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Purification and Characterization of Cinnamoyl-Coenzyme A:NADP Oxidoreductase in *Eucalyptus gunnii*[1]

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Cinnamoyl-coenzyme A:NADP oxidoreductase (CCR, EC 1.2.1.44), the entry-point enzyme into the monolignol biosynthetic pathway, was purified to apparent electrophoretic homogeneity from differentiating xylem of *Eucalyptus gunnii* Hook. The purified protein is a monomer of 38 kD and has an isoelectric point of 7. Although *Eucalyptus gunnii* CCR has approximately equal affinities for all possible substrates (*p*-coumaroyl-coenzyme A, feruloyl-coenzyme A, and sinapoyl-coenzyme A), it is approximately three times more effective at converting feruloyl-coenzyme A than the other substrates. To gain a better understanding of the catalytic regulation of *Eucalyptus* CCR, a variety of compounds were tested to determine their effect on CCR activity. CCR activity is inhibited by NADP and coenzyme A. Effectors that bind lysine and cysteine residues also inhibit CCR activity. As a prerequisite to the study of the regulation of CCR at the molecular level, polyclonal antibodies were obtained.

Lignins are complex cell wall polymers that are necessary for the growth and survival of all vascular plants (Corner, 1964; Vance et al., 1980; Wardrop, 1981; Smart and Amrhein, 1985). Lignins were a key factor in the evolution of the terrestrial habit of plants (Kubitzki, 1987), and over time they have become a major consideration in the Earth's carbon cycle because they account for approximately 30% of woody biomass and because their turnover is very low due to their complex nature (Brown, 1985). Lignins are also an important consideration in the agro-industrial utilization of plant matter (Brown, 1985; Cherney et al., 1988). For example, in the production of pulp and paper, lignins must be removed from the cellulose microfibrils in a costly and environmentally hazardous process (Whetten and Sederoff, 1991).

Lignins are aromatic polymers synthesized by the polymerization of monolignols (Higuchi, 1985; Sederoff and Chang, 1991). The biosynthesis of monolignols is a specialized branch of phenylpropanoid metabolism (Gross, 1985). Hydroxycinnamoyl-CoA esters are the end products of general phenylpropanoid metabolism and can be channeled into the biosynthesis of a diverse array of compounds (Gross, 1985). In monolignol biosynthesis, hydroxycinnamoyl-CoA esters are reduced to their corresponding alcohols by two enzymatic reactions (Gross, 1985). The second of these two enzymes, CAD (EC 1.1.1.165), has been extensively characterized at both the biochemical and molecular genetic levels (Wyrambik and Grisebach, 1975; Goffner et al., 1992; Halpin et al., 1992, 1993; Knight et al., 1992; O'Malley et al., 1992; Galliano et al., 1993a, 1993b; Grima-Pettenati et al., 1993; Hibino et al., 1993, 1994; Hawkins and Boudet, 1994). In contrast, studies of the entry-point enzyme into monolignol biosynthesis, CCR (EC 1.2.1.44), have been infrequent (Gross and Kreiten, 1975; Rhodes and Wooltorton, 1975; Wengenmayer et al., 1976; Luderitz and Grisebach, 1981; Sarni et al., 1984).

CCR catalyzes the conversion of hydroxycinnamoyl-CoA esters to their corresponding cinnamaldehydes (Gross and Kreiten, 1975). As the first committed step in monolignol biosynthesis, CCR diverts phenylpropanoid-derived metabolites into the biosynthesis of lignins. Entry-point enzymes often play key regulatory roles in biosynthesis. For example, 3-hydroxy-3-methylglutaryl-CoA reductase, another CoA reductase that is the first committed step in isoprenoid biosynthesis, plays a key regulatory role by controlling the flux of metabolites into the pathway via a complex cascade of events, including differential gene regulation (Yang et al., 1991). Based on such examples, CCR is hypothesized to play a key regulatory role in lignin biosynthesis. Although CCR has been purified and partially characterized from soybean cultures (Wengenmayer et al., 1976; Luderitz and Grisebach, 1981), spruce cambial sap (Luderitz and Grisebach, 1981), and poplar xylem (Sarni et al., 1984), little is known about the biochemical or molecular genetic regulation of this enzyme. As a prerequisite to testing the hypothesis that CCR plays a key role in regulating lignin biosynthesis, we have purified CCR from the differentiating xylem of *Eucalyptus gunnii*, characterized its physical and catalytic properties, and obtained antibodies for the protein.

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Abbreviations: CAD, cinnamoyl alcohol dehydrogenase; CCR, cinnamoyl-CoA:NADP oxidoreductase; CoASH, reduced form of CoA; DIDS, 4,4-diisothiocyano-2,2-stilbene disulfonate; MDH, malate dehydrogenase; pCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Plant Material

Trees of *Eucalyptus gunnii* Hook, clone number 800859, were clonally propagated in a plantation in Longages, France, courtesy of Association Forêt Cellulose. Differentiating xylem was obtained from actively growing branches of 5-year-old trees. Excised branches were first peeled to remove bark, and immediately cell layers corresponding to differentiating xylem were scraped directly into liquid nitrogen. The frozen xylem was stored at −80°C for further use.

Assay of CCR Activity and K_m Determinations

Feruloyl-CoA was synthesized in collaboration with Dr. H. Duran according to Duran et al. (1986). Separation of feruloyl-CoA from nonincorporated CoASH was achieved by an additional chromatography step on a G10 column (Pharmacia) (Lindl et al., 1973). Authentic *p*-coumaroyl-, caffeoyl-, and sinapoyl-CoA ester standards were the generous gifts of Drs. W. Heller (Munich, Germany), J. Negrel (Dijon, France), and U. Mattern (Freiburg, Germany). [^{14}C]Feruloyl-CoA was the kind gift of Ms. Annagrette Kohler and Dr. Heinrich Kauss (Kaiserslautern, Germany).

CCR activity was measured spectrophotometrically according to the method of Luderitz and Grisebach (1981). Briefly, the reaction mixture consisted of 100 mM sodium/potassium phosphate (pH 6.25), 0.1 mM NADPH, 70 μM feruloyl-CoA, and 5 to 100 μL of protein in a total volume of 500 μL. The reaction was started by the addition of feruloyl-CoA and run in a heated cuvette at 30°C in a spectrophotometer. The decrease in A_{366} was determined during a period of time when the reaction displayed linearity (usually between 2 and 10 min). CCR activity was calculated using the linear decrease in A_{366} and the correction factors cited by Luderitz and Grisebach (1981). The pH optimum for CCR activity was determined in preliminary experiments by assaying activity in crude protein extracts and was later confirmed using pure protein. Protein content was determined using the Bio-Rad microassay (Bradford, 1976) with γ-globulin as standard.

To verify the identity of the reaction products, feruloyl-CoA was substituted with [^{14}C]feruloyl-CoA and the reaction was run in a total volume of 50 μL. After 30 min the reaction was stopped by the addition of 5 μL of 20 mM cold coniferylaldehyde in ethylene glycol monomethyl ether. The reaction products were split and spotted on two different TLC plates (silica gel, Merck 60 F254) and developed by ascending chromatography. One plate was developed in chloroform:acetic acid (9:1), and the other was developed in butanol:acetic acid:water (4:1:1). After development in the solvent system, plates were air dried and then autoradiographed overnight. The identity of the reaction product was verified by co-chromatography of radioactivity with authentic coniferylaldehyde (which was used to stop the reaction) visualized under UV light. K_m values were determined by extrapolation from Lineweaver-Burk plots.

CCR Purification

All purification steps were carried out at 4°C. For each purification, 300 g of frozen, peeled xylem were ground in a coffee mill and the resulting powder was stirred for 30 min in 1 L of prechilled buffer containing 100 mM Tris-HCl (pH 7.6), 2% PEG 6000, 5 mM DTT, 2% polyvinylpolypyrrolidone. The crude extract was then filtered through two layers of Miracloth. The crude extract was brought to 30% saturation with crystalline ammonium sulfate, stirred for 1 h, and centrifuged at 15,000*g* for 30 min. The resulting supernatant was then brought to 70% saturation with crystalline ammonium sulfate, stirred for 1 h, and centrifuged at 15,000*g*. The resulting pellet was resuspended in 60 mL of buffer 1 (20 mM Tris-HCl, pH 7.5, 5 mM DTT, 5% ethylene glycol). The resuspended pellet was centrifuged at 10,000*g* for 15 min to clarify the extract. The extract was then desalted on PD 10 columns (pre-equilibrated in buffer 1) according to the manufacturer's recommendations (Pharmacia).

The desalted extract was loaded on a Red Sepharose (Pharmacia) column (1.5 × 19 cm) pre-equilibrated in buffer 1 at a flow rate of 10 mL/h. After rinsing the column with 50 mL of buffer 1, proteins were eluted with a linear gradient of 20 mM to 1.5 M Tris-HCl (pH 7.5) containing 5 mM DTT and 5% ethylene glycol at a flow rate of 36 mL/h (total volume = 200 mL). Fractions were assayed for CCR activity and the fractions corresponding to the peak of CCR activity were pooled and desalted on PD 10 columns in buffer 1 as described above.

The desalted fractions from the Red Sepharose column were loaded onto a Mono Q column (HR 5/5, Pharmacia) at a flow rate of 0.5 mL/min. A linear gradient of 20 to 300 mM Tris-HCl, pH 7.5, containing 5% ethylene glycol and 5 mM DTT at a flow rate of 1 mL/min (total volume = 100 mL) was used to elute the proteins. Fractions containing CCR activity were pooled and desalted as outlined above, with the exception that the PD 10 columns were pre-equilibrated in 20 mM potassium/sodium phosphate, pH 6.1, containing 5 mM DTT.

Desalted Mono Q fractions were loaded onto a HR 5/5 column (Pharmacia) packed with Mimetic Red 2 A6XL chromatography medium (Affinity Chromatography Ltd., Cambridge, UK) at a flow rate of 0.5 mL/min. The column was then washed with 20 mM potassium/sodium phosphate, pH 6.1, containing 5 mM DTT and 8 mM NAD (30 mL). CCR activity was eluted with a linear gradient of 0 to 8 mM NADP (15 mL). For complete CCR elution, further rinsing with 8 mM NADP was necessary. To ensure enzyme stability, ethylene glycol was added to each fraction as it eluted to give a final concentration of 5%. Purified CCR fractions were stored at −80°C.

Determination of Physical Properties of CCR

To determine the native molecular mass of CCR, partially purified enzyme (post-Mono Q) was subjected to Superose 6 gel filtration. Elution took place in 100 mM potassium phosphate, pH 6.25, containing 1 mM β-mercaptoethanol at a flow rate of 0.2 mL/min on a HR 10/30 Superose 6 column at 4°C. A calibration curve was constructed with proteins of known molecular masses. The protein standards used were RNase A (13.7 kD), chymotrysinogen A (25 kD), BSA (66 kD), and ferritin (440 kD). Each protein was loaded in a total volume of 200 μL and its elution was monitored by recording

Table I. *Purification of cinnamoyl-CoA reductase from Eucalyptus stems*

Purification Step	Protein	Total Activity	Specific Activity	Purification Factor	Yield
	mg	*nktal*	*nktal/mg*		%
Crude extract	1210	1888	1.6		100
Ammonium sulfate	316	824	2.6	1.6	43
Red Sepharose	28.2	276	9.8	6.1	15
Mono Q	0.66	99	151	94	5
Mimetic Red[a]	0.036	16	451	282	0.8

[a] Three fractions containing maximum CCR activity were pooled for these values.

the eluate A_{280}. The K_{AV} value was calculated for each protein using the mobility equation $K_{AV} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_o is the void volume of the column, and V_t is the total volume of the column. The K_{AV} value for each protein was plotted against the log of its molecular mass. The elution volume for CCR was determined by assaying each 200-μL fraction of the eluate for CCR activity. The relative molecular mass of CCR was determined by interpolation from the regression line of the plot with the elution volume of CCR.

The isoelectric point of CCR was determined by chromatofocusing on a HR 5/20 Mono P column (Pharmacia). Partially purified (post-Mono Q) CCR was desalted into 25 mM ethanolamine acetic acid, pH 9.2, containing 10 mM β-mercaptoethanol, by the method of Penefsky (1977) on a Sephadex G-25 (Pharmacia) spin column. The desalted extract was loaded onto the Mono P column pre-equilibrated with 25 mM ethanolamine acetic acid, pH 9.2, and subjected to chromatofocusing between pH 9 and 6. Immediately after the protein was loaded, the pH gradient was generated by switching to Polybuffer 96:acetic acid, pH 6.0 (Polybuffer:water, 1:10 [v/v]) containing 10 mM β-mercaptoethanol. The flow rate was 0.75 mL/min throughout. Fractions were collected at 1-min intervals, spiked with 100 μL of 10× concentrated assay buffer and 100 μL of ethylene glycol, and mixed thoroughly. Chromatofocusing was conducted at 4°C and fractions were held on ice prior to assay. About 450 μL of each fraction were assayed for CCR activity.

Generation of Polyclonal Antibodies

Polyclonal antibodies were raised in rabbits using 100 μg of post-Mimetic Red CCR as antigen. Immunization and bleedings were performed by Eurogentec (Seraing, Belgium).

Electrophoretic Analysis of CCR

Denaturing SDS-PAGE was performed according to Laemmli (1974). Gels contained 12% acrylamide. Proteins were stained with silver nitrate (Damerval et al., 1987). Immunoblots were performed using a semi-dry transfer apparatus (Bio-Rad) as described by Grima-Pettenati et al. (1993). Primary CCR antibodies were used at a dilution of 1/1000.

RESULTS

CCR Purification

CCR purification was achieved by the chromatographic steps indicated in Table I. The corresponding chromatograms are shown in Figure 1. A second minor peak of CCR activity was observed on Mono Q. We focused on the major peak for

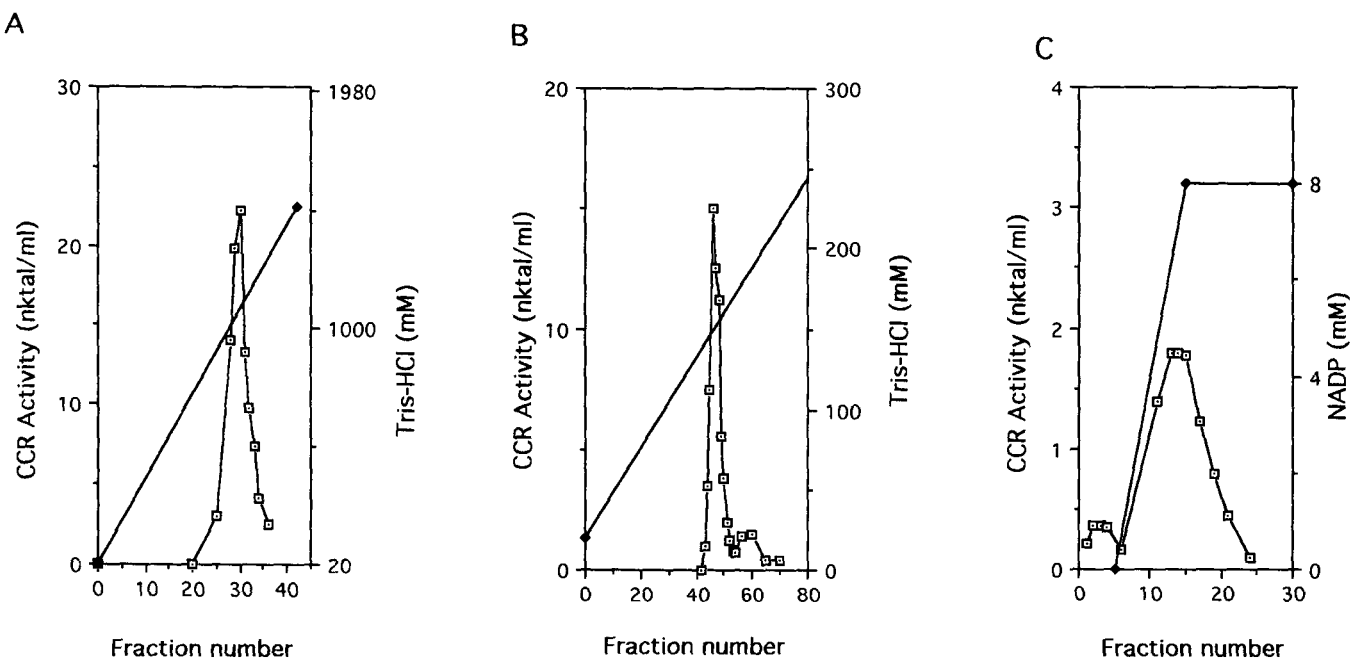

Figure 1. Purification of CCR. Chromatograms of CCR on Red Sepharose (A), Mono Q (B), and Mimetic Red (C). ⊡, CCR activity; ■, elution gradients.

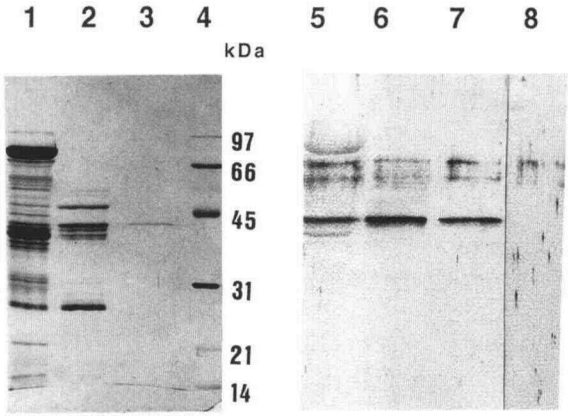

Figure 2. SDS-polyacrylamide gels of CCR-containing fractions throughout purification. Lanes 1 and 5, Red Sepharose; lanes 2 and 6, Mono Q; lanes 3 and 7, Mimetic Red. Lanes 1 to 4 were stained with silver nitrate to visualize total protein. Identical extracts were immunoblotted onto nitrocellulose and probed with CCR antibodies (lanes 5–7). Lane 4 contains the molecular mass standards: lysozyme (14 kD), trypsin inhibitor (21 kD), carbonic anhydrase (31 kD), ovalbumin (45 kD), serum albumin (66 kD), phosphorylase *b* (97 kD). Lane 8 corresponds to an immunoblot performed with loading buffer only.

our purifications. A 282-fold purification was achieved by this relatively rapid procedure. High-resolution chromatography with Mimetic Red 2, an affinity chromatography step that exploited the affinity of CCR for NADP, was particularly important in our purifications. The inclusion of an NAD wash prior to elution with an NADP gradient was necessary to eliminate MDH that persistently co-eluted with CCR in all prior purification attempts. The yield of CCR obtained by this purification procedure (36 μg for 300 g of starting material) does not reflect its relative quantity in planta (in terms of percent total protein). To maximize the elimination of contaminants, only fractions containing maximum CCR activity were pooled after each step to be used for subsequent steps. Pure CCR had a specific activity of 451 nktal/mg using feruloyl-CoA as substrate.

Figure 2 shows a silver-stained SDS-polyacrylamide gel and corresponding immunoblot of fractions from the sequential purification steps used to purify CCR. Purification of CCR to electrophoretic homogeneity was confirmed by the presence of a single polypeptide on a silver-stained SDS-polyacrylamide gel after Mimetic Red (Fig. 2, lane 3). Immunoblots indicate that anti-CCR reacts with CCR of all degrees of purity (lanes 5–7). An additional polypeptide with a slightly lower molecular mass than CCR was detected in partially purified fractions, indicating the presence of a potential isoenzyme (lane 5). Immunoreactivity was also observed in the 50- to 60-kD region. This was due to a nonspecific reaction with β-mercaptoethanol in the sample loading buffer, since loading buffer alone gave rise to the same artifact (lane 8). Indeed, immunoblots performed in the absence of β-mercaptoethanol eliminated this artifact but significantly decreased protein resolution during electrophoresis (data not shown). As a further verification of CCR purity, two-dimensional electrophoresis was performed. After silver staining, CCR was visualized as a single spot with an apparent molecular mass of approximately 38 kD and an isoelectric point of 7 (data not shown).

Identification of CCR Reaction Products

TLC of products from a reaction catalyzed by pure CCR (post-Mimetic Red) revealed a single spot that co-migrated with authentic coniferylaldehyde (Fig. 3). This was in contrast to the reaction products from the post-Mono Q activity, which included a spot that co-eluted with coniferyl alcohol (Fig. 3). Coniferyl alcohol is the reaction product of CAD, the enzyme that uses the CCR reaction product (coniferylaldehyde) as substrate. CAD must have contaminated the CCR preparation at this stage despite the fact that it could not be detected spectrophotometrically (data not shown). After purification on Mimetic Red, however, the CAD product was absent (Fig. 3).

Physicochemical Properties of CCR

The native molecular mass for CCR was determined by gel-filtration on Superose 6 (Fig. 4). The peak of CCR activity eluted in a volume corresponding to a relative molecular mass of 38 kD. The fact that subunit size on denaturing electrophoresis and native molecular mass estimation on Superose 6 both were approximately the same value suggested that CCR is monomeric.

The isoelectric point of CCR was determined by chromatofocusing on Mono P between pH 9 and 6. The elution

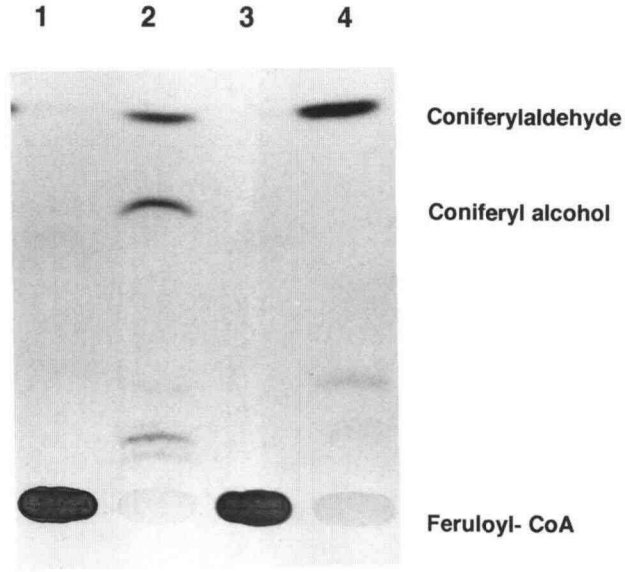

Figure 3. Autoradiogram of the thin-layer chromatogram obtained from the products of the radiometric CCR assay. Samples loaded included the post-Mono Q enzyme (boiled, lane 1; active, lane 2), which contained some contaminating CAD activity and also produced coniferyl alcohol, and the post-Mimetic Red enzyme (boiled, lane 3; active, lane 4), which produced only the reaction product. The faint bands closer to the origin are oxidation products of coniferylaldehyde.

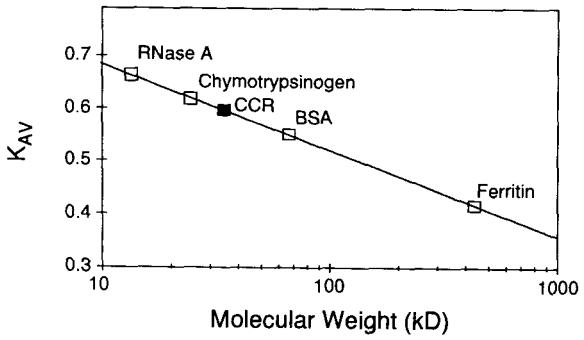

Figure 4. Determination of native molecular mass by gel filtration.

profile of CCR activity as a function of pH indicates that CCR had an isoelectric point of approximately 7 (Fig. 5).

The pH optimum of CCR activity was determined on pure CCR (post-Mimetic Red) in both 100 mM potassium/sodium phosphate and citrate/NAOH buffer (Fig. 6). A broader optimum pH range was observed in phosphate buffer (pH 5.3–6.5) versus citrate/NaOH (pH 6–6.5). In addition, CCR activity was higher in phosphate compared to citrate buffer at all pH values tested. These results indicated that the conditions used for routine spectrophotometric assays, 100 mM potassium/sodium phosphate, pH 6.25, which were based on previous reports on CCR purification, were optimum for measuring CCR activity in *Eucalyptus*.

Substrate Specificity of CCR

Apparent K_m and k_{cat} values for the three natural substrates (*p*-coumaroyl-CoA, sinapoyl-CoA, and feruloyl-CoA) as well as caffeoyl-CoA are given in Table II. K_m values for the various cinnamoyl-CoA esters were of the same order of magnitude, ranging from 28 to 45 μM, thereby indicating that CCR did not have a significantly higher affinity for any given substrate. However, the k_{cat} was approximately 3 times higher with feruloyl-CoA compared to the other substrates tested.

Inhibition of CCR Activity by Various Effectors

To further characterize CCR at the biochemical level, the effect of various chemical agents on CCR activity was determined (Table III). Product inhibition was observed by a decrease in CCR activity in the presence of 1 mM of both NADP and CoASH (20 and 53% inhibition, respectively). It was not possible to determine the effect of coniferylaldehyde on CCR activity due to interference with the spectrophotometric assay. No phenolic acid tested had any effect on CCR activity. The largest decreases in CCR activity were observed with DIDS (95% inhibition at 50 μM), which covalently and specifically binds γ-amino groups on Lys residues, and the thiol reagent pCMB (88% at 300 μM). The other effectors tested, including the chelating agent EDTA and diethylpyrocarbonate, which reacts specifically with histidyl residues, had no effect on CCR activity at millimolar concentrations.

DISCUSSION

In this paper we describe a rapid and efficient protocol to purify CCR from differentiating xylem of *E. gunnii*. CCR has been purified from either cell-suspension cultures (Wegenmayer et al., 1976; Luderitz and Grisebach, 1981) or differentiating xylem (Luderitz and Grisebach, 1981; Sarni et al., 1984) of other species. Differentiating xylem was chosen as starting material for our purifications because CCR specific activity was 3-fold higher in this tissue compared to the CCR activity found in the *E. gunnii* cell cultures routinely used in our laboratory. Furthermore, because lignins are produced in large quantities in differentiating xylem as opposed to cell cultures, xylem is a better tissue in which to establish a role of CCR in lignification.

Initial attempts to purify *E. gunnii* CCR to homogeneity were found to be unsuccessful due to the persistent co-elution of MDH (EC 1.1.1.37) with CCR. The unambiguous identification of MDH by peptide sequencing (data not shown) enabled us to develop an efficient strategy for the complete separation of these two proteins. This strategy was based on the differential affinities of MDH and CCR for NAD and NADP and the principle of separation by Mimetic Red 2

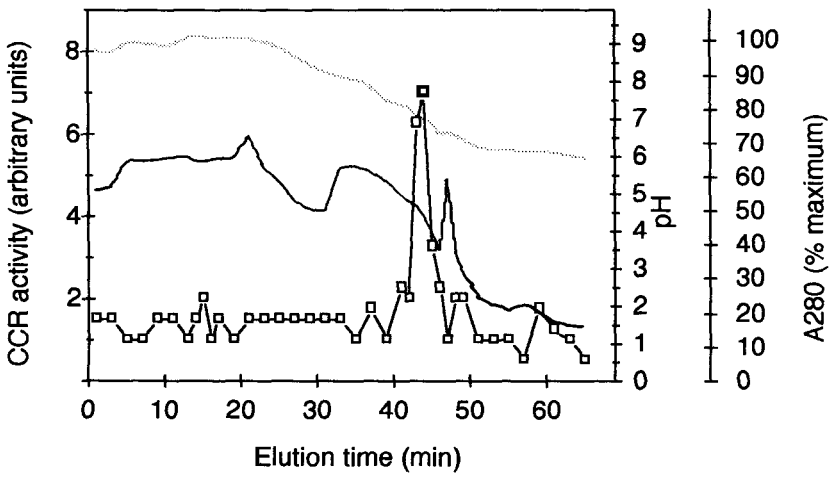

Figure 5. Chromatofocusing of semi-purified *Eucalyptus* CCR (post-Mono Q) on Mono P (fast protein liquid chromatography) between pH 9 and 6 (□). The pH gradient is represented by a dotted line and protein content (A_{280}) is represented as a continuous line.

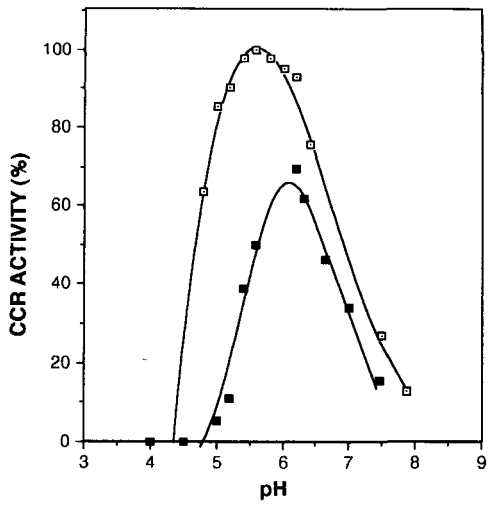

Figure 6. pH optimum of CCR activity in potassium/sodium phosphate (□) and citrate NaOH (■) buffers.

chromatography. All MDH activity was separated from CCR by washing the Mimetic Red 2 matrix with 8 mM NAD. The purity of CCR was verified not only by the presence of a single, silver nitrate-stained polypeptide of 38 kD on SDS gels, but also by the presence of a single species after two-dimensional electrophoresis. These results, in conjunction with the native molecular mass determination by Superose 6 gel filtration, demonstrated that *Eucalyptus* CCR is a monomer of approximately 38 kD. This value is in agreement with previous reports for CCR from spruce (Luderitz and Grisebach, 1981), soybean (Wengenmayer et al., 1976), and poplar (Sarni et al., 1984), indicating that CCR is a monomer in the 33- to 38-kD range. Proof that the purified protein catalyzed the CCR reaction, the conversion of feruloyl-CoA into coniferylaldehyde, was obtained by the identification of coniferylaldehyde as the reaction product of pure CCR. For this determination, TLC was carried out under conditions identical to those described for the spectrophotometric assay with the addition of radiolabeled substrate.

Several authors have hypothesized that the substrate specificities of monolignol biosynthetic enzymes play a role in dictating lignin heterogeneity. Angiosperm lignins typically contain both guaiacyl (G) and syringyl (S) units, whereas gymnosperm lignins typically contain only G units, although there are exceptions to this rule (Lewis and Yamamoto, 1990). Detailed analysis of lignin monomeric composition in *Eucalyptus* indicated an S/G ratio varying from 2.4 to 3, depending on the position of the wood from which the sample was

Table II. *Kinetic parameters of CCR from Eucalyptus*

Substrate	K_m	K_{cat}	K_{cat}/K_m
	μM	min^{-1}	$min^{-1}\ \mu M^{-1}$
Feruloyl-CoA	37 (±6.4)	12	0.32
Sinapoyl-CoA	28 (±2.3)	3.7	0.13
p-Coumaroyl-CoA	28 (±5.3)	3.3	0.12
Caffeoyl-CoA	45 (±16.2)	4.9	0.11

Table III. *Inhibition of CCR activity by effectors*

Effector	Percent Inhibition
CoASH (1 mM)	20
NADP (1 mM)	53
Phenylglyoxal (0.3 mM)	35
*p*CMB (0.3 mM)	88
DIDS (0.05 mM)	95
Diethylpyrocarbonate (≤2 mM)	0
EDTA (≤2 mM)	0
Ferulic acid (≤2 mM)	0
Cinnamic acid (≤2 mM)	0
p-Coumaric acid (≤2 mM)	0
Caffeic acid (≤2 mM)	0

taken (Monties and Lapierre, 1981). This is in keeping with the findings for other angiosperm lignins (Lapierre et al., 1986, 1988). The substrate affinity data of CCR from *E. gunnii* suggested that the protein did not prefer one cinnamoyl-CoA over another; nearly identical K_m values were obtained for the four cinnamoyl-CoA esters tested. Based on these data, no direct relationship could be established between CCR substrate specificity and lignin monomeric composition. In fact, contrary to what might be predicted, the in vitro k_{cat} and V_{max} for feruloyl-CoA was higher than that for the other substrates for *E. gunnii* CCR. This is in agreement with kinetic data for CCR from spruce and poplar (Luderitz and Grisebach, 1981; Sarni et al., 1984). CAD from *E. gunnii* also shows nearly identical substrate affinity vis-a-vis the different cinnamaldehydes (Goffner et al., 1992).

Several enzymes of the lignin biosynthetic pathway have been hypothesized to control lignin monomer composition in plants, including hydroxycinnamate:CoA ligase (Gross et al., 1975; Kutsuki et al., 1982b; Grand et al., 1983), *O*-methyltransferase (Higuchi, 1981; Gross, 1985), CAD (Mansell et al., 1976; Kutsuki et al., 1982a), and CCR (Luderitz and Grisebach, 1981). Our data are consistent with the fact that no clear-cut, consistent relationship exists between the substrate specificities of these enzymes and lignin monomer composition. To date, the only enzyme that has been unambiguously shown to play a role in dictating lignin monomer composition is ferulate 5-hydroxylase (Chapple et al., 1992).

Further biochemical characterization of CCR was carried out by determining the effect of various chemical compounds on CCR activity. Inhibition was observed by NADP and CoASH. However, because millimolar concentrations were necessary for inhibition, the physiological significance of these results remains questionable. By far the most effective inhibitors tested were DIDS and pCMB, which bind Lys and Cys residues, respectively. These results give rise to at least two hypotheses: (a) Lys and Cys are essential for catalysis at the active site, or (b) the binding of DIDS and pCMB on available residues blocks substrate access to the active site. In the future this information may be useful in defining a strategy for site-directed mutagenesis, which in turn will generate a more in-depth knowledge of CCR catalysis and thereby allow us to elucidate the role of Lys and Cys residues in the interaction between enzyme and substrate.

As a first step toward developing an understanding of the regulation of CCR at the molecular level, it is essential to generate CCR-specific probes. The antibodies obtained from this study will enable us to address questions concerning CCR regulation and the role of CCR not only in lignification but also in other physiological processes. For example, monolignols are also implicated in the biosynthesis of compounds other than lignins; some lignans are synthesized by the dimerization of monolignols (Orr and Lynn, 1992). Despite the importance of lignans in plant growth and development, little is known about the control of the biosynthesis of these compounds.

The question of how lignin quantity is regulated also remains unanswered. Studies on the inhibition of CAD activity with inhibitors (Grand et al., 1982, 1985) suggested that CAD played a key role in regulating the quantity of lignins. Results obtained with the *Sorghum* brown midrib mutant, *bmr3*, suggested that decreased CAD activity resulted in changes in lignin quality rather than quantity (Pillonel et al., 1991). Decreased CAD activity resulted in an increased incorporation of coniferylaldehyde, as opposed to coniferyl alcohol, into the lignin polymer (Pillonel et al., 1991). Recently, Halpin et al. (1993) obtained similar results with transgenic tobacco expressing the CAD gene in an antisense configuration. Antisense CAD plants had greater than 80% reduction in CAD activity (Halpin et al., 1993). Lignin levels were not decreased in these plants; rather, aldehydes were incorporated into the polymer in place of alcohols (Halpin et al., 1993). Clearly, the quantity of lignins must be regulated at some other step. The hypothesis that CCR is a point at which lignin quantity is regulated can be addressed using an approach analogous to that of Halpin et al. (1993). Using the CCR-specific tools obtained in the present study, cDNA and genomic cloning of CCR from *E. gunnii* are currently underway.

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