Opportunities for Probing the Structure and Mechanism of Porphobilinogen Synthase by Raman Spectroscopy

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Although Raman spectroscopy is capable of providing molecular detail on protein ligand contacts[1] and on enzyme−substrate intermediates,[2,3] its utility has been severely limited by problems associated with low sensitivity and spectral interference from luminescent chromophores. The latter may be intrinsic to the system under study or present as trace impurities. Due to technical innovations[4] these problems have been largely removed. We show here the first Raman difference spectroscopic data for product bound to the enzyme porphobilinogen synthase (PBGS); it is possible to detect changes in the chemistry of the product molecule upon binding to the active site and, in addition, to observe many Raman features which occur due to amino acid side chains being perturbed by product binding. The present data demonstrate the potential for obtaining Raman spectra for even recalcitrant systems. PBGS is a large enzyme, an octamer of total molecular mass ∼300 kDa involved in the tetrapyrrole biosynthetic pathway.[5] It has an intense fluorescence emission, due to impurities which may be oligopyrroles or their fragments bound to the enzyme in minute amounts, when excited by laser wavelengths in the visible spectrum. However, here we show that high-quality Raman data can be acquired for PBGS complexes using deep red excitation at 752 nm.

Porphobilinogen synthase is a metalloenzyme that catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA) to form porphobilinogen as illustrated in Figure 1.[6] This reaction is common to all tetrapyrrole biosyntheses, *e.g.* for porphyrin, chlorophyll, and vitamin B_{12}, and is essential for cellular life. The PBGS octamer contains four active sites, each of which binds two molecules of ALA that have different chemical fates. Although many details of the reaction mechanism are not well established, it is known that there is a Schiff base formed between a universally conserved lysine and one of the two ALA molecules at the active site.[5] In this study, we have reacted ALA with PBGS from *Bradyrhizobium japonicum*[7] in stoichiometric amounts and examined the resultant product complex by Raman difference spectroscopy. By running the reaction with smaller amounts of enzyme followed by ultrafiltration to remove the protein, we were able to generate the Raman spectra of free, unbound product for comparison. Spectral assignments for the product were supported by also using [4-^{13}C]ALA as substrate that gave rise to ^{13}C at the 3 and 5 positions of the product (Figure 1).

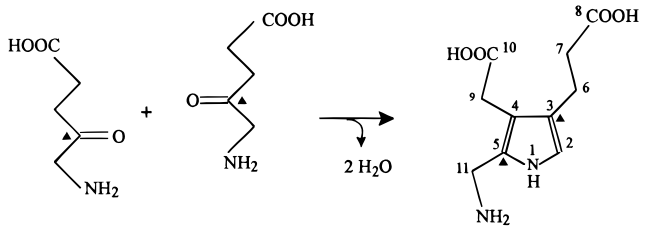

Figure 1. The PBGS-catalyzed asymmetric condensation of two molecules of ALA to form porphobilinogen. ^{13}C labeled atoms are marked ▲.

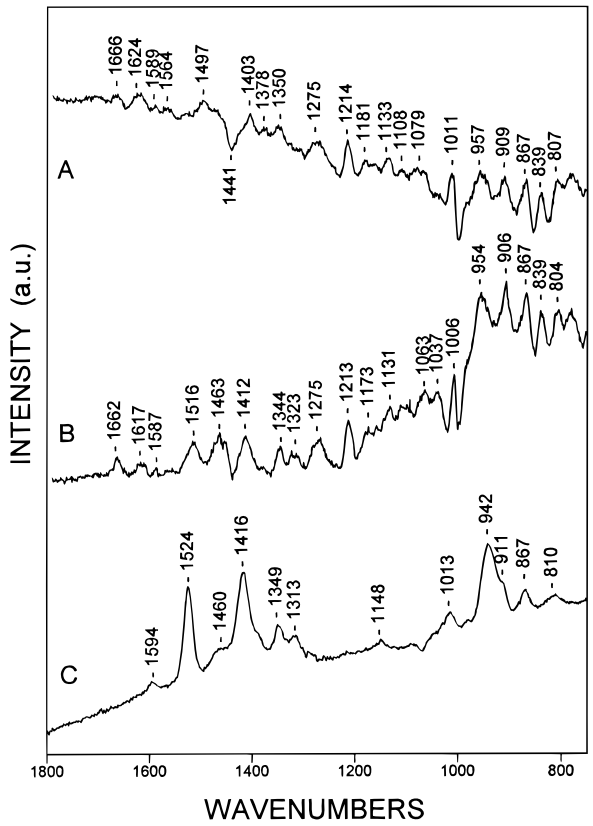

Figure 2. The Raman difference spectra of: A, product labeled with ^{13}C at the 3 and 5 positions bound to PBGS; B, bound, unlabeled product complex; and C, unlabeled free product.

The Raman difference spectra of porphobilinogen, labeled and unlabeled, bound to PBGS are compared in Figure 2 to the spectrum of the free unlabeled product. The difference spectra were obtained by undertaking a computer subtraction of the spectra: (enzyme-bound product in buffer) − (enzyme in buffer at the same concentration). For the difference spectrum of "free", a spectrum of buffer was subtracted from that of the product in buffer. Each set of spectral data was acquired in approximately 10 min using 1 W of 752 nm Kr^+ excitation. Under these conditions the features seen in the difference spectra are highly reproducible. In the spectrum of the free product (Figure 2, bottom), the intense features at 1524 and 1416 cm^{-1} are assigned to pyrrole ring stretching modes on the basis of quantum mechanical calculations (J.D. and P.R.C., unpublished work). For the bound product, the equivalent features can be seen at 1516 and 1412 cm^{-1}, unlabeled, and 1497 and 1403 cm^{-1} for enzyme-bound [3,5-^{13}C]porphobilinogen with the reduction in wavenumbers of 19 and 9 cm^{-1} supporting the assignments to pyrrole ring modes. The downshifts in the ring mode frequencies upon binding are evidence for a significant

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change of electron distribution in the pyrrole ring upon active site binding. Similar shifts are seen in modes for rings which are part of highly polarized π-electron systems such as thienylacryloyl acyl groups bound in the active sites of cysteine proteases[8] and, in the present case, could be caused by propinquity to charged groups or dipoles. The third major feature for the free product occurs at 942 cm^{-1} and may involve a contribution from the C−C stretch from the C−COO^- groups (J,D, and P.R.C. unpublished work). The feature appears to shift to near 955 cm^{-1} in the spectra of the bound products, without undergoing a significant isotope shift with 3,5-^{13}C substitution. The role of the −COO^- groups in the 942 cm^{-1} mode can be put on a firmer footing by using isotopic substitutions in one or more of the atoms involved.

B. japonicum PBGS uses a catalytic magnesium ion in contrast to other PBGSs which use zinc. ^{13}C NMR studies show high similarity between product bound to different zinc utilizing PBGSs[5] but very different chemical shifts for PBG bound to *B. japonicum* PBGS (R.M.P and E.K.J., unpublished results). Comparative Raman studies promise to assist in the interpretation of these chemical shift differences.

It is clear that the difference spectra for the enzyme−product complex contain a plethora of features which do not originate from the product. Instead, they are due to protein modes which emanate from parts of the protein which are perturbed by product binding. Since the side chains of aromatic amino acids are strong Raman scatterers, features from these are expected if the environments of these chains change. With the convention we are using to undertake Raman difference spectroscopy, an increase in intensity of a Raman band in the product complex will result in a positive feature in the difference trace, while a decease will result in a negative (below the baseline) peak. A small shift in frequency gives rise to a derivative-type peak. We have recorded the Raman spectra of the aromatic amino acids using 752 nm excitation, since we are unaware of spectra in the literature recorded at this wavelength. With this standard set and the 752 nm excited Raman spectrum of PBGS in hand (data not shown), we tentatively assign the features near 1620, 1275, 1213, and 840 cm^{-1} to the phenol ring of tyrosine, which indicates that one or more tyrosines are perturbed by product binding. Similarly, inflections in the baseline near 1005 cm^{-1} imply phenylalanine side chain(s) perturbation. PBGS contains seven phyllogenetically conserved tyrosines and six such phenylalanines.[5] The negative peak near 1441 cm^{-1} is intriguing. In some inorganic compounds, the symmetric stretch from a −COO^- group bound to a cation has been assigned to this region.[9] Thus, the negative feature in Figure 2 may mean that a −COO^- side chain on the protein is being converted to −COOH when the product binds since it implies that a putative −COO^- symmetric stretch[9] (from an amino acid side chain) disappears in the complex. This may be a result of the catalytic removal of one of the four protons which are lost from substrate and formally produce water. Interpretation of this kind is still in its infancy, and we expect it to become better defined as the number of experiments on families of related proteins increases. However, it is apparent that a wealth of molecular detail will soon become available from systems that have until now been inaccessible.[10]

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(10) Experimental conditions: Raman spectra were recorded with equipment described by Kim et al.,[4] using 1 W, 752 nm Kr^+ excitation and collecting each data set for 10 min. Concentrations are (A) 1.7 mM, (B) 1.7 mM, and (C) 21 mM. *B. japonicum* PBGS was purified according to the method of Petrovich *et al.*[7] [3,5-^{13}C]PBG was prepared in a 0.5 mL buffer using 10 μmol of [4-^{13}C]ALA-HCl and 1.5 mg of *E. coli* PBGS in 20 mM Kpi (pH 7.0), 10 μM $ZnCl_2$, 1 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. KOH (10 μmol) served to neutralize the ALA-HCl. The reaction was monitored by periodic removal of aliquots and workup with Ehrlich's reagent. After 3 h the [3,5-^{13}C]porphobilinogen was centrifuged through a 10 kDa ultrafiltration membrane to remove the protein.