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Direct evidence of the metal-free nature of sirohydrochlorin in desulfoviridin

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We have obtained direct evidence that the majority of the sirohydrochlorin chromophore in the dissimilatory sulfite reductase desulfoviridin from *Desulfovibrio gigas*, is not associated with any metal. The evidence comes from resonance Raman measurements of native and deuterated samples of desulfoviridin. The breathing mode ν_4 (or ν_4^*) at 1336 cm^{-1} in the native enzyme is downshifted to 1326 cm^{-1} upon deuteration. This mode is not sensitive to deuteration if a metal is present at the center of the chromophore inside protein or in solution. The results also establish the existence of exchangeable core hydrogen(s) at the pyrrolic nitrogen(s).

Introduction

The question of the nature of in vitro sirohydrochlorin in the dissimilatory sulfite reductase desulfoviridin (DSV) has existed for some time [1,2]. The current view based on available evidences is that sirohydrochlorin is essentially the demetallized form of siroheme, an iron-isobacteriochlorin [3]. What has not been ascertained is whether the in vitro chromophore exists in association with a metal ion since chromophore extraction always results in sirohydrochlorin [4]. Similar extractions of all other sulfite reductases yield siroheme [4]. We have studied the optical, EPR, and Mössbauer spectra of DSV and have deduced that its chromophore consists of about 80% sirohydrochlorin and 20% siroheme [5].

In a recent paper [6], we examined the resonance Raman spectrum of native DSV from *Desulfovibrio gigas*. Using the principles of perturbation theory, we were able to establish a basis for comparing the vibrational spectrum of a metallo-isobacteriochlorin with that of a metallo-porphyrin [7,8]. Of special interest was the behavior of the ν_4 breathing mode * upon changing a porphyrin into an isobacteriochlorin. It was argued that if a central metal was present, the reduction of the two adjacent pyrrole rings should not result in any substantial change in the wavenumber of the ν_4 mode. Conversely, an unusually low wavenumber for the ν_4 (or ν_4^*) mode can be interpreted as manifestation of a metal-free status. It must be emphasized that this process of deduction by elimination did not involve the comparison between a metallo-porphyrin and free-base porphyrin. Therefore, the conclusion that was reached was necessarily of an inductive rather than a predicative nature. We could not identify any peak in the Raman spectrum of DSV due to the 20% siroheme present. This is possibly due to the much lower enhancement of siroheme with 406.7 nm excitation since the Soret peak of siroheme is near 390 nm. We present here more direct evidence of the metal-free nature of in vitro sirohydrochlorin.

Materials and Methods

The experimental setup of the Raman system has been described earlier [6]. The 406.7 nm line from a

Abbreviations: DSV, desulfoviridin; TPP, tetraphenylporphyrin; TpyP, *meso*-tetrakis(4-pyridyl) porphyrin; TMpyP, *meso*-tetrakis(4-*N*-methylpyridyl) porphyrin.

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* We use the notation of Abe et al. [8] for mode assignment. Mode mixing can occur when the symmetry of the porphyrin is lowered to that of the isobacteriochlorin. However, the essential character of the ν_4 mode remains unchanged when the symmetry is lowered. The corresponding ν_4 mode of porphyrin in isobacteriochlorin is designated by ν_4^*. See Ref. 6 for more detailed discussion.

krypton ion laser was used for excitation. The samples in melting-point capillary tubes were placed transversely across the path of the laser beam. Desulfoviridin from *D. gigas* was purified as described elsewhere [5]. Deuteration of the native DSV samples was accomplished by repeated dilution with 99.99% D_2O and concentration with Centricon ultra-filtration units. Generally, only freshly prepared samples were used, although re-measurements made after one or two days did not show any changes. The reversible nature of the deuteration process was checked by re-hydrogenation of deuterated samples following the same dilution/concentration procedure as for deuteration but using H_2O.

Free bases of tetraphenylporphyrin (TPP), *meso*-tetrakis(4-pyridyl) porphyrin (TpyP) and *meso*-tetrakis(4-*N*-methylpyridyl) porphyrin (TMpyP) were obtained from Aldrich Chemical Co. and used without further purification. All solvents used were of spectrographic grade.

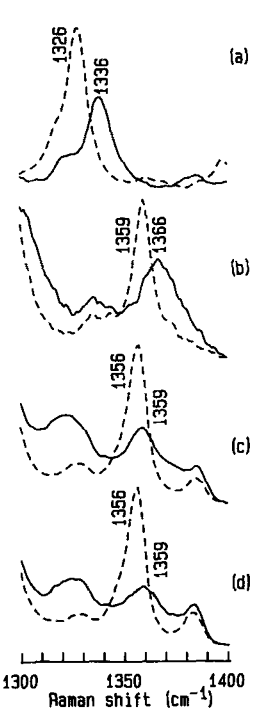

Fig. 1. Raman spectra of hydrogenated (solid line) and deuterated (dashed line) samples of (a) desulfoviridin, (b) TMpyP, (c) TpyP, and (d) TPP. Laser excitation was 406.7 nm with 6 cm^{-1} resolution. Sample was at room temperature and power at the sample was less than 15 mW. Accumulation times were 18, 5, 1 and 0.5 min, respectively.

Results and Discussions

It is known that the core hydrogens of a free-base porphyrin undergo a dynamic exchange with the surrounding hydrogen (proton) bath. Thus, by simply altering the nature of the bath one can obtain in a reversible way either deuterated or hydrogenated species. The isotopic mass change would then cause wavenumber shifts in some of the vibrational modes. The nearly pure infrared active N-H vibrational modes have indeed been observed to exhibit the expected isotopic shifts [9]. However, the corresponding Raman active modes have not been detected; the recent identification in H_2TPP is incorrect, as will be discussed later. Thus, the confirmation of isotopic substitution depends upon qualitative but consistent Raman spectral changes rather than predictable wavenumber shifts. These spectral changes can occur in three different aspects: linewidth, intensity, and wavenumber. Intensity changes occurring alone signify excited state perturbation and can thus be excluded from isotopic substitution effects. Thus, only when all three types of changes occur simultaneously can one be certain that isotopic substitution has in fact taken place.

The isotopic effect on the ν_4 or ν_4^* mode is of particular interest. The ν_4 mode in metallo-porphyrins involves predominantly the breathing motions of the four core nitrogens. In the presence of a central metal in the porphyrin, deuterium substitution can only occur in susceptible peripheral groups or in the protein matrix. The influence on the ν_4 mode would be minimal and no wavenumber shift should be observed, as was indeed confirmed by studies on Cu-TMpyP and myoglobin (data not shown). With the presence of core hydrogens, however, perturbation of the ν_4 mode can be manifested through the isotopic effect in the N-H vibrations. Thus, a wavenumber change in the ν_4 or ν_4^* mode alone would be sufficient evidence of a successful isotopic substitution which would in turn indicate the presence of core hydrogens.

Fig. 1 shows the spectral region of the ν_4 and ν_4^* modes for the hydrogenated and deuterated forms of DSV, TPP, TpyP, and TMpyP in various solvents. Deuteration was confirmed by the shift of the ν_4/ν_4^* mode and other changes. It was found that deuteration of TPP and TpyP can be achieved by vigorous mixing of the CH_2Cl_2 solutions with a few drops of D_2O. This suggests that the exchange occurs between the core hydrogens and the small amount of dissolved D_2O [10]. The wavenumber shift of the ν_4/ν_4^* mode upon deuterium substitution is approx. 10 cm^{-1} in the DSV sample and ranges from 3 to 7 cm^{-1} in the other porphyrins. This lowering of the frequencies is expected from the mass effect of the heavier deuterium, and the ratio of hydrogenated to deuterated modes is expected to be 1.35 for stretching and 1.1–1.3 for bending. Thus, the amount of shift can be interpreted qualitatively as reflecting the content of N-H vibrational modes of A_1 symmetry. The reversibility of the exchange in DSV was also confirmed by the fact that the Raman spectrum of the deuterated sample which was then re-hydrogenated was identical to that of the native enzyme.

Another prominent change is the consistent increase in the peak intensity of ν_4 and ν_4^* modes upon deuteration. This is most conspicuous in the porphyrins and is always accompanied by a reduction in the linewidths. The cause of this sharpening is unclear. It is unlikely to be due to variations in resonance enhancement since the absorption spectra for each set of H- and D-samples are identical.

In the spectrum of H_2TPP, the two peaks at 1327 cm^{-1} and 674 cm^{-1} have previously been assigned to in- and out-of-plane N-H bending modes, based upon their disappearance in D_2TPP [11]. Our measurements, however, revealed that these two modes still exist in our D_2TPP sample in the various deuterated solvents. The presence of a number of spectral changes ruled out any possibility that our samples could have remained as H_2TPP. In an effort to resolve this discrepancy, we re-measured the D_2TPP spectrum in a 5:1 dimethylformamide-D_2O mixture, the solvent that was used in the previous study. The solubility was low and the spectrum was of an inferior quality. However, the presence of the two modes is unambiguous. Furthermore, inspection of the spectra for TpyP and TMpyP showed that these two modes were constant features unrelated to any N-H vibrations. The reported disappearance of these two modes upon deuteration was probably a result of interference from the solvent spectrum, which in our case was quite strong.

The spectral changes for DSV upon deuteration coupled with the reversibility of the exchange serve to confirm the presence of core hydrogens in the sirohydrochlorin moiety. Previous optical absorption studies showed that the extracted porphyrin containing chromophore can contain 20% siroheme [5]. It did not address the question of whether or not the metal is absent in vitro. Both EPR and Mössbauer spectra can only detect the metal-containing siroheme. In EPR measurements, the amount of siroheme in DSV was deduced by comparing the intensity of the siroheme signal from DSV and that from other siroheme-containing sulfite reductases like desulforubidin. In Mössbauer experiments, the relative intensity of siroheme to that of the other iron in DSV (from the iron-sulfur clusters) was used. Finally, the amount of sirohydrochlorin is deduced from the amount of extracted sirohydrochlorin/siroheme. The conclusion on the amount of sirohydrochlorin in vitro can only be termed as indirect. The current result is by far the most direct of evidence that the majority of the sirohydrochlorin chromophore in DSV is metal free.

It is intriguing to ponder the significance of a metal-free sirohydrochlorin in DSV. Despite the absence of iron in approximately 80% of the chromophore content, the activity of DSV is still comparable to other siroheme-containing sulfite reductases. The fact that CO, a strong inhibitor of the siroheme-containing sulfite reductases [5,12–14], has no effect on DSV activity constitutes the best indication that the sirohydrochlorins are catalytically competent in this protein. Another puzzling observation is that the two types of dissimilatory sulfite reductases, the siroheme-containing desulforubidin and the sirohydrochlorin-containing DSV, have the same number of [4Fe-4S] clusters (4 per dimer). One of the two iron-sulfur clusters (per monomer) in desulforubidin is known to be coupled to the siroheme [5]. Thus, it appears that this siroheme coupled iron-sulfur cluster is still present in DSV. The question remains as to whether this cluster is similarly coupled to sirohydrochlorin and/or involved in catalysis. Clearly, more studies are necessary to fully explore the catalytic properties of sulfite reductases.

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