J. Biochem. **135**, 495–499 (2004)
DOI: 10.1093/jb/mvh057

Characterization of *Escherichia coli* Uridine Phosphorylase by Single-Site Mutagenesis

Ilaria Oliva[1], Gabriele Zuffi[2], Dorianna Barile[2], Gaetano Orsini[2], Giancarlo Tonon[2], Luca De Gioia[3] and Daniela Ghisotti[*,1]

[1]Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano, Via Celoria 26, 20133 Milano, Italy; [2]Keryos S. p. A., Divisione di Biotecnologie, Via Maritano 26, 20097 San Donato Milanese, Italy; and [3]Dipartimento di Biotecnologia e Bioscienze, Università di Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy

Received February 3, 2004; accepted February 6, 2004

The *Escherichia coli udp* gene encodes uridine phosphorylase (UP), which catalyzes the reversible phosphorolysis of uridine to uracil and ribose-1-phosphate. The X-ray structure of *E. coli* UP resolved by two different groups produced conflicting results. In order to cast some light on the *E. coli* UP catalytic site, we mutagenized several residues in UP and measured by RP-HPLC the phosphorolytic activity of the mutant UP proteins *in vitro*. Mutations Thr94Ala, Phe162Ala, and Tyr195Gly caused a drastic decrease in UP activity. These three residues were suggested to be involved in the nucleoside binding site. However, surprisingly, Tyr195Ala caused a relative increase in enzymatic activity. Both Met197Ala and Met197Ser conserved low activity, suggesting a minor role for this residue in the UP active site. Glu196Ala completely lost UP activity, whereas the more conservative Glu196Asp mutation was still partially active, confirming the importance of maintaining the correct charge in the surroundings of this position. Glu198 was mutated to either Gly, Asp and Gln. All three substitutions caused complete loss of enzymatic activity suggesting an important role of Glu198 both in ribose binding and in interaction with phosphate ions. Arg30Ala and Arg91Ala eliminated UP activity, whereas Arg30Lys and Arg91Lys presented a very low activity, confirming that these residues might interact with and stabilize the phosphate ions. Ile69Ala did not decrease UP activity, whereas His8Ala lowered the activity to about 20%. Both amino acids were suggested to take part in subunit interactions. Our results confirm the structural similarity between *E. coli* UP and *E. coli* purine nucleoside phosphorylase (PNP).

Key words: *Escherichia coli*, overlap extension PCR, RP-HPLC, uridine phosphorylase, site-directed mutagenesis.

Biochemical pathways involving nucleotides and their precursors are ubiquitous and crucial for cell functions. Among these reactions, the phosphorolysis of purine and pyrimidine nucleosides, whereby a C-N glycosidic bond is cleaved by phosphate to yield the free base and ribose 1-phosphate, is particularly relevant. The unravelling of the structural features of the enzymes capable of catalysing such a reaction is extremely important to better understand the reaction mechanism and the role of the amino acids forming the active site. In recent years it has been shown, on the basis of structural studies, that all the enzymes that catalyse the phosphorolytic cleavage of the glycosidic bond in nucleosides have either of two distinct protein folds (for a review, see Ref. *1*). The nucleoside phosphorylase-I (NP-I) family shares a common α/β subunit fold and shows either a trimeric or a hexameric quaternary structure. The enzymes of the nucleoside phosphorylase-II (NP-II) family have a dimeric quaternary structure, and each subunit is formed by a small α domain separated by a large cleft from a larger α/β domain. Sequence comparisons of NP-I enzymes suggest two NP-I subfamilies. In fact, there is significant similarity among sequences with known trimeric quaternary structure, as well as among sequences with a known hexameric quaternary structure. However, little sequence similarity is observed between the trimeric and hexameric subfamilies.

The main feature of the common subunit fold of NP-I enzymes consists of a central β-strand that forms a distorted β-barrel, surrounded by several α-helices. The active site in members of the NP-I family consists of adjacent phosphate- and nucleoside-binding pockets. These binding sites are formed by residues from the central β-strand and the interconnecting loops, as well as from residues from an adjacent subunit. Remarkably, there is a significant similarity among the active site residues of trimeric structures, while the hexameric structures appear to utilize different residues for binding the phosphate ion.

Several different mechanisms for the phosphorolysis reaction have been suggested for members of the NP-I family from different species. In particular, many details related to the catalytic properties of purine nucleoside phosphorylase (PNP) from *E. coli* have been derived both from kinetic and structural studies. Moreover, the structures of complexes between PNP and substrate analogs

[*]To whom correspondence should be addressed. Tel: +39-02-50315020, Fax: +39-02-50315044, E-mail Daniela.Ghisotti@unimi.it

have allowed the role in substrate recognition and activation of many amino acids forming the enzyme active site to be defined (*2*, *3*).

Less information is available for *E. coli* uridine phosphorylase (UP), which specifically cleaves uridine nucleosides. The results of investigation on the catalytic activity of UP from different species were not fully conclusive, since both a random-sequential mechanism (*4*) and an ordered-sequential mechanism in which phosphate is the first substrate to bind and uracil the first product to leave (*5*) were proposed.

Conflicting results were also reported on structural characteristics of *E. coli* UP. In fact, the X-ray structure of this enzyme reported some years ago (*6*) indicated relevant differences in the active site structure with respect to PNP, while an X-ray structure recently obtained at higher resolution suggested a greater conformational similarity between UP and PNP from *E. coli* (*7*).

In particular, contrary to what was previously reported, the recent UP X-ray structure indicated that Arg48 and His8 appear well ordered and in a conformation similar to Arg43 and His4 in PNP. Moreover, Glu196 and Tyr163 superimpose well with PNP Glu179 and Tyr160, respectively.

With the aim of shedding some light on the role of the amino acids forming the active site of *E. coli* UP, we have undertaken a structure-driven mutagenesis study in which selected residues of the protein were replaced by alanine and other residues. The catalytic activity of the mutated forms was evaluated experimentally and, on the basis of experimental, structural and literature data, a role for the amino acids forming the active site is proposed.

MATERIALS AND METHODS

Strains, Media and Plasmids—*Escherichia coli* DH5α (*8*) was grown in LD broth (*9*), with the addition of 100 μg/ml ampicillin when required. For the construction of pGM679, the *E. coli udp* gene was amplified from the *E. coli* K12 MG1655 (*10*) by PCR, using primers 5′ATCGGTACCATCCATGTCCAAGTCTGATGTTTTTCATCTC and 5′AGACGGTCGACAAGAGAATTACAGCAGACGACG, containing restriction sites *Kpn*I and *Sal*I, respectively, digested with *Kpn*I and *Sal*I and cloned into the polylinker region of pUC18 (*11*). Plasmid pGM859 carries the *E. coli udp* region amplified with oligonucleotides 5′TATTGTCGACGATGTCCAAGTCTGATGTT and 5′GGGAAGCTTAAGAGAATTACAGCAGACGA, digested with *Hin*dIII and *Sal*I, and cloned in the polylinker region of YCpIF1 (*12*). pGM870 was constructed by *Bam*HI-*Sal*I digestion of pGM859, and cloning of the fragment containing *udp* into the polylinker region of pUC13 (*11*). Plasmids pGM679 and pGM870 express *E. coli* UP protein fused at its N-terminal to 12 amino acids (MTMITNSSSVPS) and 13 amino acids (MTMITPSLGCRST), respectively.

Site-Directed Mutagenesis—The mutants of *E. coli udp* gene were obtained by mutagenesis of the specific portion of the gene by PCR amplification using the overlap extension technique (*13*). Briefly, two complementary primers containing the mutations were used in combination with external primers to amplify the *udp* regions upstream and downstream of the mutation. The PCR products were used as template in a third series of PCR with external primers. The PCR reactions were run with Pfu DNA polymerase (Stratagene). The final products were either a mutated portion of the *udp* gene or the whole gene, depending on the external primers used. The DNA fragments were digested with appropriate restriction enzymes, purified from a 1.5% agarose gel using the Perfectprep Gel Cleanup kit (Eppendorf) and substituted for the wild-type *udp* sequence in either pGM679 or pGM870. All the amplified regions were sequenced.

Expression of the Mutant Proteins—The expression of the mutant proteins was confirmed by checking their presence in the soluble fraction of the cell by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separations. For preparation of soluble fractions, 10 ml of LD medium containing 100 μl/ml ampicillin was inoculated with *E. coli* DH5α cells harboring a plasmid expressing the UP gene (wild type or mutant) and incubated overnight at 37°C with aeration until $O.D._{600}$ = 1.7. The cell culture was harvested by centrifugation at 4°C, and the pellet resuspended in 1 ml of 10% glycerol, 300 mM KCl, 20 mM Tris-HCl pH 7.8 buffer, cooled in ice and sonicated (Sonics & Materials Inc.) four times for 15 s. After centrifugation at 14,000 rpm for 30 min at 4°C, the soluble fractions of the cells were recovered in the supernatants. The pellets were resuspended in 7 M urea and sonicated four times for 15 s to yield the insoluble fractions. The proteins in the soluble and insoluble fractions were separated by SDS-PAGE and stained with Coomassie Blue.

Enzymatic Activity of the Mutant Proteins—The enzymatic activity of the wild-type and mutant UP proteins was assayed by reversed-phase high pressure liquid chromatography (RP-HPLC). Portions of 200 μl of the soluble fractions were added to 800 μl of 75 mM uridine in 0.1 M phosphate buffer, pH 7, and the phosphorolysis reactions were carried out at 30°C for 5 min. With extracts exhibiting a particularly low activity the reaction time was extended. Reactions were blocked by acidification with 1 ml of 0.1 N HCl, and the products were analysed by RP-HPLC on a C18-Hypersyl-100 analytical column eluted under isocratic conditions with 0.02 M K_2HPO_4 in CH_3OH-H_2O (4:96 v/v) adjusted to pH 4.5 with NH_4OH. The amount of uracil formed in the reactions was measured by comparison with a standard curve, and the enzymatic activity was calculated as μmol uracil·ml^{-1}·min^{-1} (unit/ml). The total protein content of the soluble fractions was measured by the Bradford method (*14*).

Computational Analysis—The X-ray structures of *E. coli* purine nucleoside phosphorylase and *E. coli* uridine phosphorylase were obtained from the Brookhaven Protein Data Bank (codes 1k9s and 1lx7, respectively). Protein structures were analysed using tools of the InsightII suite of programs (Biosym/MSI 9685 Scranton Road, San Diego, CA, USA).

Sequence alignments were generated using the scoring matrix Blosum as implemented in ClustalW (*15*). The gap insertion and extension penalties were set to 10 and 0.05, respectively.

A

```
UP   MSKSDVFHLGLTKNDLQGATLAIVPGDPDRVEKIAA-LMDKPVKLASHREFTTWRAELDGKPVIV 64
PNP  ----ATPHINAEMGDFA--DVVLMPGDPLRAKYIAETFLEDAREVNNVRGMLGFTGTYKGRKISV 59
          . *:.   .*:    :.::**** *.: **  :::.. :: . * :  : .  .*: : *

UP   CSTGIGGPSTSIAVEELA-QLGIRTFLRIGTTGAIQPHINVGDVLVTTASVRLDGAS-LHFAPLE 127
PNP  MGHGMGIPSCSIYTKELITDFGVKKIIRVGSCGAVLPHVKLRDVVIGMGACTDSKVNRIRFKDHD 124
      . *:* ** ** .:**  ::*::.::*:*: **: **::: **::  .:   . .: ::*   :

UP   FPAVADFECTTALVEAAKSIGATTHVGVTASSDTFYPGQERYDTYSGRVVRHFKGSMEEWQAMGV 192
PNP  FAAIADFDMVRNAVDAAKALGIDARVGNLFSADLFYSPDG--------------EMFDVMEKYGI 175
     *.*:***: .   *:***::*  ::**   *:* **. :                  ::  :  *:

UP   MNYEMESATLLTMCASQGLRAGMVAGVIVNRTQQEIPNAETMKQTESHAVKIVVEAARRLL-- 252
PNP  LGVEMEAAGIYGVAAEFGAKALTICTVSDHIRTHEQTTAAERQTTFNDMIKIALESVLLGDKE 238
     :. ***:* :  :.*. * :*  :. *  :   :* ..*   : * .. :**.:*:.
```

B

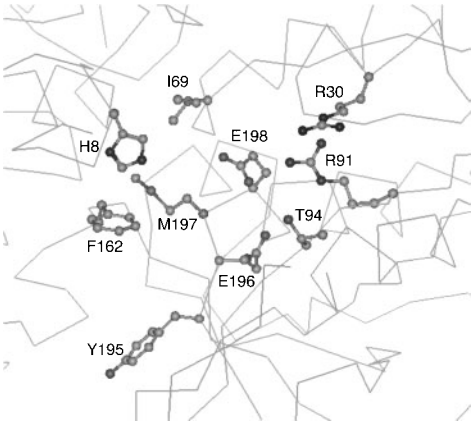

Fig. 1. **Alignment of *E. coli* uridine phosphorylase (UP) and purine nucleoside phosphorylase (PNP) proteins and tridimensional structure of *E. coli* UP.** Panel A. The amino acid sequences of *E. coli* UP (SwissProt P12758) and PNP (SwissProt P09743) were aligned with CLUSTALW, according to (*1*). The mature UP protein lacks the first methionine (*16*). However, UP residues are numbered starting from methionine, for more easy comparison with previous results. The identity or similarity of the amino acid residues is indicated by (*), (:) and (.). The UP amino acid residues mutagenized are highlighted in grey. Panel B. The structure was drawn according to the UP crystal structure of (*7*) using tools of the InsightII suite of programs (Biosym/MSI 9685 Scranton Road, San Diego, CA, USA). The mutated amino acid residues are highlighted. Part of two monomers that form the active site are visualized.

RESULTS AND DISCUSSION

Design of Mutant Forms—The first task of the present investigation was the choice of a reasonable number of mutants to be expressed in order to sample adequately the enzyme active site and define a role for the amino acids forming the catalytic pocket. The available high resolution 3D structures of *E. coli* UP do not contain bound molecules resembling either reagents or products of the catalysed reaction (*6*, *7*), and are therefore not conclusive to localize the key residues directly involved in catalysis and substrate recognition. On the other hand, the X-ray structure of *E. coli* PNP is available for the enzyme co-crystallized with either the substrate analogue formycin B and phosphate (*2*) or formycin A and phosphate (*3*), allowing the characterization of the residues involved in binding of the base, the ribose moiety and the phosphate ion. In particular, Ser90, Ser203 and Asp204 have been shown to be involved in base recognition, the side chains of His4, Arg87, and Glu181 are involved in ribose binding, and the side chains of Arg24, Arg43 and Ser90 should interact with phosphate ions. Remarkably, His4 and Arg43 of *E. coli* PNP come from a neighbouring subunit of the PNP hexameric molecule.

To evaluate quantitatively the degree of similarity between *E. coli* UP and PNP, and therefore translate to UP some structural characteristics inferred from the PNP-formycin A structure, we aligned the sequences of these two proteins (Fig. 1, panel A) as described in Methods. According to the alignment, His4, Arg24, Arg43, Arg87 and Glu181 of PNP are conserved and correspond to His8, Arg30, Arg48, Arg91 and Glu198 in UP, in agreement with previous proposals (*1*). On the other hand, Ser90, Ser203 and Asp204 of PNP are not conserved and correspond to Thr94, Ile220 and Val221 of UP, respectively. Remarkably, the latter two amino acids are strictly involved in the purine base recognition in PNP, and therefore it is not surprising that they are not conserved in UP, which has a different base substrate specificity. For this reason, these two residues were not included in the systematic mutagenesis study.

It should be also noted that Pugmire and Ealick (*1*), on the basis of sequence similarity and structural considerations for the NP-I family, proposed a role for some other amino acids of UP, such as Ile69, which might be involved in intersubunit contacts, as well as Phe162 and Tyr195, which should be located within the active site. Hence, these residues were also included in the experimental protocol. Finally, the combined analysis of the X-ray UP structure and the PNP-UP alignment suggests that Glu196 and Met197 (*E. coli* UP numbering), although corresponding to amino acids of PNP whose side chains are not directly involved in substrate recognition, might play a role in catalysis. In fact, it have been noted that Met197 is absolutely conserved in all known sequences of the NP-I family (*1*).

All the selected amino acids were first replaced by an Ala or a Gly residue; moreover, to better define the role of specific residues, other residues were replaced in a few selected positions. In particular, Lys was substituted for Arg30 and Arg91, and Asp for Glu196 and Glu198.

The UP mutagenized amino acid residues are highlighted in Fig. 1, panel A and B.

Site-Specific Mutagenesis and Activity Evaluation—The *E. coli* wild-type *udp* gene, encoding uridine phosphorylase, was amplified by PCR and cloned in either pUC18 or pUC13, creating a fusion with the first codons of *lacZ*. The resulting plasmids, pGM679 and pGM870, expressed high levels of soluble UP (data not shown).

Site-specific mutagenesis based on the overlap extension technique was used to obtain different point mutations in *E. coli udp* that caused single amino acid substitutions in UP. The different mutagenized DNA fragments were cloned either in pGM679 or in pGM870, substituting for the corresponding wild-type sequence. Strain DH5α was transformed, plasmid DNA was extracted from single colonies, and the amplified region was

Table 1. **Characterization of single amino acid mutants of uridine phosphorylase (UP).**

Expressed proteins[a]	Activity[b] (U/ml extract)	Total proteins[c] (mg/ml)	Specific activity (U/mg total proteins)	Percent of wild type activity[d]
Wild-type UP	540	24	22.5	100
–	0	12	0.0	0
His8Ala	150	31	4.8	21
Arg30Ala	5	15	0.3	1
Arg30Lys	17	14	1.2	5
Ile69Ala	900	36	25.0	111
Arg91Ala	0	13	0.0	0
Arg91Lys	11	23	0.5	2
Thr94Ala	5	14	0.4	2
Phe162Ala	0	33	0.0	0
Tyr195Ala	378	14	27.0	120
Tyr195Gly	5	12	0.4	2
Glu196Ala	0	37	0.0	0
Glu196Asp	84	11	7.6	34
Met197Ala	150	37	4.1	18
Met197Ser	32	14	2.3	10
Glu198Gly	0	23	0.0	0
Glu198Asp	0	13	0.0	0
Glu198Gln	0	32	0.0	0

[a]The activity of wild-type UP produced by pGM679 is reported. UP produced by pGM870 showed less than 2% difference. The negative control is pUC18. [b]Activity was measured by RP-HPLC. The data reported are from a single experiment. Each determination was repeated two or three times and accuracy was within ±10%. [c]Total proteins were measured by the Bradford method. [d]UP activity of the different mutants was expressed as a percentage relative to wild-type UP activity.

sequenced to identify the presence of the expected mutation and the absence of other mutations in *udp*. Cell extracts obtained from the different cultures showed that all mutant UP proteins were expressed in the soluble fraction, indicating that the amino acid substitutions do not alter UP solubility (data not shown). Furthermore, protein quantitation performed on soluble extracts by densitometry of SDS-PAGE separations indicated that both wild-type and all mutant UP enzymes were similarly expressed within a range of variation of ±20% (data not shown).

The enzymatic activities of wild-type and mutant UP proteins were assayed on soluble extracts by RP-HPLC, measuring the amount of uracil produced by uridine phosphorolysis, and the results are reported in Table 1.

Amino Acids Involved in the Interaction with the Base and the Ribose—The replacement of Thr94 with Ala caused a drastic reduction of enzymatic activity to about 2% of wild-type UP. This amino acid is not conserved in PNP, where it corresponds to Ser90, a residue located in the enzyme active site and involved in the interaction with the sugar moiety. Since both Thr and Ser residues bear a side-chain hydroxylic group involved in the formation of hydrogen bonds with the sugar moiety, it is likely that the Thr94Ala substitution eliminates this interaction, thus hindering the identification and the orientation of the substrate.

The replacement of Phe162 with Ala completely inhibited the enzymatic activity, probably because it hampered the substrate stabilization by stacking interaction between the aromatic ring of Phe and the base. For this reason, the substrate no longer bound in the active site. This residue is conserved in PNP and corresponds to Phe159, which has a key role in the identification of the base (*2*).

The replacement of Tyr195 with Ala increased UP activity by about 20%, whereas substitution of Gly for Tyr195 caused a decrease in UP activity, suggesting that the presence of Gly, which lacks a side chain, prevents the formation and/or the maintenance of the correct UP structure. Tyr195 is not conserved in PNP and in other phosphorylases, although it was proposed to be involved in the nucleoside-binding site (*1*).

The replacement of Met197 with Ala or Ser reduced the enzymatic activity to 18% and 10%, respectively. By analogy with PNP, where Met197 is conserved (Met181) and is involved in the interaction with the sugar moiety, the role proposed for this residue in UP is in a hydrophobic interaction with ribose. The substitution of Ala, a hydrophobic residue with a side chain shorter than Met, may be compatible with the low activity shown by this mutant, while the substitution of a hydrophilic residue, such as serine, is more difficult to explain. These data suggest that the function of Met197 in UP might be the stabilization and the molecular identification of ribose by means of specific hydrophobic interactions. However, our result showing the possibility of replacing Met197 with a hydrophilic residue seems to be in contrast with the conservation of Met not only in all bacterial PNP and UP enzymes but also in the mammalian trimeric PNP group (*1*).

The Glu198Asp substitution eliminated UP enzymatic activity. This result could be explained by the loss of hydrogen bond interactions with ribose, as proposed for PNP by Koellner *et al.* (*2*). To test this hypothesis, we made a conservative substitution, replacing Glu198 with Asp, an acidic residue, which resulted in the complete loss of enzymatic activity, showing the importance in this position of an acidic residue with the optimal chain length. Moreover, the substitution Glu198Gln, which dif-

fers only in the presence of an amidic group instead of a carboxylic group, also abolished the activity, indicating that this residue might have another role besides the formation of a hydrogen bond with ribose which, in principle, could be formed also by Gln. It is interesting to note that Glu, unlike Gln, may be deprotonated, suggesting that Glu198, which is localized in an Arg-rich region, could stabilize the orientation of these positively charged amino acids by means of electrostatic interactions.

Amino Acids Involved in the Interaction with Phosphate—The replacement of Arg30 and Arg91 with Ala led to the almost complete elimination of the catalytic activity, suggesting that these residues play an essential role and, in particular, that they could interact with and stabilize the phosphate ions which take part in the enzymatic reaction. Both Arg residues are conserved in PNP (as Arg24 and Arg87), where they interact with the hydroxy groups of phosphate and stabilize them electrostatically. To further investigate the role of these arginines, we replaced them with lysine residues, which have the same positive charge. These mutants showed a less drastic decrease of enzymatic activity, which was reduced to between 2% and 5% of wild-type UP activity, suggesting that the structure of the side chain is important to regulate the identification and orientation of the phosphate group in the active site. Furthermore, an effect of the different pKa of the two amino acids cannot be excluded.

Amino Acids Involved in the Subunit Interactions—Two amino acids that appear to be involved in UP subunit interactions were mutated: Ile69 and His8. The Ile69Ala mutant was completely active, whereas His8Ala conserved about 20% of the wild-type activity. The suggested function for Ile69, which is not conserved in PNP, was related to the presence of a hydrophobic side chain, common to Ile and Ala. Thus, both amino acids might support hydrophobic contacts with the adjacent subunit and support the same levels of enzymatic activity. In PNP, the correspondent Met64 has also a hydrophobic side chain involved in subunit interaction.

His8 is conserved in PNP as His4. The proposed role of this residue in PNP was not only to favour subunit interactions, but also to bind the ribose moiety (*1*, *2*). Accordingly, UP His8Ala might prevent the formation of hydrogen bonds and destabilize the interaction with the substrate.

Other Amino Acids—The replacement of Glu196 with Ala inactivated UP, while the substitution of Asp maintained about 30% of the original activity. This residue is conserved in PNP, where its role is not well defined; our data on UP suggest the importance of maintaining the correct charge in the surroundings of position 196, while dimensional effects, like the formation of hydrogen bonds, play a marginal role.

In conclusion, our results confirm the structural and functional complexity of UP, in which several residues, located in different parts of the protein, including residues located in different monomers, contribute to the formation of the active site. Several new mutants of UP have been prepared in this work and their characterization contributed to the elucidation of the possible roles of specific amino acid residues in the enzyme. Finally, the mutagenesis studies reported in this work can also be considered as an experimental confirmation of structural similarity between *E. coli* UP and PNP recently reported by Burling *et al.* (*7*) and are therefore of interest for future design of UP mutants with improved properties.

We thank Gianni Dehò for reading the manuscript and Pasquale Missineo for performing some experiments. This work was supported by a grant from Keryos S.p.A. and a grant from the Ministero della Istruzione, Università e Ricerca, Rome.

REFERENCES

1. Pugmire, M.J. and Ealick, S.E. (2002) Structural analyses reveal two distinct families of nucleoside phosphorylases. *Biochem. J.* **361**, 1–25
2. Koellner, G., Luiç, M., Shugar, D., Saenger, W., and Bzowska, A. (1998) Crystal structure of the ternary complex of *E. coli* purine nucleoside phosphorylase with formycin B, a structural analogue of the substrate inosine, and phosphate (Sulphate) at 2.1 Å resolution. *J. Mol. Biol.* **280**, 153–166
3. Koellner, G., Bzowska, A., Wielgus-Kutrowska, B., Steiner, T., Saenger, W., and Stepinski, J. (2002) Open and closed conformation of the *E. coli* purine nucleoside phosphorylase active center and implications for the catalytic mechanism. *J. Mol. Biol.* **18**, 351–371
4. Krenitsky, T.A. (1976) Uridine phosphorylase from *Escherichia coli*: Kinetic properties and mechanism. *Biochim. Biophys. Acta* **429**, 352–358
5. Kraut, A. and Yamada, E.W. (1971) Cytoplasmic uridine phosphorylase of rat liver. Characterization and kinetics. *J. Biol. Chem.* **246**, 2021–2030
6. Morgunova, E.Y., Mikhailov, A.M., Popov, A.N., Blagova, E.V., Smirnova, E.A., Vainshtein, B.K., Mao, C., Armstrong, S., Ealick, S.E., and Komissarov, A.A. (1995) Atomic structure at 2.5 Å resolution of uridine phosphorylase from *E. coli* as refined in the monoclinic crystal lattice. *FEBS Lett.* **367**, 183–187
7. Burling, F.T., Kniewel R., Buglino, J.A., Chadha, T., Beckwith, A., and Lima, C.D. (2003) Structure of *Escherichia coli* uridine phosphorylase at 2.0 Å. *Acta Cryst. Biol. Cryst.* **59**, 73–76
8. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580
9. Sabbattini, P., Forti, F., Ghisotti, D., and Dehò, G.(1995) Control of transcription termination by an RNA factor in bacteriophage P4 immunity: identification of the target sites. *J. Bacteriol.* **177**, 1425–1434
10. Singer, M., Baker, T.A., Schnitzler, G., Deischel, S.M., Goel, M., Dove, W., Jaacks, K.L., Grossman, A.D., Erickson, J.W., and Gross, A.A. (1989) Collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **54**, 1–24
11. Vieira, J. and Messing, J. (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268
12. Foreman, P.K. and Davis, R.W. (1994) Cloning vectors for the synthesis of epitope-tagged, truncated and chimeric proteins in *Saccharomyces cerevisiae*. *Gene* **144**, 63–68
13. Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R., (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reactions *Gene* **77**, 51–58
14. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
15. Higgins, D., Thompson, J., Gibson, T. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
16. Henzel, W.J., Billeci, T.M., Stults, J.T., Wong, S.C., Grimley, C., and Watanabe, C. (1993) Identifying proteins form two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. *Proc. Natl. Acad. Sci. USA* **90**, 5011–5015