FEBS Letters 354 (1994) 248–250

FEBS 14738

Effect of single-point mutations Phe[41]→His and Phe[143]→Glu on folding and catalytic properties of recombinant horseradish peroxidase expressed in *E. coli*

Irina G. Gazaryan*, Victoria V. Doseeva, Andrey G. Galkin, Vladimir I. Tishkov

Department of Chemical Enzymology, Faculty of Chemistry, M.V. Lomonosov Moscow State University, 000958 Moscow, Russian Federation

Received 21 September 1994

Abstract Wild-type recombinant and Phe[41]→His and Phe[143]→Glu mutant forms of horseradish peroxidase have been expressed in *E. coli* and reactivated from inclusion bodies with a yield of about 25%. The purified homogeneous preparations have been studied in the reaction of ABTS oxidation. The effect of mutations on heme entrapment and kinetics of ABTS oxidation demonstrates the essential role of the replaced residues in providing the hydrophobic crevice for the non-covalent heme binding.

Key words: Recombinant horseradish peroxidase; Inclusion body; Refolding; Heme entrapment; Site-specific mutagenesis

1. Introduction

Horseradish peroxidase (HRP) is widely used for analytical purposes and, thus, is of great practical importance. The recent progress in its gene expression in *E. coli* and the development of the refolding protocols [1,2] allowing the recombinant deglycosylated enzyme to be obtained in the catalytically active form, has opened up the prospect of HRP protein engineering. One of the goals of the work is the elucidation of the structure–function relationship, e.g. the details of the peroxidase catalytic mechanism. According to present knowledge, the heme protein environment provides for rapid heterolytic cleavage of the hydrogen peroxide molecule in the HRP active center. Two histidine residues, His[42] and His[170], are of key importance in the first step of peroxidase catalysis. The *E. coli* expression system has already been used to produce three heme active-site mutants: F41V, F41W and R38K [3]. In the present work we describe the production and some properties of two new mutant HRP forms to clarify the role of Phe[41], the residue immediately adjacent to the distal histidine and highly conserved in all plant peroxidases, and Phe[143] located at the predicted aromatic substrate binding site.

2. Experimental

2.1. Reagents

2,2-Azino-*bis*(3-ethyl-thiasoline-6-sulfonate) diammonium salt (ABTS), isopropyl-β-D-thiogalactopyranoside (IPTG), sodium dodecyl sulfate (SDS), Tris, glycerol, oxidized glutathione, dithiotreitol (DTT), calcium chloride, hemin, and other components of buffer and refolding solutions were purchased from Sigma, USA. Bactotryptone and yeast extract were from Difco. Restriction endonucleases, ligase and other enzymes for the mutagenesis work, as well as site-specific mutagenesis kit Sculptor IVM System and plasmid pSA261 with the HRP gene under *tac*-promoter control, were kindly provided by Amersham International plc, UK.

2.2. Activity measurements

Peroxidase activity measurements were made using a Shimadzu UV 120-02 spectrophotometer at 25°C as follows: 0.05 ml of ABTS water solution (15 mM) and an enzyme aliquot were added to 2 ml of 0.1 M Na-acetate buffer solution, pH 5.0; the reaction was initiated by the addition of 0.1 ml of a 0.5% hydrogen peroxide solution. A molar absorption coefficient at 405 nm was taken to equal 36,800 $M^{-1} \cdot cm^{-1}$ [4]. The activity was expressed in U (μmol/min) per mg protein.

2.3. Kinetics of ABTS oxidation

The apparent reaction rate constants for the wild-type and mutant HRP were determined by steady-state measurements in the following concentration range: 0.015–0.15 mM ABTS, 0.01–0.1 mM hydrogen peroxide, 0.5–80 nM peroxidase. The concentration of hydrogen peroxide was determined using a molar absorption at 240 nm of 43.6 $M^{-1} \cdot cm^{-1}$ [5], and the concentration of ABTS was estimated by a gravimetric standard.

2.4. Reactivation of the wild-type recombinant HRP from E. coli inclusion bodies and its purification

The recombinant enzyme was produced according to the previously described procedure [2] with the following modifications. *E. coli* JM109/pSA261 was grown using 300 ml LB medium in 10 mM Tris-HCl, pH 8.0, with ampicillin (100 μg/ml) and 0.4% glycerol at 30°C. Gene expression was induced by 0.2 mM IPTG added in the middle of the log-phase of cell growth. The cells were harvested and sonicated (22 kHz, 10 min) in the presence of 2 M NaCl and 10 mM DTT. The disrupted mixture was incubated for 1.5 h and then the sonication procedure was repeated. The supernatant was removed and the pellet was washed with 0.05 M Tris-HCl buffer, pH 8.5, and solubilized in 10 ml of 6 M urea containing 1 mM DTT. The solubilized protein (95% purity, 2 mg/ml) was added drop by drop to the refolding medium (2 l) containing 2 M urea, 0.7 mM oxidized glutathione, 0.1 mM DTT, 5 mM calcium chloride, 5% glycerol in 50 mM Tris-HCl buffer, pH 9.8, and incubated at 4°C. 5 μM hemin was added after the overnight incubation. The activity was measured and the active enzyme was precipitated by ammonium sulfate (60% of saturation) after the refolding was completed. The pellet was dissolved in 30 ml water and applied in 15-ml portions to a column (5.2 × 80 cm) with Toyopearl HW 55F equilibrated with 0.05 M Tris-HCl, pH 7.0, containing 0.1 M NaCl. Active fractions were collected and stored frozen.

2.5. Site-specific mutagenesis

HRP mutant forms were produced by means of site-specific mutagenesis to provide the replacements Phe[41]→His and Phe[143]→Glu using single-strand DNA mutagenesis. To replace the sequence CAT(His[40])-TTT(Phe[41])-CAT(His[42])-GAC(Asp[43]) by CAT(His[40])-CAC(His[41])-CAT(His[42])-GAC(Asp[43]) a 29-mer primer 5′-CCTT-CGT-CTA-CAT-CAC-CAT-GAC-TGC-TTTG-3′ was used. The selection procedure was based on the disappearance of the *Bsp*HI restriction site (TCATGA). To replace the sequence TTC(Phe[142])-TTC(Phe[143])-ACT(Thr[144]) by TTC(Phe[142])-GAA(Glu[143])-ACT(Thr[144]), the corre-

*Corresponding author. Fax: (7) (095) 939 2742.
E-mail: vit@fdh.phys.msu.su

SSDI 0014-5793(94)01125-7

sponding 31-mer primer 5'-CTA-CCG-GCG-CCA-TTC-GAA-ACT-CTA-CCA-CAAC-3' was synthesized. The appearance of a *Bsp*119I restriction site (TTCGAA) was used for the mutant selection. The above primers were synthesized on an Applied Biosystems 380B Synthesizer and the final constructions were sequenced using an Applied Biosystems 370A DNA Sequencer with *Taq* DNA polymerase and dye-labelled primers.

The production, purification and characterization of the mutant forms were performed in the same way as for the wild-type recombinant enzyme.

2.6. General methods

Heme content was determined by the pyridine–chromogen method [6]. Protein content was determined spectrophotometrically [7]. Homogeneity of the enzymes was tested by SDS-PAGE.

3. Results and discussion

One of the specific features of the refolding procedure used is the heme addition after the recombinant HRP apoenzyme reactivation. The yield of the active enzyme in this case is about twice as high (Fig. 1, curve 1) than that for the direct refolding of the recombinant HRP holoenzyme (Fig. 1, curve 2). The phenomenon observed is in agreement with the mechanism of native HRP renaturation and folding in the presence of guanidinium chloride proposed by Cass [8], according to which heme does not affect the HRP renaturation. The higher yield in the case of the two-step refolding procedure can be easily explained by taking into account the heme's ability to catalyse thiol group oxidation by air oxygen and, thus, to increase the possibility of wrong disulfide bond formation.

The kinetics of heme entrapment into the wild-type recombinant HRP apoprotein (Fig. 1, curve 1) shows that the formation of the holoenzyme is completed after 40–42 h incubation. In the case of the HRP mutant forms, the kinetics of heme entrapment

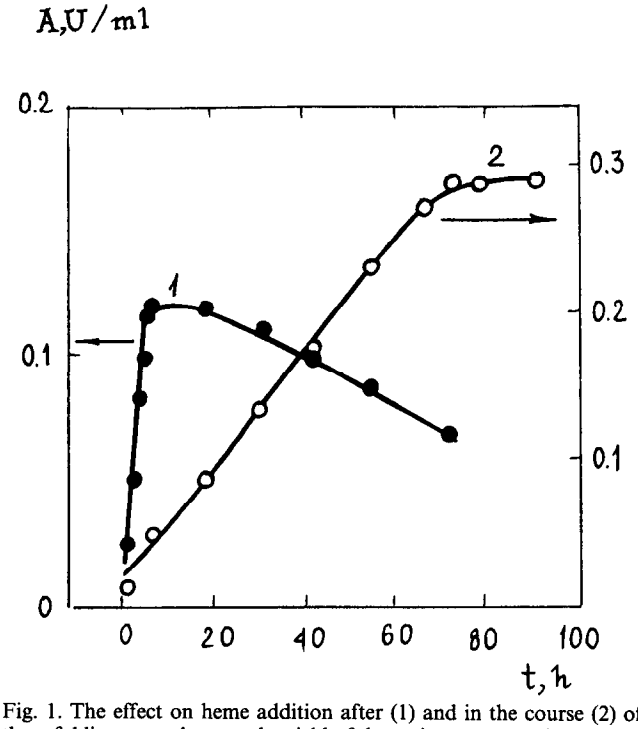

Fig. 1. The effect on heme addition after (1) and in the course (2) of the refolding procedure on the yield of the active enzyme. The concentration of the solubilised HRP apoprotein is 10 mg/l.

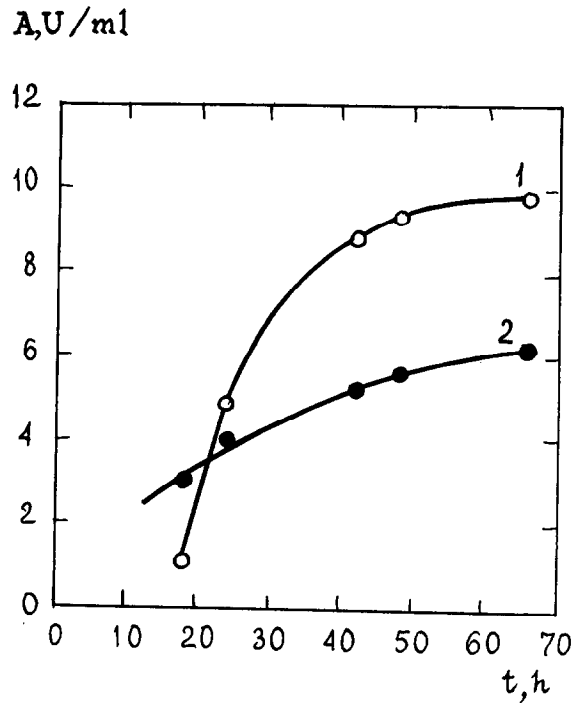

Fig. 2. Kinetics of heme entrapment into the F41H (1) and F143E (2) mutant HRPs after the refolding of the apoenzyme is completed. The protein concentration is 10 mg/l.

significantly differs from that for the wild-type enzyme and from each other (Fig. 2). Heme is easily entrapped in the case of the F41H mutant (Fig. 2, curve 1) whereas the F143E mutant is characterised by a slow kinetics of heme entrapment (Fig. 2, curve 2). The maximum yield of the active F41H mutant is observed after 5 h incubation; the prolonged incubation results in irreversible inactivation of the enzyme. Thus, the mutation also affects the enzyme's stability.

Table 1 presents the results of reactivation and purification of the wild-type recombinant and mutant HRPs. The purification data also demonstrate the instability of F41H mutant. The activity loss during the ammonium sulfate precipitation of the F143E mutant is connected to its low solubility. To dissolve the protein it is necessary to increase the water volume several times compared to the wild-type recombinant HRP and F41H mutant.

Taking into account the low specific activity of the mutants we can conclude that these single-point mutations have a dramatic effect on the catalytic properties of HRP. To distinguish the mutation effects on hydrogen peroxide cleavage and ABTS oxidation steps in the catalytic mechanism the apparent rate constants for hydrogen peroxide and ABTS have been determined from the steady-state kinetic data. The double-reciprocal plots $1/v - 1/[ABTS]$ for the recombinant forms of HRP are represented by the family of parallel lines, and the reaction rate in each case is fitted by the following equation [3]:

$$2[E_o]/v = 1/k_1[H_2O_2] + 1/k_3[ABTS] + 1/k_u,$$

where k_1 and k_3 are the apparent rate constants for H_2O_2 cleavage and ABTS binding, and k_u characterises the rate-limiting step which could be connected with the product dissociation [3]. The rate constants k_1 and k_3 are similar to those obtained by

Table 1
Refolding and purification of the HRP recombinant forms

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Activity yield (%)	Protein reactivated (%)
Wild-type HRP					
1	20	0	0	0	
2	20	20,000	1,000	100	
3	20	24,000	1,200	120	
4	5	20,000	4,000	100	25
F41H mutant HRP					
1	20	0	0	0	
2	20	240	12	100	
3	20	270	13.5	112	
4	1.5	145	90	60	7.5
F143E mutant HRP					
1	20	0	0	0	
2	20	600	30	100	
3	10	300	30	50	
4	5	250	50	25	25

Purification steps: 1, HRP apoprotein solubilization in 6 M urea; 2, reactivation in the refolding medium; 3, HRP ammonium sulfate precipitation; 4, gel filtration.

Smith [3] although k_u is much higher according to the kinetic data presented in Table 2. The F41H mutation affects mainly the step of hydrogen peroxide cleavage (k_1 is about 0.5% and k_3 is about 6% of those for the wild-type recombinant enzyme), whereas the F143E replacement affects mainly the ABTS oxidation step (k_1 is about 23% and k_3 is about 5.7% of those for the wild-type recombinant HRP). A unimolecular rate-limiting step (k_u) is not characteristic for the F41H mutant, in contrast to the F143E mutant and the previously described F41V mutant [3].

The results obtained confirm the essential roles of Phe^{41} and Phe^{143} in the HRP catalytic mechanism.

Phe^{41} is located between His^{40} and His^{42}. The latter coordinates the heme iron in the HRP active center. Thus, the F41H mutant has three histidine residues (40, 41 and 42) in the heme distal site which, probably destroy the hydrophobicity of the heme-binding pocket and provide the heme mobility inside the active center. It has been shown previously that the replacement of Phe^{41} by the hydrophobic Trp and Val [3] leads to an 8-fold decrease in their catalytic activity, mainly connected with the step of hydrogen peroxide cleavage. The presence of the easily ionised His^{41} in the novel mutant obtained causes a significant drop (44-times) in the catalytic activity affecting both hydrogen peroxide cleavage and ABTS oxidation. Moreover, the F41H replacement changes the rate-limiting step in HRP catalysis and results in a significant destabilisation of the enzyme. Thus, the results obtained demonstrate the necessity of Phe^{41} as a hydrophobic barrier separating two histidine residues and providing the firm non-covalent binding of the highly hydrophobic porphyrin ring.

Table 2
The apparent rate constants for the ABTS oxidation by hydrogen peroxide catalysed by the wild-type recombinant and mutant forms of HRP (0.1 M Na-acetate buffer, pH 5.0, 25°C; the error is about 10% of the magnitude)

Enzymes	Rate constants		
	k_1 ($\mu M^{-1} \cdot s^{-1}$)	k_3 ($\mu M^{-1} \cdot s^{-1}$)	k_u (s^{-1})
HRPrec	4.8	3.0	4,600
F41H	0.022	0.37	–
F143E	1.1	0.32	35
HRPrec [3]	5.9	3.7	850
F41V [3]	0.63	0.81	90

The proposed aromatic substrate binding site for HRP contains Phe^{142} and Phe^{143} residues in the proximal site of heme near the entrance to the heme-binding pocket [9]. Phe^{143} is in the vicinity of the proposed binding center for iodide and guaiacol [10]. Its replacement by a negatively charged glutamate group creates an electrostatic hindrance for the negatively charged molecules (heme itself and donor substrates) penetrating inside the active center. The heme content determined by the pyridine–chromogen method for the F143E mutant is about 45% in contrast to the wild-type and F41H forms (98–100%) and, thus, confirms the effect of Glu^{143} on heme entrapment. The decrease in the unimolecular reaction rate constant of ABTS product dissociation (k_u) for the F143E mutant by two orders of magnitude provides additional evidence for ABTS cation radical product interaction with the negatively charged glutamate residue at the entrance to the heme-binding pocket.

Thus, the refolding, purification and kinetic data show that the F41H and F143E single-point mutations of the recombinant HRP produced in *E. coli* affect the whole structure of the enzyme active center and cause significant changes in protein folding, stability and catalytic activity.

References

[1] Smith, A.T., Sanatama, N., Dacey, S., Edwards, M., Bray, R.C., Thorneley, R.N.F. and Burke, J.F. (1990) J. Biol. Chem. 265, 13335–13343.
[2] Egorov, A.M., Gazaryan, I.G., Kim, B.B., Doseeva, V.V., Kapeliuch, J.L., Veryovkin, A.N. and Fechina, V.A. (1994) Ann. NY Acad. Sci. 721, 73–82.
[3] Smith, A.T., Sanders, S.A., Thorneley, R.N.F., Burke, J.F. and Bray, R.C. (1992) Eur. J. Biochem. 207, 507–519.
[4] Childs, R.E. and Bardsley, W.G. (1975) Biochem. J. 145, 93–103.
[5] Arnao, M.B., Casas, J.L., del Rio, J.A., Acosta, M. and Garcia-Canovas, F. (1990) Anal. Biochem. 185, 335–338.
[6] Furhop, J.H. and Smith, K.M. (1975) Laboratory Methods. In: Porphyrins and Metalloproteins (Smith, K.M., Ed.) pp. 757–869, Elsevier, Amsterdam.
[7] Kalb, B.F. and Bernlohr, R.W. (1977) Anal. Biochem. 82, 362–371.
[8] Pappa, H.S. and Cass, A.E.G. (1993) Eur. J. Biochem. 212, 227–235.
[9] Veitch, N.C., Williams, R.J.P., Bray, R.C., Sanders, S.A., Thorneley, R.N.F., Burke, J.F. and Smith, A.T. (1992) Eur. J. Biochem. 207, 521–531.
[10] Ortiz de Montellano, P.R. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 89–107.