Phe[161] and Arg[166] variants of *p*-hydroxybenzoate hydroxylase

Implications for NADPH recognition and structural stability

Michel H.M. Eppink[a], Christine Bunthof[a], Herman A. Schreuder[b], Willem J.H. van Berkel[a,*]

[a] *Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen University Research Centre, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands*

[b] *Core Research Functions, Hoechst Marion Roussel, D-65926 Frankfurt, Germany*

Received 9 November 1998; received in revised form 16 December 1998

Abstract **Phe[161] and Arg[166] of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* belong to a newly discovered sequence motif in flavoprotein hydroxylases with a putative dual function in FAD and NADPH binding [1]. To study their role in more detail, Phe[161] and Arg[166] were selectively changed by site-directed mutagenesis. F161A and F161G are catalytically competent enzymes having a rather poor affinity for NADPH. The catalytic properties of R166K are similar to those of the native enzyme. R166S and R166E show impaired NADPH binding and R166E has lost the ability to bind FAD. The crystal structure of substrate complexed F161A at 2.2 Å is indistinguishable from the native enzyme, except for small changes at the site of mutation. The crystal structure of substrate complexed R166S at 2.0 Å revealed that Arg[166] is important for providing an intimate contact between the FAD binding domain and a long excursion of the substrate binding domain. It is proposed that this interaction is essential for structural stability and for the recognition of the pyrophosphate moiety of NADPH.**

Key words: *p*-Hydroxybenzoate hydroxylase; Flavoprotein monooxygenase; Crystal structure; NADPH binding; Sequence motif; Site-directed mutagenesis

1. Introduction

p-Hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas fluorescens* is a member of the family of NAD(P)H-dependent flavoprotein monooxygenases [2]. The enzyme catalyzes the conversion of 4-hydroxybenzoate (POHB) into 3,4-dihydroxybenzoate, an intermediate step in the degradation of aromatic compounds in soil microorganisms [3].

The crystal structure of the enzyme-substrate complex of PHBH is known in atomic detail [4] but the binding mode of NADPH is unclear. In contrast to many dehydrogenases and reductases, PHBH and related enzymes lack a well-defined domain for binding the pyridine nucleotide cofactor [5]. Site-specific modifications of PHBH have provided some insight into the mode of NADPH binding [6–8]. From these studies it was proposed that the pyrophosphate moiety of NADPH interacts with His[162] of the FAD binding domain and Arg[269] of the substrate binding domain and that helix H2 is involved in binding the 2′-phosphate ribose moiety of NADPH [8]. Earlier modelling studies suggested that the 2′-phosphate of NADPH interacts with Arg[166] [9–11], and that Phe[161] shields NADPH from entering the active site [12]. Both these residues are located near the protein surface (Fig. 1) and belong to a recently discovered sequence motif presumably involved in both FAD and NADPH binding [1]. Within this so-called DG sequence, Phe[161] is variable whereas Arg[166] is highly conserved [1].

To study their role in NADPH binding, we selectively altered Phe[161] and Arg[166] of PHBH by site-directed mutagenesis. The catalytic properties of the mutant proteins are described together with the crystal structures of F161A and R166S. It is shown that both residues are involved in NADPH binding and that Arg[166] is important for structural stability. The implications for NADPH binding are discussed. Some preliminary results have been presented elsewhere [13].

2. Materials and methods

2.1. Mutagenesis and enzyme purification

Mutations were introduced in the *pob*A gene encoding PHBH, essentially as described elsewhere [14]. The oligonucleotide 5′-GGCATCTCGXXXCAATCGATCC-3′ (where XXX describes the replacement of CGG for AAA (R166K), GAA (R166E) and AGT (R166S), respectively), and the oligonucleotide 3′-GCGATGGCXXXCACGGCATCTCGC-5′, where XXX describes the replacement of TTC for GCC (F161A) and GGC (F161G), respectively), were used for the construction of the mutant proteins. All mutations were confirmed by nucleotide sequencing according to the method of Sanger et al. [15]. Purification of PHBH variants was done according to procedures reported earlier [6,16]. The expression levels and yields of F161A, F161G and R166K were comparable to the wild-type enzyme [16]. For R166S and R166E, a rather poor expression was observed, which resulted in a 10- and 100-fold lower yield of pure enzyme, respectively.

2.2. Analytical and physical methods

Molar absorption coefficients for protein-bound FAD were determined in 50 mM sodium phosphate, pH 7.0 by recording absorption spectra in the absence and presence of 0.1% SDS [17]. Absorption difference spectra between free and substrate complexed enzymes were recorded as reported earlier [7]. Dissociation constants of enzyme-substrate complexes were determined fluorimetrically [18]. PHBH activity was routinely assayed in 100 mM Tris/sulfate, pH 8.0, containing 0.5 mM EDTA, 0.2 mM NADPH, 0.2 mM POHB and 10 μM FAD [19]. Steady-state kinetic parameters were determined at pH 8.0 [20]. Rapid-reaction kinetics were carried out using a stopped flow spectrophotometer, type SF-51, from High-Tech Scientific Inc. Rate constants for anaerobic flavin reduction were estimated from kinetic traces recorded at 450 nm (pH 8.0, 25°C) [6]. Uncoupling of substrate hydroxylation was quantified by oxygen consumption experiments performed in the absence and presence of catalase [20]. Aromatic products were identified by reverse-phase HPLC [17]. The thermal stability of PHBH variants was studied in 50 mM potassium phosphate, pH 7.0, essentially as described elsewhere [18].

2.3. Crystallization, data collection and structural refinement

Crystallization of substrate complexed F161A and R166S and collection of X-ray diffraction data were performed as reported earlier

*Corresponding author. Fax: (31) (317) 484801.
E-mail: willem.vanberkel@fad.bc.wau.nl

PII: S0014-5793(98)01726-8

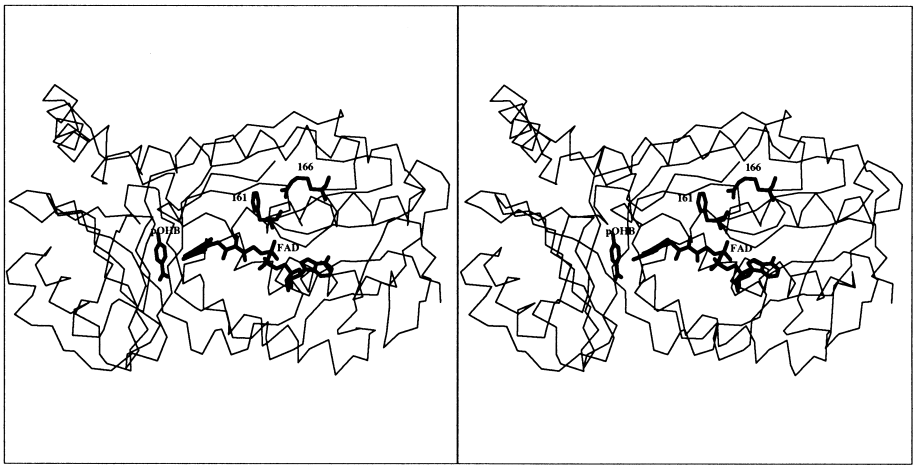

Fig. 1. Stereoview of the Cα backbone of the enzyme-substrate complex of PHBH. 4-Hydroxybenzoate (POHB), cofactor (FAD), Phe^{161} and Arg^{166} are indicated.

[6]. With R166E, no suitable crystals were obtained. A starting electron density map was calculated based on the structure of the wild-type enzyme/substrate complex [4], after a correction had been made for the slightly different cell dimensions (Table 1). The starting *R*-factor was 0.244 for F161A for data between 8.0 Å and 2.2 Å, and 0.245 for R166S for data between 8.0 Å and 2.0 Å. The F_o-F_c omit maps clearly showed the replacement of Phe^{161} by Ala in F161A (Fig. 2A), and Arg^{166} by Ser in R166S (Fig. 3A). With the graphics program O [21], the mutated residues were changed and fitted in the electron-density map. The complete protein models were inspected and corrected where necessary. Refinement was carried out by energy minimization and temperature-factor refinement using the program XPLOR [22]. For F161A overall anisotropic *B*-factors were refined and applied to the dataset, with the following *B*-factors: $B11 = 1.08$ Å^2; $B22 = -1.01$ Å^2; $B33 = -0.06$ Å^2. The final structures were obtained after five cycles of map inspection and refinement. The final *R*-factors are 0.179 and 0.183 for F161A and R166S, respectively. The X-ray diffraction data and refinement statistics are summarized in Table 1. The coordinates will be deposited in the Brookhaven Protein Data Bank.

3. Results

3.1. Structural properties

The overall structures of F161A and R166S are very similar to the structure of wild-type PHBH [4], with root mean square differences of respectively 0.18 Å and 0.22 Å for 391 equivalent Cα atoms. Fig. 2B shows that substitution of Phe^{161} for Ala results in a weaker interaction between the backbone oxygen of Phe^{161} and the NH1 atom of Arg^{166} (2.7→3.1 Å). Furthermore, a slight shift of the side chain of Arg^{269} was observed. This shift is most probably caused by the lost van der Waals contact between the NH1 of Arg^{269} and CE2 of Phe^{161} (3.1 Å) increasing the mobility of the Arg^{269} side chain as indicated by the temperature factors (52 Å^2→70 Å^2). The structure of R166S clearly revealed a backbone movement (Fig. 3B). The removal of the hydrogen bond interactions between the NH1 of Arg^{166} and the backbone oxygens of Phe^{161} and Ala^{287} results in a shift of helix H7 and adjoining loops, including residues 160–173, of about 0.5–1.0 Å. Consequently, weaker hydrogen bond interactions were present in this region, resulting in higher flexibility of this part of the protein as indicated by increased average temperature factors of 42.0 Å^2 instead of 27.5 Å^2 for wild-type PHBH.

3.2. Physical properties

Fluorescence binding studies revealed that the dissociation constant of the enzyme-substrate complex was not affected by the Phe^{161} and Arg^{166} replacements. With all mutants, K_d values of 30 ± 5 μM (pH = 7.0) were estimated, values similar to the wild-type enzyme [18]. Moreover, the nearly identical shape and intensity of flavin absorption difference spectra between the free enzymes and the enzyme-substrate complexes (not shown) suggest that the mode of flavin and substrate binding in the mutant proteins compares favorably with that of wild-type PHBH [23]. The only exception is R166E where no reliable absorption difference spectrum could be obtained due to impaired FAD binding.

3.3. Catalytic properties

The catalytic properties of the mutant proteins were studied at pH 8.0, the optimum pH for turnover of the wild-type enzyme [18]. No hydrogen peroxide production was detected in oxygen consumption experiments, showing that the Phe^{161} and Arg^{166} mutants tightly couple oxygen reduction to sub-

Table 1
Data collection and refinement statistics of the enzyme-substrate complexes of F161A and R166S

Complex	F161A	R166S
Cell dimensions (Å)		
a	71.7	72.2
b	146.3	146.8
c	88.5	88.9
Space group	$C222_1$	$C222_1$
Unique reflections	27 343	22 860
Resolution (Å)	2.0	2.2
R_{sym} (%)	4.9	5.5
Completeness (%)	94.4	92.5
Initial *R*-factor	24.4	24.5
Final *R*-factor	17.9	18.5
Water molecules	292	218
rms of bond lengths (Å)	0.009	0.009
rms of bond angles (deg)	1.43	1.46
Average *B*-factors (Å^2)		
protein	27.2	30.3
flavin ring	14.2	19.2
substrate	14.0	21.6

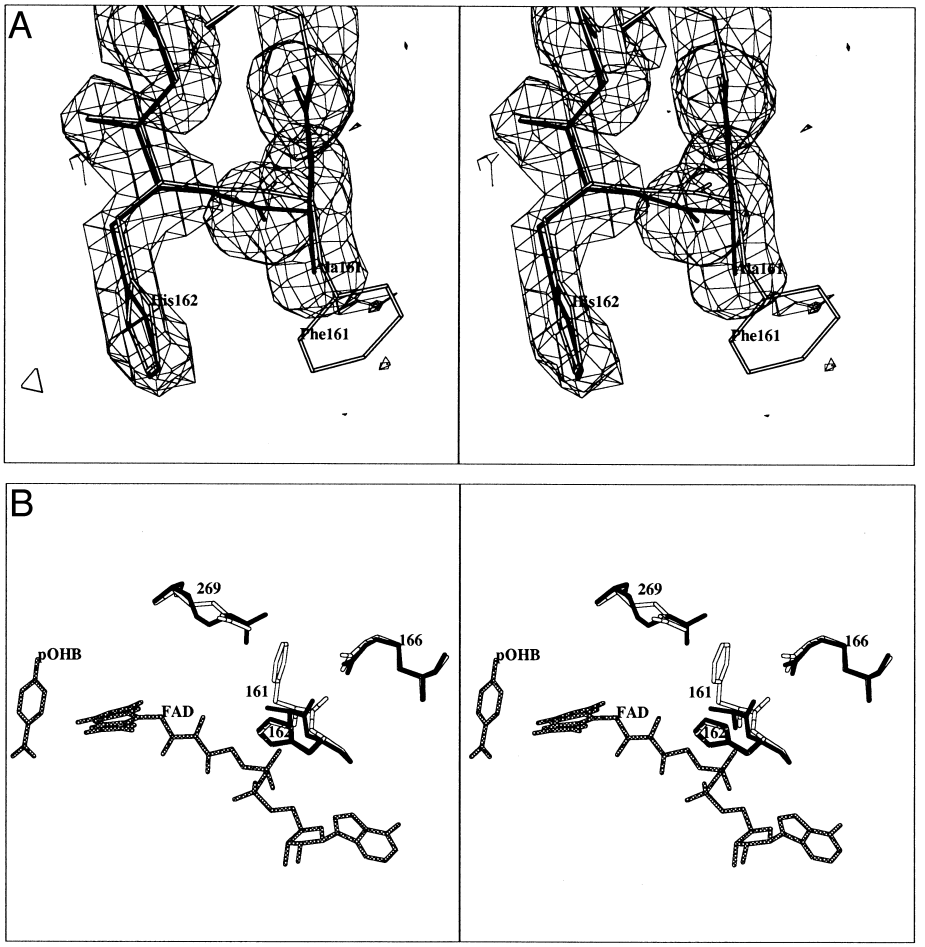

Fig. 2. Crystal structure of F161A in complex with POHB. A: Stereo diagram of the omit map of F161A, contoured at 3σ, with the residues 160–164 omitted from the map. The atomic model of F161A is drawn in bold lines, while Phe[161] of wild-type PHBH is indicated in open lines. B: Superposition of the structures of wild-type PHBH and F161A in complex with POHB. The structure of the wild-type enzyme is drawn in open bonds and the structure of F161A is shown in solid bonds.

strate hydroxylation. As can be seen from Table 2, the Phe[161] and Arg[166] replacements did not strongly affect the apparent K_m POHB. However, with some of the mutant proteins, the apparent K_m NADPH was considerably higher than with the wild-type enzyme. The most profound effects were observed with F161A, R166S and R166E, which all showed a more than five-fold increase in K_m NADPH (Table 2). With all mutants, no activity was found with NADH. With the exception of R166E, all mutants showed turnover rates comparable to wild-type enzyme. The very low turnover rate observed for R166E must be due to some structural effect since this mutant binds poorly FAD. Activity measurements in the absence and presence of varying amounts of FAD revealed an apparent K_m FAD for R166E of about 5 μM. This value is about two orders of magnitude higher than the corresponding value for the wild-type enzyme [19].

Stopped flow kinetics were performed to follow the anaerobic reduction of the enzyme-substrate complex with time as a function of the NADPH concentration. Table 2 shows that the reduction rates of the mutants are similar to that of the wild-type enzyme and not rate limiting in catalysis. However, in agreement with the results from steady-state kinetics, a 5–10-fold weaker NADPH binding was observed for F161A and R166S. As a consequence of the weak FAD binding, no reliable kinetic parameters could be determined for the reductive half-reaction of R166E.

3.4. Thermal stability

The poor expression of R166S and R166E was taken as a first indication that these mutants are less stable than the wild-type enzyme. This was confirmed by thermoinactivation studies. Fig. 4 compares the time-dependent inactivation of wild-type PHBH and the Arg[166] mutants at pH 7.0, 50°C. From this comparison it is clear that, like wild-type enzyme [18], the substrate complexed Arg[166] mutants are more thermostable than the free enzymes. Moreover, in the absence of substrate wild-type PHBH and R166K are far more stable than R166S and R166E (Fig. 4). This supports the idea that a positively charged residue at position 166 is required for structural stability.

4. Discussion

Phe[161] and Arg[166] of PHBH belong to a newly discovered conserved sequence motif in flavoprotein hydroxylases [1]. These residues are located in a surface accessible loop structure (residues 158–174), including helix H7, with a strained conformation [4]. The side chain of Arg[166] contacts the back-

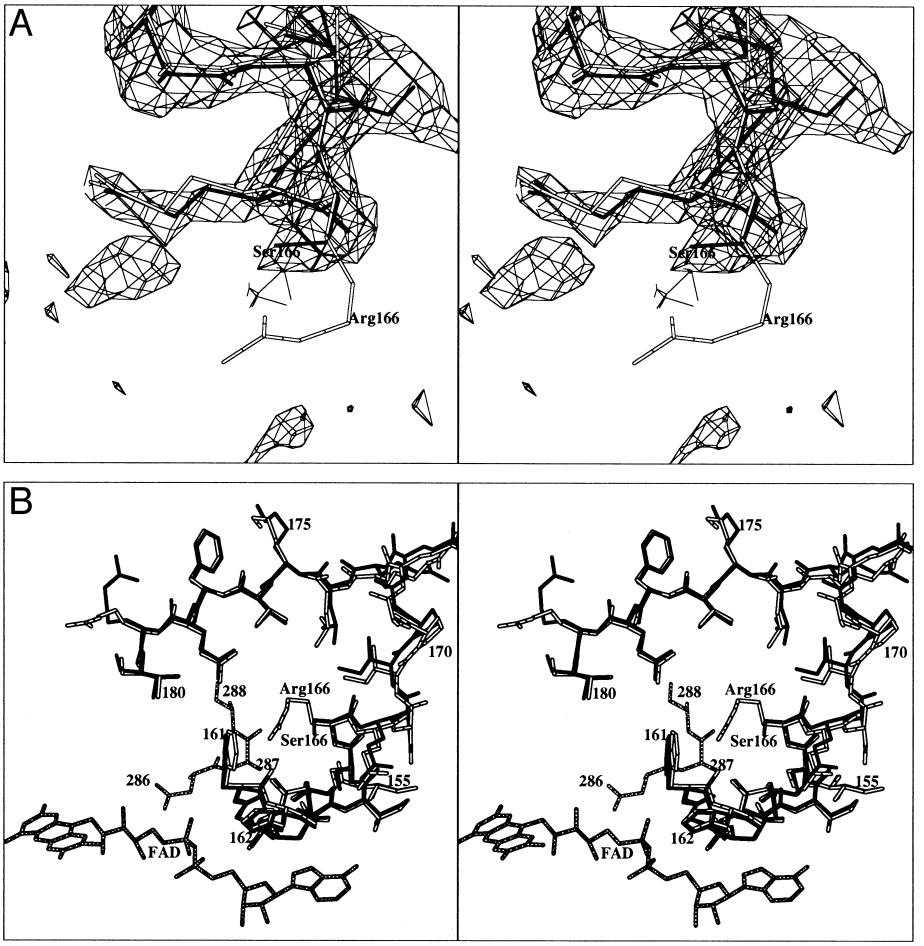

Fig. 3. Crystal structure of R166S in complex with POHB. A: Stereo diagram of the omit map of R166S, contoured at 3σ, with the residues 164–168 omitted from the map. The atomic model of R166S is drawn in bold lines, while Arg^{166} of wild-type PHBH is indicated in open lines. B: Superposition of the structures of wild-type PHBH and R166S in complex with POHB. Wild-type structure is drawn in open bonds and the structure of R166S is shown in solid bonds.

bone oxygen of both Phe^{161} (FAD binding domain) and Ala^{287} (substrate binding domain), whereas the side chain of Phe^{161} interacts with the side chain of Phe^{271} and is situated between the guanidinium groups of Arg^{166} and Arg^{269}. Removal of the aromatic side chain at position 161 slightly weakens the enzyme-NADPH interaction. The crystal structure of F161A at 2.0 Å resolution suggests that this is due to the lost contact between Arg^{269} and Phe^{161}, increasing the mobility of Arg^{269}. Recent mutagenesis studies have indicated that Arg^{269} is of crucial importance for binding the pyrophosphate moiety of NADPH [8]. The catalytic properties of Phe^{161} indicate that Phe^{161} is not essential for NADPH binding. This is in agreement with the recent finding that Phe^{161} is not conserved among flavoprotein hydroxylases [1].

More profound effects on catalysis and in particular on NADPH binding were observed with the Arg^{166} variants. R166K is an efficient enzyme, but the introduction of Ser^{166} significantly decreases the affinity for NADPH. Replacement of Arg^{166} by Glu causes structural perturbations, and impaired binding of NADPH and FAD. We therefore conclude that the catalytic performance and stability of PHBH depends strongly on the ionic character of residue 166. In this respect it is interesting to note that in phenol hydroxylase from the yeast *Trichosporon cutaneum*, the conformation and backbone interactions of the side chain of Arg^{232} are highly similar to its

Table 2
Kinetic parameters of Phe^{161} and Arg^{166} variants of PHBH from *Pseudomonas fluorescens*

Enzyme	K_m POHB (μM)	K_m NADPH (μM)	k_{cat} (s^{-1})	K_d NADPH (μM)	k_{red} (s^{-1})
Wild-type	15	34	55	230	300
F161G	31	112	37	330	300
F161A	18	180	38	1100	300
R166K	30	67	50	220	285
R166S	31	290	40	2200	240
R166E	21	360	5	n.m.	n.m.

The maximum standard error is 10%. n.m.=not measurable.

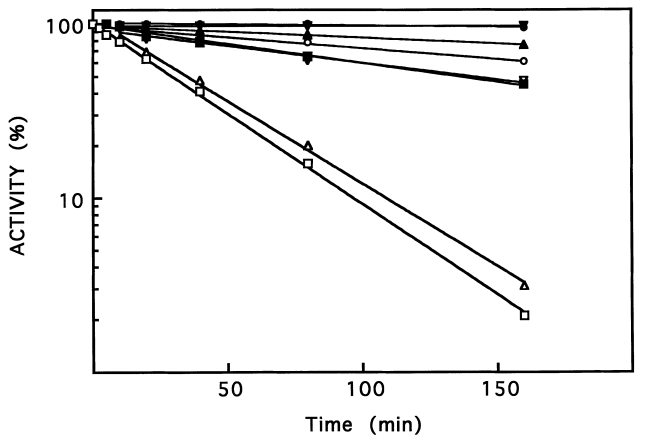

Fig. 4. Time-dependent thermoinactivation of Arg^{166} variants of PHBH. The thermoinactivation of 2 μM enzyme was studied in 50 mM potassium phosphate pH 7.0 at 50°C, in either the absence (open symbols) or presence of 1 mM POHB (filled symbols). Aliquots were withdrawn from the incubation mixtures at intervals and assayed at 25°C, pH 8.0. The remaining activity is plotted as a function of time. Wild-type PHBH (○,●), R166K (▽,▼), R166S (△,▲) and R166E (□,■).

Arg^{166} equivalent of PHBH [24]. The crystal structure of R166S shows a clear shift in the loop structure between residues 159 and 174, comprising helix H7. This movement is caused by the absence of interaction between Ser^{166} and the backbone oxygens of Phe^{161} and Ala^{287} (Fig. 2B). In R166K, the positively charged Lys side chain probably preserves the interactions with the backbone oxygens of Phe^{161} and Ala^{287}. The crystallographic analysis also established that the loop structure comprising residues 160–173 is far more flexible in the R166S mutant than in the wild-type enzyme. The flexibility of this loop decreases the overall structural stability as indicated by the low level of protein expression and the increased rate of thermoinactivation. In line with this, mutant R166E has lost the ability to bind FAD. Residue 166 is at least 10 Å away from the FAD, supporting the idea that the Glu^{166} mutation influences the structural integrity of the FAD binding domain.

In conclusion, the results presented here show that Arg^{166} in PHBH is structurally important and that both Phe^{161} and Arg^{166} are involved in NADPH recognition. However, our studies provide no evidence that Phe^{161} and Arg^{166} interact directly with NADPH. It cannot be excluded that the poor affinity for NADPH in the mutant proteins is caused by the increased flexibility of the loop structure comprising residues 160–173. This loop also contains His^{162}, a residue that is of utmost importance for binding the pyrophosphate moiety of NADPH [8].

References

[1] Eppink, M.H.M., Schreuder, H.A. and van Berkel, W.J.H. (1997) Protein Sci. 6, 2454–2458.
[2] Van Berkel, W.J.H. and Müller, F. (1991) In: Chemistry and Biochemistry of Flavoenzymes (Müller, F., Ed.), Vol. 2, pp. 1–29, CRC Press, Boca Raton, FL.
[3] Harwood, C.S. and Parales, R.E. (1996) Annu. Rev. Microbiol. 50, 553–590.
[4] Schreuder, H.A., Prick, P.A.J., Wierenga, R.K., Vriend, G., Wilson, K.S., Hol, W.G.J. and Drenth, J. (1989) J. Mol. Biol. 208, 679–696.
[5] Entsch, B. and van Berkel, W.J.H. (1995) FASEB J. 9, 476–483.
[6] Eppink, M.H.M., Schreuder, H.A. and van Berkel, W.J.H. (1995) Eur. J. Biochem. 231, 157–165.
[7] Eppink, M.H.M., Schreuder, H.A. and van Berkel, W.J.H. (1998) Eur. J. Biochem. 253, 194–201.
[8] Eppink, M.H.M., Schreuder, H.A. and van Berkel, W.J.H. (1998) J. Biol. Chem. 273, 21031–21039.
[9] Wierenga, R.K., Drenth, J. and Schulz, G.E. (1983) J. Mol. Biol. 167, 725–739.
[10] Van Berkel, W.J.H., Müller, F., Jekel, P.A., Weijer, W.J., Schreuder, H.A. and Wierenga, R.K. (1988) Eur. J. Biochem. 176, 449–459.
[11] Chaiyen, P., Ballou, D.P. and Massey, V. (1997) Proc. Natl. Acad. Sci. USA 94, 7233–7238.
[12] Van der Laan, J.M. (1986) Ph.D. Thesis, University of Groningen, Groningen.
[13] Van Berkel, W.J.H., Eppink, M.H.M., van der Bolt, F.J.T., Vervoort, J., Rietjens, I.M.C.M. and Schreuder, H.A. (1997) In: Flavins and Flavoproteins 1996 (Stevenson, K., Williams, C.H. Jr. and Massey, V., Eds.), pp. 305–314, University Press, Calgary, Alta.
[14] Eschrich, K., van Berkel, W.J.H., Westphal, A., de Kok, A., Mattevi, A., Obmolova, G., Kalk, K.H. and Hol, W.G.J. (1990) FEBS Lett. 277, 197–199.
[15] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[16] Van Berkel, W.J.H., Westphal, A., Eschrich, K., Eppink, M. and De Kok, A. (1992) Eur. J. Biochem. 210, 411–419.
[17] Entsch, B., Palfey, B.A., Ballou, D.P. and Massey, V. (1991) J. Biol. Chem. 266, 17341–17349.
[18] Van Berkel, W.J.H. and Müller, F. (1989) Eur. J. Biochem. 179, 307–314.
[19] Müller, F. and van Berkel, W.J.H. (1982) Eur. J. Biochem. 128, 21–27.
[20] Eschrich, K., van der Bolt, F.J.T., de Kok, A. and van Berkel, W.J.H. (1993) Eur. J. Biochem. 216, 137–146.
[21] Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard, M. (1991) Acta Crystallogr. A47, 110–119.
[22] Brünger, A.T. (1992) X-PLOR: Version 3.1. A System for X-ray Crystallography and NMR, Yale University Press, New Haven, CT.
[23] Gatti, D.L., Palfey, B.A., Lah, M.S., Entsch, B., Massey, V., Ballou, D.P. and Ludwig, M.L. (1994) Science 266, 110–114.
[24] Enroth, C., Neujahr, H., Schneider, G. and Lindqvist, Y. (1998) Structure 6, 605–617.