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Trapping tyrosinase key active intermediate under turnover

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Raman experiments

The attempts to detect resonance Raman enhanced signals were carried out by using a Horiba Jobin Yvon T64000 spectrometer equipped with CCD camera detector system and equipped with 1800 gr/mm gratings. CW laser was generated by Spectra-Physics Millenia diode pumped solid state laser (12 W @ 532 nm) interfaced with a Sirah Matisse combined Ti:Sa Ring (700-990 nm, 1 W), which were then used as source for the external frequency doubler Spectra-Physics Wavetrain in which LBO crystals (754 and 802 nm) were used for generating output laser line at 370 nm, 380 nm, 390 nm and 410 nm, (30-50 mW on the sample). The spectra were obtained both on frozen solution (liquid nitrogen bath finger, at T = 77 K, in a EPR cell tube) and fluid solution (spinning NMR tube). The detection was performed in back scattering for the frozen solutions at 90° for the fluid solutions. Fluorescence by the sample was excessive at any wavelength and prevented detection of resonance enhanced signals.

Materials and enzyme preparation

Commercial 3,5-difluorophenol, from Sigma, was purified by HPLC on a semi-preparative Supelco LC18 column (250×10 mm). All other chemicals were from Sigma-Aldrich unless otherwise stated. Tyrosinase was obtained from liquid cultures of *S. antibioticus* carrying the Ty pIJ703 overexpression plasmid. The enzyme was purified from the growth medium following procedures published before.[11] The protein was stored at -80 °C at a concentration of 1 mg/ml in 100 mM phosphate buffer at pH 6.8 containing 20% glycerol as a cryoprotectant. The enzyme was extensively dialyzed against Hepes buffer at pH 6.8 before running the experiments in aqueous/organic medium. For the determination of the pH of the mixed aqueous-organic solvents used for enzymatic studies we followed the procedure described before.[1]

Kinetic studies

The catalytic oxidation of L-Dopa, dopamine, tyramine, and L-tyrosine methyl ester (L-TyrOMe) by *S. antibioticus* Ty was studied at various temperatures in the mixed solvent of 34.4% methanol/glycerol (7:1, v/v) and 65.6% (v/v) aqueous 50 mM Hepes buffer at pH 6.8, saturated with atmospheric oxygen, in the same conditions used before for the study of mushroom (*Agaricus bisporus*) Ty.[1] The kinetic experiments were performed at temperatures above 8 °C using a magnetically stirred and thermostatted cell (1-cm path length), while at lower temperatures an immersible fiber-optic quartz probe (5 mm) (HELLMA) fitted to a Schlenk vessel was used. Formation of the dopachrome derivatives of the different substrates was monitored spectrophotometrically, using an HP8452A diode-array instrument, through the increase of the characteristic absorption band at 476 nm. The extinction coefficients of the various dopachrome derivatives of the substrates were assumed to be equal to that of L-Dopa (3600 M^{-1} cm^{-1}); the effect

of the cryosolvent on the intensity and position of the absorption maxima of the dopachrome derivatives was negligible.

Inhibition of the enzymatic L-Dopa oxidation by 3,5-difluorophenol

The inhibitory activity of 3,5-difluorophenol on L-Dopa oxidation catalyzed by Ty was studied spectrophotometrically, in a magnetically stirred optical cell with 1-cm path length. The temperature was kept at 21±0.1 °C by a Haake cryostat with circulating fluid and directly checked inside the solution with a thermometer with a precision of ±0.1 °C. All of the experiments were performed in 200 mM phosphate buffer, pH 6.8, with an enzyme concentration of 1.3 nM. The inhibition effect was studied by varying the concentration of L-Dopa from 1.4 to 20 mM and keeping the concentration of the 3,5-difluorophenol constant at the values of 10, 20, 30, and 40 mM; the experiments in the absence of 3,5-difluorophenol were also performed. The formation of the dopachrome derivative of L-Dopa was followed by the increase of the band at 476 nm (ε = 3600 $M^{-1}cm^{-1}$). The rate in ΔA/s units was calculated from the maximum slope of the optical trace at this wavelength in the appropriate time range, usually within 40 to 80 s after mixing of the reagents. As before, absorbance readings were corrected for drifting of the baseline by subtracting the absorbance at 800 nm. The rate and double reciprocal plots are shown in Figure S1.

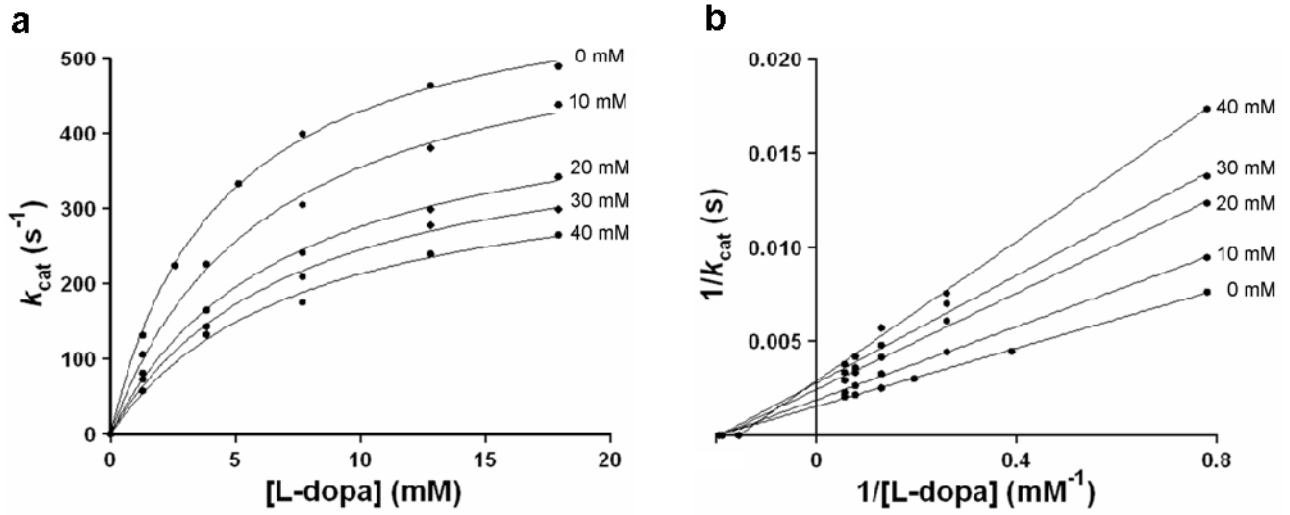


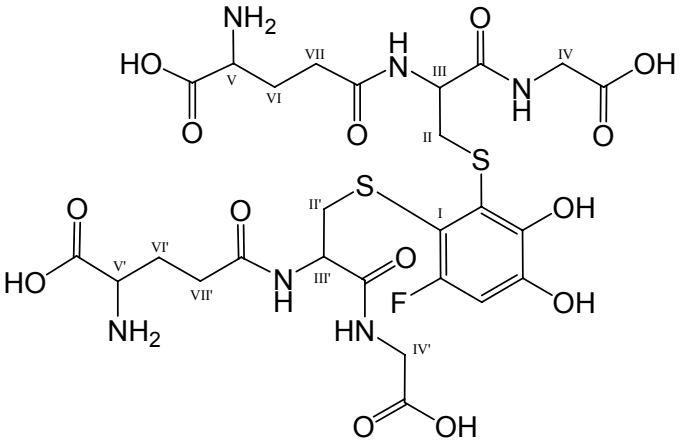
Figure S1. Inhibition of L-Dopa oxidation by 3,5-difluorophenol, studied in 200 mM phosphate buffer, pH 6.8, at 21±0.1 °C. (a) Plots of the rate *vs.* L-Dopa concentration in the presence of various concentrations of the inhibitor, and (b) the corresponding double reciprocal plots.

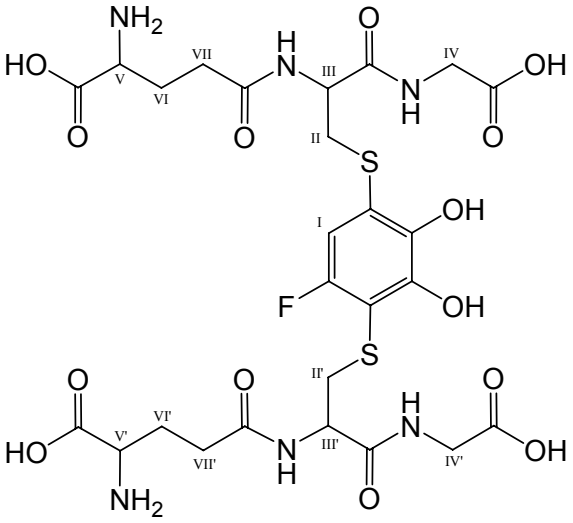
Glutathione conjugates of 3,5-difluoroquinone

The catalytic oxidation of 3,5-difluorophenol (22 mM) by tyrosinase (20 μM) was performed in the presence of L-glutathione (20 mM) in 100 mM phosphate buffer pH 6.8, at room temperature, in a magnetically stirred open vessel in order to keep the solution saturated with atmospheric oxygen. After 4 h reaction, the enzyme activity was quenched by adding a few drops of conc. HCl. The product mixture was purified by HPLC, using a Supelco LC18 semi-preparative column (250×10 mm). The elution was started with an aqueous solution containing 0.1% trifluoroacetic acid (TFA),

which was progressively changed to 80% water, 20% CH_3CN and 0.1% TFA over 20 min, and then to 100% CH_3CN containing 0,1% TFA over additional 10 min. The collected samples were rotary evaporated to dryness and analysed by MS and by ^{1}H- and ^{19}F-NMR in D_2O solutions. Small amounts of the crude reaction mixture were also directly analysed by HPLC/MS and ^{19}F-NMR. For elution in the HPLC/MS apparatus, 0.1% HCOOH in distilled water (solvent A) and 0.1% HCOOH in CH_3CN (solvent B) were used, with a flow rate of 0.2 ml/min; elution started with 100 % solvent A, followed by a linear gradient from 0 % to 100 % B in 60 min. To the sample to be used for ^{19}F-NMR analysis, 20 % D_2O was added for locking of the magnetic field, and 1000 scans were accumulated to increase the signal to noise ratio. Characterization data of the glutathione conjugates of 3,5-difluoroquinone are collected in Table S1.

Table S1. ^{1}H-NMR, ^{19}F-NMR, and MS characterization data for the glutathione conjugates of 3,5-difluoroquinone.

Product	**^{1}H-NMR (ppm)**
 **3**	6.80 (d, $J_{H\text{-}F}$ = 9.2 Hz; $H_{I,}$ 1H) 4.37 (dd, $J_{IIa\text{-}III}$ = 5.1 Hz, $J_{IIb\text{-}III}$ = 8.5 Hz; H_{III}, 1H) 4.27 (dd, $J_{II'a\text{-}III}$ = 4.5 Hz, $J_{II'b\text{-}III}$ = 8.5 Hz; $H_{III'}$, 1H) 3.75-3.65 (m, H_{IV}, $H_{IV'}$, H_V, $H_{V'}$, 4H) 3.34 (dd, $J_{IIa\text{-}IIb}$ = 14.5 Hz, $J_{IIa\text{-}III}$ = 5.1 Hz; H_{IIa} , 1H) 3.25 (dd, $J_{II'a\text{-}II'b}$= 14.5 Hz, $J_{II'a\text{-}III'}$ = 5.1 Hz; $H_{II'a}$, 1H) 3.13 (dd, $J_{IIa\text{-}IIb}$ = 14.5, $J_{IIb\text{-}III}$= 8.5; H_{IIb}, 1H) 3.05 (dd, $J_{II'a\text{-}IIb'}$ = 14.5, $J_{IIb'\text{-}III'}$ = 8.5; $H_{II'b}$, 1H) 2.36 (t, $J_{VI\text{-}VII}$= $J_{VI'\text{-}VII'}$ = 7.5, H_{VII}, $H_{VII'}$, 4H)
	^{19}F-NMR (ppm)
	-115.7 (d, $J_{F\text{-}H}$ = 9.2 Hz)
	MS (amu)
	739.23 ($M+H^+$)
	^{1}H-NMR (ppm)



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6.75 (d, $J_{H\text{-}F}$ = 9.9 Hz; $H_{I,}$ 1H)
4.22 (dd, $J_{IIa\text{-}III}$ = 4.3 Hz, $J_{IIb\text{-}III}$ = 8.5 Hz; H_{III}, $H_{III'}$, 2H)
3.74 (m, H_V, $H_{V'}$, 4H)
3.33 (dd, $_{IIa\text{-}IIb}$ = 14.5 Hz, $J_{IIa\text{-}III}$ = 4.4 Hz; H_{2a}, 1H)
3.26 (dd, $J_{II'a\text{-}II'b}$ = 14.5 Hz, $J_{II'a\text{-}III'}$ = 4,4 Hz; $H_{II'a}$, 1H)
3.16 (dd, $J_{IIa\text{-}IIb}$ = 14.5 Hz, $J_{IIb\text{-}III}$ = 8.5 Hz; H_{IIb}, 1H)
3.08 (dd, $J_{II'a\text{-}IIb'}$ = 14.5 Hz, $J_{IIb'\text{-}III'}$ = 8.5 Hz; $H_{II'b}$, 1H)
2.38 (t, $J_{VI\text{-}VII}$ = $J_{VI'\text{-}VII'}$ =7.5 Hz; H_{VII}, $H_{VII'}$, 4H)

^{19}F-NMR (ppm)

-110 (d, $J_{F\text{-}H}$ = 9.9 Hz)

MS (amu)

739.23 ($M+H^+$)

Trapping the enzyme ternary complex

The experiments were performed using a 1-cm path-length quartz cell with Schlenk connections, kept under magnetic stirring, and operating at the appropriate temperature by external fluid circulation provided by a Haake cryostat. Tyrosinase (35 μM) was dissolved in the mixed solvent of 35 % methanol, 20% glycerol and 45 % 50 mM Hepes buffer (v/v/v), pH 6.8. The exact concentration of the enzyme was determined by the absorbance at 280 nm,[2] neglecting the eventual change in extinction coefficient in the mixed solvent. The deoxy form of the protein was obtained by removing molecular oxygen from the solution through several vacuum/argon cycles at -20 °C, and then adding 10 equiv. NH_2OH.[3] Conversion to the oxy form of the enzyme was achieved by exposing the solution to air. The enzyme solution was brought to -30 °C, controlling the temperature inside the cell with a thermometer, and allowing equilibration for 30 min. Then, the reaction was started by adding a pre-chilled, concentrated (1 M) solution of 3,5-difluorophenol in the same mixed solvent, while stirring, to reach a final concentration of 70 mM. The oxidation process was monitored by recording spectra every 0.5 s in the range between 300 and 800 nm for about 10 min. The spectra of deoxyTy, oxyTy, and oxyTy immediately after the addition of 3,5-difluorophenol are shown in Figure S2.

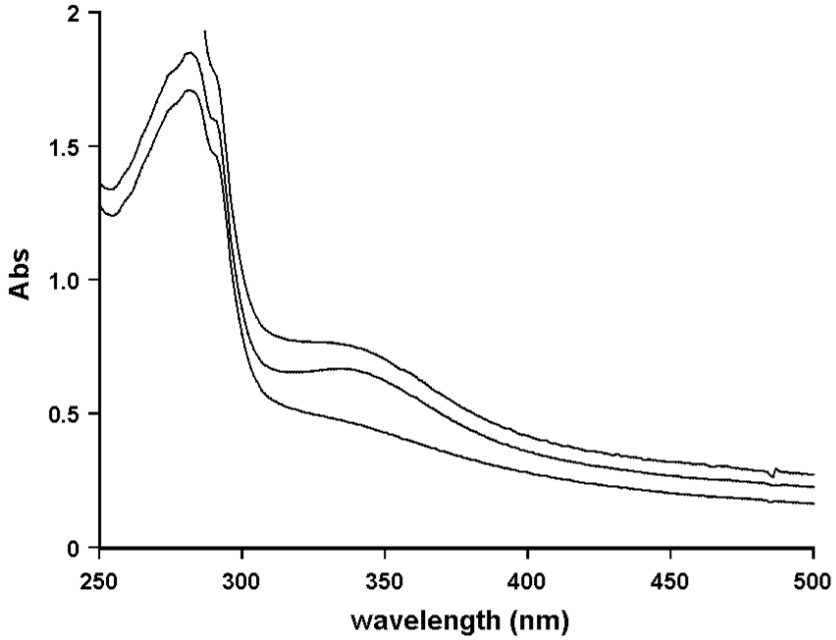

Figure S2. UV-Vis spectra recorded at -30 °C of *S. antibioticus* deoxytyrosinase (35 μM, lower trace), oxytyrosinase (middle trace), and oxytyrosinase immediately after the addition of 3,5-difluorophenol (70 mM, upper trace) in the mixed solvent of 35 % methanol, 20% glycerol and 45% 50 mM Hepes buffer pH 6.8 (v/v/v). For better reading, the middle and upper traces were slightly moved upwards.

1 A. Granata, E. Monzani, L. Bubacco, L. Casella, *Chem. Eur. J.* 2006, **12**, 2504–2514.

2 P. Jackman, A. Hajnal, K. Lerch, *Biochem. J*. 1991*,* **274**, 707-713.

3 A. W. Tepper, L. Bubacco, G. W. Canters, *J. Biol. Chem.* 2002*,* **277**, 30436-30444.