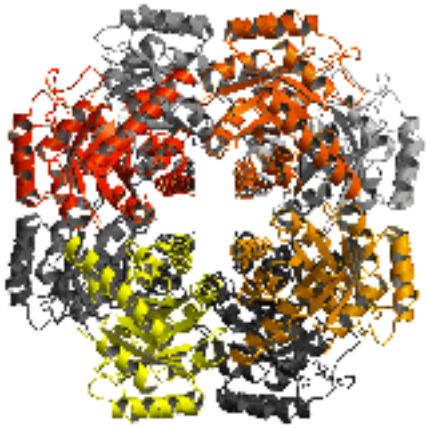


3. SUMMARY OF RESULTS AND DISCUSSION

3.1. PROTEIN CRYSTALLISATION

The Hampton Research Crystal Screens 1 and 2 and our in-house Factorial solutions were used for the initial crystallisation trials. Purified protein (6 mg ml^{-1} in 2 mM Tris/HCl pH 8.0) was mixed and equilibrated against 500 _l reservoir in a sitting drop vapour diffusion setup with a ratio 2:1 (3 _l protein:1.5 _l crystallisation solution). Several conditions at 293 K resulted in crystals with well-defined morphologies. The best diffracting crystals of PNP synthase were obtained from one condition of our in-house factorial solutions, which consisted in 10% PEG 6000 and 2 M NaCl. .The crystals belong to the orthorombic space group $C222_1$ with unit cell parameters a = 132.5 Å, b = 155.1 Å, and c = 130.1 Å. However, the main problem was to reproduce these crystals. This problem could be overcome by the use of the microseeding technique (see 1.2.1.1.), which yielded reproducible crystals of PNP synthase of suitable size and suitable quality.

3.2. STRUCTURE SOLUTION

The high resolution crystal structure of PNP synthase was solved at 2.0 Å by using the SIR method with anomalous scattering. Due to the presence of twelve well-occupied mercury binding sites, only one derivative (EMTS, also known as thiomersal) was enough to solve the phase problem and to calculate an interpretable electron density map of the protein. The monomer is folding as a $(\beta/\alpha)_8$ barrel or TIM barrel. Such a fold represents a compact domain consisting of a central barrel of 8 β-strands, which is surrounded by 8 α-helices. In PNP synthase, 3 extra helices to the basic barrel

architecture mediate the intersubunit contacts to the PNP synthase octamer. At the C-terminal end of the barrel, loops between a β-strand and its subsequent α-helix are much longer than those at the N-terminal end, and they built up the active site. One of these C-terminal loops, loop 4, was shown to be highly flexible and very important for the function of the enzyme.

The octamer can be described as a tetramer of active dimers. The monomers within a dimer are symmetrically related by a 2-fold axis, such that their barrels are approximately perpendicularly arranged to each other (Fig. 12). We believe that the active unit of the PNP synthase is the dimer because it exists a sharing in the active site between partner monomers. Arg20 protrudes to the active site of the neighboring monomer, where it is directly involved in the binding of substrates and products.

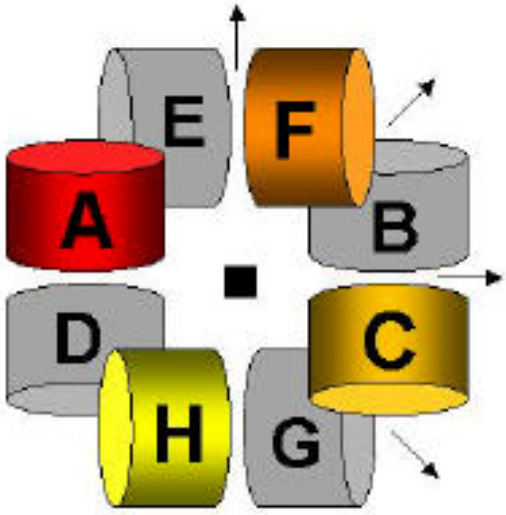


FIGURE 12. Scheme of the octamer. Here it is shown how the different oligomeric building blocks are assembled. The upper ring of tetramers is coloured from yellow to red and the correspondent partners in the lower ring are coloured in grey. The monomers within the active dimers (AE, BF, CG, DH) are almost perpendicular to each other. The 422 symmetry is indicated.

Further structural information was obtained with the solution of five enzyme-complex structures in the presence of substrates, substrate analogues, and products. These new models comprised a so-called enzyme-substrate complex, ES (DXP and GAP as an analogue of AAP); an enzyme-product complex, EP (PNP and P_i); an enzyme-substrate analogue complex, EA (GAP); and two enzyme-inorganic phosphate complexes, EPi1 (2

P_i) and EPi2 (1 P_i). All structures, the native and the various complexes, are of high stereochemical quality. Ramachandran plots (Fig. 13) confirm that the protein backbone torsion angles are almost entirely falling in the allowed regions.

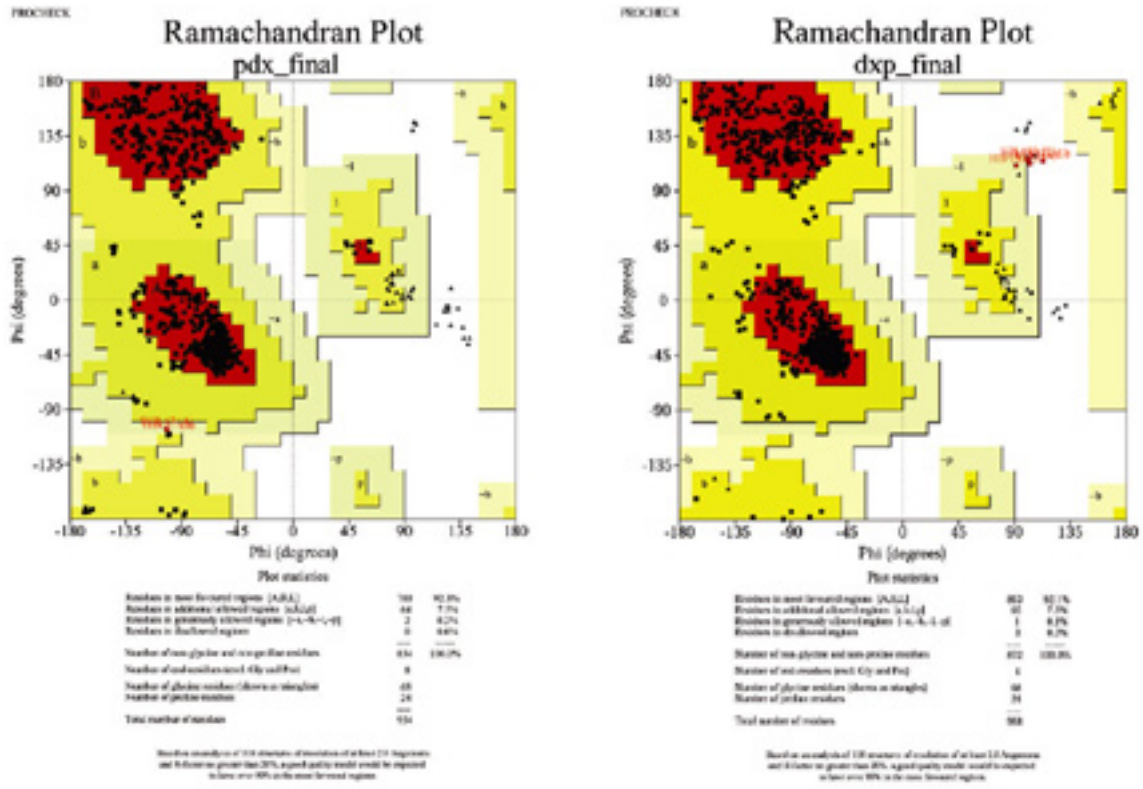


FIGURE 13. Ramachandran plots. The refined structures from the uncomplexed enzyme (left) and the ES complex (right) were choose as an example to show the reliability of the structure.

3.3. THE ACTIVE SITE

Although catalysing a remarkable variety of reactions, a common feature among all TIM barrel enzymes is the location of the active site. Its environment is provided by the loops at the C-terminal end of the β barrel core. In PNP synthase, this cavity is big enough to accommodate the substrates, AAP and DXP, the different intermediates of the reaction, and the products, PNP and P_i.

However, in order to obtain insight into the molecule mechanisms of catalysis, it is absolutely necessary to characterise the crystal structure of complexes with substrates and/or products (Fig. 14). The first solved structure of a PNP synthase complex corresponds to the enzyme-product complex, which was achieved by incubating native crystals in a solution containing PNP and P_i. The EP complex allowed an initial characterisation of the reaction mechanism and provided insight into the dynamic action of the enzyme. Further complementary information was obtained with the ES, EA, EPi1 and EPi2 complexes.

Many residues take part in the binding of substrates, intermediates of the reaction, and products. All ligands are mainly anchored at the active site through their phosphate groups, which occupy almost identical positions in different complexes. Therefore, it was possible to define a P1 (AAP/GAP, PNP) and a P2 (DXP, P_i) phosphate binding site. The main chain amides of residues Gly194, Gly215, and His216 and the side chain of Arg20* make hydrogen bonds to a phosphate bound at the P1 site. As commonly seen in phosphate binding enzymes, the P1 site is further enhanced by interaction with the macrodipole of an helix, in PNP synthase helix 8a. At the P2 site, a phosphate is fixed by strong hydrogen bonds from Arg20*, Asp11, His12, Arg47, and His52, and, when loop 4 is closed, also Thr102 and Thr103. A hydrogen bond network constructed by the side chains of Asn9, His45, Glu72, and Glu153 provide further anchoring of DXP, AAP/GAP, and PNP. The open-closed transition provides the right environment within the active site for the reaction to take place. With the shielding from solvent, the stabilisation of reaction intermediates and the prevention of side reactions are more effective.

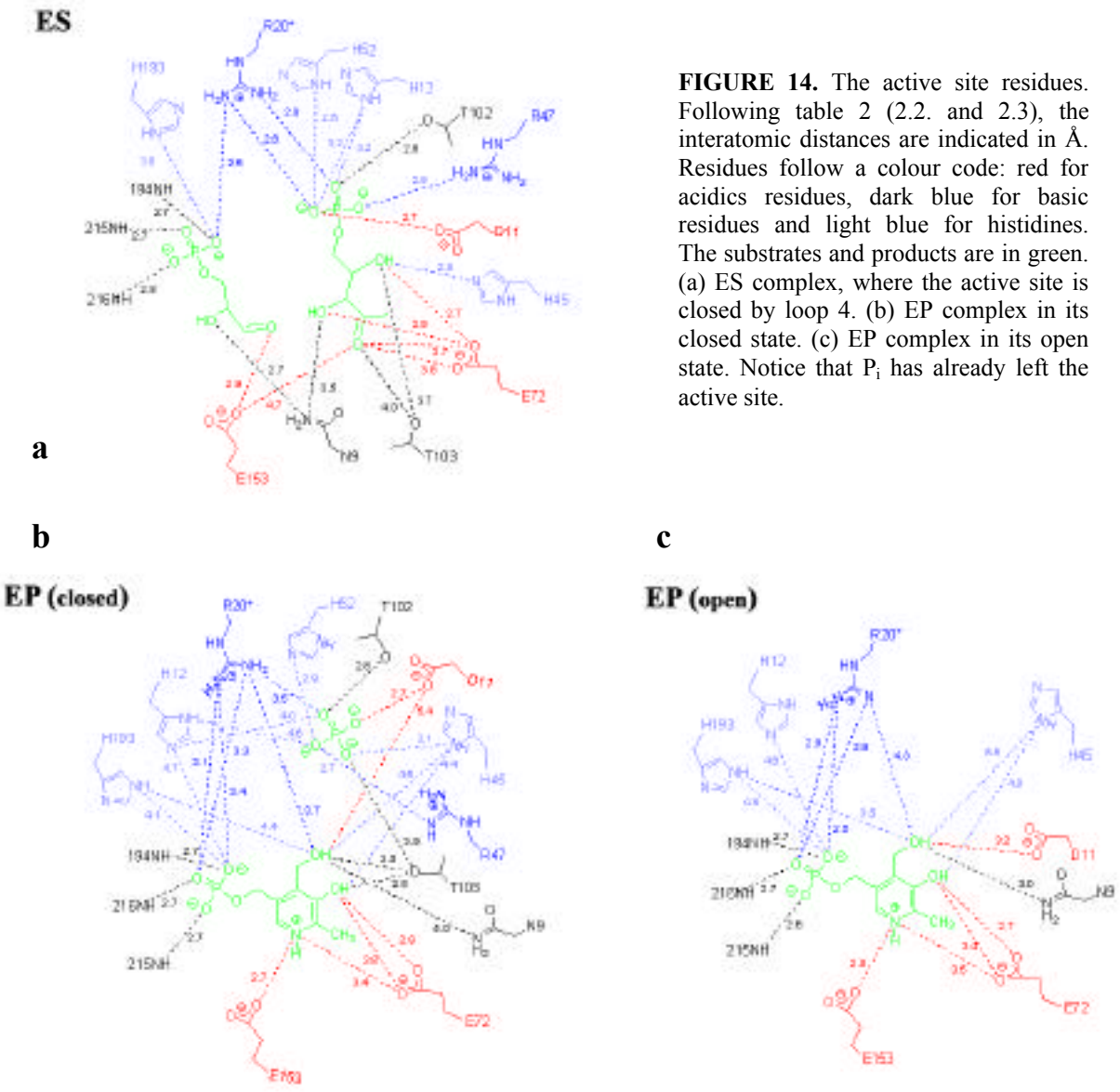


FIGURE 14. The active site residues. Following table 2 (2.2. and 2.3), the interatomic distances are indicated in Å. Residues follow a colour code: red for acidics residues, dark blue for basic residues and light blue for histidines. The substrates and products are in green. (a) ES complex, where the active site is closed by loop 4. (b) EP complex in its closed state. (c) EP complex in its open state. Notice that P_i has already left the active site.

3.4. CONFORMATIONAL CHANGES UPON COMPLEXATION

The conformational changes upon formation of the different enzyme complexes are mainly reduced to the reorientation of some active site residues and rearrangements of the flexible loop 4. In the free enzyme, loop 4 is folded away, permitting substrate entry to the active site. Once the products are formed, loop 4 flips away and PNP and P_i can be

released (Fig. 15). With the information obtained from the different complex structures we know that the occupation of the two P1 and P2 phosphate binding sites is not sufficient to trigger active site closure. Only specific interactions with both substrates will promote this transition, which can be understood as a zipper that should start at the N-terminal end of loop 4. When DXP occupies the P2 site, Arg47 reorients to hydrogen bond its phosphate group and at the same time to interact with the Glu100 side chain, which in turn fix Glu96, the N-terminal hinge residue of loop 4. After the backbone of loop 4 starts to flip, the side chain of Arg51 also reorients and helps to fix several residues of the loop. At the C-terminal hinge of loop 4, the main chain of Glu104 interacts with His193. In the EP closed form it can be seen that the bond between Glu104 and His193 is already broken, being the starting step for opening from the C-terminal end. Interestingly, His193 is an outlier in the closed forms, indicating a predisposition to change its conformation. In these complexes the backbone between residues 191-193 undergo a peptide flip, which might be the force to trigger the closed-open transition.

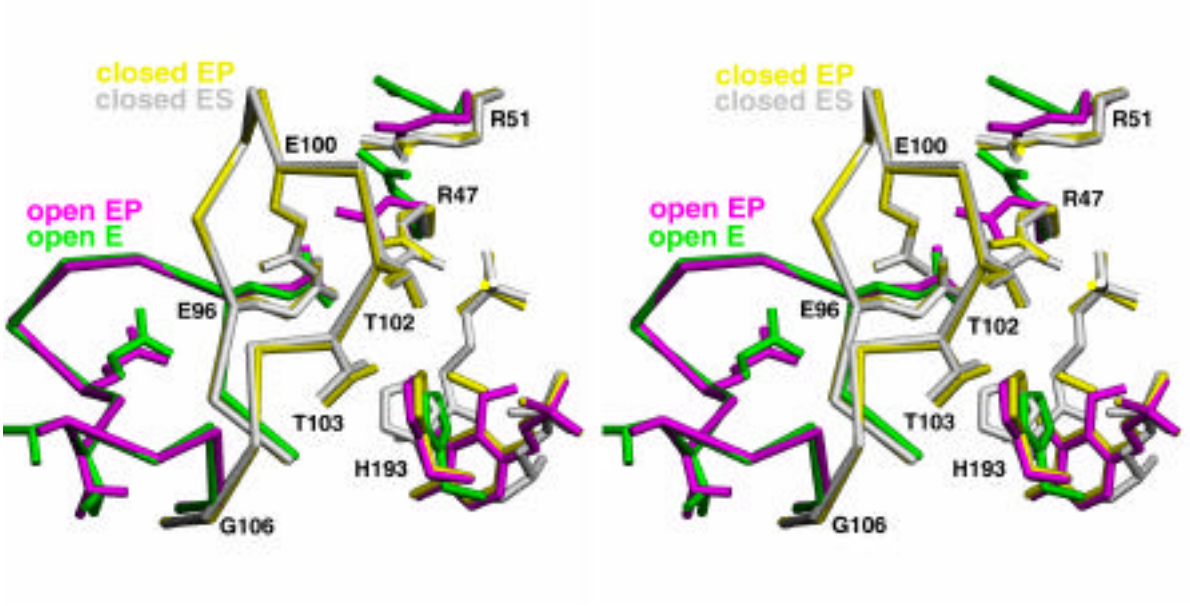


FIGURE 15. Open-closed transition of PNP synthase. Stereoview of residues involved in the conversion from open (green, uncomplexed enzyme; magenta, open EP complex) to closed (gray, EP complex; yellow, closed EP complex) conformation. The C^{-} trace of loop 4 is indicated, as well as the bound ligands.

3.5. MECHANISTIC FEATURES AND MODE OF ACTION

At this stage, it is possible to illustrate the catalytic cycle of PNP synthase with the different enzyme complexes. Thus, E + S ↔ ES1 ↔ ES2 ↔ EP1 ↔ EP2 ↔ E + P correspond to the uncomplexed enzyme, the EA complex, the ES complex, and the closed and the open EP complex, respectively (Fig. 16).

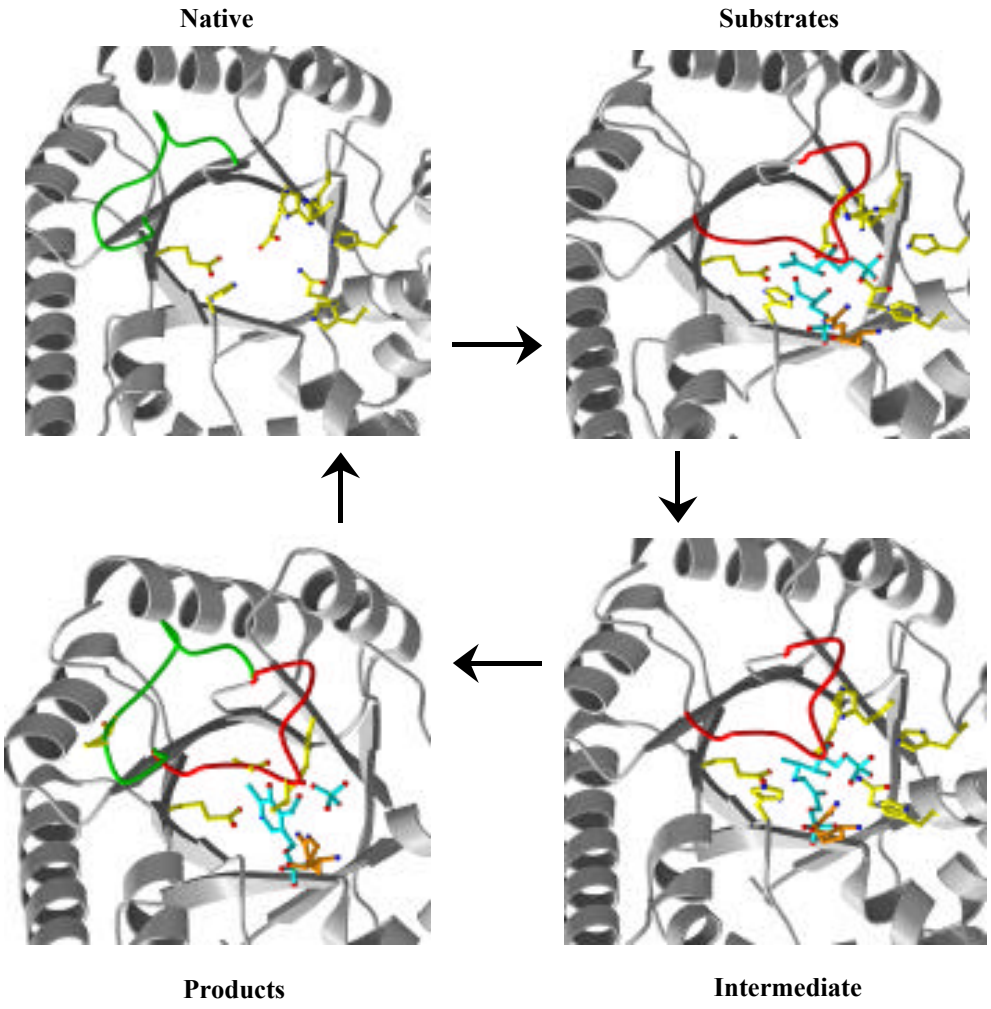


FIGURE 16. The catalytic cycle of PNP synthase. In the uncomplexed structure, loop 4 remains open, waiting for the substrates to enter the active site. Once the substrates are bound, loop 4 closes the active site establishing the right chemical environment for the reaction to take place. The different reaction transformations will occur while the loop is closed and, when the reaction comes to its end, loop 4 will be opened to allow the new molecules to left the active site. The relevant active site residues are coloured in yellow, Arg20*, coming for the neighbouring monomer, is in orange, and substrates, intermediates, and products are in cyan. Notice that loop 4 follows a colour code: green for the open and red for the closed form.

The PNP synthase acts as a two-state enzyme: in the open state the substrates can enter and be accommodated in the active site, and the products can be released; in the closed state the reaction takes place. Among the different amino acids that line the active site, Glu72 is the central acid-base catalyst. In its privileged position, it can approach each of the atoms, in substrates and intermediates, where the chemistry of the reaction occurs. Most probably, the complicated reaction is catalysed by a proton/charge-relay system that is formed by residues His45, Glu72, and Glu153 and water 1.

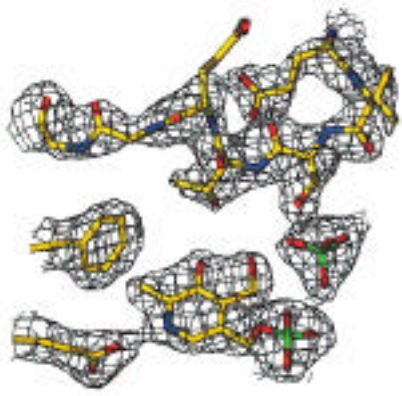
One feature of PNP synthase that represents a unique property among the TIM barrel proteins is the existence of a water channel that runs through the center of the β barrel. In this way, waters formed during the reaction can leave the active site. As the entrance is closed by loop 4, waters are released backdoors, at the N-terminal part of the β barrel. Therefore, instead of the hydrophobic character of the core in the TIM barrel structures, a hydrophilic core is observed in PNP synthase.

3.6. BIOLOGICAL RELEVANCE

Vitamin B_6 is an essential component of the human diet. The recommended daily intake for adults is ca. 2 mg that go up to 10-20 mg daily in cases of dietary deficiencies. People with vitamin B_6 deficiencies can suffer from neuronal disorders (neuritis), dermatitis and impaired aminoacid metabolism. Otherwise, a deficiency of vitamin B_6 alone is uncommon and usually the deficit occur in association with other vitamins of the B-complex.

Because coenzymatic function depends on the phosphate group for optimal binding to apoenzyme, the phosphorilated vitaminic form is needed. As coenzyme PLP, vitamin B_6, functions in numerous reactions involving the metabolism of macronutrients. Especially many PLP-dependent enzymes are involved in aminoacid transformations.

We believe that if vitamin B_6 has such a relevance in the life of human beings, it is also of vitally importance for bacteria. However, some bacteria are capable of synthesising PNP, which can be afterwards converted to PLP by a simple oxidation step. Thus, PNP synthase is the key enzyme in the bacterial *de novo* biosynthesis of vitamin B_6. Between this restricted number of bacteria we can distinguish some well-known pathogens like *Neisseria Gongonorrhoeae*, *Neisseria meningitidis*, *Salmonella typhimurium*, *Salmonella typhi*, *Vibrio cholerae*, *Yersinia pestis*, and *Brucella melitensis*. With the amount of structural and mechanistic information presented in this work it should be enough to rationally design inhibitors in the way for the development of new antibiotics.

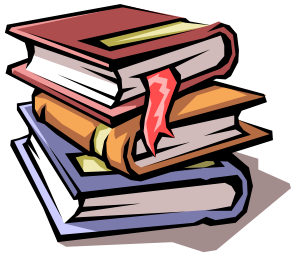


4. FINAL CONCLUSIONS

Before the use of X-rays, the obtaining of protein crystals was simply a proof of sample purity. Nowadays, crystallisation of proteins is a tool on the way of three-dimensional structure characterisation. The procedure is not as trivial as it might appear and additionally, not all the proteins are easy candidates to be crystallised. Some proteic complexes have to be divided into their subunits or smaller assemblies of them due to their huge size. There are proteins with highly flexible parts as loops and N or C terminus that can result in a not possible crystallisation without stabilisation or cleavage of those mobile components. However, to crystallise a protein is a good starting point but many other problems can arise before and after calculating the first electron density. Finding a solution of the phase problem can be many times a problem itself, as well as the acquirement of good quality data. Furthermore, the structural characterisation of enzyme complexes, i.e., with substrates, products, and/or inhibitors, may provide a huge amount of complementary information about specific interactions within the active site. However, conformational changes may occur upon complex formation thereby destroying the crystal order.

The results presented here represent a first starting point to better understand the complicated mechanism of PNP synthase, which can be regarded as the initial step to investigate the complete *de novo* biosynthetic pathway of vitamin B_6 and its vitamers. The given structural information should be used for further studies of functionality and biological implications. Nowadays, the increase in bacterial resistance against antibiotics is becoming a real problem. Therefore, it is necessary to find new targets to develop highly specific antibacterial drugs and we present PNP synthase as a possible one. Interestingly, a considerable number of bacteria containing the pdx machinery are

significant pathogens. As an example, we can distinguish among them the responsible for cholera (*V. cholerae*), typhoid fever (*S. typhi*), salmonellosis (*S. typhimurium*), meningitis (*N. meningitidis*), gonorrhea (*N. gonorrhoeae*), pest (*Y. pestis*), and brucellosis (*B. melitensis*). We believe that those bacteria can not live without vitamin B_6 and therefore we present PNP synthase as a novel drug target.



5. BIBLIOGRAPHY

B

Bilski, P., Li, M.Y., Daub, M.E., Ehrenshaft, M. and Chignell, C.F. (1999). Antioxidant properties of pyridoxine and its derivatives: quenching of singlet oxygen (1O2). *Free Radical Biology and Medicine*, **25S**, 28.

Blundell, T.L. and Johnson, L.N. (1976). *Protein Crystallography*. (London: Academic Press).

Bragg, W.L. and Bragg, W.H. (1913). The diffraction of X-rays by crystals. *Proc. Roy. Soc. Sect. A* **88**, 428-438.

Branden, C.I. (1991). The TIM barrel: the most frequently occurring folding motif in proteins. *Curr. Opin. Struct. Biol.* **1**, 978-983.

Brünger, A.T. (1992). *XPLOR (Version 3.1) Manual.* (New Haven and London: Yale University Press).

C

Cane, D.E., Hsiung, Y.J., Cornish, J.A., Robinson, J.K. and Spenser, I.D. (1998). Biosynthesis of vitamin B-6: The oxidation of 4- (phosphohydroxy)-L-threonine by PdxA. *J. Am. Chem. Soc.* **120**, 1936-1937.

Cane, D.E., Du, S.C., Robinson, J.K., Hsiung, Y. and Spenser, I.D. (1999). Biosynthesis of vitamin B-6: Enzymatic conversion of 1-deoxy-D- xylulose-5-phosphate to pyridoxol phosphate. *J. Am. Chem. Soc.* **121**, 7722-7723.

Cane, D.E., Du, S.C. and Spenser, I.D. (2000). Biosynthesis of vitamin B-6: Origin of the oxygen atoms of pyridoxol phosphate. *J. Am. Chem. Soc.* **122**, 4213-4214.

Collaborative Computing Project Number 4 (1994). The CCP4 suite - programs for protein crystallography. *Acta Cryst. Sect. D* **50**, 760-763.

D

Dempsey, W. B. (1966).Synthesis of pyridoxine by a pyridoxal auxotroph of *Escherichia coli*. *J. Bacteriol.* **92**, 333-337.

Dempsey, W. B. (1980). *Biosynthesis of control of VitaminB_6 in Escherichia coli*, G.P. Tryfiates, ed. (Westport: Connecticut: Food and Nutrition Press), pp. 93-111.

Dempsey, W.D. (1987). *Synthesis of pyridoxal phosphate*. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology, F.C. Neidhardt, J.L.

Ingraham, K.B. Low, B. Magasanik, M. Schaechter and H.E. Umbarger, eds. (Washington, D.C.: American Society for Microbiology), pp. 539-543.

Drenth, J. (1994). Principles of Protein X-ray Crystallography. C.R. Cantor, ed. (New York: Sprinter-Verlag).

Drewke, C., Klein, M., Clade, D., Arenz, A., Muller, R. and Leistner, E. (1996). 4-O-phosphoryl-L-threonine, a substrate of the pdxC(serC) gene product involved in vitamin B6 biosynthesis. *FEBS Letters* **390**, 179-182.

Ducruix, A. and Giegé, R., eds. (1992). Crystallization of Nucleic Acids and Proteins, A Practical Approach. (New York: Oxford University Press).

Dunathan, H.C. and Voet, J.G. (1974). Stereochemical Evidence For Evolution of Pyridoxal-Phosphate Enzymes of Various Function From a Common Ancestor. *Proc. Natl. Acad. Sci. U. S. A.* **71**, 3888-3891.

E

Ehrenshaft, M., Jenns, A.E., Chung, K.R. and Daub, M.E. (1998). SOR1, a gene required for photosensitizer and singlet oxygen resistance in Cercospora fungi, is higly conserved in divergent organisms. *Molecular Cell* **1**, 603-609.

Ehrenshaft, M., Bilski, P., Li, M.S., Chignell, C.F. and Daub, M.E. (1999). A highly conserved sequence is a novel gene involved in de novo vitamin B6 biosynthesis. *Proc. Natl. Acad. Sci.* **96**, 9374-9378.

Engh, R.A. and Huber, R. (1991). Accurate bond and angle parameters for X-ray protein-structure refinement. Acta Cryst. Sect. A **47**, 392-400.

Evans, P. R. (1991). *Crystallographic Computing 5*. D. Moras, A.D. Podjarni and J.C. Thierry, eds. (Oxford University Press), pp. 136-144.

Evans, S.V. (1993). Setor - hardware-lighted 3-dimensional solid model representations of macromolecules. *J. Mol. Graph.* **11**, 134-141.

Ewald, P.P. (1921). Das "rezproke Gitter" in der Strukturtheorie. *Z. Kristallogr.* **56**, 129-156.

F

Fersht, A. (1999). Structure and Mechanism in Protein Science. G.L. Hadler and M.R. Julet, eds. (New York: W.H. Freeman and Company).

French, S. and Wilson, K. (1978). Treatment of negative intensive observation. *Acta Cryst. Sect. A* **34**, 517-525.

G

Garrido-Franco, M., Huber, R., Schmidt, F.S., Laber, B. and Clausen, T. (2000). Crystallization and preliminary x-ray crystallographic analysis of PdxJ, the Pyridoxine 5'-phosphate synthesizing enzyme. *Acta Cryst. Sect. D* **56**, 1045-1048.

Garrido-Franco, M., Laber, B., Huber, R. and Clausen, T. (2001). Structural basis for the function of pyridoxine 5'-phosphate synthase. *Structure* **9**, 245-253.

H

Hill, R.E. and Spenser, I.D. (1986). In Vitamin B6 Pyridoxal Phosphate, Part A. Vol. 1. D. Dolphin, R. Poulson and O. Avramovic, eds. (New York: Wiley Interscience), pp. 417-476.

Hill, R.E. and Spenser, I.D. (eds.) (1996). *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. (Washington, DC: Am. Soc. Microbiol.).

Hockney, R.C. and Scott, T.A. (1979). The isolation and characterization of three types of vitamin B6 auxotrophs of Escherichia coli K12. *J. Gen. Microbiol.* **110**, 285-289.

Hol, W.G., van Duijnen, P.T. and Berendsen, H.J. (1978). The alpha-helix dipole and the properties of proteins. *Nature* **273**, 443-446.

J

Jansonius, J.N. (1998). Structure, evolution and action of vitamin B6-dependent enzymes. *Curr. Opin. Struct. Biol.* **8**, 759-769.

Jensen, R.A. (1976). Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.* **30**, 409-425.

John, R.A. (1998). *Pyridoxal phosphate-dependent enzymes*. In Comprehensive Biological Catalysis, Vol. 2. M. Sinnott, ed. (Accademic Press), pp.173-200.

Jones, T.A. and Kjelgaard (1991). *O-The Manual.* (Uppsala: Sweden).

Jones, T.A. (1992). *A, yaap, asap, @#*? A set of averaging programs*. In Molecular Replacement. E.J. Dodson, S. Gover and W. Wolf, eds. (Warrington: SERC Daresbury Laboratory), pp. 91-105.

K

Kleywegt, G.J. and Jones, T.A. (1994). *Halloween... Masks and Bones*. In From First Map to Final Model. S. Bailey, R. Hubbard and D. Waller, eds. (Warrington, UK: SERC Daresbury Laboratory), pp. 59-66.

Kraulis, P.J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946-950.

L

La Fortelle, E. D., Irwin, J. J. and Bricogne, G. (1997). *Crystallogr. Comput. 7*. P. E. Bourne and K. D. Watenpaugh, eds. (Oxford University Press), pp. 1-9.

Laber, B., Maurer, W., Scharf, S., Stepusin, K. and Schmidt, F.S. (1999). Vitamin B-6 biosynthesis: formation of pyridoxine 5 '-phosphate from 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose-5- phosphate by PdxA and PdxJ protein. *FEBS Lett.* **449**, 45-48.

Lam, H.M. and Winkler, M.E. (1990). Metabolic relationships between pyridoxine (vitamin B6) and serine biosynthesis in Escherichia coli K-12. *J. Bacteriol.* **172**, 6518-6528.

Lam, H.M. and Winkler, M.E. (1992). Characterization of the Complex Pdxh-Tyrs Operon of Escherichia-Coli-K-12 and Pleiotropic Phenotypes Caused By Pdxh Insertion Mutations. *J. Bacteriol.* **174**, 6033-6045.

Lu, G.G. (1999). FINDNCS: a program to detect non-crystallographic symmetries in protein crystals from heavy-atom sites. *J. Appl. Crystallogr.* **32**, 365-368.

M

Matthews, B.W. (1968). Solvent contents of protein crystals. *J. Mol. Biol.* **33**, 491-497.

McCormick, D.B., Suttie, J.W., Wagner, C. (1997). Vitamin B_6. *Met. enzymol.* **279**, 376-382.

Merrit, E.A. and Murphy, M.E.P. (1994). RASTER3D Version 2.0. A program for photorealistic molecular graphics. *Acta Cryst. Sect. D* **50**, 869-873.

N

Notheis, C., Drewke, C. and Leistner, E. (1995). Purification and Characterization of the Pyridoxol-5'-Phosphate-Oxygen Oxidoreductase (Deaminating) From Escherichia-Coli. *Biochim. Biophys. Acta* **1247**, 265-271.

O

Osmani, A.H., May, G.S. and Osmani, S.A. (1999). The extremely conserved pyroA gene of Aspergillus nidulans is required for Pyridoxine synthesis and is required indirectly for resistance to photosensitizers. *J. Biol. Chem.* **274**, 23565-23569.

Otwinowski, Z. and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307-326.

P

Patterson, A.L. (1934). A Fourier series method for the determination of the components of interatomic distances in crystals. *Phys. Rev.* **46**, 372-376.

Philippsen, A. (1999). Visualizing Structural Biology. *http://www.bioz.unibas.ch/~xray /dino*.

R

Reardon, D. and Farber, G.K. (1995). Protein motifs. 4. The structure and evolution of alpha/beta barrel proteins. *FASEB J.* **9**, 497-503.

Roa, B.B., Connolly, D.M. and Winkler, M.E. (1989). Overlap between pdxA and ksgA in the complex pdxA-ksgA-apaG-apaH operon of Escherichia coli K-12. *J. Bacteriol.* **171**, 4767-4777.

S

Schmidt, T. G. M., Koepke, J., Frank, R. & Skerra, A. (1996). Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin. *J. Mol. Biol.* **255**, 753-766.

Schoenlein, P.V., Roa, B.B. and Winkler, M.E. (1989). Divergent transcription of pdxB and homology between the pdxB and serA gene products in Escherichia coli K-12. *J. Bacteriol.* **171**, 6084-6092.

Spenser, I.D. and Hill, R.E. (1995). The Biosynthesis of Pyridoxine. *Nat. Prod. Rep.* **12**, 555-565.

T

Terwilliger, T. C. and Berendzen, J. (1999). Automated MAD and MIR structure solution. *Acta Cryst. Sect. D* **55**, 849-861.

Tong, L. and Rossmann, M. G. (1997). Rotation function calculations with GLRF program. *Methods Enzymol.* **276**, 594-611.

Tryfiates, G.P. (1986). *Pyridoxal phosphate and metabolism*. In Vitamin B6, Pyridoxal Phosphate, Part B. D. Dolphin, R. Poulson and O. Avramovic, eds. (New York: Wiley Interscience), pp. 421-448.

V

Voet, D. and Voet, J.G. (1995). Biochemistry. I. Geis, ed. (New York: John Wiley & Sons, Ltd.).

W

Willmanns, M., Schlagenhauf, E., Fol, B., and Jansonius, J.N. (1990). Crystallization and structure solution at 4 Å resolution of the recombinant synthase domain of N-(5'-phosphoribosyl)anthranilate isomerase: indole-3-glycerol-phosphate synthase from *Escherichia coli* complexed to a substrate analogue. *Protein Eng.* **3**, 173-180.

Y

Yang, Y., Zhao, G.S. and Winkler, M.E. (1996). Identification of the pdxK gene that encodes pyridoxine (vitamin B-6) kinase in Escherichia coli K-12. *FEMS Microbiol. Lett.* **141**, 89-95.

Yang, Y., Zhao, G., Man, T.K. and Winkler, M.E. (1998). Involvement of the gapA- and epd(gapB)- encoded dehydrogenases in pyridoxal 5'-phosphate coenzyme biosynthesis in Escherichia coli K-12. *J. Bacteriol.* **180**, 4294-4299.

Z

Zhao, G. and Winkler, M.E. (1996). 4-Phospho-hydroxy-L-threonine is an obligatory intermediate in pyridoxal 5'-phosphate coenzyme biosynthesis in Escherichia coli K-12. *FEMS Microbiol. Letters* **135**, 275-280.

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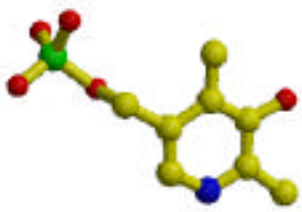

7. APPENDIX

7.1. ABBREVIATIONS

Å: Ångstrøm; 1Å = 10^{-10} m

AAP: aminoacetone 3-phosphate

AS: ammonium sulfate

ASU: asymmetric unit

ATP: adenosine 5'-triphosphate

Da, **kDa**: Dalton, kilo Dalton; 1Da = 1g mol^{-1}

DXP: 1-deoxy-D-xylulose-5-phosphate

E4P: erythrose 4'-phosphate

EA: enzyme-substrate analogue

EMTS: ethyl mercury thiosalicylate

EP: enzyme-product

ES: enzyme-substrate

FMN: flavin mononucleotide

GAP: glyceroaldehyde-3-phosphate

HPHKB: 3-hydroxy-4-phosphohydroxy-α-ketobutyrate

IGPS: indolglycerolphosphate synthase

IPTG: isoprpyl-β-D-thiogalactopyranoside

LB/Amp: Luria broth supplemented with ampicillin

MAD: Multiple-wavelength Anomalous Dispersion

MIR: Multiple Isomorphous Replacement

MPD: 2-methyl-2,4-pentanediol

NAD: nicotinamide adenine dinucleotide

NCS: noncrystallographic symmetry

4PE: 4-phosphoerythronate

4PHT: 4-(phosphohydroxy)-L-threonine

P_i: inorganic phosphate

PL: pyridoxal

PLP: pyridoxal 5'-phosphate

PM: pyridoxamine

PMP: pyridoxamine 5'-phosphate

PN: pyridoxine

PNP: pyridoxine 5'-phosphate

r.m.s.: root mean square

SIR: Single Isomorphous Replacement

SIRAS: Single Isomorphous Replacement with Anomalous Scattering

SOR1: singulet oxygen resistance 1

7.2. CODE FOR AMINOACIDS

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

7.3. INDEX OF FIGURES AND TABLES

7.3.1. Figures in the manuscript

7.3.2. Figures and Tables in the publications

Crystallization and preliminary x-ray crystallographic analysis of PdxJ, the pyridoxine 5'-phosphate synthesizing enzyme.

Structural basis for the function of pyridoxine 5'-phosphate synthase.

Enzyme-ligand complexes of pyridoxine 5'-phosphate synthase: implications for substrate binding and catalysis.

8. CURRICULUM VITAE

PERSONAL DATA

Surnames	Garrido Franco Name: Marta
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EDUCATION

1998 – to date	Ph.D. student at the Max-Planck-Institute for Biochemistry, Department of Structural Research (Dir. Prof. Dr. Robert Huber), Martinsried, Munich, Germany; and at the "Universitat Autònoma de Barcelona", Department of Biochemistry and Molecular Biology (Dir. Prof. Dr. Francesc González Sastre), Cerdanyola del Vallès, Barcelona, Spain.
1996 – 1998	Graduate student in Biochemistry at "Universitat Autònoma de Barcelona", Cerdanyola del Vallès, Barcelona, Spain.
1994 – 1996	Undergraduate student in Chemistry at "Universitat Autònoma de Barcelona", Cerdanyola del Vallès, Barcelona, Spain.

POST-GRADUATE COURSES

2001	Methodologies in Biochemistry and Cellular Biology, "Universitat Autònoma de Barcelona", Spain.
2001	Advances in Neurochemistry, "Universitat Autònoma de Barcelona", Spain.
2001	Molecular Basis of the Hormonal Action, "Universitat Autònoma de Barcelona", Spain.
2001	Structure and Dynamics of the Membrane Lipids, "Universitat Autònoma de Barcelona", Spain.

PRACTICAL EXPERIENCE

1997 Clinical Analysis Laboratory "CERBA INTERNATIONAL", Barcelona, Spain. Skills: HPLC and gas chromatography for the monitoring of drugs. June-September.

1998 Clinical Analysis Laboratory "BALAGUÉ CENTER", Barcelona, Spain. Skills: virology (ELISA) and Papanicholau analysis. July-September.

1998 Max-Planck-Institute for Biochemistry, Department of Structural Research, Martinsried, Munich, Germany. Skills: applied biochemical techniques including different methods from various aspects of Molecular Biology (cloning and insertion of genes into vectors), Biochemistry (overexpression of foreign genes in *E. coli* and *Pichia pastoris*, purification and biophysical characterisation of proteins) and Protein Crystallography (structure determination and analysis). September to date.

2001 Joslin Diabetes Center (Harvard Medical School), Department of Cellular and Molecular Physiology, Boston, USA. Skills: cloning and expression of foreign proteins in P. pastoris. September.

LIST OF PUBLICATIONS

Full papers

- **Garrido-Franco, M.**, Huber, R., Schmidt, F.S., Laber, B. and Clausen, T. (2000). Crystallization and preliminary x-ray crystallographic analysis of pdxJ, the pyridoxine 5'-phosphate synthesizing enzyme. *Acta Cryst. Sect. D*, **56**:1045-1048.
- Auerbach, G., Herrmann A., Bracher, A., Bader, G., Gutlich, M., Fischer, M., Neukamm, M., **Garrido-Franco, M.**, Richardson, J., Nar, H., Huber, R. and Bacher, A. (2000). Zinc plays a key role in human and bacterial GTP cyclohydrolase I. *P. Natl. Acad. Sci. USA*, **97**:13567-13572.
- **Garrido-Franco, M.**, Laber, B., Huber, R. and Clausen, T. (2001). Structural basis for the function of pyridoxine 5'-phosphate synthase. *Structure*, **9**:245-253.

- **Garrido-Franco, M.**, Ehlert, S., Messerschmidt, A., Marinkovic, S., Laber, B., Huber, R. and Clausen, T. (2002). Crystal structure of threonine synthase from yeast. *J. Biol. Chem.,* (In Press).
- Krojer, T., **Garrido-Franco, M.**, Huber, R., Ehrmann, M. and Clausen, T. (2002). Crystal structure of DegP (HtrA), a novel protease-chaperone machine. *Nature,* (In Press).
- **Garrido-Franco, M.**, Laber, B., Huber, R. and Clausen, T. (2002). Enzyme-ligand complexes of pyridoxine 5'-phosphate synthase: implications for substrate binding and catalysis. *J. Mol. Biol.* (Submitted).

Abstracts

- **Garrido-Franco, M.,** Laber, B., Huber, R. and Clausen, T. (2001). Pyridoxine 5'-phosphate synthase from *E. coli*: *de novo* biosynthesis of vitamin B6. FEBS Meeting, Lisbon, Portugal.

MEETINGS

1999 International School of Crystallography of Biological Macromolecules at the "Institut de Biologia Molecular de Barcelona" (IBMB) and the "Universitat Politècnica de Catalunya" (UPC), Barcelona, Spain. 9th - 15th September.

2000 EU Network Mid Term Review Meeting at the Max-Planck-Institute for Biochemistry, Martinsried, Munich, Germany. 7th July.

2000 36th Schwabinger Symposium: "Neues aus der Diabetologie für das neue Jahrhundert" at the Max-Planck Institute for Psychiatry, Munich, Germany. 2th December.

2001 FEBS Meeting, Lisbon, Portugal. 29th June - 5th July.

2001 Advance Training for Project Leaders for the Biological Security at the Max-Planck-Institute, Martinsried, Munich, Germany. 29th November - 1st December.

GRANTS AND FELLOWSHIPS

2001	American Diabetes Association summer fellowship.
2001	Travel grant from the Boehringer-Ingelheim Fonds.
1999	Grant from the International School of Crystallography of Biological Macromolecules.
1998 - 2002	Ph.D. Grant from the European Commission (ERB FMRX-CT98-0204).
1994 - 1998	Grant for university studies from the “Ministerio de Cultura", Spain.