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Thermodynamics and Kinetics of the Reaction between Pyridoxal-5-Phosphate and Hydrazides of 2-Methylfuran-3-Carboxylic and Thiophene-3-Carboxylic Acids in an Aqueous Solution

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Abstract—The stability constants of pyridoxal-5-phosphate hydrazones formed with 2-methylfuran-3-carbohydrazide and thiophene-3-carbohydrazide in an aqueous solution at pH 1.9, 6.6, 7.0, and 7.4 are determined via spectrophotometry. The kinetics of the processes of formation and hydrolysis of the Schiff bases are studied, and the constant of the direct and reverse reactions are calculated from the electronic absorption spectra. The stability constants of the Schiff bases are calculated from their ratio. The thermodynamic parameters of the reaction of formation (log K, ΔH , and $T\Delta S$) of both hydrazones at pH 6.6 are determined via calorimetry. The reasons for the differences between the equilibrium constants calculated from the data of spectrophotometric and kinetic experiments are discussed, and the reliability of the obtained results is analyzed.

Keywords: pyridoxal-5-phosphate, 2-methylfuran-3-carbohydrazide, thiophene-3-carbohydrazide, stability, enthalpy, rate constant

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INTRODUCTION

Hydrazones with different compositions and structures are of interest to researchers because of the wide possibilities for their use as sensors for anions and metal ions in analytical chemistry [1, 2], as antibacterial and antimicotic agents [2, 3], in switches of molecular machines [1] sensitive to the changes in pH and UV irradiation, and as catalysts [3]. Hydrazones, which are derivatives of vitamin B₆, pyridoxal, or its vitamer, pyridoxal-5-phosphate (PLP), have biological activity and can inhibit in particular the oxidation of ascorbic acid induced by copper(II) ions [4, 5].

The assumed useful properties of the hydrazones of pyridoxal-5-phosphate can be intensified by changing the hydrazide residue. The forecasting of biological activity performed using the PASS software [6-8] showed that the hydrazones formed by PLP and hydrazides of 2-methylfuran-3-carboxylic and thiophene-3-carboxylic acids (referred to below as 2MF3H and T3CH, respectively) are potential inhibitors of the beta-adrenergic-receptor kinase. Cardiac insufficiency is associated with the hyperactivity of beta-adrenergic receptors [9-11], and some authors consider the development of new inhibitors for them as a promising way of managing diseases of blood circulation [9].

The hydrolysis of hydrazones is capable of greatly altering their action on the body [12], so we must study the stability of Schiff bases along with the kinetics of their formation and decomposition.

The aim of this work was to determine the thermodynamic and kinetic characteristics of the reaction between PLP and 2MF3H and T3CH in an aqueous solution under conditions that mimic physiological conditions (pH 6.6–7.4 and an ionic strength of 0.25 [13]).

EXPERIMENTAL

Chemicals

Pyridoxal-5-phosphate (abcr GmbH, Germany) and 2-methylfuran-3-carbohydrazide, and thiophene-3-carbohydrazide (Sigma-Aldrich, United States) with the claimed purity of >99 wt % were used without preliminary preparation. The absence of impurities was monitored via ¹H NMR spectroscopy. Buffer solutions with pH values of 6.6, 7.0, and 7.4 were prepared using Na₂HPO₄ · 12H₂O and NaH₂PO₄ · 2H₂O (Spektr-Khim, Russia). A pH 1.9 was adjusted using preliminarily standardized solutions. The acidity of buffer solutions was controlled potentiometrically. All solutions were prepared using



Scheme 1. Formation of hydrazone as a result of the reaction between pyridoxal-5-phosphate and 2-methylfuran-3-carbohydrazide and thiophene-3-carbohydrazide.

bidistilled water ($\kappa = 1.7 \ \mu$ S/cm and pH 6.6). Ionic strength I = 0.25, close to the value of I in erythrocytes, was set using the components of a buffer mixture (at pH 6.6, 7.0, and 7.4) or KNO₃ (pH 1.9).

Instruments

Electronic absorption spectra of the solutions of PLP, 2M3FH, and T3CH with concentrations of 1.5×10^{-5} – 1.5×10^{-4} mol/L, and of solutions of PLP + 2MF3H and mixtures of PLP + T3CH, were recorded on a Shimadzu UV1800 double-beam spectrophotometer (United States) in the 190–500 nm range of wavelengths and absorbance. The corresponding buffers (distilled water for the pH 1.9) were used as blank solutions. The error in determining the wavelength did not exceed 0.5 nm, and the maximum error in measuring absorbance was ± 0.006 units. A temperature of 298.2 \pm 0.1 K was maintained using an external thermostat. Quartz cells 1 cm in size were used in this work.

The equilibrium constants and changes in the enthalpy of the reaction of formation of hydrazones at pH 6.6 were determined using a TAM III titration microcalorimeter (TA Instruments, United States) equipped with a 20-mL cell. The calorimeter was calibrated as described in [14].

Procedures for Determining Stability Constants

The formation of a hydrazone is described by Scheme 1.

The stability constants of the PLP–2MF3H and PLP–T3CH Schiff bases were determined according to the procedure described in [5], using the electronic spectra of nine to ten solutions, in which the ratio of the total concentrations PLP: 2MF3H and PLP : T3CH varied from 10 : 1 to 1 : 1. After preparation, all of the solutions were exposed for 24 h so that the system reached the equilibrium state. The spectral data were processed using the FTMT software (the principle of calculating the constants was described in

[5]). The initial concentrations of PLP were constant ((1.5 \pm 0.03) × 10⁻⁴ mol/L), while the total concentrations of 2MF3H and T3CH varied from (1.5 \pm 0.03) × 10⁻⁵ to (1.5 \pm 0.03) × 10⁻⁴ mol/L.

Calculations were performed using the experimental dependences of absorbance at three wavelengths on the total concentrations of the reagents (Figs. 1a, 1b).

The molar extinction coefficients of PLP, 2MF3H, and T3CH needed for calculations in FTMT at each pH and wavelength were preliminarily determined from the calibration plots. The values of ε were calculated by FTMT software during stability constant refining.

The stability constants and ΔH of the formation of PLP-2MF3H and PLP-T3CH hydrazones at a pH 6.6 were determined calorimetrically as described in [15]. An aliquot (15.00 mL) of a PLP solution with a concentration of $(7.29-8.66) \times 10^{-4}$ mol/L was placed into a calorimetric cell; a solution of 2MF3H or T3CH with a concentration of 0.04358-0.05949 mol/L in the corresponding buffer served as a titrant. The titrant was introduced into the cell via 15-20 injections (25 µL each). The heat effect of the dilution of the hydrazides per the volume of the cell was allowed for using a blank experiment, during which 15.00 mL of the buffer mixture was titrated with a solution of 2MF3H or T3CH. The results from each experiment were processed using the TAM Assistant program [16]. An example of data processing is presented in Fig. 2.

Procedure for Determining the Rate Constants of the Reaction

Our kinetic spectrophotometric experiments were performed in a manner similar to the one described in [15], except for the total concentrations. In this work, they were set at a level of $((1.5 \pm 0.03) \times 10^{-4} \text{ mol/L})$ for both reagents (PLP on one hand and 2MF3H or T3CH on the other). The concentrations of the hydrazone and both reagents at each point in time were calculated from the values of absorbance at a fixed wavelength using the molar extinction coefficients of the initial compounds and reaction products, determined

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Fig. 1. Electronic spectra of (a) PLP + 2MF3H and (b) PLP + T3H mixtures at pH (a) 1.9 and (b) 6.6. The total concentrations (PLP + hydrazide) are (1) 1.5×10^{-4} + 1.4×10^{-5} , (2) 1.5×10^{-4} + 2.9×10^{-5} , (3) 1.5×10^{-4} + 4.4×10^{-5} , (4) 1.5×10^{-4} + 5.9×10^{-5} , (5) 1.5×10^{-4} + 7.3×10^{-5} , (6) 1.5×10^{-4} + 8.7×10^{-5} , (7) 1.5×10^{-4} + 1.0×10^{-5} , (8) 1.5×10^{-4} + 1.2×10^{-5} , (9) 1.5×10^{-4} + 1.3×10^{-5} , and (10) 1.5×10^{-4} + 1.5×10^{-5} .

independently from the calibration curve and using the FTMT program, respectively. In order to determine the rate constants of the formation and hydrolysis of hydrazone, we processed the dependences of the concentration of PLP and a Schiff base on time using the Kinet program developed by Prof. A.V. Abramenkov [17, 18]. It was assumed that the following processes occurred:

$$PLP + Hydrazide \xrightarrow{k_1} SB,$$
(1)

$$SB \xrightarrow{\kappa_{-1}} PLP + Hydrazide,$$
 (2)



Fig. 2. Experimental (points) and calculations (solid line) curves of calorimetric titration of a 0.000866 mol/L solution of PLP with a 0.05949 mol/L solution of T3CH at pH 6.6.

where Hydrazide denotes 2MF3H or T3CH and SB denotes a Schiff base.

Examples of the calculated and experimental dependences of absorbance on time are presented in Figs. 3a, 3b, along with the kinetic curves.

At a pH 1.9, it was impossible to reliably determine the kinetic characteristics of the formation and hydrolysis of the hydrazone, due to the rapid rate of the process.

RESULTS AND DISCUSSION

The results from determining equilibrium constants from spectrophotometric data (K_{sph}), along with the rate constants of the formation (k_1) and hydrolysis (k_{-1}) of Schiff bases at different values of pH are summarized in Table 1. The table also presents the values of the equilibrium constants determined by dividing the rate constant of the direct reaction by k of the reverse reaction (K_{kin}), along with the values of the Fischer criterion (F) that characterize the adequacy of the mathematical model of the experiment. The results from our calorimetric investigation of the formation of the hydrazones at pH 6.6 are summarized in Table 2.

The values of the errors (Tables 1 and 2) are the half-width of the confidence interval at a confidence probability of 0.95 and a sample size of three experiments.

The stability of PLP–2MF3H and PLP–T3CH hydrazones was high at all considered values of pH of the medium. For the Schiff bases of pyridoxal-5-phosphate studied so far, including the products of the interaction with the derivatives of pyridine and pyra-



Fig. 3. Experimental (points) and calculations (solid line) dependences of (a) absorbance at $\lambda = 297$ nm and (b) the equilibrium concentrations of PLP, 2MF3H, and hydrazone on time at a pH 7.0: (*1*) [PLP]_{exp}, (*2*) [2MF3H]_{exp}, (*3*) [SB]_{exp}, (*4*) [PLP]_{calc}, (*5*) [2MF3H]_{calc}, and (*6*) [SB]_{calc}.

zole [5, 19], $\log K$ values of around 4.2–5.2 are characteristic as well. The equilibrium constant of the formation of the Schiff bases of pyridoxal-5-phosphate thus depended relatively weakly on the nature of the aromatic ring in the hydrazide residue.

Both Schiff bases (PLP–2MF3H and PLP– T3CH) became less stable upon moving from a pH 6.6 to one of 7.4. The rate of formation of hydrazones fell considerably upon moving from a weakly acidic medium to a weakly alkaline medium.

In a weakly acidic medium, the values of $\log K$ determined via spectrophotometry and calorimetry were in good agreement among themselves for both

hydrazones. However, the values of the equilibrium constants determined in the kinetic experiment (using a known correlation) differed:

$$K = k_1 / k_{-1}.$$
 (3)

The difference between the stability constants of the hydrazones determined in the spectrophotometric and kinetic experiments was due to aspects of calculating the value of quantity k_{-1} , which is low. Process (2) can in all cases be excluded from the mathematical model used to describe the experimental data. This alters the value of the rate constant of the direct reaction, but the change is smaller than the statistical error in determining k_1 (compare Figs. 4 to 3).

It should be noted that excluding process (2) (which indeed proceeds in the considered system) appreciably alters neither the sum of the squares of the deviations of the experimental dependence of absorbance from the calculated dependence nor the value of the dispersion of adequacy nor the value of the Fischer criterion. Both models (1, which assumes the reversibility of the formation of hydrazone, and 2, which assumes the irreversible formation of a Schiff base) are therefore satisfactory. Based solely on the results of the F-test, we could make a false negative conclusion on the hydrolysis of hydrazone proceeding in the solution.

The values of k_{-1} determined directly from the results of the kinetic experiment can be unreliable. They should most likely be calculated using Eq. (3) with the values of log $K_{\rm sph}$ and k_1 (Table 1); i.e., $k_{-1} = k_1/K_{\rm sph}$.

There is also a risk of drawing false positive conclusions. For example, when the results of the investigation of the interaction of PLP with 2MF3H at pH 7.4 are processed, the following process can be identified instead of the hydrolysis of hydrazone:

$$2PLP \xrightarrow{k_2} 2MF3H. \tag{4}$$

A model including reaction (1) and admittedly absurd process (4) allows us to calculate k_2 and is also satisfactory $(k_1 = 26.0 \text{ L mol}^{-1} \text{ min}^{-1}, k_2 = 8 \times 10^{-4} \text{ L mol}^{-1} \text{ min}^{-1}, \text{ and } F = 0.01).$

False positive conclusions can also be drawn in calculating stability constants from spectrophotometric data. For example, when calculating the stability constant of PLP–2MF3H hydrazone at a pH 7.0, we can include the following process in the mathematical model, in addition to the formation of the Schiff base (Scheme 1):

$$3(2MF3H) \Leftrightarrow PLP,$$
 (5)

which even increases the accuracy of the model somewhat (F = 0.03).

The above examples demonstrate that the accuracy of a model does not provide grounds for considering it a true model [20]. Note that when investigating a sim-

Hydrazone	рН	1.94	6.6	7.0	7.4
PLP-2MF3H	$\log K_{\rm sph}(F)$	5.30 ± 0.10 (0.04)	5.41 ± 0.08 (0.03)	5.21 ± 0.08 (0.04)	4.93 ± 0.16 (0.08)
	k_1 , L mol ⁻¹ min ⁻¹ (F)	_	81 ± 3 (0.02)	43.8 ± 1.0 (0.01)	26.5 ± 1.1 (0.03)
	k_{-1}, \min^{-1}	—	$(8\pm2)\times10^{-4}$	$(4.3 \pm 0.5) \times 10^{-4}$	$(3.8 \pm 0.7) \times 10^{-4}$
	$\log K_{\rm kin}$	—	5.01 ± 0.15	5.01 ± 0.10	4.84 ± 0.11
PLP-T3CH	$\log K_{\rm sph}(F)$	5.27 ± 0.09 (0.10)	5.39 ± 0.11 (0.06)	4.78 ± 0.10 (0.04)	4.77 ± 0.12 (0.21)
	k_1 , L mol ⁻¹ min ⁻¹ (F)	—	67.3 ± 1.5 (0.03)	39.1 ± 1.0 (0.01)	24.1 ± 0.9 (0.01)
	k_{-1}, \min^{-1}	_	$(3.1 \pm 0.8) \times 10^{-4}$	$(1.4 \pm 0.6) \times 0^{-3}$	$(2.1 \pm 0.7) \times 10^{-4}$
	$\log K_{\rm kin}$	—	5.34 ± 0.14	4.45 ± 0.22	5.06 ± 0.19

Table 1. Stability and rate constants of the reaction of formation and hydrolysis of Schiff bases, derivatives of pyridoxal-5-phosphate and 2MF3H and T3CH, at different values of pH

ple system in which only the formation and hydrolysis of hydrazones proceed, there is little possibility of making a mistake. In more complex cases, however, where a set of reactions with unknown equilibrium constants is possible, the probability of drawing false conclusions increases.

The higher stability of both hydrazones at a pH 6.6, relative to solutions with pH 7.0 and 7.4, means that 2MF3H and T3CH can bond to PLP more in tissues whose intercellular fluid has a lower pH (e.g., certain tumors [21, 22] or ischemic tissues [23], which is of additional interest when we consider the potential activity of Schiff bases as inhibitors of beta-adrener-gic-receptor kinase).

The data in Table 2 show that the main contribution to the stability of the Schiff bases at pH 6.6 comes from the enthalpic component. Negative values of the change in the entropy are expected, since a hydrazone molecule has fewer degrees of freedom than the reagents.

CONCLUSIONS

The values of ΔH of the formation of the hydrazones of pyridoxal-5-phosphate and five-membered heterocycles (Table 2) are close to the change in the enthalpy of the reaction between PLP and isoniazid [15].



Fig. 4. Experimental (points) and calculations (solid line) dependences of (a) absorbance at $\lambda = 297$ nm and (b) the equilibrium concentrations of PLP, 2MF3H, and hydrazone on time at a pH 7.0: (1) [PLP]_{exp}, (2) [2MF3H]_{exp}, (3) [SB]_{exp}, (4) [PLP]_{calc}, (5) [2MF3H]_{calc}, and (6) [SB]_{calc}. The existence of a direct reaction is only assumed.

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Table 2. Stability constants and changes in the enthalpy and entropy of the reaction of formation of the Schiff bases of pyridoxal-5-phosphate and 2MF3H and T3CH at pH 6.6 and T = 298.2 K according to the data of isothermal titration calorimetry

Hydrazone	log K	−Δ <i>H</i> , kJ/mol	$-\Delta S$, J mol ⁻¹ K ⁻¹
PLP–2MF3H	5.41 ± 0.10	49.9 ± 1.0	63.7 ± 1.1
PLP-T3CH	5.42 ± 0.09	50.8 ± 1.0	67.3 ± 1.1

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