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S-Nitrosoglutathione covalently modifies cysteine residues of human carbonyl reductase 1 and affects its activity

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ABSTRACT

Carbonyl reductase 1 (CBR1 or SDR21C1) is a ubiquitously-expressed, cytosolic, monomeric, and NADPH-dependent enzyme. CBR1 participates in apoptosis, carcinogenesis and drug resistance, and has a protective role in oxidative stress, cancer and neurodegeneration. *S*-Nitrosoglutathione (GSNO) represents the newest addition to its diverse substrate spectrum, which includes a wide range of xenobiotics and endogenous substances. GSNO has also been shown to covalently modify and inhibit CBR1. The aim of the present study was to quantify and characterize the resulting modifications. Of five candidate cysteines for modification by 2 mM GSNO (positions 26, 122, 150, 226, 227), the last four were analyzed using MALDI-TOF/TOF mass spectrometry and then quantified using the Selected Reaction Monitoring Approach on hyphenated HPLC with a triple quadrupole mass spectrometer. The analysis confirmed GSNO concentration-dependent *S*-glutathionylation of cysteines at positions 122, 150, 226, 227 which was 2–700 times higher compared to wild-type CBR1 (WT-CBR1). Moreover, a disulfide bond between neighboring Cys-226 and Cys-227 was detected. We suggest a role of these two cysteines as a redox-sensitive cysteine pair. The catalytic properties of wild-type and enzyme modified with 2 mM GSNO were also investigated by steady state kinetic experiments with various substrates. GSNO treatment of CBR1 resulted in a 2–5-fold decrease in k_{cat} with menadione, 4-benzoylpyridine, 2,3-hexanedione, daunorubicin and 1,4-naphthoquinone. In contrast, the same treatment increased k_{cat} for substrates containing a 1,2-diketo group in a ring structure (1,2-naphthoquinone, 9,10-phenanthrenequinone, isatin). Except for 9,10-phenanthrenequinone, all changes in k_{cat} were at least in part compensated for by a similar change in K_m, overall yielding no drastic changes in catalytic efficiency. The findings indicate that GSNO-induced covalent modification of cysteine residues affects the kinetic mechanism of CBR1 both in terms of substrate binding and turnover rate, probably by covalent modification of Cys-226 and/or Cys-227.

1. Introduction

Human CBR1 (carbonyl reductase 1, EC 1.1.1.184), or according to the new nomenclature SDR21C1 [1], is a ubiquitously-expressed, monomeric, cytosolic enzyme belonging to the short-chain dehydrogenase/reductase (SDR) superfamily [2]. CBR1 catalyzes the NADPH-dependent reduction of a variety of structurally diverse substrates, mostly carbonyls. The best known xenobiotic substrates include quinones (such as the vitamin K precursor menadione and 9,10-phenanthrenequinone, both often used as model substrates), anthracyclines, ketoaldehydes, aromatic aldehydes, and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), the carcinogenic nitrosamine of tobacco smoke [3,4]. CBR1 also reduces a range of endogenous substances including prostaglandins,

Abbreviations: CBR, carbonyl reductase; SDR, short-chain dehydrogenase/reductase; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; GSNO, *S*-nitrosoglutathione; GSNOR, GSNO reductase; GSH, glutathione; DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside; IAM, iodoacetamide; DMSO, dimethyl sulfoxide; ACN, acetonitrile; TFA, trifluoroacetic acid; MALDI-TOF/TOF, matrix assisted laser desorption ionization - time of flight/time of flight; MS, mass spectrometry; LC-SRM, liquid chromatography-selected reaction monitoring.

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steroids and other aliphatic aldehydes and ketones along with the endogenous indole isatin and *S*-nitrosothiol GSNO (*S*-nitrosoglutathione) [5,6]. Moreover, CBR1 plays a protective role in oxidative stress, tumor metastasis, neurodegeneration and apoptosis [7,8]. The underlying molecular mechanisms are poorly understood. One study has shown that CBR1 inactivates the lipid aldehyde 4-oxo-2-nonenal [9], a lipid peroxidation product formed during oxidative stress. Hence CBR1 might protect from oxidative stress by eliminating reactive oxygen species [10].

Before the discovery of GSNO as CBR1 substrate in 2008 [6], CBR1 had only been known to convert carbonyl groups to alcohols. GSNO reduction thus represents a new mechanism, where an NO bond is reduced. The kinetic parameters of CBR1 and GSNO are comparable to those of model CBR1 substrates as isatin or menadione [6]. CBR1 seems to be specific for GSNO, because *S*-nitrosocysteine is not reduced by CBR1. Moreover, CBR1-dependent GSNO reduction has been described in A549 lung adenocarcinoma cell lysates. This indicates that CBR1 also acts as GSNO reductase *in vivo*.

GSNO is a key endogenous *S*-nitrosothiol, which serves as a reservoir and donor of NO in organisms [11–13]. In humans, GSNO is physiologically present up to micromolar levels [11], plays a role in apoptosis [14], has a neuroprotective role [15], inhibits platelet activation [16], and has a strong bronchodilatation effect in asthma [17]. Until 2008, only GSNOR (GSNO-reductase, also termed alcohol dehydrogenase 3) was known to reduce GSNO, resulting in no NO release but in NO signaling termination [18].

The first indication that CBR1 can contain a glutathione binding site had been suggested already by Wermuth in 1981. He had found that the glutathione adduct of prostaglandin PGA1 is reduced by CBR1 while free PGA is not [2]. Another fact supporting this hypothesis was that CBR1 from human placenta was inhibited by oxidized GSH adducts [20]. Later, the same research group suggested that a cysteine residue could play an important role in glutathione binding and enzyme activity [21]. In 2008, the X-ray crystal structure of the GSH-binding site was found in close structural proximity to the active site of CBR1 [7].

Recent studies have revealed that GSNO causes covalent modification of CBR1, which results in loss of enzyme activity at a concentration around 100 μM GSNO [19]. The fact that treatment with dithiothreitol (DTT) restored the enzyme activity, while incubation with ascorbic acid did not, indicated that *S*-glutathionylation was the mechanism responsible for the enzyme inhibition. Further indirect evidence pointed towards Cys-227 as the glutathionylated residue and, in agreement with a previous study that has provided strong support for Cys-227 as the reactive residue [20], Cys-227 was hypothesized to be subject to glutathionylation [19].

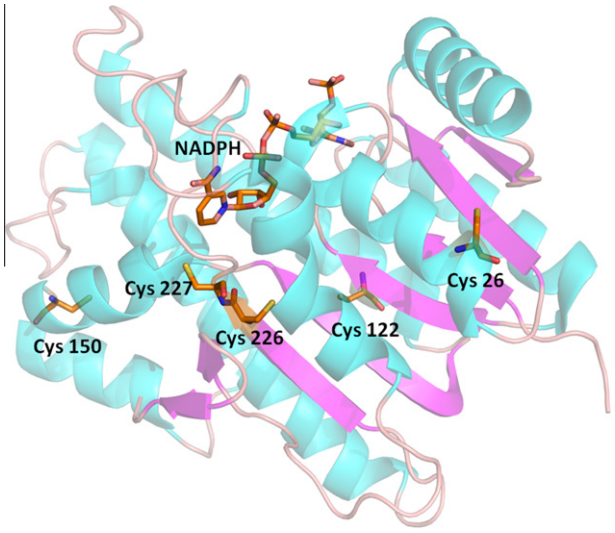

Fig. 1. The crystal structure of CBR1 (1WMA) created in PyMOL showing all cysteines in the sequence. Only Cys-227 points towards the enzyme catalytic center.

In the present study, we sought to identify the cysteine residues in CBR1 that are modified by GSNO and to characterize and quantify the modifications in a mass spectrometry approach. CBR1 contains 5 cysteine residues in its sequence (positions 26, 122, 150, 226, 227) (Fig. 1), of which mutation of two (Cys-226, Cys-227) is known to affect activities for substrates like menadione, 4-benzoylpyridine and daunorubicin [20].

To detect these modifications, we used MALDI-TOF/TOF mass spectrometry followed by a selected reaction monitoring (SRM) approach on hyphenated liquid chromatography with triple quadrupole mass spectrometer. Next, we investigated the influence of these modifications upon other substrates of CBR1 by spectrophotometric and HPLC kinetic studies.

2. Materials and methods

2.1. Cloning, protein overexpression and GSNO preparation

Cloning, overexpression and purification of CBR1 were done as described in [19] with the exceptions that PCR primers, including restriction sites, were synthesized by Generi Biotech (Hradec Kralove, Czech Republic), and the primer sequences were as follows: forward primer 5′-GGA TTC CAT ATG TCG TCC GGC ATC CA-3′, reverse primer 5′-CGC CTC GAG TCA CCA CTG TTC AAC TC-3′. Next *Nde*I and *Xho*I restriction enzymes and commercially available pET-28b(+) vector (Novagen, Darmstadt, Germany) were used. The correct sequence insertion was verified by sequencing at Generi Biotech. GSNO was synthesized according to Hart [19,21] and the purity was checked as described by Staab et al. [19] before each experiment.

2.2. Initial MALDI-TOF/TOF analysis of CBR1

2.2.1. CBR1 treatment with GSNO and trypsin digestion

CBR1 (10 μg) in 0.1 M potassium phosphate buffer (pH 7.4) and 50% glycerol was incubated either in water (control CBR1), in 2 mM GSNO, or in 200 mM GSNO at room temperature for 2 h. Thiol groups were blocked by addition of 60 mM iodoacetamide (Sigma–Aldrich, St. Louis, MO) immediately after incubation with GSNO. Blocking was performed at room temperature for 60 min in darkness. Low molecular compounds were removed and buffer was exchanged using 0.5 ml Zeba Spin Desalting Columns (7K MWCO; Thermo Scientific, Milford, MA). CBR1 in 120 μl of 50 mM ammonium bicarbonate was digested using sequencing grade trypsin (Promega, Madison, WI, USA). After overnight digestion in a thermomixer at 37 °C, the resulting peptides were desalted on 4 mm/1 ml Empore C18-SD solid phase extraction (SPE) cartridges (Sigma–Aldrich).

2.2.2. MALDI-TOF/TOF analysis and spectra evaluation

Peptides eluted from SPE columns using 80% ACN, 0.1% TFA were dried *in vacuo* and subsequently redissolved in 25 μl of 5% ACN, 0.1% TFA. Five microliters from each sample were then mixed with 5 mg/ml of α-cyano 4-hydroxycinnamic acid solution (LaserBio Labs, Sophia-Antipolis Cedex, France) in 50% ACN, 0.1% TFA in a 1:1 ratio (v/v) and spotted onto a MALDI sample plate (AB Sciex, Foster City, CA). The mass spectra and tandem mass spectra were recorded on an ABI 4800 MALDI-TOF/TOF mass analyzer (AB Sciex) and evaluated in a Peak Explorer (AB Sciex) and using mMass software [22]. Tryptic peptides resulting from CBR1 treated with 200 mM GSNO were additionally resolved on a nano-HPLC system

and spotted in time-resolved fractions onto a MALDI sample plate using a Probot fraction collector (Dionex, Sunnyvale, CA) as described earlier [23]. MS/MS spectra were evaluated using the MASCOT search engine (Matrix Science, London, UK).

2.3. Quantitative LC-SRM analysis of CBR1 modifications

2.3.1. CBR1 treatment with GSNO and trypsin digestion

CBR1 (100 μg) in 0.1 M potassium phosphate buffer (pH 7.4) and 50% glycerol was incubated in triplicate with 10 μM, 100 μM or 2 mM GSNO and H_2O_2 as well as with water (control CBR1). The incubation conditions and sample preparation were the same as described in Section 2.2.1. After buffer exchange, the protein concentration was assessed using the Bradford method. Ten micrograms from each sample was digested with sequencing grade trypsin overnight in a thermomixer at 37 °C. Subsequently, the samples were acidified using TFA and heated for 5 min at 95 °C to inactivate trypsin, dried *in vacuo*, and stored at −80 °C until analysis.

2.3.2. LC-SRM assay development

The SRM assays were developed, managed and evaluated using Skyline software (MacCoss Lab, University of Washington, Seattle, WA) [24]. The recombinant CBR1 sequence (Fig. 2) was imported into the application and digested with trypsin *in silico* to obtain individual tryptic peptides. The *S*-glutathionylation and carbamidomethylation modifications as well as a dehydro-modification on cysteinyl residues 226 and 227 (disulfide bond between neighboring cysteine residues) were set as variable cysteine modifications. In addition to the cysteinyl peptides, 9 non-cysteinyl peptides were also selected for purposes of sample processing and assay quality control.

For all peptides, doubly- and triply-charged precursors and singly-charged y and b fragment ions were included in the initial list, which was subsequently used as a starting point to identify the best responding transitions. In addition, quadruply- and quintuply-charged precursors and doubly- and triply-charged fragments were added for peptides carrying glutathionylated cysteine residue.

Transitions for carbamidomethylated peptides and for non-cysteinyl peptides were optimized using CBR1 sample incubated with water and blocked with 60 mM IAM, whereas transitions for peptides carrying either a glutathionylated cysteine residue alone or in combination with carbamidomethylation as well as transitions for the dehydro-modified peptide were optimized using CBR1 sample incubated with 200 mM GSNO followed by 60 mM IAM.

From the initial transition list, the three most intensive transitions were selected per precursor for use in the final assay. For each but one precursor, the most intensive transition with fragment ion mass higher than precursor mass was used as a quantifier transition. The remaining two qualifier transitions were used to confirm the identity of the compound of interest.

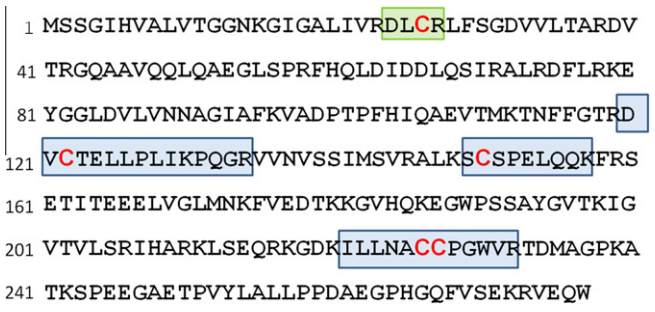

Fig. 2. Sequence of CBR1. Within frames are shown analyzed peptides (DLCR, DVCTELLPLIKPQGR, SCSPELQQK, ILLNACCPGWVR) after digestion with trypsin. The peptide DLCR is too short for MALDI-TOF/TOF and LC-SRM analysis.

2.3.3. LC-SRM analysis

The LC-SRM analyses were performed on an Agilent 1260 LC system coupled to an Agilent 6490 Triple Quadrupole mass spectrometer (both from Agilent, Palo Alto, CA), controlled by MassHunter acquisition software (version B.04.01) (Agilent).

Dry peptides were reconstituted in 5% ACN, 0.1% formic acid and injected onto a reversed phase column (Poroshell 120 SB-C18, 100 × 2.1 mm I.D., 2.7 μm core–shell particles; Agilent) which was maintained at 40 °C. Peptides were separated by a linear gradient from 5% to 40% ACN, 0.1% formic acid over 5 min at a 300 μl/min flow rate and electrosprayed using a JetStream ion source into the mass spectrometer. The acquisition method used the following parameters: drying gas flow of 15 l/min at 200 °C, nebulizing gas flow at 30 psi, sheath gas flow of 11 l/min at 250 °C, 4000 V capillary voltage, 300 V nozzle voltage, 380 V fragmentor voltage, 4 V cell accelerator voltage, and MS operating pressure of 5×10^{-5} - Torr. All transitions were acquired in positive ion mode with a dwell time of 10 ms and with both Q1 and Q3 set to unit resolution (0.7 FWHM).

For assay development experiments, approximately 1 μg of the digest was injected onto the column, whereas for the final quantification experiments, 300 ng from each sample was analyzed. All analyses for the final quantification experiments were performed in three technical replicates. All acquired data were imported into the Skyline application and evaluated. All integrated peaks were inspected to confirm proper automatic peak selection and accurate integration.

2.4. Incubation of CBR1 with GSNO and kinetics

CBR1 was treated with 2 mM GSNO for 2 h at room temperature in darkness. The enzyme was then rebuffered in 0.1 M potassium phosphate buffer (pH 7.4) using PD-10 desalting columns (GE Healthcare, Piscataway, NJ), and the concentration was determined by Bradford method with BSA as a standard.

2.4.1. Spectrophotometric assays

Catalytic properties of WT-CBR1 and CBR1 "modified" were measured spectrophotometrically at 340 nm (Cary 100 scan photometer, Varian, CA). The reaction temperature was held constant at 25 °C, and the total volume of reaction mixture was 1 ml. The reaction conditions were 0.1 mM NADPH, 1 mM DTPA (diethylene triamine pentaacetic acid), 0.1 M potassium phosphate buffer (pH 7.4), enzyme (WT-CBR1 or CBR1 "modified") dissolved in 0.1 M potassium phosphate buffer (pH 7.4) and the substrate. Daunorubicin (5–500 μM) and 2,3-hexanedione (5–1000 μM) were dissolved in water. Menadione (5–200 μM), 9,10-phenanthrenequinone (2.5–75 μM), 1,2-naphthoquinone (10–750 μM), 1,4-naphthoquinone (5–300 μM), isatin (5–200 μM) and 4-benzoylpiridine (10–1000 μM) were dissolved in DMSO. For those substrates, the final concentration of DMSO in the cuvette was 10% (v/v). A reference cuvette was composed of the reaction solution without the enzyme.

The activity was measured using at least seven different concentrations of each substrate, with each measurement being done in triplicate. The reaction velocity was calculated in mol/min per mg, corresponding to the amount of NADPH consumed in the reaction by 1 mg of enzyme per 1 min, using the NADPH extinction coefficient 6,220 M^{-1} cm^{-1}. The results were processed using GraphPad Prism software.

2.4.2. HPLC assays

Kinetic properties with oracin and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) were measured using HPLC based on production of their reduced metabolites NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) and DHO (11-dihydrooracin). The enzyme samples were first preincubated for 5 min at 37 °C in an NADPH regeneration system (0.8 mM $NADP^+$, 6 mM glucose-6-phosphate, 0.35 units of glucose-6-phosphate dehydrogenase, 3 mM $MgCl_2$) in a 10 mM sodium phosphate buffer (pH 7.4). The reaction was started by adding the substrate (oracin 0.05–1.5 mM, NNK 1–20 mM). After 30 min of incubation at 37 °C, the reaction was stopped by adding 40 μl of 25% NH_3 and the samples were kept on ice. The reduced metabolites were extracted three times with 300 μl of ethyl acetate; the upper layers were combined and the organic solvent was evaporated under vacuum. The samples were then dissolved in 200 μl mobile phase and used for HPLC analysis. For both substrates, the LiChroCART® 250–4 LiChrospher® 100 RP-18 (5 μm) column (Merck Millipore, Darmstadt, Germany) was used. The composition of the mobile phase was kept constant during the analysis. For NNK and NNAL determination, the mobile phase consisted of 10 mM phosphate buffer (pH 7.4)/ACN (82:18), and the peaks were monitored at 230 nm using a UV detector. The flow rate was 1 ml/min. The composition of the mobile phase used for oracin and DHO determination consisted of 10 mM hexanesulfonic acid and 0.1 M triethylamine adjusted to pH 3.27 with H_3PO_4/ACN (75:25). The peaks were monitored using a fluorescence detector at 340 nm excitation and 418 nm emission wavelengths. The flow rate was 1.5 ml/min.

All samples were measured in triplicate. Concentrations of NNAL and DHO were calculated with reference to corresponding calibration curves. Specific activities were expressed as μmol of NNAL or DHO/min per mg of protein. Results were analyzed using GraphPad Prism software.

3. Results and discussion

3.1. MALDI-TOF/TOF analysis of CBR1 protein

Tryptic digestion of CBR1 produces 4 cysteine-containing peptides, 3 carrying one cysteinyl residue each (positions 26, 122, and 150) and 1 peptide with two cysteines (position 226 and 227) (Fig. 2).

To verify the earlier hypothesis regarding modification of cysteine residues 226 and 227 in CBR1 by *S*-glutathionylation upon incubation with GSNO [19], we performed MALDI-TOF/TOF MS and MS/MS analyses of tryptic peptides resulting from CBR1 protein treated with GSNO. *In silico* digestion of CBR1 showed that cysteine residues 226 and 227 comprise part of the tryptic peptide ILLNACCPGWVR with m/z = 1344.7 (cysteine residues in reduced form) or m/z = 1458.7 (both cysteine residues carbamidomethylated with IAM). A gain of two glutathione residues would result in a peak at m/z = 1954.8.

We observed no substantial difference in MS spectra when comparing control CBR1 to CBR1 treated with 2 mM GSNO (Fig. 1, supplementary data). We found a high intensity peak at m/z = 1458.7 in both spectra that was identified based on MS/MS analysis and MASCOT search as the ILLNACCPGWVR peptide with both cysteine residues carbamidomethylated.

We furthermore observed a peak at m/z = 1342.7 in MALDI-TOF/TOF MS spectra, a value that agrees with an ILLNACCPGWVR peptide where the two neighboring cysteine residues form a disulfide bond. This was confirmed by MALDI-TOF/TOF MS/MS analysis and subsequent MASCOT search with dehydro-cysteine modification enabled. Intensity of the peak at m/z = 1342.7 was ~10-fold higher in CBR1 treated with 2 mM GSNO than in the control sample. Although MALDI-TOF/TOF MS analysis is not regarded as quantitative, we hypothesized that GSNO might promote formation of a disulfide bond between Cys-226 and Cys-227. While such an effect of GSNO was not expected, it is nevertheless still in line with previous experiments with DTT that restored CBR1 activity [19] and with the theory, that as soon as the mixed-disulfide bond is formed at one cysteine, it can undergo a nucleophilic attack by the neighboring cysteine. Since IAM was added to the sample right after GSNO incubation, we excluded the contribution of artificial oxidation of cysteine residues during sample preparation for MALDI-TOF/TOF analysis. Nevertheless, we cannot eliminate the possibility, that in small amount the disulfide bond is formed during the enzyme preparation.

We detected no peak at m/z = 1954.8 that would represent the ILLNACCPGWVR peptide with both cysteine residues modified by *S*-glutathionylation among those peptides resulting from CBR1 treated with 2 mM GSNO. This fact does not necessarily mean that such modification is not probable. The absence of such peak might rather be associated with the most likely low abundance or low ionization efficiency of the ILLNACCPGWVR peptide with such modifications as compared to its unmodified counterpart.

For reasons given above, we incubated CBR1 protein with extremely high concentration of GSNO (200 mM, a concentration without physiological relevance) to check if anticipated higher abundance of the ILLNACCPGWVR peptide modified by glutathione might overcome the issues associated with its detection in MALDI-TOF/TOF MS spectra. Overlay of MALDI-TOF/TOF MS spectra from control CBR1, CBR1 treated with 2 mM GSNO, and CBR1 treated with 200 mM GSNO showed a specific peak at m/z = 1954.8 in the latter sample (Fig. 3). Notably, the correlation of peak intensity at m/z = 1342.7 with increasing concentration of GSNO supports our initial hypothesis. To restore the reduced state of the ILLNACCPGWVR peptide, we treated the sample with tris(2-carboxyethyl) phosphine hydrochloride (TCEP) which effectively reduced both the disulfide bond as well as the glutathionylated cysteine residues.

The ILLNACCPGWVR peptide carries two cysteine residues, meaning that in addition to both residues being glutathionylated, GSNO incubation could also result in an ILLNACCPGWVR peptide having one glutathionylated and one carbamidomethylated cysteine. Such a peptide would correspond to m/z = 1706.8. We observed no such peak in the MALDI-TOF/TOF MS spectrum. As mentioned above, however, abundance might not be the only reason why a fragment at m/z = 1706.8 remained undetected. Thus, we performed fast nano-HPLC fractionation of the peptides directly on a MALDI plate using Probot fraction collector. Using Peak Explorer software, we extracted m/z = 1706.8 from the LC-MALDI chromatogram. Subsequent MALDI-TOF/TOF MS/MS analysis and MASCOT search confirmed this peak to carry glutathione as well as a carbamidomethyl group.

Encouraged by successful detection of the partially modified ILLNACCPGWVR peptide, we searched LC-MALDI chromatograms for the remaining predicted tryptic peptides containing cysteine: DVCTELLPLIKPQGR, SCSPELQQK and DLCR. While the latter peptide is too short for MALDI-TOF/TOF analysis, peaks corresponding to both DVCTELLPLIKPQGR and SCSPELQQK were found and identified as being modified by *S*-glutathionylation.

3.2. LC-SRM assay development

MALDI-TOF/TOF MS is valuable for initial qualitative analyses, but, due to its non-quantitative nature, it cannot easily be used for relative assessment as to the abundance of various peptide modifications. Therefore, we adopted an LC-SRM MS method to quantify the effect of GSNO treatment on the cysteine modifica-

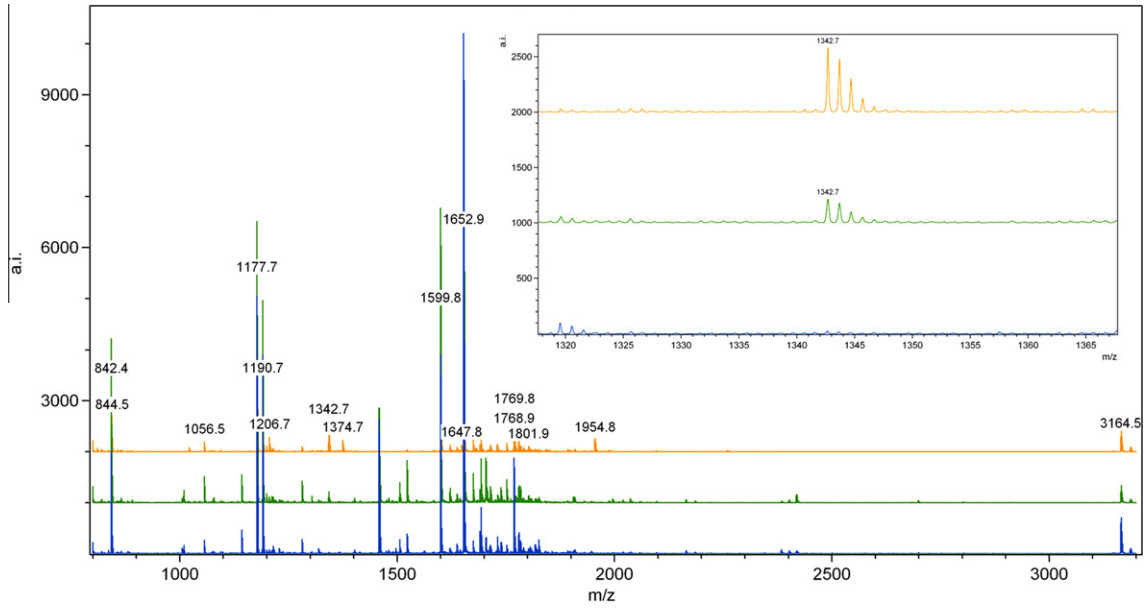

Fig. 3. MALDI-TOF/TOF mass spectrometry spectra of tryptic peptides resulting from CBR1 protein with a zoomed peak at *m/z* = 1342.7. Bottom spectrum is control CBR1, mid-spectrum was obtained from CBR1 protein treated with 2 mM GSNO, upper spectrum was obtained from CBR1 protein treated with 200 mM GSNO. Spectra are offset by 200 a.i. units.

tions that were observed in the MALDI-TOF/TOF MS and MS/MS analyses.

In order to quantify changes in CBR1 modification upon incubation with GSNO, we developed an LC-SRM assay targeting 4 out of the total 5 cysteines in the CBR1 sequence. In addition, we selected 9 non-cysteinyl peptides for use in sample processing and assay quality control (Table 1).

We successfully detected 3 tryptic peptides carrying 4 cysteine residues (DVCTELLPLIKPQGR, SCSPELQQK, ILLNACCPGWVR). Those peptides containing a single cysteine residue can be modified either by *S*-glutathionylation (adding 305 Da) or by carbamidomethylation (adding 57 Da). In addition, the cysteines in the ILLNACCPGWVR peptide were shown by MALDI-TOF/TOF analysis to be modified in a number of combinations: 1) –C[+57]C[+57]–; 2) –C[+57]C[+305]–; 3) –C[+305]C[+57]–; 4) -C[+305]C[+305]– and a variant with a disulfide bond between the neighboring peptides, 5) –C[−1]C[−1]–. While the analysis of variants 1, 4 and 5 is relatively straightforward, as individual forms differ in molecular weight and have a distinct retention on the stationary phase, the discrimination of variants 2 and 3 poses a challenge, since not only do both isoforms have an identical precursor mass and retention time, but also the masses of the majority of fragments are identical. In fact, only the y6 and/or b6 precursor fragments can distinguish between the two variants. All the aforementioned variants, including the discriminating fragments, were successfully detected and, along with the cysteinyl peptides, the developed LC-SRM assay also covers all 9 selected non-cysteinyl peptides.

3.3. LC-SRM analysis

Using the developed LC-SRM assay, we targeted 4 out of 5 cysteines in CBR1 in order to quantify changes in *S*-glutathionylation

Table 1
The developed LC-SRM assay.

Peptide sequence	Peptide MW	Precursor *m/z*	Precursor charge	Collision energy (V)	Quantifier *m/z*	Ion	Qualifier *m/z*	Ion	Qualifier *m/z*	Ion
LFSGDVVLTAR	1177.7	589.3	++	15	917.5	y9+	830.5	y8+	658.4	y6+
GQAAVQQLQAEGLSPR	1652.9	551.6	+++	11	729.4	y7+	529.3	y5+	658.4	y6+
FHQLDIDDLQSIR	1599.8	533.9	+++	10	641.3	b5+	503.3	y4+	754.4	b6+
EYGGLDVLVNNAGIAFK	1779.9	594.0	+++	12	834.4	y8+	734.3	b7+	720.4	y7+
VADPTPFHIQAEVTMK	1783.9	595.3	+++	12	806.4	y7+	379.2	y3+	478.3	y4+
TNFFGTR	842.4	421.7	++	6	627.3	y5+	480.3	y4+	333.2	y3+
VVNVSSIMSVR	1190.7	595.8	++	15	779.4	y7+	992.5	y9+	692.4	y6+
SETITEEELVGLMNK	1692.9	565.0	+++	11	661.4	y6+	562.3	y5+	919.4	b8+
EGWPSSAYGVTK	1281.6	641.3	++	17	909.5	y9+	812.4	y8+	725.4	y7+
DVC[+57]TELLPLIKPQGR	1739.0	580.3	+++	12	908.6	y8+	457.3	y4+	585.3	y5+
DVC[+305]TELLPLIKPQGR	1987.0	497.5	++++	9	454.8	y8++	567.9	y10++	511.3	y9++
SC[+57]SPELQQK	1076.5	538.8	++	12	829.4	y7+	742.4	y6+	516.3	y4+
SC[+305]SPELQQK	1324.6	442.2	+++	7	516.3	y4+	371.7	y6++	415.2	y7++
ILLNAC[+57]C[+57]PGWVR	1458.7	729.9	++	22	934.4	y7+	774.4	y6+	614.3	y5+
ILLNAC[+57]C[+305]PGWVR	1706.8	569.6	+++	11	1022.4	y6+	740.8	y10++	684.3	y9++
ILLNAC[+305]C[+57]PGWVR	1706.8	569.6	+++	11	774.4	y6+	740.8	y10++	684.3	y9++
ILLNAC[+305]C[+305]PGWVR	1954.8	489.5	++++	8	576.9	y10+++	614.3	y5+	539.2	y9+++
ILLNAC[-1]C[-1]PGWVR	1342.7	671.8	++	19	818.3	y7+	1003.4	y9+	889.4	y8+

The peptide sequence is shown including individual modifications (+57 for carbamidomethylation,+305 for *S*-glutathionylation, −1 for dehydro-modification), peptide molecular weight (MW), precursor mass-to-charge ratio (*m/z*), precursor charge and respective collision energies in volts. Moreover, both quantifier and qualifier precursor fragments are shown along with respective ion types and charges.

as a function of GSNO concentrations during incubation. CBR1 incubated with water was used as an untreated control. Moreover, we sought using our assay to verify the presence of a disulfide bridge between cysteine residues 226 and 227. In addition, we incubated CBR1 with H_2O_2 to see whether the disulfide bond would be formed as well and thus, if this formation can occur during oxidative stress when the redox-state conditions are altered. We were able to confirm our initial MALDI-TOF/TOF hypothesis, and using LC-SRM the presence of this disulfide bond was detected. Moreover, in accordance with the suggestion that increased levels of GSNO would promote the disulfide bond formation, we observed a positive correlation between the GSNO concentration used during incubation and the levels of the ILLNACCPGWVR peptide with dehydro-cysteine modification. The effect when GSNO causes disulfide bridge formation has been described, for example, for the thioredoxin system, where the reduced form of the redox active disulfide –Cys–Gly–Pro–Cys– was oxidized after addition 10 μM GSNO [25]. Due to the significantly higher sensitivity of the triple quadrupole instrument in the targeted analyses, we confidently detected the ILLNACCPGWVR peptide with a disulfide bridge not only at all examined concentrations of GSNO but even in the untreated control sample represented by WT-CBR1, which suggests the presence of the disulfide bond in a fraction of our recombinant protein sample. Our MALDI-TOF/TOF-based experiments confirmed the presence of an ILLNACCPGWVR variant with just one cysteine modified with glutathione and the other blocked by carbamidomethylation. We were able to confirm the presence of this peptide variant and, in addition, we successfully detected specific peptide fragments which, as described above, enable the discrimination of the two isoforms. We show that increasing concentration of GSNO during incubation positively correlates with increased levels of both -C[+57]C[+305]– and –C[+305]C[+57]– isoforms. Our results show that no form is preferred for *S*-glutathionylation, as the increase in the abundance is comparable for both isoforms with increasing concentration of GSNO (Table 2).

Although we detected the ILLNACCPGWVR peptide with both cysteine residues glutathionylated in our MALDI-TOF/TOF analyses, we were only able to observe this variant in CBR1 samples incubated with 200 mM GSNO. The LC-SRM analysis allowed us to detect levels of this particular ILLNACCPGWVR variant after incubation with a 100-fold lower GSNO level, but even LC-SRM failed to reliably detect this modification at lower concentrations of GSNO incubation. We speculate that either this variant arises only with increased levels of GSNO and is thus present at extremely low levels with lower GSNO concentrations or the two glutathione residues inhibit the ionization efficiency of the molecule and thus impair its detection at low concentrations. The LC-SRM chromatograms of the ILLNACCPGWVR peptide variants are shown in Fig. 2 in supplementary data. Interestingly, the variant of the ILLNACCPGWVR peptide with both cysteines blocked by carbamidomethylation can be observed at comparable levels in the control and in the samples incubated at 10 μM and 100 μM GSNO while its levels drop about threefold in the sample incubated with 2 mM GSNO. It is most likely that formation of the disulfide bridge between the neighboring cysteines and/or modification of the residues with *S*-glutathionylation underlies this drop. The results are summarized in Table 2, where the modified form is always compared to the standard, represented by WT-CBR1.

The incubation with 10 μM, 100 μM and 2 mM H_2O_2 also resulted in disulfide bond formation. The greatest increase of this formation was detected at 100 μM H_2O_2, followed by 2 mM H_2O_2 (Table 2). We explain this effect by the hypothesis that further oxidation of cysteines into sulfenic, sulfinic, and sulfonic acids at higher H_2O_2 concentrations might happen. During oxidative stress, the level of 100 μM H_2O_2 is easily reached *in vivo*. Thus, disulfide bond formation and the associated enzyme activity alteration are feasible.

While confirmation of the MALDI-TOF/TOF-based findings regarding the DVCTELLPLIKPQGR and SCSPELQQK variants carrying glutathionylated cysteines was not surprising, the fact that we detected these modifications even in the control sample certainly was. Improper sample handling and accidental contact of the CBR1 sample with GSNO in the control sample and carryover were prevented during the LC-SRM analysis.

3.4. Physiological relevance of the confirmed cysteine modifications

The formation of *S*-glutathionylated proteins (Fig. 4) is well described and often serves as a posttranslational regulatory mechanism. It is a consequence of oxidative stress, wherein it protects the protein thiol group from irreversible oxidation or plays a role in redox state regulation [26–28].

To date, no such effect has been described for CBR1. As the cysteine at position 227 had been described as the reactive residue involved in glutathione binding [19,20], we focused on modifications at this cysteine. We confirmed formation of the disulfide bond between adjacent cysteines 226 and 227. The disulfide bond formation between the two vicinal cysteines changes the 3D structure of the protein and forces the two vicinal cysteines into a distorted *trans* conformation [29]. It can be hypothesized, therefore, that the disulfide bond formation may change CBR1 conformation in a way that favors the binding of 1,2-diketo substrates into the active site. Generally, the disulfide bond formation between two vicinal cysteines is rare and has been described for only a small number of enzymes [29]. The function of disulfides is mostly conformational or the cysteines have a regulatory function as thiol-based redox-

Table 2
Relative extent of cysteine modifications after incubation with GSNO and H_2O_2.

Modification	Fold change compared to control/AUC					
Fragments of CBR1 after trypsin digestion	10 μM GSNO		100 μM GSNO		2 mM GSNO	
EYGGLDVLVNNAGIAFK	0.98	112500 ± 2200	0.98	112300 ± 2400	0.92	105600 ± 2500
DVC$^{[SSG]}$TELLPLIKPQGR	1.8	630 ± 76	47	16300 ± 4500	710	247500 ± 39100
SC$^{[SSG]}$SPELQQK	2.8	370 ± 82	61	7900 ± 270	530	68800 ± 3000
ILLNAC$^{[IAM]}$C$^{[IAM]}$PGWVR	0.85	94600 ± 7000	0.72	80600 ± 2000	0.22	24100 ± 2200
ILLNAC$^{[SSG]}$C$^{[SSG]}$PGWVR	Not detected		Not detected		Detected	8000 ± 600
ILLNAC$^{[SSG]}$C$^{[IAM]}$PGWVR	3.5	140 ± 29	52	2100 ± 110	170	6700 ± 510
ILLNAC$^{[IAM]}$C$^{[SSG]}$PGWVR	Detected	12 ± 4	Detected	83 ± 5	Detected	730 ± 9
ILLNAC$^{[Dehydro]}$C$^{[Dehydro]}$PGWVR (-S-S-)	0.90	11200 ± 670	1.8	22600 ± 1000	5.0	64500 ± 4800
	10 μM H_2O_2		100 μM H_2O_2		2 mM H_2O_2	
ILLNAC$^{[Dehydro]}$C$^{[Dehydro]}$PGWVR (-S-S-)	1.7	22900 ± 1000	4.9	66200 ± 2600	3.4	46200 ± 5200

The ratio of modification and the AUCs measured for one non-cysteinyl peptide and 4 analyzed cysteines in CBR1 after treatment with different concentrations of GSNO and H_2O_2. The ratio defines the fold increase in cysteine modification for "modified" CBR1 (treated with varying GSNO and H_2O_2 concentrations) compared to the standard, WT-CBR1. Where the modification was not found in WT-CBR1 is indicated as "not detected", and where quantification was not reliable is indicated as "detected".

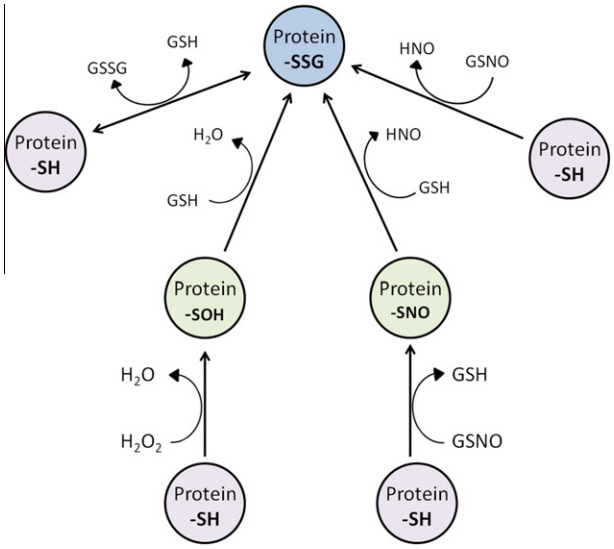

Fig. 4. GSNO structure and major mechanisms of protein *S*-glutathionylation inspired by Zaffagnini et al. [42]. The physiological levels of GSH in living cells are up to 10 mM [43] GSH is used as a cofactor for glutathione peroxidase to reduce toxic peroxides. This reaction results in the oxidized form of glutathione, GSSG, which is reduced back to GSH by glutathione reductase. In cells, the ratio of GSH:GSSG is about 100:1 [44].

switches [29–31]. According to Carugo et al. [29], the second possibility is more likely for bonds between vicinal cysteines. Those authors described several cases where the enzyme function is totally abolished when the naturally occurring disulfide bond is reduced [29,32,33]. The opposite effect was observed in another study, where formation of a disulfide bond inactivated the enzyme [34].

The unexpected detection of the disulfide bond in untreated WT-CBR1 and its increase after incubation with GSNO and H_2O_2 may indicate a role for these two cysteines in the regulation of enzyme activity and during oxidative stress. Hitherto, no redox-sensitive cysteine has been described for CBR1 and this hypothesis provides a new viewpoint on the regulation of CBR1 activity. On the other hand, despite indications as to the cysteine residue involved, we cannot be sure that disulfide bond formation is the only reason behind the changes in the activity and therefore more investigation is needed.

3.5. Kinetic assays and properties of WT-CBR1

In 2011, Staab et al. described inhibition of CBR1 with GSNO and suggested *S*-glutathionylation of Cys-227 as the reason behind the enzyme inhibition [19]. Based on the results obtained by Staab et al. together with the results from our MALDI-TOF/TOF and LC-SRM MS analysis, we decided to investigate the differences in catalytic properties of the WT-CBR1 and CBR1 "modified" forms. We incubated CBR1 with 2 mM GSNO for 2 h and then measured the catalytic properties with various substrates spectrophotometrically at 340 nm and by HPLC. Those results were always compared to the catalytic properties of WT-CBR1. The quinone substrates were represented by menadione, 9,10-phenanthrenequinone, 1,2- and 1,4-naphthoquinone. Other substrates used included isatin and the cytostatic drug daunorubicin. The ketone substrates were represented by 4-benzoylpyridine and 2,3-hexanedione, the latter of which was chosen as a ketone substrate without aromatic structure. The HPLC method was used for measurements with NNK and oracin.

To ensure we have an enzyme in active form, we measured the kinetics with GSNO and compared the data so obtained to the results published by Staab et al. [19]. These were almost identical (data not shown). For menadione, isatin and 9,10-phenanthrenequinone our recombinant WT-CBR1 revealed kinetics constants comparable to those in the literature, albeit with some differences. The K_m constants obtained in our experiments are similar to the ones published by other researchers [10,20,35–37]. For menadione, we obtained the K_m value 17.8 μM, for isatin 8 μM and for 9,10-phenanthrenequinone 31.7 μM. This indicates similar recognition of the substrates by the enzyme. In contrast, we noticed some differences in k_{cat}, which were usually lower than the published values (2–10-fold). Kinetics with 1,2- and 1,4-naphthoquinone were measured spectrophotometrically as described in the materials and methods in comparison to the modified method used by Pilka et al. [10]. As both substrates are unstable, they were dissolved in DMSO and the kinetics studies were performed immediately to avoid substrate decomposition. Kinetics constants for all substrates are summarized in Table 3.

The differences in catalytic properties can be explained by additional factors. It is known that CBR1 occurs in 3 multiple enzyme forms with differences in size and charge [2]. These differences are probably caused by various posttranslational modifications [36,38,39] and Staab et al. [19] had proposed that *S*-glutathionylat-

Table 3
Kinetics constants for WT-CBR1 and CBR1 "modified".

Enzyme form/substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($(\mu M.min)^{-1}$)
WT-CBR1 1,2-naphthoquinone	70 ± 12	208 ± 12	3.0
CBR1 "modified" 1,2-naphthoquinone	117 ± 12	402 ± 6	3.4
WT-CBR1 9,10-phenanthrenequinone	32 ± 5	214 ± 19	6.8
CBR1 "modified" 9,10-phenanthrenequinone	18 ± 2	586 ± 44	32
WT-CBR1 isatin	8.0 ± 1	10 ± 0.3	1.3
CBR1 "modified" isatin	17 ± 3	18 ± 1	1.1
WT-CBR1 4-benzoylpyridine	250 ± 38	72 ± 10	0.29
CBR1 "modified" 4-benzoylpyridine	16 ± 7	15 ± 1	0.94
WT-CBR1 1,4-naphthoquinone	7.3 ± 2	33 ± 1	4.4
CBR1 "modified" 1,4-naphthoquinone	32 ± 9	17 ± 2	0.52
WT-CBR1 menadione	18 ± 2	41 ± 0.4	2.3
CBR1 "modified" menadione	14 ± 3	19 ± 2	1.3
WT-CBR1 2,3-hexanedione	159 ± 20	308 ± 24	1.9
CBR1 "modified" 2,3-hexanedione	60 ± 5	91 ± 2	1.5
WT-CBR1 daunorubicin	67 ± 7	76 ± 1	1.1
CBR1 "modified" daunorubicin	43 ± 6	29 ± 1	0.67
WT-CBR1 oracin	96 ± 9	70 ± 1	0.73
CBR1 "modified" oracin	34 ± 5	23 ± 1	0.68
WT-CBR1 NNK	7840 ± 690	121 ± 2	0.015
CBR1 "modified" NNK	5850 ± 300	60 ± 3	0.010

CBR1 "modified" stands for CBR1 treated with 2 mM GSNO. Kinetics were measured spectrophotometrically at 340 nm and by HPLC (for oracin, NNK). The errors for k_{cat}/K_m were less than 20%.

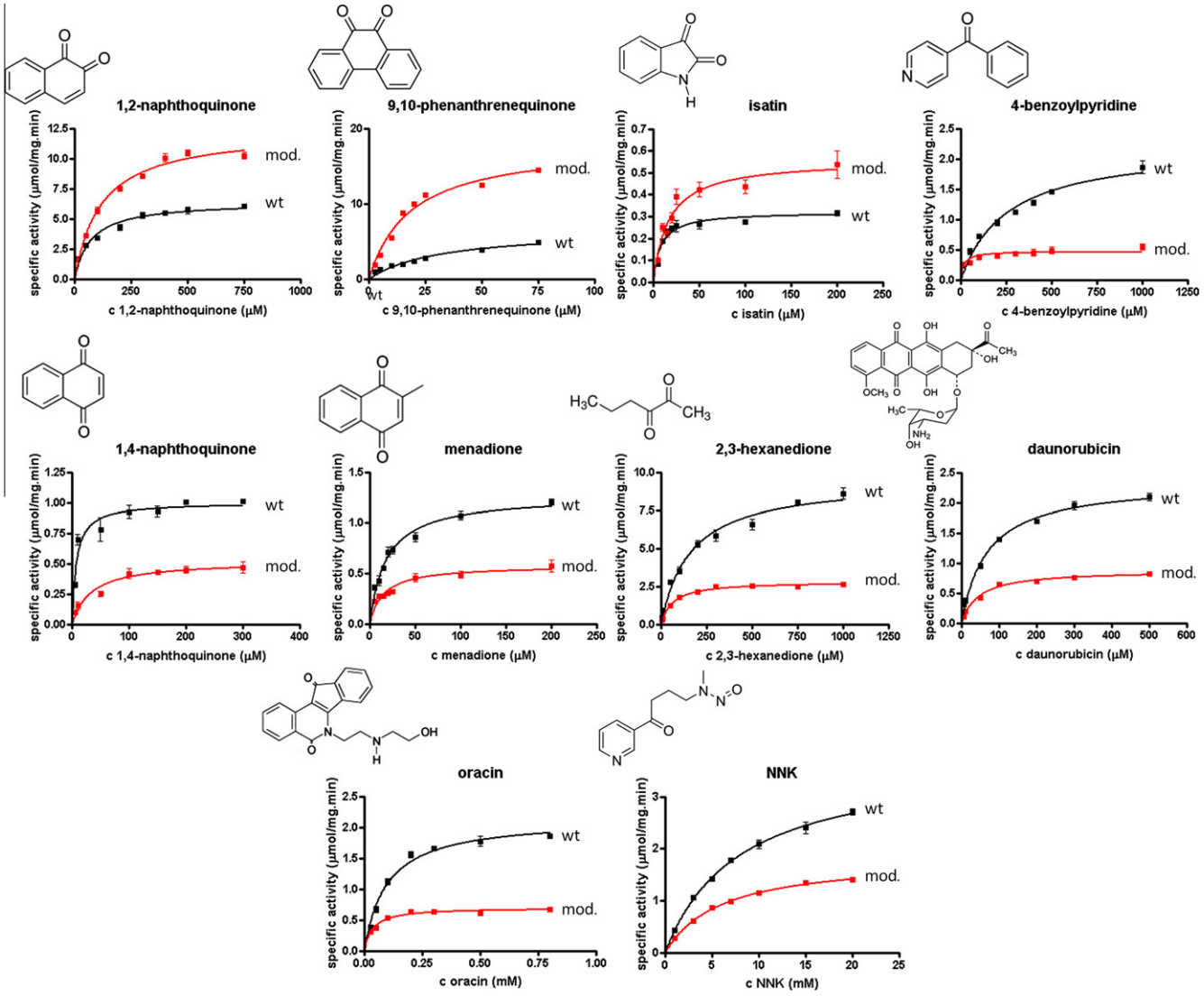

Fig. 5. Steady state kinetics measured spectrophotometrically and by HPLC. Mod.: kinetics for CBR1 "modified" (treated with 2 mM GSNO), wt: kinetics for WT-CBR1. The increased activity of the "modified" form can be seen in the cases of 1,2-naphthoquinone, 9,10-phenanthrenequinone and isatin. The lines represent the fit to the Michaelis–Menten equation.

ed CBR1 might correspond to one of the three known isoforms of CBR1 [2,19]. As we have confirmed the disulfide bond in WT-CBR1, we suggest that this modification can occur in one of those forms as well. Also, the properties of the recombinant enzyme and the enzyme purified from human tissue can differ. Next, the production of recombinant protein and the methods and chemicals used can alter the catalytic properties. Other aspects which can influence the enzyme activity is the buffer composition used for kinetics assays, the type and volume of dissolving agent for the substrate, the maximal substrate concentration used, and the substrate itself. In any case, it is not unusual to find different kinetic values published for a given substrate by different authors. As the same enzyme was used to study the differences in catalytic properties of the WT-CBR1 and CBR1 "modified," the results can be easily compared.

3.6. Comparison of catalytic properties of WT-CBR1and CBR1 "modified"

To explore whether the modification of CBR1 caused by GSNO has any effect on its activity, we performed kinetics measurements with both WT-CBR1 and CBR1 "modified" using various substrates. The activity decrease caused by GSNO-mediated *S*-glutathionylation was previously described for thioredoxin (Trx) [40], which led us to the idea that CBR1 might be influenced in a similar manner. The results we obtained were surprising, and especially so with regard to the substrate structure (Table 3, Fig. 5).

We found that, for most of the substrates investigated, k_{cat} values decreased 2–5-fold with modified CBR1. In contrast, with 1,2-naphthoquinone, 9,10-phenanthrenequinone and isatin, k_{cat} was increased by 2–3 times for the modified form. Interestingly, these three substrates all contain a 1,2-diketo group in their structures and are also metabolized by CBR3 (carbonyl reductase 3, SDR21C2), an enzyme which shares 72% identity with CBR1, including the cysteine pair at positions 226 and 227, but which displays a much more narrow substrate spectrum [10].

The observed changes in k_{cat} indicate that the modification affects the rate-limiting step of the reaction. The fact that CBR1 has different k_{cat} values for the various substrates suggests that release of $NADP^+$ is not a predominant rate-limiting step. Hence, the modification most probably affects hydride transfer or release of the hydroxyl product. Moreover, it does so differently for substrates

containing two vicinal carbonyl groups in a ring structure (like 1,2-naphthoquinone, 9,10-phenanthrenequinone, and isatin) and the other substrates, as k_{cat} is increased for the first substrate type, but decreased for all others.

The effect of the modification(s) on catalytic efficiency defined by k_{cat}/K_m, was not as obvious as that for k_{cat} values. For most substrates, a decrease in k_{cat} was totally or in part compensated for by a decrease in K_m. Hence it appears that the modification affects a kinetic step that is common to k_{cat} and k_{cat}/K_m, such as hydride transfer. Notable changes in catalytic efficiency were only observed for 4-benzoylpyridine, (k_{cat} decreased 5-fold, k_{cat}/K_m increased 3-fold), for 1,4-naphthoquinone (k_{cat} decreased 2-fold, k_{cat}/K_m decreased 8-fold), and for 9,10-phenanthrenequinone (k_{cat} increased 3-fold, k_{cat}/K_m increased 5-fold). Based on these results, we hypothesize that modification of Cys 226 and/or 227 by GSNO changes CBR1 activity by affecting the catalytic turnover rate or the nature of the rate-limiting step as well as by altering substrate binding. Another point to consider in the interpretation of the kinetic data is the fact that one would expect to observe negative cooperativity if the ''modified'' CBR1 were heterogeneous. However, the data fit the Michaelis–Menten model well (Fig. 5). This suggests that either most modified forms show similar kinetic behavior or, what is more likely, the activity of one differently modified form dominates, accounting for most of the changes in kinetic mechanism and masking the other less active or less frequent forms.

As described in the LC-SRM analysis, we have confirmed formation of GSH-mixed disulfides with all cysteines analyzed, along with the disulfide bridge between the cysteines at positions 226 and 227. Based on these findings, we cannot be certain as to which of the aforementioned modifications could cause the change in the CBR1 activity. Nevertheless, cysteines at positions 26 and 150 are apparently distant from the active site and presumably not involved in changes in activity. In case, when the cysteines 226 and 227 would be inactivated by the modification, the possible explanation is that Cys-122 may play a role in activity changes, as it is greatly modified and not so far from the active site as cysteines 26 and 150. However, Cys-227 has already been identified as the most reactive residue involved in glutathione binding [19,20], we suggest that modifications of the vicinal cysteines will be responsible for the activity changes. Although Staab et al. did not consider the possibility of the disulfide bond formation, their results with the enzyme reactivation by DTT are still in accordance with our findings, as the disulfide bridge as well as the GSH-mixed disulfide would be reduced by DTT. Also, the facts that the CBR1 Cys227Ser mutant showed a higher K_m for GSNO reduction than did WT-CBR1 together with loss of the enzyme inhibition lends support to the previous conclusions [19]. This hypothesis is also supported by the data that the Cys227Ser mutant showed 7 times higher k_{cat} and 20 times higher k_{cat}/K_m values with 9,10-phenanthrenequinone [19].

One important point to mention in this context is that treatment of CBR1 with GSNO was performed in absence of cofactor, which does not reflect the *in vivo* situation where NADPH is available in micromolar amounts. Future work will also have to elucidate whether NADPH binding affects any quantitative and qualitative aspects of the modification. However, previous work has provided indirect evidence for the modification in presence of NADPH, as we have observed inactivation of the enzyme in steady-state kinetics of GSNO reduction at GSNO concentrations >5 K_m.

4. Conclusion

Human CBR1 is a well characterized enzyme with broad substrate specificity and is known to play a role in many pathophysiological conditions [41]. Inspired by earlier studies [19,20], we analyzed 4 out of 5 cysteines of CBR1 and evaluated their modification upon incubation with GSNO. Using MALDI TOF/TOF and LC-SRM analyses, we confirmed the formation of GSH-mixed disulfides on all those cysteines analyzed, as well as the formation of a disulfide bond between the adjacent cysteines 226 and 227. Steady-state kinetics with 10 substrates showed that the modifications affect enzyme activity in terms of turnover rate and substrate binding. The direction of these changes depended on the location of the substrate carbonyl groups. For substrates with vicinal carbonyl groups in a ring structure (1,2-naphthoquinone, 9,10-phenanthrenequinone and isatin) we observed increased catalytic turnover rates, in contrast to decreased rates for all other substrates tested.

For CBR1, this work provides the first evidence for two vicinal cysteines that might function as a redox switch representing a novel regulatory mechanism of CBR1 activity. As we have detected the disulfide bond formation upon incubation with H_2O_2, a role as a redox-sensitive cysteine in oxidative stress is also probable. In conclusion, further investigation is needed on this topic, as the redox-sensitive cysteine pair would represent a new mode of the regulation of CBR1 activity [19].

5. Conflicts of interest

There are no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2012.12.011.

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