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DIFFERENCES IN THE SPECTRAL INTERACTIONS BETWEEN NADPH-CYTOCHROME P-450 REDUCTASE AND A SERIES OF CYTOCHROME P-450 ENZYMES

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The interaction between NADPH-cytochrome P-450 reductase and a series of cytochrome P-450 isozymes was investigated using UV-visible spectrophotometry. In the absence of substrate the interactions between the reductase and RLM3, RLM5, and RLM5a were tight, exhibiting sub-micromolar dissociation constants and resulted in type I spectra of varying magnitude from which the following increases in the proportion of high spin hemoprotein were calculated; RLM3 (7%), RLM5 (36%), RLM5a (6%), LM2 (29%), RLM2 (0%). Preincubation of LM2 with its type I substrate benzphetamine increased the affinity of the cytochrome for the reductase. Using initial estimates of the P-450 spin states in the absence of reductase in conjunction with the spectral binding data and equations relating these parameters to the microequilibria for the association of reductase with high or low spin P-450, RLM3, RLM5, RLM5a and LM2 were shown to bind significantly more tightly to high spin P-450. The relevance of this data to the understanding of spin state influence on P-450 reduction is discussed.

Linear correlations between the absolute amount of high spin ferric cytochrome P-450 and both the P-450 mid-point potential (1) and the kinetics of P-450 reduction by NADPH-cytochrome P-450 reductase (2-4) observed with some microsomal cytochromes P-450 have provided the basis for the spin redox coupling model for P-450 regulation (4). However, the applicability of this model to all microsomal systems has been questioned by the failure to demonstrate such correlations in some systems (5-7) in conjunction with the observation that the kinetics of the P-450 spin equilibrium are extremely fast (8). While previous studies in support of this model were performed under conditions in which the rate of complex formation between the reductase and P-450 were presumed not to be rate limiting for electron transfer, at the prevailing ratio of the two proteins in the endoplasmic reticulum between 1:10 and 1:20, other factors such as those effecting the affinity of the two

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proteins for each other should profoundly effect their functional interaction. In this study we provide spectral evidence that P-450 reductase interacts more tightly with the high spin form of some P-450 enzymes and suggest that at least under subsaturating conditions this phenomenon may in part contribute to spin-state reduction rate correlations.

MATERIALS AND METHODS

Rat hepatic microsomal P-450 RLM2 (9), RLM3 (10), RLM5 (10), RLM5a (11), NADPH-cyochrome P-450 reductase (12) and rabbit hepatic microsomal LM2 (13) were purified to electrophoretic homogeneity using the cited procedures and exhibited the following specific contents (nmol/mg protein) RLM2, 16; RLM3, 16; RLM5, 12; RLM5a, 14; LM2, 18 and NADPH-cytochrome P-450 reductase, 11. Spectral titrations of P-450 (0.56 μM) in the presence of L-α-dilauroyl phosphatidyl choline (48 μM, as vesicles) with the reductase (107 μM stock solution) were performed at 25°C and analyzed as described previously (14) yielding the spectral binding parameters Kd and ΔAmax. The spin state of the ligand-free cytochromes were calculated from the differences in A390/A417 ratio relative to RLM5 (taken as 28% high spin (15)) and using a difference absorption coefficient (Δε 390-417 nm) of 126 $mM^{-1}cm^{-1}$ for the low to high spin change (16). Estimates of the asymmetry in micro-equilibria describing the association of reductase with low or high spin P-450 were obtained using the following equations (17):

$$\Delta Amax = \frac{(K_2 - K_1) \cdot \Delta\varepsilon \cdot Et}{(K_1 + 1)(K_2 + 1)} \qquad \text{(eq. 1)}$$

$$K_2/K_1 = K_3/K_4 \qquad \text{(eq. 2)}$$

Where K_1 and K_2 are the reductase free and bound P-450 spin equilibrium constants, respectively, and K_3 and K_4 are the dissociation constants for the low and high spin reductase-P-450 complex, respectively. Et is the total P-450 concentration.

RESULTS AND DISCUSSION

The reductase-inducible type I spectral change of the P-450 isozymes investigated are shown in figure 1 and the resultant spectral binding

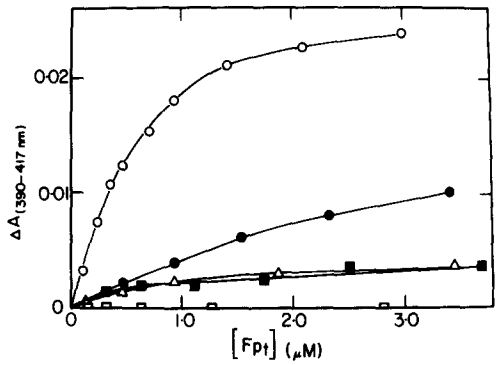

Figure 1. Spectral visualization of the interactions between NADPH-cytochrome P-450 reductase and a series of P-450 enzymes. Spectral titrations were performed at 25°C as described and cited in Materials and Methods. □ = RLM2; ■ = RLM5a; Δ = RLM3; ● = LM2; O = RLM5.

parameters are listed in table 1. Considerable variation in the magnitude of the spectral changes were observed. Thus, while RLM5 interacted tightly with the reductase yielding a large spectral change, no spectral change was evoked by the titration of RLM2. Over the reductase concentration range employed the magnitude of the LM2 type I spectra were smaller than observed with RLM5 due to the weaker affinity of LM2 for rat hepatic reductase (table 1) however, the maximal spectral change observed with LM2 was nevertheless substantial. With the exception of LM2, all of the measurable spectral interactions were tight exhibiting sub-micromolar Kd values. Pre-equilibration of LM2 with its type I substrate benzphetamine caused an 11-fold increase in affinity of the hemoprotein for P-450 reductase but did not alter the affinity of RLM5 for the flavoprotein. Due to the small magnitude

TABLE 1

Spectral binding parameters characterizing the interactions between NADPH-cytochrome P-450 reductase and a series of P-450 enzymes

P-450 enzyme	Kd (nM)	ΔAmax	Δ% Fe^{3+}_{hs}
RLM2	-	0	0
RLM3	801	0.009	7
RLM5	276	0.046	36
RLM5 + 0.49 mM BZP	296	0.019	15
RLM5a	412	0.007	5.5
LM2	3678	0.037	29
LM2 + 1.3 mM BZP	334	0.013	10

TABLE 2

Relative affinities of NADPH-cytochrome P-450 reductase for low spin (K_3) and high spin (K_4) P-450 enzyme conformations

P-450 enzyme	% Fe^{3+}_{hs} (-S)	K_1	K_2	K_3/K_4
RLM2	<5	0 - 0.053	0 - 0.053	1.0
RLM3	<5	0 - 0.053	0.075 - 0.136	∞-2.7
RLM5	28	0.390	1.78	4.6
RLM5a	<5	0 - 0.053	0.058 - 0.122	∞-2.1
LM2	<5	0 - 0.053	0.418 - 0.429	∞-8.1

of the reductase-induced spectral changes observed with the other cytochromes it was not possible to delineate with certainty the effect of substrate pre-equilibration. By comparing the A390/417 ratios of the P-450 isozymes it was calculated that in the absence of type I ligands, RLM2, 3, 5a and LM2 contained 5% or less high spin heme relative to RLM5 which contains 28% high spin heme. The figure of 5% sets the approximate upper limit for the substrate-free spin equilibrium constant at about 0.053, and since it could be much lower, even approaching zero, the possible range in K_1 values is represented in Table 2. Using this range in conjunction with the spectral binding parameter ΔAmax listed in table 1, the corresponding range of K_2 values was determined. Using eq. 2 the relative affinity of the reductase for low and high spin P-450 could be estimated. The values of K_3/K_4 calculated (table 2) show the lower limit of this fold difference in affinity. With the exception of RLM2 it was possible to demonstrate a significantly greater affinity of reductase for the high spin forms of RLM3, RLM5, RLM5a and LM2 versus the corresponding low spin states.

The possible metabolic significance of these phenomena may now be addressed. At the lower concentrations of P-450 reductase, such as are present in the endoplasmic reticulum, electron transfer between NADPH-cytochrome P-450 reductase and cytochrome P-450 is expected to be second order, limited only by the rate of translational collisions between the two proteins within the lipid bilayer. Thus, the greater affinity of the

reductase for high spin P-450 should provide a mechanism by which substrate bound (predominantly high-spin) P-450 should more effectively compete with substrate free forms of P-450 for limiting NADPH-cytochrome P-450 reductase thereby facilitating formation of a functional ternary complex. The LM2 spectral binding data would support this where in addition to a marked asymmetry in flavoprotein association to low and high spin P-450, a 10-fold increase in the overall affinity of LM2 for the reductase was observed in the presence of benzphetamine. Similar observations of substrate effects were made by French et al. (18), although the magnitude of the reductase affinity changes were much smaller and were not rationalized in terms of asymmetric association with low and high spin P-450. It is not known why a similar tightening of the reductase-RLM5 complex was not observed in the presence of benzphetamine. Another consequence of the reductase inducible spectral change is that it would cause an increase in the steady-state concentration of a reductase-bound high spin P-450 complex and thus a stimulation in the overall rate of reduction of a P-450 sub-population.

This might be particularly important for the metabolism of substrates which themselves do not ellicit type I spectral changes and for which first electron transfer would otherwise be a slow step. It is noted, at least with LM2, that the magnitude of asymmetry of association of reductase with low and high spin P-450 and consequential increase in affinity of the proteins for each other in the presence of benzphetamine could, depending on the relative P-450/reductase levels, account for a strong dependence of reduction rate upon ferric P-450 spin state due to increases in the amount of reductase-bound P-450, without necessarily evoking changes in P-450 mid-point potential.

Studies are in progress to evaluate the relative importance of spin-redox and spin-Fpt coupling in the control of P-450 reduction.

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