A pH Dependence of $3_{10}$-Helix versus Turn in M-loop Region of PDE4: Observations on PDB Entries and an Electronic Structure Study
# Contents

4.1 Abstract 137  
4.2 Introduction 138  
4.3 Details of Computational Methods 142  
4.4 Results and Discussions 145  
4.4.1 Crystal Structure Analysis 145  
4.4.2 Root Mean Square Deviation 148  
4.4.3 Importance of Secondary Structural Change and its Cause 150  
4.4.4 Influence of pH on M-loop Region 153  
4.4.5 Influence of M-loop Region on Subtype Selectivity 162  
4.5 Conclusions 163  
4.6 References 163
4.1 Abstract
4.2 Introduction

Cyclic nucleotide phosphodiesterases (PDEs) comprise a super family of metallophosphohydrolases. They specifically hydrolyze phosphodiesterase bond of cyclic adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) to produce the corresponding 5'-nucleotides (AMP and GMP) in various cells (Scheme 4.1).1,2 The second messengers (cAMP and cGMP) concentration affects the specific protein phosphorylation cascades. Hence, these isozymes play a vital role in the regulation of various physiological functions like smooth muscle relaxation, visual response, platelet aggregation, cardiac contractibility, immune response, etc.1-3

Scheme 4.1. Function of PDE enzymes

The human genome encodes 21 PDE genes that are grouped to 11 families. Alternative mRNA splicing of these genes generates over 200 splice variants or isoforms of PDE proteins.3b The PDE isozymes differ substantially in their physicochemical properties, tissue distributions, substrate and inhibitor specificities and regulatory
mechanisms.\textsuperscript{2} PDEs contain a conserved catalytic domain of about 250 amino acids and variations are observed in the regulatory domains (N- and C-terminal region).\textsuperscript{3a} They can be categorized into three groups based on substrate specificity: (a) cGMP specific (PDE5, 6 and 9), (b) cAMP specific (PDE4, 7 and 8), and (c) both cAMP and cGMP specific (PDE1, 2, 3, 10 and 11). PDEs are the therapeutic targets for cardiovascular, inflammatory, central nervous system (CNS) and erectile dysfunction diseases.\textsuperscript{4} Among the 11 PDE isozymes, PDE4 and PDE5 have received much attention in the recent years.\textsuperscript{5-7} cAMP specific PDE4 is abundant in various inflammatory cells such as eosinophils, T cells, B cells and neutrophils. Inhibition of PDE4 activity in these cells leads to higher cAMP levels and causes bronchodilation. Thus it is a keen target for the inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, allergic rhinitis, and typeII diabetes.\textsuperscript{6}

There are various classes of novel orally active PDE4 inhibitors discovered in this decade. Based on the structural motifs, PDE4 inhibitors can be broadly classified into three categories as xanthines, catechol ethers and hetrocyclics (nitraquanzone, benzofurans, indoles, isoquinoline, pyridopyrimidinones, pyrazolepyridines etc) (Scheme 4.2).\textsuperscript{8} Rolipram that belongs to catechol ether type has been reported as the first selective PDE4 inhibitor (Scheme 4.2). The first-generation PDE4 inhibitors rolipram, RO-20-1724 (mesopram) and zardaverine are discarded from clinical trials due to the side effects like nausea, vomiting, dyspepsia, headache and emesis.\textsuperscript{9a} The second-generation rolipram analogs (cilomilast, roflumilast and most potent piclamilast Scheme 4.2 B) that are now in clinical trials are also suffering from low therapeutic ratio.\textsuperscript{9b}
Phosphodiesterase4 (PDE4)

The reason for the side effects are (a) poor blood brain barrier (BBB) regions in the emetic centers of CNS allow absorption of the circulating drugs easily and lead to emesis,\textsuperscript{10a} (b) PDE4D subtype is over expressed in the emetic centers such as nucleus tractus solitarius and area postrema,\textsuperscript{10b} and (c) of the two conformation states of PDE, high-affinity rolipram binding form is stated to cause side effects.\textsuperscript{10c,d} The various strategies to overcome these side effects are (a) selectivity towards low affinity versus high affinity rolipram binding form, (b) low BBB permeable drugs, (c) disease activated drugs, (d) subtype selective PDE4 drugs, and (e) selectivity towards broader PDE family.\textsuperscript{6} Among these strategies subtype selectivity of PDE4 is a focus of present investigation.\textsuperscript{6c}

Scheme 4.2. Various classes of PDE4 inhibitors.
Lack of the 3-dimensional structure of a target protein is usually a bottleneck for rational drug design. In the last couple of years there has been a dramatic increase in the number of crystal structures of PDE4 class of enzymes in the protein data bank (PDB). Despite the availability of 39 X-ray structures along with co-crystals for PDE4, progress towards drugs without undesired side effects has been marginal. Our interest in the modeling of the subtype selective PDE4 drug candidates brought us to a close examination of these crystal structures. We found that there is a characteristic secondary structural conformation difference in the PDE4 crystal structures.\textsuperscript{11-17}

Scheme 4.3. A) The secondary structure of PDE4 in the M-loop region existing as $3_{10}$-helix (red color) and turn (green color) are shown along with the amino acids represented as capped stick model. B) Schematic 2-D representation of the $3_{10}$-helix conformation where the hydrogen bonds are shown as lines and residue numbering is given for PDE4B.

* $3_{10}$-Helix is formed when the N-H group of an amino acid forms a hydrogen bond with the C = O group of the amino acid three residues earlier ($i + 3 \rightarrow i$ hydrogen bonding).\textsuperscript{13}
Out of the 35 PDE4B and 4D crystal structures twelve of them have a $3_{10}$-helix and the rest have a turn in the M-loop region (Scheme 4.3A) which is near to the active site pocket. The four crystal structures, one for each PDE4 subtypes are deposited recently and are not included in this work. The distinct pattern of secondary structure in the highly flexible M-loop region is prominent in these crystal structures (Scheme 4.3A). According to the standard protein structural rules, three and half residues are required per turn of $\alpha$-helix. The hydrogen of the amide group of first residue forms a hydrogen bond with the oxygen of amino-terminal peptide bond of the fourth residue in a $\alpha$-helix where as in $3_{10}$-helix it forms with the third residue (Scheme 4.3 B).

Here we would like to present the factor responsible for this secondary structural change in the M-loop region. The methionine of the M-loop region has a hydrophobic interaction with inhibitors such as rolipram analogs and exists in various conformations with NVP inhibitor. Is this structural change a determining factor for the subtype selectivity? We identify that an experimental variable pH seems to be controlling this structural variation rather than the sequential differences in the PDE4. The influence of pH is studied by calculating the various protonation states of side chains or amide bonds of these structures in the gas and explicit solvent phases.

4.3 Details of Computational Methods

4.3.1 Root Mean Square Deviation (RMSD): Residue-by-residue RMSD is calculated using a FORTRAN program. RMSDs were calculated for the backbone residues of all 4B and 4D crystal structures having similar ligands. Molecular operating environment (MOE) homology module is used for structural alignment of PDE4B and
4D structures. The RMSD values were calculated using the following equation where $d_i$ is the distance between $i$th atoms and $n$ is the number of such distances.

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n} d_i^2}{n}}$$

4.3.2 Electronic Structure Calculations: We have used $ab$ initio calculations on the model structures, generated from the fragment SPMCD (Ser, Pro, Met, Cys, Asp) residue sequence of PDE4B and PDE4D PDB coordinates. The resulting dangling valencies were saturated by appropriately placing hydrogens. For uniformity and to average out the specific differences in the geometries arising from the process of formation of the crystals, only the hydrogen atom positions in each of the structures were optimized. The dihedral angles were kept constant during this process. The protonated and deprotonated states of aspartic acid, cysteine and amide bonds were calculated for seven sets of ligands. All electronic structure calculations were performed at the B3LYP/6-31+G* level of theory.23

The gas phase optimized model structures 1TB5 having $3_{10}$-helix and 1TB7 having turn were further taken and a layer of 5 angstrom thickness of water molecules is added using “soak” option in InsightII.24 The total number of water molecules present around the pentapeptide protein structure is 119. The water molecules of the proteins were generally treated as low layer with molecular mechanics in QM/MM calculations to reduce the computational cost. The ONIOM25,26 calculation is implemented for this system to analyze the explicit solvent effect on the structures. A two layer ONIOM calculation is adopted for the system, where the pentapeptide sequence SPMCD is defined quantum mechanically (high layer) with B3LYP/6-31+G* level of theory and the
water molecules were defined as low layer molecular mechanics part with universal force field.\textsuperscript{27} The ONIOM energy of the system is given as

\[ E_{ONIOM}^{(QM:MM)} = E_{QM}^{model} + E_{MM}^{real} - E_{MM}^{model} = E_{high}^{model} + E_{low}^{real} - E_{low}^{model} \]

All these calculations were done using Gaussian03 program package.\textsuperscript{28}

4.3.3 Molecular Dynamics Simulations: The simulations were performed using PMEMD module of AMBER9 version\textsuperscript{29} in IBM BLUEGENE/L machine. The deposited PDB structures 1TB5 and 1TB7 were taken for the molecular simulations. The coordinates of M-loop region (423-440) of these protein structures were taken. The FF03 force field\textsuperscript{30} was employed. The LEAP module was employed to construct a truncated octahedron solvate box (bcc) of cell length 56.46 Å with TIP3P water molecules around the protein. The solvation shell around the protein is 12Å. The total number of solute and solvent atoms present around the 1TB5 and TB7 were 12578 and 12932 respectively. The protein-solvent system was minimized with 1000 steps of steepest descent and 2000 steps of conjugate gradient method. The protein was fixed during minimization using a harmonic constraint with a force constant of 500kcal/mol/Å\textsuperscript{2}. This was done to remove any close contacts existing in the water shell with respect to protein. The whole system was also minimized after this for 200 steps steepest descent and 800 steps conjugate gradient method, so that the hydrogens of protein were optimized. The minimized structure obtained was then gradually heated up (over 40ps) from 0K to 300K with harmonic constraints on solute using SHAKE method. The dynamics was maintained under constant pressure-constant temperature (NPT) conditions using Berendsen (weak-coupling) temperature with a time step of 2fs.\textsuperscript{31} Finally, a production dynamics of 10ns with protein fixed under NPT conditions with time step of 1fs. The long range
interactions were calculated by particle mesh ewald (PME) method. The grid size of 60×60×60 with grid spacing of 1.0 Å with a direct sum tolerance of 0.00001 and 9.0 Å cutoff was used. The cubic B-spline of fourth order was interpolated in PME. The resulting trajectories of molecular simulations were analyzed using PTRAJ module of AMBER 8.0.

4.4 Results and Discussions

4.4.1 Crystal Structure Analysis

Out of the three domains of PDEs, catalytic domain and part of C-terminal containing amino acids are only crystallized. There are about 60 PDE crystal structures in the PDB and 35 of these belong to PDE4 class with various substrates such as (cAMP), AMP, 8-BrAMP and inhibitors such as rolipram analogs (rolipram, cilomilast, piclamilast, roflumilast, mesopram, and filamilast) and pyrazole analogs (Scheme 4.2). PDE4 is coded by four genes named as A, B, C and D and are called as subtypes. These subtypes depending on m-RNA splicing are of 20 splice variants. The splice variants differ in the upstream conserved region (UCR) that is UCR1 and UCR2 present in the regulatory domain (N-terminal) of the protein. Among the four genes coding for PDE4, about sixteen 4B and nineteen 4D structures are crystallized (Figure 4.1). There is one each for PDE4 subtype with NVP (4-[8-(3-nitrophenyl)-[1,7] naphthyridin-6-yl] benzoic acid) inhibitor containing structures which are deposited recently. The catalytic domain of the first crystal structure available for PDE4B contains 376 residues and explains the general architecture of PDE4. The PDE4 has basically three sub domains comprising seventeen α helices and a β hairpin. The catalytic pocket contains two divalent metal ions (Zn and Mg) that are crucial in hydrolysis of the substrate.
Figure 4.1. Details of the available X-ray crystallographic structures of PDE4 with various ligands deposited in the PDB.
Zn in the active site pocket has distorted trigonal bipyramid geometry, coordinating with Asp_{275}, Asp_{392}, His_{233}, His_{274} and a water molecule of the protein. On the other hand Asp_{275} of the protein and five water molecules surround the Mg atom. Further the co-crystals of PDE4 with various ligands also have similar three subdomains comprising sixteen α helices and a β hairpin (Figure 4.2). Other than the conservation of helices and hairpin we noticed a surprising characteristic feature in these 35 structures: Twelve structures have a 3_{10}-helix in the M-loop region. The remaining 23 of them have a turn in the M-loop region, nineteen of these twenty three belong to the PDE4D and four to PDE4B (Figure 4.1).

**Figure 4.2.** The superimposition of α–carbon of the turn having PDE4B and PDE4D and 3_{10}-helix having PDE4B structures with rolipram are shown with the yellow, green and red color secondary structure at pH= 6.5, 7.0 and 10.0 respectively (left side). The ball and stick model represents the rolipram ligand in the active site. A schematic representation of structural differences with all the other similar ligands is also shown (right side).
4.4.2 Root Mean Square Deviation (RMSD)

We superimposed all structures having the $3_{10}$-helix and the turn with similar seven sets of ligands and the RMSD of residue by residue for the backbone is calculated. There are two structures each for PDE4B and 4D with cAMP, three each with rolipram and one each with cilomilast, piclamilast, roflumilast and pyrazole analogs (Figure 4.1). The superimposition of 4B containing $3_{10}$-helix and 4D with similar ligand structures showed a major difference in three loop regions that is 10/11, 12/13 and in the M-loop region (Figure 4.2). The residues with varying RMSD are Glu$^{317}$ and Glu$^{318}$ of helix 10/11 (316-319) which interacts with UCR1 and UCR2 present in the regulatory domain (N-terminal) of the protein. The residues Ser$^{368}$ and Ser$^{369}$ of helix 12/13 (367-377) belong to extracellular signal-regulated kinases (ERK) docking site that has protein-protein interactions is also altered. In case of the M-loop region$^{13b}$ (424-437) Pro$^{430}$ and Met$^{431}$ residues are altered in the backbone of protein structure (Figure 4.3). The extent of deviation in ERK docking site varies slightly with the ligands.
Figure 4.3. Comparison of RMSD values for backbone residues of PDE4B and 4D with A) cAMP B) Rolipram C) Cilomilast, Piclamilast, Roflumilast and Pyrazole analogs crystallized X-ray structures.

On the other hand the superimposition of the PDE4B and 4D structures containing turn with cAMP and rolipram shows a difference only in the 12/13 loop region (ERK docking site) and in helix 14, particularly from the residues Lys$^{422}$ to Glu$^{427}$ that are oriented towards the solvent region (Figure 4.3 A and B). The superimposition of all the
Phosphodiesterase4 (PDE4) 4B with cAMP and rolipram structures has a difference in 10/11 and the M-loop region (Figure 4.2). In spite of the major homology sequence similarity between 4B and 4D subtypes\textsuperscript{6c} the common differences are found in 12/13-loop region and either at starting of the M-loop region or at Pro\textsuperscript{430} residue. Thus the PDE4 structures can be divided into the two groups: one with the 3\textsubscript{10}-helix and the other with the turn varying at 10/11 and in the M-loop region (Figure 4.2).

4.4.3 Importance of Secondary Structural Change and its Cause

In view of the important role of the secondary structural alterations in specific enzymatic functions of PDE5,\textsuperscript{33} the M-loop region in PDE4 is scrutinized because the methionine residue here has hydrophobic interactions with all rolipram analogs.\textsuperscript{13a,15b} The inhibitor selectivity of rolipram analogs to PDE4 is due to the hydrophobic interactions at the Q2 pocket, where methionine of the M-loop region is a constituent. The PDE4A, 4B, 4C and 4D with NVP, a subtype selective inhibitor also have shown large conformational changes of methionine having relatively high B-factors.\textsuperscript{18} The conformational changes of methionine thus can have impact on inhibitor binding. The analysis of these variations in PDE4 may help to understand the influence of the M-loop region in inhibitor selectivity.

The secondary structural difference of PDE4 crystals cannot be explained by the ligand binding or crystal packing or mutated residues. This is because there is a structural variation of 3\textsubscript{10}- helix and turn in the M-loop region within PDE4B structures with the similar ligands and the same space group (Figure 4.1). The SPMCD residue sequence is conserved in all subtypes hence cannot account for this structural difference. After an extensive search, the only difference we could find between these two sets of structures is the pH maintained during crystallization. Without any exception it was found that all the
PDE4B structures having $3_{10}$-helix are crystallized at pH=10.0 from the polar solvents such as aqueous LiSO$_4$ and NH$_4$SO$_4$ solution. In contrast, all the turn-containing PDE4B and 4D structures are obtained at pH=6.5-7.5 range with polyethylene glycol (PEG) conditions (Table 4.1). Though we do not know the reason for the use of different pH in these experiments, the results are surprising because in these examples the pH appears to dominate over the differences in sequences of PDE4B and 4D.

Table 4.1. The crystalline conditions, solvent water and ethane diol (EDO) molecules present in the various chains of 35 PDE4 crystal structures.

<table>
<thead>
<tr>
<th>PDE4 Subtype</th>
<th>PDB ID</th>
<th>pH</th>
<th>Crystalline conditions</th>
<th>Secondary Structure</th>
<th>Chain</th>
<th>Solvent (H$_2$O) near NH</th>
<th>Solvent near CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1F0J</td>
<td>7.0</td>
<td>PEG</td>
<td>II</td>
<td>a</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1ROR</td>
<td>6.5</td>
<td>PEG 3000, sodium acetate, glycerol, sodium cacodylate,</td>
<td>II</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1RO6</td>
<td>6.5</td>
<td></td>
<td>II</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1RO9</td>
<td>6.5</td>
<td></td>
<td>II</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1TB5</td>
<td>10.0</td>
<td>Ammonium sulfate, lithium sulfate</td>
<td>I</td>
<td>a</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1XMY</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1XN0</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>1XLX</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1XM4</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1XMU</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1XLZ</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>1XM6</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>1XOS</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1XOT</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1Y2J</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1Y2H</td>
<td>10.0</td>
<td></td>
<td>I</td>
<td>a, b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>1PTW</td>
<td>7.50</td>
<td>50 mm Heps, 15% PEG 3350, 25% Ethylene Glycol, 5% Methanol, 5% DMSO</td>
<td>II</td>
<td>a, b</td>
<td>c, d</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>2PW3</td>
<td>7.50</td>
<td>0.1 M HEPES, 0.1M</td>
<td>II</td>
<td>a</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Code</td>
<td>Concentration</td>
<td>Description</td>
<td>Index</td>
<td>EDO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------</td>
<td>-----</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1TB7</td>
<td>7.00</td>
<td>PEG 3000, ethylene glycol, isopropanol, DTT</td>
<td>b 2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1TBB</td>
<td>7.00</td>
<td>MgCl₂, 12% PEG3350, 30% Ethylene Glycol, 10% Isopropanol</td>
<td>b 1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Q9M</td>
<td>7.50</td>
<td>0.1 M HEPES, 20% PEG3350, 30% Ethylene Glycol, 10% Isopropanol, 5% Glycerol</td>
<td>a, b,</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1OYN</td>
<td>7.50</td>
<td>50 MM HEPES, 20% PEG3350, 25% Ethylene glycol, 20% Isopropanol</td>
<td>a, b,</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1XOM</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a 1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1XON</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a, b</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1XOQ</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a, b</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1XOR</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a 1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1MKD</td>
<td>6.5</td>
<td>PEG8000, Mg acetate, DTT,</td>
<td>a, b, c, d,</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1RKO/1ZKN</td>
<td>7.50</td>
<td>0.1 M HEPES, 20% PEG3350, 30% Ethylene Glycol, 10% Isopropanol, 5% Glycerol</td>
<td>b 1</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Y2B</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a, b</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Y2C</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a, b</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Y2D</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a, b</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Y2E</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a 2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Y2K</td>
<td>7.0</td>
<td>PEG3350, Ethylene Glycol, Isopropanol, Glycerol and DTT</td>
<td>a 1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2FM0</td>
<td>7.50</td>
<td>0.1 M HEPES, 15% PEG3350, 25% Ethylene Glycol, 5% Isopropanol, 5% Glycerol</td>
<td>a 2</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2FM5</td>
<td>7.50</td>
<td>0.05 M HEPES, 15% PEG3350, 25% Ethylene Glycol, 5% Isopropanol, 5% Glycerol</td>
<td>a, b</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.4.4 Influence of pH on M-loop Region

As a first approximation we assume that the pH dependence on the different conformations is caused by the loss or gain of a proton that leads to ionizations of carboxylates and amines. The changes in the electrostatic environment of PDE4B and 4D may alter the conformations and finally affect the secondary structure of the protein. The influence of the pH on the structure is known for lysozymes, plastocyanin, γ-chymotrypsin, azurin and insulin. Another example is of cubic insulin crystals that manifest alterations in the conformations of side chains of acidic residues as a function of the pH ranging from 7 to 11. Such an alteration in the protonation states of acidic residues influencing the enzymatic role is well known for the cytochrome P-450s. The difference in the conformation around the 10/11 loop region of PDE4 as a function of the pH might arise from the repulsion between the side chains of two glutamic acid residues that are adjacent to each other.

In the M-loop region the difference in the secondary structure is observed at the backbone of the SPMCD residue sequence that has conformation I in the 3_{10}-helix of PDE4B and II in all the turn containing PDE4B and 4D structures (Figure 4.4). The structures I and II have a conserved hydrogen bond between the CO of serine and the NH of cysteine. These two structures have a difference in the orientation of the carbonyl group of the proline in the amide bond. This difference of the carboxy group orientation of the proline helps to gain extra hydrogen bonding with NH group of the aspartic acid resulting in a twist and forming a 3_{10}-helix structure in I (Figure 4.4, Scheme 4.3B). Generally the constrained geometry of the proline initiates the turning of a protein strand, while here it brings a 3_{10}-helix structure of the conformation I.
Figure 4.4. The optimized conformations, I (3\text{10}-helix) and II (turn), of SPMCD residues at pH=10.0 and pH=6.5-7.5 respectively.

The influence of pH can lead to the existence of various protonation states of side chains or amide bonds in the structures I and II. The aspartic acid and cysteine side chains are generally known to be protonated in various protein structures. There are some examples of backbone amide bonds that undergo protonation when they are involved in interactions with a metal as in Belomycin. So, we anticipate that in SPMCD residue sequence depending on pH, aspartic acid or cysteine side chains or amide bonds can be protonated. We simulate different pH conditions by adding or removing a proton of side chains or amide bonds. The energies of the protonated and deprotonated states of aspartic acid, cysteine and amide bonds in all seven sets of similar ligands having “3\text{10}-helix” and “turn” containing structures are calculated. The protonation and deprotonation states of aspartic acid residue (isodesmic equation 4.1) do
not explain the existing structural variations (Table 4.2). The isodesmic equation 4.2 shows the energetics of the protonation and the deprotonation of the cysteine residue. Except in rolipram ligand containing structures, the formation of the protonation state of cysteine in structure II and the deprotonated state in structure I are exothermic in the gas phase (Table 4.2).

\[
\text{I}_{\text{deproasp}} + \text{II}_{\text{proasp}} \rightarrow \text{I}_{\text{proasp}} + \text{II}_{\text{deproasp}} \quad (4.1)
\]

\[
\text{I} + \text{II}_{\text{deprocys}} \rightarrow \text{I}_{\text{deprocys}} + \text{II} \quad (4.2)
\]

Table 4.2. The reaction energies in kcal/mol for equation (4.1 and 4.2)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>I</th>
<th>II</th>
<th>$\Delta H$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4.1)</td>
</tr>
<tr>
<td>cAMP</td>
<td>1TB5</td>
<td>1ROR</td>
<td>12.64</td>
</tr>
<tr>
<td></td>
<td>1TB5</td>
<td>1TB7</td>
<td>12.47</td>
</tr>
<tr>
<td>Rolipram</td>
<td>1XMY</td>
<td>1RO6</td>
<td>-1.30</td>
</tr>
<tr>
<td></td>
<td>1XMY</td>
<td>1TBB</td>
<td>3.56</td>
</tr>
<tr>
<td>R,S Rolipram</td>
<td>1XN0</td>
<td>1OYN</td>
<td>8.77</td>
</tr>
<tr>
<td>Cilomilast</td>
<td>1XLX</td>
<td>1XOM</td>
<td>-2.77</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>1XMU</td>
<td>1XOQ</td>
<td>10.17</td>
</tr>
<tr>
<td>Piclamilast</td>
<td>1XM4</td>
<td>1XON</td>
<td>8.68</td>
</tr>
<tr>
<td>Pyrazole</td>
<td>1Y2J</td>
<td>1Y2K</td>
<td>7.71</td>
</tr>
</tbody>
</table>

The exothermicity of the reaction is due to the cysteine side chain (SH) in the protonated state gains a weak hydrogen bond with the CO group of the proline in structure II whereas in the deprotonated state it is missing (Figure 4.4). On the other hand in the structure I, the deprotonated state of the cysteine side chain can have weak electrostatic interactions with the NH group of methionine. The protonated state of cysteine side chain in II and the deprotonated state in I have gained a non covalent interaction each justifying their thermodynamic stabilities.

The feasibility of protonation of backbone amide bonds is also studied. Even though the differences of pH by three units may not lead to the protonation and
deprotonation of amide bonds of PDE4 under experimental conditions, we anticipate that the protonation and deprotonation of N2 and N3 (Figure 4.4) of the structure I and II would be an indicator of the influence of the pH. The deprotonation and protonation are done at N3 (NH of methionine) and N2 (N of proline) of the amide bonds respectively where the change of conformation is observed (Figure 4.4). Reaction energies of the isodesmic equation (4.3) for seven sets of similar ligands in their protonated and deprotonated states indicate that II is more stable in the protonated form and I in the deprotonated form (Table 4.3). The isodesmic equation (4.4) shows that all turn containing PDE4 (II) on protonation at N2 are stable over 3₁₀-helix containing structures (I) and in isodesmic equation (4.5) the reverse is observed for deprotonation at N3 (Table 4.3 column 6).

\[
\begin{align*}
I_{\text{pro}} + II_{\text{depro}} & \rightarrow I_{\text{depro}} + II_{\text{pro}} \quad \text{(4.3)} \\
I_{\text{pro}} + II & \rightarrow I + II_{\text{pro}} \quad \text{(4.4)} \\
I + II_{\text{depro}} & \rightarrow I_{\text{depro}} + II \quad \text{(4.5)}
\end{align*}
\]

**Table 4.3.** The reaction energies in kcal/mol for equation (4.3, 4.4 and 4.5)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>I</th>
<th>II</th>
<th>ΔH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(4.3)</td>
<td>(4.4)</td>
<td>(4.5)</td>
</tr>
<tr>
<td>cAMP</td>
<td>1TB5</td>
<td>1ROR</td>
<td>-20.80</td>
</tr>
<tr>
<td></td>
<td>1TB5</td>
<td>1TB7</td>
<td>-10.87</td>
</tr>
<tr>
<td>Rolipram</td>
<td>1XMY</td>
<td>1RO6</td>
<td>-39.32</td>
</tr>
<tr>
<td></td>
<td>1XMY</td>
<td>1TBB</td>
<td>-24.68</td>
</tr>
<tr>
<td>R,S Rolipram</td>
<td>1XN0</td>
<td>1OYN</td>
<td>-23.36</td>
</tr>
<tr>
<td>Cilomilast</td>
<td>1XLX</td>
<td>1XOM</td>
<td>-19.79</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>1XMU</td>
<td>1XOQ</td>
<td>-23.80</td>
</tr>
<tr>
<td>Piclamilast</td>
<td>1XM4</td>
<td>1XON</td>
<td>-15.21</td>
</tr>
<tr>
<td>Pyrazole</td>
<td>1Y2J</td>
<td>1Y2K</td>
<td>-16.72</td>
</tr>
</tbody>
</table>

The order of stability of the protonated state in all II and deprotonated form in I is attributed to the increase in the number of hydrogen bonding interactions. In structure II,
the CO of the proline gains a 1,4 interaction with N2 on protonation and a weak interaction with the CH and SH of methionine and cysteine side chain residues.\textsuperscript{38,39} On the other hand the deprotonation of N3 leads to a weak C-H--N3 interaction\textsuperscript{40} in I similar to the C-H--O interaction seen in II (Figure 4.4). The thermodynamic stability of the protonated and deprotonated states in structures I and II depends on the gain in non-covalent interactions such as hydrogen bonds and electrostatic interaction with the surrounding environment. The exothermicity of the backbone amide bond protonation is quite high than cysteine protonation.

The extent of non covalent interactions in the structures I and II can also differ with respect to the surrounding solvent molecules. The 35 crystal structures are thus thoroughly examined near the M-loop region to know the influence of the water molecules. In all the crystal structures with respect to the methionine of the M-loop region a sphere radius of 10.0Å is analyzed. There are 22 turn containing structures crystallized in PEG conditions. We found that the turn containing (II) PDE4D structures with resolution lower than 2.10Å have ethane diol (EDO) and water molecules in this region (Table 4.1). The carbonyl group of the proline of the structure II has a hydrogen bonding interaction with the EDO or water molecule. The amide group of the methionine of the structure II has a hydrogen bonding network with the NH of aspartic acid through two water molecules (Figure 4.5). These additional interactions with the solvent thus stabilize the structure II. The presence of EDO near to carbonyl group of proline in these structures may be due to PEG crystallization conditions. While such type of EDO or water molecules are not found at the carbonyl group of proline in the M-loop region of four PDE4B (1F0J, 1ROR, 1RO6 and 1RO9) and in high resolution PDE4D turn
containing structures, even though they have similar crystallization conditions (Table 4.1).

![Figure 4.5](image)

**Figure 4.5.** The X-ray crystal structural conformations of A) I (3_{10}-helix 1TB5) and II (turn 1TB7) B) I (3_{10}-helix 1XM6) and II (turn 2PW3) at pH=10.0 and pH=6.5-7.5 respectively. In the SPMCD residue sequence the hydrogen bonding interaction of the amide bond with solvent molecules such as water and EDO is also shown.

Even the number of water molecules varies from 1-2 in the two chains (A and B) of the same crystal. This is also true in other crystals considered here (Table 4.1). It is possible to identify a water molecule even in the twelve low resolution structures with
3\textsubscript{10}-helix showing hydrogen bonding interaction to the amide group of methionine and carbonyl group of proline (Figure 4.5). The number of solvent molecules present around the M-loop region in these crystal structures is varied (Table 4.1).

The irregularity in the number and position of the water and the EDO molecules in the crystal structures led us to study the influence of the solvent molecules on the structures. We model this by constructing a layer of 5Å thickness of water molecules around the pentapeptide SPMCD residue sequence of structures I and II. This model system with various protonation states of the cysteine and the amide bonds of 1TB5 and 1TB7 are studied using QM/MM ONIOM method. In this model the pentapeptide is defined as QM region and water molecules in the MM region (Figure 4.6).

\begin{align*}
I + II_{deprocys} &\rightarrow I_{depro cys} + II \quad \Delta E^{\text{ONIOM}} = -6.55 \text{ kcal/mol} \ldots (4.6) \\
I_{pro} + II_{depro} &\rightarrow I_{depro} + II_{pro} \quad \Delta E^{\text{ONIOM}} = -10.06 \text{ kcal/mol} \ldots (4.7)
\end{align*}

(Where I= 1TB5 and II =1TB7)

The ONIOM energies of the protonated and the deprotonated states of the cysteine and amide bond (N2 and N3) in the structures I and II along with a water layer are given in isodesmic equation 4.6 and 4.7 respectively. The ONIOM calculation in explicit solvent shows the reaction is exothermic and similar as in the gas phase reaction (isodesmic eq 4.2 and 4.3). The water molecules forms a hydrogen bond with the NH group of methionine and the CO group of proline in the structures I and II. The gain in the hydrogen bonding interaction of amide bond in the structure II makes it more stable than I. The hydrogen bonding network of water with the residues in the crystal structures is reproduced by ONIOM calculations (Figure 4.5 and 4.7).
Figure 4.6. The ball and stick model represents the pentapeptide (SPMCD) that is considered high layer QM region and shell with of water molecules (lines) are considered as low layer MM region. The optimized geometry of I and II and their hydrogen bonding network with the solvent water molecules are also shown.

To reinvestigate the average number of water molecules that can exist near to the amide bond, CO group of the proline and NH group of the methionine, MD simulation on M-loop region of a 3_10-helix (1TB5) and turn (1TB7) containing structures are performed for 10ns in explicit solvent under NPT conditions. The simulation result also shows a similar hydrogen bonding interactions of water molecules around the CO group of the proline and the NH group of the methionine. The number of water molecules present
around the NH group of the methionine and the CO group of the proline are retained as in ONIOM model systems (Figure 4.7, Table 4.4). The gas phase, ONIOM and MD calculations of the model systems have shown non covalent interactions with adjacent residues and solvent molecules in the structures I and II are different.

**Figure 4.7.** The number of water molecules A) around the NH group B) around the CO group of methionine in the M-loop region of $3_{10}$-helix (1TB5, black color) and turn (1TB7, red color) containing structures for 10ns simulation is shown.

**Table 4.4.** The summary of number of water molecules present around in PDE4B (1TB5) and PDE4D (1TB7) structures.

<table>
<thead>
<tr>
<th>Structure</th>
<th>No of water molecules near to CO group of Proline</th>
<th>No of water molecules near to NH group of Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal</td>
<td>ONIOM</td>
<td>MD(10ns)</td>
</tr>
<tr>
<td>1TB5 (PDE4B)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>1TB7 (PDE4D)</td>
<td>1 EDO</td>
<td>2</td>
</tr>
</tbody>
</table>

Electronic structure calculations thus explain the structural anomaly that at pH=10.0 conformation I is seen in all $3_{10}$-helix PDE4B structures and at pH=6.5-7.5
Phosphodiesterase 4 (PDE4) prefer conformation II as observed in all turn containing crystal structures. The pH variation thus can have different protonation states of cysteine side chain or backbone amide bonds. The different protonated states are stabilized by the non covalent interactions of the amide bond with the surrounding environment such as adjacent residues and solvent molecules. Thus the orientation of the amide bond between proline and methionine is the deciding factor for the secondary structural change in the II or I. The pH dependence of the structures shows that there will be potential differences in the interaction at different parts of the cells in body at different pH for the same enzyme substrate combination.

4.4.5 Influence of M-loop Region on Subtype Selectivity

The secondary structural difference presented here had similar sequences and at physiological pH both subtypes PDE4B and 4D could have a turn structure. However, the variation in the sequence of residues in the M-loop region may change the interactions with the inhibitor that can help in subtype selectivity.6c A Thr$^{436}$ residue in PDE4B near to this secondary structural change is altered to Asn$^{362}$ in PDE4D. This variation of the residue does not account for secondary structural changes but influences the molecular properties of adjacent residues. The examination of molecular properties such as hydrophobicity and MOLCAD surfaces for the residues in the M-loop region were done. They show that a larger hydrophobic group containing ligands can be accommodated in the Q2 pocket of PDE4B.6c This being a collaborative project is not discussed in detail in this chapter. The essence of that analysis shows exploration of PDE4 inhibitor that can accommodate M-loop region apart from the metal$^{15b,16}$ and the solvent pocket$^{41}$ of active site can lead to better subtype selectivity.
4.5 Conclusions

The 35 X-ray crystal structures of the two subtypes of PDE4 show an influence of pH of the crystallization medium as an experimental variable on the secondary structural change in the M-loop region. The pattern of secondary structural change in the highly flexible M-loop region as a function of pH is striking in these crystal structures. Electronic structure calculations on the model sequence of the SPMCD residue of PDE4 explain the thermodynamic preferences for the secondary structure in the pattern of $3_{10}$-helix versus turn in the M-loop region. The protonated states of the cysteine side chain and amide bond of turn containing structures are exothermic according to the isodesmic equations. It is due to the increase in the number of hydrogen bonding interactions with surrounding environment such as adjacent residues and solvent molecules. The Thr$^{436}$/Asn$^{362}$ mutation in the M-loop region influenced the hydrophobic nature in Q2 pocket of active site of PDE4B and 4D subtypes. Thus the variations in local (physiological) pH at the point of interaction and hydrophobic groups of Q2 pocket can be added parameters in optimizing the specificity of PDE4 inhibitors.

4.6 References


22 MOE version **2006** Chemical Computing Group, 954, First Floor, 16th Main, BTM Layout 2nd Stage, Bangalore, India 560 076.


24 Insight II Version **2000** Molecular Modelling System 2000 Molecular Simulations. 9685 Scratan Road, San Deigo, CA.


28 *Gaussian 03*, Revision B.03, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, Jr., J. A.; Vreven, T.; Kudin, K.
[478x746]Chapter 4

N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.;
Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara,
Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.;
Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.;
Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.;
Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.;
Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.;
Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez,

29 Case, D. A.; Darden, T. A.; Cheatham, III, T. E.; Simmerling, C.; Wang, J.; Duke, R.
Brozell, S.; Tsui, V.; Gohlke, H.; Yang, L.; Tan, C.; Mongan, J.; Hornak, V.; Cui, G.;
Beroza, P.; Mathews, D. H.; Schafmeister, C.; Ross, W. S.; Kollman. P.A. AMBER9,
9th ed.; University of California: San Francisco, CA.

30 Duan, Y.; Wu, C.; Chowdhury, S.; Le, M. C.; Xiong, G.; Zhang, W.; Yang, R.;
31  Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F.; DiNola, A.; Haak J. R.  
33  Wang, H.; Liu, Y.; Huai, Q.; Cai, J.; Zoraghi, R.; Francis, S. H.; Corbin, J. D.; 
    Karplus, M. J. Phys. Chem., 1997, 101, 1663-1683. (c) Antosiewicz, J.; McCammon, 
    2006, 1764, 1227-1233. (e) Thomas, P. G.; Russell, A. J.; Fersht, A. R. Nature, 1985, 
    318, 375-376.
37  (a) Lide, D. R. Handbook of Chemistry and Physics; 84th ed.; CRC press: Boca 
    Raton, FL, 2003. (b) Thurlkill, R. L.; Grimsley, G. R.; Scholtz. J. M.; Pace, C. N. 
    Protein Science, 2006, 15, 1214–1218. (c) Dawson, R. M. C.; Elliot, D. C.; Elliot, W. 

