with the phenyl spacer in these molecules does not lead to anomalously strong D–A coupling. However, a detailed evaluation of donor–spacer–acceptor coupling must await more detailed studies. We note that analogous studies by Beyerle[12d] of phenyl linked D–A compounds suggest coupling occurs primarily through the σ framework. However, the compounds studied by Beyerle are significantly more flexible than those presented here.

Finally, we note that the diporphyrin model reacts *many* times faster (ca. 10^4) than a similar protein–protein system at similar ΔG (e.g., Zn cyt c/Fe^{III} cyt b_5 R ≅ 8 Å, $k_{max} \cong 10^6\ s^{-1}$).[16] This result minimally suggests that the protein matrix does not accelerate the electron-transfer rate.

Acknowledgment. This work was supported by grants from the National Science Foundation (CHE8303896 and PCM83-02601) and in part by the sponsors of the Laser Fusion Feasibility Project at the University of Rochester. We also acknowledge the experimental assistance of Professor Tom Nordlund and helpful discussions with Drs. Mike Wasielewski, James Collman, and C. K. Chang.

(16) Roughly 1 order of magnitude of this difference can easily be attributed to distance (8 vs. 6 Å) $k(6\ Å)/k(8\ Å) \cong \exp^{-1.2(2)}$.

Flavohemoglobin: A Semisynthetic Hydroxylase Acting in the Absence of Reductase

Toshio Kokubo,[1] Shigeru Sassa,[2] and E. T. Kaiser*[1]

Laboratory of Bioorganic Chemistry and Biochemistry
Laboratory of Metabolism and Pharmacology
The Rockefeller University, New York, New York 10021

Received August 15, 1986

Heme proteins involved in redox reactions, such as cytochrome P-450,[3] function only by single electron transfers in each step and require an electron transport system to work with the usual biological electron donor, e.g., NADPH, which acts as a two-electron donor. From the viewpoint of practical applications alteration of the heme proteins, allowing them to react directly with two-electron donors without the intervention of the electron-transport system, would be a most challenging target in the protein engineering field.

Hemoglobin is known to catalyze a wide variety of monooxygenase reactions[4] in a typical P-450-like electron-transport system consisting of NADPH, O_2, and NADPH-cytochrome P-450 reductase (EC 1.6.2.4), a major flavoprotein in the microsomal electron-transport system.[5] Therefore, we started with hemoglobin as a representative heme protein and undertook to replace the reductase in the electron-transport system by an isoalloxazine (flavin) moiety covalently attached to hemoglobin in the vicinity of the heme. We report here that the resulting molecular conjugate, flavohemoglobin (Fl-Hb^{3+}), served as a hydroxylase for aniline without requiring the P-450 reductase.

One free cysteine residue titratable with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) exists in the β-chain of hemoglobin, i.e., two exposed cysteine residues in the whole hemoglobin molecule.[6] The cysteine (β-93) is located adjacent to the histidine (β-92) which is coordinated to the heme iron. A flavin reagent designed to modify this thiol residue under mild conditions was derived from the parent 7-cyanoisoalloxazine (Scheme I), which is more reactive toward NADPH than flavin coenzymes.[7]

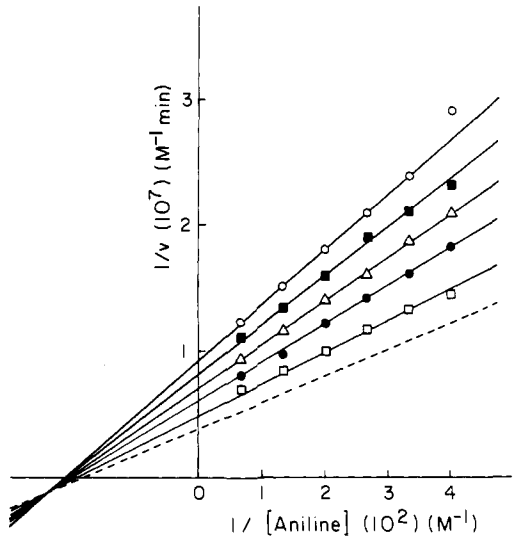


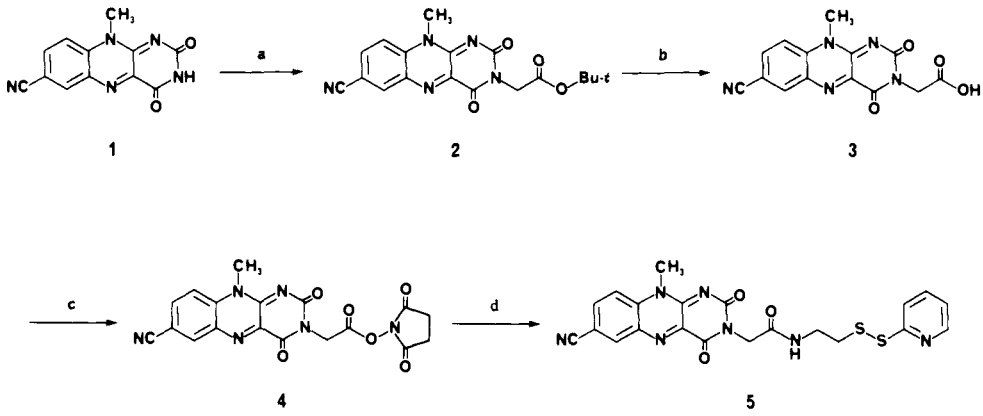
Figure 1. Double-reciprocal plots of the rate of para-hydroxylation against aniline concentration at various concentrations of NADPH. The reaction mixtures (1 mL) consisted of 1 μM Fl-Hb^{3+} (4 μM with respect to heme), 20 mM potassium phosphate, pH 7.5, aniline, and NADPH at 0.15 (○), 0.188 (■), 0.25 (△), 0.375 (●), and 0.75 mM (□). The reactions were allowed to proceed for 15 min at 37 °C and then terminated by addition of ice-cold 20% trichloroacetic acid. Product *p*-aminophenol was determined according to the phenol–indophenol method of Mieyal et al.[4a] Since substrate inhibition was significant at higher concentrations of aniline (>20 mM),[4d] the kinetic measurements were carried out at concentrations less than 3 times the value of K_m. The dashed line in the figure represents the predicted dependence of the rate at an infinite concentration of NADPH.

Hemoglobin A was prepared from normal human erythrocytes[8] and converted to carbon monoxyhemoglobin ($COHb^{2+}$). $COHb^{2+}$ (0.05 mM) was modified by treatment with 0.5 mM of **5** in 0.01 M potassium phosphate, pH 7.0 at 25 °C for 30 min. The major product, Fl-$COHb^{2+}$, was isolated by ion-exchange chromatography on CM-Sepharose.[9] Fl-$COHb^{2+}$ was then subjected to hydrophobic interaction HPLC analysis[10] using a TSK Phenyl-5PW column,[11] and the homogeneity (>96% purity) of the modified protein was confirmed. The absorption difference at 280 nm between Fl-$COHb^{2+}$ and $COHb^{2+}$ ($\Delta\epsilon_{280}$ = 95 000) corresponded to the absorbance of 1.9 equiv of the flavin, based on ϵ_{280} = 51 000 of *N*-3-alkyl-7-cyanoisoalloxazine. Together with the observed decrease in the free thiol content from 2.1 equiv in $COHb^{2+}$ to 0.1 equiv in Fl-$COHb^{2+}$, these data are consistent with the conclusion that two flavin moieties were introduced into the $COHb^{2+}$ molecule. Ferric flavohemoglobin (Fl-Hb^{3+}) was subsequently prepared by the oxidation of 0.1 mM Fl-$COHb^{2+}$ with 4 mM potassium ferricyanide at 25 °C for 1 h and isolated by gel filtration on Sephadex G-25.[4e]

(1) Laboratory of Bioorganic Chemistry and Biochemistry.
(2) Laboratory of Metabolism and Pharmacology.
(3) *Cytochrome P-450*; Sato, R., Omura, T., Eds.; Kodansha/Academic: Tokyo, 1978.
(4) (a) Mieyal, J. J.; Ackerman, R. S.; Blumer, J. L.; Freeman, L. S. *J. Biol. Chem.* **1976**, *251*, 3436–3446. (b) Golly, I.; Hlavica, P. *Biochim. Biophys. Acta 1983*, *760*, 69–76. (c) Takikawa, O.; Yoshida, R.; Hayaishi, O. *J. Biol. Chem.* **1983**, *258*, 6808–6815. (d) Ferraiolo, B. L.; Onady, G. M.; Mieyal, J. J. *Biochemistry 1984*, *23*, 5528–5539. (e) Starke, D. W.; Blisard, K. S.; Mieyal, J. J. *Mol. Pharmacol. 1984*, *25*, 467–475.
(5) Estabrook, R. W. In *Methods in Enzymology*, Fleischer, S., Packer, L., Eds.; Academic: New York, 1978; Vol. 52, pp 43–47.
(6) Hallaway, B. E.; Hedlund, B. E.; Benson, E. S. *Arch. Biochem. Biophys.* **1980**, *203*, 332–342.
(7) Kokubo, T.; Kaiser, E. T., unpublished data.
(8) Acharya, A. S.; Manning, J. M. *J. Biol. Chem.* **1980**, *255*, 1406–1412.
(9) Pharmacia Inc. The eluant was 0.01 M potassium phosphate with a linear gradient from pH 7.0 to 8.2.
(10) Kato, Y.; Kitamura, T.; Hashimoto, T. *J. Chromatogr.* **1983**, *266*, 49–54. Gooding, D.; Schmuck, M.; Gooding, K. *J. Chromatogr.* **1984**, *296*, 107–114.
(11) Toyo Soda Ltd., Japan. Fl-$COHb^{2+}$ and $COHb^{2+}$ were well resolved on a reverse linear gradient of 1–0 M $(NH_4)_2SO_4$.

0002-7863/87/1509-0606$01.50/0 © 1987 American Chemical Society

Scheme I[a]



[a] (a) *tert*-Butyl bromoacetate, K_2CO_3, DMF, 4 h (93%); (b) HCOOH, 12 h (90%); (c) *N,N'*-disuccinimidyl carbonate, pyridine, CH_3CN, 4 h (87%); (d) *o*-$(C_5H_4N)SSCH_2CH_2NH_3^+Cl^-$, $(C_2H_5)_3N$, $CHCl_3$, CH_3CN, 3 h (94%).

Table I. Aniline Hydroxylase Activity of Hemoglobin-Catalyst Systems[a]

	Fl-Hb^{3+}	Hb^{3+} + reductase	Hb^{3+}
K_m^{app}, mM	5.3 (5.5)[b]	5.3	5.6
k_{cat}^{app}, min^{-1}	0.169 (0.262)[b]	0.127	0.022
rel k_{cat}^{app}/K_m^{app}	100 (149)[b]	75	12

[a] Experiments were performed as described in Figure 1, except that the reaction mixture contained 0.375 mM NADPH and one of the hemoglobin systems. [b] Limiting K_m, k_{cat}, and k_{cat}/K_m.

Aniline hydroxylase activity of Fl-Hb^{3+} was examined (Figure 1), varying the aniline concentration at several fixed concentrations of NADPH.[12] The rate of hydroxylation of aniline was found to be dependent on the concentrations of both aniline and NADPH in a manner typical of the kinetics of two-substrate enzyme reactions. Thus, double-reciprocal plots of the rates for various concentrations of NADPH gave straight lines intersecting at a single point, clearly demonstrating that NADPH, as well as aniline, behaves as a substrate. Replots of the intercept and the slope of each line against the reciprocals of the NADPH concentrations gave the following values of k_{cat} and K_m for aniline and NADPH: k_{cat} = 0.262 min^{-1}, K_m(aniline) = 5.5 mM, and K_m(NADPH) = 0.22 mM, respectively. Microsomal cytochrome P-450 has been reported to carry out aniline hydroxylation[4c,13] with k_{cat} values ranging from 0.22 to 0.65 min^{-1}. The rate of the hydroxylase activity of Fl-Hb^{3+}, therefore, is comparable to that of microsomal cytochrome P-450.

The activity of Fl-Hb^{3+} was compared to that of ferric hemoglobin, Hb^{3+}, which was assayed in the presence and absence of 0.6 unit of NADPH-cytochrome P-450 reductase[14] (Table I). The apparent K_m values (K_m^{app}) for aniline observed in the three hemoglobin-catalyst systems with 0.375 mM NADPH were almost identical. In contrast the apparent k_{cat} (k_{cat}^{app}) of Hb^{3+} was markedly increased by reconstitution with the P-450 reductase, confirming results reported previously.[4] Nevertheless, the k_{cat}^{app} of Hb^{3+} in the reconstituted system was found to be still smaller than that of Fl-Hb^{3+}. This result demonstrates that the flavin introduced in Fl-Hb^{3+} can substitute for the reductase. The electron transfer between neighboring prosthetic groups in Fl-Hb^{3+} seems to proceed even more efficiently than that in the combined Hb^{3+} and reductase system. It is conceivable that Fl-Hb^{3+} acts in a fashion analogous to cytochrome P-450, and a detailed study of the mechanism of action of Fl-Hb^{3+} is proceeding.

Acknowledgment. This research was supported in part by NSF Grant CHE 8418878 (E.T.K.) and USPHS DK-32890 (S.S.).

(12) Preliminary product analysis by HPLC showed that *p*-aminophenol is the dominant product over the ortho isomer (p/o > 4) in the hydroxylation of aniline and that the aminophenol is susceptible to further oxidation to benzoquinoimine in the reaction mixture. The procedure of Mieyal et al.[4a] employed for the kinetic assay is specific for para-hydroxylation products including *p*-benzoquinoimine.

(13) Lu, A. Y. H.; Jacobson, M.; Levin, W.; West, S. B.; Kuntzman, R. *Arch. Biochem. Biophys. 1972, 153*, 294-297.

(14) The reductase concentration of 0.6 unit/mL was sufficient to give a rate greater than 85% of the maximal rate under the conditions employed, i.e., the reaction mixture was essentially saturated with the reductase.

Separation of Cross-Relaxation and *J* Cross-Peaks in 2D Rotating-Frame NMR Spectroscopy

Horst Kessler,*† Christian Griesinger,‡ Rainer Kerssebaum,† Klaus Wagner,† and Richard R. Ernst*‡

Institut für Organische Chemie, Universität Frankfurt D-6000 Frankfurt, Germany
Laboratorium für Physikalische Chemie Eidgenössische Technische Hochschule CH-8092 Zürich, Switzerland

Received August 5, 1986

Cross-relaxation rates measured with two-dimensional (2D) NOE spectroscopy (NOESY) have become one of the most important tools for the determination of molecular conformation in solution. However, in medium-size molecules cross-peak intensities often are close to zero, when the correlation time τ_c approaches the inverse of the Larmor frequency of the protons.[1] Then cross-relaxation in the rotating frame, measured in the CAMELSPIN experiment,[2,3] can be used because molecular reorientation rates are fast compared to the effective Larmor frequency in the rotating frame (only positive NOE's).[2] As has been shown before,[4] under conditions used for the rotating frame cross-relaxation experiment, also magnetization transfer between scalar coupled spins occurs (equivalent to isotropic mixing in the total correlation spectroscopy (TOCSY) experiment[4]), leading to so-called *J* cross-peaks. In this paper we demonstrate a method to suppress the coherent magnetization transfer through scalar coupling in rotating frame experiments, leaving more or less pure NOE cross-peaks in the spectrum.[5]

† Universität Frankfurt.
‡ Eidgenössiche Technische Hochschule.

(1) Noggle, J. H.; Shirmer, R. E. *The Nuclear Overhauser Effect*; Academic Press: New York, London, 1971.
(2) Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811.
(3) This experiment is sometimes called ROESY (rotating-frame Overhauser enhancement spectroscopy). Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207.
(4) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521.
(5) Part of: Griesinger, C. Ph.D. Thesis, Frankfurt, May 1986. Presented by H.K. at the 8th EENC, Spa, Belgium, June 1986.

0002-7863/87/1509-0607$01.50/0 © 1987 American Chemical Society