Histidine residues in rabbit liver microsomal cytochrome *P*-450 2B4 control electron transfer from NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅

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Treatment of cytochrome *P*-450 2B4 (*P*-450 2B4) with diethylpyrocarbonate to introduce 10–11 equivalents of acylating agent per polypeptide chain resulted in the selective derivatization of histidine residues characterized by differential susceptibility toward the modifier. Second-derivative spectral analysis as well as fluorescence measurements disproved gross alterations in *P*-450 2B4 structure as a consequence of labelling. The modified haemoprotein retained its ability to bind hexobarbital and catalyse cumene hydroperoxide-sustained N-demethylation of the barbiturate. However, there was a steady attenuation of NAD(P)H-driven electron flux with increasing extent of *P*-450

2B4 carbethoxylation in reconstituted systems fortified with either NADPH-cytochrome *P*-450 reductase or NADH-cytochrome *b*₅ reductase/cytochrome *b*₅ as the redox partners, with 50% inhibition occurring when 6–7 histidines were blocked. Hampered *P*-450 2B4 reductase activities recovered to differing degrees upon treatment of the acylated mono-oxygenase with neutral hydroxylamine. Spectral data indicated that docking of the redox components to derivatized *P*-450 2B4 was not perturbed, so that disruption of the electron flows most likely resulted from some injury of the electron-transfer mechanisms.

INTRODUCTION

The cytochrome *P*-450 enzymes (EC 1.14.14.1) are a superfamily of mono-oxygenases, which have adopted diverse roles in the oxidative metabolism. The common catalytic function of these enzymes is the two-electron reduction of molecular oxygen to form water and a reactive oxygen species, which serves for insertion into a diversity of endogenous and exogenous substrates to yield hydroxylated products [1]. Although NADPH-cytochrome *P*-450 reductase has long been recognized to be an essential electron-transfer component of the microsomal mixed-function oxygenase system [2], the role of cytochrome *b*₅ is more complex, as it can either enhance or inhibit cytochrome *P*-450-catalysed activities in an isoform-specific manner [3–6]. Interaction of cytochrome *b*₅ with the various cytochrome *P*-450 species has been shown to involve electrostatic attraction by cytochrome *b*₅ carboxylate groups surrounding the haem pocket [7]. Similarly, the vast majority of available data hints at a charge-pairing mechanism governing the association of cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase [8–12]. In contrast to this concept, a recent report claims that charged residues on the two redox proteins rather destabilize the intermolecular electron-transfer complex [13].

Elucidation of the molecular basis of the specificity of interaction of the electron donors with cytochrome *P*-450 requires identification of amino acid residues in the haemoprotein that constitute the binding site(s). Conserved positive charges on the surface of the cytochrome *P*-450 molecule have been advocated to participate in salt-bridge contacts with the redox partners [14]. Indeed, lysine and arginine residues in the diverse cytochrome *P*-

450 subforms have been detected to serve in the donor–acceptor recognition [8,15–18]. The present study focuses on the importance of histidine(s) in the cytochrome *P*-450 2B4 (*P*-450 2B4) polypeptide in electron transfer from NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅.

MATERIALS AND METHODS

Chemicals

The chemicals used in the present study were from the following sources. NADH, NADPH, catalase (EC 1.11.1.6), glucose oxidase (EC 1.1.3.4) and trypsin (EC 3.4.21.4) were from Boehringer Mannheim. Dimyristoyl- α -phosphatidylcholine, hexobarbital, cumene hydroperoxide and diethylpyrocarbonate (DEPC) were from Sigma Chemie. The purity of DEPC was tested as detailed elsewhere [19]. Sephadex G-25 (medium) was purchased from Pharmacia.

Purification of rabbit liver microsomal haemoproteins and reductases

P-450 2B4 was purified to apparent homogeneity by established procedures [20] from hepatic microsomes of male New Zealand White rabbits pretreated with phenobarbital (50 mg/kg) for 7 consecutive days; the specific content of the final preparations averaged 17.8 nmol/mg of protein.

Cytochrome *b*₅ was prepared from untreated rabbit liver

Abbreviations used: DEPC, diethylpyrocarbonate; *P*-450 2B4, cytochrome *P*-450 2B4, the major phenobarbital-inducible rabbit liver microsomal isoenzyme.

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microsomal membranes by the method of Strittmatter et al. [21] to a specific content of 47 nmol/mg of protein.

NADPH-cytochrome *P*-450 reductase (EC 1.6.2.4) and NADH-cytochrome *b*₅ reductase (EC 1.6.2.2), isolated from rabbit liver microsomal fractions as reported previously [20,22], had specific activities of 40 μ mol of cytochrome *c* reduced/min per mg of protein and 970 μ mol of K₃Fe(CN)₆ reduced/min per mg of protein respectively. All protein preparations were subjected to analysis by SDS/PAGE on slab gels containing 7.5 % (w/v) acrylamide, as described by Laemmli [23].

Chemical modification of *P*-450 2B4

To assess the kinetics of carbethoxylation of *P*-450 2B4, DEPC was dissolved directly before use in a small volume of absolute ethanol, and aliquots of the solution were added to assay medium containing 2 μ M haemoprotein in 100 mM sodium phosphate, pH 7.25, fortified with 20 % (v/v) glycerol. Reactions were followed at 25 °C as a function of time by monitoring the increase in absorbance at 240 nm relative to 275 nm with an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode. The number of *N*-carbethoxyhistidine residues per *P*-450 2B4 polypeptide was evaluated on the basis of an absorption coefficient of 3200 M⁻¹·cm⁻¹ [24].

For the production of carbethoxylated *P*-450 2B4 on a preparative scale, the pigment was diluted with 100 mM sodium phosphate, pH 7.25, containing 20 % (v/v) glycerol to a final concentration of 44 μ M. DEPC was added, the final concentrations ranging from 240 μ M to 600 μ M. The mixture was stirred for 20 min at 25 °C. Subsequently, the sample was passed twice through a Sephadex G-25 column (0.8 cm × 18 cm) equilibrated with the incubation buffer to remove excess reagent. The extent of modification was assessed as described previously [24]. Controls in the absence of DEPC were run simultaneously. Partial regeneration of derivatized *P*-450 2B4 was achieved by reacting the haemoprotein with 0.1 M neutral hydroxylamine at 25 °C for 2 h; controls were treated analogously. Excess hydroxylamine was removed by gel filtration of the enzyme preparations on a Sephadex G-25 matrix as detailed above.

In some experiments, native and derivatized *P*-450 2B4, both electrophoresed on polyacrylamide gels [23], were subjected to tryptic digestion [25] followed by resolution of the fragments by reverse-phase HPLC and N-terminal sequence analysis of the peptide material using an Applied Biosystems 477A instrument [26].

Optical measurements

Spectral analysis was carried out at 25 °C using a Shimadzu UV-265FW spectrophotometer. The standard conditions for measuring second-derivative spectra were: scan speed, 60 nm/min; slit width, 1 nm; and $\Delta\lambda$, 2 nm. The assay mixtures consisted of 2 μ M native or carbethoxylated *P*-450 2B4 in 100 mM sodium phosphate, pH 7.4, containing 20 % (v/v) glycerol. Excitation and emission fluorescence spectra were taken with 0.3 μ M native or acylated haemoprotein in 20 mM sodium phosphate, pH 7.4, on a Perkin-Elmer MPF-4 spectrofluorimeter.

The pre-steady-state kinetics of association of hexobarbital, NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅ with native and modified ferric *P*-450 2B4 were measured at 25 °C with an Aminco Dasar/DW-2 spectrophotometer equipped with an Aminco-Morrow stopped-flow apparatus; dead time of the instrument was 4 ms. Formation of the individual high-spin spectral complexes was assessed by monitoring the increase in absorbance at 386 nm relative to 418 nm in the mixing chamber

containing 4 μ M *P*-450 2B4, 40 μ M dimyristoyl phosphatidylcholine and either 2 mM hexobarbital, 2 μ M reductase or 4 μ M cytochrome *b*₅ in 100 mM sodium phosphate, pH 7.4.

Enzyme assays

NAD(P)H-supported cytochrome *P*-450 reductase activity was assayed at 25 °C in reaction mixtures containing 2 μ M ferric *P*-450 2B4 (either native, carbethoxylated or partially regenerated), 0.2 μ M NADPH-cytochrome *P*-450 reductase (or 0.5 μ M NADH-cytochrome *b*₅ reductase and 2 μ M cytochrome *b*₅), 40 μ M dimyristoyl phosphatidylcholine (sonicated until clarification was observed), 1 mM hexobarbital, 100 mM glucose, glucose oxidase (400 μ g/ml) and catalase (80 μ g/ml) in 100 mM sodium phosphate, pH 7.4. The mixtures were allowed to stand at room temperature for 30 min; this time period was adequate for efficient incorporation of the proteins into the lipid micelles [27]. Subsequently, the samples were evacuated and gassed for two 5-min cycles with CO; this served to establish anaerobiosis, as determined with an oxygen electrode. Reactions were initiated by the rapid addition of NAD(P)H to give a final concentration of 0.33 mM using a set of plunger cuvettes. When the chemical reduction of *P*-450 2B4 was tested, the components of the natural electron-transfer systems were omitted, and the haemoprotein was reduced by the addition of Na₂S₂O₄ to yield a final concentration of 10 mM. Absorbance changes at 450 nm relative to 500 nm were recorded with an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode.

Hexobarbital N-demethylase activity was determined in assay medium containing 2 μ M native or acylated *P*-450 2B4, 40 μ M sonicated dimyristoyl phosphatidylcholine, 5 mM hexobarbital and 5 mM cumene hydroperoxide in 100 mM sodium phosphate, pH 7.4. Reactions were carried out for 20 min at 37 °C. The release of formaldehyde was quantified by the method of Nash [28].

Miscellaneous procedures

Cytochromes *P*-450 and *b*₅ were measured as indicated previously [29]. NADH-cytochrome *b*₅ reductase and NADPH-cytochrome *P*-450 reductase were quantified from their absorbance at 456 nm assuming absorption coefficients of 10000 M⁻¹·cm⁻¹ and 21400 M⁻¹·cm⁻¹ respectively [30,31]. Protein assaying was carried out by the method of Lowry et al. [32].

RESULTS AND DISCUSSION

Characteristics of *P*-450 2B4 modification by DEPC

DEPC was chosen as the modifying reagent, since it appears to be reasonably specific for reaction with histidine residues in proteins in the pH range from 5.5 to 7.5 [33]. The extent of derivatization, in the absence of denaturant, of rabbit liver microsomal *P*-450 2B4 was assessed spectrophotometrically from the specific absorbance change originating from the *N*-carbethoxylation of histidines. As depicted in Figure 1(A), acylation was highly dependent on the concentration of DEPC used. Irrespective of whether *P*-450 2B4 was labelled to the extent of a statistical average of 3.5 or 11.2 carbethoxy groups introduced per polypeptide within the time frame of the experiment, biphasic kinetic patterns were observed (Figures 1B and 1C). Unless the particular p*K*_a values for the individual histidines or specificities in solvent accessibility accounted for this dualism, the slow-reacting species might have participated in hydrogen bonding with proton donors [34]; as is well known, the electrophilic DEPC molecule interferes preferentially with the unprotonated

Figure 1 Kinetics of modification of *P*-450 2B4 as a function of DEPC concentration

(A) Solutions containing 2 μ M *P*-450 2B4 were supplemented with various amounts of DEPC at pH 7.25, and the time course of carbethoxylation of the enzyme was followed by monitoring the increase in absorbance at 240 nm. Inset: difference spectrum of haemoprotein acylated in the presence of 430 μ M modifier. Panels (B) and (C) show semi-logarithmic re-plots of the kinetic data displayed in panel (A). The symbols used throughout the Figure indicate derivatization performed in the presence of 170 μ M (●) or 430 μ M (○) DEPC. Pseudo-first-order rates (k_{app}) of modification were computed from $k_{app} = 0.693/t_{0.5}$, where $t_{0.5}$ represents the half-time of the reaction. The points are the means of three measurements.

form of histidine [24]. From the tracings depicted in Figure 1(C), the proportions of fast- and slow-reacting material were computed as 35 and 65 % respectively, characterized by pseudo-first-order rate constants of 0.3 s⁻¹ and 0.06 s⁻¹. Comparison of the kinetic data presented in panels B and C of Figure 1 suggested that the rate constants for the fast and slow phases of derivatization were independent of the DEPC concentration applied. This was tentatively interpreted to mean that accessibility of the histidines rather than the level of acylating agent constituted the rate-limiting factor in the process of enzyme labelling.

As lysine, cysteine and tyrosine residues in proteins can undergo modification by DEPC to certain extents [24,33], attempts were made to delineate specificity of the reagent for the carbethoxylation of histidines under the routine conditions employed in this study. To this end, derivatized *P*-450 2B4 was treated with neutral hydroxylamine; this procedure is known to remove the carbethoxy group from histidine, but not from lysine or cysteine residues [24]. The high degree of NH₂OH-triggered regeneration of *P*-450 2B4, which had not come to completion during the observation period (Figure 2), thus argued against significant labelling of the latter two amino acid residues. In accord with this, the difference spectrum of the ferrous-carbonyl adduct of haemoprotein bearing 11.2 carbethoxy groups per polypeptide indicated that the cytochrome *P*-420 content was lower than 7 %, suggesting that the haem thiolate ligand was not appreciably attacked by DEPC. In addition, second-derivative spectroscopy was used, offering a means for high resolution of spectral bands, in the middle-UV region, ascribable to tyrosine [35]. Thus the trough-to-peak difference at the wavelength pair 280.5 nm/285 nm was almost identical with native and modified *P*-450 2B4 (Figure 3A), disproving significant derivatization of tyrosine residues. Similarly, the zero-order difference spectrum of labelled pigment did not exhibit a trough around 275 nm (Figure 1A, inset), as would have occurred if *O*-carbethoxy-tyrosine had been produced [33]. Summarizing these results, histidine residues appeared to constitute the selective target for modification by DEPC under the conditions adopted throughout our experiments.

One complication that needs to be considered in the chemical modification of proteins is the possibility of the induction of

Figure 2 Regeneration of carbethoxylated *P*-450 2B4 by hydroxylamine treatment

P-450 2B4 was reacted with DEPC at pH 7.25 to introduce a maximum of 10.5 carbethoxy groups per polypeptide chain. At this stage, neutral hydroxylamine was added to the assay medium to yield a final concentration of 0.1 M, and the time-dependent decarbethoxylation of the pigment was followed by scanning the decrease in absorbance at 240 nm (○); controls in the absence of hydroxylamine (●) were run simultaneously. The data represent the means of three experiments.

conformational changes serving to perturb protein function. Comparison of the Soret bands in the absolute spectra of native ferricytochrome *P*-450 2B4 and pigment containing 11.2 *N*-carbethoxyhistidines per mol of enzyme revealed peaks uniformly centred around 416 nm. The A_{416}/A_{390} ratios for the two preparations had values of 1.40 and 1.44 respectively, suggesting identity of the spin states. The CO-reduced-difference spectra did not display aberrations with respect to wavelength (450.8 nm) or magnitude of the peaks. Derivatization also did not markedly affect the net solvent exposure of tyrosine, as judged from the nearly identical ratios (a/b) between two peak-to-trough second-derivative absorbance differences obtained with untreated and labelled haemoprotein (Figure 3A), indicating that microenvironmental polarity was not perturbed [35]. It has to be noted that

Figure 3 Spectral characterization of native and DEPC-treated *P*-450 2B4

(A) Second-derivative spectra of solutions containing 2 μ M native *P*-450 2B4 (—) or pigment bearing 10.3 *N*-carboxyhistidine groups per polypeptide (---) in 100 mM sodium phosphate, pH 7.4. a and b indicate peak-to-trough second-derivative absorbance differences. Fluorescence excitation (B) and emission (C) spectra were recorded of solutions containing 20 mM sodium phosphate, pH 7.4, and 0.3 μ M unmodified *P*-450 2B4 (—) or haemoprotein modified (---) to the extent indicated for panel (A). Excitation spectra were monitored at the emission wavelength 320 nm; emission spectra were run at the excitation wavelength 280 nm.

Figure 4 Pre-steady-state kinetics of binding of hexobarbital to native and carbethoxylated *P*-450 2B4

Lipid-incorporated native *P*-450 2B4 (●) or enzyme containing 10.3 blocked histidines per polypeptide (○) were rapidly mixed with hexobarbital dissolved in sodium phosphate, pH 7.4. Complex formation was monitored at 386 nm relative to 418 nm; for details see the Materials and methods section. The data points represent the means of three experiments.

tyrosine accessibility has been shown to be closely related to the tertiary structure of proteins, as determined by X-ray analysis [35]. Furthermore, fluorescence excitation and emission spectra were taken with unmodified and modified pigment as a sensitive probe for conformational changes. However, we were unable to detect relevant differences in the spectral behaviour of these preparations (Figures 3B and 3C). Finally, analysis by SDS/PAGE showed identical migration patterns for all samples, whether DEPC-treated or not, with bands corresponding to a molecular mass of 54 kDa, ruling out cleavage or polymerization [33] during derivatization. We believe these results provide little or no evidence to support the notion that carbethoxylation grossly altered the global conformation of *P*-450 2B4.

It was important to determine whether the modified *P*-450 2B4 retained its ability to bind and metabolize drugs, as a catalytically active enzyme is essential if meaningful conclusions concerning the structure–function relationship are to be made. Therefore association of hexobarbital with native and derivatized *P*-450 2B4 was assessed in terms of type I spectral complex formation. As evidenced by Figure 4, the kinetic tracings measured with the

two preparations overlap, permitting the calculation of a common pseudo-first-order rate constant of 86 s^{-1} . Accordingly, rates of cumene hydroperoxide-sustained *N*-demethylation of the barbiturate were equivalent: turnover numbers of 0.23 ± 0.02 (S.E.M.) min^{-1} and 0.35 ± 0.03 (S.E.M.) min^{-1} , respectively, were obtained for untreated haemoprotein and pigment containing 10.3 *N*-carboxyhistidine groups per mol of enzyme. This finding is at variance with a previous report, indicating that exhaustive carbethoxylation of phenobarbital-inducible rat liver microsomal cytochrome *P*-450, sharing 77% structural identity with *P*-450 2B4 [36], results in hampered binding of benzphetamine [37], another type I compound.

Ultimately, studies were designed to identify at least some of the histidine residues susceptible to carbethoxylation. To this purpose, unmodified *P*-450 2B4 was used to standardize histidine-containing peptides after tryptic cleavage of the enzyme and *N*-terminal sequence analysis of the fragments (results not shown). When this set of experiments was conducted with haemoprotein originally bearing 10.3 *N*-carboxyhistidines per polypeptide, available evidence indicated that a considerable portion of the DEPC-modified peptides had undergone decomposition during the work-up procedure [19], so that the quantity of labelled material that we were able to collect and repurify was insufficient to permit unequivocal identification of acylated histidines. A similar lability of the label has been previously observed with other types of carbethoxylated proteins [24,33].

Impact of derivatization of *P*-450 2B4 on NAD(P)H-dependent electron transfer

Our interest focused on the influence of chemical modification of *P*-450 2B4 on the NAD(P)H-driven electron-transfer pathways. This necessitated preparation of labelled pigment on a preparative scale, as was brought about by reacting the haemoprotein with 240–600 μ M DEPC followed by gel filtration. Taking into account the small amount of apoprotein present in the final preparations, this procedure consistently introduced 4.5–10.3 carbethoxy groups per *P*-450 2B4 molecule. When the DEPC concentration was raised above a level of 600 μ M, the extent of derivatization did not substantially increase, suggesting that 10–11 of the 14 available histidine residues per mol of enzyme [36] were accessible to the reagent. Measurements of

Table 1 Loss of NAD(P)H-supported *P*-450 2B4 reductase activity upon carbethoxylation and reversal by neutral hydroxylamine

P-450 2B4 was treated with DEPC to introduce 10.3 carbethoxy groups per mol of enzyme. Part of the preparation was reacted with 0.1 M neutral hydroxylamine as described in the Materials and methods section. Excess reagents were removed by gel filtration. NAD(P)H-dependent, hexobarbital-stimulated anaerobic *P*-450 2B4 reduction was measured in reconstituted systems containing either NADPH-cytochrome *P*-450 reductase or NADH-cytochrome *b*₅ reductase plus cytochrome *b*₅. Data represent calculated means $\pm$ S.E.M. derived from three to four experiments. The figures in parentheses indicate percentage of native activity.

<i>P</i> -450 2B4 preparation	<i>P</i> -450 2B4 reductase activity (nmol/min per nmol of <i>P</i> -450)	
	NADH-dependent pathway	NADPH-dependent pathway
Native	2.39 $\pm$ 0.14 (100)	2.31 $\pm$ 0.42 (100)
Carbethoxylated	0.12 $\pm$ 0.01 (5)	0.73 $\pm$ 0.14 (32)
Partially regenerated	1.08 $\pm$ 0.05 (45)	2.00 $\pm$ 0.49 (87)

Figure 5 Dependence of NAD(P)H-sustained *P*-450 2B4 reduction on the extent of histidine modification

P-450 2B4 was reacted with various amounts of DEPC at pH 7.25 to introduce the number of carbethoxy groups per polypeptide as specified on the abscissa. NAD(P)H-driven, hexobarbital-stimulated anaerobic reduction of the derivatized ferric enzyme preparations was measured at pH 7.4 in reconstituted systems containing either NADPH-cytochrome *P*-450 reductase (●) or NADH-cytochrome *b*₅ reductase plus cytochrome *b*₅ (○) as the electron donors; for details see the Materials and methods section. The data indicate relative initial velocities of reduction; control rates averaged 2.39 ($\pm$ 0.14 S.E.M.) and 2.31 ($\pm$ 0.42 S.E.M.) nmol/min per nmol of *P*-450 2B4 for the NADH- and NADPH-dependent pathways respectively. The points represent the means derived from three experiments.

NAD(P)H-driven *P*-450 2B4 reduction were conducted under strictly anaerobic conditions to avoid autoxidation of the terminal acceptor, so that the observed velocities of accumulation of ferrous haemoprotein primarily reflected rates of electron transmission. The assay mixtures contained native or modified *P*-450 2B4 reconstituted with either NADPH-cytochrome *P*-450 reductase or NADH-cytochrome *b*₅ reductase plus cytochrome *b*₅ embedded in a phospholipid matrix. It has to be observed that differences in the reactivity of NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅ toward *P*-450 2B4, as existing at the ionic strength of the buffer system (100 mM sodium phosphate) used throughout our experiments [10,13,38], were compensated for by adapting the levels, in the assay medium, of the respective redox proteins in such a way as to permit balanced rates of NADH- and NADPH-sustained electron transfer to native *P*-450 2B4 (Table 1), so that any DEPC-induced change in the basic fluxes could be ascribed solely to manipulation at histidines.

As documented by Figure 5, increases in the extent of histidine modification were paralleled by a steady attenuation of the initial

Figure 6 Pre-steady-state kinetics of association of NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅ with untreated and carbethoxylated ferric *P*-450 2B4

Lipid-incorporated native *P*-450 2B4 (●) or pigment bearing 10.3 labelled histidines per polypeptide (○) were rapidly mixed with solutions containing either NADPH-cytochrome *P*-450 reductase (A) or cytochrome *b*₅ (B) in sodium phosphate, pH 7.4. Adduct formations were followed by recording the absorbance changes at 386 nm relative to 418 nm. The points are the means of three experiments.

velocities of electron flow with both transfer chains. The apparently linear relationship between the two parameters suggested that each derivatized residue might have contributed equally to lowering reductase activity, 50% inhibition being observed when 6–7 histidines were acylated. At this level of inhibition, the kinetics of *P*-450 2B4 reduction still exhibited biphasic characteristics with predominance of the slow phase (results not shown). At the highest degree of labelling attainable, the cytochrome *b*₅-linked route was almost completely inhibited, whereas the rate of the NADPH-cytochrome *P*-450 reductase-sustained flux was decreased by some 68%. These findings were interpreted to mean that some of the carbethoxylated histidines were crucial for productive interactions with cytochrome *b*₅, whereas additional amino acid residues appeared to play a definite, albeit minor role in the functional coupling of NADPH-cytochrome *P*-450 reductase. This view was endorsed by the fact that lysines in the *P*-450 2B4 polypeptide had been found to serve in ionic interactions with the flavoprotein [10].

The deleterious effect of modification on NAD(P)H-promoted *P*-450 2B4 reductase activity was partially reversed by enzyme treatment with 0.1 M neutral hydroxylamine (Table 1). However, recovery, during the relatively short period of regeneration, of the hexobarbital-stimulated electron fluxes related to the individual branches of the transfer systems tested proceeded to differing extents. This was ascribed to the possibility that coupling to *P*-450 2B4 of NADPH-cytochrome *P*-450 reductase and cytochrome *b*₅, respectively, might have involved participation, to a certain degree, of distinct histidine residues characterized by aberrant sensitivity toward hydroxylamine.

Attempts were made to identify in more detail the defect in carbethoxylated *P*-450 2B4 responsible for its impaired enzymic reduction, placing special emphasis on the ability of the haemoprotein to form high-spin spectral adducts with the two redox partners investigated. Analysis of the binding kinetics revealed monophasic tracings for NADPH-cytochrome *P*-450 reductase and biphasic ones for cytochrome *b*₅ irrespective of the mode of pretreatment (Figure 6). The time courses for association, in the pre-steady state, with native *P*-450 2B4 and enzyme containing 10.3 *N*-carbethoxyhistidines per polypeptide were of comparable magnitude for both redox proteins, suggesting that simple docking of the electron donors to derivatized *P*-450 2B4 was not hampered. Similarly, the basic electron-acceptor properties of modified ferric *P*-450 2B4 were shown to be equivalent to those of untreated pigment, as evidenced by the Na₂S₂O₄-dependent

Figure 7 Time course of chemical reduction of native and modified *P*-450 2B4

Solutions of untreated ferric *P*-450 2B4 (●) or haemoprotein containing 10.3 derivatized histidines per polypeptide (○) were fortified with hexobarbital and the O_2 -scavenging system as detailed in the Materials and methods section. After gassing the mixtures with CO, enzyme reduction was initiated by the addition of excess $Na_2S_2O_4$. The data points represent the means of three experiments.

reduction kinetics (Figure 7). Thus disruption of the electron fluxes (Figure 5) most likely resulted from some defect in a step supposed to govern electron transmission mechanisms. In this regard, the well-known DEPC-induced shift of the pK_a of histidine to about 4 [19] might have caused neutrality of the derivatized entities at the experimental pH of 7.4 to preclude complementary electrostatic contacts responsible for recognition of the electron-transfer interfaces of the redox partners. Clearly, further work is needed to substantiate this hypothesis. It has to be pointed out that both electrostatic [7,9,10,12,38,39] and hydrophobic [40,41] forces have been previously detected to foster electron-transfer events between the cytochrome *P*-450 2B subfamily and its electron donors.

Collectively, we have provided conclusive evidence of the importance of histidine residues in controlling electron flow to ferricytochrome *P*-450 2B4 from NADPH-cytochrome *P*-450 reductase and cytochrome b_5 . It seems noteworthy that suicidal inactivation of *P*-450 2B4 through pretreatment of rabbits with allylisopropylacetamide has been previously detected to be associated with a loss of selective histidines responsible for electron transfer from cytochrome b_5 to the oxyferrous enzyme [42]. Similarly, mutation experiments have shown that His-163 in cytochrome *P*-450 1A2 serves in catalytic functions associated with cytochrome b_5 [43].

Unfortunately, we were unable to identify at least some of the critical histidines, owing to instability of the carbethoxylated material. Therefore, work is underway to examine the influence on the electron-transfer pathways of replacement of conserved cytochrome *P*-450 2B histidines with other amino acid residues via site-directed mutagenesis.

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