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Purification and Catalytic Properties of a Tetrameric Carbonyl Reductase from Rabbit Heart

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An enzyme responsible for the ketone-reduction of 4-benzoylpyridine (4BP) was purified 350-fold to homogeneity from the cytosolic fraction of rabbit heart. The purified enzyme exhibited a molecular mass of 110 kDa on gel filtration, and 27 kDa on SDS-PAGE, indicating that it is a tetrameric protein composed of four identical-size subunits. Aromatic aldehydes, ketones, and menadione were effective substrates for the enzyme. Flavonoids were potent inhibitors of the enzyme, but barbiturates or pyrazole was not. Based on this substrate specificity and inhibitor sensitivity, the enzyme was taken to be a carbonyl reductase. Kinetic studies led us to conclude that the reduction of 4BP by the enzyme follows an ordered Bi Bi mechanism. The enzyme also appeared to catalyze the redox (oxidation-reduction) cycling of menadione to produce the superoxide radical. Furthermore, we provide evidence that a hydrophobic pocket, which corresponds to a straight-chain alkyl group of five carbon atoms in length, is located in the substrate-binding site of the enzyme.

Key words: carbonyl reductase, drug-metabolizing enzyme, rabbit heart, redox cycling, tetrameric enzyme.

Carbonyl reductase [EC 1.1.1.184], aldehyde reductase [EC 1.1.1.2], and aldose reductase [EC 1.1.1.21] catalyze the NADPH-dependent reduction of endogenous and exogenous carbonyl compounds to the corresponding alcohol metabolites (*1-3*). In particular, carbonyl reductase is known to play an important role in the reductive metabolism of many drugs such as acetohexamide, an oral antidiabetic drug, and daunorubicin, an anticancer drug, having a ketone group within their chemical structures (*1, 2*). Recently, carbonyl reductase, unlike aldehyde reductase and aldose reductase, which belong to the aldo-keto reductase superfamily (*4, 5*), was demonstrated to be a member of the short-chain dehydrogenase/reductase superfamily, based on its primary structure (*6-8*).

A variety of carbonyl reductases have been purified from the liver, kidney, testis, ovary, lung, and brain of mammalian species, and the catalytic properties of the purified enzymes have been examined (*9-18*). In addition to these tissues, the heart may contain carbonyl reductase, and carbonyl reductase may catalyze the redox (oxidation-reduction) cycling of quinones such as menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) to produce the superoxide radical in the heart (*19, 20*). However, little information about carbonyl reductase purified from the heart is available. In a preliminary study we found that both 4-benzoylpyridine (4BP), which is widely used as a substrate for carbonyl reductase, and acetohexamide are efficiently reduced in the cytosolic fraction of rabbit heart. Thus, it was of interest to purify carbonyl reductase from rabbit heart using 4BP or acetohexamide as a substrate. We have already purified a tetrameric enzyme from the cytosolic fraction of rabbit heart using acetohexamide as a substrate (*21*). Unexpectedly, the purified acetohexamide-reducing enzyme exhibited no ability to reduce 4BP (*21*), even though carbonyl reductases generally exhibit a broad substrate specificity for xenobiotic ketones and aldehydes. It is likely that the 4BP-reducing enzyme present in rabbit heart is distinguishable from the purified acetohexamide-reducing enzyme. The purpose of this study was to purify a new carbonyl reductase from the cytosolic fraction of rabbit heart using 4BP as a substrate, and to elucidate the catalytic properties of the purified enzyme.

MATERIALS AND METHODS

Materials—4BP was purchased from Wako Pure Chemicals (Osaka). Acetohexamide (Shionogi, Osaka), befunolol (Kaken Seiyaku, Tokyo), daunorubicin (Meiji Seika, Tokyo), and loxoprofen (Sankyo, Tokyo) were supplied by the respective manufacturers. Metyrapone was purchased from Aldrich (Milwaukee, WI, USA). 4-Acetylpyridine analogs were synthesized as described previously (*12*). Steroids and Cibacron Blue (3GA) were obtained from Sigma (St. Louis, MO, USA). Other substrates and inhibitors were purchased from Wako Pure Chemicals, Nacalai Tesque (Kyoto), and Tokyo Kasei (Tokyo). DEAE-Sephacel, Red Sepharose CL-6B, and Sephacryl S-200 and S-300

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Abbreviations: 4BP, 4-benzoylpyridine; 4BPH, reduction product of 4BP.

(high resolution) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and hydroxylapatite from Bio-Rad Laboratories Japan (Tokyo). NADPH, NADP, and NADH were obtained from Oriental Yeast (Tokyo). Protein markers were products of ICN Biomedicals (Aurora, OH, USA). Superoxide dismutase (human recombinant Cu,Zn-superoxide dismutase) was a gift from Nippon Kayaku (Tokyo), and cytochrome *c* was purchased from Sigma. All other chemicals were of reagent grade.

Synthesis of α-Phenyl-4-pyridylmethanol (4BPH)—4BPH (a reduction product of 4BP) was synthesized from 4BP by the method of Inui and Asada (*22*). A solution of $NaBH_4$ dissolved in methanol was added to a solution of 4BP dissolved in methanol by means of a dropping funnel. The mixture was stirred for 2 h at room temperature. The reaction mixture was acidified with some drops of acetic acid and then evaporated *in vacuo*. The residue was dissolved in water and then extracted with ethyl acetate. The extract was dried, filtered, and then evaporated to give 4BPH. The reduction product was recrystallized from water/methanol. The chemical structure of the reduction product was confirmed by elemental analysis, IR and NMR.

Determination of Partition Coefficients—The partition coefficients of 4-acetylpyridine and its analogs were determined according to the method of Hansch *et al.* (*23*).

Enzyme Purification—All procedures were performed at 3-5°C unless otherwise stated. Adult male rabbits (Japanese white strain) were killed by exsanguination. Their hearts were immediately removed, and homogenized in 3 volumes of 10 mM sodium potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol. The homogenate was centrifuged at $10,000 \times g$ for 20 min and the supernatant was recentrifuged at $105,000 \times g$ for 60 min. The resulting supernatant (cytosolic fraction) was dialyzed against 10 mM sodium potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer A), and then applied to a DEAE-Sephacel column (2.5×15 cm) equilibrated with 100 mM sodium potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer B). A linear salt gradient of 0 to 0.3 M NaCl was passed through the column to elute the bound proteins. The 4BP-reducing enzyme fractions were pooled, concentrated by ultrafiltration, dialyzed against 10 mM sodium potassium phosphate buffer (pH 6.4) containing 0.5 mM EDTA and 5 mM 2-mercaptoethanol (buffer C), and then applied to a Red Sepharose CL-6B column (1.5×25 cm) equilibrated with buffer C, followed by elution with a linear salt gradient of 0 to 3.0 M KCl. The enzyme fractions were pooled, concentrated by ultrafiltration, dialyzed against buffer A without EDTA, and then applied to a hydroxylapatite column (1.5×10 cm) equilibrated buffer A without EDTA. The enzyme was absorbed on the hydroxylapatite column and eluted with 250 mM sodium potassium phosphate buffer (pH 7.4) containing 5 mM 2-mercaptoethanol (buffer D). The enzyme fractions were pooled, dialyzed against buffer A, concentrated by ultrafiltration, and then applied to a Sephacryl S-300 column (1.5×50 cm) equilibrated with buffer A. The enzyme fractions were pooled, concentrated by ultrafiltration, and then stored at 2-4°C in 20% (v/v) glycerol until use for the present study. The purified enzyme was stable for at least one month under these conditions.

Estimation of Molecular Weights—The native molecular weight of the enzyme was estimated by gel filtration on a Sephacryl S-200 column (1.5×50 cm). The subunit molecular weight of the enzyme was determined by SDS-PAGE. SDS-PAGE was performed using Multi Gel 10/20 (Daiichi Pure Chemicals, Tokyo) according to the method of Laemmli (*24*).

Enzyme Assay—Enzyme activity was assayed spectrophotometrically by monitoring NADPH oxidation at 340 nm. The reaction mixture, in a total volume of 0.7 ml, consisted of 100 mM sodium potassium phosphate buffer (pH 6.0), 0.25 mM NADPH, the substrate at various concentrations, and the purified enzyme. To determine the optimal pH, the following 100 mM buffers were used (pH 4.5 to 5.5, citrate/sodium phosphate buffer; pH 5.5 to 8.0, sodium potassium phosphate buffer; pH 8.0 to 8.5, Tris/HCl buffer). The reaction was initiated by addition of the purified enzyme. One unit of the enzyme activity was defined as the amount causing a decrease in absorbance at 340 nm corresponding to the oxidation of 1 μmol of NADPH/min at 30°C. Protein concentrations were determined with bovine serum albumin as the standard by the method of Lowry *et al.* (*25*).

Kinetic Analysis—The K_m and V_{max} values of the enzyme for substrates were determined by means of least-squares linear regression of double reciprocal plots. The inhibition constants, K_{is} and K_{ii}, were determined from replots of the slopes and intercepts, respectively, of the double-reciprocal plots in the presence of inhibitors.

Determination of the Superoxide Radical—The superoxide radical was determined by the method of McCord and Fridovich (*26*) using cytochrome *c*. The absorbance of cytochrome *c* at 550 nm was measured in the enzyme reaction system described above.

RESULTS

Purification of the 4BP-Reducing Enzyme—We attempted to purify an enzyme from the cytosolic fraction of rabbit heart using 4BP as a substrate. When the cytosolic fraction of rabbit heart was applied to a DEAE-Sephacel column, the activity of the 4BP-reducing enzyme appeared as a single peak (Fig. 1). Furthermore, the activity peak was found to be completely separated from that of the acetohexamide-reducing enzyme, indicating clearly that different enzymes catalyze the ketone-reduction of 4BP and acetohexamide in the cytosolic fraction of rabbit heart. After the subsequent chromatographic steps, the 4BP-reducing enzyme was purified 350-fold in terms of specific activity, with a yield of 33%, from the cytosolic fraction of rabbit heart (Table I). The enzyme obtained at the final step was a homogeneous protein on SDS-PAGE, as shown in Fig. 2.

Molecular Weight—The molecular weight of the purified enzyme was estimated to be 27 kDa by SDS-PAGE and 110 kDa by gel filtration on Sephacryl S-200. These results suggest that the native form of the enzyme is composed of four identical-size subunits.

Substrate Specificity—The purified enzyme required NADPH as a cofactor. The K_m value for the oxidation of NADPH by the enzyme was 6.09 ± 0.96 μM (mean ± SD, $n=3$) in the presence of 1.0 mM 4BP, whereas the enzyme activity was not detected when NADPH was replaced by NADH. The optimum pH of the enzyme for the reduction of

4BP was around 6.0. The enzyme did not catalyze the reverse reaction from 4BPH to 4BP at pH 6.0.

Table II summarizes the substrate specificity of the enzyme for various carbonyl compounds. The enzyme effectively reduced aromatic aldehydes (pyridine-3-aldehyde and pyridine-4-aldehyde), ketones (benzoylpyridines, 4-acetylpyridine and 4-nitroacetophenone) and menadione. Of the acetylpyridines and nitroacetophenones tested, only 4-acetylpyridine and 4-nitroacetophenone, respectively, were effective substrates for the enzyme. However, the enzyme had no ability to reduce D-xylose and D-glucuronic acid, which are representative substrates for aldose reductase and aldehyde reductase, respectively. As expected, acetohexamide was not reduced by the enzyme. Furthermore, the drugs with a ketone group (befunolol, metyrapone, daunorubicin, and loxoprofen) or steroids (4-androstene-3,17-dione, 5α-androstane-3,17-dione, 5β-androstane-3,17-dione, 5α-androstan-17β-ol-3-one, 5α-androstan-3α-ol-17-one, and 5α-androstan-3β-ol-17-one) tested in this study were inactive as substrates for the enzyme.

Inhibitory Effects—Table III shows the effects of various inhibitors on the purified enzyme. 4BP was used as a substrate, and the concentrations of the inhibitors were as reported previously (*21*). Flavonoids (quercetin and quercitrin), well-known inhibitors of carbonyl reductase, strongly inhibited the enzyme. Quercetin inhibited the enzyme more strongly than quercitrin (quercetin-3-L-rhamnoside), suggesting the contribution of hydrophobicity to their inhibitory effects. On the other hand, pyrazole, a potent inhibitor of alcohol dehydrogenase, and barbiturates (barbital and phenobarbital), specific inhibitors of aldehyde

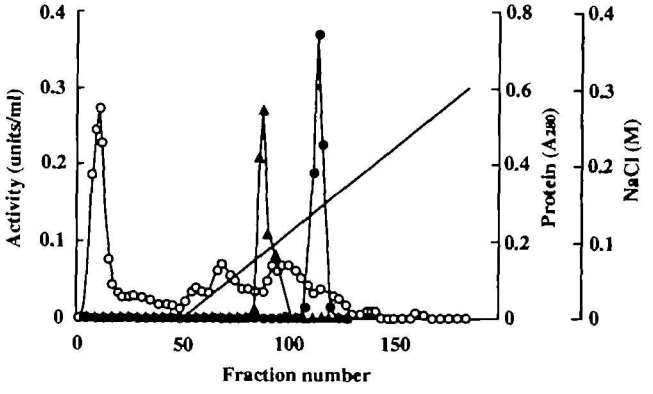

Fig. 1. **Elution pattern of two enzyme activities on a DEAE-Sephacel column.** The DEAE-Sephacel column was eluted with a linear 0-0.3 M NaCl gradient. Each fraction was analyzed for protein (○), 4BP-reducing enzyme activity (▲), and acetohexamide-reducing enzyme activity (●).

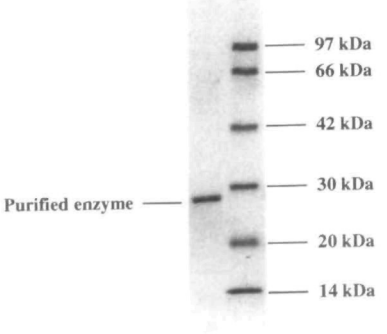

Fig. 2. **SDS-PAGE of the purified enzyme.** The molecular weight markers used were phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

TABLE II. **Substrate specificity of the purified enzyme for ketones, aldehydes, and menadione.** Values are the means±SD for three experiments. The values in parentheses indicate the activity with 1.0 mM substrate.

Substrate	K_m (mM)	V_{max} (units/mg)
D-Xylose	–	(0)
D-Glucuronic acid	–	(0)
D-Glucuronolactone	–	(0)
DL-Glyceraldehyde	–	(0)
Pyridine-3-aldehyde	1.80±0.31	3.64±0.13
Pyridine-4-aldehyde	0.90±0.28	10.5±2.60
2-Benzoylpyridine	1.70±0.16	0.73±0.04
3-Benzoylpyridine	0.33±0.03	0.76±0.04
4-Benzoylpyridine (4BP)	0.63±0.02	2.98±0.07
2-Acetylpyridine	–	(0.01)
3-Acetylpyridine	–	(0.01)
4-Acetylpyridine	1.55±0.20	1.76±0.13
2,6-Diacetylpyridine	–	(0.14)
2-Nitroacetophenone	–	(0.09)
3-Nitroacetophenone	–	(0)
4-Nitroacetophenone	0.59±0.03	1.73±0.07
Acetophenone	–	(0.09)
Menadione	0.18±0.01	2.47±0.03

TABLE III. **Effects of various inhibitors on the purified enzyme.** Values are the means±SD for three experiments. 4BP (1.0 mM) was used as the substrate.

Inhibitor	Concentration (mM)	Inhibition (%)
Pyrazole	10	17.1±2.4
Quercetin	0.05	88.1±1.5
Quercitrin	0.05	56.0±1.0
Barbital	1	15.6±5.2
Phenobarbital	1	20.1±5.9
$HgCl_2$	0.01	1.2±0.0
$CuSO_4$	0.01	9.9±2.0

TABLE I. **Purification of the 4-benzoylpyridine-reducing enzyme from rabbit heart.**

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Cytosol	2,014	14.7	0.007	1.0	100
DEAE-Sephacel	251	13.4	0.053	7.3	91
Red Sepharose CL-6B	29.3	10.6	0.361	49	72
Hydroxylapatite	2.94	6.67	2.26	309	45
Sephacryl S-300	1.87	4.78	2.55	350	33

reductase, exhibited no significant inhibitory effect against the enzyme. $HgCl_2$ had no ability to inhibit the enzyme. In addition, $CuSO_4$ had little effect on the enzyme activity, although it strongly inhibited the carbonyl reductases purified from rabbit liver and kidney at the same concentration (*12, 27*).

Kinetics Mechanism—Initial velocity analyses of the purified enzyme were performed as to the reduction of 4BP. When NADPH was the variable substrate and 4BP was kept constant at concentrations of 0.2-1.0 mM, double-reciprocal plots of the initial velocity against NADPH gave a series of intersecting lines (Fig. 3A). Replots of the slopes and intercepts against the reciprocals of fixed 4BP concentrations were linear, as shown in Fig. 3B. Similar initial velocity analysis results were obtained when 4BP was the variable substrate. These results indicate that the reduction of 4BP catalyzed by the purified enzyme proceeds through a sequential mechanism, in which both substrates (4BP and NADPH) bind to the enzyme prior to the release of the products.

In order to obtain more information on the kinetic mechanism of the purified enzyme, the product inhibition patterns were examined with 4BPH and NADP as inhibitors, and 4BP and NADPH as variable substrates. Table IV summarizes the product inhibition patterns and the inhibition constants. When 4BPH was used as the product

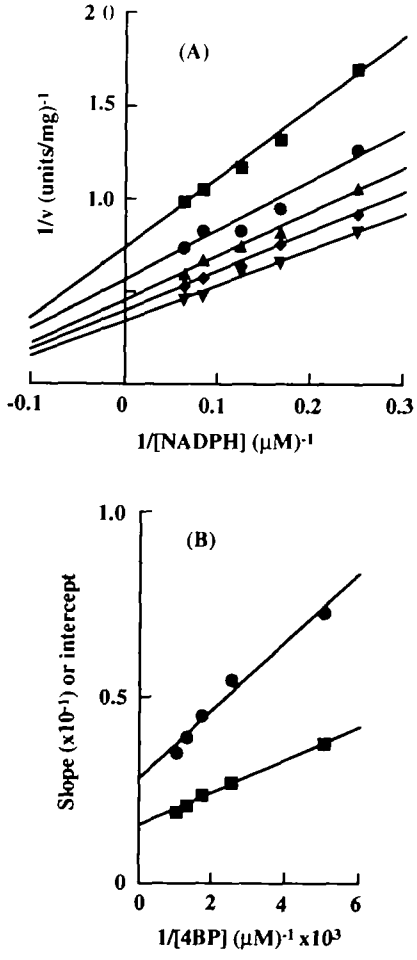

Fig. 3. **Initial velocity analysis of the purified enzyme.** (A) The 4BP concentrations used were 0.2 mM (■), 0.4 mM (●), 0.6 mM (▲), 0.8 mM (◆), and 1.0 mM (▼). (B) Replots of the slope (●) and intercept (■).

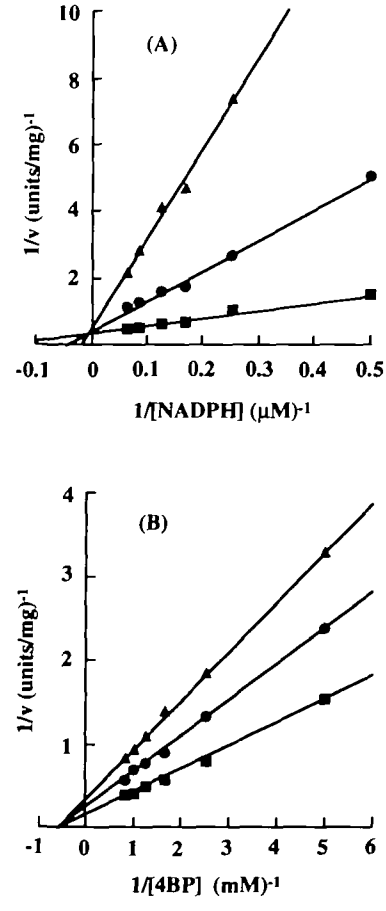

Fig. 4. **Dead-end inhibition of the purified enzyme by Cibacron Blue.** (A) The concentration of 4BP was 1.0 mM. (B) The concentration of NADPH was 0.25 mM. The concentrations of Cinacron Blue were 0 μM (■), 0.5 μM (●), and 1.0 μM (▲).

TABLE IV. **Product inhibition patterns for the reduction of 4BP catalyzed by the purified enzyme.** The inhibition constants, K_{is} and K_{ii}, were determined from replots of the slopes and intercepts, respectively, of double-reciplocal plots with the inhibitors, and are expressed as the means for duplicate analyses.

Inhibitor	Varied substrate	Fixed substrate	Inhibition pattern	Inhibition constant	
				K_{is} (μM)	K_{ii} (μM)
NADP	4BP	10 μM NADPH	Noncompetitive	115	68
NADP	NADPH	1 mM 4BP	Competitive	58	–
4BPH	4BP	10 μM NADPH	Noncompetitive	3,140	5,410
4BPH	NADPH	1 mM 4BP	Noncompetitive	7,200	7,010

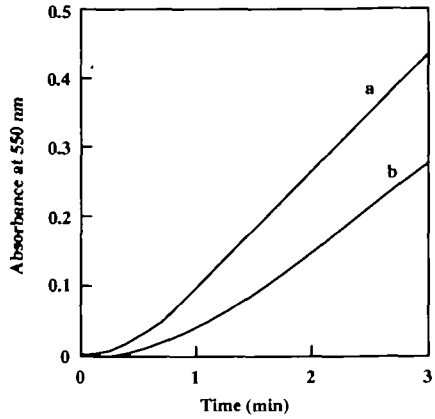

Fig. 5. **Menadione-mediated reduction of ferricytochrome *c* in the reaction system of the purified enzyme.** The reaction mixture, in a final volume of 0.7 ml, consisted of 100 mM sodium potassium phosphate buffer (pH 6.0), 0.1 mM NADPH, 0.1 mM EDTA, 50 μM cytochrome c, and 1.0 μg enzyme protein. The reaction was started by the addition of 50 μM menadione. The reduction of ferricytochrome *c* to ferrocytochrome *c* in the complete reaction system was measured by recording the absorbance at 550 nm. Superoxide dismutase (100 units) was added to the complete reaction system. a, complete reaction system; b, complete reaction system + superoxide dismutase.

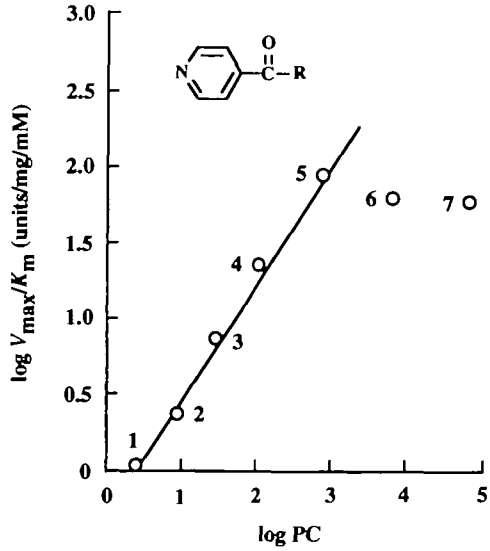

Fig. 6. **Relationship between the common logarithm of the V_{max}/K_m values of the purified enzyme for 4-acetylpyridine and its analogs and the common logarithm of their partition coefficients.** R: 1, methyl (4-acetylpyridine); 2, ethyl; 3, *n*-propyl; 4, *n*-butyl; 5, *n*-pentyl; 6, *n*-hexyl; 7, *n*-heptyl. PC, partition coefficient in *n*-octanol/0.1 M sodium potassium phosphate buffer (pH 6.0).

inhibitor, the enzyme was inhibited noncompetitively with respect to 4BP at a nonsaturating concentration of NADPH and with respect to NADPH at a nonsaturating concentration of 4BP. Furthermore, NADP was a noncompetitive inhibitor with respect to 4BP at a nonsaturating concentration of NADPH, whereas it was a competitive inhibitor with respect to NADPH at a nonsaturating concentration of 4BP. The product inhibition patterns of the purified enzyme were consistent with that for an ordered Bi Bi mechanism, as described by Cleland (*28, 29*).

Cibacron Blue interacts with the coenzyme-binding sites of many NAD(P)H-dependent enzymes and is widely used as a dead-end type inhibitor (*30-32*). As shown in Fig. 4, Cibacron Blue was found to inhibit the purified enzyme competitively with respect to NADPH and noncompetitively with respect to 4BP. These results also support the idea that the reduction of 4BP catalyzed by the purified enzyme follows an ordered Bi Bi mechanism.

Formation of the Superoxide Radical—The redox cycling of quinones is known to produce the superoxide radical. Menadione is a quinone compound and an effective substrate for the enzyme purified from rabbit heart, as described above. Thus, we examined whether or not the reduction of menadione catalyzed by the enzyme is associated with the formation of the superoxide radical. As shown in Fig. 5, the absorbance of cytochrome *c* at 550 nm increased with time in the complete enzyme reaction system, indicating that ferricytochrome *c* (Fe^{3+}) was reduced to ferrocytochrome *c* (Fe^{2+}). However, the reduction of ferricytochrome *c* was not induced in the enzyme reaction system without the enzyme or menadione. Furthermore, superoxide dismutase was found to inhibit the reduction of ferricytochrome *c*. Based on these results, we conclude that the enzyme purified in this study catalyzes the redox cycling of menadione thereby producing the superoxide radical.

Structural Requirements for 4-Acetylpyridine Analogs—4-Acetylpyridine analogs substituted with a straight-chain alkyl group instead of the methyl group were synthesized, and the structural requirements of the purified enzyme for these synthesized substrates were examined. Figure 6 shows the relationship between the common logarithm of the V_{max}/K_m values of the enzyme for 4-acetylpyridine and its analogs and the common logarithm of their partition coefficients. A significant regression line was obtained from the plots for 4-acetylpyridine and its analogs with a straight-chain alkyl group of up to five carbon atoms. However, the plots for 4-acetylpyridine analogs with a straight-chain alkyl group of six and seven carbon atoms were distant from the regression line. It is reasonable to assume that a hydrophobic pocket, which corresponds to a straight-chain alkyl group of five carbon atoms in length, is located in the substrate-binding site of the enzyme.

DISCUSSION

A 4BP-reducing enzyme was purified to homogeneity from the cytosolic fraction of rabbit heart. The 4BP-reducing enzyme is a tetrameric protein with a molecular mass of 110 kDa. Furthermore, judging from its substrate specificity and specific inhibitor sensitivity, the 4BP-reducing enzyme was deduced to be a tetrameric carbonyl reductase. So far, most carbonyl reductases purified from the liver, kidney, testis, ovary, and brain are known to be monomeric enzymes (*9-14, 18*). Thus, it is noteworthy that the heart carbonyl reductase is a tetrameric enzyme. We recently purified a tetrameric enzyme responsible for the ketone-reduction of acetohexamide from the cytosolic fraction of rabbit heart (*21*). However, the tetrameric acetohexamide-reducing enzyme was found to be clearly different from the tetrameric carbonyl reductase purified in the present study. Nakayama *et al.* (*15-17*) have purified some tetrameric carbonyl reductases from the lungs of guinea pig,

mouse, and pig. These lung carbonyl reductases were potently inhibited by pyrazole, whereas the heart carbonyl reductase was inhibited little by pyrazole. The lung enzymes, unlike the heart enzyme, utilized both NADPH and NADH as the cofactor. The lung carbonyl reductases are probably distinguishable from the heart carbonyl reductase. Therefore, it is concluded that the enzyme purified from rabbit heart in the present study is a new tetrameric carbonyl reductase.

The results obtained in the initial-velocity, product inhibition and dead-end type inhibition experiments led us to the assumption that the reduction of 4BP catalyzed by the carbonyl reductase purified from rabbit heart follows an ordered Bi Bi mechanism, in which NADPH binds to the enzyme first and NADP leaves the enzyme last. This type of mechanism has been proposed for human brain carbonyl reductase (*33*). We have also demonstrated that the rabbit liver and kidney carbonyl reductases catalyze the ketone-reduction of acetohexamide through the same kinetic mechanism (*34, 35*). In many cases, racemic reduction products have been used as substrates in product inhibition experiments. In the present study, we used a racemate of 4BPH as an inhibitor. However, xenobiotic ketones have been reported to be stereoselectively converted to the corresponding reduction products through enzyme reactions (*36-38*). Thus, use of the optically active 4BPH is preferable in the product inhibition experiment. We are currently investigating whether (−)-4BPH or (+)-4BPH is produced on the reduction of 4BP catalyzed by the purified enzyme.

The carbonyl reductase purified from rabbit heart appeared to be associated with the generation of the superoxide radical through the redox cycling of menadione in the presence of NADPH. It should be noted in connection with the cardiotoxicity of quinones that the heart carbonyl reductase functions as a quinone reductase. Quinone reductases are divided into two groups. One-electron transfer reductases lead to redox cycling with the formation of semiquinones; NADPH-cytochrome P450 reductase [EC 1.6.1.4] is a one-electron transfer reductase. Two-electron transfer reductases, unlike one-electron transfer reductases, catalyze the reduction of quinones to the corresponding hydroquinones. A well-known two-electron transfer reductase is NAD(P)H:quinone oxidoreductase (DT-diaphorase) [EC 1.6.99.2]. In general, hydroquinones formed on two-electron reduction of quinones are thought to be relatively stable as to autoxidation. However, recent studies have provided evidence that carbonyl reductases catalyze two-electron reduction and mediate the redox cycling of various quinones (*19, 20*). For example, Jarabak (*19*) has shown that human placental carbonyl reductase catalyzes the redox cycling of menadione through a two-electron transfer mechanism. Consequently, it is possible to assume that the rabbit heart carbonyl reductase purified in the present study catalyzes the redox cycling of menadione through a mechanism similar to that in the case of the human placental carbonyl reductase.

The present study provides evidence that a hydrophobic pocket, which corresponds to a straight-chain alkyl group of five carbon atoms in length, is located in the substrate-binding site of the carbonyl reductase purified from rabbit heart. A similar result was obtained for the monomeric carbonyl reductases from rabbit liver and kidney (*12, 39*). Hara *et al.* (*10*) have also proposed the presence of a hydrophobic pocket with a chain length of four carbon atoms in the substrate-binding site of the dimeric carbonyl reductase from dog liver. More recently, we demonstrated that the acetohexamide-reducing enzyme, which was isolated from rabbit heart using acetohexamide as a substrate, lacks such a hydrophobic pocket, but has a strongly hydrophobic cleft at the opposite position across the catalytic site (*40*). Further studies are in progress to determine whether or not a strongly hydrophobic cleft, in addition to the hydrophobic pocket with a chain length of five carbon atoms, is located in the vicinity of the substrate-binding site of the carbonyl reductase purified from rabbit heart.

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