a tighter beam focus), we observed much smaller shifts (+2 to +5 cm^{-1}). No dependence upon protein concentration or laser repetition rate (over a 2-20-Hz range) was detected in any of our spectra.

Only minimal changes are evident in the comparison of the high-frequency spectra of steady-state and transient cytochrome oxidase. Changes in ν_4[27] (from 1358 to 1359 cm^{-1}) and the heme a_3 ν_2 mode (from 1569 to 1573 cm^{-1}) may be indicative of a decrease in both porphyrin π^* electron density and core size, respectively, in the transient heme a_3 species. However, the former speculation is tenuous since the certainty of a 1-cm^{-1} shift is questionable at our spectrometer resolution. Also noteworthy is the lack of a shift in the frequency of the formyl mode (at 1667 cm^{-1}) in the transient spectra.

Our present data indicate the heme a_3 site of cytochrome oxidase remains in a transient conformation for at least 10 ns after ligand photolysis. This is most clearly demonstrated in the behavior of the Fe-His vibrational mode in cytochrome oxidase transients. It shifts to higher frequency in a manner analogous to Hb transients.[16-19] This increase can be interpreted as resulting from a proximal perturbation induced by ligand binding. The Fe-His behavior of photolytic transients of Hb can be assigned to geometric constraints in the distal pocket (i.e., heme-histidine tilt) by correlating crystallographic and transient Raman data.[17] Recent resonance Raman data obtained by Rousseau et al.[13] from a steady-state carbon monoxide cytochrome oxidase indicates that the heme a_3 Fe-His bond is highly strained in both five- and six-coordinate geometries. Thus it is not surprising that we find significant reorganization of the heme a_3 proximal pocket subsequent to CO photolysis. We believe that such behavior is a generic property of the dynamics of the low-spin, in-plane to high-spin, out-of-plane transition that occurs subsequent to ligand photolysis. However, variability in the energetics involved in this process may provide an important means of regulating ligand affinity in a large class of proteins.

The possible changes observed in the high-frequency spectra may indicate specific differences between Hb and cytochrome oxidase photolytic transients. While the ν_2 mode of the heme a_3 photolytic transient is clearly at a high-spin value within 10 ns of ligand photolysis, it appears at a frequency indicative of a contracted porphyrin core relative to the heme a_3 equilibrium. This contrasts with the expanded core found in picosecond hemoglobin photolytic transients and the lack of a difference in core size between steady-state deoxyhemoglobin and nanosecond hemoglobin photolytic transients.[15] Furthermore, the position of ν_4 has been found to inversely correlate with the Fe-His mode frequency in a variety of hemoglobin photolytic transient species[19] and steady-state, deoxyhemoglobins.[22] Our cytochrome oxidase photolytic transient spectra indicate either no change in ν_4 frequency or a shift to higher frequency with increasing Fe-His frequency. The lack of a difference in the formyl frequency between steady-state and transient cytochrome oxidase species is, at first, surprising in view of the mechanistic importance ascribed to the formyl group. However, Rosseau et al.[23] have found only a small ($\leq$3 cm^{-1}) difference in the frequency of this mode between unliganded and CO liganded cytochrome a_3. Evidently even this difference has dissipated within 10 ns of CO photolysis.

The absence of any distinct bands assignable to the CO liganded heme a_3 site in transient spectra generated with moderate laser power ($\leq$0.5 mJ/pulse) contrasts with the behavior of HbCO under similar conditions, where the rapid geminate recombination of CO produces a significant amount of HbCO within 10 ns of photolysis.[24] The heme-carbon monoxide geminate recombination rate in cytochrome oxidase is evidently slow on a 10-ns time scale. This behavior may be a direct result of either the specific geometric constraints imposed upon the bound CO by the distal heme a_3 pocket[13] or a secondary binding of the photolyzed CO to the Cu_B site[25] and is not totally unexpected in view of the lower overall CO affinity of cytochrome oxidase relative to HbA.[26]

In conclusion, we believe that this preliminary investigation has established the following: (1) Time-resolved resonance Raman investigation of the transient heme species generated by ligand photolysis is a viable technique for the study of heme-ligand dynamics in proteins other than hemoglobin. (2) A transient proximal geometry leading to a strengthened Fe-His bond is present in cytochrome oxidase photolytic transients. (3) The interplay of porphyrin core size, π^* electron density, and Fe-His bonding as modulated by heme-protein dynamics is different for the ligand binding sites of Hb and cytochrome oxidase. An extensive series of investigations utilizing extended time resolution and a variety of cytochrome oxidase species has been initiated in our laboratory in order to expand upon these promising initial results.

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Registry No. Cytochrome a_3, 72841-18-0; heme a_3, 58916-42-0; carbon monoxide, 630-08-0; cytochrome oxidase, 9001-16-5.

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Biosynthesis of Porphyrins and Corrins. Direct Observation of an Enzyme-Substrate Complex by Tritium NMR Spectroscopy

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Recent studies from this laboratory[1-5] have demonstrated the power of CMR spectroscopy in defining the structure and stereochemistry of enzyme-inhibitor and enzyme-substrate complexes, using proteases of modest molecular weight (<30000). In this communication we describe NMR experiments with porphobilinogen (PBG) deaminase (EC 4.3.1.8) which catalyzes the head-to-tail condensation of 4 mol of PBG (**1**) to pre-uro'gen, whose release and stabilization as the (hydroxymethyl)bilane (HMB, **7**) has been the subject of extensive investigation.[6-10]

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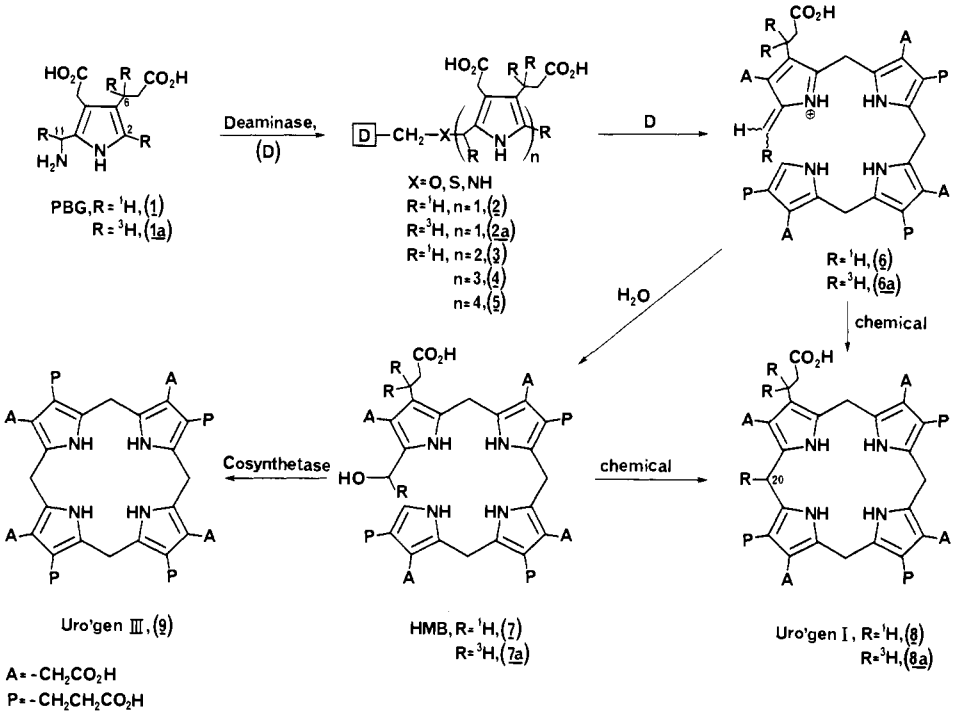
Scheme I



HMB may cyclize chemically to uro'gen I (**8**) or serve as the substrate for uro'gen III cosynthetase (EC 4.2.1.75) to form uro'gen III (**9**) (Scheme I).

Deaminase (MW $\simeq$ 36 000) from human sources,[11] *Rhodopseudomonas* spheroides,[12-14] and *Euglena gracilis*[15,16] forms stable covalent complexes bearing up to four condensed PBG units. It has been suggested[11-16] that the amine function at C-11 of PBG is replaced by a nucleophilic "X" group at the active site of deaminase, where X = O, S, or NH to form **2–5** (Scheme I). The introduction of ^{13}C enrichment at C-11 should allow observation of the chemical shift for the new CH_2X group in the region 25–60 ppm. In our hands, experiments[17] using [2,11-$^{13}C_2$]PBG to label the complexes **2–5** provided no ^{13}C NMR evidence for enrichment above natural abundance either in the above complexes or in the proteolytic digests of the mono-PBG complex **2**[11,12,17] due to the combination of large line widths and high density of resonances in the region of interest. This result contrasts with a recent claim[16] for ^{13}C-signal enhancement near 42 ppm, consistent with amine functionality at the active site (**2**, X = NH). In view of the high natural abundance profile near 40 ppm in the ^{13}C spectrum of deaminase[16,17] and the resultant ambiguity of assignment in the CH_2X region, we sought a general solution to structural problems in covalent enzyme–substrate complexes where the lack of a "window region" precludes rigorous assignment and now describe the first application of tritium, a nucleus of high sensitivity and low ($<10^{-16}$) natural abundance,[18] as an NMR probe of chemical shift in the environment of a productive covalent complex of enzyme and substrate.

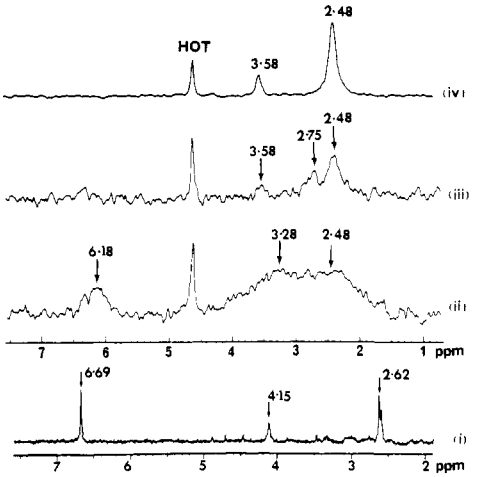


Figure 1. (i) ^1H-decoupled 320-MHz ^3H NMR spectrum of [2,6,6,11,11-$^3H_5$]PBG at pH 8.0, 25 °C. (ii) ^1H-decoupled 320-MHz ^3H NMR spectrum of [2,6,6,11,11-$^3H_5$]PBG deaminase complex at pH 8.0, 5.5 °C. (iii) Same as (ii) but at 23 °C. (iv) Same as (ii) plus unlabeled PBG (950 μg) and after 40 min at 3.5 °C and 6 h at 23 °C.

[2,6,6,11,11-$^3H_5$]PBG (**1a**)[19] (132 Ci/mmol) was synthesized from [3,3,5,5-$^3H_4$]-5-aminolevulinic acid (ALA)[20] by the action of ALA dehydratase from *R. spheroides*.[22] The ^1H-decoupled ^3H NMR spectrum of **1a** (Figure 1i) confirmed the position of ^3H labeling by comparison with the ^1H NMR spectrum of PBG. The resonances occur at 6.69 (C-2, CT), 4.15 (C-11, HCT), 2.62 (C-6, HCT), and 2.56 ppm (C-6, CT_2). The tritiated PBG was rapidly mixed with highly purified (>95%) deaminase[22] (4000 units[23]) from *R. spheroides* and the covalent complexes separated from small molecules by gel filtration on G-50 Sephadex. Analysis

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(19) The [^3H]PBG employed contains all multiply tritiated species up to the fully tritiated [2,6,6,11,11-$^3H_5$]PBG but consisted mainly of [2-$^3H_1$,6,6-$^3H_2$,11-$^3H_1$]PBG.
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of the concentrate by analytical gel electrophoresis showed it to be >90% of the monopyrrole complex[11,12] (**2a**; 16 mCi). The ^3H spectrum (Figure 1ii) of **2a** exhibits resonances[24] at 6.18 (C-2, CT), 3.28 ± 0.1 (pyrrole-CHT-X-Enz), and 2.48 ± 0.1 ppm (pyrrole-$CT_2CH_2CO_2H$) at 5.5 °C. At 23 °C (Figure 1iii) the signals at 6.18 and 3.28 ppm disappear and are replaced by new resonances at 3.58 ± 0.1 (meso methylenes (CHT) of uro'gen I (**8a**) and methylenes (CHT) of complexes **3–5**, R = ^3H), 2.75 ± 0.1 ($CT_2CH_2CO_2H$ of **3–5**, R = ^3H), 2.48 ± 0.1 ($CT_2CH_2CO_2H$ of uro'gen I (**8a**)), and 4.69 ppm (HOT, exchanged from C-2—also present in Figure 1, spectra ii and iv). The formation of uro'gen I (**8**) (whose ^3H chemical shifts are identical with the corresponding CH_2 resonances in the ^1H NMR spectrum) in absence of free PBG is ascribed to disproportionation of complex **2a** via **3–5** whose methylene (CHT) and side-chain groups (C-$T_2CH_2CO_2H$) can be observed along with those of uro'gen I in the signals at 3.58, 2.75, and 2.48 ppm. Such a disproportionation also accounts for the disappearance of the signal at 3.28 ppm, which would be expected to lose up to 90% of its original intensity, on the basis of the statistical randomization of ^3H label, from **2** → **3** → **4** → **5** → **8**.

To demonstrate the catalytic competence of the monopyrrole complex **2**, *unlabeled* PBG (**1**) was added to the ^3H complex **2a** at 3.5 °C and the formation of uro'gen I monitored by ^3H NMR. At 3.5 °C a transient low-intensity signal was observed at 4.76 ppm, a chemical shift consistent with the vinyl (R) hydrogen of the azafulvene **6a**.[25] On warming to 23 °C (Figure 1iv), sharp resonances for unbound uro'gen I (**8a**) appeared at 3.58 (20-meso CHT) and 2.48 ppm (propionate CHT) corresponding to the ^1H chemical shifts in an enzyme-free sample of uro'gen I.[22]

The large line widths of the spectrum in Figure 1 (spectra ii and iii) (~150–300 Hz) reflect an environment in which the active site of the enzyme is probably buried within the protein. The line width of the propionate side chain also suggests that it is covalently attached or ionically associated with the protein, which is consistent with literature reports on the inhibitory effects of PBG analogues.[26-30] The ^3H chemical shift (3.28 ± 0.1 ppm) of the methylene directly attached to the enzyme allows conclusions to be drawn as to the nature of the nucleophilic group "X" in Scheme I. That the methylene could be bound to the oxygen of a serine residue is ruled out, since HMB (**7**) and its methyl ether have chemical shifts of 4.4 and 4.2 ppm, respectively.[22] Model studies[31] predict δ 3.9 for methylene attached to amine while the observed value (3.28 ± 0.1 ppm) is more consistent[33] with a thioether linkage (**2a**, X = S). We therefore suggest that the active-site nucleophilic group in deaminase is a cysteine thiol residue or, less probably, an amino group (**2a**, X = NH) which on covalent binding to C-11 of PBG leads to an upfield shift of ~0.6 ppm from the anticipated value of 3.9 ppm. The former possibility is supported by the observation that deaminase is reversibly inhibited by sulfhydryl blocking reagents.[34]

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(24) ^3H NMR referencing was performed as follows: A ^1H NMR spectrum of [2,6,6,11,11-$^3H_5$]PBG containing internal $Me_3SiCD_2CD_2CO_2Na$ was recorded and the PBG ^1H resonances referenced. The same sample was then examined by ^3H NMR in the same probe and referenced to the ^1H spectrum ($\delta_{^3H} = \delta_{^1H}$). The absolute field frequency was then used from this to calculate the chemical shifts of subsequent spectra.

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(31) PBG (**1**) displays a resonance at 4.16 ppm for the C-11 methylene protons and shifts 0.05 ppm upfield on deprotonation.[22] The (aminomethyl)bilane (**7** where $CH_2OH = CH_2NH_2$) displays a resonance at 3.92 ppm at pH 12,[32] which implies a methylene chemical shift of 3.97 ppm at pH 8.0.

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6A6B-, 6A6C-, and 6A6D-Disulfonates of α-Cyclodextrin

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Recently, specific preparation of primary two- (or more) substituted β-cyclodextrins has been attained to construct refined and sophisticated models of enzymes.[1] Transannular disulfonate-capping methods have been developed in order to activate 6A6C and/or 6A6D primary hydroxyls of β-cyclodextrin.[2] Although α-cyclodextrin shows different molecular recognition as an inclusion host from that of β-cyclodextrin, there have been a few studies on specific activation of two (or more) hydroxyls of α-cyclodextrin. Sulfonation on three (6A6C6E) primary hydroxyls[3] and transannular sulfonation on two primary hydroxyls,[4] whose positions were not determined, were reported.

We wish to demonstrate here a novel and absolute strategy of isomer determination through the first isolation of 6A6B-, 6A6C-, and 6A6D-disulfonates of α-cyclodextrin. A solution of α-cyclodextrin (3 g, 3.1 mmol) and mesitylenesulfonyl chloride (6 g, 27 mmol)[5] in pyridine (230 mL) was stirred for 2 h at room temperature followed by addition of water (1 mL) and concentration in vacuo. The crude concentrated mixture was applied on a reversed-phase column (Lobar column LiChroprep RP 8, Merck Ltd., 25 × 310 mm). After a stepwise elution from 900 mL of 10% aqueous MeOH to 100 mL of 20% aqueous MeOH, a gradient elution with 1 L of 40% aqueous MeOH–1 L of 60% aqueous MeOH was applied to give 6-deoxy-6-mesitylenesulfonates **1** (298 mg, 7.3%), **2** (374 mg, 9.1%), and **3** (555 mg, 13.6%) (Figure 1).[6] ^1H NMR of **1–3**[7] and **4–6**[8] obtained from $NaBH_4$ reduction of **1–3** demonstrated that **1–3** were primary dimesitylenesulfonates. Positional assignments of **1–3** were carried out

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(5) We added mesitylenesulfonyl chloride to the pyridine solution, monitoring the formation of the desirable products. The amount of the sulfonyl chloride was dependent on the dryness of pyridine and α-cyclodextrin.

(6) These isolated compounds were shown to be pure by HPLC (TSK GEL LS-410 ODS SIL column, 4 × 300 mm, 5 μm, Toyo Soda, Japan).

(7) They differed from one another in chemical shifts of aromatic protons. **1**, δ 7.00–7.20 (4 H, m); **2**, δ 7.04 (2 H, s) and 7.08 (2 H, s); **3**, δ 7.07 (4 H, m).

(8) The deoxy derivatives **4–6** showed doublet absorptions of two methyl groups at δ 1.2 in their ^1H NMR and parent ions (M + H^+) at m/z 957 in their FABMS.

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