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ARTICLE *in* FEBS LETTERS · OCTOBER 2003

Impact Factor: 3.17 · DOI: 10.1016/S0014-5793(03)00901-3 · Source: PubMed

Distal side tryptophan, tyrosine and methionine in catalase–peroxidases are covalently linked in solution

Christa Jakopitsch[a], Daniel Kolarich[b], Gabriele Petutschnig[a], Paul Georg Furtmüller[a], Christian Obinger[a,*]

[a] *Institute of Chemistry, Metalloprotein Research Group, BOKU, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria*

[b] *Glycobiology Group, BOKU, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria*

Received 25 June 2003; revised 4 August 2003; accepted 5 August 2003

First published online 22 August 2003

Edited by Judit Ovádi

Abstract Distal side tryptophan and tyrosine have been shown to be essential in the catalase but not the peroxidase activity of bifunctional catalase–peroxidases (KatGs). Recently published crystal structures suggest that both residues could be part of a novel adduct including in addition a conserved methionine. A mass spectrometric analysis of the tryptic peptides from recombinant wild-type *Synechocystis* KatG and the variants Trp122Phe, Tyr249Phe and Met275Ile confirms that this novel adduct really exists in solution and thus may be common to all KatGs. Exchange of either Trp122 or Tyr249 prevents cross-linking, whereas exchange of Met275 still allowed bond formation between Trp122 and Tyr249. It is proposed that the covalent bond between Trp and Tyr may form before that between Tyr and Met. The findings are discussed with respect to the mechanism of cross-linking and its role in KatG catalysis.

Key words: Catalase–peroxidase; *Synechocystis* PCC 6803; Novel covalent bonds; Catalase activity; Mass spectrometry; Peptide mass mapping

1. Introduction

On the basis of sequence similarities with fungal cytochrome *c* peroxidase (CCP) and plant ascorbate peroxidases (APXs), catalase–peroxidases (KatGs) have been shown to be members of class I of the superfamily of plant, fungal, and bacterial heme peroxidases [1]. KatGs are found in archaebacteria, eubacteria and fungi and are homomultimeric proteins with monomers approximately double in size compared with those of CCP or APXs, a phenomenon ascribed to gene duplication [2]. From both CCP and APX the crystal structures have been solved [3,4]. Recently, the 2.0 Å crystal structure of the KatG from the archaebacterium *Haloarcula marismortui* (*Hm*KatG) [5] and the 1.7 Å crystal structure of the KatG from *Burkholderia pseudomallei* (*Bp*KatG) have also been published [6]. Comparison of these structures clearly showed that in class I peroxidases both the conserved proximal triad His, Asp and Trp and the conserved distal triad Trp, Arg and His have very similar coordinates (Fig. 1). Despite this homology, class I peroxidases differ dramatically in their reactivities towards hydrogen peroxide and one-electron donors as well as in the electronic and spectral features of their redox intermediates [7–9]. KatGs have a predominant catalase activity but differ from monofunctional catalases in also exhibiting a substantial peroxidatic activity with broad specificity. On the contrary, substantial catalase activity has never been reported for either CCP or APX.

A structural peculiarity of KatGs is a proposed covalent link between three conserved distal side amino acids (Fig. 1). These novel bonds, indicated by continuous electron density [5,6] apparently link the side chain of the distal Trp (found in all class I peroxidases) with that of a tyrosine, which is found in all so far sequenced KatGs but neither in CCP nor in APXs. Finally, this tyrosine could be linked with the side chain of a methionine found in KatGs and also in APXs but not in CCP. In detail, bonds between $C^{\varepsilon 1}$ of Tyr218 (*Hm*KatG numbering) and $C^{\eta 2}$ of distal Trp95 as well as between $C^{\varepsilon 2}$ of Tyr218 and S^{δ} of Met244 were suggested (Fig. 1) [5,6]. The distances between the covalently bonded atoms Tyr $C^{\varepsilon 1}$ to Trp $C^{\eta 2}$ are 1.68 Å (*Hm*KatG) and 1.69 Å (*Bp*KatG), and between Tyr $C^{\varepsilon 2}$ and Met S^{δ} are 1.72 Å (*Hm*KatG) and 1.78 Å (*Bp*KatG), respectively (Fig. 1B). These bond lengths are somewhat long in comparison to most covalent bonds and so far it has not been demonstrated by mass spectrometric analysis that these covalent links really exist. In case of *Hm*KatG the authors definitely state they were unable to positively prove the existence of these covalent bonds in the enzyme in solution [5]. The corresponding residues in *Synechocystis* KatG are Trp122, Tyr249 and Met275.

However, in order to understand the structure–function relationships of the bifunctional KatGs it is very important to clarify this issue. This is underlined by the following facts: (i) exchange of the Trp122 in *Synechocystis* KatG led to the total loss of the catalase activity but to an increased peroxidase activity [10,11], (ii) the *Synechocystis* KatG Tyr249Phe variant also lost its catalase activity but retained its peroxidase activity and, interestingly, for the first time showed a clear transition of compound I to an oxoferryl-type compound II (which was never observed so far with wild-type KatGs or other variants) [9], and (iii) the proposed covalent bonds seem to fix two KatG-specific long loops on the surface of

*Corresponding author. Fax: (43)-1-360066059.
E-mail address: christian.obinger@boku.ac.at (C. Obinger).

Abbreviations: KatG, catalase–peroxidase; *Hm*KatG, catalase–peroxidase from *Haloarcula marismortui*; *Bp*KatG, catalase–peroxidase from *Burkholderia pseudomallei*; APX, ascorbate peroxidase; CCP, cytochrome *c* peroxidase; LC-ESI-MS, liquid chromatography coupled with electrospray ionization mass spectrometry

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00901-3

the enzyme that cover the substrate access channel to the active site [5].

Here, we report a mass spectrometric analysis of the tryptic peptides from recombinant wild-type *Synechocystis* KatG and the variants Trp122Phe, Tyr249Phe and Met275Ile. The peptide mass mapping unequivocally demonstrates that these novel adducts really exist in *Synechocystis* wild-type KatG in solution and thus may be common to all KatGs. By contrast it was not found in the three variants, whereas the peptide pattern of the Met275Ile variant indicated the existence of a link between Trp122 and Tyr249. Fascinating questions about the role of these adducts and the mechanism of their formation can be raised.

2. Materials and methods

2.1. Materials

Standard chemicals and biochemicals were obtained from Sigma Chemical Co. at the highest grade available. Expression, purification of KatGs from *Synechocystis* and spectrophotometric characterization of wild-type and mutant proteins were described previously [11,12].

2.2. Mutagenesis

Oligonucleotide site-directed mutagenesis was performed using polymerase chain reaction (PCR)-mediated introduction of silent mutations as described [12]. A pET-3a expression vector, that contained the cloned KatG gene from the cyanobacterium *Synechocystis* PCC 6803 was used as the template for PCR. The variant Met275Ile was produced as follows: At first unique restriction sites were selected flanking the region to be mutated. The flanking primers were 5′-AATGATCAGGTACCGGCCAGTAAAG-3′ containing a *Kpn*I restriction site and 5′-AGTCAGCTATTCGAACG-3′ containing a *Spe*I restriction site. The following mutant primer with the desired mutation and a silent mutation introducing a restriction site (*Pau*I) were constructed (point mutations italicized and restriction sites underlined): 5′-TGGCGATGCGCGCAAAGGTAGTG-3′ and 5′-CTACCTTTGCGCGCATCGCCATGAATGAC-3′ changed Met275 to Ile. The fragment defined by the *Kpn*I and *Spe*I restriction sites was replaced by the new construct containing the point mutation. The construct was sequenced to verify DNA changes using thermal cycle sequencing.

2.3. Carboxymethylation and tryptic digestion

The purified recombinant proteins were dried in a vacuum centrifuge. About 1 mg was dissolved in 200 μl 0.5 M Tris–HCl, pH 8.5, containing guanidinium chloride (7 M) and EDTA (10 mM). After reduction by 3 mM dithiothreitol for 2 h at room temperature, iodoacetamide was added to a final concentration of 7 mM. The reaction mixture was incubated for 1 h in the dark and at room temperature. After overnight dialysis against 10 mM NH_4HCO_3, pH 8.0, 2 μg TPCK-trypsin (Sigma Chemical Co.) was added to the protein solution for overnight digestion at 37°C. Finally, the incubation mixture was adjusted to pH 2.0 by addition of HCl (6 M) and centrifuged. The clear supernatant was recovered and its pH adjusted to pH 8.0 by ammonia. Before analysis by liquid chromatography-mass spectrometry (LC-MS) it was diluted 1:1 with double distilled water.

2.4. Mass spectrometry

Molecular masses were determined on a liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) apparatus (Waters Micromass Q-TOF Ultima Global). LC was performed on a Waters CAP-LC with a reversed phase C_{18} column (Thermo Hypersil, 150×0,18 mm) using 0.1% formic acid in double distilled water and 0.1% formic acid in acetonitrile as the mobile phase components. A linear gradient of acetonitrile (0–80%) was used and detection was performed by on-line mass measurements of positive ions with the following settings: spray potential 3000 V; cone potential 150 V; 40 scans/min; flow rate 2 μl/min; acquisition range 280–3000 μm. External calibration was achieved using the following

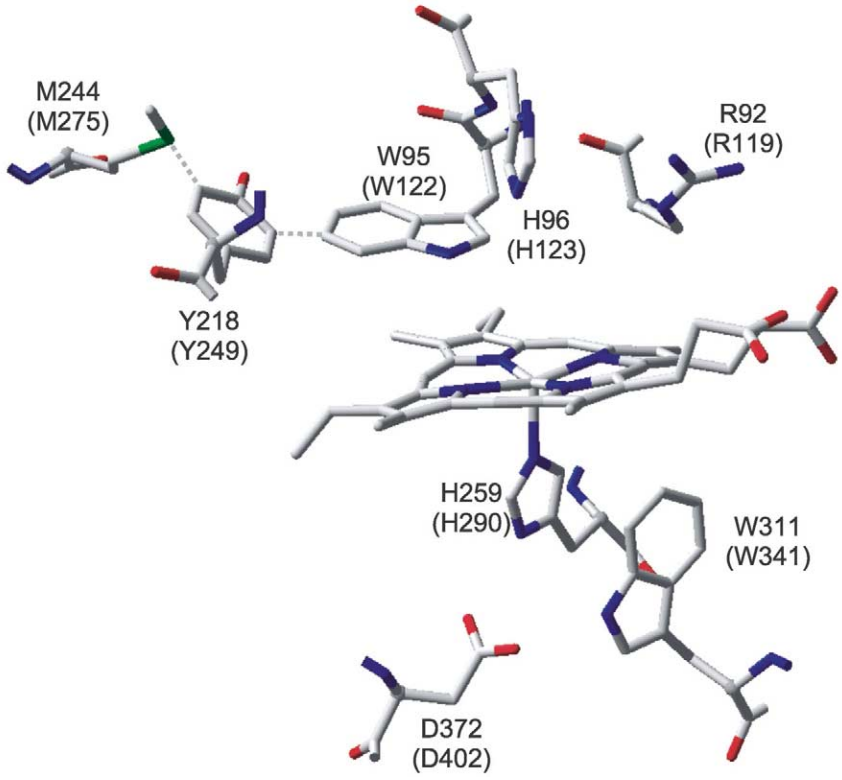

Fig. 1. Conserved distal and proximal residues in KatG from *H. marismortui*. The figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1ITK) for *H. marismortui* KatG [5]. The amino acid numbering is for *H. marismortui* KatG, but numbers in parentheses denote numbering for *Synechocystis* KatG. The proposed adduct formed among the side chains of Trp95, Tyr218 and Met244 in *H. marismortui* KatG is shown by orange dotted lines.

standard peptides and proteins: methionine enkephalin, angiotensin II, [Glu[1]]-fibrinopeptide B human, dabsylated fibrin4 GnGn, mellitin (bee venom) and bovine insuline chain B.

The proteolytic digest patterns were compared with virtual digests performed by the PeptideMass software [13]. The software is freely available via the ExPASy World Wide Web server, at the URL address http://www.expasy.ch/www/tools.html.

3. Results and discussion

The structural prerequisites of an enzyme to catalyze hydrogen peroxide reduction and oxidation (i.e. the catalatic activity) are still under discussion. The only heme peroxidase with substantial catalase activity is KatG. Recently, several papers on site-directed mutagenesis of *Escherichia coli* [14] and *Synechocystis* KatG [8–11] have been published showing that at least three distal side residues could be important in the hydrogen peroxide oxidation reaction, namely aspartate [8], tryptophan [10,14] and tyrosine [9]. These residues are found in all KatGs sequenced so far. Their exchange had a dramatic impact on the catalase but not the peroxidase activity. Most interestingly, the recently published three-dimensional structures of *Hm*KatG [5] and *Bp*KatG [6] strongly suggest that two of these residues, namely Trp and Tyr are part of a novel covalent link including also a conserved methionine (Fig. 1). But so far mass spectrometry evidence supportive of the existence of this adduct in solution was not performed [5].

Since Trp122 in *Synechocystis* KatG has been proposed to be essential for the two-electron reduction of KatG compound I by hydrogen peroxide [10,14], exchange of Tyr249 could presumably modify the coordinates of the distal Trp122 and disturb the hydrogen-bond network on the distal side, if assuming that a covalent link of Tyr249 to Trp122 and Met275 really exists. It should be noted that the H-bond network was shown to be necessary for the catalase pathway of the enzyme [15].

This motivated us to investigate the questionable link by tryptic digestion of KatG and mass spectrometry of the peptides [2]. Since most of the kinetic investigations on KatG were performed with the cyanobacterial enzyme, wild-type KatG from *Synechocystis* as well as the variants Trp122Phe, Tyr249Phe and Met275Ile were produced in order to answer this question. Protein purification and spectroscopic characterization of wild-type KatG, Trp122Phe and Tyr249Phe was described recently [9,11]. The A_{406}/A_{280} ratios (i.e. Reinheitszahl) of the actual recombinant proteins were 0.59 (wild-type), 0.51 (Trp122Phe), 0.52 (Tyr249Phe) and 0.50 (Met275Ile). The purified recombinant proteins [12] were digested by trypsin and the obtained peptide patterns were analyzed by LC-

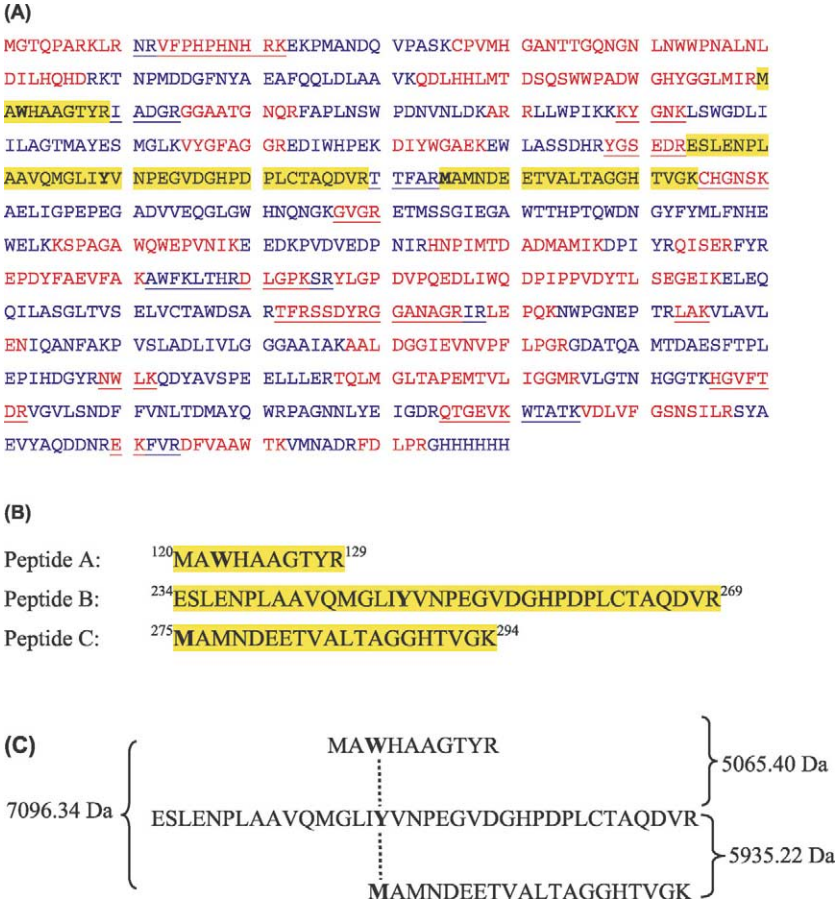

Fig. 2. A: Amino acid sequence of KatG from *Synechocystis* PCC 6803. The peptide masses obtained by virtual digestion by trypsin are in blue and red. Those peptides which contain Trp122, Tyr249 and Met275 are highlighted in yellow (peptides A, B and C). In addition Trp122, Tyr249and Met275 are bold. Those peptides which could not be identified unequivocally by LC-ESI-MS are underlined. B: Trp122, Tyr249 and Met275 are found in peptides A, B and C with isotopic masses of the corresponding singly charged species, $[MH]^+$, of 1163.54 Da, 3904.88 Da and 2031.95 Da, respectively. C: The dashed lines indicate the possible covalent link between peptides A, B and C. The monoisotopic size of the peptides that would result from a Trp-Tyr, Tyr-Met and Trp-Tyr-Met covalent linkage are shown on the right.

ESI-MS as described above. With this method more than 88% of the proposed peptides could be identified unequivocally.

Fig. 2 shows the amino acid sequence of wild-type *Synechocystis* KatG and the peptides obtained by digestion with trypsin under the assumption that the proposed covalent link does not exist. Each of the key residues in the structure (Trp122, Tyr249 and Met275) is located on a separate tryptic peptide fragment (peptides A, B and C) (Fig. 2B), with the corresponding monoisotopic peptide masses of the singly charged species, $[MH]^+$, being 1163.54 Da, 3904.88 Da and 2031.95 Da, respectively, calculating that all cysteines formed carbamidomethyl-cysteines upon treatment with iodoacetamide. If the covalent link between Trp122, Tyr249 and Met275 exists in solution, a peptide of $[MH]^+$ with the monoisotopic mass of 7096.34 Da should be found, calculated from the sum of masses of peptides A, B and C minus the protons released during bond formation between Tyr $C^{\varepsilon 1}$ to Trp $C^{\eta 2}$ and Tyr $C^{\varepsilon 2}$ and Met S^{δ} (Fig. 2C). The LC-MS analysis demonstrated the existence of this peptide in wild-type *Synechocystis* KatG. At least two higher charged species of this peptide, namely $[M4H]^{4+}$ (1774.84 Da) and $[M5H]^{5+}$ (1420.07 Da), could be detected unequivocally by LC-MS analysis. Fig. 3 demonstrates this finding showing the isotopic pattern of the $[M5H]^{5+}$ species. By contrast, the digest of the three variants did not contain this peptide strongly suggesting the absence of an adduct involving all three candidates. It has to be noted that the digest of wild-type KatG also showed to some extent the presence of higher charged species derived from individual peptides A, B and C indicating that in a small fraction of wild-type KatG the covalent link did not exist. Assuming

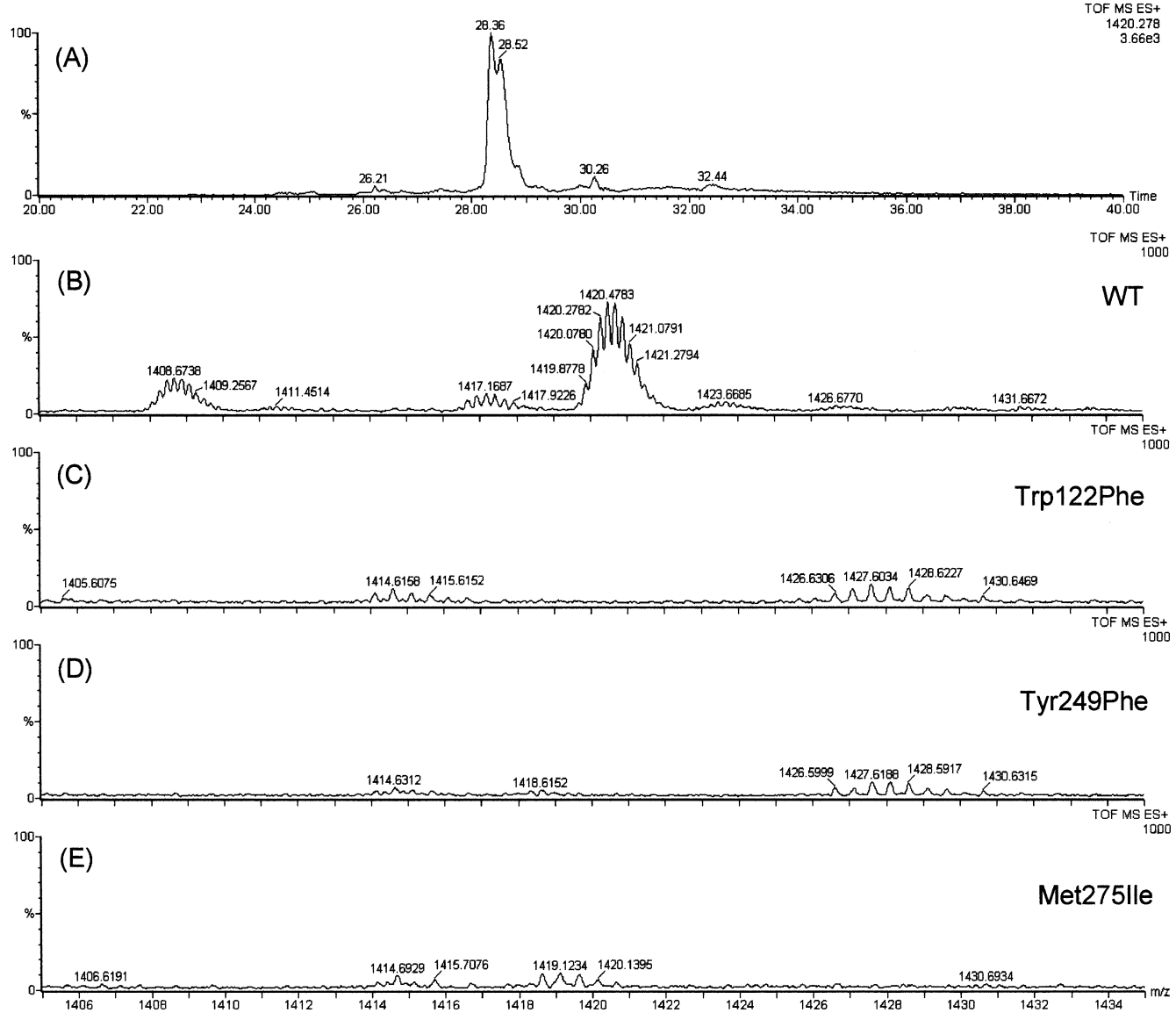

Fig. 3. Mass spectrometric analysis of peptides obtained by tryptic digestion of wild-type *Synechocystis* KatG and the variants Trp122Phe, Tyr249Phe and Met275Ile. A: HPLC elution profile of the ion at *m/z* 1420.28 Da. Sample: tryptic digest of wild-type protein. B: Mass spectrum of the tryptic digest of wild-type KatG in the *m/z* range 1405–1435 Da. Only wild-type KatG shows the isotopic pattern of $[M5H]^{5+}$ (i.e. ion cluster at monoisotopic *m/z* of 1419.88 Da) deriving from $[MH]^+ = 7096.34$ Da, which corresponds to the mass of the covalently linked peptides A, B and C. C–E: Peptides in the mass/charge range of 1405–1435 Da from the same section of the HPLC elution profile are shown for the tryptic digest of the variants Trp122Phe (C), Tyr249Phe (D) and Met275Ile (E). Spectra were normalized to the same intensity (1000 counts).

that bond formation is a redox-linked process involving the heme iron, as was recently demonstrated for a novel tryptophan-tyrosine cross-link in a mutant of CCP [16], this observation would be consistent with the fact that (i) KatG is known to easily lose heme, and (ii) normally not all active sites are totally occupied by the prosthetic group [14]. However, the proof of this hypothesis needs the incorporation of a redox-inactive Zn-protoporphyrin into the recombinant protein produced in *E. coli* under anaerobic conditions.

In the digest of the Tyr249Phe more than 90% of the peptides shown in Fig. 2 could be identified and a more or less identical peptide pattern was obtained with Trp122Phe. This ruled out a possible covalent link between Tyr249 and Met275 in the Trp122Phe variant. A peptide derived from covalently linked peptides B and C (theoretical monoisotopic masses: $[MH]^+ = 5935.22$ Da; $[M2H]^{2+} = 2968.12$ Da; $[M3H]^{3+} = 1979.08$ Da; $[M4H]^{4+} = 1484.56$ Da; $[M5H]^{5+} = 1187.85$ Da; $[M6H]^{6+} = 990.04$ Da) was definitely not detected.

In the digest of the Met275Ile variant and wild-type KatG a novel peptide was identified deriving from covalently linked peptides A and B with a theoretical monoisotopic mass of the singly charged species, $[MH]^+$, of 5065.40 Da, and of higher charged species of 2533.21 Da, 1689.14 Da, 1267.11 Da, 1013.89 Da and 845.07 Da, respectively. The inset in Fig. 4 depicts the presence of a peptide with exactly the same mass in a small fraction of wild-type KatG. The typical isotopic pattern of the $[M5H]^{5+}$ species (monoisotopic mass of 1013.89 Da) can be seen. In the Met275Ile variant, this peptide is prominent, whereas in Trp122Phe and Tyr249Phe variant it is completely missing. However, in Met275Ile the monoisotopic mass of the corresponding singly charged species is 5063.40 and thus 2 Da smaller than in wild-type KatG. This difference was clearly seen by comparing all detected multiple charged species of this peptide (not shown). Fig. 4 shows the isotopic pattern of the $[M5H]^{5+}$ species of Met275Ile variant, which is – compared to wild-type KatG – 0.4 Da shifted to lower masses. How this discrepancy is related to differences in the cross-linking between Trp122 and Tyr249 in wild-type KatG and Met275Ile is not clear at the moment.

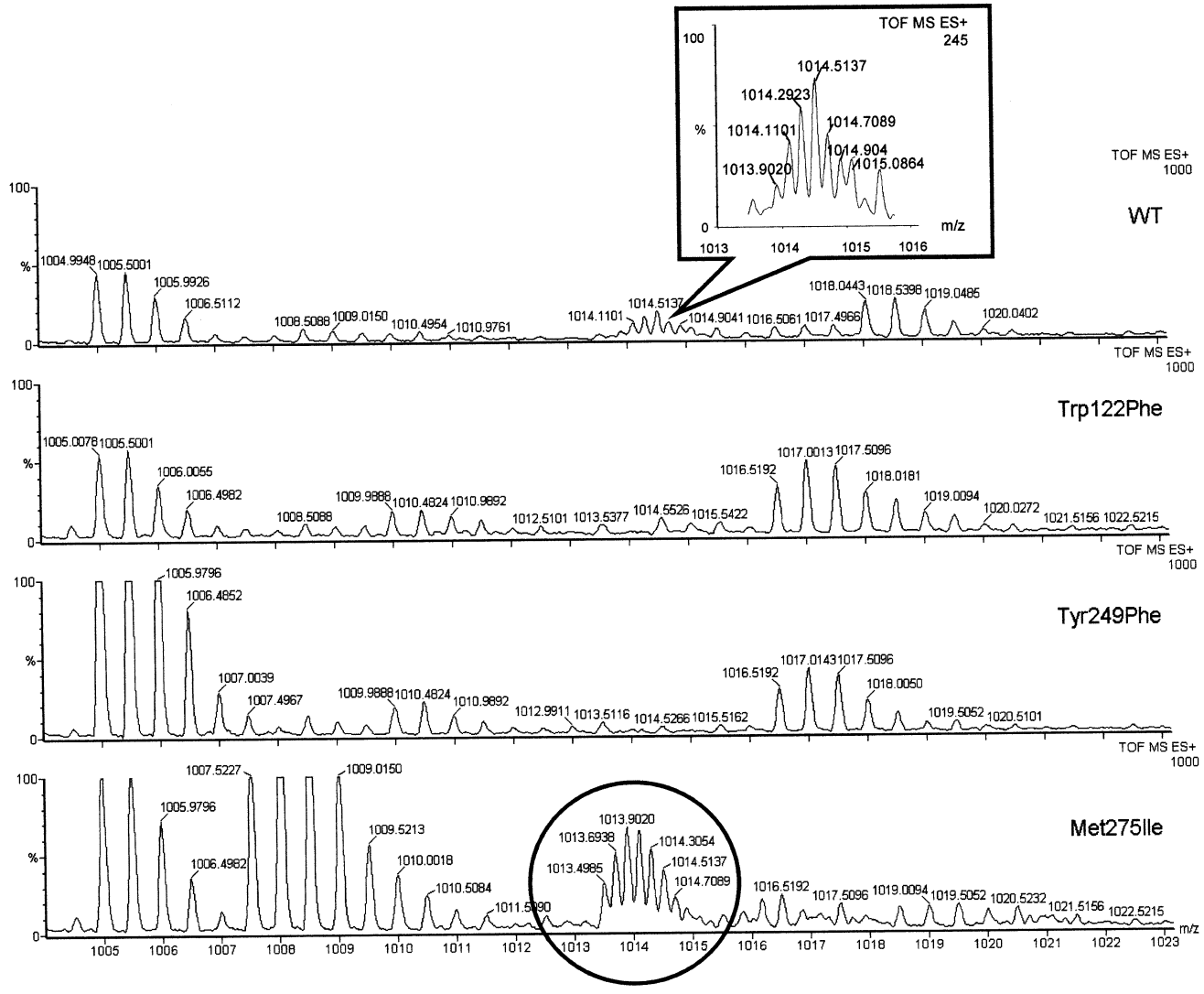

Fig. 4. Mass spectrometric analysis of peptides obtained by tryptic digestion of wild-type *Synechocystis* KatG and the variants Trp122Phe, Tyr249Phe and Met275Ile. Peptides in the mass/charge range of 1004–1023 Da are shown. Spectra were normalized to the same intensity (1000 counts). The isotopic pattern of $[M5H]^{5+}$ (i.e. ion cluster at monoisotopic *m/z* of 1013.5 Da) deriving from $[MH]^+ = 5065.40$ Da, which corresponds to the mass of the covalently linked peptides A and B, is found in Met275Ile and in some fraction of wild-type KatG (inset).

4. Conclusion

The present paper provides supporting evidence for the existence of novel covalent bonds in KatGs as originally deduced from the electron density maps of *H. marismortui* and *B. pseudomallei* KatGs. It is shown that in *Synechocystis* KatG the distal side residues Trp122, Tyr249 and Met275 are covalently linked in *Synechocystis* KatG and that bond formation depends on the presence of the distal side tryptophan. The results suggest that the covalent bond between Trp122 and Tyr249 may form before the bond between Tyr249 and Met275. Though not proven so far, it is reasonable to assume that bond formation is a redox-linked process, and that at the beginning of these coupling reactions Trp122 and concomitantly or subsequently Tyr249 are oxidized in the enzyme cycle by the redox intermediate compound I, which is two oxidizing equivalents above the ferric protein and forms readily in the presence of hydrogen peroxide.

KatG offers an interesting model system for studying the role of this crosslinking chemistry in connection with the biological activity of an enzyme. In the present case this is evident by the fact that both Trp122 and Tyr249 in *Synechocystis* KatG are essential in the two-electron reduction process of the redox intermediate compound I but not in the one-electron reduction process. Apparently the intact adduct is essential in the catalase activity of KatG, but its definite role is not clear at the moment. It could contribute to a greater rigidity of the peroxidase, since Tyr249 anchors a KatG-specific loop to the molecular surface by forming the covalent bonds with Trp122 and Met275. Additional stabilization is achieved by a hydrogen bond of an arginine (Arg439) with the hydroxyl-oxygen of Tyr249 and the amide nitrogen atom of Met275. How this cross-linking offers a suitable binding and redox environment for a KatG to catalyze both H_2O_2 reduction and subsequent oxidation is a fascinating question for future research.

Acknowledgements: This work was supported by the Austrian Science Funds (FWF project P15417). We thank Friedrich Altmann to use the Q-TOF Global, a donation of the Austrian Council for Research and Technology Development, and Dubravko Rendic for carefully reading the manuscript and fruitful discussion.

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