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CYTOCHROME $P450_{cam}$: SS- DIMER AND -SH DERIVATIVE REACTIVITIES

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SUMMARY: N-ethyl-maleimide alkylation converts ferric $P450_{cam}$ to a (succinimido-cys)$_4$ protein with native optical and EPR spectra but insensitive to substrate induced shift of iron to high spin with Soret absorption to higher energy and inactive in putidaredoxin ferric-ferrous reduction. On photo or chemical reduction the ferrous protein oxygenates and, with reduced putidaredoxin, converts substrate to product. Mild oxidation of $P450_{cam}$ yields a disulfide dimer whose properties on alkylation of 3 sulfhydryls equal the succinimido$_4$ monomer; additional alkylation converts either monomer or dimer to a P420.

Sulfhydryls assume a central importance in P450 cytochrome monoxygenase structure and function. The unique spectral and redox properties are attributed to an axial, or z, cysteinyl sulfur ligand (1,2); additional -SH groups modulate substrate affinity, ferric ion spin state and ferric-ferrous reduction by the native monoxygenase electron transport systems.

Crystalline $P450_{cam}$, cyt m, contains 6 to 8 cysteine sulfhydryl groups per molecule of ∿45,000 daltons (3,4). They differ in autoxidation rates, substitution by mercurial or alkylating reagents, and essentiality to individual reactions in the P450 monoxygenase cycle (5,6). PMB (para-chloromercuribenzoate) reacts with four to five sulfhydryls to form a modified protein with the spectral and catalytic response of the original enzyme; more rigorous PMB treatment yields a P420 molecule (5).

N-ethyl maleimide is more selective (7,8), and yields proteins of reproducible composition and activity (7-9). We outline here the preparation, and

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Abbreviations: PMB, parachloromercuribenzoate; NEM, N-ethyl maleimide; NES, N-ethyl succinimido; cyt m, cytochrome $P450_{cam}$; Pd, putidaredoxin; SDS, sodium dodecyl sulfate, βME, β-mercaptoethanol.

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the chemical and optical properties of the (N-ethyl-succinimido-cys)$_4$ P450$_{cam}$, termed m-NES$_4$.

MATERIALS AND METHODS

The three protein components of the P450$_{cam}$ monoxygenase system, cyt m, Pd, and reductase fp, were prepared from Pseudomonas putida strain PpG786 (ATCC 29607) as described previously (10-12). The cytochrome was crystallized once prior to use.

P450 activity was estimated: a) from the rate of DPNH oxidation in the reconstituted three enzyme system (11,13) and b) by product formation starting from the oxyferrous substrate oxygenated ternary complex, $m^{rs}_{O_2}$ (13,14). In a) we follow DPNH oxidation at 340 nm in 1 ml of 50 mM K^+PO_4, pH 7, containing the micromolar concentrations DPNH 250, camphor 1000, reductase 0.5, Pd 16, and cyt m 0.05. In b) we measure product formed in the same buffer with 30 nMoles of P450$_{cam}$ and 600 µM camphor plus the photoreduction system, 4 µM proflavin and 10 mM EDTA. The system is degassed and photoreduced under argon, O_2 is admitted to form $m^{rs}_{O_2}$, and 1.5 µM Pd added immediately, see (14). Product formation is quantitated by vapor phase chromatography on a dichloromethane extract using a 3% QF 1 column.

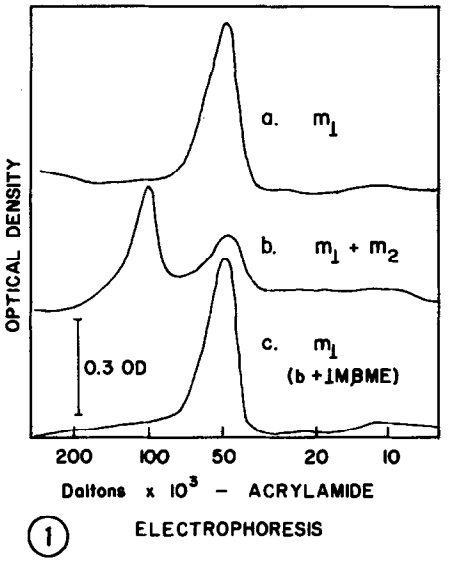
The -SH titrations, unless otherwise noted, were on pure ferric P450$_{cam}$, m^o, or m^{os} (the substrate complex), at 150 µM in 50 mM K^+PO_4 buffer, pH 7.1, at 0°C with 2 mM N-ethyl maleimide (NEM) (Pierce Chemical Co.) containing 0.2 µCi/ml of 1-[^{14}C] NEM from New England Nuclear. The m^{os} alkylation contained in addition 250 µM D-camphor. Aliquots removed from the reaction vessels at the indicated intervals were freed of residual NEM by transfer to 10 mM β-mercaptoethanol (βME) and immediate separation on a 1 x 5 cm Biogel P-10 column, followed by dialysis where necessary to remove the last traces of NES. The rate of alkylation and ratio of NEM incorporated per P450 were monitored by scintillation counter and amino acid analyses after hydrolysis in 6 N HCl at 110°C for 72 hours.

The P450 response to substrate, $m^o \rightarrow m^{os}$, was estimated from the absorbance at 417 and 391 nm (isosbestic at 406 nm) on adding 600 µM camphor in 50 mM K^+PO_4 buffer, pH 7 ($\varepsilon^{417}_{mM} = 115$; $\varepsilon^{391}_{mM} = 102$, Table 1). The level of substrate insensitive cytochrome, termed m-417, can be calculated from the extinction coefficients, and the ferrous CO spectra ($\varepsilon^{446}_{mM} = 120$) used to correct for the P420 content (12).

RESULTS AND DISCUSSION

m^{os}-NES$_4$ Preparation and Optical Spectra. Table 1 indicates the substrate effects on the native and NES$_4$ P450$_{cam}$ ferric spectra. The alkylated protein retains the 417 nm Soret and lacks the 645 nm charge transfer band indicative of a high spin ferric heme. On chemical reduction to the ferrous state the absorption spectra are equivalent for the free proteins and for their complexes with substrate, O_2, and CO.

Dimer Formation. A dimer $(m)_2$ accumulates during P450$_{cam}$ purification in the absence of reducing agents, or on freeze-thaw cycles, as shown by SDS polyacrylamide gel electrophoresis (15), Figure 1. βME treatment reforms monomer, $(m)_1$. Sedimentation at 20°C, 60K rpm, gives two $s_{20,w}$ values, 5.08



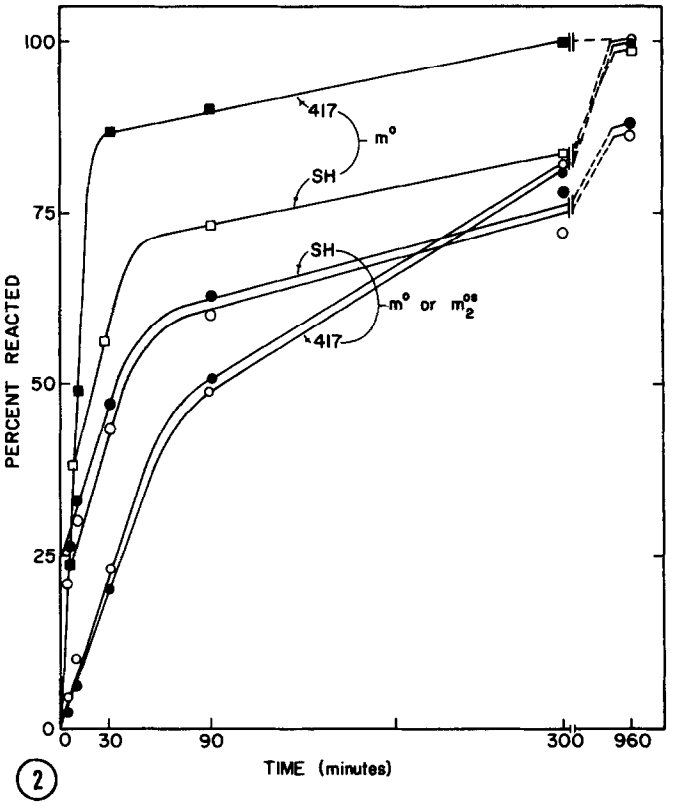


Fig. 1. An Active $P450_{cam}$ Dimer.

After electrophoresis of 100 µg of each protein in 5 x 50 mm polyacrylamide gel, 7.5% 100 mM Na^+PO_4 buffer, pH 7, +0.1% SDS, densitometry traces were taken at 417 nm. (a) and (b) proteins from anaerobic and aerobic isolation, 100 µM camphor present; (c) = (b) after 30 min in 1 M βME at 25°C.

Fig. 2 R-SH Titration Kinetics and Ferric P450 Spin State.

Ferric $P450_{cam}$, 150 µM, in 150 mM K^+PO_4 buffer, pH 7.1, treated at 0°C with 2 mM N-ethyl maleimide containing 0.2 µCi/ml of 1-^{14}C-NEM. Aliquots plus 10 mM βME at intervals indicated, passed through Biogel P-10 columns and assayed, see Methods.

and 3.46, and calculated molecular weights (16) compatible with those indicated by the SDS polyacrylamide gels. Molecular sieve chromatography on Sephadex G-100 provides pure $(m)_1$ and $(m)_2$ with identical catalytic properties; both are recovered unchanged after the reaction cycle in the reconstituted DPNH driven system. The dimer did not, on treatment with the isothiocyanate, form the fluorescein derivative used to measure Pd·cyt m affinities

Table 1. Absorption Fe^{3+} $P450_{cam}$--Native vs. NES_4

Form		m^o native	m^o NES_4	m^{os} native	m^{os} NES_4
γ:	λ (nm)	417	417	391	417
	ε (mM)	(115)	(115)	(102)	(105)
β:	λ (nm)	535	535	540	536
	ε (mM)	(11.6)	(11.6)	(11.2)	(11.6)
α:	λ (nm)	569	569	646*	569
	ε (mM)	(11.9)	(11.9)	(5.4)	(11.8)

50 mM K^+PO_4 buffer, pH 7; s = 600 μM D-camphor.

*Charge transfer band.

(17). We deduce that a highly reactive exposed sulfhydryl participates in the formation of the dimeric hemoprotein, leaving one fewer free -SH group per 45,000 daltons--2 per dimer. The retention of full catalytic activity further distinguishes the rapidly reacting mercapto group from those modified more slowly by NEM, with parallel loss of spectral response to substrate and ferric-ferrous reduction by Pd^-, as shown below.

Sulfhydryl Alkylation and m-417 Formation. The rates of alkylation of $(m)_1$ and $(m)_2$ and the effect of substrate are shown in Figure 2. The 100% abscissa corresponds to 4 NEM molecules reacted with cysteinyl -SH groups. Substrate free cyt m, as both monomer and dimer, is converted more rapidly to the low spin m-417 than are the substrate complexes, halftimes approximately 10 min vs. ∿90 min. The kinetics approximate first order for the first mole of NEM per P450, and are not affected by the presence of substrate. If one assumes that the $(m)_2$ dimer formation used the most reactive sulfhydryl and plots SH at t = 0 from 25% (1 SH reacted) the data for m^{os} and $(m^{os})_2$ become parallel. The rates of alkylation differ markedly from SH_1 to SH_4, but those of SH_2 and SH_3 are too similar to permit simple kinetic analysis; both appear. however, to influence the substrate induced spectral shifts. Without sub-

strate the half-conversion of the monomer to m-417 requires about 2 SH groups reacted, but approaches completion only near 3; in the substrate complexes half m-417 formation approximates 2.5 SH groups reacted. Thus we draw the qualitative conclusion that the reaction of NEM with the second sulfhydryl, and very likely the third, conditions the loss of substrate induced spectral shift and the retention of low spin state of the heme ferric iron.

EPR Spectra: m^{os}-NES_4 and -NES_6. The iron spin state as defined by the EPR spectra taken at 13°K, Figure 3, confirms the inferences from the optical data (18,13). The g values of the native low-spin protein, m^{o}, observed at 2.45, 2.26, and 1.91 (18) are essentially reproduced by the m^{os}-NES_4 low-spin ferric substrate complex, g = 2.46, 2.25 and 1.91. These values are attributed to tetragonal and rhombic symmetry components of a heme crystal field associated with a single mercaptide axial ligand (1,2,18-21). Thus we infer that 4 sulfhydryl groups in cyt m can react with NEM without disturbing the presumed cysteinyl axial ligand, strongly indicated an appreciable resistance to alkylation by this ligand at pH 7.

Figure 3 shows that, in contrast, the m^{os}-NES_6 has a high-spin axial symmetry, g = 6, resembling the P420 formed from mammalian P450 cytochromes by treatment with sulfhydryl reagents (22) but distinct from the rhombically distorted high-spin signal of the native m^{os}. One should recall that a P420 prepared from $P450_{cam}$ by acetone treatment retains a low-spin EPR spectrum resembling m^{o}. Thus again one concludes that a protected SH group either is an axial ligand in the cyt m or is in a position to greatly affect the iron ligand geometry.

Hydroxylating Activities of m-NES_4. Catalysis by hemeproteins $(m)_1$, $(m)_2$, and m-NES_4 was compared as indicated in Methods, starting from a) m^{os} and b) $m^{rs}_{O_2}$.

a) DPNH oxidation decreased in parallel with m-417 formation--i.e. stable low spin (LS) state in the presence of substrate. The defect was traced to an impaired ferric-ferrous electron transfer, e_1, to cyt m from Pd^{-}. Substrate

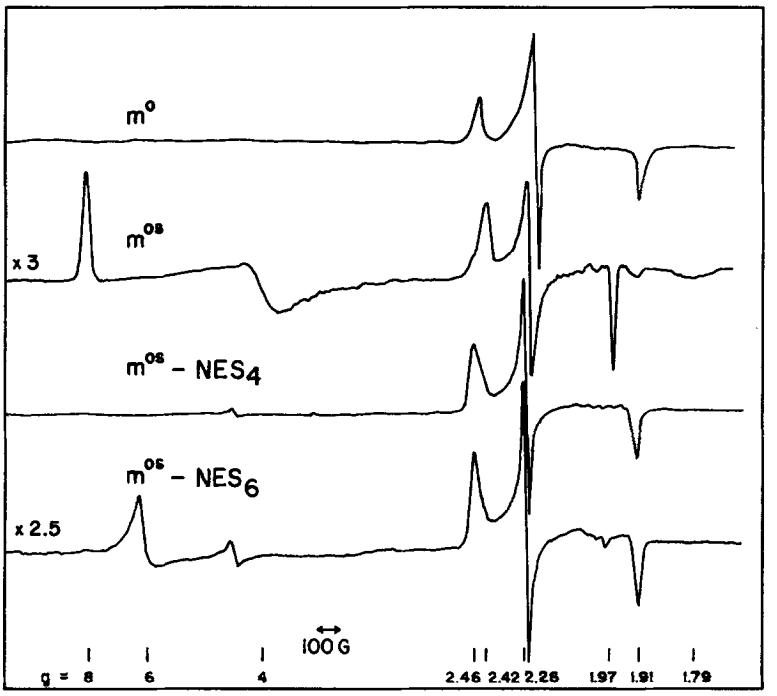


Fig. 3. EPR Spectra of Native and NES $P450_{cam}$.
P450, 1 mM, and camphor, 250 μM, in buffer, Figure 2, scanned in Varian E-9 spectrophotometer; 13°K; modulation frequency 100 kHz; amplitude 12.5 G; microwave power 0.5 mW; time constant 0.3 sec; base receiver gain of 500 was multiplied by the factors shown. $m\text{-}NEM_6$ sample was ≥ 60% P420 as estimated from the ferrous CO spectrum.

addition to native cyt m--m^{os} formation--shifts the E_o' from -340 to -170 mv; in m^{os}-NES_4 the midpoint potential increased only to -266 mv. The E_o' of the Pd^o/Pd^- redox couple, -235 mv (23), is increased to -195 mv in the tightly coupled $Pd{\cdot}m^{os}$ ternary complex (24). The analogous potential and an association constant for $Pd{\cdot}m^{os}$-NES_4 have not been measured.

b) the e_2 transfer with product formation from the $Pd{\cdot}m^{rs}_{O_2}$ complex occurred at ca. 95% the rate with native $m^{rs}_{O_2}$ and with similar product yield. The affinities of Pd^- have not been measured for $m^{rs}_{O_2}$, nor have those of camphor and O_2--but near saturation levels of all three must have been achieved to support the observed reaction rates.

Thus with a modified P450 a separation of the two reduction steps, e_1 and

e_2, has been achieved in the complete hydroxylation system, including O_2. That is, the reduction of cyt m^{os} to m^{rs} is clearly resolved from the hydroxylation cycle from m^{rs} through $m^{rs}_{O_2}$ formation, and with added Pd^- the oxygen cleavage-oxygenation reaction sequence. The active center of the monoxygenase complex is thus opened to further elucidation. One can inquire as to the sulfhydryl groups essential to the activated coupled spin state modulation and potential shift, and very likely the affinity constants among the several proteins and substrates. Additional kinetic, structural, and mechanistic details will appear in a full manuscript.

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