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SOME PROPERTIES OF AN INHIBITORY CYTOCHROME P-450 METABOLIC-INTERMEDIATE COMPLEX EXISTING IN A SPIN-STATE EQUILIBRIUM

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Abstract—Incubation, in the presence of NADPH/O_2, of the type II compound revenast with a partially solubilized, phenobarbital-induced rat liver microsomal cytochrome P-450 system results in the formation of a difference spectrum exhibiting a Soret band at 407 nm and a trough at 423 nm. Experiments with N_2 and metyrapone suggest this spectral perturbation to originate from binding to the hemoprotein of a metabolic intermediate derived from the amine substrate. The percentage of pigment complexed can be assessed to be about 7%, with half-maximal complexation occurring at a revenast concentration of 125 μM. Adduct formation is inhibitory to the N-hydroxylation of 4-chloroaniline and N-demethylation of *N,N*-dimethylaniline; kinetic analysis suggests a competitive interaction at the catalytic site of the reactive revenast derivative with the tertiary arylamine. Inhibition of the two monooxygenation reactions is reversible, indicating weak heme bonding of the active intermediate. This behaviour differs from that of most other product adducts so far examined. The aberrant functional properties of the metabolic adduct appear to reflect complex molecular organization of this compound, as is also evidenced by the unique position of the Soret region absorption maximum at 407 nm; this spectral data hints at the presence of a mixture of high- and low-spin forms. Indeed, chemical reduction or oxidation of the product complex reveals the existence of a low-spin component hidden in the metabolic spectrum. Model studies suggest that the product adduct possibly arises from N-oxidation of the revenast molecule.

There exist numerous examples of cytochrome P-450 metabolic-intermediate complexes formed during NADPH-dependent oxidative biotransformation of certain types of xenobiotics [1, 2]. Except for carbene and carbanion species, nitroso or nitroxide products, generated from diverse classes of nitrogenous compounds, represent the major group of reactive ligands tightly bound to the heme iron of cytochrome P-450 [3, 4]. Hence, substrates capable to form metabolic-intermediate adducts usually are potent inhibitors of cytochrome P-450-catalysed monooxygenations through blocking oxygen association [5].

All the ligand interactions exerted by intermediates derived from nitrogen-containing compounds hold the heme iron of cytochrome P-450 in the low-spin, hexacoordinated state, and the complexes thus formed exhibit difference spectra with Soret region absorption maxima at 445–457 nm [1, 2]. With the use of revenast (1,5-diphenyl-2-[3-(4-(2-pyridyl)-piperazin-1-yl)propyl]pyrazolin-3-one; Scheme 1), a novel nitrogenous antiallergic agent, we now report for the first time on the production of a spectrally detectable metabolic adduct characterized by a Soret peak at 407 nm and a concomitant loss in absorbance at 423 nm.

MATERIALS AND METHODS

Chemicals. The chemicals used in the present study were obtained from the following sources: NADP$^+$, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) from Boehringer (Mannheim, F.R.G.); polyethylene glycol 6000, 4-chloroaniline and *N,N*-dimethylaniline from Merck AG (Darmstadt, F.R.G.). Revenast (compound KC6300) and its metabolic derivatives were kindly donated by Kali-Chemie (Hannover, F.R.G.).

Synthesis of revenast N-*oxide.* Synthesis of revenast *N*-oxide was carried out by reacting the parent amine with H_2O_2 in hot glacial acetic acid [6]. The crude oil was purified by preparative thin-layer chromatography using glass plates (20 × 20 cm) precoated with silica gel 60 F_{254} (Merck AG, Darmstadt, F.R.G.); the solvent system was acetone/methanol/glacial acetic acid (1:2:0.02, v/v). Compounds were visualized under UV light, and material characterized by a R_f value of 0.23 was scraped from the plates and eluted from the silica gel powder with a mixture of methanol and chloroform (1:1, v/v) for 1 hr at room temperature. Finally, the solvent was removed under reduced pressure.

The purified oxy product was subjected to mass-spectral analysis in a Finnigan MAT HSQ 30 spectrometer. Analysis by the fast-atom-bombardment mode of detection revealed a quasi molecular ion $[M + 1]^+$ at m/z 456 and a fragment at m/z 440 originating from loss of an oxygen atom. Selective analysis of the quasi molecular ion in the collision–activation mode showed a peak at m/z 277 corresponding to ($[M + 1]^+$—$C_9H_{13}N_3O$). These findings indicate that an oxygen atom had been

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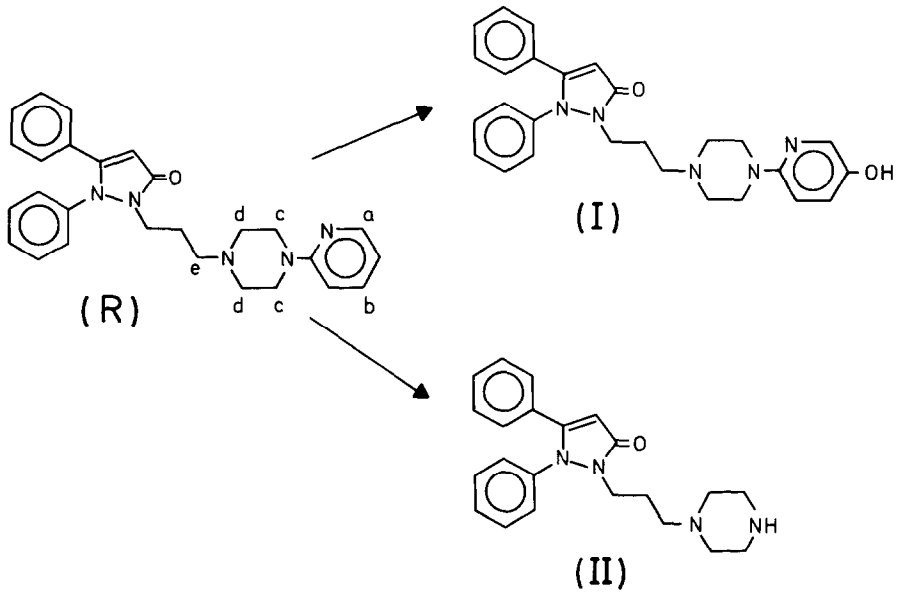

Scheme 1. The chemical structures of revenast (R) and its biliary metabolic derivatives (I, II).

introduced into the pyridyl piperazinyl moiety of the revenast structure.

Placement of the oxygen atom was confirmed by ^{1}H-NMR spectroscopy. Spectra were recorded at 500 MHz with a Bruker AM-500 spectrometer using $CDCl_3$ as a solvent. Proton assignments (see Scheme 1) of the critical signals for standard revenast (A) and its oxidised derivative (B) were made as follows. A: δ8.19, d, J = 3 Hz: H_a; δ7.48, t, J = 6 Hz: H_b; δ3.54, s: H_c; δ2.57, s: H_d; δ2.46, s: H_e. B: δ8.21, d, J = 3 Hz: H_a; δ7.55, t, J = 7 Hz: H_b; δ3.74 (3.36), t(s), J = 9 Hz: H_c; δ4.16 (3.74), d(t), J = 12(9) Hz: H_d; δ2.35, m: H_e. As can be seen, there is no significant difference between the standard and the oxidised product in the patterns of the aromatic protons a and b associated with the pyridyl nucleus. Chemical oxidation, however, induces nonequivalence of the protons c and d of the piperazinyl ring and causes a pronounced downfield shift of the resonance peak associated with the protons d. It is clear from these results that the oxygen was added to the piperazinyl nitrogen bearing the substituted propyl sidechain.

The UV spectrum of the ethanolic solution of the *N*-oxide showed an absorption maximum at 246.5 nm and a shoulder around 300 nm.

Preparation of microsomal fraction and partial purification of cytochrome P-450. Hepatic microsomal fractions from male Sprague–Dawley rats, pretreated with phenobarbital (50 mg/kg of body wt) for 7 consecutive days, were prepared by the method of Jagow *et al.* [7]. Cytochrome P-450 was partially purified by carrying out the initial steps of the procedure described by Hlavica and Hülsmann [8]. In short, the microsomal fractions were treated with cholate, and the solubilized hemoprotein was fractionated by the gradual addition of polyethylene glycol 6000 to give a final concentration of 20% (w/v). The precipitate was dissolved in 150 mM phosphate buffer (pH 7.4), containing 20% (v/v) glycerol and 0.1 mM dithiothreitol. This crude cytochrome P-450 preparation (5 nmol P-450/mg of protein) still contained cytochrome b_5 (0.37 nmol/nmol of P-450) and NADPH-cytochrome *c*(P-450) reductase (29 units/nmol of P-450; 1 unit is defined as 1 nmol cytochrome *c* reduced/min).

Measurement of metabolic-intermediate complex formation. The crude enzyme preparation was diluted with 150 mM phosphate buffer (pH 7.4), containing 20% (v/v) glycerol and 0.1 mM dithiothreitol, to give 4 μM cytochrome P-450, 1.48 μM cytochrome b_5 and 116 units of NADPH–cytochrome *c*(P-450) reductase/ml of mixture. The solution was supplemented with NADPH (final concentration 1 mM) and divided between two cuvettes. After establishing the baseline, revenast (final concentration 0.1 mM) was added to the sample cell and the difference spectrum recorded between 380 and 500 nm by repetitive scanning at room temperature, using a Shimadzu UV-265FW spectrophotometer; optical path length was 1 cm. Further additions to the basic assay media as specified in the appropriate figure legends.

Measurement of monooxygenase activities. The reaction mixtures for measuring *N*-demethylase and *N*-oxidase activity in the absence and presence of 1 mM revenast contained: microsomal protein equivalent to 0.5 and 2 μM cytochrome P-450, respectively, 1.2 mM $NADP^+$, 10 mM glucose-6-phosphate, 6 mM $MgCl_2$, glucose-6-phosphate dehydrogenase (5 μg/ml) and 1 mM substrate (*N*,*N*-dimethylaniline or 4-chloroaniline) in 150 mM phosphate buffer (pH 7.4). Reactions were carried out for 20 min at 37°.

Analytical procedures. Cytochromes P-450 and b_5 as well as NADPH–cytochrome *c*(P-450) reductase activity were measured as described previously [8]. Formaldehyde production from *N*,*N*-dimethylaniline was assayed by the method of Nash [9]; 4-chlorophenylhydroxylamine was quantified as indicated by Herr and Kiese [10].

RESULTS AND DISCUSSION

Addition, in the presence of NADPH, of revenast

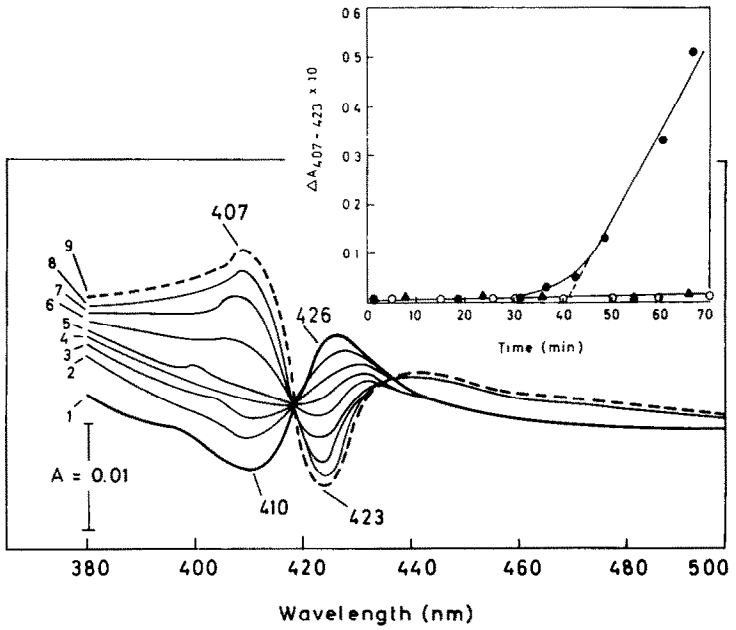

Fig. 1. Optical changes associated with the oxidative metabolism of revenast by partially solubilized rat liver microsomal cytochrome P-450. The assay mixtures contained 4 μM cytochrome P-450, 1.48 μM cytochrome b_5, 116 units of NADPH-cytochrome *c*(P-450) reductase/ml, 1 mM NADPH and 0.1 mM revenast in 150 mM phosphate buffer (pH 7.4). Spectra, recorded between 0.5 min (trace 1) and 70 min (trace 9) after substrate addition were analysed for time-dependent development of the 407 nm-asborbing product adduct (inset) in the presence of air (●), N_2 (○), or 1 mM metyrapone (▲) in the sample and reference cell.

to an aerobic reaction mixture containing cytochrome P-450, cytochrome b_5 and NADPH-cytochrome *c*(P-450) reductase originally results in the formation of a so-called type II spectral change characterized by a Soret band at 426 nm and a trough at 410 nm (Fig. 1), as is typical of heme liganding with a sterically unhindered substrate nitrogen [2, 11].

Repetitive scanning of the sample reveals the progressive transformation with time of the initial optical perturbation to a difference spectrum exhibiting a Soret region absorption maximum at 407 nm and a minimum at 423 nm (Fig. 1); there is also an isosbestic point at 418 nm. This spectrum does not arise from incomplete reduction of cytochrome b_5 in the sample cuvette, since addition to the experimental cells of excess dithionite fails to abolish the optical change (Fig. 2B). Similarly, accumulating free metabolite(s) of revenast do not appear to account for the 407 nm-absorbing species, since analysis of the spectral properties of compounds I and II, arising from biotransformation of the parent amine by cytochrome P-450 (Scheme 1)*, reveals absorption bands located in the UV region of the spectrum (I: λ_{max}-EtOH 202.5 and 249 nm, shoulder around 330 nm; II: λ_{max}EtOH 203.5 and 254.5 nm). Alternatively, metabolic derivative(s) formed could displace revenast from the prosthetic heme iron such as to disclose type I interactions of the parent amine with the protein moiety of cytochrome P-450 [12]. This mechanism, however, does not appear to be operative, since metyrapone, a strong heme ligand of the pigment, added to the assay media in 10-fold excess over the antiallergic drug fails to mimic the metabolic spectrum (Fig. 1, inset). Moreover, there is no measurable production of cytochrome P-420 or loss of CO-reactive hemoprotein during exposure to revenast, indicating that the spectral transition observed is not due to complex formation with structurally altered cytochrome P-450.

In the light of these findings, the 407 nm-absorbing species is concluded to reflect the binding to cytochrome P-450 of a reactive intermediate originating from oxidative transformation of revenast. Support in favour of this comes from the fact that there is no product adduct formation when the experiments are conducted under an atmosphere of N_2 or when metyrapone is incorporated into the contents of the sample and reference cuvette (Fig. 1, inset). The time course of complexation exhibits a fairly long lag phase (Fig. 1, inset). Unless one assumes that the metabolic intermediate responsible for adduct formation is a secondary product slowly generated, the most plausible explanation is that the high background of substrate-induced type II spectral change initially obscures the spectral manifestation of the metabolic complex (cf. Fig. 1), which forms at a relatively low rate of 26 pmol/min/nmol P-450. Catalysis appears to be brought about preferentially by phenobarbital-inducible species of rat liver microsomal cytochrome P-450 such as form *b*, since pretreatment of the animals with the barbiturate produces an approximately 6-fold increase in magnitude of the metabolic spectrum relative to controls (data not shown).

* G. Achtert, unpublished work.

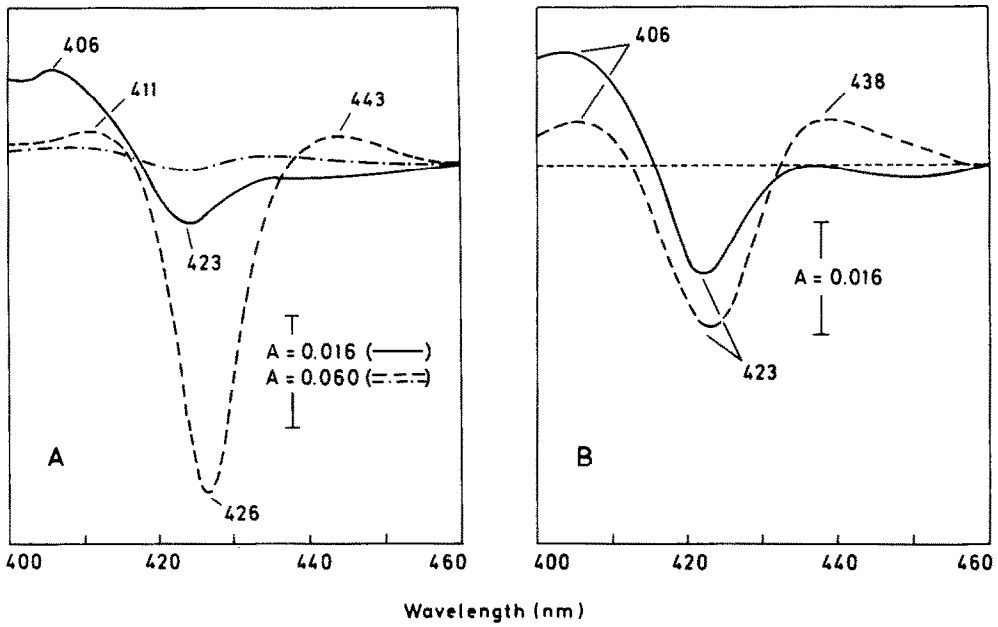

Fig. 2. Spectral perturbations arising from chemical oxidation or reduction of the 407 nm-absorbing metabolic complex. The reaction mixtures were as described in the legend to Fig. 1. After maximal development of the metabolic spectrum (——), either 100 μM $K_3Fe(CN)_6$ (A) or excess solid $Na_2S_2O_4$ (B) were added to the reaction mixtures and spectra run 1 min (– – –) and 3 min (– · –) later.

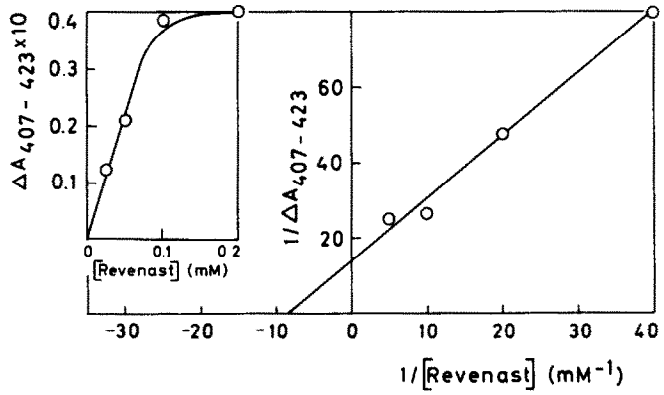

Fig. 3. Dependence of metabolic-adduct formation on the revenast concentration. The assay media were as described in the legend to Fig. 1, except that 8 μM cytochrome P-450 was present. Optical data were taken after an apparent steady state of complex formation was achieved.

The extent of metabolic-intermediate complex formation is dependent upon the amount of revenast present, with half-maximal complexation occurring at a concentration of 125 μM (Fig. 3). Since the adduct formed does not appear to exist fully in the high-spin state (see below), the percentage of cytochrome P-450 complexed can be but roughly assessed from the ΔA_{max} value (Fig. 3) by tentatively using the extinction coefficient of 126/mM/cm for complete high-spin transition of the pigment [13]. This calculation permits one to conclude that at least 7% of the total amount of hemoprotein present participate in the production of the 407 nm-absorbing species.

The preponderant formation of a cytochrome P-450 metabolic-intermediate complex characterized by a strongly blue-shifted Soret region absorption maximum is unique in that all product adducts so far examined are of the low-spin type [1, 2]. The position at 407 nm of the Soret band reported in the present study deviates from that usually found for pure high-spin complexes, 385–390 nm [14]. Our data, therefore, hint at the presence of a mixture of high and low-spin adducts being at thermal equilibrium [15]. Indeed, the existence of a latent low-spin component is revealed by the response of the metabolic complex to dithionite treatment: the chemical reductant preserves the absorption band at 406 nm and triggers the appearance of a second peak centred at 438 nm (Fig. 2B); this spectrum displays considerable stability. Clearly, our data do not permit us to decide

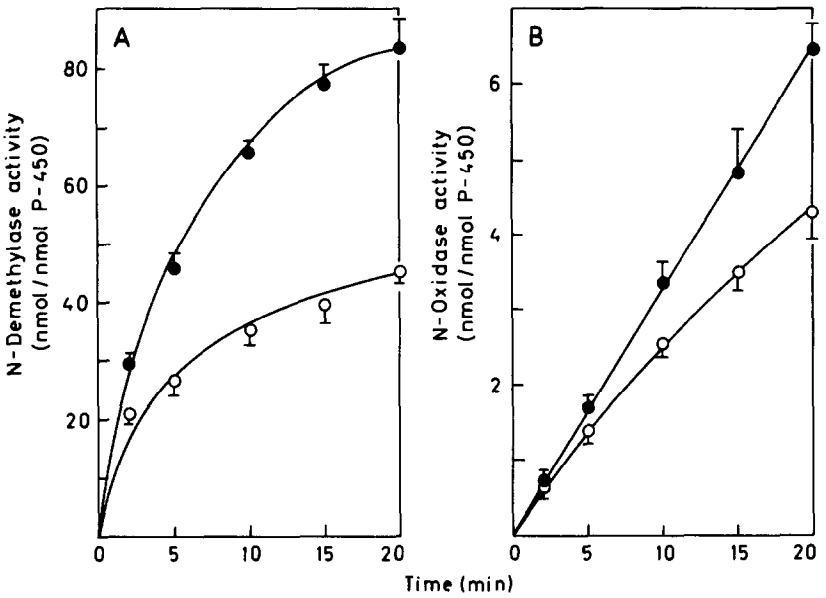

Fig. 4. Effect of revenast pretreatment on liver microsomal N-demethylation of *N*,*N*-dimethylaniline (A) and N-hydroxylation of 4-chloroaniline (B). Assay mixtures containing microsomal protein equivalent to 0.5 μM (A) or 2 μM (B) cytochrome P-450 were fortified with NADPH-generating system and pre-incubated for 10 min in the absence (●) or presence (○) of 1 mM revenast before initiating reactions A and B by the addition of 1 mM amine substrate. All incubations were carried out at 37°. The data are the means ± SE of three experiments.

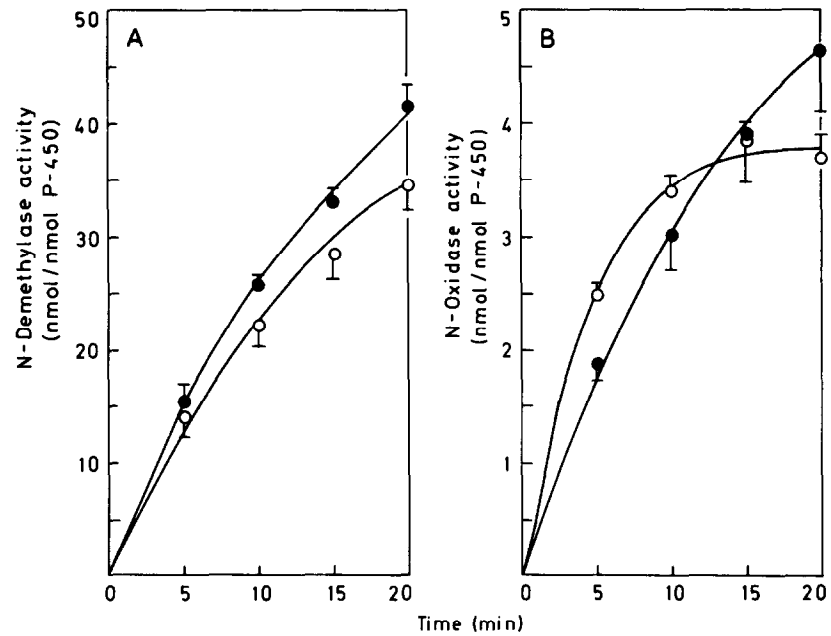

Fig. 5. Restoration of *N*-demethylase (A) and *N*-oxidase (B) activities after dissociation of the metabolic adduct. Microsomal membranes were preincubated for 45 min at 37° with NADPH in the absence (●) or presence (○) of 1 mM revenast. The suspensions were centrifuged and the microsomal pellets washed three times with an ice-cold solution of 150 mM KCl. The final preparations were analysed for monooxygenase activities as described in the legend to Fig. 4. The data are the means ± SE of three experiments.

whether the dithionite-induced co-existence of different spin forms might be effected with a single ferrous cytochrome P-450 isozyme or multiple types of the pigment present in our enzyme preparations. The question arises as to why there is preference for the formation of the 407 nm-absorbing species when NADPH is the sole source of reducing equivalents. Answer to this might come from the fact that

NADPH, apart from converting ferric to ferrous cytochrome P-450, elicits a selective conformational change in the active site of the hemoprotein through functional coupling of the NADPH-cytochrome P-450 reductase segment [16]; this might serve to weaken liganding of the reactive intermediate to the prosthetic heme group to such an extent as to make hydrophobic protein interactions predominate.

Most interestingly, artificial oxidation of the product complex also gives rise to the formation of a transient dual-chromophore difference spectrum exhibiting maxima at 411 and 443 nm (Fig. 2A); instability of the spectral complexes in the ferric state is shared by pure ligand-type metabolic adducts generated from a variety of nitrogenous compounds [1].

Since formation of the metabolic-intermediate complex is also observed when intact microsomes are used instead of the partially purified enzyme preparations (data not shown), it was of interest to examine the influence of revenast on some microsomal monooxygenations. Thus, preincubation of phenobarbital-induced rat liver microsomal fractions with 1 mM drug to allow the complex to form decreases the initial rates of 4-chloroaniline N-oxidation and *N*,*N*-dimethylaniline N-demethylation by 20 and 49%, respectively, as calculated from the progress curves depicted in Fig. 4. No relevant adverse effect is seen if revenast is added simultaneously with the individual arylamine, ruling out substrate competition (data not shown). The extent of loss in *N*-demethylase activity, as observed under the specific experimental conditions (Fig. 4A), suggests an IC_{50} of about 1 mM. Comparing this data with the 'K_S' value derived from Fig. 3 on the basis of the different cytochrome P-450 concentrations used in the two assay systems, it turns out that affinity of revenast for the operative enzyme is approximately 80-times higher than its inhibitory potency towards the N-demethylation reaction, hinting at a competitive site I interaction of the active revenast metabolite with *N*,*N*-dimethylaniline.

Impaired arylamine turnover can be restored: when microsomal preparations, pretreated with revenast in the presence of NADPH/O_2, are washed several times with an isotonic aqueous solution of KCl, *N*-oxidase and *N*-demethylase activities in the membranes are close to those in controls preincubated in the absence of the modifier (Fig. 5), suggesting that the washing procedure largely dissociates the revenast-dependent product adduct. This finding adds support in favour of the previously advanced concept of weak heme bonding of the inhibitory intermediate, as is also documented by the relatively moderate perturbation of metabolism of the type II substrate 4-chloroaniline (Fig. 4B). It has to be noted that in pure ligand-type metabolic complexes coordination usually occurs in a quasi-irreversible manner [5], although exception to this rule has been observed [17].

The unique spectral and functional properties of the metabolic complex described in the present paper create interest in further studies concerning the molecular organization of this adduct. With respect to this, knowledge of the chemical nature of the intermediate(s) serving for complexation is of particular

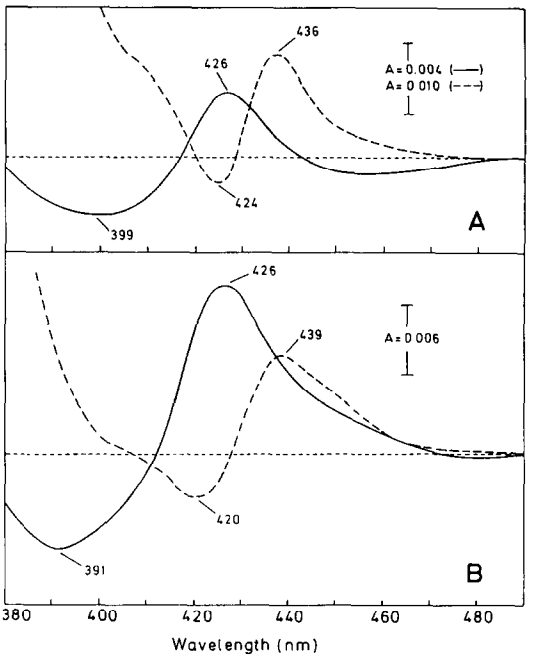

Fig. 6. Binding of metabolites I and II to ferric and ferrous cytochrome P-450. The assay mixtures were as described in the legend to Fig. 1, except that 8 μM cytochrome P-450 was present. Metabolite I (A; final concentration 0.1 mM) or metabolite II (B; final concentration 1 mM) was added to the sample cuvette and the difference spectrum recorded (——). Subsequently, NADPH (final concentration 1 mM) was incorporated into the contents of both experimental cells, and scanning was repeated immediately after the addition of the reductant (– – –).

importance. Assuming that there is sufficient structural resemblance between reactive intermediates and their stable end products, precursors of metabolites I and II (Scheme 1) can be dismissed as being involved: addition of the latter compounds to ferric cytochrome P-450 yields fairly stable type II spectra with Soret bands at 426 nm (Fig. 6); subsequent reduction of the hemoprotein with NADPH causes a bathochromic shift of the absorption peaks to 436 and 439 nm, respectively, with faint shoulders appearing around 405 and 410 nm.

It is, therefore, interesting to speculate that the crucial metabolite giving rise to the 407 nm-absorbing complex might result from nitrogen oxidation in the revenast molecule; such a cytochrome P-450-mediated oxidative attack may occur on the pyridine [18] as well as on the piperazine [19] ring nitrogens. Since revenast and its metabolic derivative lacking the pyridly nucleus (II) produce analogous low-spin spectra with ferric cytochrome P-450 (cf. Figs 1 and 6B), the heterocyclic nitrogen can be ruled out to associate with the catalytic heme site. On the other hand, chemical oxidation of the nitrogen at position 1 of the piperazinyl structure to yield a *N*-oxide obviously blocks complexation of the oxidised product with ferric cytochrome P-450 (Fig. 7). This suggests that the parent amine combines with the prosthetic heme group via the free pair of electrons of the piperazinyl nitrogen bearing the substituted

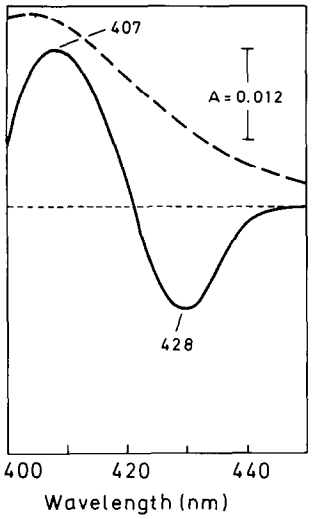

Fig. 7. Binding of synthetic revenast *N*-oxide to cytochrome P-450. The basic assay media were as described in the legend to Fig. 1. Optical changes were recorded after the addition of revenast *N*-oxide (final concentration 1 mM) to the sample cell (– – –). Subsequently, NADPH (final concentration 1 mM) was incorporated into the contents of the sample and reference cuvette, and monitoring of the spectral perturbations was repeated immediately after the addition of the reduced pyridine nucleotide (——).

propyl sidechain. Such an orientation of the nitrogen atom towards the heme iron might favour N-oxidation under mixed-function conditions. Indeed, addition, in the presence of NADPH, of the synthetic revenast *N*-oxide to cytochrome P-450 provokes a difference spectrum closely resembling that generated by the metabolic-intermediate adduct (cf. Figs 1 and 7). The dissociation of the latter complex following chemical oxidation (Fig. 2A) is consistent with the poor reactivity of the stable *N*-oxide towards ferric cytochrome P-450 (Fig. 7).

It is noteworthy that tiaramide *N*-oxide, another 1,4-disubstituted piperazine *N*-oxide, elicits a pronounced ligand-type spectral perturbation with ferrous cytochrome P-450 characterized by a Soret band at 442 nm [20]. The unusual position at 407 nm of the absorption maximum in the revenast *N*-oxide-induced spectrum thus might reflect specific substituent effects supporting lipophilic protein interactions such as to shift the spin equilibrium of cytochrome P-450 to an intermediate state.

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