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Original scientific paper

A calorimetric investigation for the bindings of mushroom tyrosinase to *p*-phenylene-bis(dithiocarbamate) and to alkyl xanthates

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Abstract: A comprehensive, simple and rapid thermodynamic study on the interaction of mushroom tyrosinase, MT, with three isoalkyldithiocarbonates (xanthates) as sodium salts, $C_3H_7OCS_2Na$ (**I**), $C_4H_9OCS_2Na$ (**II**), and $C_5H_{11}OCS_2Na$ (**III**), with *p*-phenylene-bis(dithiocarbamate) (**IV**), was performed using isothermal titration calorimetry to clarify the thermodynamics of these bindings as well as structural changes of the enzyme due to its interaction with inhibitors at 300 K in phosphate buffer (10 m mol L^{-1}; pH 6.8). The extended solvation theory was used to elucidate the effect of the inhibitors on the stability of the enzyme. The obtained results indicated that there are two identical and non-cooperative binding sites for these inhibitors.

Keywords: mushroom tyrosinase; isopropyl xanthate; isobutyl xanthate; isopentyl xanthate; *p*-phenylene-bis(dithiocarbamate); extended solvation theory.

INTRODUCTION

Tyrosinase is a bifunctional, copper-containing mono-oxygenase catalysing the *o*-hydroxylation of monophenols to the corresponding catechols and the oxidation of catechols to the corresponding *o*-quinones.[1] *o*-Quinones undergo some reactions that result in the formation of biopolymers such as melanin.[2] Tyrosinase is ubiquitously distributed among animals, plants and fungi. Mushroom tyrosinase (MT) is popular among researchers as it is commercially available and inexpensive.[3] In mushrooms as well as in fruits and vegetables, this enzyme is responsible for browning, a commercially undesirable phenomenon.[4] Therefore, tyrosinase inhibitors have attracted interest recently due to undesired browning in vegetables and fruits in post-harvest handling. Among the inhibitors, a distinction

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can be made between chelators of two Cu^{2+} in the active site.[5] The well-known heavy metal chelators, derivatives of dithiocarbamate, have been found to possess a wide range of biological activities of mushroom tyrosinase. Special interest in the study of metaldithiocarbamates was aroused because of the striking structural features presented by this class of compounds and also because of their diverse applications, such as high pressure lubricants in industry, fungicides and pesticides and as accelerators in vulcanization.[6] Bismuth and chromone dithiocarbamate complexes show antitumor activity.[7,8] The inhibitory effects of xanthates on mushroom tyrosinase was elucidated, which is related to the chelating of the copper ions at the active site by a negative head group (S^-) of the xanthate anion. The inhibitory effects of three synthetic *n*-alkyl xanthates sodium salts with C_3, C_4 and C_5 aliphatic tails were described. Lineweaver–Burk plots showed different patterns of mixed inhibition for *p*-phenylene-bis(dithiocarbamate) and isopropyl xanthate and competitive inhibition for isobutyl and isopentyl xanthate.[4,9] In view of the increasing importance of controlling tyrosinase activities, we applied isothermal titration calorimetry (ITC) was applied in the present study to obtain thermodynamic parameters for the interaction between mushroom tyrosinase and inhibitors.

EXPERIMENTAL

MT was obtained from Sigma and the inhibitors were synthesized. All other materials and reagents were of analytical grade and solutions were made in 10 mmol L^{-1} phosphate buffer (pH 6.8) using double-distilled water.

The isothermal titration calorimetric experiments were performed with a four-channel commercial microcalorimetric system, Thermal Activity Monitor 2277 (Thermometric, Sweden). The microcalorimeter is composed of two identical cells, a reference cell and a sample cell of 1.8 mL in volume, which were made of a highly efficient thermal conducting material surrounded by an idiabetic jacket. The sample cell was loaded with mushroom tyrosinase solution (8.3 μmol L^{-1}) and phosphate buffer solution (10 mmol L^{-1}) and the reference cell contained buffer solution. The titration of MT with isopropyl xanthate (**I**), isobutyl xanthate (**II**), isopentyl xanthate (**III**) or *p*-phenylene-bis(dithiocarbamate) (**IV**) involved 20 consecutive injections and each injection included 20 μL of inhibitor. To correct the thermal effects due to ligand dilution, control experiments were performed in which identical aliquots were injected into the buffer solution with the exception of enzyme. The precision of measured heats were ±0.1 μJ or better.

RESULTS AND DISCUSSION

It was shown previously that the heats of interactions between a biomolecule and ligand in aqueous solvent systems could be analyzed by the following equation:[10–14]

$$q = q_{max} x'_B - \delta^{\theta}_A (x'_A L_A + x'_B L_B) - (\delta^{\theta}_B - \delta^{\theta}_A)(x'_A L_A + x'_B L_B) x'_B \quad (1)$$

where:

$$x'_B = \frac{px_B}{x_A + px_B} \quad (2)$$

x'_B is the fraction of the inhibitor bound to the binding sites and $x'_A = 1 - x'_B$ is the fraction of unbound inhibitor. x_B can be expressed as the concentration of the inhibitor after each injection, $[L]$, divided by the maximum concentration of the inhibitor, $[L]_{max}$, upon saturation of the entire enzyme.

$$x_B = \frac{[L]}{[L]_{max}} \quad (3)$$

p = 1 means that the inhibitor binds at each site independently and that the binding is non-cooperative. L_A and L_B are the relative contributions of unbound and bound inhibitor in the heats of dilution with the exclusion of MT and can be calculated from the heats of dilution of inhibitor in buffer as follows:

$$L_A = q_{dilut} + x_B\left(\frac{\partial q_{dilut}}{\partial x_B}\right),\ L_B = q_{dilut} + x_A\left(\frac{\partial q_{dilut}}{\partial x_B}\right) \quad (4)$$

The agreement between the experimental data and the data calculated *via* Eq. (1) (Fig. 1) supports the extended solvation model. The values of δ_A^θ and δ_B^θ obtained from the coefficients of the second and third terms of Eq. (1) are the

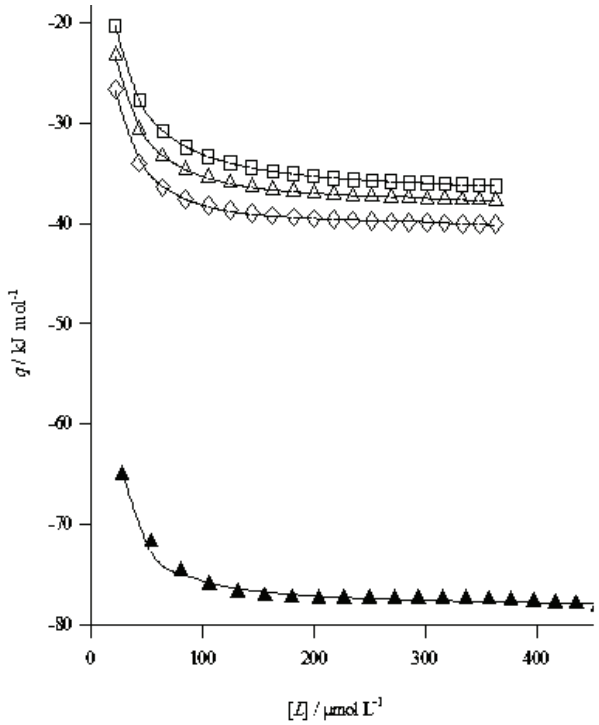

Fig. 1. Comparison between the experimental heats, q, for the interaction of mushroom tyrosinase with isopropyl (□), isobutyl (Δ) and isopentyl (◊) xanthate, and *p*-phenylene-bis(dithiocarbamate) (▲) and the data calculated *via* Eq. (1) (lines).

indexes of MT structural changes due to the reaction with the inhibitor in low and high concentrations, respectively. The superscript θ in all cases refers to the quantities at infinite dilution of the solute. The negative values of δ_A^θ and δ_B^θ show that isopropyl, isobutyl, isopentyl xanthate and *p*-phenylene-bis(dithiocarbamate) destabilize the MT structure.

In competitive inhibition, the amount of enzyme inhibition depends upon the inhibitor concentration; hence, the large changes in the δ_A^θ and δ_B^θ values for isobutyl and isopentyl xanthate with increasing inhibitor concentration can be attributed to the competitive manner of inhibition, which are in good agreement with the kinetic results.

In mixed inhibition, the inhibitor can bind to the enzyme at the same time as its substrate, although, the binding of the inhibitor affects the binding of the substrate, and *vice versa*. This type of inhibition can be reduced, but not overcome, by increasing the concentration of the substrate. Although it is possible for mixed-type inhibitors to bind in the active site, this type of inhibition generally results from an allosteric effect where the inhibitor binds to a different place on the enzyme. Inhibitor binding to this allosteric site changes the conformation of the enzyme so that the affinity of the substrate for the active site is reduced. Mixed inhibition occurs when contributions of competitive and uncompetitive are both available. In mixed inhibition with a predominantly competitive component, the obtained δ_A^θ and δ_B^θ values are different, such as in the inhibition of MT by *p*-phenylene-bis(dithiocarbamate), but when the uncompetitive component is dominant, the obtained δ_A^θ and δ_B^θ values are similar to each other, such as in the mixed mode of inhibition of MT by isopropyl xanthate (Table I). Double reciprocal Lineweaver–Burk plots confirmed the mixed inhibition by *p*-phenylene-bis(dithiocarbamate) and *iso*-propyl xanthate.[4,9]

TABLE I. Thermodynamic parameters for the interaction of isopropyl (I), isobutyl (**II**) and isopentyl (**III**) xanthates, and *p*-phenylene-bis(dithiocarbamate) (**IV**) with mushroom tyrosinase. The same values of δ_A^θ and δ_B^θ indicate mixed inhibition with the same contribution of competitive and uncompetitive component of MT by **I** and the different values for **IV** show a larger contribution of the competitive component in the mixed inhibition of MT by **IV**. The great changes of δ_A^θ and δ_B^θ are in good agreement with competitive inhibition of MT by **II** and **III**

Compound	**I**	**II**	**III**	**IV**
p	1	1	1	1
g	2±0.02	2±0.02	2±0.02	2±0.01
K_a / mol^{-1} L	9.07×10^4±24	1.26×10^5±12	1.68×10^5±12	3.3×10^5±56
ΔH / kJ mol^{-1}	–18.70±0.06	–19.30±0.07	–1.16±0.03	–39.23±0.12
ΔG / kJ mol^{-1}	–28.47±0.12	–29.28±0.14	–30.02±0.13	–31.7±0.09
ΔS / kJ mol^{-1} K^{-1}	0.03±0.01	0.03±0.01	0.10±0.02	–0.025±0.002
δ_A^θ	–4.99±0.02	–4.47±0.06	–4.23±0.06	–7.4±0.05
δ_B^θ	–4.23±0.02	–6.58±0.08	–8.66±0.08	–16.7±0.04

For a set of identical and independent binding sites, there are three different methods of ITC data analysis for providing the dissociation-binding constant (K_d). In the first method, using Eq. (5):[15–17]

$$\frac{\Delta q}{q_{max}}[M] = \frac{\Delta q}{q}[L]\frac{1}{g} - \frac{K_d}{g} \quad (5)$$

where $\Delta q = q_{max} - q$. q Represents the heat value at a certain inhibitor and MT concentration, $[M]$ and $[L]$ are the concentrations of MT and the inhibitor, respectively and q_{max} represents the heat value upon saturation of all MT molecules. The obtained results suggest a set of two binding sites (g = 2) with non-cooperativity for each inhibitor. The related plot for the binding of MT to *p*-phenylene--bis(dithiocarbamate) is shown in Fig. 2 as an example of the employment of Eq. (5). The best linear plot was obtained using −1171.5 μJ (equal to −78.4 kJ mol^{-1}). If q and q_{max} are calculated per mole of MT, then the molar enthalpy of binding for each binding site (ΔH) will be:

$$\Delta H = \frac{q_{max}}{g}$$

The calculated K_a ($1/K_d$), g and ΔH are reported in Table I.

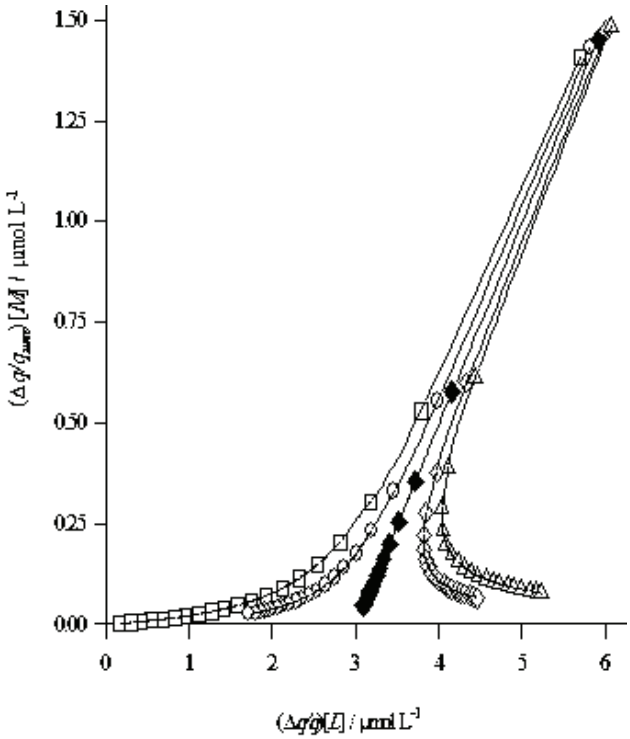

Fig. 2. The best linear plot of ($\Delta q/q_{max}$) [M] *vs.* ($\Delta q/q$) [L], according to Eq. (5), using the values of –1164 μJ ( ), –1168 μJ (○), –1171.5 μJ (♦), –1175 μJ (◊) and –1176 μJ (Δ) for q_{max} to obtain the best correlation coefficient value for a linear plot.

In the second ITC data analysis method, a simple linear plot of q/[L] *vs.* q can be used for the determination of the association equilibrium constant and the molar enthalpy of binding by using Eq. (6):

$$\frac{q}{[L]} = K_a(\Delta H - q) \tag{6}$$

A plot of q/[L] *vs.* q for *p*-phenylene-bis(dithiocarbamate) is shown Fig. 3 as a typical example. The values of K_a and ΔH obtained from the axis intercept and slope are in good agreement with the results obtained from Eq. (5).

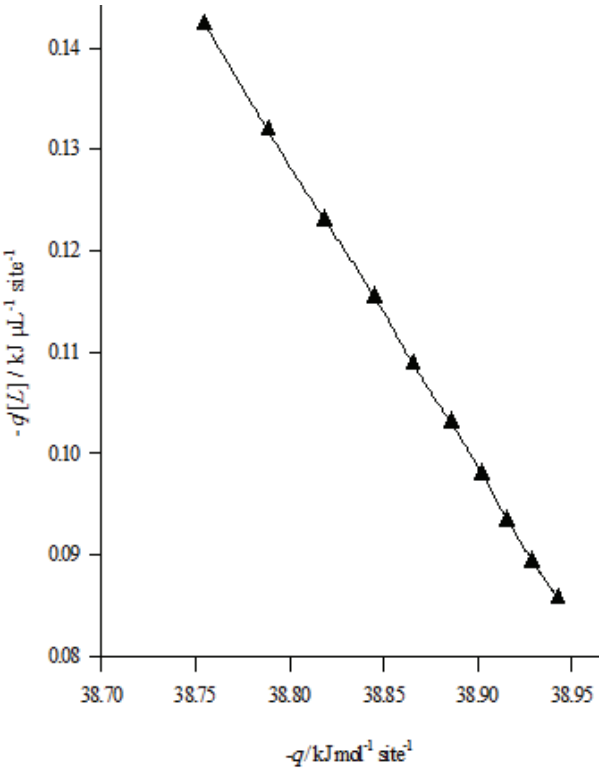

Fig. 3. The linear plot of q/[L] *vs.* q according to Eq. (6) for the interaction between mushroom tyrosinase and *p*-phenylene-bis-(dithiocarbamate).

In third method, by a double reciprocal linear plot of $1/q$ *vs.* $1/[L]$, the values of K_d and ΔH can be calculated from the intercept of the axis using Eq. (7):

$$\frac{1}{q} = \frac{1}{\Delta H} + \frac{K_d}{\Delta H} \times \frac{1}{[L]} \tag{7}$$

As an example, the plot for *p*-phenylene-bis(dithiocarbamate) is shown in Fig. 4. The obtained results are markedly consistent with the results from the analysis of the ITC data *via* Eqs. (5) and (6).

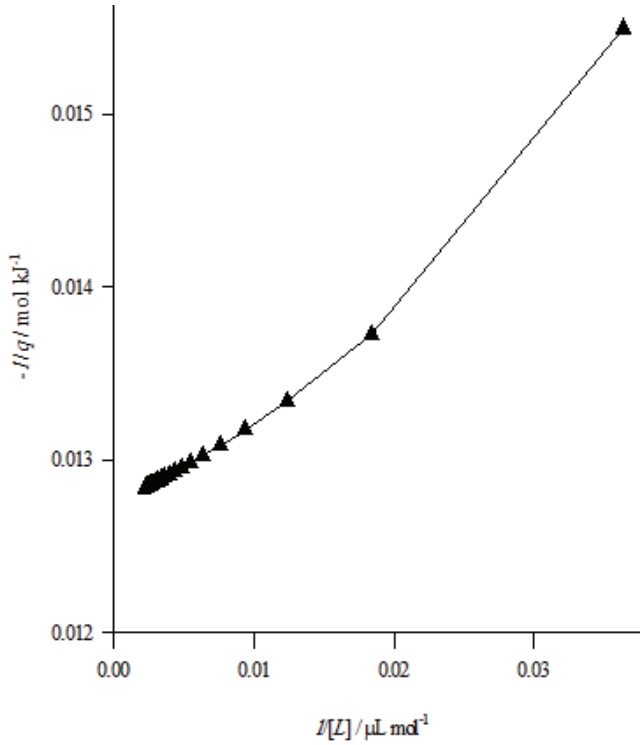

Fig. 4. The double reciprocal linear plot of 1/q *vs.* 1/[L] according to Eq. (7) for the interaction between mushroom tyrosinase and *p*-phenylene-bis(dithiocarbamate).

The change in the standard Gibbs free energy of binding (ΔG) was determined using K_a, the association-binding constant (the inverse of K_d) from the equation:

$$\Delta G = -RT\ln K_a \tag{8}$$

The change in standard entropy (ΔS) of this binding can be calculated as follows:[18,19]

$$\Delta S = \frac{\Delta H - \Delta G}{T} \tag{9}$$

The obtained values of ΔG and ΔS are given in Table I.

CONCLUSIONS

The extended solvation theory was used to determine the effects of the studied inhibitors on the thermal stability of MT. The agreements between the experimental heats and the results calculated *via* Eq. (1) are striking and support the extended solvation model. The results of this study also revealed the molar enthalpy and entropy, the standard Gibbs free energy changes and non cooperativity between two identical binding sites on MT for the three xanthates and *p*-phenylene-bis(dithiocarbamate). All the studied binding processes were spon-

taneous ($\Delta G < 0$). The obtained values of δ_A^θ and δ_B^θ were attributed to the type of inhibition. The different values of δ_A^θ and δ_B^θ showed the competitive inhibition of MT by **II**, **III** and **IV**.

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ИЗВОД

КАЛОРИМЕТРИЈСКО ИСПИТИВАЊЕ ВЕЗИВАЊА ТИРОЗИНАЗЕ ИЗ ПЕЧУРАКА ЗА *p*-ФЕНИЛЕН-BIS(ДИТИОКАРБАМАТ) И КСАНТАТ

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Једноставно и брзо термодинамичко испитивање интеракције тирозиназе из печурака, MT, са три изо-алкилдитиокарбоната (ксантата), као соли натријума, $C_3H_7OCS_2Na$ (**I**), $C_4H_9OCS_2Na$ (**II**), $C_5H_{11}OCS_2Na$ (**III**) и *p*-фенилен-бис(дитиокарбамат) (**IV**), применом изотермалне титрационе калориметрије, је урађено са циљем да се објасни термодинамички аспект формираних веза као и структурне промене ензима при интеракцији са инхибиторима на 300 K у фосфатном пуферу (10 m mol L^{-1}; *p*H 6,8). Проширена теорија солватације је коришћена да би се објаснио ефекат инхибитора на стабилност ензима. Добијени резултати указују да постоје два идентична и не-кооперативна везујућа места за испитиване инхибиторе.

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