Probing an Acyl Enzyme of Selenosubtilisin by Raman Spectroscopy

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Replacement of the catalytically essential serine in subtilisin by selenocysteine dramatically alters the properties of the protease. In addition to affording it novel peroxidase activity,[1,2] the single atom mutation (from oxygen to selenium) converts the enzyme into an acyl transferase, favoring aminolysis over hydrolysis of the acyl enzyme intermediate formed in reactions with activated esters and imidazolides.[3] The latter properties (and the lack of amidase activity) make selenosubtilisin a potential candidate for the development of a peptide ligase.[4−6] We have initiated spectroscopic investigations of enzyme-bound seleno esters to establish the origins of the altered hydrolytic chemistry.[7] This report describes the characterization of a typical intermediate by Raman spectroscopy and shows that selenosubtilisin polarizes the carbonyl of the acyl enzyme to a much lesser extent than the native enzyme. These results have important implications for the design of the more active variants.

Acyl enzyme intermediates of selenosubtilisin (Scheme 1) can be formed for spectroscopic study using reactive substrates and protocols based on those employed for serine and cysteine proteases.[8]

The acyl group (5-methylthienyl)acryloyl (5-MTA) was chosen as an initial active site probe for selenosubtilisin BPN′ [2] because of the extensive spectroscopic knowledge on this moiety.[8−10] As is evidenced in Figure 1, Raman difference spectroscopy[11,12] furnishes detailed, high signal-to-noise Raman spectra of the enzyme-bound acyl group for the unlabeled intermediate and for the acyl group labeled with $^{13}C{=}O$ and, separately, $C{=}^{13}C{-}C(=O)$. The spectra in Figure 1 represent "raw" data, which have not been smoothed to artificially suppress noise. The Raman difference spectra were obtained using 488 nm laser excitation, and since this wavelength is 130 nm from λ_{max} of the bound acyl group, the data were obtained under nonresonance Raman conditions. However, the highly polarizable nature of the acyl group's π-electron chain means that this group is a strong Raman scatterer and, upon performance of the subtraction [Raman spectrum, acyl enzyme] − [Raman spectrum, enzyme], only features due to the bound acyl moiety are seen in the difference spectrum. Acquisition of the data under nonresonance conditions has the added advantage that possible photochemical effects due to the laser beam used to generate the Raman spectrum[8,9] are absent.

In this communication we focus on three areas where analysis of the Raman data can provide unique insight into events in the active site, *viz.*, hydrogen bonding of the acyl C═O group,

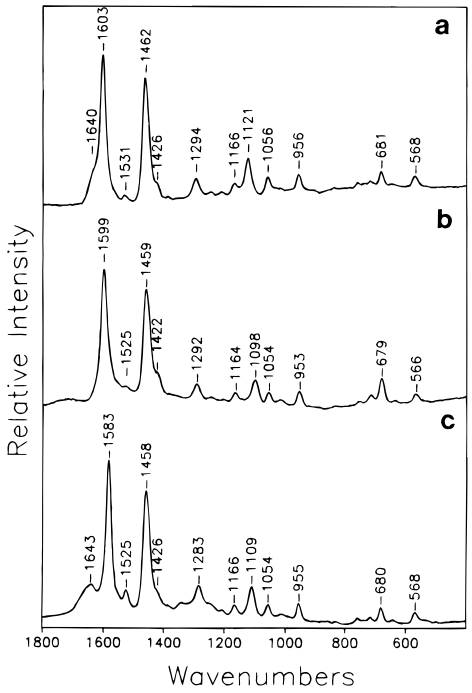
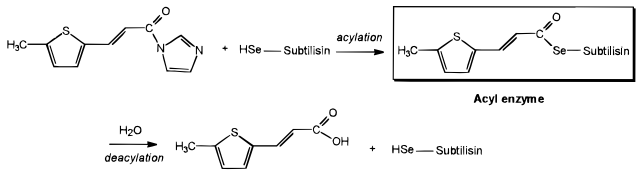


Figure 1. Raman difference spectra for (5-methylthienyl)acryloyl-selenosubtilisin BPN′: (a) unlabeled, (b) $^{13}C{=}O$, (c) $C{=}^{13}C{-}C(=O)$. Each spectrum is obtained by subtracting the spectrum of enzyme in buffer from that of acyl enzyme in buffer. Spectral conditions: 400 mW, 488 nm, 30 s × 60 acquisition time. Note: the Raman peak near 680 cm^{-1} is due to a small amount of dimethyl sulfoxide used to carry the substrate into solution, which travels with the enzyme during column chromatography. The Raman spectrometer is described in Kim *et al.*[21] Acyl enzyme: 1 mM, pH 6.5 in 100 mM phosphate buffer. At pH 6.5 the acyl enzyme has a half-life of 33 min. To prepare the acyl enzymes, oxidized selenosubtilisin in water (1 mM) was degassed by passage of argon for 15 min in a closed reaction vial and then reduced with 1 or 2 drops of aqueous sodium borohydride (1 M) for 20 min. Excess sodium borohydride was destroyed by reducing the pH to between 4.5 and 5.0 using 0.5 M sodium acetate solution. The pH was then increased to 6.5 by adding 1 M potassium phosphate buffer. All reactions were carried out under Ar, unless stated otherwise, and buffers were degassed by bubbling Ar for 15 min. The enzyme was acylated using 5-MTA-imidazolide in DMSO (final concentration, 2.5 mM) for 1 min and separated from unreacted substrate by low-speed centrifugation in a Sephadex G-25 column (1 mL) previously equilibrated with 100 mM phosphate buffer pH 6.5. The acyl enzyme was passed through a 0.45 μm filter prior to obtaining Raman data.

Scheme 1



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electric field effects experienced by the substrate, and the conformation of the bound acyl group. The carbonyl stretching feature is seen clearly at 1643 cm^{-1} in Figure 1c, where ^{13}C labeling at C2 is used to shift the more intense $\nu_{C=C}$ peak to lower wavenumbers. The same peak is seen as a shoulder near 1640 cm^{-1} in the spectrum of the unlabeled intermediate (Figure 1a) and is likely unresolved near 1620 cm^{-1} in the $^{13}C{=}O$ labeled intermediate (Figure 1b) (on the basis of unpublished work on α,β-unsaturated thiol esters we expect $\nu_{C=O}$ to downshift by about 25 cm^{-1} upon $^{13}C{=}O$ substitution). The ^{13}C labeling at C2 may shift $\nu_{C=O}$ to lower wavenumbers due to vibrational coupling; however, this effect is minimal since $^{13}C{=}O$ substitution is seen to shift $\nu_{C=C}$ down by only 3.5 cm^{-1} (Figure 1b). Thus, we can confidently place $\nu_{C=O}$ for the unlabeled intermediate in the range 1640 ± 3 cm^{-1}. For the model compound (5-methylthienyl)acryloyl ethyl thio ester in CCl_4 solution, $\nu_{C=O}$ occurs in the normal Raman spectrum at 1655 cm^{-1} (unpublished data, this laboratory). This approximately 15 cm^{-1} shift upon going from a non-hydrogen-bonding environment to the active site represents the formation of only weak hydrogen bond(s) to the C=O oxygen. Quantitative data on the relationship between the $\nu_{C=O}$ shift and the enthalpy of hydrogen bonding are not yet available for α,β-unsaturated thiol esters (or the analagous seleno esters), but a semiquantitative estimate can be deduced from the corresponding data for α,β-unsaturated esters.[13,14] For the latter, a shift in $\nu_{C=O}$ of 15 cm^{-1} corresponds to a change in hydrogen-bonding strength of approximately 8 kJ mol^{-1}.[13] On the basis of literature comparisons of simple esters and thiol esters,[15] we expect a similar value for the strength of the hydrogen bond(s) to the acyl carbonyl in the active site. Without doubt, the carbonyl oxygen is hydrogen bonded only weakly. The value of approximately 8 kJ mol^{-1} in selenosubtilisin compares to the value of 27 kJ mol^{-1} for the hydrogen bonding in the oxyanion hole of subtilisin itself.[13] Substitution of selenium for oxygen engenders steric crowding in the active site and presumably limits productive interactions of the acyl intermediate with the oxyanion hole. It may be possible to relieve this crowding by mutating additional residues near the active site. Analogous experiments with thiolsubtilisin have yielded a highly active peptide ligase.[5,6]

The position of the Raman peaks reflects the degree of electron polarization in the ground electronic state of the acyl chromophore, which in turn is determined by the strength of the electric field in the active site.[16−18] A particularly useful probe is the intense $\nu_{C=C}$ band, which moves to lower wavenumbers with increasing electron polarization. Thus, for 5-MTA-papain, where there is strong polarization, $\nu_{C=C}$ occurs near 1570 cm^{-1}.[9] In the present case $\nu_{C=C}$ occurs at 1603 cm^{-1}, very close to the value of 1604 cm^{-1} for the model compound 5-MTA-SC_2H_5 in CCl_4 (unpublished data, this laboratory). Thus, the 5-MTA-acyl group in the active site of selenosubtilisin is not polarized and is not experiencing an electric field from active site charges or dipoles. This finding is the same as that for thiolsubtilisin[9] and subtilisin[19] (and unpublished work, this laboratory) based on earlier resonance Raman studies.

α,β-Unsaturated thiol esters usually exist as a mixture of *s-cis* and *s-trans* conformational isomers about the C=C−C(=O) single bond.[20] *Ab initio* and vibrational calculations have shown that a marker band, having a contribution from the stretching motion of the same C−C bond, occurs for the *s-trans* isomer in the 1150 cm^{-1} region while the *s-cis* isomer gives rise to a corresponding feature near 1040 cm^{-1}.[20] The feature seen in Figure 1a at 1121 cm^{-1}, which moves to 1098 and 1109 cm^{-1} upon $^{13}C{=}O$ and $^{13}C2$ substitution, respectively, is evidence that the 5-MTA-acyl group in the active site is assuming the *s-trans* conformation about the C2−C1 bond.

Raman spectroscopy has become a powerful tool for probing subtle interactions within enzyme−substrate complexes. In the present study, this technique has revealed substantial differences in the active site properties of subtilisin and selenosubtilisin, the most important of which may be the disruption of productive hydrogen-bonding interactions with the oxyanion hole. A more complete assignment of the 5-MTA-selenosubtilisin Raman spectrum will greatly aid future studies to probe the chemistry of the C−Se bonds in the active site, to follow conformational changes which occur upon activating subtilisin's charge relay system, and to compare the active sites of natural and recombinant selenosubtilisin variants.

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