The role of the T-loop of the signal transducing protein P_{II} from *Escherichia coli*

Rene Jaggi[a], Wendy Ybarlucea[a], Eong Cheah[b], Paul D.Carr[b], Karen J. Edwards[b], David L. Ollis[b], Subhash G. Vasudevan[a,*]

[a]*Department of Biochemistry and Molecular Biology, School of Molecular Sciences, James Cook University of North Queensland, Townsville, Qld 4811, Australia*

[b]*Centre for Molecular Structure and Function, Research School of Chemistry, Australian National University, Canberra, Australia*

Received 29 May 1996; revised version received 26 June 1996

Abstract The 3D structure of P_{II}, the central protein that controls the level of transcription and the enzymatic activity of glutamine synthetase in enteric bacteria revealed that residues 37–55 form the 'T' loop, part of which protrudes from the core of the protein. Within this loop are the only two tyrosine residues that occur in the polypeptide, and one of them, Tyr-51, has been shown by chemical modification studies to be the site of uridylylation. Since tyrosine at position 46 is conserved in all known P_{II} proteins, oligonucleotide directed mutagenesis was used to investigate the role of the two residues. Changing Tyr-51 to phenylalanine or serine abolished uridylylation. Altering tyrosine at position 46 to phenylalanine affected the rate of uridylylation of the protein. This latter mutation does not alter the structure of P_{II} but the reduction in the uridylylation efficiency suggests a role for this residue in recognition and binding of the sensor enzyme uridylyl transferase.

Key words: Bacterial signal transduction; Uridylylation; Nitrogen regulation; 3D structure

1. Introduction

Nitrogen availability is critical for survival of microorganisms and hence the key enzyme for assimilation of ammonia, glutamine synthetase (GS), is very tightly regulated by feedback regulation, activity control by covalent modification and gene expression. The latter two regulatory mechanisms involve the small signal transducing protein P_{II} which serves as a relay centre between the nitrogen sensor and GS.

In *Escherichia coli*, cellular nitrogen status is sensed by the water soluble bifunctional enzyme, uridylyl transferase/removase (UT/UR; *glnD* gene product). In the event of nitrogen excess ($[NH_3] > 1$ mM) the uridylyl removase activity of UT/UR is stimulated so that P_{II} is predominantly in the unmodified form. Unmodified P_{II} interacts with the bifunctional enzyme adenylyl transferase (ATase) to inactivate GS by covalent attachment of AMP group to the hydroxyl residue of Tyr at position 397 of each subunit [1]. Also, unmodified P_{II} interacts with NR_{II} (nitrogen regulatory protein with phosphatase and kinase activity) to dephosphorylate the transcriptional enhancer binding protein NR_I-P to reduce the concentration of GS in the cell. Thus, as a consequence of protein-protein interaction between unmodified P_{II} and ATase or NR_{II}, GS is inactivated and its cellular concentration is reduced.

During nitrogen starvation ($[NH_3] < 1$ mM), UT/UR catalyses the covalent attachment of UMP groups onto P_{II}. Subsequently, P_{II}-UMP interacts with ATase to deadenylylate GS to restore its enzymatic activity. The absence of unmodified P_{II} stimulates the kinase activity of NR_{II} to phosphorylate NR_I. Phosphorylated NR_I and the nitrogen regulated sigma factor (σ^{54}) act in concert to increase the concentration of GS by enhanced transcription from the nitrogen regulated promoter *glnAp2* [2–4].

While much is known about the genetics of nitrogen assimilation and also the order of the events that lead to the control of activity and level of GS, our knowledge of the functionally significant conformational changes that take place during the signalling is limited. As a first step towards addressing this imbalance the 3D structure of unmodified P_{II} has been obtained [5,6]. P_{II} is a highly conserved protein in bacteria and the recent discovery of a second P_{II}-like protein, GlnK, in *E.coli* [7] as well as the P_{II} homologues in other bacterial strains have increased the interest in the structure and function studies of the protein [8].

P_{II} is a compact trimer consisting of an inner core of three antiparallel β-sheets that interlock the subunits (each consisting of 112 amino acids, 12.4 kDa). An outer ring of six α-helices surround the central core of β-sheets. The most intriguing feature of the 3D structure of P_{II} is the exposed T-loop formed by residues 37–55 of each subunit that is connected by β-strands 2 and 3 [6]. This loop also contains two highly conserved Tyr residues, at positions 46 and 51, found in P_{II} from various different species. The hydroxyl group of Tyr at position 51 which lies at the apex of the loop has been identified by protein chemical studies to be the site of uridylylation [9]. Interestingly, Tyr-46 is completely conserved in all P_{II} proteins [5] including the P_{II} homologue from *B. subtilis* which has an Ile in place of Tyr at position 51 [10].

In order to confirm the site of uridylylation and determine the significance of the invariant Tyr 46 in the signalling function of this protein, we have used site-directed mutagenesis to change the residues to Phe individually. We also show that the 3D structure of the Y46F mutant is not distinct from the wild-type P_{II} structure and discuss the role of the T-loop with respect to uridylylation.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

E. coli K12 strain AN1459 containing plasmid pCG647 has been described previously [11]. *E. coli* strain RB9040 (*glnD99*::Tn10; [12]) and plasmid pWVH55 containing the *glnD* gene were kindly provided by Drs van Heeswijk and Westerhoff [13]. *E. coli* strain TG1 [14] was provided with the Sculptor site-directed mutagenesis kit (Amersham).

*Corresponding author. Fax: (61) 77 25 1394.
E-mail: Subhash.Vasudevan@jcu.edu.au

PII S0014-5793(96)00737-5

Phagemid pMA200U which has the tandem bacteriophage lambda promoters P_R and P_L has been described previously [15]. Cells were grown at 37°C in LB medium at pH 7.0 with ampicillin (100 μg ml^{-1}) where appropriate. Cells bearing phagemid pMA200 or derivatives, plasmid pCG647 and plasmid pPL450 or derivatives were grown at 30°C.

2.2. DNA manipulations

Standard methods were used for in vitro DNA manipulations essentially as described in Sambrook et al. [16]. Oligonucleotide directed mutagenesis using primers Y46F (5′ GCCGCGGAACAGCTCGGT), Y51F (5′ ATCCACCATAAACTCCGC) and Y51S (5′ ATCCACCATGGACTCCGC) were carried out using phosphorothioate nucleotides by the Eckstein method [17,18] with modifications outlined in the manufacturer's protocol (Amersham). Single-stranded phagemid DNA was produced and purified according to Vieira and Messing [19]. Mutations were selected by hybridisation screening and confirmed by Sanger dideoxy chain termination sequencing [20] of the *glnB* gene to ensure that no other changes had occurred during mutagenesis. Sequencing was carried out using the mutagenic primer or primers P_{II}-FSP (5′ AATGAATTCGCGTTATGT) and P_{II}-RSP (5′ AATGCTTTGGCCCGCAT).

2.3. Plasmid constructions

2.3.1. Phagemid pRJ 1. Plasmid pCG647 was digested with restriction endonucleases *Bam*HI and *Sma*I to obtain a 1472 bp fragment containing the *glnB* gene which was cloned into similarly cut sites in phagemid pMA200U [15].

2.3.2. Plasmid pNV101. An ~2.8 kb fragment containing the *glnD* gene was isolated from plasmid pWVH55 after digestion with restriction endonuclease *Bam*HI and *Kpn*I and cloned into similarly cut expression plasmid pPL450 [21].

2.4. Expression of P_{II} and UTase

The overproduction of proteins coded by genes borne on plasmids under the control of the strong lambda P_R and P_L promoters was induced by a temperature shift from 30 to 42°C. Transcription from the lambda promoters is repressed at 30°C by the thermolabile repressor supplied by the temperature-sensitive *cI857* gene carried on the expression plasmid, and derepressed at 42°C. Over-expression of proteins by induction was monitored by SDS-PAGE.

2.5. Purification of P_{II}

E. coli strain RB9040 bearing plasmid pRJ1 or pRJ1 with appropriate single site mutations was used for production of wild-type P_{II} and mutants. The production and purification of the proteins were essentially as described previously [11] with the exception that the cell-free extract obtained after French press lysis was treated with DNase (10 μg/ml) for 1 h at 37°C. Similarly, UT/UR produced by the direction of plasmid pNV101 was partially purified by ammonium sulfate precipitation followed by DEAE ion exchange chromatography.

2.6. Uridylylation assay

The centrifugation assay method used for radioligand binding to membrane receptor proteins was essentially as described by Hulme [22]. The assay components were made up as two cocktails: cocktail I (stored as 80-μl aliquots at −20°C) consisted of HEPES pH 7.5 (25 mM), α-ketoglutarate (1.25 mM), $MgCl_2$ (25 mM), DTT (1.25 mM) and KCl (125 mM); cocktail II (stored as 5-μl aliquots at −70°C) consisted of 40 mM ATP and [α-^{33}P]UTP diluted with unlabelled UTP (4 mM). Purified P_{II} (5 μl, 16 μg) was added to 80 μl of cocktail I and 5 μl of cocktail II and incubated at 30°C for 2 min. The uridylylation reaction was started by adding partially purified UTase (10 μl, ~1 μg as judged visually on SDS-PA gel) and incubated at 30°C for the required time (2–120 min). The reaction was terminated by addition of 0.5 ml ice-cold BSA (0.1 mg/ml solution) and 0.5 ml trichloroacetic acid (20% w/v). The mixture was left standing on ice for 30 min and protein precipitate was pelleted by centrifugation at 14000 rpm for 10 min. The supernatant was poured off. The pellet and the sides of the tube was washed by carefully immersing the tube serially into two large beakers containing 1 l deionised water each. The tubes were air dried in an inverted position to remove excess liquid and the pellet was suspended in 100 μl of water by ultrasonication in a water bath for 2 min. After addition of 1 ml of scintillation fluid (BCS, Amersham) the mixture was vortexed briefly and counted in a scintillation counter (Wallac 1410, Pharmacia). The assay was performed in duplicates and the final counts were within 5%. Protein concentration was determined by the method of Bradford [23]. Non-denaturing PAGE to examine the extent of uridylylation was carried out essentially as described by Forchhammer and Tandeau de Marsac [24].

2.7. Crystallisation

P_{II} Y46F was crystallised from a mixture of 18 mg/ml protein and 0.4–0.8 M phosphate buffer, pH 7.1 placed over a 2.1 M phosphate reservoir. Hexagonal crystals isomorphous with the native crystals appeared after 2 weeks. Attempts to crystallise P_{II} Y51F from a variety of conditions have so far only produced small crystals unsuitable for data collection.

2.8. Crystallographic methods

X-ray data were collected on a Rigaku RAXIS-IIC imaging plate detector mounted on a Rigaku RU200 rotating anode X-ray generator operating at 50 kV and 100 mA. The space group and unit cell dimensions were determined using RAXIS data processing software. A total of 22128 reflections were reduced and merged to give 8783 unique reflections with a merging R factor of 9.5% on intensities. The twin fraction for the data was calculated to be 0.24 and a correction for twinning applied to the data [6]. This gave a final total of 7984 unique reflections. The data are 83.7% complete to 2.1 Å resolution (86.6% complete in the 8–2.5 Å shell).

2.9. Structure solution and refinement

The program X-PLOR v3.1 [25] was used for all calculations. The native 1.9 Å P_{II} structure [6] was used as an initial model for fitting to the Y46F mutant data. A positional refinement using data between 8 and 2.5 Å resolution with $F > 2\sigma(F)$ (3573 reflections) gave an initial R factor of 21.9%. Inspection of difference Fourier maps at this stage showed the presence of negative density over the OH of Tyr-46. This is indicative of the absence of an oxygen atom and confirmation of the mutation to a phenylalanine amino acid residue. Tyr-46 was then replaced by a Phe residue in the model. Minor changes were made to the model which was refined further by simulated annealing (4000–300 K in 25 K and 0.05 fs steps), individual B factor and positional refinement. This gave a final R factor of 20.9% for data between 8 and 2.5 Å resolution and $F > 2\sigma(F)$.

3. Results and discussion

3.1. Expression of P_{II} and mutants

Previously we over-expressed P_{II} and separated the unmodified P_{II} from the uridylylated form [5,11]. In this work

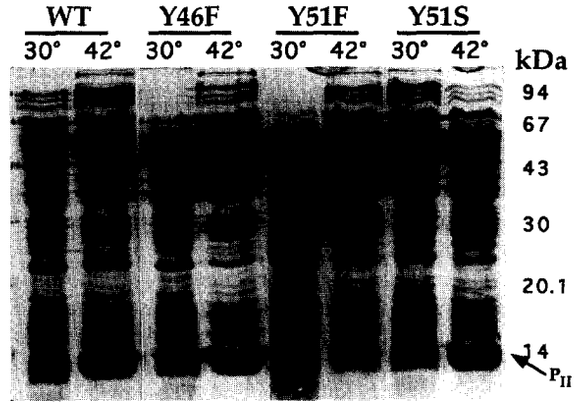

Fig. 1. Overproduction of wild-type P_{II} and P_{II} mutants Y46F, Y51F and Y51S. Coomassie blue stained SDS-PA gel (15%) showing whole cell lysate of indicated samples withdrawn before induction when cells were grown at 30°C to an OD_{595} of ~0.5 and samples withdrawn 2 h post induction by growth at 42°C. The cells were suspended in SDS-gel loading buffer to an OD_{595} of 10. The arrow indicates the position of P_{II}.

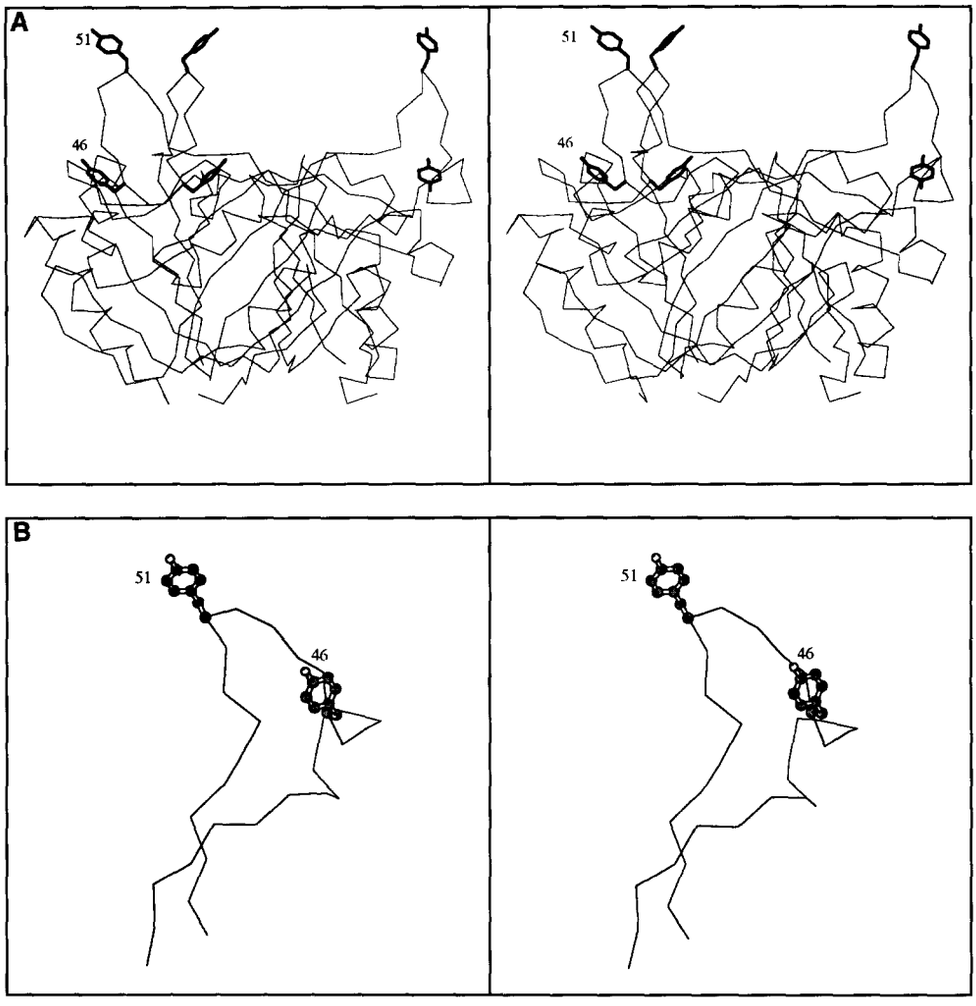

Fig. 2. (A) Stereo view of the C_α trace of P_{II} trimer showing Y46 and Y51. (B) Stereo view of the C_α trace of T-loop of P_{II} monomer showing the bend in the loop. The figure was prepared with the program MOLSCRPT [27].

the expression of P_{II} and mutants were carried out in *E. coli* strain RB9040 (UT/UR deficient strain) so that only unmodified P_{II} was formed. The expression levels of wild-type P_{II} directed by plasmid pCG647 was identical to that previously reported in strain AN1459 and furthermore the expression level was not affected when the DNA insert bearing the *glnB* gene obtained from pCG647 was cloned into phagemid pMA200U (data not shown). The fragment was subcloned into phagemid pMA200U [15] to facilitate the direct expression of the mutants after they were identified by hybridisation or DNA sequencing. Fig. 1 shows the SDS-PAGE analysis of whole cell samples prior to induction and 2 h post induction. This result indicates that the Y46F mutant is expressed at the same level as wild-type P_{II}. The Y51F mutant, on the other hand, only expressed around 10–20% compared to wild-type or Y46F. Initially, it was thought that the reduced expression was due to mutations in the *glnB* gene acquired during the oligonucleotide directed mutagenesis reaction. The nucleotide sequence of *glnB*Y51F revealed that there were no changes in open reading frame other than the intended. Also, the native ribosome binding site in the 5′ untranslated region of *glnB* was unchanged. In order to exclude the possibility that the reduced expression may be due to mutations in the promoter region of the expression vector, the *glnB* gene with the Y51F mutation was subcloned into freshly cut phagemid vector pMA200U. This, however, did not change the level of expression.

A plausible explanation for the reduced level of expression may be the hydrophobicity of Phe at position 51. The hydropathy index for tyrosine is −1.3 compared to phenylalanine for which it is 2.8 [26]. From the 3D structure of P_{II} we know that Tyr at position 51 is at the apex of the T-loop and its hydroxyl group is about 13 Å away from the flat surface of the P_{II} trimer [5]. The hydroxyl group of Y51 is probably critical for solvation of the polypeptide chain during folding. If this is so then replacement with the more hydrophobic Phe at position 51 may result in misfolding of the protein and consequently subject the protein to rapid proteolysis. One may point out that this argument is inconsistent with the natural occurrence of a hydrophobic amino acid, Ile, at position 51 in a P_{II} homologue from *B. subtilis* [10]. However, the *B. subtilis* protein has the lowest amino acid identity (41%) to *E.coli* P_{II} [5] so its signalling mode and 3D structure of the loop may be quite different. The substitution of Tyr at position 51 with Ser restored the expression to wild-type level (Fig. 1). This result indirectly supports our hypothesis that changing the highly exposed Tyr-51 residue to more hydrophobic residues may lead to misfolding and proteolysis, since

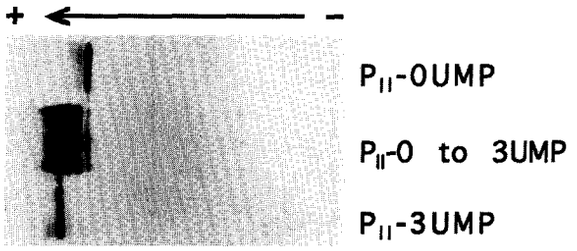

Fig. 3. Analysis of uridylylation of P_{II} by non-denaturing PAGE. Lanes: 1, ununidylylated P_{II} (P_{II}-0UMP); 2, partially uridylylated P_{II} showing all the four molecular forms, P_{II}-0UMP to P_{II}-3UMP; 3, >95% uridylylated P_{II}-3UMP. The uridylylation reaction (see Section 2) for lane 2 was stopped at 10 min and for lane 3 at 20 min. The samples were separated on a 8% polyacrylamide gel and visualised by Coomassie blue staining.

it is unlikely that a single base change in the middle of the *glnB* ORF can result in a lower level of transcription. The high level of expression of Y46F in comparison suggests that this residue is not as highly exposed to the solvent as Y51. Fig. 2A shows the position of tyrosine residues of the P_{II} polypeptide in the trimer. The 20 residue long T-loop is bent in the middle to form an 'L'-shaped structure (Fig. 2B) with several interacting residues including a sharp turn resulting from a hydrogen bond between Glu-40 and Gly-41. Although Y46 itself is not hydrogen bonded the interaction of the other amino acids within the loop places it in a partially solvent-excluded pocket [6].

3.2. *Uridylylation of P_{II} and mutants*

The in vitro uridylylation of cell-free extracts from UT/UR deficient cells(RB9040) expressing the wild-type or mutant proteins gave a high background due to non-specific binding of radioactivity (data not shown) hence the proteins were purified and analysed by uridylylation assay. Purification of P_{II} and the mutants was achieved using the three-step procedure [11] that varied slightly from the protocol of Son and Rhee [9] but was based on the ß-mercaptoethanol fractionation procedure. Fig. 3 clearly shows that P_{II} purified by this method is around 95% modified by uridylylation (i.e. suitable substrate for UT/UR) and can be resolved from unmodified P_{II} by native PAGE.

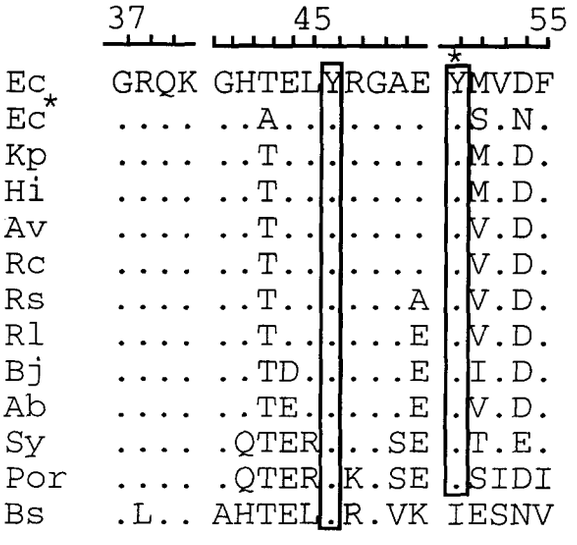

Fig. 5. Alignment of the P_{II} 'T-loop' sequences from various species. Ec, *Escherichia coli glnB*, Ec*, *Escherichia coli glnK*; Kp, *Klebsiella pneumoniae*; Hi, *Haemophilus influenzae*; Av, *Azotobacter vinelandii*; Rc, *Rhodobacter capsulatus*; Rs, *Rhodobacter sphaeroides*; Rl, *Rhizobium leguminosarum*; Bj, *Bradyrhizobium japonicum*; Ab, *Azospirillum brasilense*; Sy, *Synechococcus* strain; Por, *Porphyra purpurea* and Bs, *Bacillus subtilis*.

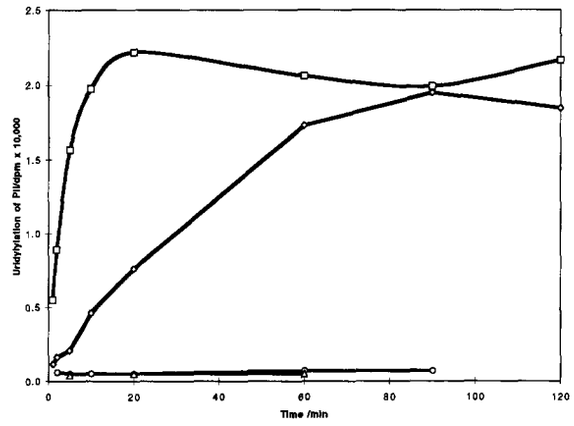

Fig. 4. In vitro uridylylation of P_{II} and mutants. Time course of uridylylation of P_{II} and mutants (5 µg each) were carried out as described in Section 2. The mutants P_{II}Y51F (△) and P_{II}Y51S (○) are not uridylylated whereas P_{II}Y46F (◇) is not as efficiently uridylylated as wild-type P_{II} (□).

The uridylylation profile of purified P_{II} or the mutants shown in Fig. 4 indicates that Y51F or Y51S mutant P_{II} is not uridylylated. Son and Rhee [9] have previously shown that Y51 was the site of uridylylation based on protein chemical studies, and our data obtained from studies with mutants at this position confirm the result. The weakly acidic hydroxyl group of Ser at position 51 cannot replace the phenoxyl group of Tyr as the acceptor of the UMP group via a phosphate ester linkage. The rate of uridylylation of P_{II}-Y46F mutant is significantly slower than wild-type P_{II}. The profile in Fig. 4 shows that under conditions of the in vitro assay when wild-type P_{II} is maximally uridylylated after 20 min, only 40% of P_{II} Y46F was uridylylated. The exposed structure of the T-loop (amino acid residues 37–55) and the very high degree of

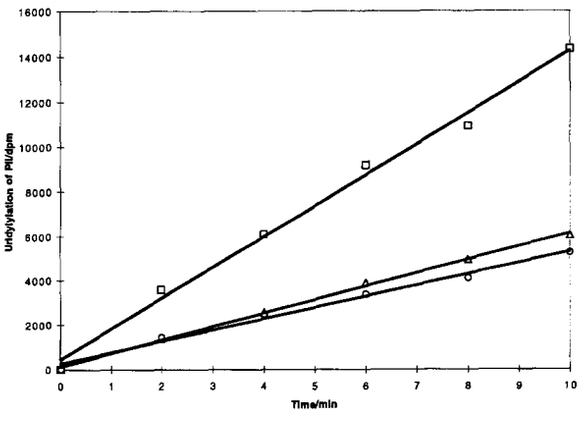

Fig. 6. Inhibition of uridylylation of P_{II} by the P_{II}Y51F and Y51S. 5 µg of wild-type P_{II} (□) was used in the uridylylation assay which was carried out as described in Section 2. Competition experiments using 5 µg of P_{II}Y51F (△) or P_{II}Y51S (○) were incubated with wild-type PII in the uridylylation assay.

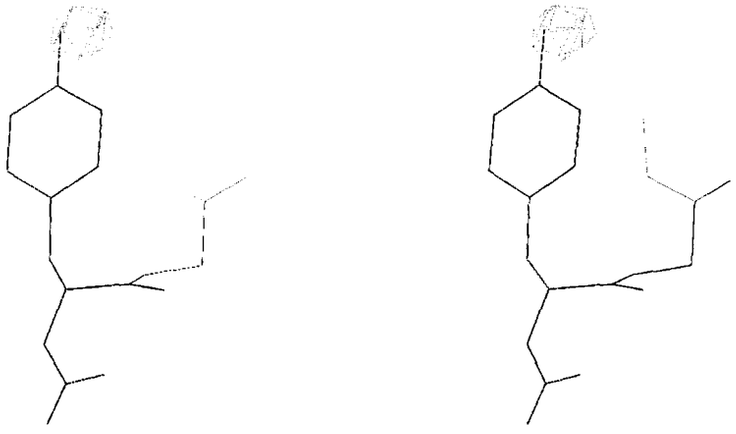

Fig. 7. Negative density map of P_{II}Y46F contoured at -3.5σ.

sequence conservation in this region (Fig. 5) of P_{II} from various species suggest that this may be the recognition site for UT/UR. Recently, Ninfa and colleagues have demonstrated that ATP and α-ketoglutarate bind to P_{II} [2]. The binding of ATP and α-ketoglutarate must play a role in presentation of the correct loop confirmation to the active site of the sensor enzyme. The reduction of uridylylation efficiency of Y46F supports this notion and it is likely that mutations of other amino acids in the loop may similarly affect the rate of uridylylation. Indeed, Holtel and Merrick [28] have obtained the nucleotide sequence of a glutamine auxotroph of *K. pneumoniae glnB502* where the only change in the *glnB* ORF results in E50K mutation. In vitro uridylylation has not been attempted on this mutant but the glutamine auxotrophy phenotype may be due to lack of P_{II}-UMP formation. Other highly conserved residues within the T-loop may play a role in the recognition and binding to UT/UR and also be involved in the enzymatic mechanism during uridylylation or deuridylylation. Also note that in *B. subtilis* residue 50 is a Lys, which again points to the likelihood that the structure and function of this P_{II}-like protein especially in the loop region, may be quite different.

3.3. Inhibition of UT/UR

Little is known about UT/UR due to its labile nature. The preliminary data presented in Fig. 6 show that both P_{II} Y51F and Y51S inhibit UT/UR. The observed (probably competitive) inhibition suggests that the mutant proteins can interact with UT/UR at the same docking site as wild-type P_{II}. Furthermore, this result complements the data presented in Fig. 4, in showing that the lack of uridylylation of the two mutants is not due to their inability to interact with UT/UR. A more complete analysis of this inhibition is currently under way and this may be useful for co-crystallisation of P_{II}-Y51S with UT/UR.

3.4. Crystallisation of P_{II} mutants and structure of P_{II} Y46F

The P_{II} Y46F crystals are similar to the native P_{II} crystals and belong to the hexagonal $P6_3$ space group with unit cell dimensions of $a = b = 61.55$ Å and $c = 56.28$ Å. The presence of negative density over the absent phenolic oxygen of Tyr-46 in the initial model confirms the presence of a Phe residue at position 46 in the Y46F mutant (Fig. 7). There are no structural differences between the mutant Y46F and native P_{II}. The hydroxyl group of Y46 is not involved in hydrogen bonds interactions within P_{II} and also is not as highly exposed as Y51.

4. Conclusion

The 3D structure of unmodified P_{II} provided an initial view of how some of the protein-protein interactions that are critical for signal transduction in the nitrogen assimilation cascade may occur. Here we have shown that Y51 is the site of uridylylation of P_{II} and that the solvation of this residue may be important for the folding of the protein, at least under the conditions of high level protein expression. While little is known about the sites of interactions of P_{II} with either UT/UR or the other proteins in the cascade, this work has for the first time using site-directed mutagenesis shown that the T-loop is involved in recognition and binding to UT/UR. This inference is drawn from the Y46F mutation which does not prevent uridylylation of P_{II} but is nonetheless not uridylylated as efficiently as wild-type P_{II} and the isolation of the glutamine auxotroph phenotype of *K. pneumoniae, glnB502* which has an E50K mutation. Neither Y46 nor E50 is the uridylylated residue. The T-loop is not a random structure and the phenoxyl group of Tyr-51 is more solvent exposed than that of Tyr-46.

Acknowledgements: We thank Hans Westerhoff and Wally van Heeswijk (Free University, Netherlands), and Nick Dixon (ANU) for providing strains and plasmids. The ANU Supercomputer Facility is thanked for a grant of time on the Fujitsu VP2200. R.J. is supported by an Australian Postgraduate Research Award. The Australian Research Council is thanked for financial support.

References

[1] Almassy, R.J., Janson, C.A., Hamlin, R., Xuong, N.-H. and Eisenberg, D (1986) Nature 323, 304–309.
[2] Kamberov, E.S., Atkinson, M.R., Feng, J., Chandran, P. and Ninfa, A.J. (1994) Cell. Mol. Biol. Res. 40, 175–191.
[3] Magasanik, B. (1993) J. Cell. Biochem. 51, 34–40.
[4] Reitzer, L.J. and Magasanik, B. (1986) Cell 45, 785–792.

[5] Cheah, E., Carr, P.D., Suffolk, P.M., Vasudevan, S.G., Dixon, N.E. and Ollis, D.L. (1994) Structure 2, 981–990.
[6] Carr, P.D., Cheah, E., Suffolk, P.M., Vasudevan, S.G., Dixon, N.E. and Ollis, D.L. (1996) Acta Crystallogr. D52, 93–104.
[7] Van Heeswijk, W., Stageman, B., Hoving, S., Molenaar, D., Kahn, D. and Westerhoff, H. (1995) FEMS Microbiol. Lett. 132, 153–157.
[8] Merrick, M J. and Edwards, R.A. (1995) Microbiol. Rev. 59, 604–622.
[9] Son, H.S. and Rhee, S.G. (1987) J. Biol. Chem. 262, 8690–8695.
[10] Wray, L.V., Atkinson, M.R. and Fisher, S.H. (1994) J.Bacteriol. 176, 108–114.
[11] Vasudevan, S.G, Gedye, C., Dixon, E., Cheah, E., Carr, P.D., Suffolk, P.M., Jeffrey, P.D. and Ollis, D.L. (1994) FEBS Lett. 337, 255–258.
[12] Bueno, R., Pahel, G. and Magasanik, B. (1985) J. Bacteriol. 164, 816–822.
[13] Van Heeswijk, W., Rabenberg, M., Westerhoff, H. and Kahn, D. (1993) Mol. Microbiol. 9, 443–457.
[14] Gibson, T.J. (1984) Ph.D Thesis, Cambridge University, Cambridge, UK.
[15] Elvin, C.M., Thompson, P.R., Argall, M.E., Hendry, P., Stamford, N.P.J., Lilley, P.E. and Dixon, N.E. (1990) Gene 87, 123–126.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[17] Sayers, J.R., Krekel, C. and Eckstein, F. (1992) BioTechniques 13, 592–596
[18] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765–8785.
[19] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[20] Sanger, F., Niklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[21] Lilley, P.E., Stamford, N.P.J., Vasudevan, S.G. and Dixon, N.E. (1993) Gene 129, 9–16.
[22] Hulme, E.C. (1990) Receptor Biochemistry, A Practical Approach. Oxford University Press, Oxford.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Forchhammer, K. and Tandeau de Marsac, N. (1994) J. Bacteriol. 176, 84–91.
[25] Brünger, A.T. (1992) X-PLOR Manual Version 3.1, Yale University Press, New Haven, CT.
[26] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[27] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.
[28] Holtel, A. and Merrick, M. (1988) Mol. Gen. Genet. 215, 134–138.