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Activities and Kinetic Mechanisms of Native and Soluble NADPH–Cytochrome P450 Reductase

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Native yeast NADPH–cytochrome P450 oxidoreductase (CPR; EC 1.6.2.4) and a soluble derivative lacking 33 amino acids of the NH_2-terminus have been overexpressed as recombinant proteins in *Escherichia coli*. The presence of a hexahistidine sequence at the N-terminus allowed protein purification in a single step using nickel-chelating affinity chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis confirmed the predicted molecular weights of the proteins and indicated a purity of >95%. Protein functionality was demonstrated by cytochrome *c* reduction and reconstitution of CYP61-mediated sterol Δ^{22}-desaturation. Steady-state kinetics of cytochrome *c* reductase activity revealed a random Bi-Bi mechanism with NADPH donating electrons directly to CPR to produce a reduced intermediary form of the enzyme. The kinetic mechanism studies showed no difference between the two yeast CPRs in mechanism or after reconstitution with CYP61-mediated 22-desaturation, confirming that the retention of the NH_2-terminable membrane anchor is functionally dispensable.

Key Words: **NADPH–cytochrome P450 oxidoreductase; cytochrome P450; purification; reconstitution; reaction mechanism.**

NADPH–cytochrome P450 oxidoreductase (CPR; EC 1.6.2.4) is a membrane-bound flavoprotein involved with cytochrome P450 (CYP) in hydroxylation of its substrates by transferring electrons from NADPH to the CYP molecule (1, 2). In fungi, individual CYP substrates can be pollutants (for review, 3) or steroids (4); additionally, CYPs can be biosynthetic enzymes of, for instance, terpene, alkaloid, aflatoxin or sterol biosynthesis (for review, 5). CPR also has the ability to transfer electrons to several other heme proteins (heme oxygenase, cytochrome *c* and cytochrome b_5), potassium ferricyanide, therapeutically important compounds such as anticancer drug mitomycin *c* and the herbicide paraquat (for review, 6). As noted above CPR participates in sterol biosynthesis and in the yeast *Saccharomyces cerevisiae* is defined as Ncrp1p donating electrons to squalene epoxidase, sterol 14α-demethylase (CYP51) and sterol Δ^{22}-desaturase (CYP61) (7).

Yeast cytochrome $P450_{14dm}$ (sterol 14α-demethylase; CYP51) catalyzes the removal 14α-methyl group from lanosterol in the ergosterol biosynthetic pathway and is the target for azole antifungal drugs, which are commonly used in agriculture and medicine (8). Two other enzymes of yeast ergosterol biosynthesis require CPR, CYP61 a sterol 22-desaturase (9–11) and a non-CYP monooxygenase, squalene epoxidase (12). The former may also be present in other Kingdoms of Life where sterol 22-desaturation is observed unlike in animals, but the latter is present in all organisms producing sterol (13). A yeast *NCPR1* gene disruption is not lethal, unlike *CYP51* gene disruption (14), indicating the presence of an alternative electron donating system (7). However *NCPR1* gene disrupted strains are 200-fold more sensitive to ketoconazole (azole drug) than the non-disrupted strain (15). This indicates the reductase may have an indirect involvement in tolerance of fungus to azole drugs.

Here the proteins were produced as N-terminally appended his-tagged derivatives following heterologous expression in *Escherichia coli*, purified to homogeneity and demonstrated both to be catalytically active in cytochrome *c* reduction and reconstitution of ergosterol biosynthesis in cell lysates of a CPR disrupted yeast strain. Understanding the structure/function/mechanism of this enzyme at the molecular level will help in the development of antifungal drugs blocking essential electrons transfer during ergosterol biosynthesis, to understand the mechanism of fungal CYP mediated xenobiotic metabolism and to unravel

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the mechanisms for reductive metabolism of pesticides and pollutants.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemical Company (Poole, UK), unless otherwise stated. Chemicals for the preparation of phosphate buffers were obtained from Fisher Scientific (Loughborough, UK). Sodium cholate was obtained from Acros Fine Chemicals (Loughborough, UK).

Cloning of native and soluble yeast CPRs for expression in E. coli. CPR:pET15b expression vector was constructed by subcloning a 2.1 kb *Sal*I–*Hin*dIII fragment containing *CPR* gene, released from CPR:YEp51 (16), into *Xho*I–*Bam*HI site of pET15b using a 0.35 kb *Hin*dIII–*Bam*HI fragment, obtained from YEp51 plasmid, as a linker. Similarly CPR (Δ33):pET15b expression vector was produced by subcloning a 2.3 kb *Sal*I–*Bcl*I fragment containing CPR (Δ33) gene, released from CPR (Δ33):YEp51 (16), into the unique *Xho*I–*Bam*HI site of the *E. coli* expression vector, pET15b. All recombinant DNA manipulations and bacterial transformations were carried out as described previously (17).

Overexpression and purification of native and soluble yeast CPRs in E. coli. Protein expression was carried out by following the procedure described previously (18). The expression vectors were transformed into BL21DE3 (pLysS) strain. The transformed strains were grown overnight at 37°C and with shaking at 150 rpm in Luria broth (LB) containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). Heterologous protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 20 h at 25°C and with shaking at 190 rpm after the cell density had reached to an optical density of 0.5–0.7 at 660 nm. The cells were harvested at 4°C by centrifugation at 5000*g* for 10 min. The cell pellet of 1 L culture was resuspended in 40 ml of buffer A (50 mM KPi [pH 7.5] buffer containing 0.5 M NaCl). Cells were lysed by freeze–thawing. The cell lysate were centrifuged at 4°C for 10 min at 5000*g* to remove cell debris and then at 100,000*g* at 4°C for 60 min to separate membrane fractions (pellet) from cytosolic fractions (supernatant). The membrane fractions were resuspended in buffer A. Protein concentration was determined by the bicinchoninic acid method (BCA; Sigma) using bovine serum albumin standards.

Purification of native and soluble CPR. His_6-tagged native and soluble CPR were purified by a single step using nickel-chelating affinity chromatography. Membrane bound CPR was solubilized with 2% (w/v) sodium cholate as described previously (19). Both solubilized, native CPR and the cytosolic fraction containing soluble CPR were applied onto a 5 ml Ni^{2+}-NTA affinity chromatography column (Qiagen) previously equilibrated with 5 volumes of binding buffer (20 mM Tris–HCl, pH 7.5, containing 500 mM NaCl and 50 mM glycine). The column was washed with 10 volumes of binding buffer and 10 volumes of wash buffer (binding buffer containing 20 mM imidazole). Highly purified CPR protein was obtained when a linear gradient of 5 to 100 mM imidazole (10 ml each of wash buffer and elution buffer) was used for elution. The fractions containing purified native and soluble CPR (monitored by cytochrome *c* reduction assay) were pooled, dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.5, concentrated by ultrafiltration using 30 kDa membrane (Whatman) to 30 mg/ml and stored at −80°C. All steps were carried out at 4°C and 1 ml/min flow rate. SDS–PAGE was performed as described by Laemmli (12). A set of SDS–protein standards (Sigma) were used and consisted of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). The electrophoresed gel was stained with Coomassie Blue R-250 to visualize protein bands.

Reconstitution of Sterol Δ^{22}-desaturase activity with native and soluble CPR. Yeast cytochrome sterol Δ^{22}-desaturase (CYP61) was purified from yeast microsomal fractions as described previously (10). Each reaction mixture contained 0.5 nmol purified CYP61 and 1U of purified native or soluble yeast CPR in a total volume of 50 μl. To this, 50 μg dilauroylphosphatidylcholine (DLPC) was added and the reaction volume adjusted to 950 μl with 100 mM potassium phosphate buffer, pH 7.2. Ergosta-5,7-dienol was added at the appropriate concentration and the mixture sonicated until a white suspension had formed. Ergosta-5,7-dienol was purified from a polyene-resistant *erg5* mutant of *S. cerevisiae* (20). NADPH was added to the mixture to start the reaction. All reactions were incubated at 37°C for 20 min in a shaking water bath. In control experiments, the involvement of CPR was examined by omission of P450, NADPH or CPR to the reconstituted system. Sterol substrate and metabolite were extracted following the addition of 3 ml methanol, 2 ml (0.5% w/v) pyrogallol in methanol and 2ml 60% (w/v) potassium hydroxide (in water) and incubation at 90°C for 2 h in a preheated water bath. After cooling the saponified mixture was extracted with 3 × 5 ml hexane and dried under nitrogen. A Hewlett/Packard GC/MS was used to confirm sterol identities. An Ultra 1 capillary column was used (10 m × 0.2 mm) on a temperature program 50°C (1 min) increased by 40°C/min to 290°C with a run time of 17 min. Injection port temperature was 280°C (splitless) and the carrier gas was helium at 40 kPa.

Determination of the kinetic mechanism of native and soluble yeast CPR. Kinetics assays relied on the change in absorbance at 550 nm when oxidized cytochrome *c* is converted into reduced cytochrome *c* with an extinction coefficient of 21.1 mM^{-1}. The kinetics assay system contained an enzymatic NADPH regeneration system and consisted of 0.1 M Tris–HCl, pH 7.8, 2 mM glucose-6-phosphate, 3 units of glucose-6-phosphate dehydrogenase (Sigma) in a total volume of 1 ml. The cytochrome *c* concentration was 50 μM when NADPH was the variable substrate and the NADPH concentration was 50 μM when cytochrome *c* was the variable substrate. The change in absorbance at 550 nm was monitored against time at 26°C. Protein contents of 212 and 41 ng were used per assay for the native and soluble enzymes. To determine the enzyme mechanism of each CPR, substrate saturation experiments were performed varying the cytochrome *c* concentration at three different fixed NADPH concentrations of 2.5, 5, and 50 μM. Velocities are expressed as picomoles reduced cytochrome *c* produced per minute per picomole of enzyme. Kinetic parameters were determined by nonlinear regression using the Michaelis–Menten equation to determine k_m and v_{max} values. Linear regression was used to analyze the Lineweaver–Burk plots constructed for the enzyme mechanism studies. The analyses were performed using ProFit 5.01 (QuantumSoft, Zurich, Switzerland).

RESULTS

Expression of Native and Soluble Yeast CPR Proteins in E. coli

The yeast CPR contents were determined by using the cytochrome *c* reduction assay. As expected native CPR localized in the membrane fraction of *E. coli* and the amino-terminal truncated and soluble CPR form located in the cytosolic fraction (also confirmed by Western blot, not shown). The specific contents and yields are shown in Table 1. Soluble CPR protein expressed at very high levels compared to the native CPR protein. No CPR was detected in the cytosolic fractions of *E. coli* expressing native CPR protein. The results clearly indicated that the N-terminal truncated yeast CPR localized in the cytosol following expression in a

TABLE 1

The Specific Contents and Protein Yields of Native and Soluble CPR Following Heterologous Expression in *Escherichia coli*

	Amount (nmol)		
	per mg protein		
CPR	Cytosol	Membranes	per L culture
Native	ND	0.02 ± 0.005	7.5 ± 2.1
Soluble	0.35 ± 0.06	ND	112 ± 11.7

similar manner to previous reports for mammalian CPR following trypsin-cleavage experiments (21).

Purification and Characterization of Native and Soluble Yeast CPR

SDS–PAGE of purified native and soluble CPRs (Fig. 1) revealed the soluble enzyme consisted of predominantly (ca. 95%) one polypeptide of M_r 75 kDa and that the native CPR contained one major polypeptide of M_r 81 kDa. Both purified CPRs were yellow in color, indicating the presence of flavin. Further confirmation was obtained through UV/visible spectral analysis. The native and soluble CPRs produced a spectrum with maximal absorption at 455 and 380 nm. Addition of NADPH to pure CPR followed by air reoxidation (by incubating at room temperature for 10 min) resulted in a decrease in the absorption at 455 nm and the appearance of a broad absorption band 550–650 nm characteristic of the air-stable semiquinone (not shown). The oxidized and reduced spectra of both CPRs were indistinguishable from those of other microsomal CPRs (22, 23).

Purified native and soluble CPR were assayed by their ability to reduce cytochrome *c*. The specific activity of the purified native CPR in reducing cytochrome *c* was 52 μmol/min/mg protein. This value is consistent with published values for CPRs purified from various organisms, i.e., 50–60 μmol/min/mg protein (for review, 6). The specific content of reductase was calculated as 17 nmol/mg protein (by assuming 1 nmol CPR reduces 3 μmol of cytochrome *c*).

Sterol Δ^{22}-Desaturase (CYP61) Activity Following Reconstitution with Native and Soluble CPR

Ergosta-5,7-dienol was aerobically metabolized by a reconstituted monooxygenase system containing CYP61 and either native and soluble yeast CPR. Soluble CPR could drive CYP61 mediated sterol Δ^{22}-desaturation giving a k_m of 25 μM and a maximal enzymatic rate (v_{max}) of 3.1 nmol ergosterol formed/min/nmol CYP61. These values compared with a k_m of 20 μM and a maximal enzymatic rate (v_{max}) of 2.1 nmol ergosterol formed/minute/nmol CYP61 for sterol Δ^{22}-desaturation driven by native CPR. No significant difference in k_m was observed coupled with only a slight reduction in CYP61 catalytic activity. The identity of the sterol peaks in gas chromatography were confirmed by mass spectroscopy and showed conversion of the sterol substrate into ergosterol. Control experiments showed P450, CPR and NADPH dependency for the reaction.

Kinetic Mechanism Determination for Native and Soluble Yeast CPR

Both yeast native and soluble CPRs obeyed Michaelis–Menten kinetics with respect to both cytochrome *c* and NADPH (Fig. 2). The kinetic parameters

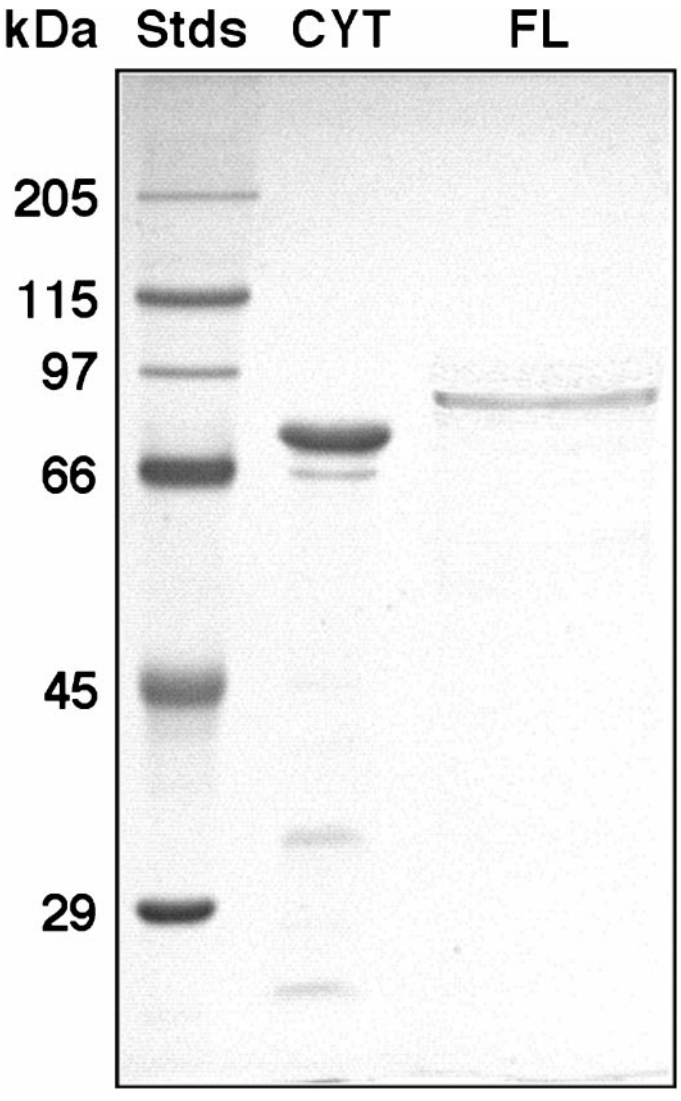

FIG. 1. SDS–PAGE of native and soluble yeast CPRs. An 11% acrylamide resolving gel was used and protein bands were detected by staining the gel with Coomassie blue R-250. Protein loadings were ca. 5 μg for each sample and each protein standard band.

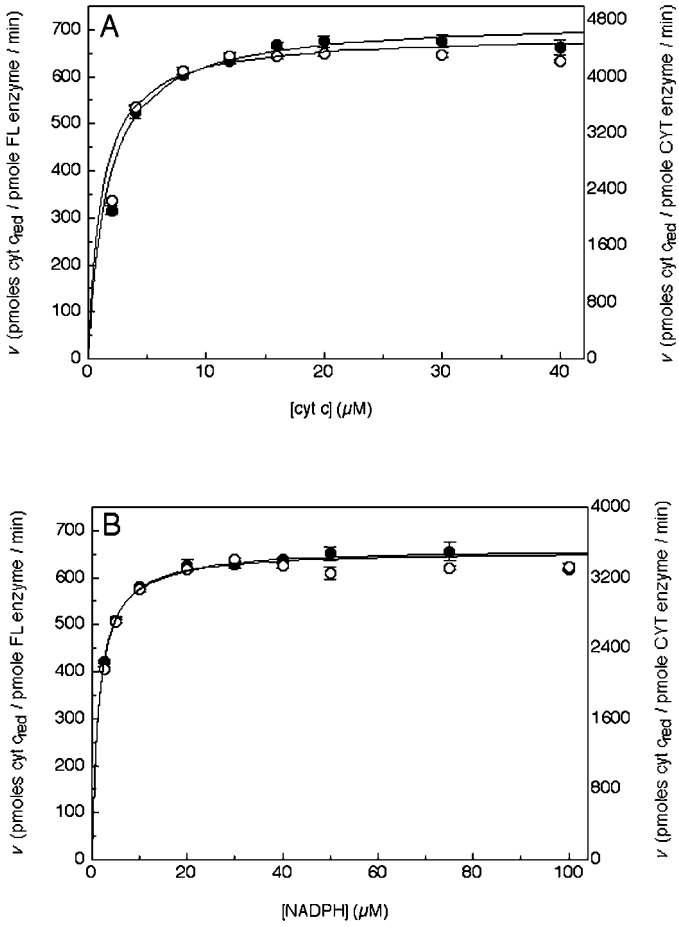

FIG. 2. Substrate saturation of yeast native and soluble CPRs with increasing cytochrome *c* concentrations at 50 μM NADPH (A) and increasing NADPH concentrations at 50 μM cytochrome *c* (B). The data points are means of three replicates with standard error bars shown. Protein present per assay was 212 ng for the native enzyme (●) and 41 ng for the soluble enzyme (○). Velocities are expressed in picomoles reduced cytochrome *c* produced per minute per picomole of enzyme.

derived (Table 2A) were similar for both yeast CPRs. The k_m values for cytochrome *c* were similar at 1.59 and 1.12 μM and the k_m values for NADPH were nearly identical at 1.46 and 1.41 μM for the native and soluble CPRs. Consequently, both native and soluble CPRs have identical affinities for both cytochrome *c* and NADPH. The maximum turnover numbers were determined to be 75 molecules per second for the soluble CPR and 11 molecules per second for the native CPR.

Kinetic studies showed no difference in the catalytic mechanism for yeast native and soluble CPRs. Lineweaver–Burk plots of the substrate saturation data where cytochrome *c* was the variable substrate at different fixed concentrations of NADPH gave a distinctive pattern of near parallel lines (Fig. 3). This is indicative of a random Bi-Bi- ping-pong kinetic mechanism in which the first product must be released before the second substrate can bind and with no ternary complex being involved. Table 2B lists the apparent kinetic parameters derived from the Lineweaver–Burk plots in Fig. 4. A plot of $1/v_{max}$ against 1/NADPH concentration (Fig. 4) allowed further determinations of k_m for NADPH to be made and were calculated to be 2.22 and 2.80 μM for the native and soluble CPRs.

DISCUSSION

Since there is only one CPR gene in eukaryotes with the exception of some plant species (24), CPR must be able to interact with and reduce the widely divergent cytochromes P450 which exist in each organism. Therefore understanding the process by which this interaction occurs is an important biological goal. Furthermore, establishing the molecular structure for CPR and its relation to the many properties inferred from spectral, electrochemical, spectroscopic, kinetic and site-specific mutagenesis studies at the atomic

TABLE 2

The Determined Kinetic Constants Derived from Substrate Saturation Experiments (A) and the Determined Kinetic Constants Derived from the Enzyme Mechanism Experiments (B)

A

CPR	k_m for cyt *c* (μM)	v_{max} [a]	k_m for NADPH (μM)	v_{max} [a]
Native	1.59 ± 0.2	721 ± 11	1.46 ± 0.1	662 ± 4.0
Soluble	1.12 ± 0.07	4597.0 ± 32.0	1.41 ± 0.06	3506 ± 13.0

B

	k_m for cyt *c* (μM)			v_{max} [a]		
CPR	2.5	5	50	2.5	5	50
Native	1.02	1.24	1.49	431	560	781
Soluble	0.55	1.12	1.20	2347	3240	4704

[a] v_{max} is expressed in units of picomoles of reduced cytochrome *c* produced per minute per picomole CPR.

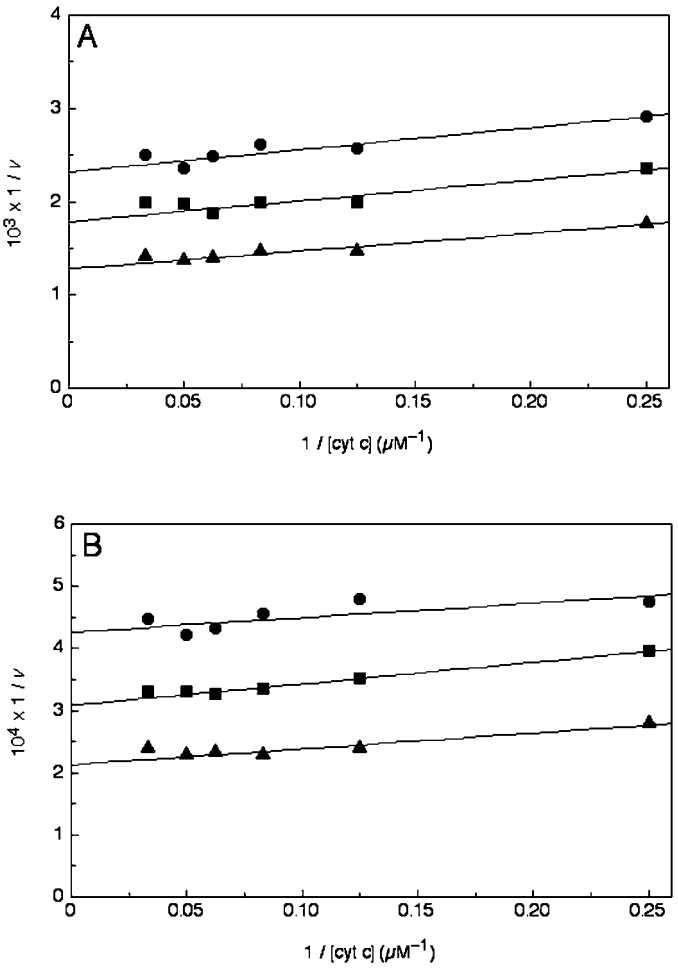

FIG. 3. Initial velocity patterns obtained from enzyme mechanism substrate saturation experiments. Substrate saturation experiments using of yeast native and soluble CPRs with increasing cytochrome *c* concentrations were performed at three fixed NADPH concentrations of 2.5 μM (●), 5 μM (■), and 50 μM (▲). Lineweaver–Burk plots of these data were constructed for the native (A) and soluble (B) CPRs. Velocities (*v*) are expressed as picomoles reduced cytochrome *c* produced per minute per picomole of enzyme. All data points are means of three replicates.

level is essential (for review, 25). Understanding such aspects can only be possible through X-ray crystallographic studies. However, crystallization of membrane proteins is still a difficult task and to date is still overcome by making membrane proteins water-soluble. According to the currently accepted model, CPR inserts into the membranes of the endoplasmic reticulum through the N-terminal hydrophobic domain, which acts as a membrane-binding anchor (26). Based on this model soluble CPR can be obtained by deleting the N-terminal domain of CPR. Even though soluble CPR can be prepared by the trypsinolysis of CPR (21), in order to eliminate the possible side effects of proteolysis on the CPR soluble catalytic domain, recombinant DNA techniques were utilized. Furthermore the truncated CPR expressed at very high levels compared to full-length CPR with the expression system used in this study.

Both the native and soluble yeast CPRs obeyed Michaelis–Menten kinetics with respect to both cytochrome *c* and NADPH when the concentration for one of the substrates was varied at a fixed level of the second substrate. This is in agreement with the kinetics displayed by CPRs isolated from insects, plants and animals (1, 27–29, 31). The turnover numbers for the native and soluble CPRs were 11 molecules per second compared to 75 molecules per second, respectively. This compares with published turnover numbers of 50 to 6100 per second for other CPRs (29). The specific activities of the native and soluble CPRs were 8.3 and 60 μmol/min/mg, respectively. This compares to previously published specific activities of 15 to 180 μmol/min/mg (2, 16, 27, 29, 32–34).

The derived kinetic parameters for native and soluble CPRs were almost identical. The k_m values for cytochrome *c* were 1.59 and 1.12 μM and the k_m values for NADPH were 1.46 and 1.41 μM for the native and soluble CPRs. These values were within the 1 to 10 μM values of those previously published (1, 27–29, 30–31). There was no difference in the kinetic mechanism between the native and soluble CPRs. Substrate saturation experiments using both CPRs where cytochrome *c* concentrations were varied at different fixed NADPH concentrations gave Lineweaver–Burk plots consisting of a series of near parallel lines. This is indicative of a random bi-bi- ping-pong kinetic mechanism in which the first product must be released before the second substrate can bind with no ternary complex being formed. This type of kinetic mechanism was also displayed by rat liver NADPH (P450) reductase (31). Murataliev *et al.* (29) however, established that overexpressed house-fly CPR followed a random bi-bi

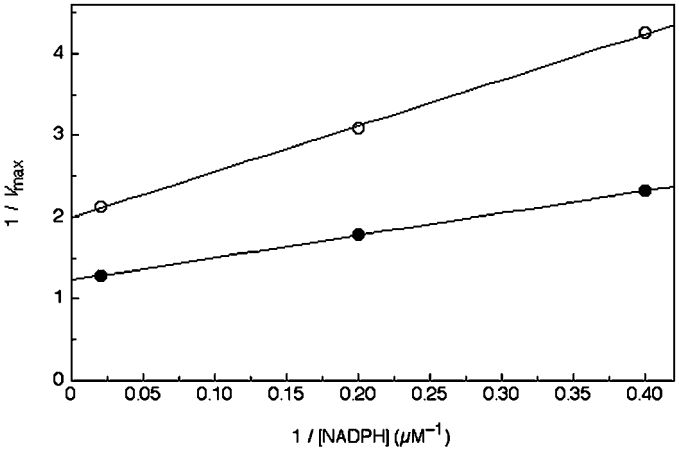

FIG. 4. Intercept ($1/v_{max}$) replots for the initial velocity patterns obtained from Fig. 4 for native CPR (●) and soluble (○) CPRs. The $1/v_{max}$ values shown have been multiplied by 1000 for the full-length CPR and by 10,000 for the cytosolic CPR.

mechanism that involved a ternary enzyme-substrate complex.

A ping-pong mechanism would involve the formation of an enzyme conformation that carries the reducing equivalence obtained by the conversion of NADPH to $NADP^+$, and is therefore likely to be unstable. In comparison, an enzyme mechanism that involved a ternary complex would allow a more direct transfer of reductant from NADPH to cytochrome *c* or cytochrome P450 as both substrates must bind to the CPR molecule prior to the reaction proceeding. In free solution a CPR mechanism that involved a ternary complex would therefore be favored.

CPR, *in vivo*, is located in the E.R. membrane in eukaryotes with the ratio of CPR to cytochrome P450 approximately 1 to 10 (26). Therefore, the advantage the ternary complex mechanism holds over the ping-pong mechanism will be diminished because CPR would be in close proximity to one of its natural substrates, cytochrome P450. The reduced form of the CPR enzyme formed in a ping-pong mechanism could quickly transfer the reducing equivalence to the cytochrome P450 in the E.R. membrane, and the stability of the reduced enzyme conformation thus becomes less important. The efficiency of both enzyme mechanisms in the E.R. membrane now becomes primarily dependent on the availability of external NADPH. In free solution, a mechanism that involved a ternary complex would be favored over a ping-pong mechanism. However, *in vivo*, in the E.R. membrane, there is less reason to prefer one mechanism against the other on efficiency grounds. Further kinetic studies are required on purified CPRs from a wide range of organisms to establish whether there is any evolutionary preference for one catalytic kinetic mechanism over another.

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