Stability of Thermophilic Cytochrome P450 Modified with Poly(ethylene oxide) in Ionic Liquid

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Cytochrome P450 from the thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7 (P450st) was chemically modified with activated poly(ethylene oxide) (PEO) to make it more soluble in ionic liquid and in which Raman spectra results show the structural stability in the five coordinate high-spin state even at a temperature of 120 °C.

Cytochrome P450s (P450s) are heme-containing monooxygenases involved in a variety of oxidative metabolic reactions, including synthesis and degradation of many physiologically important compounds.[1] P450s are potentially useful catalysts for bioreactors and biosensors because of their molecular diversity.[2] However, neither their industrial nor their medical application has been successful due to both instability and the requirement of a cofactor to mediate the electron transfer. Considerable efforts have been made to explore methods that can improve the stability of proteins. We have achieved the overexpression and purification of a P450 from the thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7 (P450st), which showed a redox response even when the temperature was elevated to 80 °C.[3] A number of cases of improved stability of enzymes were also made in non-aqueous media.[4] We have already reported that solutions of salt-containing polyethers, especially poly(ethylene oxide) (PEO), are non-aqueous media useful as a reaction field for biomaterials over a wide temperature range[5] and long-term stability.[6] Recently, room temperature ionic liquids (ILs) have been gaining attention as green, multi-use reaction media due to unique features such as high thermal, chemical, and electrochemical stability.[7] ILs are also being tested for a variety of bio-applications including as media for biocatalytic reactions, biosensors, and protein stabilization.[8] However, native proteins are usually insoluble in organic solvents and ILs. In most reports, the enzyme was in a finely dispersed state and therefore acting as a heterogeneous catalyst. Recently, it has been shown that homogeneous solutions of proteins in organic solvents can be prepared by the chemical modification of amino groups on the protein's surface with PEO.[9] Since the PEO chains have high affinity for ions, PEOs are expected to be soluble in ILs.[10]

In the present study, we describe the first attempt to modify P450st with PEO (PEO–P450) to solubilize it in 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([EMIm][TFSI]) which is a typical IL. Structural information on PEO–P450 in [EMIm][TFSI] was obtained by using UV–vis and resonance Raman spectroscopies. P450st was chemically modified with activated PEO (average molecular weight of 2000) according to the method reported previously.[9] The average number of PEO molecules bound to one molecule of P450st was estimated to be 16 from the titration results of amino groups of P450st by 2,4,6-trinitrobenzenesulfonic acid.[11] The modification was confirmed not to affect the conformation of P450st based on the spectroscopic results.

At 25 °C, PEO–P450 in aqueous solution was a low-spin state with a Soret absorbance maximum at 412 nm (Figure 1a). The Soret absorption of PEO–P450 was shifted to 419 nm in [EMIm][TFSI], as shown in Figure 1b. This suggested that the solvent's polarity affects the electronic absorption spectrum of PEO–P450. When the temperature was raised to 90 °C, the absorbance of Soret bands both in aqueous solution and in [EMIm][TFSI] slightly decreased. These results indicate the major characteristic of thermophilic P450s, the ability to withstand high temperatures. However, a distinctive difference in Soret absorption between the PEO–P450 in aqueous solution and that in [EMIm][TFSI] was observed at 25 °C after preincubation at high temperature (up to 90 °C). The Soret absorption of PEO–P450 in [EMIm][TFSI] was shifted and remained entirely at 408 nm, while the Soret absorption of PEO–P450 in aqueous solution was shifted at high temperature but return to 412 nm when the temperature was decrease to 25 °C. To explain this observation, resonance Raman spectroscopy was used to provide further information on the ligand coordination and heme pocket of PEO–P450.

The Raman spectrum of PEO–P450 solubilized in [EMIm][TFSI] is shown in Figure 2a. This spectrum has contributions from the vibrational modes of both the protein itself and [EMIm][TFSI]. The difference spectrum obtained by subtracting the Raman spectrum of [EMIm][TFSI] (Figure 2b) from that of PEO–P450 in [EMIm][TFSI] (Figure 2a) presents a clearer picture of the resonance-enhanced modes of PEO-modified P450st (Figure 2c).

The difference Raman spectrum of PEO–P450 in

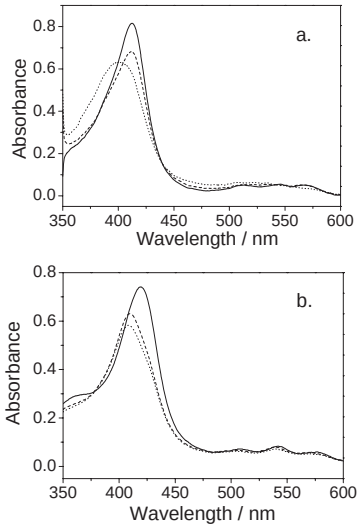

Figure 1. UV–vis absorption spectra of PEO-modified P450st in aqueous solution (a) and in [EMIm][TFSI] (b) at 25 °C (solid line), 90 °C (dotted line), and 25 °C after preincubation at 90 °C (dashed line).

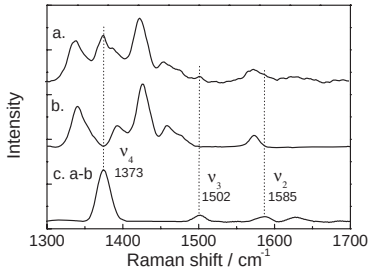

Figure 2. Raman spectra of PEO-modified P450st with 413.1-nm excitation. (a) Spectrum of PEO-modified P450st in [EMIm][TFSI]. (b) Spectrum of [EMIm][TFSI]. (c) Difference spectrum of (a) minus (b).

[EMIm][TFSI] showed intense bands at 1373, 1502, and 1585 cm^{-1}, which indicate that iron is in the ferric and low-spin state (Figure 3b). This characteristic was similar to that of PEO–P450 in water, as shown in Figure 3a, suggesting that the solubilizing of PEO–P450 in [EMIm][TFSI] does not cause structural change around the heme iron. At high temperature up to 90 °C, several distinct differences can be recognized in Figure 3c: The spin marker bands at 1502 and 1585 cm^{-1} from the 25 °C sample were shifted to 1493 and 1578 cm^{-1} in the spectrum of PEO–P450 at 90 °C, respectively. Instead, the oxidative state marker band (1373 cm^{-1}) remained at the same position. This result indicated that PEO–P450 in [EMIm][TFSI] at high temperature is in the ferric five coordinate high-spin state. It is worth noting that this characteristic was similar to that in the case of aqueous P450st at high temperature (data not shown). This observation is also consistent with that of CYP119, a thermophilic P450 from *S. solfataricus.* It is suggested that the evaporation of water from the system results in a change in the heme distal pocket structure of the heme iron.[12] Next, we attempted to characterize the structure of PEO–P450 in [EMIm][TFSI] at higher temperature. It was found that the Raman spectrum of PEO–P450 in [EMIm][TFSI] remained almost unchanged even when the temperature was elevated to 120 °C (Figure 3d).

Furthermore, to explain the difference in UV–vis spectra at 25 °C after preincubation at 90 °C between the aqueous solution and IL, the structure of PEO–P450 was characterized by Raman spectroscopy. In aqueous solution, the Raman spectrum showed intense bands that indicate that iron is in the six coordinate low-spin state (data not shown). On the other hand, in IL, PEO–P450 shows the same Raman spectrum as at high temperature. These results indicate that even at 25 °C after preincubation at high temperature, the water molecule in aqueous PEO–P450 was again coordinated at the sixth position of the heme iron, whereas the water molecule of PEO–P450 in IL was dissociated from the sixth position and the heme iron remained in a five coordinate high-spin state.[13]

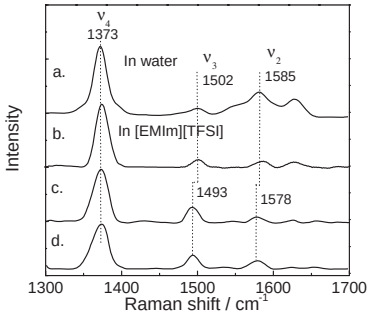

Figure 3. Difference Raman spectra of PEO-modified P450st minus [EMIm][TFSI] in water (a) and in [EMIm][TFSI] at 25 °C (b), 90 °C (c), and 120 °C (d). $\lambda_{max} = 413.1$ nm.

The spin state of P450 plays an important role in enzymatic activity. Ortiz de Montellano and co-workers found that the conformation of CYP119 correlates with the catalysis of styrene epoxidation.[12] The author claimed that the dissociation of water at high temperature results in a change in the heme distal pocket from a low to high-spin state and leads to more catalytic activity at the active site of heme in CYP119. According to this observation, the stabilizing of PEO–P450 in [EMIm][TFSI], having the five coordinate high-spin state, even after preincubation at high temperature may contribute to the catalytic efficiency of P450st. The structural features obtained in this study should provide important information for the development of reasonable designs for biodevices based on cytochrome P450 in the future.

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13 Structural diagrams of the heme for PEO-modified P450 in ionic liquid are available from Supporting Information.

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