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Rapid Scanning Stopped-Flow Absorption Studies of the Effect on Tryptophanase of a Change in pH or K^+ Concentration: Evidence for a Slow Conformational Change[1]

Sir:

Tryptophanase, a pyridoxal-P-dependent enzyme requiring monovalent cations for optimum catalytic activity[2-4] has absorption maxima at 337 and 420 nm which have been attributed[4] to active and inactive forms, respectively. The amplitudes of these interconvertible absorptions depend upon pH and the type and concentrations of monovalent cations.[5] We now report that their interconversion following a change in pH or K^+ concentration occurs on the stopped-flow time scale. This new result indicates the occurrence of either enzyme conformational changes in the interconversion process or slow protonation-deprotonation steps. Such slow changes have important implications for the mechanism of catalytic action of a large class of pyridoxal-P-dependent enzymes. For example, the amplitudes and rates of biphasic formation of an enzyme bound quinonoid observed with tyrosine phenol lyase,[6] serine hydroxymethylase,[7] and tryptophanase[8] vary with pH[7,8] and monovalent cations.[8] It is likely that the slow changes described here contribute to this kinetic complexity.

Holotryptophanase from *Escherichia coli* B/1t 7-A, prepared as described previously,[9,10] was studied at 24 °C in three experiments:[11] (A) a pH drop from 8.53 to 6.72; (B) a pH jump from 7.38 to 9.30; (C) a sudden change (K^+ jump) from 0.1 M Na^+ to 0.05 M Na^+ and 0.05 M K^+ at pH 8.0.

A computerized double-beam rapid-scanning absorbance stopped-flow system[12] was used to collect 150 spectra per second over the wavelength range 280-550 nm. The abrupt spectral changes observed in these experiments occurred within the 6.5-ms dead time (1.85-cm-path-length cell), while the slower changes required seconds to minutes. The absorbance-wavelength-time data as well as spectra from control experiments (enzyme spectra, the change in the pyridoxal-P spectrum with pH[13] and background spectra) were collected with a PDP 8/I computer. The software permits substraction of the contribution of free pyridoxal-P, determination of the enzyme absorbance, $A_0(\lambda)$ (the expected spectrum after mixing in the absence of change), and construction of difference spectra.

Most of the effects of pH can be rationalized by Scheme I which assumes rapid protonation and deprotonation (involving one or more protons) of two slowly interconvertible enzyme forms. The high pH form, E′, has an absorption peak at 337 nm (Figure 1, ○), while the low pH form, EH^+, has its absorption maximum at 420 nm (Figure 1, ▲). After a pH drop, E′ forms $E'H^+$ to give an abrupt decrease in absorbance at ~330 nm ($\Delta A = -0.075$) and a corresponding increase at 390 nm ($\Delta A = +0.033$) (Figure 1, ●). These changes are followed by first-order decay at ~340 nm ($\Delta A = -0.054$) and simultaneous growth at ~420 nm ($\Delta A = +0.071$) with $k_2 = 0.43 \pm 0.03\ s^{-1}$. This slow change is indicative of a conformational change from $E'H^+$ to EH^+.

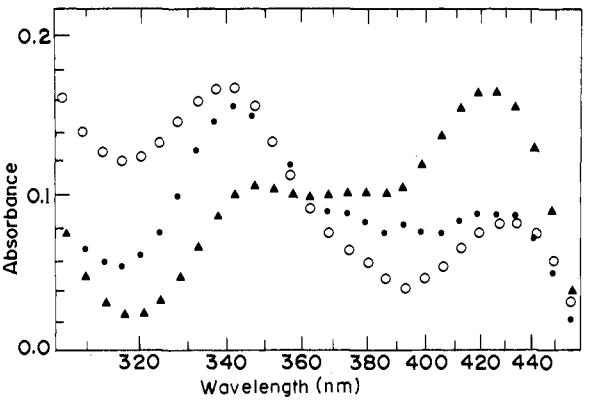

Figure 1. Tryptophanase spectra (path length 1.85 cm) corrected for free pyridoxal-P absorption, observed in the pH drop experiment: zero time, ○; first spectrum after mixing (t = 6-12 ms), ●; spectrum after completion of the first-order process (t = 8 s), ▲.

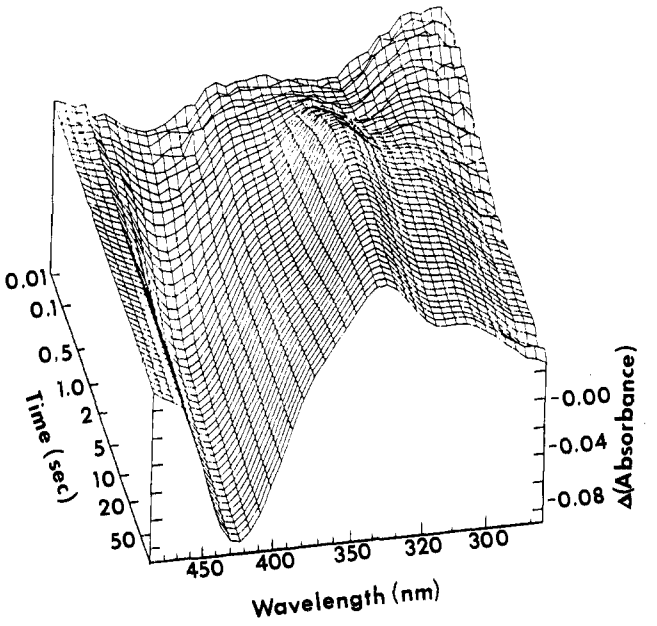

Figure 2. Absorbance difference-wavelength-time surface observed in the pH jump experiment. The spectra of free pyridoxal-P and of tryptophanase at zero time ($A_0(\lambda)$) have been substracted to give these difference spectra.

The overall changes for the pH jump experiment are the reverse of those observed for the pH drop, but the time sequence and the intermediates involved are different. Figure 2 is a view of the three-dimensional surface which shows the time evolution of the difference spectrum, $A(\lambda) - A_0(\lambda)$. After the pH jump, EH^+ is converted to E yielding an abrupt absorbance increase at ~300 nm ($\Delta A = 0.056$) and a decrease at 420 nm ($\Delta A = -0.047$). Then a first-order growth at ~340 nm ($\Delta A = +0.106$) and simultaneous decay at ~420 nm ($\Delta A = -0.133$) occur as E is converted to E′ with $k_1 = 0.56 \pm 0.03\ s^{-1}$. This is followed by a slower first-order decay of absorbance

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Scheme I

$$\begin{array}{ccc} EH^+ & & E'H^+ \\ (\text{fast})\uparrow\downarrow\pm H^+ & \overset{k_1}{\underset{k_2}{\rightleftharpoons}} & \pm H^+\uparrow\downarrow(\text{fast}) \\ E & & E' \end{array}$$

at ~325 nm ($\Delta A = -0.012$) and at ~425 nm ($\Delta A = -0.012$) and growth at ~360 nm ($\Delta A = +0.036$) with a rate constant of 0.062 ± 0.002 s^{-1}. No further changes in the spectrum were seen over a time span of several minutes.

The K^+-jump experiment also showed slow conversion of the 420-nm peak to the 340-nm absorption, but the kinetics are not cleanly first order. An approximate first-order rate constant of 0.3–0.5 s^{-1} can be obtained from the data at short times, but the analysis is complicated by continuing changes in absorbance.

Scheme I, or a modified version to include the effects of Na^+–K^+ exchange, accounts for the major observed spectral changes. However, other changes also occur which will require additions to this simple phenomenological scheme. Full analysis of these data including the use of principal component techniques[14] is underway and should further our understanding of the mechanism of tryptophanase catalysis.

References and Notes

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(11) Experiment A: tryptophanase (2.04 mg ml^{-1}) in 1 mM *N*,*N*-bis(2-hydroxyethyl)glycine, pH 8.53, 0.2 M KCl, 15 μM pyridoxal-P, 1 mM EDTA, and 0.2 mM dithiothreitol was pushed against 50 mM 2(*N*-morpholino)ethane sulfonate, pH 6.72, 0.2 M KCl, 1 mM EDTA, and 15 μM pyridoxal-P. Experiment B: tryptophanase (2.06 mg ml^{-1}) in 1 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate, pH 7.38, 0.2 M KCl, 15 μM pyridoxal-P, 1 mM EDTA and 0.2 mM dithiothreitol was pushed against 50 mM 2-(*N*-cyclohexylamino)ethane sulfonate, pH 9.30, 0.2 M KCl, 1 mM EDTA, and 15 μM pyridoxal-P. Experiment C: tryptophanase (2.4 mg ml^{-1}) in 50 mM *N*-2-hydroxyethylpiperazine propanesulfonate, pH 8.0, in 1 mM EDTA, 0.2 mM dithiothreitol, 100 mM NaCl, and 80 μM pyridoxal-P was pushed against 50 mM *N*-2-hydroxyethylpiperazine sulfonate, 1 mM EDTA, 0.2 mM dithiothreitol, and 100 mM KCl.
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Resonance Raman Studies on Pyrocatechase

Sir:

The intradiol dioxygenases, pyrocatechase and protocatechuate 3,4-dioxygenase, catalyze the cleavage of catechols to yield *cis,cis*-muconic acids.[1] These enzymes contain high-spin ferric iron in the active site and are characterized by a broad absorption band centered near 450 nm (ϵ_M ~3000/Fe). Upon substrate binding, the absorption maximum shifts to the red, accompanied by a significant increase in absorbance in the region above 600 nm.[2,3] Recently, resonance Raman experiments on protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* have shown the presence of tyrosine in the iron coordination site.[4-6] Furthermore, Felton et al.,[6] using 514.5-nm excitation, observed the appearance of new Raman peaks in the spectra of enzyme–substrate and enzyme–inhibitor complexes which were assignable to the respective ligating species, while retaining the tyrosine peaks observed in the native enzyme. They concluded that tyrosine ligation was not altered upon substrate or inhibitor binding.

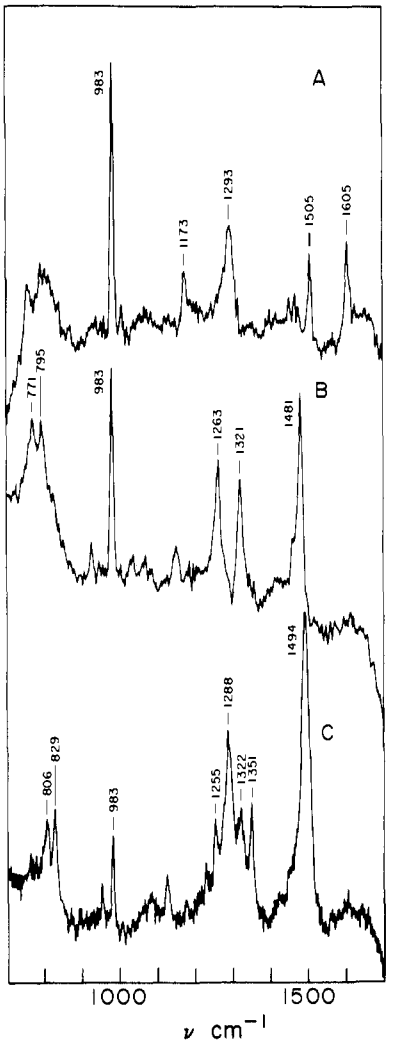

Figure 1. Raman spectra of (A) pyrocatechase; (B) the enzyme-catechol complex, prepared anaerobically, [catechol] = 10 mM; and (C) the enzyme-4-nitrocatechol complex, [4-nitrocatechol] = 0.5 mM. Spectra were obtained using 647.1-nm excitation from a Coherent Radiation Model 500K krypton ion laser and recorded on a Spex 1401 spectrometer interfaced to an Interdata 70 computer. Conditions: 100–150-mW power, 4-cm^{-1} slit width, 20–25 mg/mL of protein in Tris-OAc pH 8.5 buffer, 4 °C sample temperature. SO_4^{2-} was used as internal standard.

We report here the resonance Raman spectra of pyrocatechase from *Pseudomonas arvilla* C-1 and its complexes with catechol and 4-nitrocatechol. The enzyme was prepared according to the procedure of Fujiwara et al.[7] Spectra were obtained using the 647.1-nm line of a krypton laser; this wavelength was selected because of fluorescence problems at shorter wavelengths. Figure 1A shows the Raman spectrum of the native enzyme with peaks at 1173, 1293, 1505, and 1605 cm^{-1}. These vibrations are assigned to tyrosine, analogous to those observed for protocatechuate 3,4-dioxygenase[4,5] and the transferrins.[8,9] Pyrocatechase, thus, joins the list of iron proteins having tyrosine coordination.

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