FEBS Letters 355 (1994) 220–222

FEBS 14793

In vivo and in vitro effects of mutagenesis of active site tyrosine residues of mercuric reductase

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Received 23 September 1994; revised version received 19 October 1994

Abstract X-ray crystal structure analysis of mercuric reductase suggested that the binding site for Hg^{2+} consisted of two tyrosine residues, Tyr^{264} and Tyr^{605}, as well as two cysteine residues, Cys^{207} and Cys^{628}. We have previously shown that mutagenesis of Tyr^{605} to Phe lowered the k_{cat} of the enzyme 6-fold, whereas the same mutation of Tyr^{264} resulted in a reduction of 160-fold [(1993) Biochemistry 32, 7475–7478]. Tyr^{605} occupies the same position in mercuric reductase as the active site His residue in the related enzyme glutathione reductase. The mutation of Tyr^{605} of mercuric reductase to a His residue produced a 24-fold decrease in k_{cat} and a 15-fold decrease in K_m. The in vivo resistance to Hg^{2+} of *E. coli* strains carrying wild type or mutant *merA* genes correlated with the in vitro measurements of k_{cat}/K_m for mercuric reductase activity.

Key words: Mercuric reductase; Flavoprotein; Mutagenesis

1. Introduction

Mercuric reductase (MerA), one of the proteins encoded by the mercury resistance (*mer*) operon, reduces Hg^{2+} to mercury metal Hg^0 using NADPH as a source of reducing power [1]. It is a member of the family of flavin-dependent disulfide oxidoreductases, which includes glutathione reductase. All of these enzymes are active as homodimers and catalyse the transfer of electrons from NADPH via an enzyme-bound FAD into a disulfide bond at the active site.

The X-ray crystal structure of the enzyme from *Bacillus* sp. strain RC607 suggested that the probable binding site for Hg^{2+} was formed by two cysteine residues, Cys^{207} and $Cys^{208'}$ (i.e. Cys^{628} from the other subunit) and two tyrosine residues, Tyr^{264} and $Tyr^{605'}$ [2]. Cys^{207} is one of the active site cysteine residues present in other members of this family, whereas Cys^{628} forms part of a C-terminal extension unique to MerA. Although Tyr^{264} is also conserved in other members of this family, Tyr^{605} is located at the homologous position in MerA as the active site His residue in glutathione reductase [2]. Site-directed mutagenesis shows that the roles of the two tyrosine residues are not equivalent and that the substitution of Phe for Tyr^{605} had a much less drastic effect on enzyme activity in vitro than a similar substitution of Tyr^{264} [3]. We postulated that the change from the strongly coordinating imidazole side chain of His to the more weakly coordinating side chain of Tyr, which could occur by only a single base change from CA(C/T) for His to TA(C/T) for Tyr at this position, could have been an important change in enabling MerA to bind Hg^{2+} loosely enough to be able to reduce it. To investigate this possibility, we constructed a mutant of MerA, in which Tyr^{605} is changed to His, and characterized the effect of this mutation on enzyme activity in vitro. In addition, we have examined the effect of this and 3 other mutations (Y264F, Y605F and Y264,605F) on in vivo resistance to mercuric chloride.

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2. Materials and methods

2.1. *Mutagenesis of* merA

Site-directed mutagenesis of the *merA* gene was performed as previously described [3]. A single base change in codon 605 from Tyr (TAT) to His (CAT) was introduced using the oligonucleotide 5′ GAT TCC ACA TCT AAC AAT 3′. The presence of the mutation was confirmed by sequence analysis. A 540 bp *ClaI*/*Hin*dIII fragment containing the mutation was ligated into the wild type *merA* gene in expression vector pKK223-3 (Pharmacia, Piscataway, NJ), which had been previously cut with the same enzymes. The final construct was checked by restriction analysis and the *merA* gene was sequenced.

2.2. *Overexpression, purification and characterization of MerA*

MerA protein was expressed in the *E. coli* strain XL1Blue (Stratagene, La Jolla, CA). Cultures were grown in 2 × TY media, supplemented with 100 μg/ml ampicillin at 37°C until an OD_{600} of 2 was reached. Expression was induced by the addition of IPTG to a final concentration of 0.2 mM and the cells were grown a further 3.5 h before harvesting. MerA was purified as previously described [4], except that the buffer used was 50 mM potassium phosphate, pH 7.5, containing 0.5 mM EDTA and 1% (v/v) 2-mercaptoethanol (Buffer A) and the desalted protein was loaded onto a column of DEAE-Sepharose, washed extensively with Buffer A and eluted with a gradient of 0–0.3 M NaCl in buffer A. The purified protein was characterized by SDS-polyacrylamide gel electrophoresis and kinetic analysis as previously described [3].

2.3. *Assay of in vivo resistance to* $HgCl_2$

A functional Hg^{2+} transport system was constructed by ligating a 1.2 kb *Bam*HI/*Hin*dIII fragment of the plasmid pUB3466.BS2 (a generous gift of Prof. N. Brown, University of Birmingham) into pACYC184 which had been cut with the same enzymes. This fragment contains *merR*, *merT* and *merP* genes from transposon Tn*501* which together constitute a Hg^{2+}-inducible Hg^{2+} transport system [1]. The resulting plasmid pDR1 was introduced into the *E. coli* strain XA90, together with the expression plasmid pKK223-3 containing wild type or mutant *merA* genes, and cells containing both plasmids were selected with ampicillin and chloramphenicol. Sensitivities in vivo to mercuric chloride were measured by minimum inhibitory concentration (MIC) and efficiency of plating (EOP) assays as described previously [5], except that the LB plates used contained 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 0.2 mM IPTG and varying concentrations of $HgCl_2$. The MIC was defined as the lowest concentration of Hg^{2+} tested that severely affected colony growth and the EOP was the ratio of number of viable cells at the Hg^{2+} concentration tested to the number of viable cells in the absence of Hg^{2+}.

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SSDI 0014-5793(94)01180-X

Table 1
Kinetic constants for reduction of Hg^{2+} and transhydrogenation of Thio-NADP$^+$ by wild type and Y605H MerA enzymes[a]

Enzyme	K_m (Hg^{2+}) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1}) (Transhydrog.)	K_m (NADPH) (μM) (Transhydrog.)
Wild type[b]	30	12	4.0×10^5	1.3	0.3
Y605H	1.9	0.50	2.6×10^5	0.8	1.8
Y605F[b]	39	1.9	4.9×10^4	0.9	0.6
Y264F[b]	6	0.073	1.2×10^4	1.8	2.9
Y264,605F[b]	6	0.011	1.8×10^3	1.2	1.4

[a]Values of k_{cat} and K_m are apparent values, as in each case only one substrate was varied.
[b]Data taken from [3].

3. Results and discussion

3.1. Analysis of in vitro activity of MerA Y605H

The results of kinetic analysis of MerA Y605H are shown in Table 1. Analysis of Hg^{2+} reductase activity of MerA Y605H showed that the k_{cat} of the enzyme had been reduced by 24-fold compared to wild type enzyme and the K_m of the enzyme for Hg^{2+} had been lowered by about 15-fold. The effect of this mutation at position 605 on Hg^{2+} reductase activity differed markedly from a mutant previously produced in which Tyr^{605} was changed to Phe. This mutant showed only a 30% increase in K_m for Hg^{2+} compared to wild type and the k_{cat} was lowered only 6-fold. These results agree with the proposition that the replacement of the His residue which is found at the equivalent of position 605 in other members of this family of enzymes with the Tyr residue found in MerA would result in an enzyme that would bind Hg^{2+} more loosely, and increase the ability of the enzyme to reduce it to Hg^0. It is interesting, however, that the value of k_{cat}/K_m for MerA Y605H is reduced less than 2-fold compared to the wild type enzyme.

Members of the flavin-dependent disulfide oxidoreductase family are able to catalyse the reduction of thio-NADP$^+$ by NADPH. This reaction is believed to involve the reduction and reoxidation of the enzyme-bound flavin and is a useful control to check that any mutation made has not grossly affected protein folding [6]. MerA Y605H differed only minimally from the wild type enzyme in transhydrogenation activity and the small changes observed did not correlate with changes in Hg^{2+} reductase activity seen in this and other mutant MerA enzymes [3].

Table 2
Minimum inhibitory concentration (MIC) of mercuric chloride for *E. coli* XA90 expressing wild type or mutant MerA enzymes

Hg^{2+} transport system present	MerA enzyme expressed	MIC (μM $HgCl_2$)
None[a]	None[b]	50–75
pDR1	None[b]	16
pDR1	Wild type	>300
pDR1	Y605H	300
pDR1	Y605F	100
pDR1	Y264F	50
pDR1	Y264,605F	18–20

[a]pACYC184 vector present.
[b]pKK223-3 vector present.

3.2. Measurements of in vivo resistance to mercuric chloride

Hg^{2+} resistance in bacteria involves the transport of Hg^{2+} into the cell, followed by its reduction by MerA to Hg^0, which then diffuses out of the cell. To investigate the in vivo effect of mutations affecting MerA, the proteins responsible for Hg^{2+} transport into and within the cell must be present. To allow this, we constructed a plasmid (pDR1) carrying *merT* and *merP*, two genes encoding Hg^{2+} transport proteins, and *merR* which encodes the protein responsible for their Hg^{2+}-dependent transcription [1]. This plasmid was constructed using pACYC184, which carries the chloramphenicol acetyltransferase gene and is compatible with the plasmid used to express wild type or mutant MerA proteins.

The results of the minimum inhibitory concentration assay for Hg^{2+} resistance are shown in Table 2. The MIC for XA90 cells carrying only the two vectors pACYC184 and pKK223-3 was 50–75 μM Hg^{2+}. The introduction of a Hg^{2+}-transport system reduced the MIC to 20 μM Hg^{2+}. Cells containing both a functional Hg^{2+}-transport system and expressing wild type MerA had MIC values greater than the highest Hg^{2+} concentra-

Table 3
Efficiencies of plating for *E. coli* XA90 containing pDR1 and expressing wild type or mutant MerA enzymes

μM Hg^{2+}	Enzyme expressed				
	Wild type	Y264F	Y605F	Y605H	Y264,605F[a]
10	1.1	1.1	1.0	0.94	0.90
20	1.0	0.93	1.1	0.91	5.2×10^{-7}
50	0.94	1.3×10^{-5}	0.070	0.86	$<2.6 \times 10^{-7}$
75	0.82	$<2.6 \times 10^{-6}$	2.6×10^{-4}	0.58	$<2.6 \times 10^{-7}$
100	0.93	$<2.6 \times 10^{-6}$	2.1×10^{-4}	0.082	$<2.6 \times 10^{-7}$
200	0.95	$<2.6 \times 10^{-6}$	1.4×10^{-4}	4.1×10^{-5}	$<2.6 \times 10^{-7}$
300	0.96	$<2.6 \times 10^{-6}$	1.0×10^{-4}	1.2×10^{-5}	$<2.6 \times 10^{-7}$

[a]The lowest dilution of cells expressing MerA Y264,605F tested was 100-fold, compared to 1,000-fold for the other enzymes. Therefore, the lowest limit for efficiency of plating for these cells was 10-fold less than for cells expressing wild type enzyme or the other mutants tested.

tion tested (300 μM). The MIC of cells expressing the mutations tested fell between these two values and correlated with the value of k_{cat}/K_m for Hg^{2+} reductase activity measured in vitro for each mutant. Hence, MerA Y605H which had the highest k_{cat}/K_m of the mutants tested had a MIC of 300 μM. Similarly, the double mutant MerA Y264,605F which had the lowest k_{cat}/K_m had a MIC that on initial testing did not differ from that of cells carrying the Hg^{2+}-transport system alone. When testing was repeated over a narrower range of Hg^{2+} concentrations, there was a difference in MIC for cells expressing MerA Y264,605F compared to those lacking MerA of 2–4 μM. The MIC for cells expressing the mutations MerA Y264F and MerA Y605F were 50 μM and 100 μM, respectively. A more sensitive estimation of the effect of mutations of MerA on the resistance of cells to mercuric chloride is the efficiency of plating assay. The results of this assay (Table 3) confirmed those of the MIC assay. The in vivo resistance to Hg^{2+} of *E. coli* strains carrying wild type or mutant *merA* genes agreed well with the in vitro measurements of k_{cat}/K_m for mercuric reductase activity and suggest that purely kinetic measurements have a validity for interpreting possible in vivo implications of mutations in this enzyme.

Although the mutation of Tyr^{605} to His in mercuric reductase resulted in a decrease in both K_m and k_{cat} for the enzyme, supporting the proposition that the presence of an imidazole side chain at position 605 could lead to tighter binding of the Hg^{2+} substrate, the overall catalytic efficiency of the enzyme was decreased by less than 2-fold. This lack of effect on the catalytic efficiency of the mutation of Tyr^{605} to His in MerA Y605H was supported by measurements of in vivo resistance to mercuric chloride of cells expressing this mutant of MerA. This result makes it unlikely that the change from a His residue at position 605 to Tyr was a crucial change in the evolution of the ability to reduce and detoxify Hg^{2+} by a member of the flavin-dependent disulfide oxidoreductase family of enzymes.

Acknowledgements: This work was supported by a SERC grant to M.B. and a grant from the Edna McConnell Clark Foundation (M.P.). We would like to thank Prof. N. Brown for the generous gift of the plasmid pUB3466.BS2 and John Probert for excellent technical assistance.

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