Human Carbonyl Reductase Catalyzes Reduction of 4-Oxonon-2-enal†

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ABSTRACT: 4-Oxonon-2-enal (4ONE) was demonstrated to be a product of lipid peroxidation, and previous studies found that it was highly reactive toward DNA and protein. The present study sought to determine whether carbonyl reductase (CR) catalyzes reduction of 4ONE, representing a potential pathway for metabolism of the lipid peroxidation product. Recombinant CR was cloned from a human liver cDNA library, expressed in *Escherichia coli*, and purified by metal chelate chromatography. Both 4ONE and its glutathione conjugate were found to be substrates for CR, and kinetic parameters were calculated. TLC analysis of reaction products revealed the presence of three compounds, two of which were identified as 4-hydroxynon-2-enal (4HNE) and 1-hydroxynon-2-en-4-one (1HNO). GC/MS analysis confirmed 4HNE and 1HNO and identified the unknown reaction product as 4-oxononanal (4ONA). Analysis of oxime derivatives of the reaction products via LC/MS confirmed the unknown as 4ONA. The time course for CR-mediated, NADPH-dependent 4ONE reduction and appearance of 4HNE and 1HNO was determined using HPLC, demonstrating 4HNE to be a major product and 1HNO and 4ONA to be minor products. Simulated structures of 4ONE in the active site of CR/NADPH calculated via docking experiments predict the ketone positioned as primary hydride acceptor. Results of the present study demonstrate that 4ONE is a substrate for CR/NADPH and the enzyme may represent a pathway for biotransformation of the lipid. Furthermore, these findings reveal that CR catalyzes hydride transfer selectively to the ketone but also to the aldehyde and C═C of 4ONE, resulting in 4HNE, 1HNO, and 4ONA, respectively.

Carbonyl reductase (CR;[1] secondary-alcohol/$NADP^+$ oxidoreductase; EC 1.1.1.184) is a cytosolic, monomeric enzyme belonging to the short-chain dehydrogenase/reductase family that is ubiquitously expressed in human tissues (*1*−*3*). CR has been shown to catalyze NADPH-dependent reduction of a wide variety of compounds including anthracycline drugs (e.g., daunorubicin) and quinone-containing xenobiotics (*4*, *5*). Aldehydes and ketones derived from lipid peroxidation were found to be substrates for the enzyme (*6*). Activity toward carbonyl-containing prostaglandins and steroids has been demonstrated for CR; however, the low catalytic efficiency reported raises skepticism that these compounds are endogenous substrates and whether steroid metabolism represents a physiological role for the enzyme (*1*, *7*−*9*).

Cellular oxidative stress and lipid peroxidation can yield toxic lipids with reactive carbonyls (*10*) that modify proteins with deleterious consequences (*11*, *12*). Examples of such compounds are 4-hydroxynon-2-enal (4HNE) and acrolein, which contain an α,β-unsaturated aldehyde capable of reacting with cysteine, histidine, and lysine via Michael addition (*13*, *14*). Routes of metabolism for these carbonylic lipids include aldehyde dehydrogenase-catalyzed oxidation (*15*), reduction mediated by aldose reductase (*16*), and spontaneous and enzyme-catalyzed glutathione (GSH) conjugation (*17*).

Previous work has demonstrated 4-oxonon-2-enal (4ONE) to be a major product of lipid peroxidation (*18*, *19*). Studies have shown that the compound modifies DNA and the protein nucleophiles cysteine, histidine, lysine, and arginine (*20*−*23*) and is highly reactive toward thiols (i.e., $k = 145$ M^{-1} s^{-1} for GSH; *21*). A recent paper demonstrated that aldose reductase (AR; AKR 1B1; EC 1.1.1.21) efficiently catalyzes NADPH-dependent reduction of 4ONE, as well as the conjugate of GSH and 4ONE (GS-4ONE), indicating that the enzyme may play a significant role in 4ONE metabolism and detoxication (*24*). Currently, other pathways of 4ONE biotransformation are unknown.

The present study was undertaken to determine whether CR catalyzes reduction of 4ONE, identify products produced, and ascertain the ability of the reactive lipid aldehyde to inhibit the enzyme. Recombinant human CR was produced,

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[1] Abbreviations: 1HNA, 1-hydroxynonan-4-one; 1HNO, 1-hydroxynon-2-en-4-one; 4HNA, 4-hydroxynonanal; 4HNE, 4-hydroxynon-2-enal; 4ONA, 4-oxononanal; 4ONE, 4-oxonon-2-enal; ADH, alcohol dehydrogenase; AR, aldose reductase; C═C, carbon−carbon double bond; CR, carbonyl reductase; DHN, 1,4-dihydroxynon-2-ene; GS-4ONE, glutathione−4ONE conjugate; GSH, glutathione; IPTG, isopropyl-β-D-thiogalactopyranoside; TIC, total ion chromatogram.

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and its activity was measured with 4ONE and GS-4ONE. Products were identified using TLC, GC/MS, and LC/MS analysis, and the time course for 4ONE disappearance and product appearance was monitored via an HPLC method. Molecular modeling was performed with CR/NADPH and 4ONE to determine the probable orientation of the lipid substrate in the enzyme active site and to provide rationale for production of products. Inhibition of CR was assessed with 4ONE and identified reaction products. For the first time, activity of CR toward the lipid peroxidation product 4ONE is documented, thus suggesting a potential physiological role for the enzyme. Furthermore, a novel activity for the enzyme is reported, namely, carbon−carbon double bond (C=C) reduction.

MATERIALS AND METHODS

Materials. Equine liver alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (Baker's yeast), GSH, hydroxylamine hydrochloride, menadione, NADH, NADP, and NADPH were purchased from Sigma (St. Louis, MO). Dess−Martin periodinane, D-glucose-1-*d*, and phosphomolybdic acid (20% w/w solution in ethanol) were obtained from Aldrich (Milwaukee, WI). (*S*)-NADPD was synthesized according to previously described procedures (*25*, *26*). Briefly, glucose-6-phosphate dehydrogenase (50 units) was incubated with 10 mM NADP and 16 mM D-glucose-1-*d* in 60% 83 mM sodium phosphate buffer (pH 8) and 40% DMSO (v/v) at 37 °C. Progress of the reaction was monitored by measuring absorbance at 340 nm, corresponding to NADPD formation, which reached a maximum at 4 h (92% yield). To remove DMSO, 1 part NADPD solution was mixed with 3 parts acetone and incubated at −20 °C for 30 min, resulting in precipitation. The suspension was spun at 10 000 × *g* for 10 min, and the supernatant was removed. The precipitate was dried using a gentle stream of nitrogen and reconstituted in 50 mM sodium phosphate, pH 7.4. Concentration of NADPD was assessed by measuring absorbance at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$).

Synthesis of 4ONE and Derivatives. 4HNE was synthesized as the dimethyl acetal and liberated from the acetal via acid hydrolysis according to procedures described elsewhere (*27*, *28*). 4ONE was prepared by oxidizing 4HNE with Dess−Martin periodinane (*21*). 1,4-Dihydroxynon-2-ene (DHN) and 1-hydroxynon-2-en-4-one (1HNO) were synthesized as previously described (*23*). GS-4ONE was prepared by incubation of 1 mM 4ONE with 1.5 mM GSH in 50 mM sodium phosphate buffer, pH 7.4, for 30 min at room temperature (~23 °C). Conjugation of 4ONE with GSH was confirmed by an absence of a sharp peak at 228 nm, corresponding to loss of the 4ONE enone system.

Cloning of Human CR. The cDNA for human CR was amplified from a human cDNA library (Stratagene, Uni-ZAP XR cDNA library) by PCR. The primers used were designed to anneal at the 5'- and 3'-ends of the coding region and had the following sequences: 5'-primer = 5'ATGTCGTCCGGCATCCATGTA3'; 3'-primer = 5'TCACCACTGTTCAACTCTCTT3'. For further cloning, the cDNA obtained was extended with the required restriction sites in a second PCR. For cloning into the bacterial expression vector pET15b, a *Xho*1 site was introduced. The correct sequence and open reading frame of the plasmid obtained was confirmed by DNA sequencing.

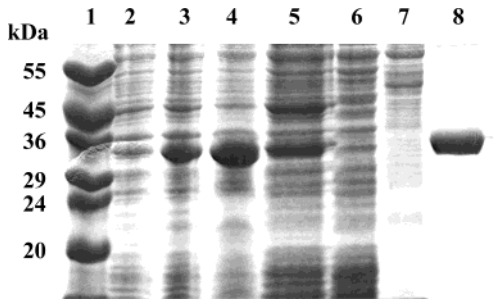


FIGURE 1: Overexpression and purification of recombinant human carbonyl reductase in *E. coli*. The purity of the enzyme is shown in a reducing 12% SDS−PAGE: lane 1 = molecular mass marker; lane 2 = cell lysate from uninduced BL21(DE3) cells; lane 3 = cell lysate from IPTG (3 h)-induced BL21(DE3) cells; lane 4 = cell debris after ultracentrifugation; lane 5 = supernatant that was loaded on the His-Trap column; lane 6 = breakthrough fraction; lane 7 = wash eluate; lane 8 = purified recombinant carbonyl reductase, eluted at about 200 mM imidazole.

Overexpression of Human CR. The bacterial overexpression of human CR was performed in *Escherichia coli* strain BL21(DE3). After transformation of *E. coli* with the plasmid construct and subsequent plating on LB−agar (100 μg/mL ampicillin), a single colony was picked to inoculate a 500 mL culture in TB medium (200 μg/mL ampicillin). The cells were grown at 37 °C. At an OD_{600} between 0.6 and 0.8, overexpression of recombinant human CR was induced with 1 mL of 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) solution and incubated at 37 °C for 3 h. The cells were harvested (Sorvall GSA, 6000 × *g*, 15 min, 4 °C) and washed once with 100 mL of KCl solution. After centrifugation (Sorvall SS-34, 6000 × *g*, 15 min, 4 °C), the bacterial pellet was resuspended in 30 mL of buffer (20 mM phosphate, 100 mM NaCl, 10 mM imidazole, pH = 7.4). The cells were mechanically disrupted using a French press. The cell debris was sedimented by ultracentrifugation (Beckman Ti50.1, 173 000 × *g*, 45 min, 4 °C). The supernatant was directly applied to a Ni-agarose affinity column.

Purification of Recombinant Human CR. CR overexpressed in *E. coli* was purified by affinity chromatography (ÄKTA Purifier, Pharmacia Biotech). For this purpose, 10 mL of bacterial extract was applied to a 5 mL Ni^{2+} HiTrap Chelating column, which has been equilibrated with 15 mL of starting buffer (20 mM phosphate, 100 mM NaCl, 10 mM imidazole, pH = 7.4). After loading, the column was washed with 20 mL of starting buffer. The His-tagged CR was then eluted by using an imidazole gradient ranging from 10 to 500 mM. Fractions containing the purified protein were pooled and dialyzed against a 20 mM phosphate buffer, pH = 7.4. The successful overexpression and purification was monitored by SDS−PAGE (Figure 1).

Enzyme Activity. All assays were performed in 50 mM sodium phosphate buffer, pH 7.4, at 37 °C. Activity was assessed by monitoring the disappearance of NADPH at 340 nm using a Molecular Devices SpectraMax 190 plate-reader (Sunnyvale, CA). Lipid aldehyde substrates were used at concentrations varying from 10 to 1000 μM and 76−760 μM for 4ONE and GS-4ONE, respectively. Menadione was used at concentrations varying from 10 to 100 μM.

For enzyme inhibition experiments, 0.75 μg of CR (±500 μM NADPH) was incubated with 100 μM 4ONE, 4HNE, or 1HNO at 37 °C in 50 mM sodium phosphate buffer, pH

7.4. After 1 h, an equal volume of substrate solution was added (final volume = 100 μL; $[4ONE]_{final}$ = 1 mM; $[NADPH]_{final}$ = 1 mM), and residual activity was measured compared to a control using a Molecular Devices SpectraMax 190 plate reader (Sunnyvale, CA).

Determination of Enzyme Kinetic Parameters. Enzyme assays were performed in triplicate using eight different concentrations of 4ONE and GS-4ONE. Kinetic parameters and statistical analysis were obtained via nonlinear regression analysis of Michael−Menten plots using GraphPad Prism, version 3.02 (GraphPad Software, San Diego, CA).

Determination of CR/NADPH/4ONE Product(s). CR (5−10 μg) was incubated in phosphate buffer, pH 7.4, containing 2 mM 4ONE and 5 mM NADPH for 60 min at 37 °C. Incubations with (*S*)-NADPD were allowed to proceed longer (i.e., 2 h). Products were extracted three times with two volumes of ethyl acetate or methylene chloride. The solvent was dried with $MgSO_4$ and evaporated to dryness with a gentle stream of nitrogen. The reaction product was reconstituted in acetonitrile and stored at −20 °C until analysis. TLC analysis was performed to identify the product using 3:1 ether/*n*-hexane with standards 4ONE, 4HNE, and 1HNO and ethyl acetate with standards 1HNO and DHN. To visualize the compounds, the plates were stained with phosphomolybdic acid (20% w/w solution in ethanol) and allowed to dry with gentle heating. Additional confirmation of the reaction product was performed via GC/MS analysis using a HP 5973 GC/MSD system.

For LC/MS analysis, CR reaction products (in acetonitrile) were reacted with hydroxylamine to yield oxime derivatives (*18*). Briefly, the acetonitrile solution was dried under nitrogen and reconstituted in 0.2 M hydroxylamine-hydrochloride. The solution was heated for 20 min at 80 °C and allowed to cool. Products were extracted three times with two volumes of methylene chloride. The solvent was evaporated under nitrogen, and derivatized products were reconstituted in deionized water.

LC/ESI/MS analysis was accomplished using an Agilent 1100 series capillary LC and MSD Ion Trap SL (Agilent Technologies, Palo Alto, CA). Eight microliters of the derivatized products was injected via an autosampler, and separation was accomplished with gradient elution using a Phenomenex C18 column (150 mm × 1 mm ID; 300 Å) (Torrance, CA) at a flow rate of 50 μL/min. Solvents used were 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) with gradient conditions as follows: 20% B at 0 min, 20% B at 5 min, 70% B at 35 min, 90% B at 38 min and held for 2 min, and 20% B at 42 min and held for 3 min. Mass spectrometric detection and analysis was accomplished using positive ion mode with a capillary voltage of 3.5 kV. Nebulizer pressure was set at 20 psi and dry gas flow at 8 L/min with the temperature of the dry gas set to 350 °C. The scanning range for all analyses was 50−300 *m*/*z*. MS/MS analysis was accomplished using the Auto MS^n feature with fragmentation amplitude set to 1.5 V.

HPLC Analysis of Time Course for CR/NADPH/4ONE Reaction. The reaction of CR with 4ONE was initiated by incubating 4 μg of enzyme with 2 mM NADPH and ∼0.5 mM 4ONE at 37 °C. Aliquots were removed over time and incubated with a 20-fold excess of 0.1 M hydroxylamine-hydrochloride at 80 °C for 30 min. The samples were allowed to cool and analyzed using a VP series Shimadzu HPLC system (Columbia, MD). Briefly, 20 μL of sample was injected via an autoinjector, and separation was achieved using gradient elution with a Phenomenex C18 column (150 mm × 1 mm ID; 300 Å) (Torrance, CA) at a flow rate of 70 μL/min. Solvents used were 5 mM formic acid in 95% water/5% acetonitrile (A) and 5 mM formic acid in 90% acetonitrile/10% water (B) with gradient conditions as follows: 20% B at 0 min, 20% B at 5 min, 70% B at 30 min, 90% B at 33 min and held for 2 min, and 20% B at 37 min and held for 3 min. Detection of compounds was achieved using a photodiode array detector set at 190−300 nm. For quantitation, a calibration curve was generated using concentrations of 0.1, 0.2, 0.5, and 1 mM for 4HNE and 4ONE. Values for peak area were calculated using a major peak in the chromatogram: ∼16 min for 4HNE-oxime and ∼18 min for 4ONE-oxime. Because the absorptivity of 1HNO-oxime was identical to that for 4HNE-oxime, concentrations of 1HNO were calculated using the calibration curve for 4HNE.

GS-4ONE Reduction by CR/NADPH. GS-4ONE (1 mM) was incubated with 2 mM NADPH and 8 μg of CR for 2 h at 37 °C. One microliter was injected via an autosampler, and separation was accomplished with gradient elution using a Phenomenex C18 column (150 mm × 1 mm ID; 300 Å) (Torrance, CA) at a flow rate of 50 μL/min. Solvents used were 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) with gradient conditions as follows: 5% B at 0 min, 5% B at 5 min, 70% B at 35 min, 90% B at 38 min and held for 2 min, and 5% B at 42 min and held for 3 min. Mass spectrometric detection and analysis was accomplished using positive ion mode with a capillary voltage of 3.5 kV. Nebulizer pressure was set at 20 psi and dry gas flow at 8 L/min with the temperature of the dry gas set to 350 °C. The scanning range for all analyses was 50−600 *m*/*z*. MS/MS analysis was accomplished using the Auto MS^n feature with fragmentation amplitude set to 1.5 V.

Molecular Modeling of 4ONE in CR Active Site. An SGI Octane computer (Mountain View, CA) with InsightII 2002 software and Affinity, Biopolymer, Builder, and Discover modules (Accelyris, San Diego, CA) was used for docking experiments. The crystal structure of CR complexed with $NADP^+$ (*29*, 1N5D.pdb) was downloaded from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb/). It should be noted that this structure shares ∼85% sequence identity with human CR. Crystallographic waters were removed, and the $NADP^+$ was converted to NADPH using the Biopolymer module. Missing residues and hydrogen atoms were added (pH 7.4) with the Biopolymer module. With use of the Builder module, 4ONE was generated and manually docked in the active site of CR. The hydroxyl oxygen of Tyr193, the proton donor in CR catalysis, was tethered to the nicotinamide ring of NADPH to maintain the enzyme in an active conformation (*30*). 4ONE and a subset of CR/NADPH atoms located within a 10 Å radius of the lipid were designated as flexible, while the rest of the enzyme was held rigid. 4ONE was confined to a 2 Å radius to prevent it from drifting out of the active site. Docking was accomplished via a two-phase energy minimization process followed by a molecular dynamics simulation (500−300 K) and a final round of energy minimization (1000-step). With the exception of the initial phase of energy minimization, a

Table 1: Kinetic Constants for CR/NADPH-Catalyzed Reduction of Substrates

	kinetic parameters[a]			
substrate[b]	K_m (μM)	V_{max} (μmol/min/mg)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
4ONE	345 ± 53.7	7.33 ± 0.521	220 ± 15.6	638 ± 109
GS-4ONE	329 ± 77.5	0.214 ± 0.0216	8.56 ± 0.864	26.2 ± 6.73
4HNE	*c*	*c*	*c*	*c*
1HNO	*c*	*c*	*c*	*c*
menadione	25.0 ± 0.520	8.20 ± 0.900	246 ± 27.0	9840 ± 1100

[a] Kinetic parameters were determined as described in Materials and Methods section. All assays were performed in 50 mM sodium phosphate buffer, pH 7.4, at 37 °C. [b] Substrates were synthesized as described in Materials and Methods section. [c] Not determined. No activity was detected with these substrates.

distance-dependent dielectric was used ($\epsilon = 4.0$) to simulate solvation by water.

RESULTS

Reduction of 4ONE Catalyzed by CR. To establish the potential of CR in 4ONE reduction, the recombinant enzyme was incubated with NADPH and substrate. Activity was assessed by monitoring change in absorbance at 340 nm, corresponding to enzyme-catalyzed oxidation of the cofactor. The enzyme was found to catalyze reduction of 4ONE with values for k_{cat} and K_M of 220 min^{-1} and 345 μM, respectively (Table 1). It was anticipated that the reduced products of this reaction would be 4HNE and 1HNO. When recombinant CR was incubated with NADPH and either of these products, no activity was detected. Furthermore, the reverse reaction of 4ONE reduction (i.e., CR-catalyzed oxidation of 4HNE and 1HNO to 4ONE in the presence of $NADP^+$) was not observed.

CR activity toward menadione was measured to verify that the recombinant enzyme, containing a His-tag, used in the present study had kinetic properties similar to that previously reported. Kinetic constants determined for menadione were found to be similar to those documented in prior studies (Table 1; refs *9*, *31*, and *32*). Such a result demonstrates that the recombinant CR used in the present report is not kinetically distinct from enzyme used in previous studies and the activity toward 4ONE is relevant to native CR.

The ability of 4ONE to efficiently form a Michael addition product with GSH has been reported (*20*), and the abundance of this soluble thiol/antioxidant in physiological systems suggests that GS-4ONE would form readily in most cells. The possibility that GS-4ONE is a substrate for CR/NADPH was confirmed, and the reaction was found to proceed with calculated kinetic constants k_{cat} of 8.56 min^{-1} and a K_M value of 329 μM (Table 1). Compared to 4ONE kinetic parameters, the K_M measured for GS-4ONE (i.e., 329 μM) was not significantly different; however, the turnover for GS-4ONE was 26-fold lower (i.e., 8.56 min^{-1}).

Identification of the Product Resulting from CR-Mediated 4ONE Reduction. It is predictable that, as a result of the CR-mediated reaction, 4ONE could be reduced at the 4-oxo group yielding 4HNE. Likewise, it is also expected that reduction could occur at the aldehyde moiety resulting in the production of 1HNO. To identify the CR reaction products, 4ONE was added to CR and NADPH at pH 7.4, 37 °C, with $[4ONE]_{final} = 2.0$ mM. The product was isolated by solvent extraction using ethyl acetate or methylene chloride and analyzed using TLC and the standards 4ONE, 4HNE, and 1HNO (Figure 2). Four spots were evident, indicating the presence of residual substrate (i.e., 4ONE) and three reaction products. Based on R_f values, two of the compounds were identified as 4HNE and 1HNO with the darkest staining spot representing 4HNE. The absence of DHN as a product was demonstrated using TLC with ethyl acetate as solvent and the standards 1HNO and DHN (results not shown).

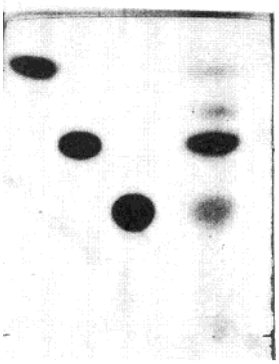


FIGURE 2: TLC analysis of the reaction of CR/NADPH with 4ONE: lane A, 4ONE standard; lane B, 4HNE standard; lane C, 1HNO standard; lane D, CR/4ONE products. The products were identified as 4HNE, 1HNO, and an unknown (with $R_f = 0.70$).

Interestingly, one spot was apparent that did not correspond to R_f values for 4ONE or any carbonyl-reduced derivative of 4ONE (i.e., 4HNE, 1HNO, or DHN). Given this result, it was surmised that the unknown product could be the result of C=C reduction for 4ONE. Such a product, lacking an α,β-unsaturated carbonyl, would not be a Michael acceptor, and therefore, not reactive toward Michael donors, such as thiols. The CR products (in acetonitrile) were dried of solvent, reconstituted in an equal volume of 10 mM *N*-acetyl cysteine in 50 mM sodium phosphate buffer, pH 7.4, and allowed to react at 37 °C. After 1 h, the products were extracted using and analyzed via TLC (3:1 ether/*n*-hexane) with standards 4ONE and 4HNE. The unknown was the only compound found to be resistant toward the *N*-acetyl cysteine treatment, indicating that it lacks an α,β-unsaturated carbonyl.

GC/MS analysis of the CR reaction products demonstrated the presence of 4HNE, 1HNO, and residual 4ONE substrate. In the total ion chromatogram (TIC), a peak was found that did not match any of the standards (i.e., 4ONE, 4HNE, or 1HNO) and was unresolved with the 4ONE peak. The compound had a molecular ion (M^+) at 156 *m*/*z* and major fragment ions at *m*/*z* 57, 72, 85, 99/100, and 128. Compared

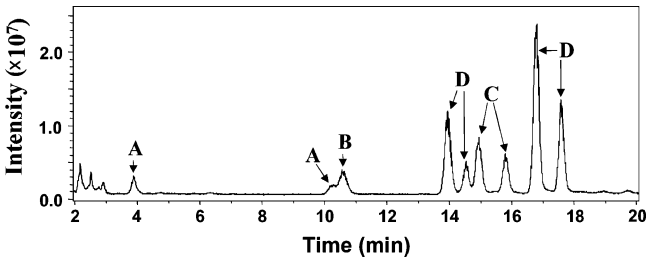


FIGURE 3: TIC chromatogram for CR/4ONE products demonstrating presence of 4HNE, 1HNO, 4ONA, and residual 4ONE: (A) 1HNO monooxime derivative (MH^+ at *m*/*z* 174); (B) 4ONA bisoxime derivative (MH^+ at *m*/*z* 187); (C) 4HNE monooxime derivative (MH^+ at *m*/*z* 174); (D) 4ONE bisoxime derivative (MH^+ at *m*/*z* 185).

with 4ONE, which has a M^+ at *m*/*z* 154 and fragments at *m*/*z* 55, 70, 83, 100, and 125, a 2 Da shift in mass is evident, thus demonstrating reduction of the 4ONE molecule and that the unknown is 4-oxononanal (4ONA).

Additional confirmation of compound identity was afforded via LC/ESI/MS analysis of oxime derivatives of the CR reaction products. As shown in Figure 3, several major peaks were evident in the TIC and in agreement with standards for 4ONE, 4HNE, and 1HNO. The compounds in the peaks corresponded to the syn and anti isomers for monooxime derivatives of 4HNE and 1HNO and the bisoxime of 4ONE, as previously observed for 4ONE and 4HNE (*17*). The MH^+ for 4HNE and 1HNO (monooximes) was at 172 *m*/*z* and at 185 *m*/*z* for 4ONE (bisoxime), and fragmentation patterns were consistent with standards. A peak was observed with retention time of ~11 min, and the eluted compound had a MH^+ at 187 *m*/*z*, consistent with that for the bisoxime of 4ONA.

Further confirmation of CR reaction products was obtained from LC/ESI/MS analysis of CR reaction products using (*S*)-NADPD as a substituted cofactor. A shift in mass of 1 Da was evident for all compounds, with the exception of 4ONE (bisoxime), which had a MH^+ at 185 *m*/*z* (data not shown), demonstrating that 4HNE, 1HNO, and 4ONA are generated from CR/NADPH-mediated hydride transfer. For 4HNE and 1HNO, the monooxime derivatives had a MH^+ at 173 *m*/*z*, while 4ONA exhibited MH^+ at 188 *m*/*z*.

To verify that the spots on the TLC plate were correctly identified, a TLC plate was prepared with CR products and developed using 3:1 ether/*n*-hexane as before (see Figure 2). One-quarter of the plate was cut lengthwise and stained with phosphomolybdic acid and compared to the other portion of the plate. Strips, which presumably contained the CR products, were cut (widthwise) and soaked in 500 μL of ethyl acetate. The ethyl acetate was evaporated using a gentle stream of nitrogen, and 100 μL of 0.2 M hydroxylamine-hydrochloride added to each vile. The reaction was allowed to proceed at 80 °C for 20 min, and products were extracted three times with two volumes of methylene chloride. The methylene chloride was evaporated using nitrogen, and 50 μL of 20% acetonitrile in water was added to each vial. Eight microliters was used for LC/ESI/MS analysis as described in Materials and Methods. Spots with R_f values of 0.85, 0.60, and 0.40 were identified as 4ONE, 4HNE, and 1HNO, respectively, consistent with standards (Figure 2). In the TIC for the spot with R_f value of 0.70, a major peak was observed

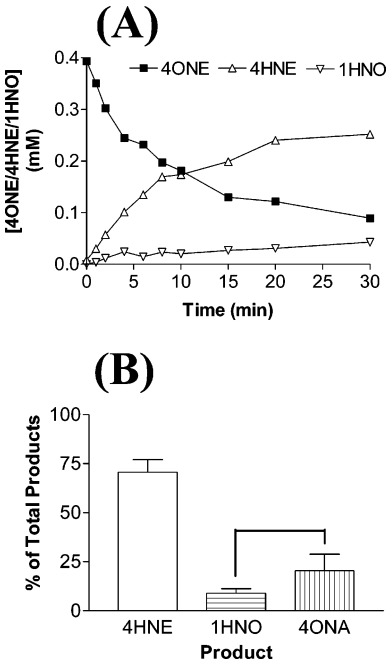


FIGURE 4: Quantitative analysis of CR-mediated product formation. Panel A shows a representative time course for CR-mediated reduction of 4ONE to the products 4HNE and 1HNO. See Materials and Methods for details on the HPLC analysis. Panel B shows the percentage of each product produced at 30 min on a molar basis compared to 4ONE consumption ($n = 4$). Values are significantly different ($p < 0.05$) unless connected by a bar. Note that 4HNE corresponds to ~70% of the total products.

representing a compound with MH^+ at *m*/*z* 187 and identified as 4ONA.

Time Course for the Reaction of CR/NADPH with 4ONE. The potential of CR to generate multiple reaction products raises important questions concerning the quantities and time course of the individual products produced. To monitor the time course for CR-mediated 4ONE reduction and quantitatively identify the major product, an HPLC assay was developed (as described in the Materials and Methods section). Oxime derivatives of 4ONE and 4HNE/1HNO exhibited λ_{max} values at 268 and 230 nm, respectively, and were detected using a photodiode array detector. As shown in Figure 4, 4HNE was found to be the major product, representing 71% ± 6.4% of CR/NADPH activity. 1HNO was demonstrated to be a minor product, accounting for only 11% ± 3.0% of the final product. This leaves 18% ± 4.4% of enzyme activity unaccounted that may be attributed to CR-catalyzed C═C reduction.

LC/ESI/MS Analysis of Product for CR-Mediated GS-4ONE Reduction. To confirm that CR catalyzed reduction of the GS-4ONE conjugate, 1 mM GS-4ONE was incubated with CR/NADPH, and the resulting products were analyzed via LC/ESI/MS as described in the Materials and Methods section. Two major peaks were evident in the TIC (Figure 5A), both of which contained compounds with MH^+ at *m*/*z* 446.2 (major) and 464.1 (minor), as shown in Figure 5B. Compared to the GS-4ONE conjugate (MH^+ at *m*/*z* 462.1), these compounds represent the reduced product and the product − H_2O (18 Da). Collectively, these results confirm that CR/NAPDH catalyzes reduction of GS-4ONE.

Molecular Modeling of 4ONE in the CR Active Site. For the top two structures (lowest energy), the ketone moiety of

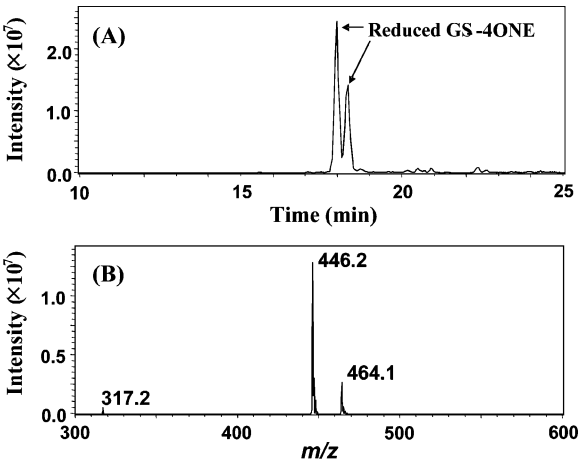


FIGURE 5: TIC chromatogram (A) of CR/GS-4ONE products, demonstrating enzyme-catalyzed reduction of GS-4ONE carbonyls, and mass spectrum (B) for peak with retention time of 18.3 min. The peak at *m*/*z* 464.1 corresponds to reduced GS-4ONE (MH^+ at *m*/*z* 462.1), and the peak at *m*/*z* 446.1 represents the reduced product − H_2O (18 Da).

Table 2: Bond Distances (Å) between 4ONE and CR

4ONE	CR	model 1[a]	model 2[a]
ketone C	C4, NADPH	3.9	4.8
aldehyde C	C4, NADPH	6.7	6.4
ketone O	−OH, Tyr193	1.9	4.8
aldehyde O	−OH, Tyr193	3.7	3.8
C═C	−SH, Cys226	9.0	9.6
C_5H_{11} "tail"	indole, Trp228	3.7	3.8

[a] Bond distances determined for the molecular models generated as described in Materials and Methods section.

4ONE was positioned as the hydride acceptor. See Table 2 for bond distances from 4ONE to active site components of CR. It should be noted that the bond distances predicted (Table 2) are comparable to those observed in the crystal structure of mouse lung CR complexed with substrate (i.e., 2-propanol) and NADPH (*33*; 1CYD.pdb). In the mouse lung enzyme structure, the distance between C2 (substrate) and C4 (cofactor) was calculated to be 3.4 and 3.0 Å between the active site tyrosine hydroxyl and the hydroxyl of substrate (see Table 2 for comparison).

Much variability was evident in the models for the structure(s) of substrate in the CR active site, demonstrating conformational freedom in the binding of 4ONE, and no specific or consistent contacts between enzyme and substrate were observed. Taken together, these results provide rationale for the selective but nonspecific hydride transfer catalyzed by CR/NADPH.

The active site of CR contains a free thiol (i.e., Cys226) that could be modified by 4ONE; however, the C═C of 4ONE was predicted to be distant from the sulfhydryl moiety (i.e., ≥9 Å). Such a result may provide the basis for resistance of the enzyme toward inactivation by the lipid aldehyde (see following section).

4ONE-Mediated Inhibition of CR. The high reactivity of 4ONE toward cysteine, histidine, and lysine residues of peptides and proteins has been documented (*20*) and suggests that this electrophilic aldehyde could function as an enzyme inhibitor. To evaluate this possibility, CR was incubated with 100 μM 4ONE at 37 °C with and without 500 μM NADPH. At 1 h, both 4ONE and NADPH were added to final concentrations of 1 mM, and initial rates were measured. CR in the absence of cofactor was completely resistant toward 4ONE; however, enzyme in the presence of NADPH lost ∼50% activity (Figure 6). Such a finding suggests that the CR−NADPH binary complex is required for inactivation and that 4ONE may be a mechanism-based inhibitor of the enzyme. The reaction products 4HNE and 1HNO (both at 100 μM) were incubated for 1 h with CR at 37 °C with and without 500 μM NADPH, and interestingly, the enzyme was resistant toward inactivation in the presence or absence of cofactor (Figure 6). This result indicates that inhibition of CR is due to 4ONE and not the products 4HNE, 1HNO, or both.

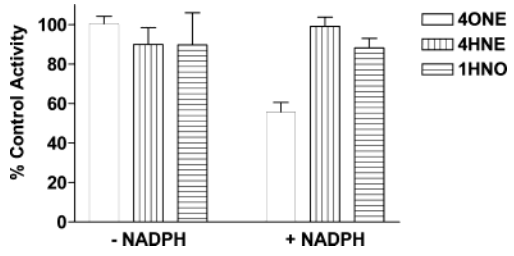


FIGURE 6: Result of treatment of CR with 100 μM 4ONE, 4HNE, and 1HNO (±500 μM NADPH) for 60 min. Values shown represent the mean ± SE of $n = 2-4$ experiments (i.e., $n = 3$ or 4 for 4ONE, 3 for 4HNE, and 2 for 1HNO). Residual activity was measured as described in Materials and Methods section. In the absence of 500 μM NADPH, CR was completely resistant toward inactivation by 4ONE. However, enzyme incubated with 100 μM 4ONE in the presence of cofactor lost ∼50% activity.

It should be noted that $NADP^+$ may be at a maximum concentration of 50 μM in the final assay. However, such a level of $NADP^+$ was found to have no effect on activity of CR toward 1 mM 4ONE and 1 mM NADPH, thus ruling out the possibility that product inhibition by $NADP^+$ is responsible for the reported results (data not shown).

DISCUSSION

CR has been demonstrated to catalyze the reduction of several carbonyl-containing compounds, including quinones of polycyclic aromatic hydrocarbons, anthracycline drugs such as daunorubicin, and aldehydes and ketones derived from lipid peroxidation (*4*−*6*). The enzyme was found to have activity toward physiologic compounds such as steroids and prostaglandins; however, the high K_M and low k_{cat} values raised skepticism about these ketone-containing molecules being endogenous substrates (*1*, *7*−*9*).

In the present study, CR was demonstrated for the first time to have activity toward a particular product (i.e., 4ONE) of lipid peroxidation, an endogenous reaction that yields toxic carbonylic lipids. 4ONE was found to be a substrate for the enzyme in the presence of NADPH with k_{cat} and K_M of 220 min^{-1} and 345 μM, respectively. The k_{cat}/K_m values determined in the present study suggest that CR could play a significant role in the reduction of 4ONE generated as a consequence of radical-mediated lipid peroxidation in cellular systems. A novel observation of the current study is that the reduction of 4ONE by CR gives rise to multiple products, some of which retain substantial electrophilic properties and bioactivities (e.g., 4HNE).

Previous reports demonstrated that CR has a GSH binding site close in proximity to the catalytic center (*34*−*36*) and that GSH conjugates (e.g., GSH-bound quinones and pros-

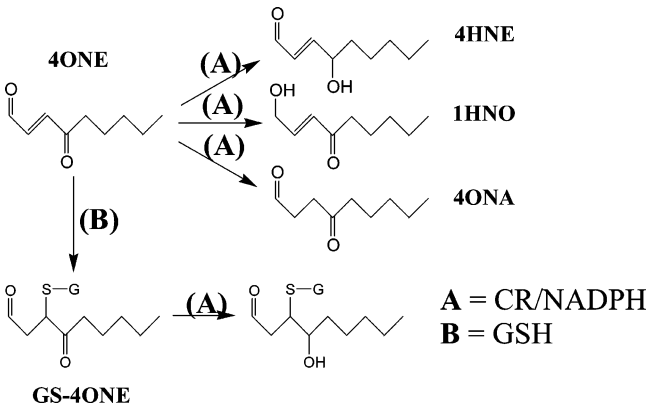
taglandin A_1) are good substrates for the enzyme. Moreover, the GSH conjugate of prostaglandin A_1 was reported to be a better substrate than the parent molecule (*37*, *38*). In the present study, we have extended these observations by demonstrating that GS-4ONE was a substrate for the enzyme with k_{cat} and K_M of 8.56 min^{-1} and 329 mM. GS-4ONE was found to have a K_M similar to that of 4ONE but a 26-fold lower k_{cat}, thus exhibiting a decrease in turnover for the GSH conjugate compared to the parent compound. Such a result may indicate that the GS moiety of GS-4ONE does not make specific or favorable contacts or both with the GSH binding site of the enzyme. Another possibility is that the 4ONE portion of GS-4ONE is oriented poorly in the CR active site due to constraints from interaction of the GS moiety with the GSH binding site (*39*). It appears that there are alternative enzymatic pathways for reduction of GS-4ONE in that a recent study found that both 4ONE and GS-4ONE were substrates for human AR (EC 1.1.1.21) in the presence of NADPH (*24*). Kinetic constants for 4ONE (K_M = 42 μM; k_{cat} = 92 min^{-1}) and GS-4ONE (K_M = 4.6 μM; k_{cat} = 46 min^{-1}) demonstrated that the enzyme is an efficient catalyst for reduction of these compounds. Compared to CR, AR exhibits a higher k_{cat}/K_M (due to a lower K_M) and appears to be the superior catalyst for 4ONE and GS-4ONE reduction.

It should be noted, however, that AR has activity toward a wide range of aldehydic products of lipid peroxidation, as well as the GSH conjugates, such as 4HNE and GS-4HNE (*40*, *41*). Therefore, AR activity toward 4ONE and GS-4ONE may be competitively inhibited in the presence of 4HNE and other aldehydes (e.g., 2-nonenal), which are expected to be at significant concentrations during lipid peroxidation (*10*) or produced from the reaction of CR/NADPH with 4ONE and GS-4ONE. For example, consider AR/NADPH in the presence of 4ONE and other lipid aldehydes formed, such as 4HNE, 2-nonenal, 2-octenal, hexanal, and pentanal, and assume a concentration of 10 μM for each (*10*). It is estimated that specific activity of AR for 4ONE would be reduced to 120 nmol/min/mg of protein based on a K_M and V_{max} of 42.0 μM and 2.58 μmol/min/mg of protein, respectively (*24*), and previously measured K_M values for the specified aldehydes (*16*).[2] These calculations suggest that the activity of AR toward 4ONE may be inhibited to a significant degree in the presence of other lipid aldehydes expected to be present following lipid peroxidation.

In contrast, it was determined that 4HNE is not a substrate for CR, and the activity of the enzyme toward 4ONE was not inhibited by 4HNE (i.e., 100 μM). Based on kinetic constants, the activity of CR toward 10 μM 4ONE is calculated to be 206 nmol/min/mg of protein. Such activity and specificity toward 4ONE indicates that the lipid aldehyde may be a physiologically relevant substrate for CR and that following lipid peroxidation, the enzyme may play an equally important or unique role in 4ONE metabolism and detoxification compared to other pathways.

[2] Activity of AR/NADPH toward 4ONE was estimated using the following equation for enzyme inhibition in the presence of multiple inhibitors for K_M values (in μM) of 28.0, 19.0, 16.0, 7.00, and 10.8 for 4HNE, 2-nonenal, 2-octenal, hexanal, and pentanal, respectively (*16*): $v_o = V_{max}[S]/\{K_M(1 + A + B + C + D + E) + [S]\}$, where V_{max} = 2.58 μmol/min/mg of protein (*24*), [S] = [4ONE] = 10 μM, K_M = 42.0 μM (*23*), and *A*, *B*, *C*, *D*, and $E = [I]/K_M$ for the specified aldehydes (*48*, *49*). The concentrations of all competitive substrates (i.e., [I]) were assumed to be 10 μM.

Scheme 1



While AR was found to catalyze specific reduction of the 4ONE aldehyde (*24*), results of experiments in the present study demonstrate that the reaction of CR/NADPH and 4ONE yields three distinct products. CR was determined to catalyze reduction of the 4ONE ketone, aldehyde, and C═C (Scheme 1). The ketone-reducing activity of the enzyme was found to predominate, 4HNE representing ~70% of the total product. Previous studies have shown that CR catalyzes reduction of ketones (e.g., steroids, quinones; refs *4*−*9*), and it is therefore no surprise that 4HNE is a major product. However, it is interesting that the C═C of 4ONE serves as hydride acceptor as well. While it is known that other members of the short-chain dehydrogenase/reductase superfamily can catalyze reduction of both C═O and C═C, this is the first report (to our knowledge) that CR has activity toward a C═C (*30*). Such a finding raises speculation that the enzyme may catalyze reduction of the C═C for other physiologically relevant substrates.

The finding that three products result from the reaction of CR and 4ONE indicates that several different orientations of the substrate in the active site are possible resulting in hydride transfer. The measured K_M (345 μM) and results of molecular modeling experiments (Table 2) support such a contention and demonstrate that 4ONE may have freedom in the CR active site to adopt several active conformations. No specific contacts (e.g., hydrogen bonding) between substrate and enzyme were evident in calculated models (Table 2). However, it should be noted that the nonspecific hydride transfer is unique to 4ONE. Neither 4HNE nor 1HNO were found to be substrates for the enzyme in the presence of NADPH, indicating that both carbonyls of 4ONE are necessary for binding and reductive catalysis.

The characteristic of CR to enzymatically generate products that are potential substrates for further reduction prompted us to evaluate the potential of this enzyme to sequentially reduce functional groups on 4ONE. The results presented here demonstrate that CR does not catalyze subsequent reduction of the product(s) 4HNE and 1HNO, yielding the inactive diol (i.e., DHN). While both of the compounds are much less reactive toward thiols than 4ONE (i.e., 55-fold for 1HNO and 110-fold for 4HNE), 1HNO and 4HNE are still electrophilic Michael acceptors (*21*, *24*). Therefore, one potential consequence of generating electrophilic intermediates such as 4HNE and 1HNO is the modification of nucleophilic sites on cellular proteins. Previous studies have shown that modification of proteins by 4HNE, the major product of CR-mediated 4ONE reduc-

tion, can result in deleterious consequences (*11*, *12*). Therefore, the net physiologic effect of 4HNE generation via CR/NADPH/4ONE may or may not be beneficial to a cell. Other mechanisms would be required for complete metabolism and detoxification of 4ONE, yielding an inactive product (e.g., 4HNE oxidation or reduction).

4ONA has two carbonyls that could react with protein amines to form adducts; however, this reaction product may undergo subsequent reduction catalyzed by CR/NADPH. A small peak was observed in the TIC for GC/MS and LC/ESI/MS analysis with mass corresponding to that of 4-hydroxynonanal (4HNA) or 1-hydroxynonan-4-one (1HNA), with M^+ at 158 *m*/*z* for GC/MS and MH^+ at 176 *m*/*z* (monooxime derivative) for LC/ESI/MS. Such a finding suggests that the product 4ONA is a substrate for CR/NADPH and undergoes carbonyl reduction, yielding 4HNA or 1HNA. At this point, however, the reactivity of 4HNA and 1HNA toward proteins is unknown. It is conceivable that these carbonyl compounds could modify proteins; however, 4HNA and 1HNA probably exist primarily as a lactol and, therefore, may not have a free aldehyde to react with amines (*42*).

4ONE is a reactive electrophile capable of modifying cellular nucleophiles (*20*−*23*); however, CR is resistant toward inactivation by the lipid aldehyde. Previous studies have shown that the enzyme active site contains a free thiol (i.e., Cys226/227) important for catalysis and that modification of this residue results in impairment of activity (*9*, *31*, *43*). On the basis of this, one would expect CR to be potently inhibited by 4ONE and other lipid aldehydes, but surprisingly, only partial activity is lost using high 4ONE concentration (i.e., 100 μM) and long incubations (i.e., 1 h). Results of molecular modeling experiments demonstrated that the C═C of the 4ONE substrate is not positioned to react with Cys226 (Table 2). Furthermore, preliminary data of experiments involving treatment of CR with 4ONE and subsequent tryptic digestion and LC/MS analysis reveal that Cys226/227 is not adducted by 4ONE (data not shown).

Interestingly, the presence of cofactor is necessary for inhibition of the enzyme, which indicates the following. First, the NADPH binding site, which contains an essential lysine (*44*), is not modified by 4ONE. Second, inactivation of CR by the lipid aldehyde may be mechanism-based, requiring "activation" of 4ONE to an inhibitory product. It is unlikely that 4HNE or 1HNO is the inhibitory product, given that CR was resistant toward inactivation by these compounds in the presence or absence of cofactor (Figure 6). Studies are currently underway in this laboratory to identify the inhibitor and elucidate the specific mechanisms by which the inhibition occurs. Previous work has demonstrated autocatalytic modification of Lys238 by 2-oxocarboxylic acids, and it is conceivable that this residue is a target for adduction (*45*−*47*).

In summary, results of the present study demonstrate 4ONE and its GSH conjugate to be substrates for CR/NADPH, which is the first report of the enzyme having activity toward the lipid peroxidation product. The enzyme was determined to catalyze nonspecific reduction of 4ONE functional groups, resulting in multiple products (Scheme 1). Ketone-reduction of 4ONE represented the major activity (~70%) of CR, and reduction of the 4ONE aldehyde was observed as well. Surprisingly, CR mediated hydride transfer to C═C, yielding 4ONA as a product. C═C reduction has not been previously reported for this enzyme. The activity of CR was specific for 4ONE, and subsequent reduction of products (e.g., 4HNE → DHN) was not observed. Taken together, results of the present study demonstrate novel activities for CR and suggest a role of the enzyme in 4ONE biotransformation.

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