Emergence of pyridoxal phosphorylation through a promiscuous ancestor during the evolution of hydroxymethyl pyrimidine kinases

Victor Castro-Fernandez, Felipe Bravo-Moraga, Cesar A. Ramirez-Sarmiento, Victoria Guixe*

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

A R T I C L E   I N F O

Article history:
Received 7 April 2014
Revised 5 June 2014
Accepted 6 June 2014
Available online 19 June 2014
Edited by Miguel De la Rosa

Keywords:
HMP kinase
Pyridoxal kinase
Bifunctional enzyme
Enzyme evolution
Resurrection ancestral enzyme

Abstract

In the family of ATP-dependent vitamin kinases, several bifunctional enzymes that phosphorylate hydroxymethyl pyrimidine (HMP) and pyridoxal (PL) have been described besides enzymes specific towards HMP. To determine how bifunctionality emerged, we reconstructed the sequence of three ancestors of HMP kinases, experimentally resurrected, and assayed the enzymatic activity of their last common ancestor. The latter has ~8-fold higher specificity for HMP due to a glutamine residue (Gln44) that is a key determinant of the specificity towards HMP, although it is capable of phosphorylating both substrates. These results show how a specific enzyme with catalytic promiscuity gave rise to current bifunctional enzymes.

© 2014 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Although textbooks and research articles traditionally highlighted the remarkable specificity of enzyme action, the promiscuity of enzymes has received considerable attention in the past few years due to its role in the evolution of new functions. Several observations support the hypothesis that evolutionary predecessors and intermediates display broad specificity or high promiscuity. Therefore, bifunctional or promiscuous proteins could reflect ancestral forms from which two different specificities split and originated through a gene duplication event during evolution. Catalytic promiscuity can be defined as a secondary catalytic activity, since either affinity or intracellular substrate concentrations are not physiologically relevant [1], while bifunctionality refers to catalytic activities that have physiological relevance.

The family of ATP-dependent vitamin kinases from the ribokinase superfamily features a single domain with a conserved Rossmann-like fold [2], thus structurally diverging from other enzymes of this superfamily that have, in addition, a small domain composed of a β-sheet that may also include some α-helical insertions [3]. This family includes hydroxymethylpyrimidine kinases (HMPK, EC 2.7.1.49), pyridoxal kinases (PLK, EC 2.7.1.35) and hydroxethylthiazole kinases (THZK, EC 2.7.1.50), enzymes that participate in the biosynthesis de novo of active (phosphorylated) forms of vitamin B1 (thiamine pyrophosphate) and B6 (PLP) in bacteria, essential cofactors for several enzymes involved in the metabolism of amino acids [4] and carbohydrates [5]. Although at first sight it seems that these enzymes can be easily split in different groups based on their substrate specificities, the fact is that several members of this protein family are able to phosphorylate more than one substrate. Some enzymes have been classified based either in enzyme kinetics or in structural comparisons as being specific for HMP, such as Escherichia coli and Salmonella typhimurium HMPKs derived from the thiD gene [6,7], or for pyridoxal (PL), like the protein codified by the pdxY gene of E. coli, where the PL moiety is so tightly bound to the enzyme that it is not released unless the protein is subjected to denaturation [8]. However, several bifunctional HMP/PLK enzymes that are able to phosphorylate both PL and HMP have been described, such as the protein encoded by the pdxK gene of E. coli [9,10], Trypanosoma brucei and Plasmodium falciparum [11], and the protein encoded by the thiD gene of Bacillus subtilis [12]. Also, the crystal structures of bifunctional kinases from B. subtilis and Staphylococcus aureus have been recently solved [13,14]. Both enzymes have been described as possessing significant activity towards both PL and the thiamine...
precursor HMP, thus conferring to these enzymes a dual function in both the pyridoxal and thiamine biosynthesis pathways. Moreover, the *thiD* gene of *B. subtilis* has been found not to be essential for bacterial survival [15] and located outside the operon where thiamine biosynthetic genes are usually clustered [13,16]. Although this evidence sheds light about the emergence of PLK activity inside the group of HMPK enzymes as a late event of convergence in the evolution of these kinases, there is still no proof that the trait of their last common ancestor corresponded to the specificity towards HMP.

In order to test the evolutionary history of the ATP-dependent vitamin kinases and the role of bifunctionality in the appearance of the HMPK and PLK activities, we performed phylogenetic analysis, ancestral enzyme reconstruction, molecular modeling and docking, and we also tested experimentally the kinetic features of the resurrected last common ancestor of the HMPK enzymes. Our results show that this ancestor is able to phosphorylate both PL and HMP, but has a 8-fold higher preference for HMP. Also, the expected catalytic rate at near-physiological concentrations is higher for HMP than PL. These results provide strong evidence of how PLK activity emerged during the evolution of this protein family.

### 2. Materials and methods

#### 2.1. Structural alignment and phylogenetic tree of PLKs and HMPKs

Crystal structures of PLKs and HMPKs (PDB ID: 2I5B, 4C5L, 1JXI, 1UBO, 1TD2, 2YXT, 1HJP and 2DDM) were structurally aligned using STAMP [17], and the resulting alignment was used to construct a phylogenetic tree with MrBayes 3.1.2 [18]. For the analysis we performed 2 runs with 4 chains per run and heating temperature set to 0.2 for 1.0 simulation using the mixed analysis of fixed amino acid substitution models with fixed site-specific rates. Samples were collected every 100 steps and the initial 40% of samples were removed before summarizing trees and parameters, ensuring that the average standard deviation of split frequencies was less than 0.001 upon convergence. The consensus tree was processed in Dendroscope 3.2.10 [19].

#### 2.2. Multiple sequence alignment

Sequences were collected from the non-redundant protein database (nr) using a PSI-BLAST algorithm with 3 iterations through Protein BLAST Server (blast.ncbi.nlm.nih.gov), using HMPKs and PLKs with known structure as templates (PDB ID: 2I5B, 1JXI, 1UBO, 1TD2, 2YXT, 1HJP and 2DDM). An initial multiple sequence alignment (MSA) was constructed using MAFFT [20], then redundant sequences were removed using QR Sequence tool of Multiseq in VMD [17] with a PID cutoff of 88%. A second MSA was constructed based on three-dimensional and secondary structure constraints using Promals3D [21]. Misaligned C-terminal, N-terminal and loop positions were manually corrected based on the structural alignment obtained previously. The final MSA used in this work is available in the Supplementary information.

#### 2.3. HMPKs phylogenetic tree

Sequences of HMPK were selected from MSA and their phylogeny was inferred with MrBayes version 3.1.2 [18], where human PLK was used as outgroup. For the analysis we used WAG as the fixed model, based on its posterior probability of 1.0 in the mixed model analysis, and gamma-shaped rate variation across sites with a proportion of invariable sites. Runs were performed as previously described, but increasing the number of generations to 1 × 10^7 and only removing the initial 30% of samples before summarizing trees and parameters. The average standard deviation of the split frequencies was less than 0.01 upon convergence.

#### 2.4. Ancestral sequence reconstruction

We used a modified method described by Hall [22]. Briefly, ancestral sequences were inferred through a hierarchical Bayes approach implemented in MrBayes 3.1.2 with model and parameters used for phylogeny inference [23]. Each target node was constrained and probabilities for each amino acid were calculated in each position of alignment, where the aminoacid with highest probability in each position were selected. Sequence gaps were inferred as described by Hall [22].

#### 2.5. Gene synthesis, protein expression and purification

The gene of the last common ancestor was codon-optimized for expression in *E. coli* and synthesized by GENESCRIP (Piscataway, NJ, USA), then cloned into a modified pET-28b vector and verified by DNA sequencing. *E. coli* BL21(DE3) were transformed and grown in LB broth containing 35 μg/ml kanamycin at 37 °C until OD600 reached ~0.8. Expression of the recombinant protein was induced with 1 mM of isopropyl-β-D-thiogalactopyranoside overnight. Cells were harvested by centrifugation, resuspended in binding buffer (50 mM Tris–HCl pH 7.6, 500 mM NaCl, 20 mM imidazole and 5 mM MgCl2) and disrupted by sonication. After centrifugation (18514 g for 45 min), the soluble fraction was loaded onto a Ni²⁺-NTA affinity column (HisTrap HP, GE Healthcare, UK). Protein was eluted with a linear gradient between 20 and 500 mM imidazole and fractions with enzyme activity were pooled and stored at 4 °C with 1 mM ATP and 5% glycerol. Enzyme purity was analyzed by SDS–PAGE stained with Coomassie blue.

#### 2.6. Enzyme activity assays

HMPK activity was measured following the appearance of ADP with a coupled assay containing pyruvate kinase/lactate dehydrogenase. Briefly, enzyme preparation was mixed with reaction buffer containing 25 mM Tris–HCl pH 7.8, 0.8 U/ml of pyruvate kinase, 2.4 U/ml lactate dehydrogenase, 0.3 mM phosphoenolpyruvate (PEP), 125 mM KCl, 0.2 mM NADH, 10 mM ATP, 15 mM HMP and 15 mM MgCl2.

PLK activity was measured spectrophotometrically by following PLP formation at 388 nm. The reaction mixture consisted of 10 mM ATP, 15 mM PL, 15 mM MgCl2 and 25 mM PIPES pH 6.5. An extinction coefficient of 6.22 mM⁻¹ cm⁻¹ for NADH and 2.886 mM⁻¹ cm⁻¹ for PLP were used, and the enzymatic unit (U) was defined as μmol min⁻¹. Both activities were measured at 37 °C.

#### 2.7. Homology modeling and docking

Fifty models were constructed for each ancestral protein with MODELLER 8 [24]. The best 10 models were chosen based on DOPE potential, and its quality evaluated with PROSA2003 [25], Procheck [26] and VERIFY3D [27].

Docking assays were performed with AutodockVina 1.0 [28], the protonation state of the ionizable residues was calculated using the web server H++ [29] and partial charges were derived with Gasteiger method using AutoDockTools [30]. Hydrogens of HMP and PL substrates were added and optimized with Gaussian [31]. Docking results with the lowest interaction energy and the phosphoryl acceptor hydroxyl oriented towards the OGXD(C) motif were selected, since the aspartic acid (or cysteine) is considered the catalytic base for the phosphate group transfer in all ribokinase superfamily members [32].
3. Results and discussion

3.1. PLK and HMPK activity, evidence for bifunctional enzymes

The relationship between PLKs or HMPKs and bifunctional enzymes was analyzed by constructing a phylogenetic tree based on structural alignment (Fig. 1). The resulting dendrogram shows that enzymes HMPK or PLK appear as separate groups, while bifunctional enzymes cluster inside the HMPK group, as described previously [13,14]. Therefore, the HMPK group can be divided into specific HMPKs (Thermus thermophilus and S. typhimurium) and bifunctional HMPK/PLKs (B. subtilis and S. aureus) whereas the PLK group includes enzymes from prokaryotes (pxdY and pxdK) and eukaryotes (human and sheep). It is interesting to analyze the evolutionary history of the HMPK group, since the ability to phosphorylate PL within the group can be an event of convergent evolution, in which PLK activity reappears in the HMPK group as proposed by Newman et al. [13] or, conversely, the bifunctionality is the ancestral trait [33] and HMP specificity would be a recent event in the evolutionary history of these enzymes. These two hypotheses can be scrutinized by reconstructing the last common ancestor of the HMPK group (Fig. 1). The experimental resurrection of this ancestor and its kinetic characterization, regarding its ability to phosphorylate PL and HMP, will reveal if this ancestor was either specific for HMP (convergent evolution) or bifunctional (ancestral conservation of PLK activity) (see Fig. 2).

3.2. HMPK phylogeny and ancestral sequence reconstruction

In order to reconstruct the last common ancestor of HMPK enzymes, we established the phylogeny of bacterial HMPKs by Bayesian method (Fig. S1), using the human PLK as outgroup to root the tree. As expected, these enzymes cluster in two groups; one that contains the sequences of HMPK/PLK from B. subtilis and S. aureus (putative bifunctional group) with a posterior probability (pp) of 1.0, and other that includes specific HMPK enzymes from S. typhimurium and T. thermophilus (HMPK specific group) with a support of 0.92. The posterior probability for the node of the last common ancestor for all HMPKs was 0.92. We inferred the ancestral sequences of the last common ancestor of HMPKs enzymes (common ancestor, ancC), of the specific HMPK enzymes (ancS) and of the putative bifunctional group of enzymes (ancB) using the hierarchical Bayesian approach [23]. Sequences of ancestors ancS and ancB presented a very high support of posterior probability of reconstruction; ancS (Fig. S2A) shows 94% of residues over pp = 0.90 (254 of 268 aa) (Fig. S2A), while ancB (Fig. S2B) displays 92% of residues over pp = 0.90 (245 of 266 aa) (Fig. S2B). AncC was more ambiguously reconstructed (mean pp = 0.84 overall) (Fig. S2C), with 55% of its residues having pp ≥ 0.90 (Fig. S2C). Nevertheless, the reconstruction of ligand-contacting sites was considerably more robust (mean pp = 0.94) (Table S1). This mean posterior probability is a good support for the validity of the ancestral sequence reconstruction, as reported in other publications [34,35].

3.3. Experimental resurrection of the last common ancestor of HMPK enzymes

We expressed and purified the last common ancestor of HMPK enzymes (ancC). The ancestral enzyme showed activity with both substrates (PL and HMP) with an optimum pH of 8.0 and 6.5 for the HMPK and PLK activity, respectively (Fig. S3). The \( K_m \) for ATP using HMP as co-substrate was 1.00 mM; this substrate also shows a slight inhibition with a \( K_i \) of 44 mM. A \( K_m \) of 7 mM was obtained when HMP was used as substrate, a value very similar to those reported for current enzymes, like the one present in B. subtilis (\( K_m \) of 2 mM) [12]. At HMP concentrations above 10 mM, substrate inhibition was observed with a \( K_i \) of 31 mM. When employing PL as substrate a \( K_m \) of 28 mM, a \( k_{cat} \) of 0.2 s\(^{-1}\) and a \( K_i \) of 85 mM for substrate inhibition (Table 1) were obtained. The \( K_m \) value for PL is much higher than the ones reported for current enzymes (Table S2); both the pdxK-derived enzyme from E. coli as well as the thiD-derived enzyme from B. subtilis presents \( K_m \) values for PL in the micromolar range. Comparison of the \( k_{cat}/K_m \) values for HMP and PL show that ancC displays a HMP preference of 8 times over PL, which results mainly due to its high \( K_m \) for PL. This suggest that the catalytic activity of ancC with PL corresponds to catalytic promiscuity since, due to the high chemical reactivity of this metabolite with proteins [36], its intracellular concentration in

![Fig. 1. Dendrogram of PLK and HMPK activities.](image)

| Parameters   | HMP     | PL      | Mg-ATP |
|--------------|---------|---------|--------|
| \( K_m \) (mM) | 7 ± 2   | 28 ± 9  | 1 ± 0.2|
| \( k_{cat} \) (s\(^{-1}\)) | 0.40 ± 0.06 | 0.20 ± 0.04 | 0.080 ± 0.005 |
| \( K_i \) (mM) | 31 ± 17 | 85 ± 54 | 44 ± 19 |
| \( k_{cat}/K_m \) (M\(^{-1}\) s\(^{-1}\)) | 57 | 7 | 80 |
E. coli is 27 μM total and only 10 μM free, not-protein bound [37], thus suggesting that it would not be physiologically relevant. In fact, in this concentration range, the catalytic rate is significantly higher when HMP is used as substrate compared to PL. This idea is reinforced by the fact that if $k_{cat}/K_m$ values are calculated from initial velocity slopes the difference between HMP versus PL as substrates is 11-fold (Fig. S4). In the case of the bifunctional enzyme from B. subtilis, there is a ~4-fold preference of PL over HMP that is accompanied by a $K_m$ for PL in the micromolar range, thus exhibiting a higher catalytic rate for PL for the expected intracellular concentrations (Fig. S5) [12]. Moreover, in vivo covalent modification by analogs of the antibacterial compound rugulactone of the enzyme encoded by the thiD gene of S. aureus, which has a 65-fold preference for PL over HMP and possess a millimolar $K_m$ for HMP [14], leads to inhibition of thiamine biosynthesis [38], thus exhibiting a higher catalytic rate for PL for the expected intracellular concentrations (Fig. S5) [12].

In vivo covalent modification by analogs of the antibacterial compound rugulactone of the enzyme encoded by the thiD gene of S. aureus, which has a 65-fold preference for PL over HMP and possess a millimolar $K_m$ for HMP [14], leads to inhibition of thiamine biosynthesis [38], thus exemplifying a higher catalytic rate for PL for the expected intracellular concentrations (Fig. S5) [12].

3.4. Molecular modeling of ancestral HMPKs

In order to find the structural determinants that account for the specificity of ancC by HMP, we built homology models for this ancestor and for the ancestral ancS and ancB sequences (Table S3), and then performed docking assays with PL and HMP. Docking results showed the orientation for both ligands were similar to those present in crystalized enzymes. For ancC, the closest residues to PL and HMP moieties are: Met81, Ser13, Val43, Gln45, His50, His211, Val108 and Cys215 (Fig. 3). For ancB these residues are Met80, Thr12, Val42, Gln44, Val49, His209, Val107 and Cys213, and for ancS with HMP the residues are the following: Met81, Thr12, Val42, Glu44, Val49, His209, Val107 and Cys214. A comparison of the active sites of current bifunctional HMPK/PLK and specific HMPKs enzymes, shows that the main differences between them correspond to residues in positions 44 and 52 where the bifunctional enzyme from B. subtilis has Met and His respectively, while in the specific HMPK enzyme from S. typhimurium Glu44 and Val49 are found in equivalent positions [13]. In the ancC ancestor we found glutamine in position 45 and histidine in position 50, so that Gln44 can explain the HMP specificity and His50 could...
account for the promiscuous activity with PL, highlighting the role of Gln in HMP specificity. Moreover, ancS presents glutamine (Gln44) and valine (Val49) in the corresponding positions, which also stressed the importance of glutamine for this specificity. Interestingly, ancB presents glutamine (Gln44) and valine (Val49) in the corresponding positions, which does not match with the active sites of current bifunctional enzymes since S. aureus and B. subtilis enzymes present Met and His in those positions (Fig. 4). A search for residue conservation at the active site inside the group of bifunctional enzymes shows that some of them (enzymes from Megamonas funiformis, Caldalkalibacilus thermarum, Exiguobacterium sp, Acetonema longum) retain a glutamine residue like the ancestral enzyme. These enzymes are found in different branches of the bifunctional group in the phylogenetic tree (Fig. S1), therefore suggesting some of these members may also use HMP as the preferred substrate.

4. Conclusions
The experimental resurrection of the last common ancestor of the HMPK group showed that probably this protein was not able to use PL under physiological conditions. Therefore, the PLK activity present in the current bifunctional enzymes must have appeared in a convergent event independently of the PLK activity of pdxY and pdxK genes, as was proposed by Newman et al.[13]. The ability of ancC to phosphorylate pyridoxal, which is 8-times less preferred than the phosphorylation of HMP, was considered by us as a promiscuous activity, since its high \(K_{m}\) value for PL would not be physiologically relevant. The promiscuous activity of enzymes has been proposed as the starting point for new activities [1,39]. In our case, this trait would have allowed the appearance of an activity already present in this family, in a convergent and independent manner.

![Fig. 4. Structure-based alignment of models of ancestral enzymes and structures of modern protein. BsHMPK PLK (B. subtilis, PDB: 2IBB), ShHMPK PLK (S. aureus, PDB: 4C5L), ThHMPK (T. thermophile, PDB: 1UTC), EsEcdy (E. coli, PDB: 1TFD), EsEcdk (E. coli, PDB: 2DDM), OaPLK (O. aries, PDB: 1LHP), HsPLK (H. sapiens, PDB: 2YXT). Alignment was performed with Stamp. The similarity coloring scheme is based on blosum62 matrix. Residues of the active site of ancC are highlighted in green and the secondary structure elements are annotated based on the homology model of ancC.](image-url)
Acknowledgment

This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt, Chile) Grant 1110137.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.033.

References

[1] Allewell, N.M. (2012) Thematic minireview series on enzyme evolution in the post-genomic era. J. Biol. Chem. 287, 1–2.
[2] Sigrell, J.A., Cameron, A.D., Jones, T.A. and Mowbray, S.L. (1998) Structure of Escherichia coli ribokinase in complex with ribose and dinitroaniline determined to 1.8 A resolution: insights into a new family of kinase structures. Structure 6, 183–193.
[3] Rivas-Pardo, J.A., Herrera-Morande, A., Castro-Fernandez, V., Fernandez, F.J., Vega, M.C. and Guixé, V. (2013) Crystal structure, SAXS and kinetic mechanism of hyperthermophilic ADP-dependent glucokinase from Thermococcus litoralis reveal a conserved mechanism for catalysis. PLoS One 8, e66687.
[4] Eliot, A.C. and Kirsch, J.F. (2004) Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. Annu. Rev. Biochem. 73, 383–415.
[5] Pohl, M., Sprenger, G.A. and Müller, M. (2004) A new perspective on thiamine catalysis. Curr. Opin. Biotechnol. 15, 335–342.
[6] Cheng, G., Bennett, E.M., Begley, T.P. and Ealick, S.E. (2002) Crystal structure of 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase from Salmonella typhimurium at 2.3 A resolution. Structure 10, 225–235.
[7] Reddicka, J.J., Kinslanda, C., Nicewongera, R., Christianb, T., Downsb, D.M., Cheng, G., Bennett, E.M., Begley, T.P. and Ealick, S.E. (2002) Crystal structure of Thermococcus litoralis pyridoxal kinase provides a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucieic Acids Res. 30, 3059–3066.
[8] Yang, Y., Tsui, H.C., Moss, D.S. and Thornton, J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291.
[9] Mizote, T. and Nakayama, H. (1989) Purification and properties of K-12. J. Bacteriol. 180, 1814–1821.
[10] Yang, Y., Tsui, H.C., Man, T.K. and Begley, M.E. (1998) Identification and inhibition of thiamine biosynthesis. Curr. Opin. Biotechnol. 10, 157–164.
[11] Wrenger, C., Eschbach, M.-L., Müller, I.B., Laun, N.P., Begley, T.P. and Walter, M.E. and Begley, T.P. (1998) Overexpression, purification and determination to 1.8 A resolution: insights into a new family of kinase ribokinase in complex with ribose and dinucleotide. J. Bacteriol. 180, 1814–1821.
[12] Ronquist, F. and Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
[13] Huuson, D.H. and Scorpanavacc, c. (2012) Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst. Biol. 61, 1061–1067.
[14] Nodwell, M.B., Koch, M.F., Alte, F., Schneider, S. and Sieber, S.A. (2014) A subfamily of bacterial ribokinases utilizes a hemithioacetal for pyridoxal phosphate salvage. J. Am. Chem. Soc. 136, 4992–4999.
[15] Kobayashi, K., Ehrlich, S.D., Albertini, A., et al. (2003) Essential Bacillus subtilis genes. Proc. Natl. Acad. Sci. USA 100, 4678–4683.
[16] Petersen, L.A. and Downs, D.M. (1997) Identification and characterization of an operon in Salmonella typhimurium involved in thiamine biosynthesis. J. Bacteriol. 179, 4894–4900.
[17] Wadeson, E., Eargle, J., Wright, D. and Luthey-Schulten, Z. (2006) MultiSeq: unifying sequence and structure data for evolutionary analysis. BMC Bioinformatics 7, 382.
[18] Katoh, K., Misawa, K., Kuma, K. and Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucieic Acids Res. 30, 3059–3066.
[19] Hall, B.C. (2006) Simple and accurate estimation of ancestral protein sequences. Proc. Natl. Acad. Sci. USA 103, 5431–5436.
[20] Huelsenbeck, J.P. and Bollback, J.P. (2001) Empirical and hierarchical Bayesian estimation of ancestral states. Syst. Biol. 50, 351–366.
[21] Hall, B.C. (2006) Simple and accurate estimation of ancestral protein sequences. Proc. Natl. Acad. Sci. USA 103, 5431–5436.
[22] Sali, A. and Blundell, T.L. (1993) Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815.
[23] Sippl, M.J. (1993) Recognition of errors in three-dimensional structures of proteins. Struct. Funct. Genet. 17, 355–362.
[24] Wada, T., Takagaki, T., Tsuda, M., Ishihara, T., Okada, Y., Ishii, K., Kubo, S. and Ohno, S. (2005) A5 residue: a server for estimating pKas and adding missing hydrogens to macromolecules. Nucieic Acids Res. 33, W368–W371.
[25] Sippl, M.J. (1993) Recognition of errors in three-dimensional structures of proteins. Struct. Funct. Genet. 17, 355–362.
[26] Huelsenbeck, J.P. and Bollback, J.P. (2001) Empirical and hierarchical Bayesian estimation of ancestral states. Syst. Biol. 50, 351–366.
[27] Li, D. and Yang, Y. (2005) The effect of amino acid replacements on the ratios of amino acid frequencies in the protein sequences of eukaryotes. BMC Evol. Biol. 5, 9.
[28] Caflisch, A., et al. (2004) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31, 455–461.
[29] Li, D. and Yang, Y. (2005) The effect of amino acid replacements on the ratios of amino acid frequencies in the protein sequences of eukaryotes. BMC Evol. Biol. 5, 9.
[30] Caflisch, A., et al. (2004) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31, 455–461.
[31] Daniel, R.M. and Arcus, V.L. (2012) On the origin and evolution of thermophily: an overview of the active site residue Asp235 of human pyridoxal kinase. Biochim. Biophys. Acta 991, 109–113.
[32] Daniel, R.M. and Arcus, V.L. (2012) On the origin and evolution of thermophily: an overview of the active site residue Asp235 of human pyridoxal kinase. Biochim. Biophys. Acta 991, 109–113.
[33] Huelsenbeck, J.P. and Bollback, J.P. (2001) Empirical and hierarchical Bayesian estimation of ancestral states. Syst. Biol. 50, 351–366.
[34] Wrenger, C., Eschbach, M.-L., Müller, I.B., Laun, N.P., Begley, T.P. and Walter, R.D. (2006) Vitamin B1 de novo synthesis in the human malaria parasite Plasmodium falciparum depends on external provision of 4-amino-5-hydroxymethyl-2-methylpyrimidine. Biol. Chem. 387, 41–51.