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STEROID 11β-HYDROXYLATION BY A FUNGAL MICROSOMAL CYTOCHROME *P*450

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Summary—The steroid 11β-hydroxylase activity of the fungus *Cochliobolus lunatus* was increased about 100-fold by cultivation of mycelia for 4–5 h with 20-hydroxymethyl-1,4-pregnadien-3-one. Cell-free extracts revealed a maximum activity of 45 nmol 11β-hydroxyprogesterone/h · mg protein in the 100,000 *g* pellet fraction. The 11β-hydroxylation was dependent on NADPH. The formation of 11β-hydroxyprogesterone correlated linearly with the cytochrome *P*450 concentration. The fungal 11β-hydroxylase transformed both 21-methyl and 21-hydroxymethyl steroids. The enzyme showed a broader substrate specificity and lower regioselectivity as compared with the adrenal cytochrome *P*45011β system. The fungal cytochrome *P*450 was partially purified to a specific content of 700 pmol *P*450/mg protein. Western blots showed that polyclonal antibodies against cytochrome *P*45011α from *Rhizopus nigricans* cross-react with a 60 kD protein of partially purified fractions. The NADPH–cytochrome *c* reductase was enriched up to a specific activity of 20 U/mg protein. Polyclonal antibodies against NADPH–cytochrome *P*450 reductases from *Candida maltosa* and rat liver cross-reacted with the fungal reductase. It is concluded that the 11β-hydroxylase of *Cochliobolus lunatus* represents a microsomal two-component monooxygenase system which is composed of a cytochrome *P*450 (M_r 60 kD) and a NADPH–cytochrome *P*450 reductase (M_r 79 kD).

INTRODUCTION

The hydroxylation of steroids in the 11β-position is an essential step in the synthesis of glucocorticoids. A few eukaryotic microorganisms such as *Curvularia lunata* and *Cunninghamella elegans* are capable of catalyzing 11β-hydroxylation of steroids [1, 2]. 11β-Hydroxylase activity is also found in the fungus *Cochliobolus lunatus* [3]. The components constituting these hydroxylases have not been characterized biochemically up to now. Other microbial steroid hydroxylases are composed either of cytochrome *P*450 and a flavoprotein, e.g. the 14α-demethylase of *Saccharomyces cerevisiae* [4], or with a ferredoxin in addition (*Rhizopus nigricans* [5], *Bacillus megaterium* [6]). The present paper demonstrates the involvement of a cytochrome *P*450 monooxygenase system in 11β-hydroxylations by *Cochliobolus lunatus*. The 11β-hydroxylase system of this fungus is characterized with respect to inducibility, intracellular localization, coenzyme dependence, substrate specificity, and enzyme components.

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EXPERIMENTAL

Organism and growth conditions

Cochliobolus lunatus m 118 was obtained from the Culture Collection of the Central Institute of Microbiology and Experimental Therapy (Jena, Germany). Lyophilized mycelia maintained on agar slants for 11 days were used as inocula for cultivation in flasks (500 ml) with 200 ml medium on a rotatory shaker at 240 rpm as described previously [7]. After two precultures (64 h, 24 h) at 28°C 20-hydroxymethyl-1,4-pregnadien-3-one (C_{22}-alcohol) was added as inducer (0.15 g l^{-1}) and the fermentation was continued for different times.

Preparation of cell-free fractions

Subcellular fractions were prepared by differential centrifugation of the cell homogenate after mechanical disruption of washed mycelia with a Dyno mill. The disruption buffer was composed of 100 mM Tris–HCl, pH 8.0, 250 mM sucrose, 10 mM EDTA, 1 mM glutathione, 1 mM KCl, 200 μM phenylmethylsulfonylfluoride, 10% glycerol, 20 μM 11-deoxycortisol.

Enzyme assays

The incubation mixture for the determination of the 11β-hydroxylase activity contained: 0.3–1.0 mg protein, 0.5 mM NADPH or/and 0.5 mM NADH, 0.25 mM glucose-6-phosphate, 2 U glucose-6-phosphate-dehydrogenase II from Boehringer, 0.5 mM $MgCl_2$, 200 μM steroid (added in 20 μl ethanol), and disruption buffer in a total volume of 2 ml. The reaction was started with pyridine nucleotides and stopped after shaking for 1 h at 22°C by addition of 2 ml dichloromethane.

The 11β-hydroxylase activity was assayed by HPLC analysis of the steroids extracted into dichloromethane from the incubation mixture (HIC 6A, Shimadzu, reversed phase C_{18} column, Nucleosil 120-5, 13 × 4 mm, Macherey and Nagel). The products of the various substrates were identified by comparison with reference steroids. The specific 11β-hydroxylase activity is expressed as nmol 11β-hydroxysteroid/h·mg protein.

The cytochrome *P*450 content was calculated from carbon monoxide-difference spectra [8] recorded with a Shimadzu UV 300 spectrophotometer. The NADPH– and NADH–cytochrome *c* reductase activity was determined according to the method of Williams and Kamin [9] with slight modifications. One activity unit (U) is defined as the reduction rate of one μmol cytochrome *c* per min. Proteins were determined by a modified method according to Lowry *et al.* [10] with Serulat EG as calibration standard.

Electrophoresis and immunoblot analysis

Subcellular fractions were subjected to SDS–PAGE (10% gel) by the method of Laemmli [11]. Following electrophoresis proteins were electrotransferred to nitrocellulose paper (50 mA for 2 h at 25°C) by the method of Towbin *et al.* [12]. The detection of immunocross-reactive proteins was carried out by incubation with polyclonal antibodies raised in rabbits (10 μg ml^{-1} of IgG fraction) against either purified cytochrome *P*45011α from *Rhizopus nigricans* or NADPH–cytochrome *P*450 reductase from *Candida maltosa* or rat liver and with horseradish peroxidase-conjugated anti-rabbit IgG using 4-chloro-1-naphthol as substrate. The antibodies against the reductase from rat liver were obtained from pooled antisera by ammonium sulfate precipitation and purified by DEAE-cellulose chromatography [13].

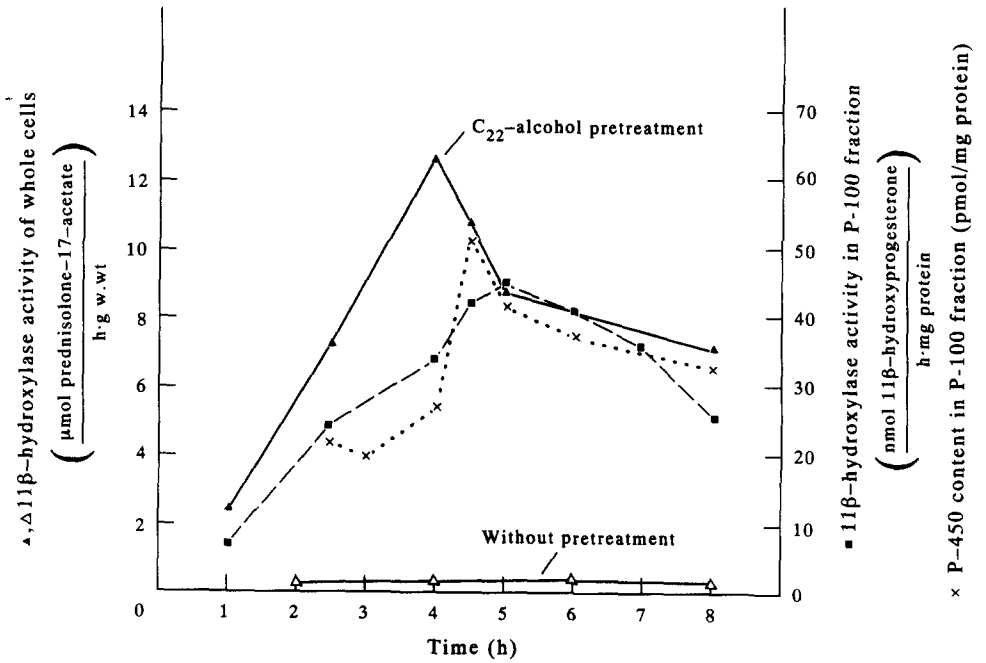

Fig. 1. Time course of 11β-hydroxylase activity of mycelia and 100,000 *g* sediment fraction (P-100) of *Cochliobolus lunatus* and cytochrome *P*450 content on treatment with 20-hydroxymethyl-1,4-pregnadien-3-one (C_{22}-alcohol). The determination of the 11β-hydroxylase activity of whole cells was performed with 1-dehydro-17α-acetoxy-11-deoxycortisol (0.75 g ml^{-1}) as substrate in 0.05 M phosphate buffer, pH 5.5, with 1% glucose and 10 μg cycloheximide ml^{-1} for 1 h at 28°C. The 11β-hydroxylase activity and the cytochrome *P*450 content of the P-100 fractions were determined as described in the experimental part.

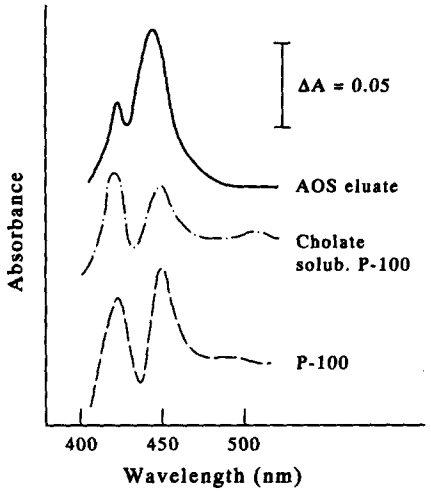

Fig. 2. Carbon monoxide-difference spectra of cell-free fractions of *Cochliobolus lunatus*. P-100: 100,000 *g* sediment fraction; cholate solub. P-100: sodium cholate solubilized P-100 fraction, AOS eluate: aminooctyl-Sepharose eluate.

RESULTS AND DISCUSSION

Untreated cells of *Cochliobolus lunatus* contain a very low 11β-hydroxylase activity (Fig. 1). It can be enhanced about 100-fold by treatment of the fungus with C_{22}-alcohol for 4–5 h. Differential centrifugations of the cell-free extracts reveal concomitantly a maximum activity in the 100,000 *g* pellet fraction (P-100) at a cultivation time of 4–5 h. The P-100 fraction contains an 11β-hydroxylase activity of 45 nmol 11β-hydroxyprogesterone/h·mg protein which is 20-fold higher as compared to the 100,000 *g* supernatant (data not shown). The distribution of the activity indicates a microsomal localization of the hydroxylase. This suggestion is supported by specific contents of up to 50 pmol cytochrome *P*450/mg protein as calculated from carbon monoxide-difference spectra of P-100 fractions (cf. Fig. 2) as compared to 2.0 pmol *P*450/mg protein in the supernatant. As shown in Fig. 3, the formation of 11β-hydroxyprogesterone by P-100 fractions of *Cochliobolus lunatus* correlates with the spectrophotometrically determined cytochrome *P*450 concentration indicating a functional linkage between the 11β-hydroxylase activity and the cytochrome *P*450 content of the samples. A molecular activity of about 45 nmol 11β-hydroxyprogesterone $(\text{min}\cdot\text{nmol } P450)^{-1}$ was estimated from this correlation. This activity is comparable to the cytochrome *P*450-dependent 11α-hydroxylation of progesterone by *Rhizopus nigricans* [5].

Inhibition experiments with cytochrome *P*450 specific inhibitors such as miconazole, ketoconazole, metyrapone and carbon monoxide further characterize the 11β-hydroxylation of steroids by *Cochliobolus lunatus* as a cytochrome *P*450-catalyzed reaction. The activity

Table 1. Inhibition of the steroid 11β-hydroxylase in the microsomal fraction of *Cochliobolus lunatus*

Compound	Concentration (μM)	Inhibition (%)
Control	—	0
Miconazole	1	20
	10	44
	50	71
Ketoconazole	1	56
	50	67
Metyrapone	100	5
	500	34
	1000	56
	2500	68
Carbon monoxide	*	20
Cytochrome *c*	5	65
	10	76
	20	90

The results are expressed as the percentage of original 11β-hydroxylase activity (100% represent 52.7 nmol cortisol/h·mg protein) remaining after incubation.
*Carbon monoxide-bubbled buffer solution was used as incubation medium; N_2- resp. O_2-bubbled buffer was used in the control.

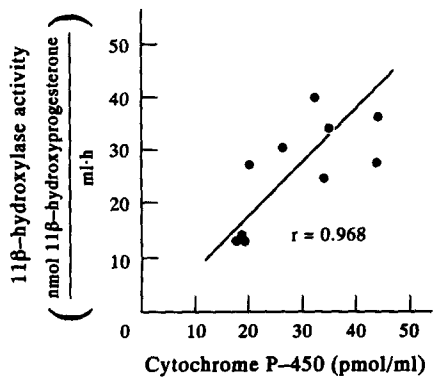

Fig. 3. Correlation between the cytochrome *P*450 content and the 11β-hydroxylase activity in P-100 fractions of *Cochliobolus lunatus*.

Table 2. Comparison of the substrate specificity of the 11β-hydroxylases from *Cochliobolus lunatus* and adrenal mitochondria

Substrate	Relative activity (%)* Fungal	Adrenal
Deoxycorticosterone	100	100
Progesterone	105 ± 2	10 ± 2
17α-Hydroxyprogesteone	80 ± 2	5 ± 1
Deoxycortisol	96 ± 3	80 ± 2
17α-Acetoxy-11-deoxycortisol	55 ± 5	33 ± 3
1-Dehydro-11-deoxycortisol	40 ± 2	62 ± 2

The fungal hydroxylation mixture contained 0.2 ml microsomal protein solution representing 20 pmol cytochrome *P*450 and 400 nmol steroid in a total volume of 2 ml. Further conditions are given in the experimental part. The reconstituted adrenal 11β-hydroxylase system consisting of 200 pmol cytochrome *P*450 11β, 10 nmol adrenodoxin and 400 pmol adrenodoxin reductase in 2 ml was incubated for 5 min at 22°C.
*Molecular activities for deoxycorticosterone of 43.5 min^{-1} for the fungal system and of 62.0 min^{-1} for the adrenal one were taken as 100%.

Table 3. Partial purification of cytochrome *P*450 from microsomes of *Cochliobolus lunatus*

Fraction	Protein (mg)	*P*450-Content Specific (pmol/mg protein)	*P*450-Content Total (pmol)	Yield (%)
Microsomes	211	39	8160	100
Cholate solubilized fraction	116	26	3000	37
DEAE-cellulose eluate	31	93	2870	35
AO-Sepharose[a] eluate	12	220	2637	32
Sepharose 6B filtrate	1.8	700	1260	15

[a] Aminooctyl-Sepharose.

decreases with increasing concentrations of the compounds tested (Table 1). The ID_{50} value amounts to 10 μM for miconazole and to about 1 mM for metyrapone. The value for miconazole is about 100-fold higher in the fungal system than in the adrenal cytochrome *P*45011β [14], but it corresponds to the value found for the liver microsomal steroid 16β-hydroxylase [15]. The inhibition by cytochrome *c* which is an artificial substrate for reductases [16] suggests the involvement of a reductase as electron transfer component of the fungal 11β-hydroxylase. The about 20-fold higher product formation in the presence of NADPH as compared to NADH (data not shown) clearly indicates the 11β-hydroxylase as a NADPH-dependent system.

Analyses of the conversion of different steroids by the fungal cytochrome *P*450 show about the same activity for deoxycorticosterone, progesterone, and 11-deoxycortisol and a slightly lower one for a 17α-hydroxyprogesterone (Table 2). Thus the extent of oxygen substitution in the side chain of the steroid substrates only slightly influences the steroid conversion by *Cochliobolus lunatus*. However, 17α-acetylation of 11-deoxycortisol results in a 45% lower conversion and the introduction of a second double

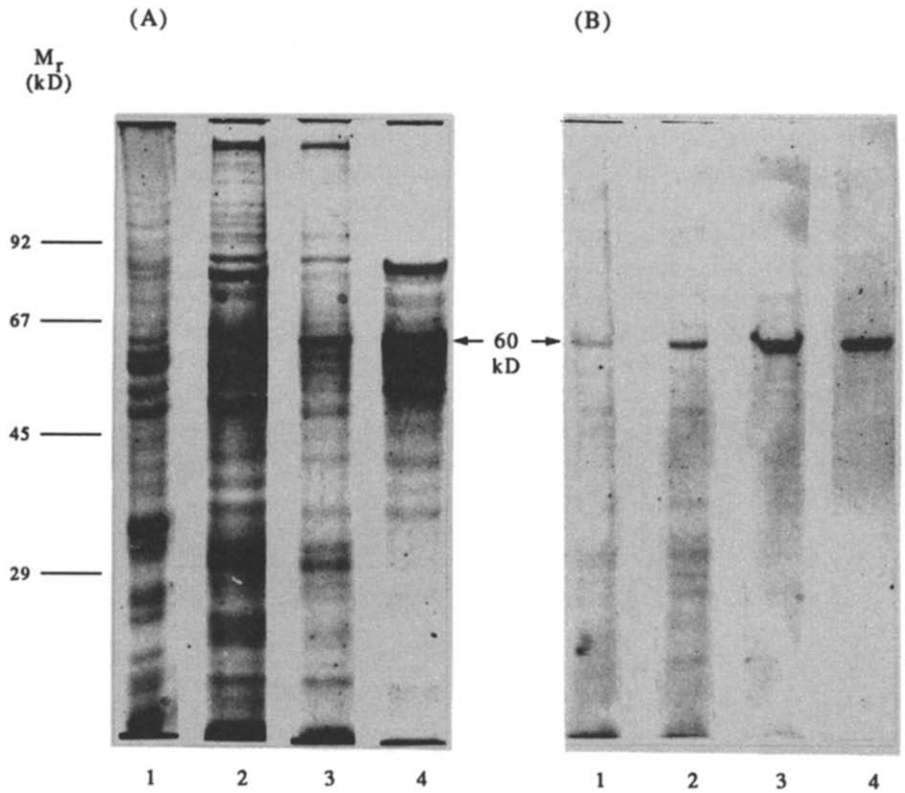

Fig. 4. SDS–PAGE of cell-free fractions of *Cochliobolus lunatus* (A) and immunoblotting with polyclonal antibodies against cytochrome *P*45011α from *Rhizopus nigricans* (B). Conditions: (A) P-100 fraction (lane 1, 10 μg), DEAE-cellulose eluate (lane 2, 10 μg), aminooctyl-Sepharose eluate (lane 3, 10 μg), and Sepharose 6B filtrate (lane 4, 16 μg); 10% gel concentration, Coomassie brilliant blue staining. (B) After electrotransfer to nitrocellulose membranes the same proteins as in (A) were immunostained with 10 μg anti-cytochrome *P*450 11α IgG ml^{-1} for 2 h at 25°C and with horseradish peroxidase-conjugated anti-rabbit IgG. Arrows indicate 60 kD proteins.

Table 4. Purification of NADPH–cytochrome *c* reductase from microsomes of *Cochliobolus lunatus*

Fraction	Protein (mg)	Reductase activity: Specific (U/mg protein)	Reductase activity: Total (U)	Yield (%)
Microsomes	600	0.12	71	100
Cholate solubilized fraction	273	0.22	60	84
AO-Sepharose[a] unbound protein	142	0.20	28	39
DE 52-cellulose eluate	30	0.60	18	25
ADP-Sepharose eluate	0.6	20.00	12	17

[a] Aminooctyl-Sepharose.

bond into the A-ring (1-dehydro-11-deoxycortisol) decreases the activity to 40% as compared to deoxycorticosterone. In contrast to the fungal system by which progesterone and 17α-hydroxyprogesterone are converted to 105 and 80%, respectively, as compared to deoxycorticosterone the adrenal system exhibits a remarkably lower capability to convert progesterone and 17α-hydroxyprogesterone with an extent of 10 and 5%. In addition to the 11β-hydroxylation a cytochrome *P*450-dependent 14α-hydroxylation of some steroid substrates has been found. The ratio of 11β- and 14α-hydroxylated products formed from progesterone by the P-100 fraction amounts to about 2 to 1 (not shown). In comparison with the adrenal cytochrome *P*45011β system the data show a broader substrate specificity and lower regioselectivity for the fungal cytochrome *P*450.

To purify the components of the 11β-hydroxylase system of *Cochliobolus lunatus* the P-100 fraction was solubilized with sodium

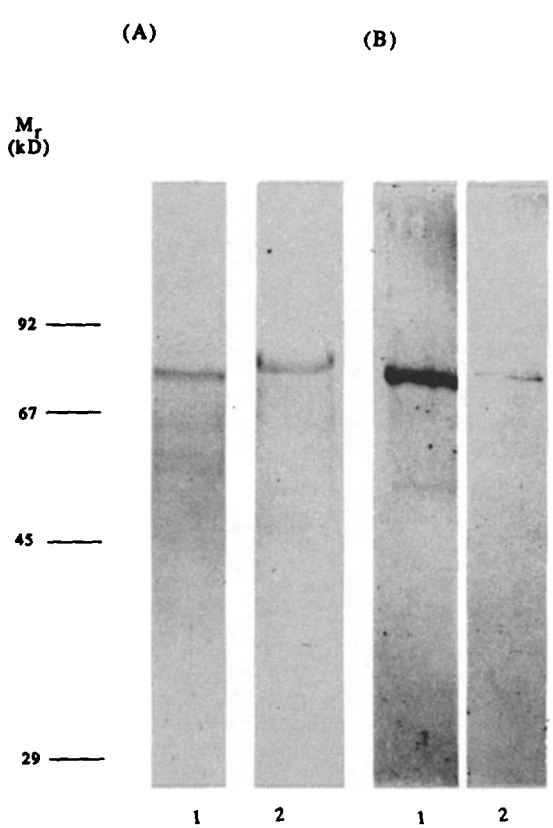

Fig. 5. (A) SDS–PAGE of NADPH–cytochrome *P*450 reductase from *Candida maltosa* (lane 1) and from *Cochliobolus lunatus* (lane 2), 1 μg protein of each. (B) Immunoblotting of NADPH–cytochrome *P*450 reductases from *Candida maltosa* (lane 1, 0.01 μg) and from *Cochliobolus lunatus* (lane 2, 0.25 μg) with polyclonal antibodies against NADPH–cytochrome *P*450 reductase of *Candida maltosa* (10 μg IgG ml^{-1}).

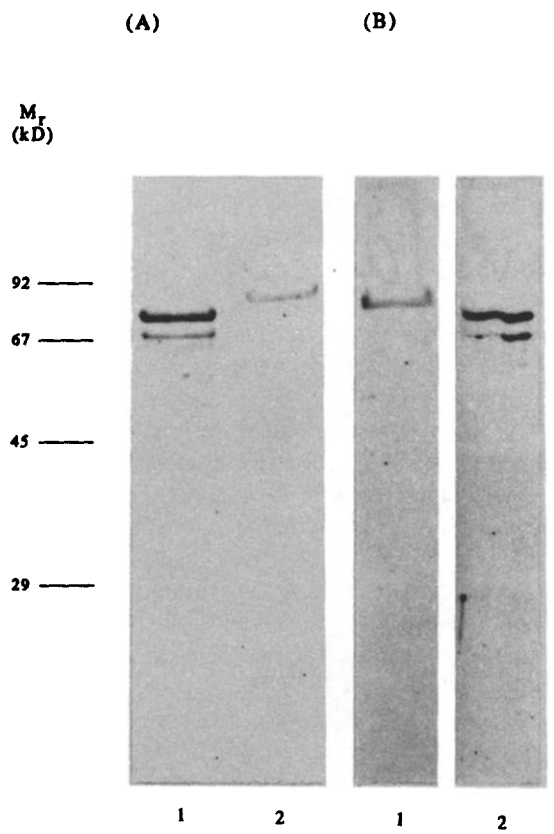

Fig. 6. (A) SDS–PAGE of NADPH–cytochrome *P*450 reductase from rat liver microsomes (lane 1, 1 μg protein) and from *Cochliobolus lunatus* (lane 2, 0.5 μg protein). (B) Immunoblotting of NADPH–cytochrome *P*450 reductases from rat liver microsomes (lane 1, 0.25 μg) and from *Cochliobolus lunatus* (lane 2, 1 μg) with polyclonal antibodies against rat liver NADPH–cytochrome *P*450 reductase (20 μg IgG ml^{-1}).

Table 5. Inhibition effect of antibodies against rat liver microsomal NADPH–cytochrome *P*450 reductase on the NADPH–cytochrome *c* reductase activity of rat liver microsomes and *Cochliobolus lunatus* microsomes

System	Anti-*P*450 reductase IgG (mg protein)	NADPH–cytochrome *c* reductase activity[a] (U/mg protein)	(%)
Fungal microsomes	—	19.9	100
	1.52	18.0	90
	4.89	14.0	70
	4.89 (preimmune)	24.1	121[b]
Rat liver microsomes	—	2.84	100
	1.06	2.30	81
	3.36	1.08	35
	1.55 (preimmune)	4.48	158[b]

Antibodies were incubated with 1 μg antigen for 2.5 h at room temperature before measurements of the reductase activity.

[a]Values are the mean from duplicate determinations.

[b]The increased cytochrome *c* reductase activity with preimmune IgG may be due to unspecific protein–protein interactions [17].

cholate (2 mg per mg protein) and subjected to chromatographic separations. The cytochrome *P*450 can be enriched 18-fold up to a specific content of 700 pmol *P*450/mg protein by chromatography on DEAE-cellulose and omega-aminooctyl Sepharose and gelfiltration (Table 3, Fig. 2). The low specific content is probably due to a loss of heme during the purification procedure. Western blot experiments show that polyclonal antibodies against cytochrome *P*45011α from *Rhizopus nigricans* cross-react with a 60 kD protein of the partially purified cytochrome *P*450 preparation of *Cochliobolus lunatus* [Fig. 4(B)]. The amount of this cross-reacting protein increases along with the purification of the cytochrome *P*450 suggesting a molecular mass of 60 kD of the fungal cytochrome *P*450.

After cholate solubilization of the P-100 fraction the NADPH–cytochrome *c* reductase activity is increased 90-fold up to a value of 20 U/mg protein essentially by affinity chromatography on ADP-Sepharose (Table 4). Western blot analyses indicate an immunochemical relatedness of this fungal reductase with FAD-FMN containing cytochrome *P*450 reductases. Antibodies against NADPH–cytochrome *P*450 reductase from *Candida maltosa* cross-react with a 79 kD protein [Fig. 5(B)]. Furthermore, antibodies against rat liver NADPH–cytochrome *P*450 reductase show this cross-reactivity [Fig. 6(B)]. The fungal reductase did not cross-react with antibodies raised against the FAD-containing NADPH–adrenodoxin reductase from adrenal mitochondria (not shown).

Immunoinhibition experiments with antibodies against the rat liver NADPH–cytochrome *P*450 reductase show an inhibition of the NADPH–cytochrome *c* reductase activity of the reductase from *Cochliobolus lunatus* in comparison to the control with preimmune IgG (Table 5).

Taken together the data suggest that the fungal 11β-hydroxylase from *Cochliobolus lunatus* represents a microsomal two-component monooxygenase system which is composed of a cytochrome *P*450 and a NADPH–cytochrome *P*450 reductase.

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