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CARBONYL REDUCTASE PROVIDES THE ENZYMATIC BASIS OF QUINONE DETOXICATION IN MAN*

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Abstract—Enzymes catalyzing the two-electron reduction of quinones to hydroquinones are thought to protect the cell against quinone-induced oxidative stress. Using menadione as a substrate, carbonyl reductase, a cytosolic, monomeric oxidoreductase of broad specificity for carbonyl compounds, was found to be the main NADPH-dependent quinone reductase in human liver, whereas DT-diaphorase, the principal two-electron transferring quinone reductase in rat liver, contributed a very minor part to the quinone reductase activity of human liver. Carbonyl reductase from liver was indistinguishable from carbonyl reductase previously isolated from brain (B. Wermuth, *J. biol. Chem.* **256**, 1206 (1981)) on the basis of molecular weight, isoelectric point, immunogenicity, substrate specificity and inhibitor sensitivity. The purified enzyme from liver catalyzed the reduction of a great variety of quinones. The best substrates were benzo- and naphthoquinones with short substituents, and the K-region ortho-quinones of phenanthrene, benz(*a*)anthracene, pyrene and benzo(*a*)pyrene. A long hydrophobic side chain in the 3-position of the benzo- and naphthoquinones and the vicinity of a bay area or aliphatic substituent (pseudo bay area) to the oxo groups of the polycyclic compounds decreased or abolished the ability of the quinone to serve as a substrate. Non-k-region orthoquinones of polycyclic aromatic hydrocarbons were more slowly reduced than the corresponding K-region derivatives. The broad specificity of carbonyl reductase for quinones is in keeping with a role of the enzyme as a general quinone reductase in the catabolism of these compounds.

A number of quinones, including various quinonoid drugs, are supposed to exert toxic effects in biological systems via oxygen reduction. These compounds are reduced by single-electron transfer to the corresponding semiquinones which readily donate the electron to molecular oxygen, yielding superoxide anions and other cytotoxic, active oxygen species. Thus, a cycle is formed of O_2 uptake at the expense of cellular reducing equivalents, notably NADPH, generating further active oxygen species (reviewed in [1]).

Alternatively, the quinones may be reduced by two-electron transfer to the corresponding hydroquinone derivatives. In principle, these may also undergo autooxidation and generate active oxygen species. However, in the presence of second-phase detoxication enzymes, the hydroquinones are rapidly converted to the glucuronyl or sulfate conjugates, which prevents their reoxidation to quinones. As first postulated by Ernster and associates [2] enzymes catalyzing the two-electron reduction of quinones to hydroquinones may, therefore, protect the cell against the oxidative stress induced by quinonoid compounds by competing with the single-electron reduction pathways.

Probably the most widely distributed two-electron transferring quinone reductase is NAD(P)H: quinone oxidoreductase (EC 1.6.99.2), otherwise known as DT-diaphorase [3]. It occurs in animals, plants and bacteria, and it was from work on rat liver DT-diaphorase that Ernster and coworkers deduced their hypothesis.

Another enzyme which catalyzes the reduction of menadione and other quinones to the corresponding hydroquinones is carbonyl reductase (EC 1.1.1.184). It has been purified from human [4–6], pig [7, 8] and chicken tissues [9], and its chemical and physical properties have been investigated. Little, however, is known about its physiological role, although its partiality for quinones suggests that it may be involved in the metabolism of these compounds. In view of the postulated protective effect of two-electron transferring quinone reductases against quinone-induced oxidative stress, we have compared its distribution relative to that of DT-diaphorase in human liver and further investigated its specificity for quinones. We show that carbonyl reductase constitutes the major NADPH-dependent quinone reductase of human liver and provides the enzymatic basis for the reduction of a great variety of natural and man-made quinones.

MATERIALS AND METHODS

Materials

Human liver from both sexes was obtained from legal medical autopsies. The tissue was frozen 6–20 hr post mortem and stored at −20°. NADPH and dicoumarol were obtained from Sigma (St. Louis, MO), daunorubicin was purchased from Serva (Heidelberg, F.R.G.) and flavonoids were supplied by Roth (Karlsruhe, F.R.G.). The following compounds were gifts: menadione, vitamin K_1 and ubiquinones from F. Hoffmann-La Roche AG

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(Basel, Switzerland), prostaglandin E_1 from Wander AG (Bern, Switzerland) and mitomycin from Bristol-Myers (Syracuse, NY). Quinones which were not commercially available were synthesized as previously described [10, 11], with the exception of the quinones of chrysene and picene which were synthesized by different methods (Seidel *et al.*, manuscript in preparation). All other chemicals were obtained from Fluka (Buchs, Switzerland) or from Merck (Darmstadt, F.R.G.). Cellulose resins were obtained from Whatman (Maidstone, U.K.), Sephadex G-100 was purchased from Pharmacia (Uppsala, Sweden) and 8-(6-aminohexyl)-amino-2'-phosphoadenosine diphosphoribose-Sepharose was synthesized according to Lopez-Barea and Lee [12].

Methods

Enzyme assay. Quinone reductase activity was assayed spectrophotometrically by recording the change of NADPH absorbance at 340 nm. Reaction mixtures consisted of 0.1 M sodium phosphate buffer (pH 7.0), 0.08 mM NADPH and 0.2 mM menadione, and the reaction was started by the addition of enzyme. Controls without enzyme, NADPH or substrate were routinely included. Carbonyl reductase activity was estimated from the fraction of the total enzyme activity which was inhibited by 10 μM rutin. Similarly, DT-diaphorase activity was determined by including 1 μM dicoumarol in the assay medium (cf. Results).

Quinones tested as substrates for carbonyl reductase were assayed under the same conditions with the exception that the assay medium contained 1% bovine serum albumin. Quinones which were not soluble in water were dissolved in dimethyl sulfoxide. Aqueous stock solutions (50–200 μM) were prepared immediately before use by diluting the dimethyl sulfoxide solution 100-fold with 0.1 M sodium phosphate (pH 7.0), containing 1% bovine serum albumin. The final concentration of dimethyl sulfoxide in the assay mixture did not exceed 0.5% and had no effect on the enzyme activity as determined with menadione as substrate.

Purification of carbonyl reductase

The whole purification was carried out at 4°. Elution of u.v. light-absorbing material from chromatography columns was monitored using an Uvicord II (LKB Produkter AB).

Extraction. Human liver (250–300 g) was homogenized with the same amount of 50 mM Na_2HPO_4, containing 10 mM EDTA, 1% 2-mercaptoethanol and 50 mg phenylmethanesulfonyl fluoride (added immediately before homogenization), in a Waring blender. The homogenate was centrifuged for 30 min at 20,000 *g*, the lipid layer carefully removed and the remaining supernatant re-centrifuged for 90 min at 80,000 *g*.

Gel filtration. The second supernatant (maximum 250 ml) was applied to a Sephadex G-100 column (7 × 200 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0), containing 100 mM KCl and 1 mM EDTA. Fractions containing carbonyl reductase activity were pooled and dialyzed against 30 l. of 10 mM Tris/phosphate buffer (pH 7.4), containing 1 mM EDTA.

DEAE-cellulose chromatography. The dialyzed fractions were applied to a DEAE-cellulose column (2 × 40 cm) equilibrated with dialysis buffer. Carbonyl reductase did not bind to the ion exchanger under these conditions and was recovered in the eluate.

Affinity chromatography. The eluate was applied to a column (1 × 50 cm) of 8-(6-aminohexyl)-amino-2'-phosphoadenosine diphosphoribose-Sepharose equilibrated with 10 mM Tris/phosphate buffer (pH 7.4), containing 1 mM EDTA. The resin was washed with several column volumes of the same buffer, before elution of carbonyl reductase was initiated by the addition of 20 μM NADPH. Fractions containing carbonyl reductase activity were pooled and stored at 4°.

RESULTS

Liver contains a multiplicity of enzymes which, in the presence of reduced pyridine nucleotides, catalyze the reduction of menadione and other quinones to the corresponding semi- and hydroquinones. An estimate of the contribution of carbonyl reductase and DT-diaphorase to the total menadione reductase activity of the tissue was obtained from the decrease in the rate of reduction caused by the selective inhibitors rutin [4] and dicoumarol [3], respectively. The studies suggested, in agreement with previous findings [4], that carbonyl reductase activity accounts for up to 70% of the NADPH-dependent activity of human liver homogenate. For DT-diaphorase, on the other hand, a contribution of less than 10% was determined. Essentially the same proportions were obtained when liver homogenates from eight different preparations were subjected to gel filtration and the eluate was analyzed for menadione reductase activity (Fig. 1). Carbonyl reductase activity made up 55–70% of the applied activity, but no clearly definable dicoumarol-sensitive enzyme, represen-

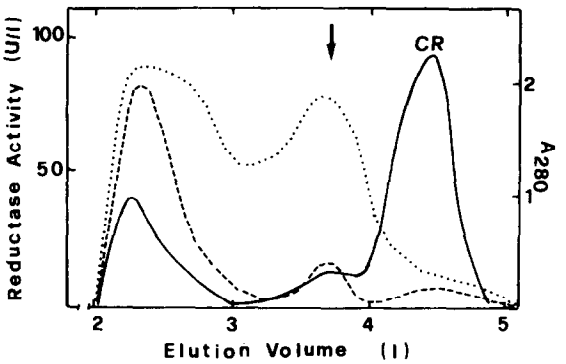

Fig. 1. Fractionation of human liver menadione reductase activity by gel filtration. Human liver homogenate (250 ml), prepared as described under Materials and Methods, was applied to a Sephadex G-100 column (7 × 200 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M KCl and 1 mM EDTA. Fractions of 15 ml were collected and assayed for menadione reductase activity. Solid line: NADPH-dependent activity; dashed line: NADH-dependent activity; dotted line: A_{280} (trace from Uvicord). CR: Carbonyl reductase. The arrow marks the position of DT-diaphorase from rat liver.

tative of DT-diaphorase, was detectable in the eluate. However, the occurrence in human liver of such an enzyme, which contributed a very minor part to the total quinone reductase activity, could be demonstrated by passing the homogenate over azodicoumarol-Sepharose as described for the purification of DT-diaphorase from rat liver [13]. In contrast to the findings with human tissue, DT-diaphorase made up the bulk of the NAD(P)H-dependent menadione reductase activity when rat liver homogenate was passed over Sephadex G-100, and no carbonyl reductase activity was detectable in the eluate. Thus, DT-diaphorase is the principal quinone reductase in rat liver, whereas carbonyl reductase appears to hold this position in human liver.

Further purification of carbonyl reductase from human liver by ion-exchange and affinity chromatography resulted in an enzyme preparation that contained no contaminating proteins as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Although carbonyl reductase has been purified from human liver before [5, 6], it has not systematically been compared with the enzyme from brain [4], and the degree of similarity between the two enzymes has not been established. Here we report the results of a comparative study. The liver enzyme consisted, as described for the brain enzyme, of multiple molecular forms which were indistinguishable from the corresponding forms of the brain enzyme by polyacrylamide gel electrophoresis and by isoelectric focusing (cf. [4]). Antibodies raised against the purified liver enzyme [14] showed complete cross-reactivity with the enzyme from brain on Ouchterlony plates. Moreover, the two enzymes exhibited essentially the same relative rates of reduction with menadione, phenylglyoxal, 4-nitrobenzaldehyde, hydrindantin, daunorubicin, prostaglandin E_2, 5α-dihydrotestosterone and dichlorophenolindophenol as substrates, and were equally susceptible to inhibition by the flavonoids rutin, quercetin and quercitrin (cf. [4]). In contrast to our previous report that ferricyanide is a substrate for the brain enzyme [4], no ferricyanide reductase activity was detectable with either the brain or the liver enzyme in this study. We suspect that a small amount of mercaptoethanol had accidentally been included in the assay medium in the previous study. Contrary to the report by Sawada and coworkers, however, who observed no reduction of dichlorophenolindophenol in the presence of human liver carbonyl reductase [6], this compound was a substrate for both enzymes. Thus, carbonyl reductase from brain and liver are indistinguishable on the basis of physiochemical properties, immunoreactivity, substrate specificity and sensitivity to inhibitors.

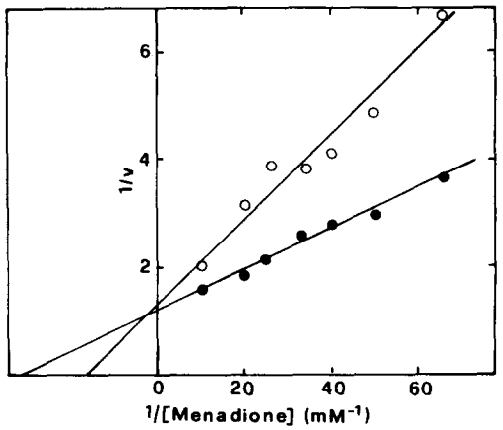

Fig. 2. Effect of bovine serum albumin on the reduction of menadione. Carbonyl reductase activity was assayed in 0.1 M sodium phosphate (pH 7.0) containing 0.08 mM NADPH and varying concentrations of menadione in the presence (○) and absence (●) of 1% bovine serum albumin.

To further characterize the enzyme and to investigate its capacity for serving as a general quinone reductase, a variety of natural and man-made quinones were tested as substrates for the liver enzyme (cf. Table 1). Many of the compounds are very little soluble in water. They could successfully be kept in solution in the aqueous assay medium by the addition of bovine serum albumin. The protein was superior to other solubilizers, e.g. the nonionic detergents Triton X-100 and Tween 80, and the organic solvents methanol and dimethylsulfoxide, which, at concentrations giving sufficient solubilization of the quinones, caused enzyme inactivation. The effect of albumin on the reduction of quinones was tested using menadione as substrate. Figure 2 shows that the maximum rate of quinone reduction was little affected, but that about twice the amount of substrate was needed to obtain half maximum activity in the presence of the protein. The increase in the K_m value most probably reflects a decrease in the concentration of free quinone due to its absorption by albumin [15]. In order to minimize the risk of false negative results because of too low concentrations of free substrate to produce a detectable photometric response, quinones yielding ambiguous results were also assayed in the presence of oxidized cytochrome *c* which rapidly reoxidizes the reduced quinones [3]. By this method we were able to measure the reduction of submicromolar concentrations of quinones.

Table 1 summarizes the results of the study. Generally, oxo groups at chemically reactive positions (K-region) were more efficiently reduced than those at more inert positions. The best substrates were the K-region ortho-quinones of the polycyclic aromatic hydrocarbons phenanthrene, pyrene, benz(*a*)anthracene and benzo(*a*)pyrene. Lower rates of reduction were obtained with compounds in which one carbonyl group of the quinone function is at the same time part of a bay region, e.g. chrysene-5,6-quinone, picene-5,6-quinone and dibenz(*a*, *h*)anthracene-5,6-quinone, or, as in the case of the 5,6-quinones of 7-methyl- and 7,12-dimethylbenz(*a*)anthracene, of an area which is similarly crowded because of a methyl substituent (pseudo bay region). Picene-13,14-quinone in which both carbonyl groups of the quinone are part of bay regions and 3-methylcholanthrene-11,12-quinone with a non-aromatic ring close to the dioxo group were very poor substrates. Table 2 lists the kinetic constants, K_m and k_{cat}, for a number of K-region *o*-quinones of polycyclic aromatic hydrocarbons.

Table 1. Specificity of carbonyl reductase for quinones

Quinone	Concentration (μM)	Relative activity (%)
1,4-Benzoquinone*	200	20
2-Methyl-1,4-benzoquinone (toluquinone)*	200	50
Ubiquinone-1*	200	115
Ubiquinone-3	130	58
Ubiquinone-4	110	8
Ubiquinone-10	50	n.d.†
1,2-Naphthoquinone*	200	100
1,4-Naphthoquinone*	200	65
2-Methyl-1,4-naphthoquinone (menadione)	250	100
Vitamin K_1	50	n.d.
1,2-Anthraquinone	30	95
9,10-Anthraquinone	15	22
1,2-Phenanthroquinone	20	9
3,4-Phenanthroquinone	25	11
9,10-Phenanthroquinone	50	140
1,4-Phenanthroquinone	70	90
Benz(*a*)-anthracene-1,2-quinone	12	5
Benz(*a*)anthracene-3,4-quinone	10	<2
Benz(*a*)anthracene-5,6-quinone	35	110
Benz(*a*)anthracene-8,9-quinone	30	18
7-Methylbenz(*a*)anthracene-5,6-quinone	15	7
7,12-Dimethylbenz(*a*)anthracene-5,6-quinone	20	11
Pyrene-4,5-quinone	45	130
Benzo(*a*)pyrene-4,5-quinone	40	95
Benzo(*a*)pyrene-7,8-quinone	30	5
Benzo(*a*)pyrene-9,10-quinone	25	3
Chrysene-1,2-quinone	20	4
Chrysene-3,4-quinone	20	2
Chrysene-5,6-quinone	50	70
Picene-1,2-quinone	18	<2
Picene-3,4-quinone	20	<2
Picene-5,6-quinone	42	30
Picene-13,14-quinone	20	<2
Dibenz(*a,h*)anthracene-5,6-quinone	40	17
3-Methylcholanthrene-11,12-quinone	18	<2
Daunorubicin (quinone)	200	n.d.
Mitomycin C	100	n.d.

Enzyme activity was determined as described under Materials and Methods. The activity obtained with menadione was arbitrarily set as 100%, corresponding to 2.3 U/mg protein.

* Assayed in the absence of bovine serum albumin.

† No activity detectable.

Table 2. Apparent kinetic constants K_m and k_{cat} of carbonyl reductase for K-region quinones of polycyclic aromatic hydrocarbons

Quinone	K_m (μM)	k_{cat} (sec^{-1})
9,10-Phenanthroquinone	4	1.9
Pyrene-4,5-quinone	10	1.9
Benz(*a*)anthracene-5,6-quinone	12	1.8
Benzo(*a*)pyrene-4,5-quinone	14	1.35
Chrysene-5,6-quinone	6	0.95
Picene-5,6-quinone	15	0.55
Dibenz(*a,h*)anthracene-5,6-quinone	16	0.3
7,12-Dimethylbenz(*a*)anthracene-5,6-quinone	9	0.2
7-Methylbenz(*a*)anthracene-5,6-quinone	13	0.15

Kinetic constants were estimated from direct linear plots according to Eisenthal and Cornish-Bowden [16]. The concentration of NADPH was 0.08 mM. K_m and k_{cat} for the standard substrate menadione under the same conditions were 67 μM and 1.35 sec^{-1}, respectively.

Although the Michaelis constants represent only apparent values, we can extrapolate that the true values are in the micromolar or even lower concentration range.

Among the non-K-region ortho-quinones, 1,2-naphthoquinone and 1,2-anthraquinone were the best substrates. Non-K-region *o*-quinones derived from angular polycycles generally were poor substrates. As an example, 1,2- and 3,4-phenanthroquinone were reduced at about 10% of the rate obtained with 1,2-anthraquinone. Even lower rates of reduction or essentially no detectable enzymatic activity were obtained with the structurally homologous 1,2- and 3,4-quinones of chrysene and picene. Benz(*a*)anthracene-7,8-quinone, in which the quinone function is apart from the angular ring, was more readily reduced by carbonyl reductase.

Among the para-quinones, benzo- and naphthoquinones, such as the model substrate menadione, were readily reduced. Substitution of the aromatic nucleus with methyl and methoxy groups or with one isoprene unit had little effect on the quinone's activity as electron acceptor, but the activity rapidly decreased with the chain length of the substituent in the 3-position, and the physiologically important derivatives ubiquinone-10 and vitamin K_1 were inactive. A marked decrease in the rate of reduction was also observed when an additional aromatic ring was added to the quinone-bearing ring of the naphthoquinone moiety. Structurally even more complex *p*-quinones, such as the antitumor drugs daunorubicin and mitomycin C, were not reduced. In this context it may be noted, however, that carbonyl reductase catalyzes the reduction of daunorubicin to daunorubicinol, a reaction affecting an aliphatic carbonyl group [5, 17].

DISCUSSION

Carbonyl reductase was first demonstrated in human brain by Ris and von Wartburg on the basis of its affinity for aromatic aldehydes, and designated as aldehyde reductase-1 [18]. Later, when the enzyme was found to catalyze the reduction of a variety of other carbonyl compounds, including quinones, the more general name of carbonyl reductase was suggested [4]. Reductases which closely resemble, and most probably are identical with, carbonyl reductase have been purified independently from placenta [19] and liver [5] in the course of studies on the metabolism of 9-ketoprostaglandins and the anthracycline antibiotic daunorubicin, respectively. Enzymes similar to carbonyl reductase from human tissues have also been isolated from pig [7, 8] and chicken [9] tissues and most probably also occur in the rabbit, monkey and pigeon (reviewed in [20]). On the other hand, as observed in this study, carbonyl reductase appears not to be present in rat liver. This finding is in agreement with earlier observations by Bachur and coworkers, who were unable to detect daunorubicin reductase (carbonyl reductase) in rat and mouse liver [17].

Although carbonyl reductase is widely distributed in animal tissues and catalyzes the reduction of a great variety of carbonyl compounds, its physiological role is not known. The enzyme most efficiently catalyzes the reduction of quinones, suggesting that it may take part in the metabolism of these compounds. Quinones play an important role as redox catalysts in several biological processes: ubiquinones are a constitutive part of the respiratory chain, and phylloquinones and menaquinones act as coenzymes in the carboxylation of glutamic acid to γ-carboxyglutamic acid in prothrombin and other proteins. The lack of activity of carbonyl reductase with long-chain ubiquinones and phylloquinones *in vitro* and the fact that *in vivo* these compounds are primarily associated with membranes, however, makes the participation of the enzyme in these processes unlikely. More promising in view of the enzyme's wide specificity for quinones is the hypothesis put forward by Ernster and colleagues that two-electron transferring quinone reductases function as a cellular device, protecting the cell against quinone-induced oxidative stress by converting the potentially toxic quinones to the reduced conjugatable excretion products [2]. Quinones which can form toxic intermediates are widely distributed in nature, and human exposure to them is extensive [21]. The quinones of polycyclic aromatic hydrocarbons, e.g. are abundant in all burnt organic material, including cigarette smoke and automobile exhaust [22], and may also arise *in vivo* from the parent hydrocarbons [23]. In the last few years experimental evidence has been presented that DT-diaphorase indeed protects rat and mouse cells against quinone-induced oxidative stress [24–27]. Whether carbonyl reductase fulfils a similar protective role in human tissues remains to be established. As a first step towards that goal we have investigated the specificity of the enzyme for quinones and show that it provides the enzymatic basis for the reduction to hydroquinones of many natural and man-made quinones.

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