Fluorescence Resonance Energy Transfer Analysis of Cytochromes P450 2C2 and 2E1 Molecular Interactions in Living Cells*

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The molecular organization of microsomal cytochromes P450 (P450s) and formation of complexes with P450 reductase have been studied previously with isolated proteins and in reconstituted systems. Although these studies demonstrated that some P450s oligomerize in vitro, neither oligomerization nor interactions of P450 with P450 reductase have been studied in living cells. Here we have used fluorescence resonance energy transfer (FRET) to study P450 oligomerization and binding to P450 reductase in live transfected cells. Cytochrome P450 2C2, but not P450 2E1, forms homo-oligomeric structures, and this self-association is mediated by the signal-anchor sequence. Because P450 2C2, in contrast to P450 2E1, is directly retained in the endoplasmic reticulum (ER), these results could suggest that oligomerization may prevent transport from the ER. However, P450 2C1 signal-anchor sequence chimera defective in ER retention also formed oligomers, and chimera containing the cytoplasmic domain of P450 2C2, which is directly retained in the ER, did not exhibit self-oligomerization, which indicates that oligomerization is not correlated with direct retention. By using FRET, we have also detected binding of P450 2C2 and P450 2E1 to P450 reductase. In contrast to self-oligomerization, the catalytic domain can mediate an interaction of P450 2C2 with P450 reductase. These results suggest that microsomal P450s may differ in their quaternary structure but that these differences do not detectably affect interaction with the reductase or transport from the ER.

The molecular organization of the cytochrome P450 (P450)1-containing monooxygenase system in the microsomal membrane is not well understood despite many studies using a wide variety of biophysical methods. This system consists of P450s, NADPH-cytochrome P450 reductase (P450 reductase), and for some P450s, cytochrome b5. If P450 levels in the endoplasmic reticulum (ER) are induced to maximal concentrations, there are 20–30 P450s for each P450 reductase molecule. The presence of multiple forms of P450s in the ER membranes combined with limiting amounts of P450 reductase has important implications for the association of the P450s with each other and the reductase and for electron transfer from the reductase to P450. It has been postulated that either a multimeric complex of P450s binds to a molecule of P450 reductase (1, 2) or that the interaction results from random collisions of independently diffusing monomeric proteins that have high rotational and lateral mobility (3–5). Formation of large complexes by P450s has been observed with both isolated proteins and reconstituted systems (6–8). Studies on rotational mobilities of P450s in microsomal membranes were also consistent with either self-oligomerization or association of P450s with other components of the membranes (9–11). The second model in which monomeric P450 interacts with P450 reductase is supported by observations that high concentrations of some P450s in membranes result in their aggregation and immobilization, but the addition of reductase increases rotational mobility, thereby disaggregating P450 (3, 4, 12). These data would suggest that the binding of a P450 to P450 reductase competes for P450 self-association, as would be expected if the monomer binds with reductase.

The molecular architecture of the microsomal monooxygenase system in the ER membranes is further complicated by the presence of multiple P450s that metabolize a variety of different substrates. Formation of heteromeric multienzymatic P450 complexes, demonstrated in a reconstituted system and in microsomes (13–15), could have dramatic consequences for the catalytic properties of the individual P450s (13–16). Heteromeric complexes of different P450s, which catalyze parallel or consecutive steps in metabolism of some substrates, for example steroids or benzopyrene, could have significant kinetic and catalytic consequences, so that analysis of their formation under physiological conditions (in cells and tissues) has important implications for drug metabolism and activation of carcinogens.

In addition to interactions with P450 reductase, oligomerization potentially could be an important determinant of the localization of P450 in the ER. One mechanism that has been proposed to explain retention of ER resident proteins is that they form very large immobile complexes or networks that prevent entry of the proteins into transport vesicles. P450 2C2 is directly retained in the ER (17); however, based on fluorescence recovery after photobleaching (FRAP) studies, it has high mobility in the ER membrane, similar to that of a model protein transported from the ER (18). It is still possible that oligomerization of P450 2C2 into smaller homomeric complexes, which remain mobile in the membrane, prevents it from entering the transport vesicles. In contrast to P450 2C2, P450 2E1 is not directly retained in the ER but is retained by a retrieval mechanism (19), and mutations in the signal-anchor (SA) region of P450 2C2 have been described that also result in transport from the ER (20). If oligomerization is important for direct ER retention, transport from the ER should correlate...
with decreased oligomerization.

In the present study we have used the fluorescence resonance energy transfer (FRET) technique to study the quaternary structure of P450s 2C2 and 2E1 and their interactions with P450 reductase in the membranes of living cells. FRET takes place if the energy from a donor (blue-shifted fluorophore) is transferred to an acceptor (red-shifted fluorophore) when both fluorophores are in close physical proximity. The FRET technique is a powerful non-invasive approach to study protein-protein interactions in intact living or fixed cells. The recent introduction of green fluorescent protein (GFP) mutants, cyan and yellow variants (CFP and YFP), which have favorable spectral characteristics for FRET, resulted in the broad application of this technique for studies on interactions of a diverse array of proteins (21-24). CFP- and YFP-mediated FRET has also been used successfully for studies on homooligomerization and multimeric complex formation (25-29).

Our studies show that the N-terminal SA of P450 2C2 facilitates formation of the oligomeric structures by this P450, whereas P450 2E1 exists mostly in a monomeric form. However, there is no apparent correlation between the formation of oligomeric structures and either direct ER retention of P450 2C2 or the interaction of P450 with P450 reductase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture materials were purchased from Invitrogen, and CFP (ECFP) and YFP (EYFP) vectors were from Clontech.

**Construction of Fluorescent Chimeras**—The positive control fusion protein CFP-YFP was constructed by amplifying the YFP coding sequence with primers designed so that 5′ primer introduced a NotI site and eliminated the original BsrGI site. The PCR product was digested with BsrGI and NotI and inserted into the ECFP vector digested with the same enzymes.

To construct chimeric proteins tagged at the C terminus with either YFP or CFP, we have used GFP chimeras described previously (18-20, 30), in which we have replaced the GFP part of the chimera with YFP or CFP. To make full-length 2C2/CFP and 2C2/YFP, a KpnI/BamHI fragment from 2C2/CFP (18) containing the P450 2C2 cDNA was inserted into the vectors pECFP and pEYFP, respectively, that had been digested with KpnI and BamHI. 2E1/CFP and 2E1/YFP were constructed by inserting a KpnI/BamHI fragment from 2E1/CFP containing the 2E1 cDNA into the KpnI-BamHI site of the CFP and YFP vectors, respectively (20). To make fusions of CFP and YFP to the C terminus of P450 2C2 (1-28), P450 2C1 (1-21), and P450 2E1 (1-31), BglII-HindIII inserts encoding the P450 fragments were ligated with HindIII-BglII-digested CFP and YFP vectors. Construction of OEC/CFP and YFP was made by substituting the BamHI-Ncol fragment encoding CFP or YFP from their respective vectors for the BamHI-Ncol fragment encoding GFP in OEC/GFP (18). A chimera of P450 reductase/YFP was made by amplifying the P450 reductase cDNA insert from the plasmid reductase/CMV55 by PCR using a 5′ primer introducing a BglII site and a 3′ primer engineered to insert an HindIII site. The amplified DNA was digested with BglII and HindIII and inserted into the YFP vector digested with the same enzymes.

**Spectrofluorometry**—COS1 cells were transfected on 6-well plates as described (30). 1 μg of plasmid DNA was used for each chimera expressing the full-length proteins (C2, 2E1, OEC, and reductase), whereas for chimeras with the signal anchor sequence only (P450 2C1 (1-28), P450 2C1 (1-21), C2 6L, and P450 2E1 (1-31)) 0.2 μg of each plasmid DNA was used. Twenty-four h later cells were washed with PBS, scraped, and collected by centrifugation for 2 min in microcentrifuge tubes. Additional washes were performed in order to remove residual detergent and has catalytic activity similar to the untagged protein (18), encoding the coding sequence with primers designed so that 5′ primer introduced a NotI site and eliminated the original BsrGI site. The PCR product was digested with BsrGI and NotI and inserted into the ECFP vector digested with the same enzymes.

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COS1 cells were transfected with equal amounts of the indicated plasmids encoding either CFP chimeras (black lines) or with both CFP and YFP chimeras (gray lines), and 24 h later cells suspensions were analyzed by emission scanning spectrofluorimetry using excitation at 425 nm. The emission spectra were corrected for the background by subtracting the emission of mock-transfected cells. The fluorescence resulting from co-excitation of YFP at 425 nm was also subtracted. The curves were normalized to the CFP fluorescence level at 475 nm detected in cells expressing CFP chimeras only. C1–21, P450 2C1-(1–28), C1–28, P450 2C1-(1–28).

The spectral studies described above analyze fluorescence emission spectra of transfected COS1 cells. COS1 cells were transfected with plasmids encoding either CFP chimeras only (gray lines) or with both CFP and YFP chimeras (black lines), and 24 h later cells suspensions were analyzed by emission scanning spectrofluorimetry using excitation at 425 nm. The emission spectra were corrected for the background by subtracting the emission of mock-transfected cells. The fluorescence resulting from co-excitation of YFP at 425 nm was also subtracted. The curves were normalized to the CFP fluorescence level at 475 nm detected in cells expressing CFP chimeras only. C1–21, P450 2C1-(1–28), C1–28, P450 2C1-(1–28).

These results indicate that while the linker sequence, 21–28, is required for direct retention in the ER, it is not required for oligomerization.

To further establish whether there is a correlation between oligomerization and ER retention, we also tested self-association, as detected by the FRET assay, of a P450 2C1 SA mutant, which was not retained in the ER and was transported in cells through the secretory pathway to the plasma membrane (20). In this mutant six leucines inserted after residue 18 extend the hydrophobic core of the SA on the C-terminal side. As shown in Fig. 1, the spectrum from cells expressing both the 6L/CFP and 6L/YFP fusions has a prominent peak at 525 nm, characteristic of FRET, although the efficiency of FRET is decreased relative to the wild type P450 2C1-(1–28) form. Interestingly, only a shoulder in the 525-nm region was observed for the pair 6L/CFP and P450 2C1-(1–28)/YFP indicating that there is a weak interaction between the wild type and 6L mutant SA. The oligomerization of both 6L and P450 2C1-(1–21), which are not directly retained in the ER, further support the conclusion that the oligomerization is not the main determinant of direct ER retention of P450 2C2.

P450 2C2 Oligomerization Analysis by Confocal Microscopy—The spectral studies described above analyze fluorescence of bulk cells suspended in PBS. Thus, although living cells are used, it is possible that some of the protein aggregation is due to changes in the cells after removal from the normal growth environment. To examine the fluorescence of the cells in normal growth media, individual COS1 cells, grown and transfected on multichamber dishes with coverslip bottoms, were used for imaging 24 h after transfection. First, we adjusted optical conditions to minimize the bleed through of light between bandwidths selected for specific detection of either CFP or YFP emission. To accomplish this, we analyzed cells transfected either with one fluorophore or with both. Cells were observed after excitation at 458 nm and detection in the bandwidth of 465–510 nm (CFP channel), after excitation at 514 nm and detection in the bandwidth of 530–563 nm (YFP channel), or after excitation at 458 nm and detection in the bandwidth at 530–563 nm (FRET channel). In cells expressing YFP, fluorescence is strongly visible in the YFP channel but not in either the CFP or FRET channels (Fig. 2). In cells expressing CFP, fluorescence is strongly visible with the CFP channel and not with the YFP channel, but substantial bleed-through fluorescence is observed with the FRET channel (Fig. 2). To correct for this bleed-through fluorescence, a ratio image was obtained by dividing the image obtained with FRET channel by the image.
detected with the CFP channel using Leica confocal software version 2.0, as described (25). The ratio image represents a net FRET signal. By using this method we detected a strong intramolecular FRET signal in cells expressing tandem CFP-YFP as a control (Fig. 3). This is demonstrated by the higher fluorescence intensity recorded with the FRET channel than with the CFP channel, which results in an intense FRET signal in the ratio image. High efficiency of FRET is also manifested by the decrease in fluorescence from the donor CFP, which is quenched by transfer of energy to YFP. A weaker, but visible, intermolecular FRET signal could be detected for chimeras of both full-length P450 2C2 and chimeras with the SA sequence as a control (Fig. 3). This is demonstrated by the higher fluorescence intensity recorded with the FRET channel than with the CFP channel, which results in an intense FRET signal in the ratio image. High efficiency of FRET is also manifested by the decrease in fluorescence from the donor CFP, which is quenched by transfer of energy to YFP. A weaker, but visible, intermolecular FRET signal could be detected for chimeras of both full-length P450 2C2 and chimeras with the SA sequence only (Fig. 3). In contrast, no FRET was observed for OEC partners (Fig. 3), in agreement with the spectrophotometric assay method.

As an independent and more stringent assay of FRET, we have used selective photobleaching of the acceptor (YFP), which should lead to an increase of the donor (CFP) fluorescence, if FRET is occurring, because quenching of the donor by the acceptor is eliminated by the bleaching. This technique alleviates concerns of bleed-through fluorescence between fluorophores, so that the brighter signal from the donor cannot result from co-excitation of the acceptor, which was bleached. First, the images were collected with both CFP and YFP channels, and subsequently, the YFP partner was partially photobleached at the YFP-specific wavelength (514 nm) for 2 min, after which images were re-acquired with both CFP and YFP channels. Similar optical conditions were used for acquiring both the CFP and YFP images so that the YFP images appear overexposed because of the higher intrinsic fluorescence of YFP even though the same amounts of expression vectors were transfected for both. The CFP image was also converted to a pseudocolor scale to better illustrate the changes in CFP intensity. For both full-length P450 2C2 and P450 2C1-(1–28), bleaching of the acceptor caused an increase of donor fluorescence, consistent with their interaction (Fig. 4A). To measure the level of CFP and YFP fluorescence change, fluorescence intensities in selected regions of interest were quantified, and background values were subtracted. The level of the YFP fluorescence was decreased after bleaching by about 41 and 47%, whereas the level of CFP fluorescence increased about 29 and 38% for the full-length and P450 2C1-(1–28) chimeras, respectively (Fig. 4B). It is not clear whether the slightly higher relative increase in CFP fluorescence for SA chimeras results from increased oligomerization or from more efficient FRET because of the possibility that the fluorophores are closer to each other when fused to the short SA sequence than to full-length P450 2C2. For chimeras of OEC, the ratio of CFP fluorescence after bleaching compared with before bleaching was 0.95 ± 0.15 (n = 8), which is consistent with no FRET in agreement with the spectral and ratio image method results.

The ER retention-defective chimera 6L, which showed weaker oligomerization than wild type P450 2C1-(1–28) as assayed by scanning fluorimetry, was also analyzed by acceptor photobleaching technique and showed a slight CFP intensity increase (ratio of 1.18 ± 0.09, n = 8) (Fig. 4B), but this was dependent on the cellular location. We observed negligible FRET at the plasma membrane. The increase of CFP fluorescence was more pronounced in the intracellular compartment (compare CFP fluorescence in the perinuclear region of the cell before and after bleaching as shown in Fig. 4A). This result suggests that the intracellular pool of the mutant protein consists of self-associating molecules, but proteins that reached the cell surface are either in a monomeric form or in smaller aggregates. Interestingly, no FRET was observed between P450 2C1-(1–28) and 6L (not shown) in agreement with the spectral method (Fig. 1), which suggests either that these proteins are targeted to different subcompartments of the ER or that the affinity for homo-oligomerization for each is higher than the affinity for hetero-oligomerization.

Thus, from three independent methods designed to detect FRET, we conclude that in live transfected cells P450 2C2 forms oligomers and that this process is mediated by the SA sequence only. Chimera OEC, which like wild type C2 is directly retained in the ER in transfected cells (17), remains in a monomeric state. The mutated P450 2C2 (6L) and P450 2C1-(1–21), which, unlike P450 2C1-(1–28), are not directly retained in the ER, still formed homomeric complexes, although the former oligomerized less efficiently. Therefore, oligomerization per se does not limit transport beyond the ER.

FRET Assay for Oligomerization of P450 2E1—P450 2E1 has been shown to recycle between the ER and the intermediate compartment (20), and it can also be detected in the Golgi and plasma membrane (32, 33). We tested whether this P450, which is competent for transport beyond the ER, can also form oligomers in transfected cells. Full-length chimeras of 2E1 tagged at the C terminus with CFP and YFP were expressed in COS1 cells, and whole cells were analyzed by scanning spectrophotometry. Little or no increase in fluorescence was observed at 525 nm when both of the CFP and YFP forms were expressed (Fig. 5A). Consistent with this observation, analysis by the confocal microscopy method also did not detect net FRET in the ratio image (Fig. 5B). Finally, photobleaching of 2E1/YFP did not increase fluorescence of P450 2E1/CFP (not shown). The absence of FRET in cells co-expressing 2E1/CFP and 2E1/YFP is also demonstrated by stronger CFP fluorescence...
cence in these cells than in cells expressing a pair of P450 2C2 chimeras, which indicates a lack of CFP quenching by the YFP chimera.

We next tested whether mixed heterologous complexes between P450s 2C2 and 2E1 are formed in transfected cells, but no FRET was detected in cells co-expressing fluorescent chimeras of 2C2 and 2E1 (Fig. 5B). When the pair of chimeras containing only the N-terminal SA of 2C2, P450 2C1-(1–28)/CFP, and of 2E1, P450 2E1-(1–31)/YFP, were assayed, only weak FRET was observed (not shown). These data suggest that P450 2E1 does not efficiently form homo-oligomers nor is there formation of mixed hetero-oligomers between either full-length 2C2 and 2E1 or chimeras containing only their N-terminal SAs.

Detection of Interaction between P450 and P450 Reductase Using FRET Assay—Although there are multiple forms of P450 in the ER membrane, there is only one P450 reductase, which must interact with all the P450s. P450 2C2 and P450 2E1 differ in their efficiency of homo-oligomerization, which might affect the efficiency of the interaction of each with P450 reductase. To examine the interaction of the P450s with P450 reductase by FRET analysis, we co-transfected COS1 cells with either 2C2/CFP or 2E1/CFP and P450 reductase tagged at the C terminus with YFP (reductase/YFP). The scanning spectrofluorimetry assay suggested that an interaction between either P450 and the reductase was taking place, as indicated by the increase in fluorescence at 525 nm when either 2C2/CFP or 2E1/CFP was expressed with reductase/YFP (Fig. 6). Similar results were observed for OEC (Fig. 6). Consistent with these results, after photobleaching of the P450 reductase YFP donor, fluorescence of CFP was increased by full-length P450 2C2, full-length P450 2E1, and the cytoplasmic domain of P450 2C2 (Fig. 7A).

These experiments demonstrate that both P450 2C2 and P450 2E1 form complexes with P450 reductase when expressed at similar levels (based on CFP fluorescence). Although the cytoplasmic domain chimera (OEC) did not form homo-oligomers efficiently, it did form complexes with P450 reductase (Fig. 7). We could not determine whether the SA sequence of P450 2C2 contributes to the interaction with P450 reductase directly because the differences in sizes of the two proteins, P450 2C1-(1–28) compared with the full-length reductase, probably would prevent FRET even if the two chimeras interacted, because the distance between chromophores would be too great. For example, full-length P450 2C2 did not exhibit significant FRET with P450 2C1-(1–28) (data not shown) even
though it is highly likely that interaction between these two chimeras occurs.

**DISCUSSION**

The present work demonstrates the feasibility and advantages of fluorescent techniques for studies of the organization of different P450s in the membranes of the endoplasmic reticulum of living cells. Unlike reconstituted systems, this method allows for visualization and analysis of protein-protein interactions in a natural membrane environment. Our results show that different P450s expressed in COS1 cells have different homo-oligomerization affinities resulting in different quaternary structures. Analysis by FRET indicated that P450 2C2 formed oligomeric complexes, whereas oligomerization of P450 2E1 was not detected. In contrast, based on gel filtration, isolated P450 2E1 was estimated to be in 10-mer complexes (34). Isolated P450 2B4 (34) and P450 2C3 (35) have also been shown to aggregate into complexes of hexamers and greater than octamers, respectively. Clearly, the aggregation of an isolated P450 in vitro does not correlate with the propensity to form homo-oligomers in the membrane of a living cell.

The oligomerization of P450 2C2 in the cell was mediated by the N-terminal transmembrane peptide. Chimeras containing only the N-terminal sequence interacted strongly, but oligomerization of the cytosolic domain (chimera OEC) was not detected. Although it could be argued that oligomerization of OEC in fact takes place but cannot be detected with FRET as a result of an unfavorable orientation of fluorescent tags, we consider it very unlikely. Because the enzymatic activity of OEC/GFP is very similar to that of the full-length P450 2C2/GFP (18), both enzymes must interact functionally with P450 reductase, and in fact interaction of OEC/CFP with reductase/YFP can be demonstrated by FRET. Thus, the heterologous transmembrane domain does not significantly affect folding of the P450s nor their membrane topology. The decreased FRET signal most likely results from decreased oligomerization. This result differs from the in vitro studies referenced above on P450 2E1, P450 2B4, and P450 2C3 in that deletion of the N-terminal hydrophobic region did not eliminate aggregation of isolated proteins in the absence of detergent. However, these isolated proteins, missing the N-terminal hydrophobic sequence, were disaggregated at much lower concentrations of detergent than the wild type proteins indicating a significant role for the N-terminal region in aggregation in vitro. The N-terminal sequence thus plays a central role in aggregation of the P450s both in the cell and in vitro.

The formation of large complexes has been proposed to explain the direct retention of membrane proteins in the ER. However, we have previously established by FRAP analysis that P450 2C2/GFP has high lateral mobility in the ER membranes of transfected COS1 cells and that OEC/GFP has similar, but slightly lower, mobility (18). The aggregation of P450 2C2 observed in the present study, therefore, does not decrease its mobility, as detected by FRAP. These results suggest that parameters other than the state of oligomerization primarily determine lateral mobility in the membranes, consistent with the proposal that there is only a weak correlation between the size and the membrane diffusion coefficient of a protein (36).

Unlike P450 2C2, P450 2E1 is not directly retained in the ER but is transported to the intermediate compartment and then retrieved to the ER (19), and unlike P450 2C2, P450 2E1 does not appear to form homo-oligomers. This raised the possibility that oligomerization was critical for direct retention in the ER. However, this possibility is not supported by several other observations. First, chimera OEC is directly retained in the ER despite its monomeric structure; second, the chimera with N-terminal amino acids 1–21 is not directly retained in the ER, although it oligomerizes; and third, the 2C2 SA chimera mutant 6L, which can be transported from the ER to the cell
surface, shows weaker, but detectable, FRET in the Golgi region consistent with oligomer formation. The self-association in intracellular Golgi-like structures is consistent with the competency of oligomeric forms of SA mutant 6L for transport from the ER. Therefore, these observations indicate that the different mechanisms of ER retention for P450 2C2 and 2E1 most likely are not related to their different quaternary structures.

The different mechanisms of ER retention for P450 2C2 and P450 2E1 could be the result of targeting to different domains in the ER so that C2 is excluded from regions of transport vesicle formation and 2E1 is not. Consistent with this idea, no heteromeric complexes were observed with either full-length or N-terminal SA chimeras of P450 2C2 and 2E1. However, the possibility that P450 2C2 and P450 2E1 are incompatible for heteromer formation cannot be eliminated by these studies. High specificity in the formation of multimeric structures composed from different classes of P450s has been demonstrated (13, 15, 37). Additional studies will be required to determine whether the lack of FRET between P450 2C2 and P450 2E1 is due to this incompatibility for heterocomplex formation or to targeting to separate subcompartments of the ER.

The interactions of both P450 2C2 and P450 2E1 with P450 reductase were detected by FRET. In contrast to self-aggregation, the cytoplasmic domain of C2 could mediate an interaction with P450 reductase. This result is consistent with in vitro studies showing that P450 reductase binding to P450s is mediated by the cystolic domain (38–40). The decreased efficiency of FRET for OEC compared with full-length P450 2C2 suggests that the SA may also contribute to the interaction. In this regard, differences in the SA of P450s have been shown to affect the affinity to P450 reductase (41).

We have shown previously that the chimera OEC/GFP has enzymatic activity similar to that of wild type P450 2C2 (18), yet it does not oligomerize when expressed in COS1 cells, which indicates that oligomerization is not a prerequisite for enzymatic activity. This would imply that interactions with the substrate and the electron donor, P450 reductase, are not dependent on oligomerization. However, the possibility that P450 2C2 and P450 2E1 all interact with P450 reductase but differ in their state of homo-oligomerization. Studies of rotational mobilities of P450 indicate that P450 oligomers may dissociate when P450 binds to the reductase (3, 12), which might explain the lack of correlation of oligomerization with the binding to reductase.

The functional significance of homo-oligomerization of P450s and hetero-oligomerization has not been established. Heteromers could be important for the flux of metabolites for substrates that can undergo multiple modifications by different P450s in which case the presence of two P450s in the same complex, possibly including P450 reductase, would facilitate

**Fig. 7.** Detection of FRET between the reductase and P450s 2C2 and 2E1 by acceptor photobleaching in transfected cells. COS1 cells were co-transfected with expression vectors for reductase/YFP and for either P450 C2/CFP, 2E1/CFP, or OEC/CFP. A, images of the cells were taken using the YFP and CFP channels, before and after photobleaching of the acceptor YFP with the 514 nm laser light for 2 min. The pseudocolored images for the CFP before and after bleaching are also shown. Bar, 20 μm. B, ratios of the fluorescence emissions before and after bleaching, calculated as described in the legend to Fig. 4, are shown.
the multiple catalyses. The specificity of the interaction of P450s with P450 reductase and effects of substrates on the interaction in natural membranes is an important question that has not been addressed. Our work demonstrates the great potential utility of FRET analysis in studies on the molecular interactions of multiple P450s and P450 reductase.

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