site.

As previously determined,[7] we found that adenosine produced little inhibition of mMDH, and thus we did not include it in our molecular modeling study.

Minimized Structures of the Enzyme-Coenzyme and Enzyme-Inhibitor Complexes

The minimized structure of the coenzyme NAD bound in the active site of mMDH is depicted in Fig. 2. We found the very small RMS value of 0.70 upon superimposing all the atoms of the critically active-site amino-acid residues of eMDH with those of mMDH. This observation is consistent with the finding that the apo-form of eMDH is isomorphous with the bound coenzyme form of the enzyme.[1] We found that the bulky hydrophobic residue of Ile-116 covers the B-face of the nicotinamide ring. This characteristic was proposed to induce the anisotropic puckering motion of the nicotinamide ring, placing the hydrogen to be transferred to the substrate in an energetically favorable pseudoaxial position.[11]

As seen in Figs. 3 and 4 and as indicated by the calculated interaction energies in Table 1, the results of energy minimization of the four solvated enzyme-inhibitor complexes verified that the inhibitors bind favorably to the coenzyme site of mMDH. During minimization, the conformation and orientation of the various components of the complexes changed only slightly, and the overall shapes of the complexes stayed about the same. For example, during minimization, the adenine base and phosphate moieties maintained about the same position and orientation as the minimized coenzyme structure (compare Figs. 2 and 4). These results suggest that the

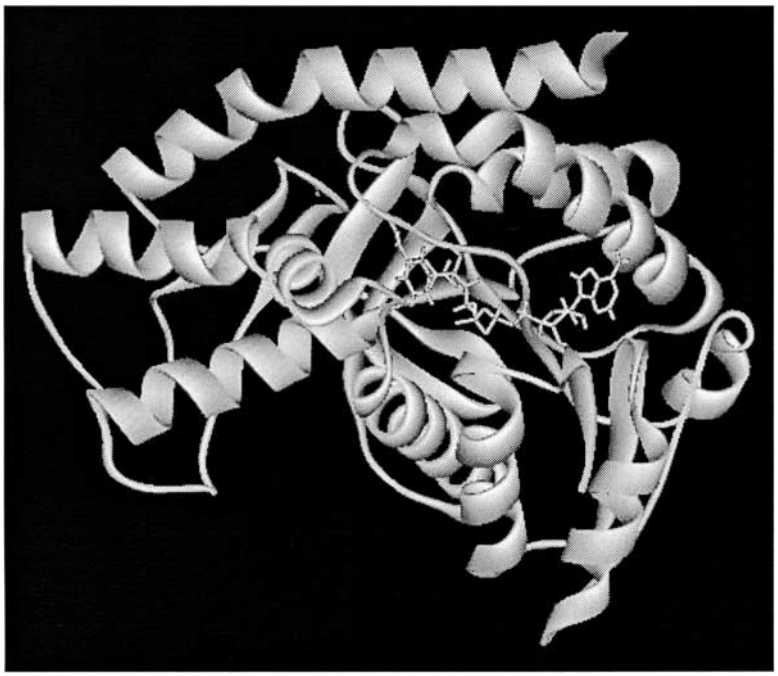

Figure 2. Minimized structure of NAD bound in the active site of mMDH.

structures we selected as the starting conformations for the inhibitors are stable in the coenzyme site of mMDH.

Binding Energies

Table 1 shows the binding energy ΔE_B for cAMP, AMP, ADP, and ATP to the enzyme and the relative binding energy $\Delta\Delta E_B$ between the cAMP complex and the other complexes. These values indicate that the binding of all nucleotides to the enzyme is energetically favorable, and because the $\Delta\Delta E_B$ values for the AMP, ADP, and ATP are positive, the binding of cAMP is more favorable than that for the other nucleotides. The trend in the calculated binding energy (cAMP < AMP < ADP < ATP) qualitatively agree with the observed kinetic results, as shown by comparing the K_i and $\Delta\Delta E_B$ values in Table 1.

We note that the experimental K_i values of all the nucleotides studied here are within experimental error, and in particular, that ADP and ATP are indistinguishable, whereas the computed binding energies show that cAMP binds most favorably and that ADP binds more favorably than ATP. Thus, the molecular modeling provides insight into the actual order of binding.

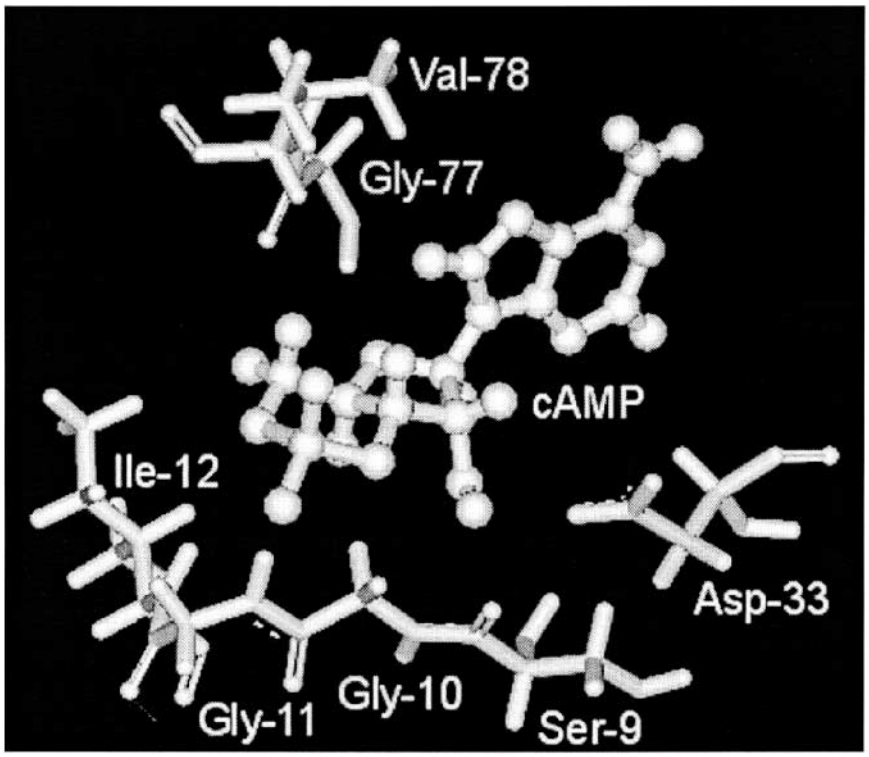

Figure 3. Minimized structure of cAMP in the coenzyme-binding site of mMDH, showing only key residues of the enzyme.

Our general results are similar to those found in a study[12] of the binding energies of inhibitors to HIV-1 protease.[12] The calculated binding energies in that study ranged from -76 to -105 kcal/mol, not too dissimilar to our range of -49 to -73 kcal/mol.

Another related study[13] made the important point that the calculation of the binding energy only reflects the affinity of the enzyme for a potential inhibitor structure once it is within the binding site of the "closed" protein structure and that various other factors are involved in the binding process that are not reflected in the binding energy term. We have made an attempt to partially account for one of these factors, namely, desolvation, by including water in our calculations. In fact, desolvation is a major factor in determining the order of binding of the nucleotides to mMDH.

We also include in Table 1 the strain energy (E_{strain}) associated with the nucleotides within the binding site. The strain energy is defined as the energy difference between the nucleotide structures in the bound conformation and in water. This energy term contributes to the order of the binding energies: the lower (more favorable) the binding energy, the lower the strain energy.

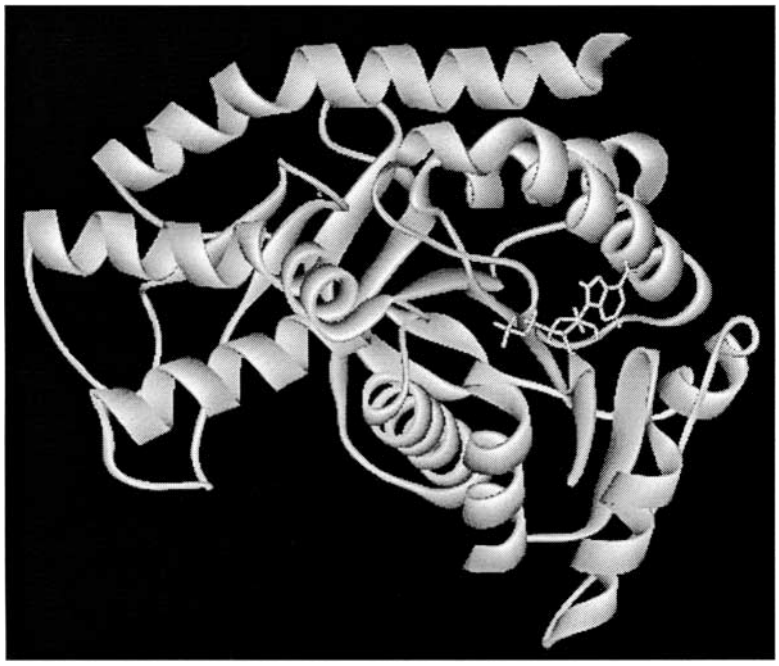

Figure 4. Minimized structure of AMP in the coenzyme-binding side of mMDH.

Thus, a low strain energy contributes to the fact that cAMP binds more favorably to mMDH than the other nucleotides.

CONCLUSIONS

We used molecular modeling to study the binding of cAMP, AMP, ADP, and ATP to the coenzyme site of mMDH. Using a crystal structure from eMDH to help generate starting conformations, positions, and orientations of the coenzyme and inhibitors in the coenzyme-binding site of mMDH proved successful in finding low-energy complexes and in producing results consistent with the kinetics.

Our results suggest that structurally and energetically, cAMP binds in a similar orientation as the adenine portion of NAD in the coenzyme site. Furthermore, the calculated ΔE_B and $\Delta\Delta E_B$ values for the binding of the inhibitors show that cAMP binds favorably to the enzyme, and more favorably than AMP, ADP, ATP, accounting for the experimental kinetics results. With the success of the molecular modeling methodology in determining the correct order of affinity between enzyme and inhibitor in this familiar system, we are ready to apply this methodology to less familiar systems involving drug-enzyme complexation.

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