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CINCHONINONE: NADPH OXIDOREDUCTASES I AND II—NOVEL ENZYMES IN THE BIOSYNTHESIS OF QUINOLINE ALKALOIDS IN *CINCHONA LEDGERIANA*

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Key Word Index—*Cinchona ledgeriana*; Rubiaceae; suspension cultures; alkaloids; biosynthesis; cinchoninone; NADP(H) oxidoreductase; isoenzymes.

Abstract—An enzyme (cinchoninone: NADPH oxidoreductase) which catalyses the reduction of cinchoninone to an unequal mixture of cinchonine and cinchonidine has been isolated from cells of *Cinchona ledgeriana* in suspension culture and its properties have been studied. It is present in two isoenzymic forms which can be separated by ion-exchange chromatography on DEAE-cellulose. Both forms of the enzyme are cytosolic and have an absolute requirement for NADPH. They both catalyse reversible reactions. Isoenzyme I acts specifically on cinchoninone in the forward direction and cinchonidine, cinchonine, cupreine and cupreidine in the reverse direction, while isoenzyme II has a broad specificity acting on all the quinoline alkaloids of *Cinchona*. The kinetic properties of these two isoenzymes are presented. This is the first description of an enzyme wholly committed to the biosynthesis of *Cinchona* quinoline alkaloids.

INTRODUCTION

The major alkaloids accumulating in *Cinchona* plants are the stereoisomeric quinoline alkaloids quinine and quinidine and their 6′-demethoxy derivatives, cinchonidine and cinchonine (Scheme 1). The two series differ in their stereochemistry at C-8 and C-9, the quinine series being 8*S*,9*R* and the quinidine series being 8*R*,9*S*. Biosynthetically, these alkaloids are derived from tryptamine and secologanin [1, 2] by the action of the enzymes tryptophan decarboxylase [3] and strictosidine synthase (Walton, Skinner, Robins and Rhodes, unpublished work). These early stages are common with indole alkaloid formation in many species [4] but subsequently strictosidine is converted by an undescribed pathway to the quinoline intermediate, cinchoninone. This compound appears to be an important intermediate leading to the two steric series of products [5] since cinchoninone, labelled in C-11 and fed to *Cinchona* tissue, was incorporated into both cinchonine and cinchonidine and their methoxylated derivatives [6].

This paper reports two NADP(H)-dependent enzymes, the first activities described specific to the *Cinchona* quinoline alkaloid pathway, isolated from suspension cultures of *C. ledgeriana* [7]. Both of these catalyse the reversible reduction of cinchoninone to form both cinchonine and cinchonidine, and one of them also shows reversible activity with its 6′-methoxy derivative, quinidinone, forming quinine and quinidine.

RESULTS

Activity in crude enzyme preparations

Soluble enzyme preparations from *C. ledgeriana* cultures, incubated with NADPH and either cinchoninone or quinidinone, showed a NADPH-dependent, substrate-dependent progressive decrease in fluorescence. The stoichiometry of product formation was studied by incubating until the reaction reached equilibrium. The disappearance of NADPH was quantified fluorometrically; the products were extracted and analysed by HPLC [8]. Cinchoninone as substrate led to the accumulation of cinchonidine and cinchonine, while quinidinone similarly was reduced to quinine and quinidine (Table 1). The molar stoichiometry of the reaction (Table 1) (NADPH oxidized: alkaloid synthesized) was approximately unity for cinchoninone and quinidinone but the steric ratio of products (Table 1) favoured the 8*S*,9*R*-series from both substrates.

These reactions were found to be essentially reversible. When cinchonine and cinchonidine were presented together, the rate of NADPH formation was comparable to that obtained with either substrate alone, indicating that a single activity is responsible for the oxidation of both isomers.

Thus it appears that, schematically, the reactions are:

$$\text{Cinchoninone} + \text{NADPH, H}^+ \rightleftharpoons (\text{cinchonidine} + \text{cinchonine}) + \text{NADP}^+$$

and

$$\text{Quinidinone} + \text{NADPH, H}^+ \rightleftharpoons (\text{quinine} + \text{quinidine}) + \text{NADP}^+.$$

The two forward reactions show different effects of pH on the rate of reduction (Fig. 1). With cinchoninone a

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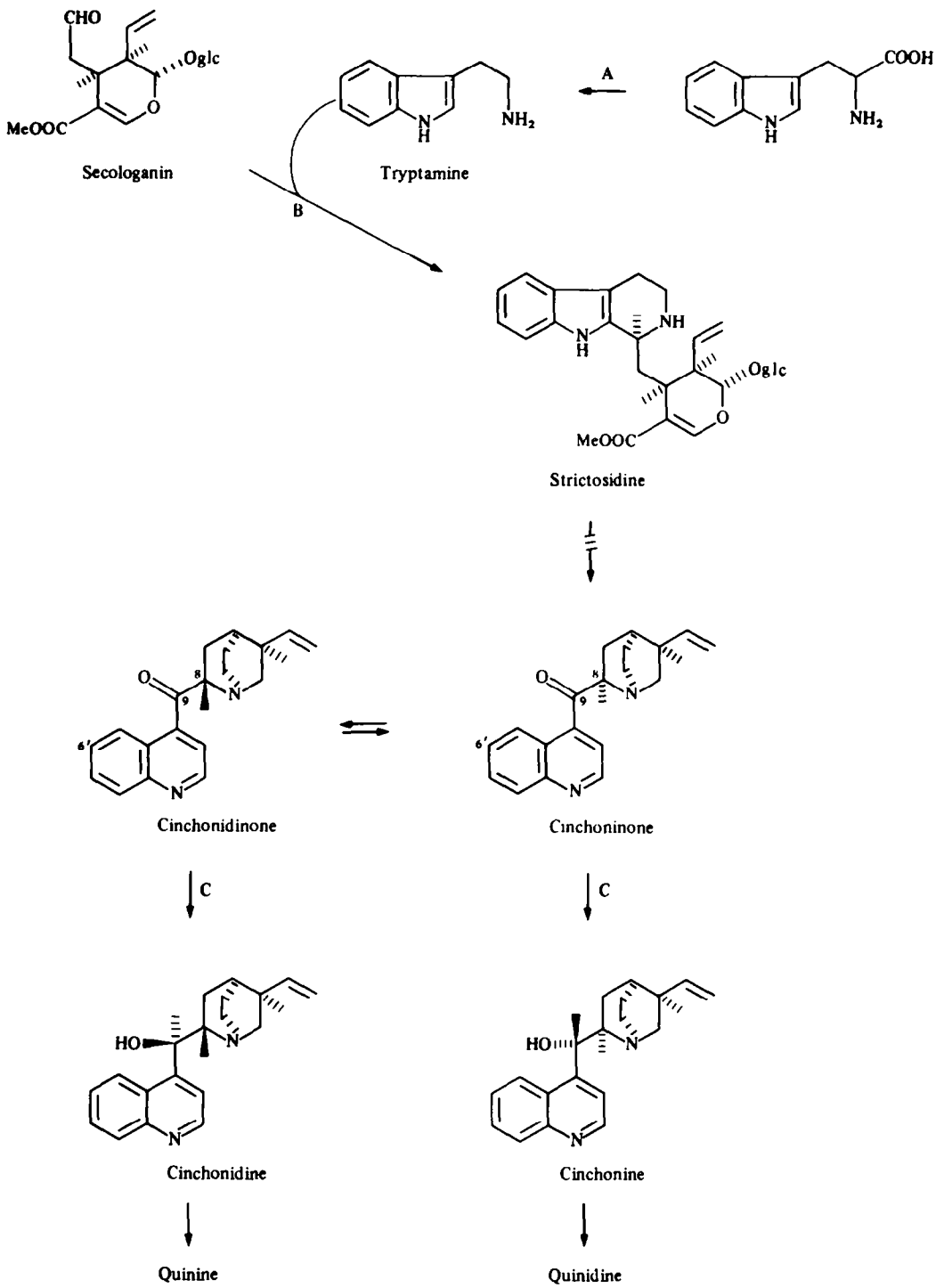

Scheme 1. Outline of the pathway for the biosynthesis of the *Cinchona* quinoline alkaloids. A = Tryptophan decarboxylase; B = strictosidine synthase; C = cinchoninone oxidoreductases.

broad optimum around pH 7.5–8.5 is found, while quinidinone shows a sharp optimum at pH 7.2. For further analysis, pH 7.5 was used.

Purification

The activities involved in these reactions were concentrated by (a) ammonium sulphate precipitation followed by desalting on Sephadex G25 or (b) dialysis followed by ion-exchange chromatography on DEAE-cellulose. Procedure (b) rendered two, separable, fractions able to reduce the ketones of the quinoline alkaloids, designated isoenzymes I and II (Fig. 2). These preparations showed variable stability at −20°, with (a) retaining 80% activity after 1 month, and (b) losing activity completely after only 3 days.

Table 1. Stoichiometric and steric ratios of the reaction in a crude extract

Reactant alkaloid	NADPH oxidized (pmol)	Product formed (pmol)	Stoichiometric ratio (NADPH oxidized: alkaloid formed)	Steric ratio of products (8*S*,9*R*:8*R*,9*S*)
Cinchoninone	11250	Cinchonidine 7000 Cinchonine 5120	1.08	1.36
	11250	Cinchonidine 6126 Cinchonine 4074	0.91	1.50
	22240	Cinchonidine 11150 Cinchonine 6067	0.77	1.84
		Mean:	0.92 ± 0.13	1.57 ± 0.20
Quinidinone	5930	Quinine 3465 Quinidine 2017	1.08	1.72
	7500	Quinine 4068 Quinidine 2986	0.94	1.36
	8750	Quinine 5504 Quinidine 2384	1.11	2.34
	11250	Quinine 6790 Quinidine 4939	1.04	1.37
		Mean:	0.99 ± 0.07	1.70 ± 0.39

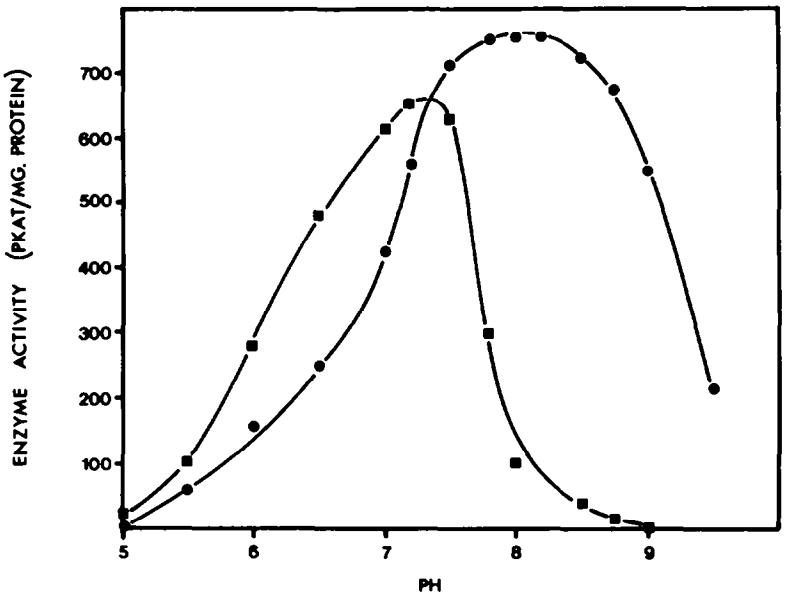

Fig. 1. Effect of pH on the activity of a crude extract against cinchoninone (●) and quinidinone (■) in the presence of 0.5 mM NADPH.

Substrate specificity

Neither of the activities against quinoline alkaloids is due to general alcohol dehydrogenases. NAD(H)-dependent ethanol dehydrogenase (EC 1.1.1.1) [9] coincided with isoenzyme II (Fig. 2) but was absolutely specific for NAD(H). Both isoenzymes, however, showed absolute specificity for NADP(H) as cofactor (Table 2). Aromatic alcohol dehydrogenase (EC 1.1.1.2) [10, 11] partially overlapped, but was not coincident with, isoenzyme I.

Isoenzyme I is specific for cinchoninone and unable to reduce quinidinone (Table 3). In the reverse direction, it catalyses the oxidation of cinchonine and cinchonidine, cupreine and cupreidine (the 6'-hydroxy derivatives) at approximately 25% of the rate but not quinidine or quinine (the 6'-methoxylated derivatives) or 4-quinoline carboxaldehyde. On the other hand, isoenzyme II has a broad substrate specificity, acting on hydroxylated, methoxylated and unsubstituted alkaloids.

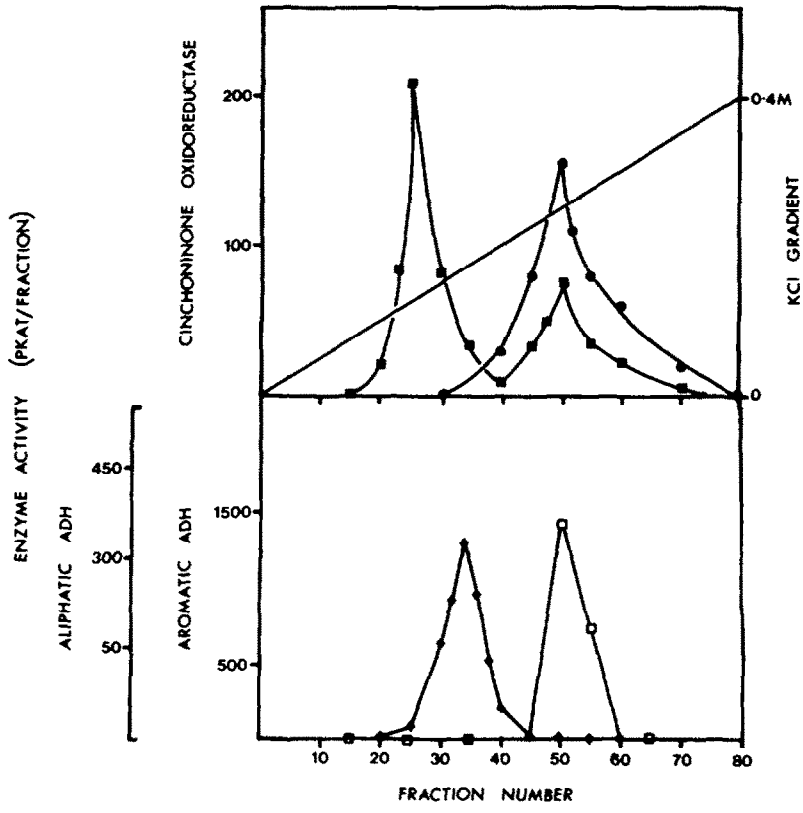

Fig. 2. Elution profiles for a *C. ledgeriana* cell extract on DE-52 DEAE-cellulose, eluted with KCl (0–0.4 M). Enzyme activities are shown with NADPH as cofactor against cinchoninone (■), and quinidinone (●), with $NADP^+$ and coniferyl alcohol (◆) and with NAD^+ and ethanol (□). The values given for quinidinone are comparative, not absolute (see Experimental).

Table 2. Cofactor requirement of cinchoninone:NADPH oxidoreductase

Reactants		Rate (pkat)*
1 mM Cinchoninone	+0.5 mM NADPH	182
	+0.5 mM NADH	~0
1 mM Quinidinone	+0.5 mM NADPH	152
	+0.5 mM NADH	~0
0.5 mM Cinchonidine	+0.5 mM $NADP^+$	170
	+0.5 mM NAD^+	6
0.5 mM Cinchonine	+0.5 mM $NADP^+$	154
	+0.5 mM NAD^+	8

*Determined using dialysed crude extract (see Experimental).

Table 3. Substrate specifity of cinchoninone:NADPH oxidoreductase isoenzymes

Substrate (1 mM)	Isoenzyme I (pkat)	Isoenzyme II (pkat)
Cinchoninone	205	88
Quinidinone	n.d.*	180
Cinchonidine	217	35
Cinchonine	207	77
Quinidine	n.d.	89 (n.m.)†
Quinine	n.d.	80 (n.m.)
Cupreine	51	33
Cupreidine	49	34
4-Quinoline carboxaldehyde	n.d.	46
Coniferyl alcohol	42	n.d.

Enzymes assayed at pH 7.5 in the presence of 0.5 mM $NADP^+$ or NADPH. No activity with either enzyme was observed with benzaldehyde or cinnamaldehyde as substrates in the presence of NADPH or with ethanol and $NADP^+$.

*n.d. Not detectable.

†n.m. Not precisely measurable due to fluorescence of substrate (see text).

Kinetic properties of isoenzyme I

Isoenzyme I only reacts with cinchoninone as substrate and NADPH in the forward direction. The kinetics show a simple Michaelis–Menten pattern (Table 4) but in the reverse direction at concentrations of cinchonidine, but not cinchonine, higher than 0.5 mM there is strong substrate inhibition (Fig. 3). The affinity of the isoenzyme for the cofactor NADP(H) is independent of the substrate and the direction of the reaction (K_m = 0.14 mM), and follows simple Michaelis–Menten kinetics. Furthermore, the affinity of the isoenzyme is much higher for cinchonidine (K_m = 80 μM) than for cinchonine (K_m = 580 μM).

The different kinetic properties of isoenzyme I with cinchonine and cinchonidine could be interpreted to

Table 4. Kinetic constants for the activities of cinchoninone:NADPH oxidoreductase isoenzymes with various substrates

Substrate	Isoenzyme I K_m (mM)	Isoenzyme I V_{max} (pkat/μg protein)	Isoenzyme II K_m (mM)	Isoenzyme II V_{max} (pkat/μg protein)
(a) Forward direction (reduction):				
Cinchoninone*	0.6	3.2	0.4	1.0
Quinidinone*	—†	—†	0.4	3.3
NADPH (+cinchoninone‡)	0.14	1.0	1.0	3.2
(b) Reverse direction (oxidation):				
Cinchonine*	0.58	1.14	1.0	0.44
Cinchonidine*	0.08	0.76	0.4	1.0
$NADP^+$ (+cinchonine‡)	0.14	0.73	1.0	0.27
$NADP^+$ (+cinchonidine‡)	0.14	0.94	1.0	0.6

*Plus 0.5 mM cofactor.
†Activity I inactive with quinidinone (see Table 2).
‡Plus 1.0 mM alkaloid.

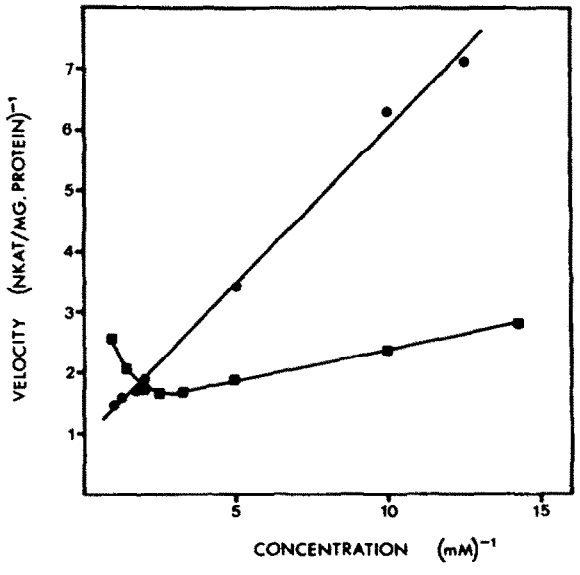

Fig. 3. Double reciprocal plot showing the kinetics of the reverse reaction of cinchoninone oxidoreductase peak I with cinchonine (●) or cinchonidine (■) and $NADP^+$ (0.5 mM) as cofactor.

suggest that there are two enzymes present, one acting on cinchonine and the other on cinchonidine. As found with the crude preparation, however, isoenzyme I did not show additive rates of reaction with cinchonine and cinchonidine. Furthermore, attempts to separate these activities using Blue Sepharose and specific elutions with either substrate were not able to resolve this activity. It is probable, therefore, that isoenzyme I is a single enzyme which can bind both the 8*S*,9*R* and 8*R*,9*S* products, though with differing affinities.

Kinetic properties of isoenzyme II

Isoenzyme II reacts in the forward direction with either quinidinone or cinchoninone as substrates and NADPH. In the reverse direction, it was not possible to conduct detailed kinetic studies with quinine and quinidine owing to the high background fluorescence (see Experimental). In both directions the kinetics for both substrates and cofactor follow the simple Michaelis–Menten pattern (Table 4). In the forward direction, the affinity for the substrate was not affected by the methoxyl substituent at C-6', though the V_{max} was greater for the methoxylated alkaloid. In the reverse direction, as with isoenzyme I, isoenzyme II showed a higher affinity for the 8*S*-stereoisomer. The affinity for cofactor (K_m = 1.0 mM) was lower than for isoenzyme I (K_m = 0.14 mM), and the V_{max} varied considerably depending on the substrate exhibited.

DISCUSSION

Alkaloid-producing suspension cultures of *C. ledgeriana* [7] have been shown to contain two isoenzymes which catalyse the formation of both cinchonidine (8*S*,9*R*) and cinchonine (8*R*,9*S*) from cinchoninone in the presence of NADPH. This is an important synthetic reduction in the biosynthesis of quinoline alkaloids and provides enzymological evidence supporting the postulated intermediacy of cinchoninone in quinine and quinidine biosynthesis [5]. The reversibility of the reaction is compatible with radiolabelling experiments in which cinchonine was incorporated into cinchoninone [6]. The isoenzymes are separable from other plant alcohol dehydrogenases which require NADP(H) [10, 11] and distinct from the NAD(H)-dependent ethanol dehydrogenase [9]. The alkaloid oxidoreductases I and II described here may therefore be committed to alkaloid metabolism, and their absolute dependence on NADP(H) fits the patterns of cofactor requirement becoming established for oxidoreductases in pathways of secondary metabolism [12, 13].

The isoenzymes differ in their substrate specificities and kinetic properties, in particular in relation to the presence of a 6'-substituent on the alkaloid. The affinity of the isoenzymes for the substrates differ in both directions, although only with cinchonidine is a major difference in K_m determined. Isoenzyme I, however, shows a much higher affinity for cofactor and a narrower substrate range, which might indicate this to be the more important activity under physiological conditions. The reduction by isoenzyme II of quinidinone, however, indicates that, biosynthetically, methoxylation could occur before racemization, although there is no evidence to support this.

Purely on the basis of the observed kinetic values (Table 4), cells should accumulate cinchonine and cinchoninone, with smaller amounts of cinchonidine. Nevertheless, the very low endogenous levels of cinchoninone relative to the reduced forms and the ratio in which cinchonine and cinchonidine accumulate [7, 8] are inconsistent with the observed high apparent K_m values and the 8*R*:8*S* stoichiometry of the reaction (Table 1). The final mixture of products formed in fact depends on a number of additional inter-related factors. Tautomerism of the ketone substrates causes racemization at C-8 [14], generating a mixture of species dominated by the 8*S*- and 8*R*-keto forms [15]. As a result, the true substrate for the enzymes is at lower concentration than the total solution value, causing aberrant K_ms to be determined *in vitro*. With other substrates of biological importance which tautomerize, such as oxaloacetate [16] or dihydroxyacetone phosphate [17], the keto is the true enzymatic substrate. With cinchoninone, the mixture is only 63% keto [15], so the K_ms can be adjusted accordingly, giving values of 0.38 and 0.25 mM for isoenzymes I and II, respectively. Furthermore, the racemic mixture is dominated by the 8*S*-keto (49%), the 8*R* being only 14% [15], and so the actual substrates are at even lower concentrations, further decreasing the K_m values in the forward direction. Since, as adjudged by the K_ms in the reverse direction, the affinities of both isoenzymes for the steric isomers are not equivalent, the relative rates with the 8*S*- and 8*R*-keto forms cannot be determined but the affinities for the keto forms may be high enough to favour the forward reaction.

In vivo, the common intermediate for both 8*R*- and 8*S*-alkaloids is the 8*R*-ketone, cinchoninone (Scheme 1), and the extent to which the 8*S*-series is synthesized will depend on the relative rates of racemization to cinchonidinone and reduction to cinchonine and cinchonidine. As racemization of cinchoninone is naturally a relatively slow reaction [14], there may be a specific racemase involved as for oxaloacetate [18]. This is yet to be determined, but might be inferred from the approximately unitary 8*R*:8*S* ratio obtained [7, 8]. The resulting 8*S*:8*R* ratio may also be dependent on the subsequent methoxylation of cinchonine and cinchonidine and on the transport of alkaloids into the vacuole and extracellular spaces [19]. Hence, the final balance of alkaloids obtained is unlikely to be determined by the kinetic properties of the cinchoninone: NADPH oxidoreductases I and II alone, despite their key regulatory position.

EXPERIMENTAL

Materials. Suspension cultures of *Cinchona ledgeriana* (FRIN-CL2A6) transformed with *Agrobacterium tumefaciens* strain A6 were developed and maintained as described in ref. [7]. Cells were harvested, frozen and kept at −20° until required. HEPES, $NADP^+$, NADPH, DTE, cinchonine·HCl, cinchonidine·HCl and quinine·HCl were obtained from Sigma. Cinchoninone was a gift from Professor Battersby, and quinidinone was provided by ACF Chemifarma (The Netherlands). Polyclar AT and Kollidon 40 (soluble) were from BDH and DE-52 was from Whatman.

Enzyme extraction. The extraction medium contained 0.2 M HEPES (pH 7.5), 2 mM EDTA and 5 mM DTE (buffer A). To every gram of frozen cells, 2 ml extraction medium, 100 μg Polyclar AT and 50 μg Kollidon were added. Following homogenization and filtration, the extract was centrifuged at 16000 *g* for 25 min, yielding the crude extract.

Partial purification of the enzyme. (a) Solid $(NH_4)_2SO_4$ was added up to 70% (w/v) to the crude extract while monitoring the pH. The extract was centrifuged at 16000 *g* for 25 min, the pellet resuspended in 5–10 ml buffer A and desalted on a Sephadex G25 column (37 × 2.6 cm) eluted at 0.5 ml/min with buffer A. A fraction representing 1.2 × the vol. of the applied fraction was collected following the elution of the void vol.

(b) DEAE-cellulose chromatography. The crude extract was dialysed against 10 × its vol. with 10 mM HEPES (pH 7.5) containing 1 mM DTE and 1 mM EDTA (buffer B). The dialysed crude extract was then applied to a DE-52 DEAE-cellulose column (12 × 1.6 cm), equilibrated with buffer B. After washing with buffer B, the enzymes were eluted using a linear gradient (0–0.4 M KCl in B) at a flow rate of 0.1 ml/min and fractions (1.2 ml) were collected.

Enzyme assays. The determination of enzyme activities fluorimetrically by the change in fluorescence of the $NADP^+$/NADPH cofactor can potentially be impaired by any native fluorescence in the substrates and products. The fluorescence intensity of the alkaloids was therefore determined in reaction buffer (pH 7.5). With the unmethoxylated forms the fluorescence was only *ca* 1% that of NADPH at comparable concn. Furthermore, the intensity change on reduction or oxidation of the alkaloid was negligible (i.e. cinchoninone = cinchonine = cinchonidine). Thus, accurate kinetic measurements were possible. With the methoxylated forms, however, quinine and quinidine fluoresced as intensely as comparable concns of NADPH, while quinidinone was only *ca* 5% as fluorescent. Thus, the oxidation of the alkaloid at high concn will contribute almost as much to the nett change in fluorescent intensity as that of the nucleotide, making absolute quantification difficult to achieve. Values of a comparative nature can be given (as in Fig. 1) since a change determined in the fluorescence does indicate a reaction to be taking place. Only with high concns of NADPH and quinidinone is an absolute determination of reduction possible (see Table 1).

(i) Cinchoninone oxidoreductases were assayed by monitoring the change in fluorescence of NADPH (ex = 340 nm; em = 458 nm) using a Perkin–Elmer fluorimeter LS5. The reaction rate at 25° was initiated by adding the alkaloid and the nucleotide simultaneously. The reaction rates were checked for dependence on the presence of substrate. For studies on cinchoninone and quinidinone, stock solns were allowed to equilibrate overnight (18 hr) before use in the assay in order to effect mutarotation fully [14, 15] (see Discussion). Unless stated otherwise, the reaction mixture contained the following in a 1 ml cuvette: 1 mM alkaloid; 0.5 mM nucleotide; enzyme preparation as appropriate in elution buffer (A or B); to 1.0 ml with 0.2 M HEPES (pH 7.5).

(ii) Aliphatic alcohol dehydrogenase was assayed as described previously [9].

(iii) Aromatic alcohol dehydrogenase was assayed as described previously [10].

Stoichiometry of the reaction. For this purpose, cinchoninone and quinidinone were re-purified by prep. TLC on silica gel F60

eluted with $CHCl_3$–Me_2CO– 15% NH_4OH in EtOH (5:4:1). Following the enzyme reaction, the reaction mixture was made alkaline with NH_4OH and the alkaloids were extracted with Et_2O and quantified as described previously [8].

Protein determination. Proteins were determined using the Coomassie Blue dye binding method [20] and bovine serum albumin as a standard.

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