Single Molecule Activity Measurements of Cytochrome P450 Oxidoreductase Reveal the Existence of Two Discrete Functional States

ARTICLE *in* ACS CHEMICAL BIOLOGY · DECEMBER 2013

Impact Factor: 5.33 · DOI: 10.1021/cb400708v · Source: PubMed

9 AUTHORS, INCLUDING:

Natalie O'Donnell
Purdue University
11 PUBLICATIONS **67** CITATIONS
SEE PROFILE

Jonas Borch
University of Southern Denmark
33 PUBLICATIONS **1,079** CITATIONS
SEE PROFILE

Dimitrios Stamou
University of Copenhagen
91 PUBLICATIONS **2,274** CITATIONS
SEE PROFILE

Birger Møller
University of Copenhagen
258 PUBLICATIONS **7,876** CITATIONS
SEE PROFILE

ACS chemical biology

Letters

pubs.acs.org/acschemicalbiology

Single Molecule Activity Measurements of Cytochrome P450 Oxidoreductase Reveal the Existence of Two Discrete Functional States

Tomas Laursen,[†,¶,Δ] Aparajita Singha,[‡,¶,Δ] Nicolai Rantzau,[‡,¶] Marijonas Tutkus,[‡,¶] Jonas Borch,[§] Per Hedegård,[⊥] Dimitrios Stamou,[‡,¶] Birger Lindberg Møller,*[,†,¶] and Nikos S. Hatzakis*[,‡,¶]

[†]Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsenvej 40, DK-1871 Frederiksberg C, Denmark

[‡]Bio-Nanotechnology Laboratory, Department of Chemistry, Nano-Science Center, Lundbeck Foundation Center Biomembranes in Nanomedicine, University of Copenhagen, 2100 Copenhagen, Denmark

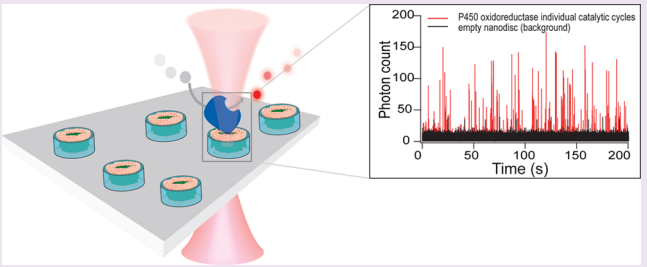
[§]Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK−5230 Odense M, Denmark

[¶]bioSYNergy, Center for Synthetic Biology, University of Copenhagen, Denmark

[⊥]Nano-Science Center, Niels Bohr Institute, University of Copenhagen, Denmark

Supporting Information

ABSTRACT: Electron transfer between membrane spanning oxidoreductase enzymes controls vital metabolic processes. Here we studied for the first time with single molecule resolution the function of P450 oxidoreductase (POR), the canonical membrane spanning activator of all microsomal cytochrome P450 enzymes. Measurements and statistical analysis of individual catalytic turnover cycles shows POR to sample at least two major functional states. This phenotype may underlie regulatory interactions with different cytochromes P450 but to date has remained masked in bulk kinetics. To ensure that we measured the inherent behavior of POR, we reconstituted the full length POR in "native like" membrane patches, nanodiscs. Nanodisc reconstitution increased stability by ∼2-fold as compared to detergent solubilized POR and showed significantly increased activity at biologically relevant ionic strength conditions, highlighting the importance of studying POR function in a membrane environment. This assay paves the way for studying the function of additional membrane spanning oxidoreductases with single molecule resolution.



Electron transfer between membrane spanning oxidoreductases controls an immense number of vital cellular processes in nature ranging from energy conversion of photosynthesis and respiration to metabolism. Improved knowledge of these processes is crucial for advances in medicine and biotechnology but to date is limited by conventional bulk kinetics of the enzymes involved. Single molecule activity measurements on water-soluble enzymes on the other hand have revealed the existence of a number of interconverting functional states underlying their function and regulation that were masked in bulk kinetics.[1−4] To date however there is no assay for single molecule functional characterization of membrane appended oxidoreductases.

An important member of the oxidoreductase super family is the 78 kDa single transmembrane helix spanning NADPH-dependent cytochrome P450 oxidoreductase (POR). It is present in animals and plants and thus involved in, e.g., steroid hormone biosynthesis, xenobiotic drug catabolism in animals,[5] and terpenoid and flavonoid synthesis in plants.[6,7] Single point mutations in human *POR* cause disordered steroidogenesis and skeletal dysplasia.[8−11] POR is the omnipresent donor of electrons to all microsomal (>50) type II cytochrome P450 (CYP) enzymes.[12,13] POR contains an FAD and an FMN domain connected via a flexible hinge region; the domain motions are anticipated to be important for catalysis.[14] Within each catalytic cycle POR is proposed to undergo sequential transition from the "extended" (FAD reduction by NADPH) to a "compact" conformation (electron transfer from FAD to FMN) and back to the extended (electron transfer from FMN to CYP). The specificity of activating each of the CYP partners is proposed to originate from the existence of multiple states in the free energy landscape of POR with different oscillating frequencies and activity rates.[11,15] The direct observation however of such functional states, their lifetime and activity, is averaged out in current bulk measurements and can only be measured by single molecules studies.

Received: September 14, 2013
Accepted: December 23, 2013

   dx.doi.org/10.1021/cb400708v | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

Current POR functional characterization primarily relies on indirect bulk kinetics measuring the amount of reduced water-soluble non-native protein electron recipients such as cytochrome *c* (Cyt C) for enzymes solubilized in detergents. Electron relaying capacity depends on the employed recipient as observed for each of the pathogenic mutants.[8,11,16] These differences may originate from distorted electrostatic interaction between POR and the protein electron recipients.[17] The behavior of POR and almost any other transmembrane protein is often considered identical for measurements in solubilized (by detergents), truncated (transmembrane domain cleaved), and native (membrane appended) constructs. Nevertheless both activity[18] and conformational equilibrium of POR is critically affected by reconstitution medium.[14,15,19,20] Therefore, to understand the native POR function it is essential to perform measurements in membranes.

Here we present the first single molecule functional measurements on POR oxidoreductase. Reconstituting in membrane patches, nanodiscs (NDs), allowed monitoring activity of the full length POR in "native-like" conditions. We initially evaluated the critical role of membrane POR stability, activity, and optimum ionic strength. We then followed for the first time the activity of full length POR with single turnover resolution. Our findings suggest POR to sample two major functional states that may provide means for its efficient regulation and selectivity of downstream partner activation.[3]

To study the function of full-length POR, we employed NDs,[21] nanoscale biomimetic lipid bilayer discs encircled by two membrane scaffold proteins (MSPs) (Figure 1A).[20−22] We

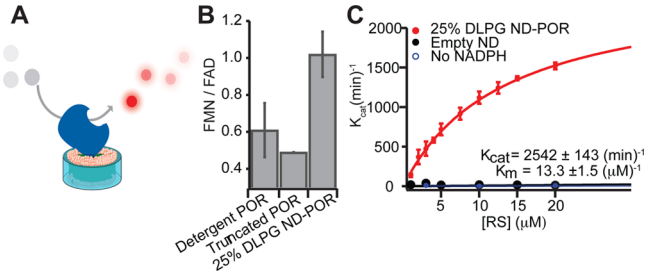


Figure 1. A novel functional assay for cytochrome P450 oxidoreductase (POR). (A) POR is reconstituted in "native-like" membranes in the form of nano discs (ND), and its activity is monitored using the prefluorescent substrate resazurin, which upon reduction generates the highly fluorescent product resorufin. (B) Bar plots showing the FMN:FAD ratio in detergent solubilized, truncated, or ND reconstituted POR. Error bars are the SD of 11, 3, and 14 experiments, respectively. (C) Resazurin reduction by POR follows Michaelis−Menten kinetics. Control experiments using empty ND or in the absence of NADPH shows no activity. Error bars are the SD from 3 independent experiments.

used a mixture of DLPC and 25 mol % negatively charged DLPG lipids to achieve physiologically relevant conditions.[23] To eliminate the release of POR from NDs reconstituted with the MSP1 as recently reported,[20] we used the extended MSP1E3 forming NDs with diameters of 13 nm (see Supplementary Figure S1; see Supporting Information for POR and NDs dimensions).[24] We initially compared detergent solubilized, membrane truncated, and ND reconstituted POR by quantifying the loss of flavin mononucleotide (FMN) cofactor associated with POR inactivation.[25] Flavin content was determined on the basis of the differential fluorescence of FMN and FAD (see Supporting Information). Upon ND reconstitution, POR preserved a 1:1 ratio of FMN:FAD following incubation with free FMN in solution (Figure 1B and Supporting Information). In contrast, solubilized and truncated POR only bound 0.6:1 and 0.5:1 FMN:FAD, respectively (Figure 1B), equivalent to an up to ∼2-fold loss in catalytically active enzyme. This documents that membrane reconstitution is crucial for maintaining the structural integrity of POR.

To enable monitoring the activity of POR with sensitive fluorescence detection, resazurin was introduced as a small molecule electron acceptor that is primarily noncharged (Figure 1A). Upon reduction, resazurin is converted into the bright fluorescent product resorufin (see Supplementary Figure S2). The reaction followed first-order Michaelis−Menten kinetics with a K_m of 13.3 μM and K_{cat} of 2542 molecules min^{-1} (Figure 1C) comparable to previously published Cyt C reduction rates.[26] Empty NDs did not reduce resazurin, validating that the reaction was POR-dependent (Figure 1C).

Ionic strength affects conformational gating and thus the function of POR.[19,26] Reduction of resazurin followed the expected non-monotonous dependence on salt concentration,[27] with an optimum at 316 mM salt (Figure 2A). In contrast, the

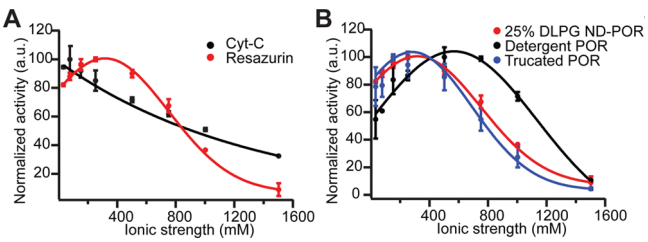


Figure 2. POR functional readout is critically dependent on the choice of substrate and reconstitution medium. (A) The small substrate molecule resazurin is able to report the presence of an optimum ionic strength for POR in NDs. This property is not revealed using Cyt C as electron acceptor. (B) Effect of ionic strength on POR activity of reducing resazurin using detergent solubilized, truncated, or ND reconstituted POR. Error bars are SD from 3 independent experiments.

Cyt C reduction assay did not identify an optimum but displayed a continuous decay of K_{cat} as ionic strength increased (Figure 2A). This indicates that the widely employed Cyt C reduction assay probes the electrostatic interaction of Cyt C with the membrane rather than the inherent electron relaying capacity of POR in agreement with earlier calculations.[28] To examine the effect of solubilization medium on POR behavior, we compared the function of solubilized, truncated and ND reconstituted POR at varying ionic strength conditions. All three POR preparations displayed a nonmonotonous activity dependence on ionic strength (Figure 2B). Incrementing salt concentrations resulted in all cases in increasing the reaction rate followed by an optimum and decay at high ionic strengths. Both ND-POR and truncated POR had similar ionic strength optimum K_{cat} at ∼300 mM salt, whereas the optimum for detergent solubilized POR was shifted to ∼565 mM salt, an unphysiologically high ionic strength (Figure 2B), further demonstrating the deleterious effect of detergent solubilization on POR function.

The design of the POR assay system enabled monitoring the function of individual POR enzymes with single molecule resolution, the proof of principle of which is shown here. To image and monitor enzymatic turnovers from individual molecules of the POR enzyme, a cysteine mutant POR variant

dx.doi.org/10.1021/cb400708v | *ACS Chem. Biol.* XXXX, XXX, XXX−XXX

(cysPOR) was prepared and conjugated to the fluorescent label Cy5 label (see Supplementary Figure S3) before ND reconstitution. NDs fluorescently labeled with DiO were tethered on a passivated glass surface at low densities. Imaging with confocal microscopy enabled the identifications of POR loaded ones (Figure 3A) (see Supporting Information).[3,20,29−31] NDs both provided a "native-like" membrane environment for POR and furthermore minimized possible nonspecific interactions of POR with glass that may alter its behavior.[32,33] The low POR to ND ratio (7.76%) made it statistically unlikely ($P < 1\%$) for a ND to harbor a second POR as confirmed by the single step bleaching behavior of Cy5 (Figure 3B and Supplementary Figure S4). Each single enzymatic POR turnover produced a burst of fluorescent photons before the resorufin molecule diffused away from the confocal volume, providing a trajectory of stochastic single turnover events.[1−3,34] In Figure 3C we depict a typical trajectory of events out of the ∼15 examined. Positioning the beam on empty NDs verified that the reaction was POR mediated and allowed the independent measurement of the background signal (Figure 3C). Earlier single reductase studies used changes in the fluorescent properties of cofactors to assay activity, curtailing the length of the trajectories to a few seconds due to cofactor photobleaching.[4,33,35] Here, a new substrate is probed for each turnover cycle, and the length of the trajectories have no limitation except substrate depletion.

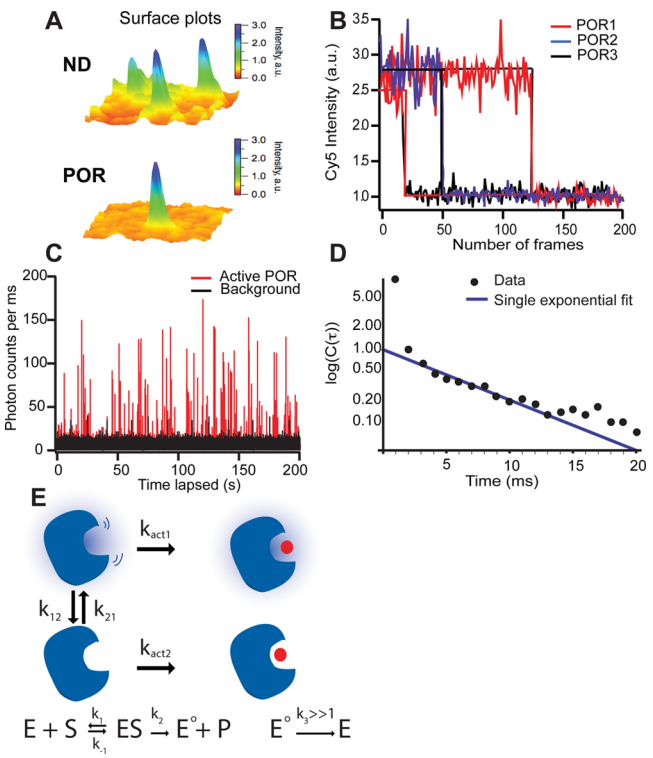


Figure 3. Single turnover measurements of POR. (A) Representative three-dimensional plots of fluorescence intensity from DiO labeled ND (top) and Cy5 labeled POR (bottom). (B) Single step bleaching of Cy5 labeled POR verifies the single POR reconstitution into NDs in our conditions (see Supporting Information). (C) Representative time trace of resorufin formation catalyzed by a single POR molecule reconstituted into a ND (red). Each spike corresponds to photon counts from a single resorufin molecule formed. Control experiment on empty ND shows background photon counts (black). (D) The intensity autocorrelation for POR decays monoexponentially, indicating the existence of two major functional states. (E) Cartoon representation of two-state model describing POR behavior. Each state may have different oscillating frequencies and specificities for each downstream CYP enzyme, providing cues for their efficient activation.

To reliably extract information from the stochastic trajectory of events in Figure 3C, the enzyme contribution to the intensity autocorrelation $C_s(\tau)$[3,4] was initially investigated. $C_s(\tau)$ is independent of the applied threshold, which may cause artifactual results for noisy data[36] (see Supporting Information and Supplementary Figure S5). We found $C(\tau)$ to decay monoexponentialy (Figure 3D), consistent with the presence of two discrete functional states.[1,3] Similarly the waiting time distribution follows a double exponential decay supporting the existence of two functional states (see Supporting Information and Supplementary Figure S5).

We propose here that POR samples two major functional states (see Figure 3E). Each of these functional states may correspond to different oscilating frequencies.[14,37] While POR enzyme resides in one functional state it may perform a catalysis cycle with rate k_{act1}. After product formation the enzyme rapidly recovers to its original state where it can either catalyze a second cycle with the same activity or interconvert to the other functional state with different activity k_{act2} (Figure 3E).

Our single molecule data allowed us to characterize both the lifetimes and the activity of each of the two POR discrete functional states. The exponent of $C_s(\tau)$ (0.16 ± 0.04 ms^{-1}) of the trace in Figure 3C equals the sum of the interconversion rates showing lifetimes in the low millisecond time scale, as anticipated.[19] Statistical analysis of the waiting times showed the activity of the two states to differ by a factor of >20 (k_{act1} = 0.21 ms^{-1} and k_{act2} < 0.011 ms^{-1}, respectively; see Figure 3E and Supporting Information). Similarly the interconversion rates between the two functional states were k_{21} = 0.16 ms^{-1} and k_{12} = 0.002 ms^{-1}. Under our experimental conditions we thus found POR to spend 5% of its time in the highly active state. The average turnover rate was 21 s^{-1}, in agreement with our bulk measurements (∼26 s^{-1}) (Figure 1C) and earlier reports.[38] Our data are consistent with bulk studies proposing POR to sample multiple states anticipated to have different activities.[19] Each of these states could have different specificities for each downstream CYP partners providing cues for their selective activation.[15] However, before this study the existence of these functional states remained masked in bulk kinetics due to ensemble averaging.

The comparative studies presented here demonstrate that reliable interpretation of POR functional readouts, e.g., in the analysis of pathogenic POR mutants,[8,11,39] requires the incorporation of POR in a "native-like" membrane systems as well as use of a proper electron acceptor.

Our single turnover resolution experiments revealed the existence and quantified the activity and lifetime of two major functional states for POR. It would be exciting to investigate how regulatory cofactors alter the free energy landscape and the characteristics of these states and POR specific activation of CYPs. Employing "native-like" membrane systems, nanodiscs[20] or liposomes,[3] would be ideal for performing these single-molecule measurements due to the convenient control of chemical environment. We thus anticipate this assay to be generically applied for understanding the parameters underlying function and regulation[40] of multiple membrane appended oxidoreductases.[13]

dx.doi.org/10.1021/cb400708v | ACS Chem. Biol. XXXX, XXX, XXX−XXX

■ ASSOCIATED CONTENT

Supporting Information

Protein purification and nanodisc preparation protocols, activity assay conditions and single molecule setup and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: blm@life.ku.dk.
*E-mail: hatzakis@nano.ku.dk.

Author Contributions

[Δ]These authors contributed equally to this work and are listed alphabetically.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the UNIK research initiative of the Danish Ministry of Science, Technology and Innovation through the "Center for Synthetic Biology" at the University of Copenhagen, by the VILLUM Center of Excellence "Plant Plasticity", and the Lundbeck foundation center of excellence "Biomembranes in nanomedicine".

■ REFERENCES

(1) English, B. P., Min, W., van Oijen, A. M., Lee, K. T., Luo, G., Sun, H., Cherayil, B. J., Kou, S. C., and Xie, X. S. (2006) Ever-fluctuating single enzyme molecules: Michaelis-Menten equation revisited. *Nat. Chem. Biol. 2*, 87−94.

(2) Hatzakis, N. S., Engelkamp, H., Velonia, K., Hofkens, J., Christianen, P. C., Svendsen, A., Patkar, S. A., Vind, J., Maan, J. C., Rowan, A. E., and Nolte, R. J. (2006) Synthesis and single enzyme activity of a clicked lipase-BSA hetero-dimer. *Chem. Commun. (Cambridge, U. K.)*, 2012−2014.

(3) Hatzakis, N. S., Wei, L., Jorgensen, S. K., Kunding, A. H., Bolinger, P. Y., Ehrlich, N., Makarov, I., Skjot, M., Svendsen, A., Hedegard, P., and Stamou, D. (2012) Single enzyme studies reveal the existence of discrete functional states for monomeric enzymes and how they are "selected" upon allosteric regulation. *J. Am. Chem. Soc. 134*, 9296−9302.

(4) Kuznetsova, S., Zauner, G., Aartsma, T. J., Engelkamp, H., Hatzakis, N., Rowan, A. E., Nolte, R. J., Christianen, P. C., and Canters, G. W. (2008) The enzyme mechanism of nitrite reductase studied at single-molecule level. *Proc. Natl. Acad. Sci. U.S.A. 105*, 3250−3255.

(5) Guengerich, F. P. (2005) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) 3rd ed., pp 377−530, Plenum, New York.

(6) Hall, D. E., Zerbe, P., Jancsik, S., Quesada, A. L., Dullat, H., Madilao, L. L., Yuen, M., and Bohlmann, J. (2013) Evolution of conifer diterpene synthases: diterpene resin acid biosynthesis in lodgepole pine and jack pine involves monofunctional and bifunctional diterpene synthases. *Plant Phys. 161*, 600−616.

(7) Niehaus, T. D., Okada, S., Devarenne, T. P., Watt, D. S., Sviripa, V., and Chappell, J. (2011) Identification of unique mechanisms for triterpene biosynthesis in Botryococcus braunii. *Proc. Natl. Acad. Sci. U.S.A. 108*, 12260−12265.

(8) Fluck, C. E., Tajima, T., Pandey, A. V., Arlt, W., Okuhara, K., Verge, C. F., Jabs, E. W., Mendonca, B. B., Fujieda, K., and Miller, W. L. (2004) Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. *Nat. Genet. 36*, 228−230.

(9) Huang, N., Pandey, A. V., Agrawal, V., Reardon, W., Lapunzina, P. D., Mowat, D., Jabs, E. W., Van Vliet, G., Sack, J., Fluck, C. E., and Miller, W. L. (2005) Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. *Am. J. Hum. Genet. 76*, 729−749.

(10) Shen, A. L., O'Leary, K. A., and Kasper, C. B. (2002) Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J. Biol. Chem. 277*, 6536−6541.

(11) Xia, C., Panda, S. P., Marohnic, C. C., Martasek, P., Masters, B. S., and Kim, J. J. (2011) Structural basis for human NADPH-cytochrome P450 oxidoreductase deficiency. *Proc. Natl. Acad. Sci. U.S.A. 108*, 13486−13491.

(12) Hannemann, F., Bichet, A., Ewen, K. M., and Bernhardt, R. (2007) Cytochrome P450 systems–biological variations of electron transport chains. *Biochim. Biophys. Acta 1770*, 330−344.

(13) Murataliev, M. B., Feyereisen, R., and Walker, F. A. (2004) Electron transfer by diflavin reductases. *Biochim. Biophys. Acta 1698*, 1−26.

(14) Ellis, J., Gutierrez, A., Barsukov, I. L., Huang, W. C., Grossmann, J. G., and Roberts, G. C. (2009) Domain motion in cytochrome P450 reductase: conformational equilibria revealed by NMR and small-angle x-ray scattering. *J. Biol. Chem. 284*, 36628−36637.

(15) Laursen, T., Jensen, K., and Moller, B. L. (2011) Conformational changes of the NADPH-dependent cytochrome P450 reductase in the course of electron transfer to cytochromes P450. *Biochim. Biophys. Acta 1814*, 132−138.

(16) Pandey, A. V., and Fluck, C. E. (2013) NADPH P450 oxidoreductase: structure, function, and pathology of diseases. *Pharmacol. Ther. 138*, 229−254.

(17) Tamburini, P. P., and Schenkman, J. B. (1986) Differences in the mechanism of functional interaction between NADPH-cytochrome P-450 reductase and its redox partners. *Mol. Pharmacol. 30*, 178−185.

(18) Vermilion, J. L., and Coon, M. J. (1978) Purified liver microsomal NADPH-cytochrome P-450 reductase. Spectral characterization of oxidation-reduction states. *J. Biol. Chem. 253*, 2694−2704.

(19) Hay, S., Brenner, S., Khara, B., Quinn, A. M., Rigby, S. E., and Scrutton, N. S. (2010) Nature of the energy landscape for gated electron transfer in a dynamic redox protein. *J. Am. Chem. Soc. 132*, 9738−9745.

(20) Wadsater, M., Laursen, T., Singha, A., Hatzakis, N. S., Stamou, D., Barker, R., Mortensen, K., Feidenhans'l, R., Moller, B. L., and Cardenas, M. (2012) Monitoring shifts in the conformation equilibrium of the membrane protein cytochrome P450 reductase (POR) in nanodiscs. *J. Biol. Chem. 287*, 34596−34603.

(21) Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2002) Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. *Nano Lett. 2*, 853−856.

(22) Bayburt, T. H., Carlson, J. W., and Sligar, S. G. (1998) Reconstitution and imaging of a membrane protein in a nanometer-size phospholipid bilayer. *J. Struct. Biol. 123*, 37−44.

(23) Musgrave, A., Walters, J., Sargent, J. A., and Powell, G. (1976) Microsomal membrane lipids from pea shoots and Ranunculus sceleratus petioles. *Phytochemistry 15*, 1219−1223.

(24) Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., and Sligar, S. G. (2004) Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled size. *J. Am. Chem. Soc. 126*, 3477−3487.

(25) Marohnic, C. C., Panda, S. P., McCammon, K., Rueff, J., Masters, B. S., and Kranendonk, M. (2010) Human cytochrome P450 oxidoreductase deficiency caused by the Y181D mutation: molecular consequences and rescue of defect. *Drug Metab. Dispos. 38*, 332−340.

(26) Lamb, D. C., Warrilow, A. G., Venkateswarlu, K., Kelly, D. E., and Kelly, S. L. (2001) Activities and kinetic mechanisms of native and soluble NADPH-cytochrome P450 reductase. *Biochem. Biophys. Res. Commun. 286*, 48−54.

(27) Sem, D. S., and Kasper, C. B. (1995) Effect of ionic strength on the kinetic mechanism and relative rate limitation of steps in the model NADPH-cytochrome P450 oxidoreductase reaction with cytochrome *c*. *Biochemistry 34*, 12768−12774.

(28) Spaar, A., Flock, D., and Helms, V. (2009) Association of cytochrome c with membrane-bound cytochrome c oxidase proceeds parallel to the membrane rather than in bulk solution. *Biophys. J. 96*, 1721−1732.
(29) Larsen, J., Hatzakis, N. S., and Stamou, D. (2011) Observation of inhomogeneity in the lipid composition of individual nanoscale liposomes. *J. Am. Chem. Soc. 133*, 10685−10687.
(30) Hatzakis, N. S., Bhatia, V. K., Larsen, J., Madsen, K. L., Bolinger, P. Y., Kunding, A. H., Castillo, J., Gether, U., Hedegard, P., and Stamou, D. (2009) How curved membranes recruit amphipathic helices and protein anchoring motifs. *Nat. Chem. Biol. 5*, 835−841.
(31) Christensen, S. M., Bolinger, P. Y., Hatzakis, N. S., Mortensen, M. W., and Stamou, D. (2012) Mixing subattolitre volumes in a quantitative and highly parallel manner with soft matter nanofluidics. *Nat. Nanotechnol. 7*, 51−55.
(32) Cisse, I., Okumus, B., Joo, C., and Ha, T. (2007) Fueling protein DNA interactions inside porous nanocontainers. *Proc. Natl. Acad. Sci. U.S.A. 104*, 12646−12650.
(33) Goldsmith, R. H., Tabares, L. C., Kostrz, D., Dennison, C., Aartsma, T. J., Canters, G. W., and Moerner, W. E. (2011) Redox cycling and kinetic analysis of single molecules of solution-phase nitrite reductase. *Proc. Natl. Acad. Sci. U.S.A. 108*, 17269−17274.
(34) De Cremer, G., Roeffaers, M. B. J., Baruah, M., Sliwa, M., Sels, B. F., Hofkens, J., and De Vos, D. E. (2007) Dynamic disorder and stepwise deactivation in a chymotrypsin catalyzed hydrolysis reaction. *J. Am. Chem. Soc. 129*, 15458−+.
(35) Lu, H. P., Xun, L., and Xie, X. S. (1998) Single-molecule enzymatic dynamics. *Science 282*, 1877−1882.
(36) Terentyeva, T. G., Engelkamp, H., Rowan, A. E., Komatsuzaki, T., Hofkens, J., Li, C. B., and Blank, K. (2012) Dynamic disorder in single-enzyme experiments: facts and artifacts. *ACS Nano 6*, 346−354.
(37) Pudney, C. R., Khara, B., Johannissen, L. O., and Scrutton, N. S. (2011) Coupled motions direct electrons along human microsomal P450 Chains. *PLoS Biol. 9*, e1001222.
(38) Brenner, S., Hay, S., Munro, A. W., and Scrutton, N. S. (2008) Inter-flavin electron transfer in cytochrome P450 reductase - effects of solvent and pH identify hidden complexity in mechanism. *FEBS J. 275*, 4540−4557.
(39) Huang, N., Agrawal, V., Giacomini, K. M., and Miller, W. L. (2008) Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. *Proc. Natl. Acad. Sci. U.S.A. 105*, 1733−1738.
(40) Hatzakis, N. S. (2013) Single molecule insights in conformational selection and induced fit mechanism. *Biophys. Chem.*, DOI: 10.1016/j.bpc.2013.11.003.