Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 98-10

OXIDATIVE C4-DEMETHYLATION OF 24-METHYLENE CYCLOARTANOL BY A CYANIDE-SENSITIVE ENZYMATIC SYSTEM FROM HIGHER PLANT MICROSOMES

Sophie PASCAL, Maryse TATON and Alain RAHIER

Département d'Enzymologie Moléculaire et Cellulaire de l'IBMP - CNRS UPR 406
Institut de Botanique, 28 rue Goethe 67083 - Strasbourg, France

Received July 4, 1990

SUMMARY : Microsomes isolated from corn embryos (*Zea mays*) were shown to catalyse the C-4 monodemethylation of 28-[^{3}H],24-methylene cycloartanol 1, leading to the corresponding 4α-methyl sterol, cycloeucalenol 5. An enzymatic assay has been developed for the 4,4-dimethyl sterol 4-demethylase in higher plants. The demethylation process was shown to involve a 4-methyl, 4-hydroxymethyl derivative 2 which can be considered as the immediate metabolite of 1 by the 4-methyl oxidase. Compound 2 is further metabolized into 5 through a 4-methyl-4-carboxylic acid 3 and a 3-keto-4α-methyl intermediate 4 which were identified. The conversion of 1 into 5 requires NADPH and molecular oxygen. The initial oxidative step was strictly dependent upon molecular oxygen, NADPH or NADH, and strongly inhibited by cyanide, whereas the overall process was completely insensitive to CO and to specific inhibitors of cytochrome P-450. It is concluded that in *Zea mays* microsomes, the C-4 demethylation of 1 results from a multistep process involving a terminal oxygenation system sensitive to cyanide which is distinct from cytochrome P-450 and in particular from that involved in the 14α-demethylation of obtusifoliol.

Biosynthesis of cholesterol in mammals and ergosterol in fungi involves the oxidative removal of three methyl groups from lanosterol : the 14α-methyl is first removed and is followed by two successive demethylations of both C-30 and C-31 methyl groups of the 4,4-dimethyl precursor (1,2). In contrast, results from biosynthesis studies in higher plants indicate that the first methyl to be removed during the conversion of cycloartenol to phytosterols is located at the C4-position (3,4). The 14α-methyl group is

ABBREVIATIONS

GC : gas chromatography ; TLC : thin layer chromatography ; GC-MS : gas chromatography-mass spectrometry ; I_{50} : concentration producing 50% inhibition.

24-methylene-cycloartanol 1 : 4,4,14α-trimethyl-5α-ergosta-9β,19-cyclo-24(28)-en-3β-ol ; cycloeucalenol 5 : 4α,14α-dimethyl-5α-ergosta-9β,19-cyclo-24(28)-en-3β-ol ; obtusifoliol : 4α.14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol ; 24-methylene lophenol : 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol.

0006-291X/90 $1.50

lost next, at the obtusifoliol level (5), after cleavage of the 9β,19-cyclopropane ring of cycloeucalenol 5. In addition, results from these studies suggest that 24-methylene lophenol is the most probable substrate of the second C-4 methyl group elimination (4).

In this initial report, we describe for the first time a microsomal preparation isolated from a higher plant which contains a cyanide-sensitive enzyme and is functional in the oxidative conversion of 24-methylene cycloartanol 1 to cycloeucalenol 5. In addition, we report the successful isolation by manipulating cofactors, of three intermediates in the demethylation process.

MATERIALS AND METHODS

Authentic materials : Cycloartenol was isolated from Indian opium marc as described previously (13) (mp=99-100°C, MeOH). Cycloeucalenol 5 was extracted from tallow wood (*Eucalyptus mycrocorys*) kindly provided by Prof. R.A. Massy Westropp (Adelaïde, Australia) (mp=139-140°C, MeOH). Cycloeucalenone 4 was synthesized as described previously (7) (mp=81-83°C, MeOH). N[(1,5,9)-trimethyl-decyl]-4α,10-dimethyl-8-aza-trans-decal-3β-ol 6 was synthesized as previously described (14).

24-methylene cycloartanol 1. This compound was synthetized starting from cycloartenol (to be reported) ; (mp=120-122°C, MeOH) ; >98% GC purity). All control data, IR, MS, ^{1}H-NMR are in full agreement with the structure of 1 and literature data.

28-[^{3}H]-24-methylene cycloartanol : This compound was prepared by enzymatic methylation of cycloartenol in the presence of [methyl-^{3}H]-Adomet (200 µCi/µMole) (Amersham) and purified as described before (15). The resulting 28-[^{3}H]-24-methylene cycloartanol was diluted with cold material to a specific radioactivity of 1.0 µCi/µMole. It showed a single peak in GC and a single radioactive band on TLC.

4,4-dimethyl 4-demethylase assay : Microsomes (pH 7.6) were prepared from maize seedlings as described previously (5). In the standard procedure the microsomes (0.75 ml = 4 mg protein) were incubated aerobically in the presence of 24-methylene cycloartanol (100 µM), emulsified with Tween 80 (final concentration 1 g/l), 1 mM NADPH, 1 unit glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate and other additions as indicated in tables and figures. Unless otherwise specified, the reaction mixture contained 0.2 µM N-[(1,5,9)-trimethyl-decyl]-4α-10-dimethyl-8-aza-trans-decal-3β-ol 6 to eliminate concomitant metabolization of cycloeucalenol 5 by cycloeucalenol-obtusifoliol isomerase and subsequent sterol metabolizing enzymes present in our system (7). Incubations were continued aerobically at 30°C with gentle stirring for 4 h, during which the reaction proceeded linearly.

Assay with tritiated substrate 1 : Tritiated 1 (0.15 µCi, 100 µM) was incubated with the aforementioned conditions and sterols were extracted as described previously (5). The residue was analyzed by TLC on silica gel using CH_2Cl_2 as solvent (two migrations). The 3-keto derivative 4 (band e) (Rf = 0.55) was separated from 4,4-dimethyl sterols (band d) (Rf = 0.44), 4α-methyl sterols (band c) (Rf = 0.37), 4-demethyl sterols (band b) (Rf = 0.26) and from the mixture of the 4-hydroxymethyl 2 and 4-carboxylic derivative 3 when present (band a) (Rf = 0.02-0.08) (Fig. 1; A,B). These five fractions were eluted separately and the associated radioactivity determined. The overall conversion ratio was calculated from radioactivity associated with remaining substrate (band d) and with the products formed (bands a,b,c and e) ; for standard incubation times (4 h), a very low amount of radioactivity was associated with band b attesting the blockade by 6 of the metabolization of 5. The rate of 24-methylene cycloartanol demethylation activity was calculated from the amount

of 1 added and the conversion ratio. Compounds present in each of the three aforementioned bands d,e and c were identified by i) comigration with authentic standards on TLC, ii) t_R relative to cholesterol and coinjection with authentic standards in GC (OV17, WCOT 25mx0.25 mm), iii) GC-MS analysis (LKB 9000F, 70 eV) and comparison with MS of authentic standards.

Assay with unlabelled substrate 1 : In the case of incubations under conditions where both compounds 2 and 3 (band a) and demethyl sterol metabolites (band b) were not detectable or negligible, a routine assay was performed using unlabelled 1. In that case the aforementioned bands e,d and c were eluted together and the mixture of substrate and products analyzed by GC using a silica-fused capillary column (WCOT, 25 m x 0.25 mm) coated with OV 17, (230°C to 280°C), (2°C/min) using hydrogen as carrier gas. 24-methylene cycloartanol 1 (t_R = 1.428), its demethylation metabolites cycloeucalenone 4 (t_R = 1.340) and cycloeucalenol 5 (t_R = 1.284) were clearly separated on this column (Fig. 1; C,D). The overall conversion ratio was calculated from the areas of the peaks of compounds 1, 4 and 5 and corrected from the value obtained in the boiled assay (<0.05 for a concentration of 1 = 100 µM). The overall 4-demethylase activity was deduced from this conversion ratio and the amount of substrate added.

Identification of metabolites 2 and 3 : Experiments directed to the accumulation of 4 hydroxymethyl- 2 and 4-carboxylic acid intermediate 3 were carried out with the 100000 x g supernatant resulting from centrifugation of microsomes (30 ml) treated with a mixture of sodium cholate and Emulphogen (to be published elsewhere). This solubilized fraction (30 ml) was strictly depleted from endogenous NAD^+ and $NADP^+$ by preincubation (15 min., 30°C) in the presence of the NADH and NADPH generating systems. It was incubated in the presence of 1 (100 µM ; 4 µCi) with the standard procedure used for the microsomal preparation with the three following modifications : i) a 10 µM concentration of NADPH was used instead of 1 mM, ii) the reaction was stopped by adding 1 ml methanol and iii) the pH was adjusted to pH 3 before extraction with diethyl-ether. Band a (Rf = 0.02-0.08) (1.1 µCi) was eluted and treated with 200 µl of a fresh solution of diazomethane (CH_2N_2, 0.15 M in ether), for 2 h at 0°C. Following evaporation to dryness of CH_2N_2 and Et_2O, the residue was acetylated using a 1/1 mixture of acetic anhydride and pyridine under standard conditions. The reaction mixture was chromatographied (SiO_2, hexane/AcoEt 6/4) leading to a radioactive band (Rf = 0.75) (0.9 µCi) which was eluted. GC-MS analysis of this fraction showed only 2 components in a 1 to 2 ratio which were respectively identified as 4,14α-dimethyl-4acetoxymethyl-5α-ergosta-9β,19-cyclo-24(28)-en-3β-yl-acetate 7 and 4,14α-dimethy-4carbomethoxy-5α-ergosta-9β,19-cyclo-24(28)-en-3β-yl acetate 8 (Table 2).

RESULTS

24-methylene cycloartanol C4-monodemethylase : assay conditions and identification of 24-methylene cycloartanol metabolites

The ubiquitous presence of cycloeucalenol 5 in higher plants and the observation that cycloartenol is from afar the best substrate of Adomet-Δ^{24}-sterol C24-methyltransferase in higher plants (6) led us to consider 24-methylene cycloartanol 1 as the most probable physiological substrate of the first C4 demethylation in these organisms. Incubation of 1 with microsomes from *Zea mays* in the presence of NADPH, a NADPH regenerating system, molecular oxygen, and 0.2 µM of 6 a potent inhibitor of cycloeucalenol-obtusifoliol isomerase (7), allowed identification of a 4-monomethyl sterol metabolite, cycloeucalenol 5, demonstrating that conditions have been obtained to study

removal of one of the 4-methyl group of 1. Under these conditions the use of radiolabelled 1 allowed preparative resolution of the remaining substrate 1, cycloeucalenol 5 and another metabolite produced in low amount which could be identified as cycloeucalenone 4 (see Materials and Methods) (Table 1, Fig. 1). In the case of incubation in absence of 6 the overall conversion of 1 was similar, but product 5 was further metabolized into obtusifoliol, 32-NOR-obtusifoliol, 24-methylene lophenol and 4-demethyl sterols by the microsomes (Datas not shown). In order to attempt to accumulate any possible intermediate metabolites and to assess precisely the requirements of the demethylase system, we manipulated the cofactors composition and conditions of the incubation. The results of such modifications on the nature and amount of metabolites produced, and on the overall demethylation rate are described in Table 2. In the presence of its regenerating system, NADPH was shown to be able to sustain the overall demethylation process. In contrast, absence of NADPH regenerating system as well as elimination of NADPH and substitution by NADH as electron donor, led to the accumulation of the 3-keto, 4α-monomethyl derivative, cycloeucalenone 4, at the expense of 5, without reduction of the overall demethylation rate of 1. In addition, results from Table 2 indicate that endogenous pyridine nucleotides, after complete reduction by their regenerating systems, are sufficient to sustain the demethylation process. However, under these conditions, the reduction of 4 was strongly decreased. In order to strictly eliminate any

Table 1 . GC-MS[a] analysis of substrate and metabolites by the C4 demethylase system

Component	t_R relative to cholesterol in OV 17	OV 1	Molecular ion percent relative abundance	Prominent fragment ion m/e (%)		
24-methylene cycloartanol 1	1.428	1.389	440(45)	425(58) 379(43) 300(70)	422(94) 357(6) 285(20)	407(100) 353(24)
4-methyl, 4 acetoxymethyl 3β-acetate derivative 7	2.437	1.943	540(10)	525(11) 465(11) 405(13)	480(18) 420(44) 300(25)	466(9) 407(11)
4-methyl, 4 carbomethoxy, 3β-acetate derivative 8	2.286	1.833	526(24)	511(6) 451(26) 341(30)	467(49) 407(9) 300(24)	466(100) 401(5)
cycloeucalenone 4	1.340	1.294	424(100)	409(43) 326(74) 297(21)	381(38) 300(32) 285(13)	341(36) 299(74)
cycloeucalenol 5	1.284	1.265	426(31)	411(50) 343(3) 285(41)	408(100) 301(25) 283(20)	393(83) 300(41)

[a]) 70 eV

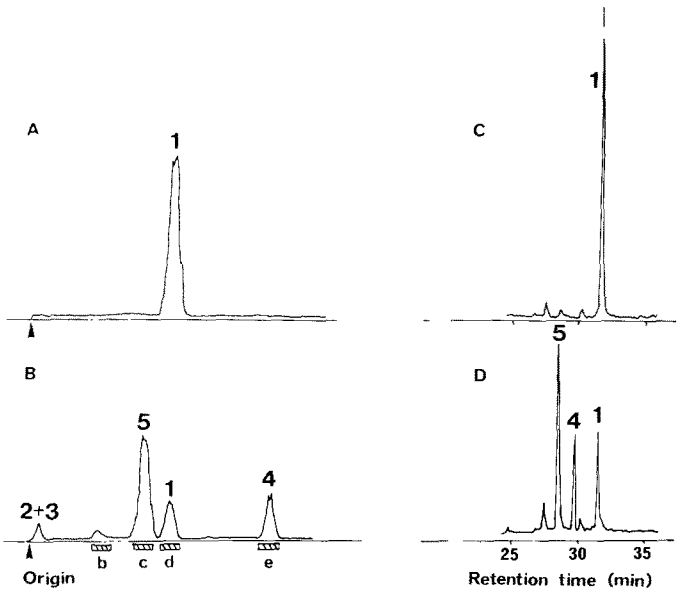

Figure 1 . Radiochemical- (A,B) and Gas chromatographic assay (C,D) of 24-methylene cycloartanol-4-demethylase.
Tritiated 1 (0.15 µCi, 100 µM) was incubated in the standard assay conditions (see Materials and Methods), 0.75 ml of microsomes, 4 mg of protein.
A,B : Radiochromatogram scan of T.L.C. plate showing production of cycloeucalenol 5, cycloeucalenone 4, 4 methyl, 4-hydroxymethyl- 2 plus 4-methyl and 4-carboxylic acid intermediate 3 from tritiated 1. Unlabelled standards : b) sitosterol ; c) cycloeucalenol ; d) 24-methylene cycloartanol ; e) cycloeucalenone. A : boiled microsomes ; B : 4 hr. incubation in the presence of 7 (0.2 µM). The overall amount of radioactivity recovered was 70% ; C,D : corresponding gas chromatogram (OV17 column) of the pooled fractions migrating as c, d, and e. C : boiled microsomes ; D : 4 hr. incubation in the presence of 6 (0.2 µM).

traces of oxidized pyridine nucleotides the 4-methyl demethylase complex was solubilized from the microsomes by mean of detergents. Incubation of the 100000 g supernatant in the presence of both NADH and NADPH generating systems, and of a limiting concentration of NADPH (10 µM), led to the accumulation of two other intermediates which after derivatization were identified as the 4-hydroxymethyl- 2 and the 4-carboxylic acid derivatives 3. (see Materials and Methods). In addition data from Table 2 show that the initial step of the C4-demethylation process was fully dependent upon molecular oxygen since none labelled metabolite could be detected in anaerobic conditions. Finally, this initial oxidative step was shown to be sustained by the presence of either NADPH or NADH.

Effects of inhibitors on the enzymic demethylation system

In order to get more insight about the nature of the enzymatic system involved in the demethylation process, the 4-demethylase was challenged with a

Table 2 . Influence of cofactors on 24 methylene-cycloartanol-4 methyl demethylase activity and repartition of metabolites

ncubation content[A]	Percent metabolization of tritiated 1 in			
	4-methyl,4-hydroxymethyl derivative 2, 4-methyl, 4-carboxylic acid 3	cyclo-eucalenone 4	cyclo-eucalenol[B] 5	Overall relative demethylation rate
	%	%	%	
crosomes + air + 1 mM NADPH + RS1	3[D]	12	56	100
iled microsomes + air + 1 mM NADPH + RS1	0	0	0	0
crosomes + air + 1 mM NADH + 1 mM NADPH RS1 + RS2	7	4	57	96
crosomes + Argon + 1 mM NADPH + RS1 + RS2 6Lu + 6Lu 1-oxidase	0	0	0	0
crosomes + air	4	6	0	14
crosomes + air + RS1	3	18	0	30
crosomes + air + RS2	3	70	2	105
crosomes + air + RS1 + RS2	5	53	9	94
.crosomes + air + 1 mM NADH + RS2	4	70	3	108
crosomes + air + 1 mM NADH + RS2 + RS1	0	49	21	99
.crosomes + air + 1 mM NADPH	0	85	4	125
D^+ and $NADP^+$ depleted (100000g)[C] + air + 10 µM NADPH RS1 + RS2	31	0	0	44
D^+ and $NADP^+$ depleted (100000g) + air + 20 µM NADH RS1 + RS2	10	40	0	70
D^+ and $NADP^+$ depleted (100000g) + air	0	0	0	0

) All incubations are performed in the presence of 6 (0.2 µM). [B]) Including, when present, the low amount (<3%) of 4-demethyl sterol formed. [C]) 100000g supernatant resulting from the centrifugation of solubilized microsomes. [D]) Data represent the mean of two to four experiments, the standard deviation was 2%.
S1 : NADPH regenerating system = Glu6P + Glu6P dehydrogenase
S2 : NADH regenerating system = EtOH + alcohol dehydrogenase.

Table 3 . Effects of inhibitors on 24-methylene-cycloartanol 4-methyl demethylase activity

Inhibitors	demethylase activity[A]
none	100
CN^- 8 x 10^{-6} M	50
CN^- 50 x 10^{-6} M	5
menadione 5 x 10^{-6}M	95
menadione 50 x 10^{-6}M	39
CO saturated	100
LAB 170250F 100 x 10^{-6}M	100
Tetcyclacis 100 x 10^{-6}M	100

[A]) normalized activity

series of inhibitors. Data from Table 3 show that the 4-demethylation was potently inhibited by cyanide (I_{50} = $8x10^{-6}$M). In addition the electron acceptor menadione proved to be an inhibitor presumably by diverting the flow of electrons from NADPH to the oxidase. In contrast the 4-demethylation was insensitive to carbone monoxyde and to azole derivatives known to ligand the ferric iron atom of the protohem of Cyt.P-450 such as LAB 170250F, a potent inhibitor of the plant obtusifoliol 14α-demethylase (9) or Tetcyclacis, a potent inhibitor of kaurene hydroxylase (10).

DISCUSSION

We have described herein, for the first time, a microsomal preparation isolated from a higher plant which can demethylate one C-4 methyl group of 24-methylene cycloartanol 1, yielding cycloeucalenol 5 under conditions blocking its concomitant metabolization by COI. Under these interrupted conditions the very low amount of labelling incorporated in the 4-demethyl sterol fraction, when compared to a higher incorporation in this fraction in absence of COI inhibition, supports the idea that elimination of the second methyl group at C-4 is carried out by a distinct enzymatic system, with a distinct substrate specificity. This situation would be in contrast with that observed in mammals where the removal of both C-4 methyl groups is carried out successively by the same enzymatic system (1). We could determine experimental conditions which allowed accumulation and characterization of three intermediates (2, 3 and 4) involved during the demethylation process of 1. This result is clearly indicating the following sequence of reactions for the oxidative demethylation of 1 : 1->2->3->4->5 (Fig. 2). Moreover, compound 2 can be considered as the immediate metabolite of 1 by the terminal oxygenase involved in the 4,4-dimethyl sterol 4-methyl demethylation process ; compound 2 is most probably further oxidized into the 4-carboxylic acid derivative 3 by this oxidative system. The question to know whether the 4α or 4β methyl group is loss during this *in vitro* demethylation process is now under investigation (3). Studying the effects of the presence, nature, and oxidative state of pyridine nucleotides on the nature and amount of each of these metabolites led to the following conclusions : i) these metabolites are produced by a succession of at least three distinct enzymatic reactions with distinct

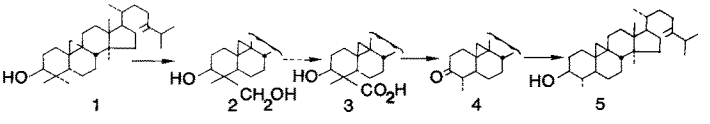

Figure 2 . Pathway for the demethylation of 24-methylene cycloartanol 1 by *Zea Mays* microsomes.

cofactor requirements, ii) reducing equivalents are transferred from either NADH or NADPH to the oxidase. This last result support the idea that two electron transfer pathways are operating as it has been previously proposed for other oxidation reactions (11), iii) reduction of the 3-keto intermediate is strictly NADPH dependent and apparently strongly inhibited by $NADP^+$. When challenged with a series of inhibitors, the 4-demethylase system responded in a way indicating that cytochrome P-450 is not involved in the removal of the C-4 methyl group which in turn involves the participation of a cyanide sensitive enzyme. A possibility would be the participation of cytochrome b_5 as an intermediate electron carrier associated with the reaction. Indeed cyt.b_5 was detected by spectral measurements (12) in the microsomal preparation (0.15 nmole/mg protein) and in the solubilized fraction (0.18 nmole/mg protein). These results clearly show that two distinct oxidative systems are responsible respectively for the oxidative removal of the C14α- and C4-methyl group of plant sterol precursors. Taken together the results presented herein indicate that in *Zea mays* microsomes, the removal of the first C-4 methyl group of 4,4-dimethyl-sterols involves a mechanism similar in many respects with that functioning in mammals (1). The properties and characterization of the particulate plant enzymes responsible for these individual reactions are now being investigated.

ACKNOWLEDGMENTS

We wish to thank warmly Pr. P. Benveniste for his continuous interest in this work. We are indebted to B. Bastian for kindly typing the manuscript.

REFERENCES

(1) Tratskos J.M. and Gaylor J.L. (1985) in "The Enzymes of Biological Membranes", Eds, 2, 177-204.

(2) Mercer E.I. (1984) Pest. Sci. 15, 133-155.

(3) Ghisalberti E.L., De Souza N.J., Rees H.H., Goad L.J. and Goodwin T.W. (1969) Chem. Commun. 1403-1405.

(4) Benveniste P. (1986) Ann. Rev. Plant Physiol. (37), 275-308.

(5) Rahier A. and Taton M. (1986) Biochem. Biophys. Res. Comm. 140 (3), 1064-1072.

(6) Rahier A., Taton M., Bouvier-Navé P., Schmitt P., Benveniste P., Schuber F., Narula A.S., Cattel L., Anding C. and Place P. (1986) Lipids 21, (1), 52-62.

(7) Rahier A., Taton M. and Benveniste P. (1989) Eur. J. Biochem. 181, 615-626.

(8) Aoyama Y., Yoshida Y., Sato R., Susani M. and Ruis H. (1981) Biochim. Biophys. Acta 663, 194-202.

(9) Taton M., Ullmann P., Benveniste P. and Rahier A. (1988) Pestic. Biochem. Physiol. 30, 178-189.

(10) Graebe J.E. (1982) in Plant Growth Substances (P.F. Wareing ed.) Academic Press, London.

(11) Hildebrandt A. and Estabrook R.W. (1971) Arch. Biochem. Biophys. 143, 66-79.

(12) Omura T. and Sato R. (1964) J. Biol. Chem. 239, 2379-2385.

(13) Narula A.S. and Dev S. (1971) Tetrahedron 27, 1119-1127.

(14) Taton M., Benveniste P. and Rahier A. (1986) Biochem. Biophys. Res. Commun. 138, 764-770.

(15) Rahier A., Génot J.C., Schuber F., Benveniste P. and Narula A.S. (1984) J. Biol. Chem. 259 (24), 15215-15223.