Engineering of microheterogeneity-resistant *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*

Klaus Eschrich[1,*], Willem J.H. van Berkel[1], Adrie H. Westphal[1], Arie de Kok[1], Andrea Mattevi[2], Galina Obmolova[2], Kor H. Kalk[2] and Wim G.J. Hol[2]

[1] *Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands* and [2] *BIOSON Research Institute, Department of Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands*

Received 5 November 1990

By site-directed mutagenesis, Cys-116 was converted to Ser-116 in *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) from *Pseudomonas fluorescens*. In contrast to wild-type enzyme, the C116S mutant is no longer susceptible to oxidation by hydrogen peroxide and shows no reactivity towards 5,5'-dithiobis(2-nitrobenzoate). Crystals of the C116S mutant are isomorphous with the crystal form of wild-type enzyme. A difference electron density confirms the mutation made.

p-Hydroxybenzoate hydroxylase; Protein-engineering; Site-directed mutagenesis; Thiol-reactivity; Crystallization

1. INTRODUCTION

Microheterogeneity of purified enzymes is a common and often puzzling problem when studying the physical or catalytical properties of these proteins in detail. Microheterogeneity may occur in varying degrees depending on the purification and the actual state of the enzyme [1].

For *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* it was shown that during purification Cys-116, located near the enzyme surface, is slowly oxidized in air. This leads to a variety of oxidation products with normal catalytic properties [2]. Oxidation of Cys-116 however strongly hampers the crystallization of the enzyme [3].

p-Hydroxybenzoate hydroxylase from *P. fluorescens* is a homodimer with an apparent M_r of about 89000, each subunit containing a non-covalently bound FAD molecule [4]. The reaction pathway has been established in detail [5] and crystallographic studies have resulted in high resolution three-dimensional models of the enzyme-substrate and enzyme-product complexes [6]. Fundamental features such as, e.g. the exact mechanism of hydroxylation [7,8] or the binding mode of NADPH [9] are still unclear.

Recently, the genes encoding *p*-hydroxybenzoate hydroxylase from both *P. aeruginosa* [10] and *P. fluorescens* [11] have been cloned in *Escherichia coli*. This now allows modification of the enzymes by site-directed mutagenesis. We describe here the effects of replacing Cys-116 in the *P. fluorescens* enzyme with a serine residue, thereby creating a mutant protein, which is expected to show resistance against this type of microheterogeneity.

Correspondence address: W.J.H. van Berkel, Department of Biochemistry, Agricultural University, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands

* On leave from the Institute of Biochemistry, School of Medicine, University of Leipzig, Germany

2. MATERIALS AND METHODS

2.1. *Genetic manipulation, mutagenesis and DNA sequencing*

The 1.65 kbp fragment containing the gene encoding *p*-hydroxybenzoate hydroxylase from *P. fluorescens* [11] was subjected to site-directed mutagenesis in the bacteriophage M13mp18 according to the method of Kunkel [12]. *E. coli* JM109 [13] was used for methylation of phage DNA. Uracil containing single-stranded DNA was generated in *E. coli* RZ1032 [12]

The mutagenic oligonucleotide 5'-GCGAAGCCTCCGGCGCC-AC-3' (C116S, 10 pmol) was annealed to 1 μg single-stranded DNA and extended using 250 μM of each of the dNTPs, 5 mM DTT, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 1.5 U Klenow DNA polymerase I in a final volume of 20 μl for 2 h at 16°C. After addition of 1 μl 10 mM ATP and 1 U T4 DNA-ligase the incubation was continued for 3 h at 16°C. *E. coli* TG2 was transformed with the reaction mixture and single-stranded DNA was isolated.

Screening for mutants was performed by sequencing the region where the mutation was expected using an oligonucleotide (bp 318-334) as sequencing primer. Clones carrying the mutation were then sequenced entirely to screen for undesired mutations using the universal sequencing primer and 6 oligonucleotides (*His-22*, bp120-136; *Leu-88*, bp 318-334; *Asp-159*, bp 530-546; *Val-231*, bp 748-763; *Gly-298*, bp 948-964; *Glu-367*, bp 1154-1171).

From a positive clone double-stranded DNA was isolated and digested with *Hin*dIII and *Eco*RI. The resulting 1.65 kbp insert was then ligated into pUC9 (pAW46), transformed in *E. coli* TG2 and resulting clones were screened for production of *p*-hydroxybenzoate hydroxylase.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.2. *Purification of enzymes*

Wild-type and mutant (C116S) *p*-hydroxybenzoate hydroxylase were purified from *E. coli* TG2 transformed with the appropriate expression plasmid as described in [11].

2.3. *Analytical methods*

SDS-PAGE was carried out in 12.5% polyacrylamide slab gels [2]. Analytical gel filtration (Superose 12) and analytical anionic exchange chromatography (Mono Q) were performed using FPLC equipment, essentially as described in [2].

2.4. *Chemical modification of sulfhydryl groups*

Labeling of wild-type and mutant enzyme with 5,5'-dithiobis(2-nitrobenzoate) (Ellman's reagent) under non-denaturing conditions was carried out as described earlier [2,4].

Chemical modification of wild-type and mutant enzyme by H_2O_2 was performed at pH 8.0 as reported in [2].

2.5 *Crystal structure analysis*

Crystals of the mutant (C116S) *p*-hydroxybenzoate hydroxylase were obtained by the free interface diffusion method as described by Van der Laan et al. [3]: 5 μl of a solution containing 10 mg/ml protein, 10 mM *p*-hydroxybenzoate, 0.3 mM EDTA, 30 mM sodium sulphite were layered on top of 5 μl of a 65% saturated ammonium sulfate solution in 50 mM phosphate buffer (pH 7.5). The temperature was gradually increased from 4°C to 20°C in 3 weeks.

Diffraction data were collected on a FAST area detector and processed by the MADNES program package [14].

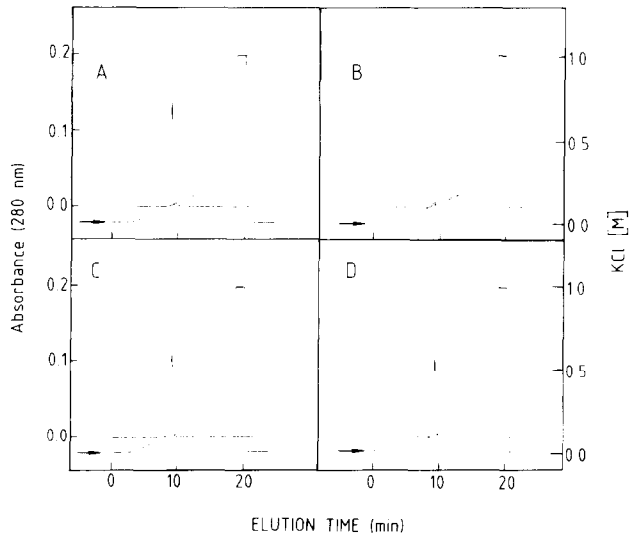

Fig. 1. Chemical modification of wild-type and C116S mutant *p*-hydroxybenzoate hydroxylase by hydrogen peroxide as monitored by FPLC analysis (Mono Q column). (A) Chromatogram of wild-type enzyme. (B) Chromatogram of wild-type enzyme after 120 min reaction time. (C) Chromatogram of C116S mutant. (D) Chromatogram of C116S mutant after 120 min reaction time.

3. RESULTS

3.1 *Biochemical properties of mutant* p-*hydroxybenzoate hydroxylase*

The mutant (C116S) *p*-hydroxybenzoate hydroxylase was purified from extracts of *E. coli* TG2 cells overexpressing the protein from the plasmid vector pAW46. The yield of mutant enzyme was comparable to that of wild-type enzyme, overexpressed in this host [11]. In contrast to wild-type enzyme [11], the mutant protein eluted from the preparative DEAE anion-exchanger in one symmetrical peak. When subjected to SDS-PAGE, the mutant enzyme migrated as a single band with an apparent M_r of about 43 000, identical to the value found for fully Cys-116 reduced wild-type enzyme [11].

Gel filtration through Superose 12 in the absence of dithiothreitol clearly showed that both wild-type and mutant enzyme migrated as dimers. No higher-order quaternary structures, as found under nonreducing purification conditions [3,4], were detectable.

The specific activity of the C116S mutant (50 $U \cdot mg^{-1}$) compared favourably with the value obtained for the wild-type enzyme [11], confirming previous results from chemical modification studies [4,15].

3.2 *Chemical modification of sulfhydryl groups*

p-Hydroxybenzoate hydroxylase from *P. fluorescens* contains 5 sulfhydryl groups [2], but only Cys-116 is accessible to 5,5'-dithiobis(2-nitrobenzoate) and *N*-ethylmaleimide [14].

Both wild-type and mutant enzyme were analyzed for their sulfhydryl content under nondenaturing conditions. In contrast to wild-type enzyme (about 1.1 mol SH/mol FAD) the C116S mutant showed almost no reactivity (less than 0.1 mol SH/mol FAD) with Ellman's reagent.

After chemical modification of *p*-hydroxybenzoate hydroxylase from *P. fluorescens* by H_2O_2, at least 6 apparently different forms of enzyme molecules could be separated by ultrathin isoelectric focusing and/or anionic exchange chromatography [2]. It was suggested that the different products of oxidation represent (hybrid) forms of dimeric enzyme containing sulfenic, sulfinic and sulfonic acid derivatives of Cys-116.

We now have repeated the oxidation experiments with both wild-type and mutant enzyme. From Fig. 1D it is clear that even after 120 min of incubation in the presence of excess H_2O_2 the C116S mutant eluted in one symmetrical peak, showing the same retention time as the reference sample (Fig. 1C). In contrast, in the wild-type enzyme Cys-116 was almost completely converted to the sulfonic acid state (Fig. 1B).

3.3. *Structure determination*

Crystals of the mutant (C116S) *p*-hydroxybenzoate hydroxylase grew to a maximum size of $0.2 \times 0.5 \times 0.8$ mm^3 in a period of 40 days. They appear to be isomorphous to those of wild-type protein [3] having space group $C222_1$ and cell dimensions $a = 71.5$ Å, $b = 145.8$ Å, and $c = 88.2$ Å. A total of 59 381 intensities were collected which yielded to 25 172 independent reflections ($F > \sigma_F$) with $R_{sym} = 3.5\%$ ($R_{sym} = \Sigma |I - I_{av}| / \Sigma I_{av}$). This corresponds to 91% of all the possible reflections between 100 Å and 2.1 Å.

A difference Fourier map calculated with coefficients $|F_{native}| - |F_{C116S}|$ and model phases [6] had the highest

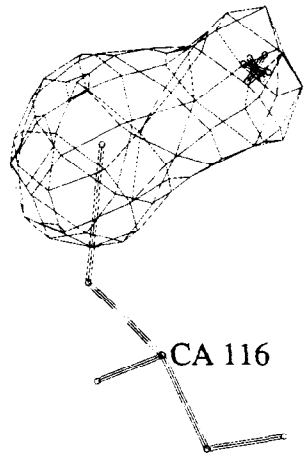

Fig. 2. The peak in the difference $|F_{native}| - |F_{C116S}|$ Fourier map at the position of the side chain of Cys-116. Due to the replacement of the cysteine residue also a water molecule (OHH 505) changes its position. The map is contoured at 5σ level.

peak (2 times higher than the second one) at the position of $S\gamma 116$ (Fig. 2). This suggests that no other significant differences, except from those due to the replacement of the cysteine residue, are present in the structure of the mutated enzyme.

4. DISCUSSION

In *p*-hydroxybenzoate hydroxylase from *P. fluorescens* the Cys-116 residue is part of a surface loop connecting helix H6 and β-strand A3, and is situated about 2.5 nm away from the active site [3,6]. In the absence of reducing agents, Cys-116 is slowly oxidized in air yielding either sulfenyl derivatives or an intermolecular disulfide bond [2,3]. The fully reduced state of Cys-116 was shown to be essential in order to obtain high quality crystals [3].

The mutation of Cys-116 to a serine residue has now been shown to generate a *p*-hydroxybenzoate hydroxylase no longer sensitive towards oxidation causing microheterogeneity and aggregation. Chemical modification by H_2O_2 and Ellman's reagent was completely blocked in the C116S mutant. Replacement of the cysteine residue by an uncharged residue did not influence the crystal packing.

The mutated (C116S) gene is an attractive starting frame for further modification studies with one restriction. The reactivity of Cys-116 allowed covalently binding of the enzyme to Sepharose 5,5′-dithiobis(2-nitrobenzoate) [16]. In this way it was possible to prepare large amounts of highly stable apoprotein, a prerequisite for NMR studies on ^{13}C- and ^{15}N-FAD reconstituted enzymes [17]. Therefore, for preparation of mutant apoenzymes, the presence of Cys-116 is advantageous.

Acknowledgements: We gratefully acknowledge the support of Dr H.A. Schreuder regarding the crystallographic experiments. This research was supported in part by the Netherlands Foundation of Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for Scientific Research (N.W.O.).

REFERENCES

[1] Sadana, A. and Malhotra, A. (1987) Biotechnol. Bioeng. 30, 1041-1056.
[2] Van Berkel, W.J.H. and Müller, F. (1987) Eur. J. Biochem. 167, 35-46.
[3] Van der Laan, J.M., Swarte , M.B.A., Groendijk, H., Hol, W.G.J. and Drenth, J. (1989) Eur. J. Biochem. 179, 715-724.
[4] Müller, F., Voordouw, G., Van Berkel, W.J.H., Steennis, P.J., Visser, S. and Van Rooijen, P. (1979) Eur. J. Biochem. 101, 235-244.
[5] Husain, M. and Massey, V. (1979) J. Biol. Chem. 254, 6657-6666.
[6] Schreuder, H.A., Prick, P., Wierenga, R.K., Vriend, G., Wilson, K.S., Hol, W.G.J. and Drenth, J. (1989) J. Mol. Biol. 208, 679-696.
[7] Van Berkel, W.J.H. and Müller, F. (1989) Eur. J. Biochem. 179, 307-314.
[8] Entsch, B. and Ballou, D.P. (1989) Biochim. Biophys. Acta 999, 313-322.
[9] Van Berkel, W.J.H., Müller, F., Jekel, P.J., Weijer, W., Schreuder, H.A. and Wierenga, R.K. (1988) Eur. J. Biochem. 176, 449-459.
[10] Entsch, B., Nan, Y., Weaich, K. and Scott, K.F. (1988) Gene 71, 279-291.
[11] Westphal, A.H., Van Berkel, W.J.H., Benen, J.A.E., Van Dongen, W.M.A.M. and De Kok, A. (1990) FEBS Lett., submitted.
[12] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367-382.
[13] Yanish-Perron, C., Viera, J. and Messing, J. (1985) Gene 33, 103-119.
[14] Pflugrath, J.W. and Messerschmidt, A. (1986) MADNES User's GUIDE, Max-Planck-Institut für Biochemie, Martinsried, Germany.
[15] Van Berkel, W.J.H., Weijer, W.J., Müller, F., Jekel, P.A. and Beintema, J.J. (1984) Eur. J. Biochem. 145, 245-256.
[16] Van Berkel, W.J.H. and Müller, F. (1982) Eur. J. Biochem. 128, 21-27.
[17] Vervoort, J.J.M., Van Berkel, W.J.H., Moonen, C.T.W. and Müller, F. (1990) Eur. J. Biochem. (submitted).