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Study of pH and temperature-induced transitions in urate oxidase (Uox-EC1.7.3.3) by microcalorimetry (DSC), size exclusion chromatography (SEC) and enzymatic activity experiments

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Abstract

Purified recombinant urate oxidase (urate oxygen oxidoreductase EC 1.7.3.3 re-Uox) has been studied by means of differential scanning calorimetry (DSC) in correlation with enzymatic activity measurements and size exclusion chromatography. Differential scanning calorimetry curves versus pH show two endothermal effects in the pH range 6–10. The first endotherm reveals a maximum stability between pH 7.25 and pH 9.5 corresponding to a temperature of transition T_{m1} of 49.0° C and an enthalpy of transition of 326 kJ mol^{-1}. This value dramatically decreases below pH 7.25. The behavior of the second endotherm is more complex but the temperature of transition T_{m2} is constant between pH 9 and 7.25 and a maximum for the corresponding enthalpy is obtained near pH 8 with $\Delta H_2 = 272$ kJ mol^{-1}. An optimal pH of 8.0 for the stability of the enzymatic activity at elevated temperature was also found which was in good agreement with calorimetric results. Reversibility of the first endotherm is obtained from 20 to 51.5° C. The calorimetric result is correlated to enzymatic activity, purity by size exclusion chromatography (SEC) and protein concentration measurements. In contrast, for the second endotherm, after heating up to 68.9° C, no reversibility was found. Interaction with structural analogues of urate has been studied by DSC. 8-Azahypoxanthine has only a small effect and caffeine has no effect at all. With 8-azaxanthine, a rapid increase of the T_{m1} function of the concentration is obtained. At high concentration T_{m1} reached the T_{m2} value which remained unaffected.

Keywords: Urate oxidase; Thermal behavior; pH; Inhibitor interaction; Differential scanning calorimetry; Size exclusion chromatography; Enzymatic activity

1. Introduction

Urate oxidase (urate oxygen oxidoreductase EC 1.7.3.3 Uox), an enzyme derived from *Aspergillus flavus* which oxidises uric acid to allentoin [1] has been used therapeutically for the prevention or treatment of hyperuricemia during chemotherapy [2] for the last twenty years.

The cDNA encoding this protein has been cloned recently and expressed in *E. coli* [3], *A. flavus* [4] and *S. cerevisiae* [5]. The higher yield obtained with genetically engineered *S. cerevisiae* has favoured the

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choice of yeast for the production of recombinant urate oxidase (re-Uox). The recombinant enzyme accumulated intracellularly in a soluble and active form is extracted then purified to pharmaceutical grade level. The enzyme, a tetrameric protein N4 with identical subunits of a molecular weight of about 32000 is identical to the natural *A. flavus* Uox [6]. The monomer has no disulfide bridges and is N-terminal acetylated [3].

During the early development of peptide and protein drugs, knowledge of the stability of the active ingredient is necessary. To detect product degradation many different analytical methods can be employed. However, the three-dimensional structure of the protein may complicate the interpretation of the results. Each protein is unique and has to be studied on a case by case basis. To detect rapidly the conformational changes in solution, we find that differential scanning calorimetry is a good initial approach. The presence of an endothermal effect is generally attributed to the disruption of the tertiary structure [7], and an exothermic effect may be correlated with aggregation of the protein [8].

In this work, a microcalorimetric study was performed in order to determine the thermal behavior of re-Uox solutions at different pH's. Correlation to several techniques such as size exclusion chromatography (SEC) and enzymatic activity studies was carried out. Finally, interaction with structural analogues of uric acid was studied.

2. Materials and methods

2.1. Materials

Purified recombinant re-Uox was obtained from Sanofi Recherche (Labège, France). All other reagents were analytical grade.

2.2. Differential scanning calorimetry (DSC)

2.2.1. Effect of pH

Calorimetry scans were performed with a Mettler TA 2000 B heat flow apparatus equipped with a 'home-made' sensor. The performance and calibration of the system (sensitivity 0.2 μW) have been previously described [9]. The volume of the cell was 120 μl. The instrument was flushed with argon. All experiments were performed with a heating rate of 1° C min^{-1}.

For stability studies over a wide pH range, the following 50 mM buffers were used: sodium acetate, pH 4.0 to 6.0; sodium phosphate, pH 6.0 to 8.0; Tris–HCl, pH 8.5 to 9.0, and sodium carbonate, pH 9.5. The concentration of re-Uox was 19.55 mg ml^{-1}.

2.2.2. Reversibility of endothermic effect

For these calorimetric studies, a micro-DSC 'batch and flow' Setaram calorimeter was used with a 1-ml cell in order to have, after scanning, a sufficient volume for further analysis. Experiments were performed with a heating rate of 0.1 and 1° C min^{-1} at pH 8.0.

2.2.3. Interaction with structural analogues of uric acid

The same apparatus (micro-DSC Setaram) was used with a heating rate of 0.1° C min^{-1}. Solutions had a concentration in re-Uox of 4.5 mg ml^{-1} for both studies.

2.3. Size exclusion chromatography (SEC)

The protein content and its purity were measured by SEC with a Superose-12 column (Pharmacia). The sample was eluted with a 50 mM sodium phosphate buffer pH 8.0 at a flow-rate of 0.5 ml min^{-1}. Elution was monitored at 280 nm.

2.4. Enzymatic activity

Urate oxidase activity was assayed spectrophotometrically with a cell chamber regulated at 30° C, or other temperatures as necessary, by monitoring the disappearance of uric acid at 292 nm [3].

3. Results

3.1. Effect of the pH on the thermal denaturation and degradation of re-Uox by DSC

For the sample prepared at pH 7.8 (Fig. 1), two endothermic responses were observed. The thermo-

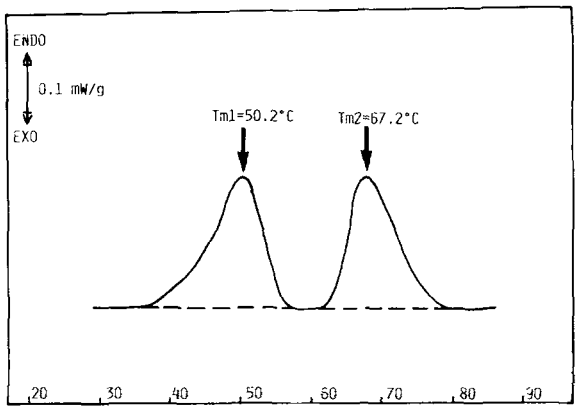

Fig. 1. Representative DSC curve of re-Uox at pH = 7.8 with a heating rate of 1° C min^{-1} and 19.55 mg of protein per ml (120 μl in a Mettler TA 2000 B apparatus).

gram shows a temperature T_{m1} at the top of the first endotherm of 50.1° C and a T_{m2} temperature at the top of the second endotherm of 68.1° C. The corresponding calorimetric enthalpies (ΔH_{cal}) were 346 kJ mol^{-1} and 273 kJ mol^{-1} (MW = 32000) respectively. The precision of the determination was 5%. All results are given in Table 1.

Figs. 2 and 3 show plots of the observed temperatures and the calorimetric enthalpies as a function of pH. The following comments can be made:

- The first endotherm shows a maximum stability between pH 7.25 and pH 9.5 and a dramatic decrease below pH 7.25.
- The behavior of the second endotherm is more complex, nevertheless the results reveal a plateau for T_{m2} between pH 7.25 and pH 9.0, and a maximum for the calorimetric enthalpy near pH 8.0.

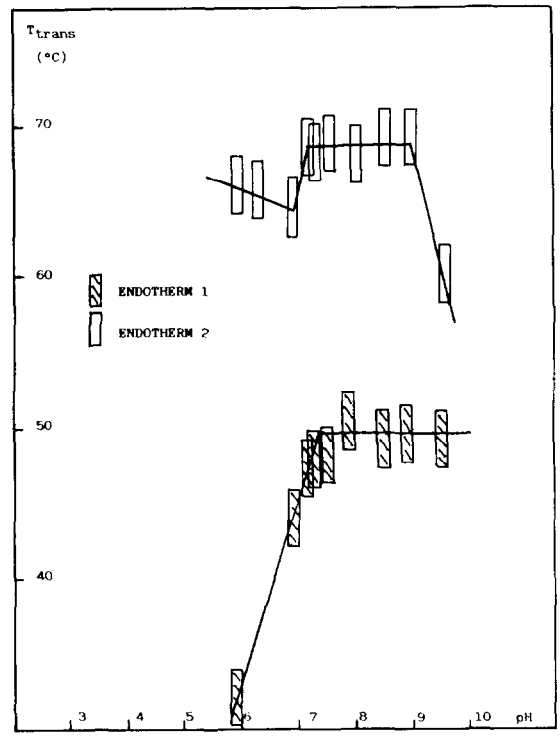

Fig. 2. Temperature of transition of re-Uox versus pH.

Table 1
Temperature and enthalpy of transition of re-Uox versus pH

pH	Buffer	T_{m1} (° C)	$\Delta H1$ (kJ mol^{-1})	$\Delta H_{v'H}$ (kJ mol^{-1})	T_{m2} (° C)	$\Delta H2$ (kJ mol^{-1})
9.50	Carbonate	49.7	344	500	60.6	247
9.05	Tris–HCl	49.7	321	536	68.9	112
8.80	Tris–HCl	49.9	335	514	69.3	107
8.50	Tris–HCl	49.7	330	521	69.3	124
7.80	Sodium phosphate	50.1	346	499	68.1	273
7.45	Sodium phosphate	48.1	310	550	68.4	242
7.25	Sodium phosphate	47.8	301	565	68.7	223
7.20	Sodium phosphate	47.7	317	536	68.9	201
6.95	Sodium phosphate	44.7	214	–	65.0	68
6.10	Sodium phosphate	40.0	18	–	66.1	264
5.95	Sodium acetate	32.6	29	–	65.4	281
5.80	Sodium acetate	–	–	–	66.2	262
4.45	Sodium acetate	–	–	–	–	–
4.00	Sodium acetate	–	–	–	–	–

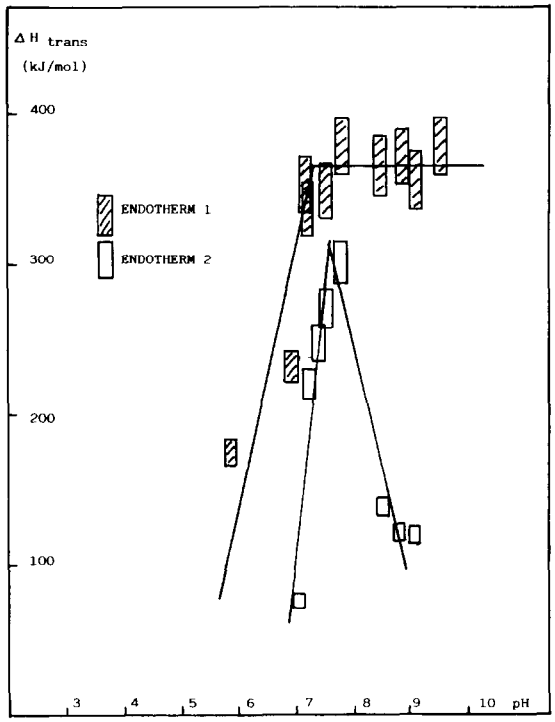

Fig. 3. Enthalpy of transition of re-Uox versus pH.

In a different experiment, samples of re-Uox were submitted to an accelerated stability study at 35° C for 2 hours, and 5 and 9 days. They showed a strong pH dependence on their degradation profile when analysed by isoelectrofocusing. Acidic pH's produced basic compounds, whereas basic pH's produced acidic compounds (results not shown). Size exclusion chromatography analysis revealed an accumulation of higher molecular weight aggregates under these extreme conditions. The results in Fig. 4

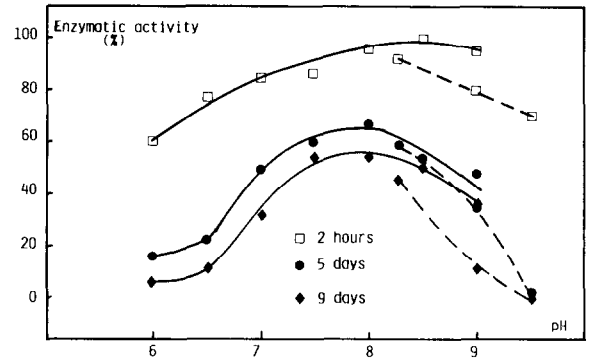

Fig. 4. Effect of pH on the enzymatic activity after incubation at 35° C. (———) Phosphate buffer, (– – –) carbonate buffer.

show the residual enzyme activity and reveal an optimum pH of 8.0 for stability; showing agreement with the calorimetry results.

3.2. Reversibility of endothermic responses by DSC

Studies of the reversibility of the first endotherm were performed from 20° C to 51.5° C at a heating rate of 0.1° C min^{-1} or 54.5° C at 1° C min^{-1} — the final temperature being the end of the first endothermic response. The temperature of the sample was then lowered to 20° C at the same rate. The enthalpy of the phenomenon decreases with the number of cycles in a time dependent manner (results not shown). T_{m1} was not changed, but visual observations of the cell content revealed that the protein was partially precipitated.

The reversibility of the second response was tested under the same conditions, but with a final temperature of 69° C, representing the end of the second response. No reversibility was observed.

Table 2
Study of the reversibility of the first endotherm

Final temperature (° C)	Re-Uox enzymatic activity (%)	Re-Uox SEC (%)	Specific activity (%)	Purity by SEC (%)
43.8	99.3	105.0	94.6	97.4
48.3	95.3	97.0	98.3	98.5
51.0	94.0	98.6	95.3	98.6
54.5	99.6	123.3	80.7	95.1
59.5	57.3	96.6	59.4	98.2
71.9	7.4	22.5	32.9	99.5

3.3. Correlation with enzymatic activity and purity by size exclusion chromatography

This study was conducted in a different manner in order to minimize losses due to the residence time of the sample in the calorimeter. For the study of the first endotherm the temperature was increased from 20° C to the chosen final temperature at a heating rate of 1° C min^{-1} and then rapidly decreased to 20° C at 9.9° C min^{-1}. The sample was then removed from the cell and enzymatic activity, the protein concentration and purity by size exclusion chromatography were determined.

Good reversibility was obtained for these parameters (Table 2) below 55° C. Above this temperature, enzymatic activity and protein concentration decreased until they became negligible at about 70° C, end of the exothermic response. The purity and retention time did not differ from that of the untreated control indicating that losses were due to precipitation. However, the decrease in specific activity indicated that soluble inactive material was formed during the inactivation process. Conley et al. [6] have studied the reversibility of *A. flavus* Uox denaturation by measuring the enzyme activity after preincubation at different temperatures. They also found that reversibility was reasonably good only up to 45° C. Similar results were obtained by Pitts et al. [10] with porcine Uox.

A study of the second endothermic response was also performed at a heating rate of 0.1° C min^{-1}. Under these experimental conditions the residence time in the calorimeter was longer, and as expected, the enzyme activity decreased faster than at the 1° C min^{-1} rate. As in the first experiment, the purity of the enzyme was the same as the untreated sample, and the specific activity dropped.

3.4. Enzymatic activity and aggregation as a function of temperature

The effect of temperature on the rate of uric acid oxidation by re-Uox was determined over a temperature range of 20 to 60° C (Fig. 5). The optimum temperature was found to be 30° C, in agreement with the value found by Laboureur and Langlois [1]. The enzyme was inactive at 50° C. Interestingly, this

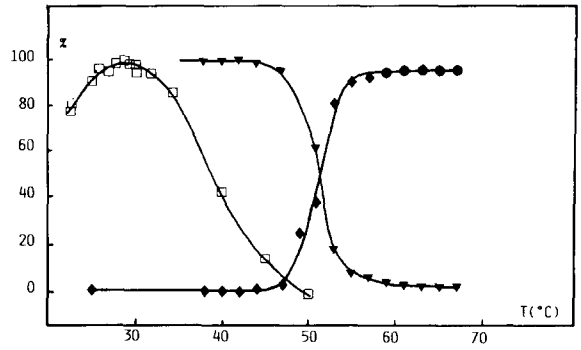

Fig. 5. Enzymatic activity (□), Uox native form percentage (▼), intermediate state in percentage (◆) and aggregate state (●) by SEC versus temperature.

temperature was near the end of the first endothermic response.

Size exclusion chromatography was carried out

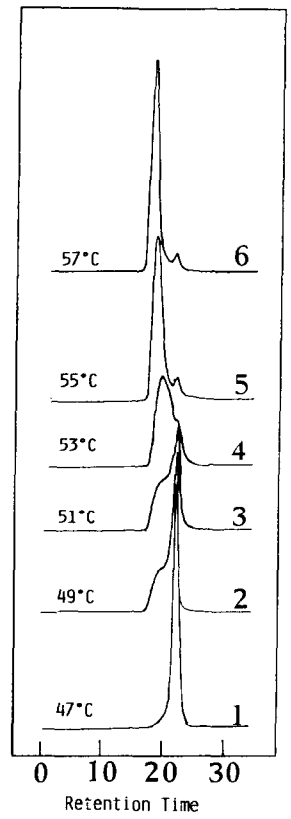

Fig. 6. Evolution of chromatographic profiles obtained for re-Uox at 6 temperatures.

with a completely calorifuged system (column, injector, detector). This method revealed that the area of the main peak decreased as a function of temperature to reach a negligible value at 55° C. At the same time, a new product appeared, with a hydrodynamic volume higher than the untreated product (Fig. 6). Based on the results obtained above, up to 55° C this new form is probably inactive and reversibly convertible to the active form when the temperature is lowered to 20° C. The presence of a higher molecular weight form was also noted as an impurity by Alamillo et al. [11] for the Uox from *Chlamydomonas reinhardtii* obtained by affinity chromatography, but there is no evidence to indicate that it was produced by the same denaturation process. Above 55° C, we found that the size of the aggregates increased to the point where they were excluded from the column.

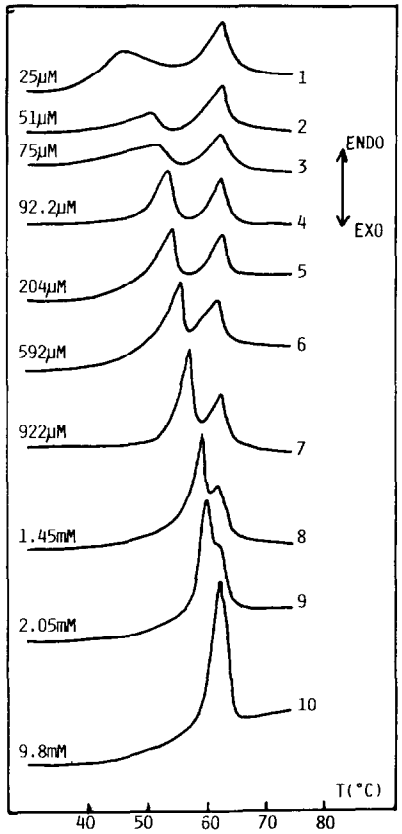

Fig. 7. DSC curves of re-Uox versus concentration of 8-azaxanthine at a heating rate of 0.1° C min^{-1} and 4.5 mg of protein per ml (850 μl in a micro-DSC Setaram apparatus).

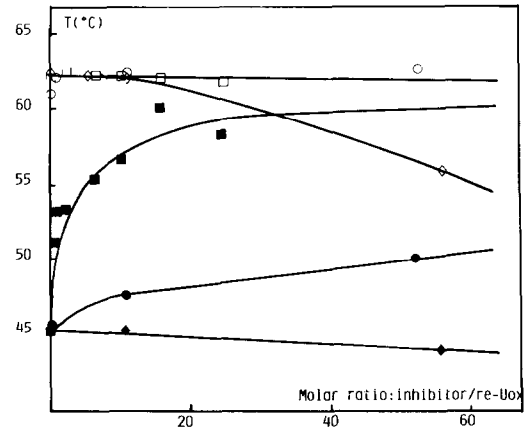

Fig. 8. Evolution of the temperatures of transition of the two endotherms versus molar ratio urate analogue/re-Uox (same experimental conditions as in Fig. 7). (■) T_{m1} 8-Azaxanthine, (□) T_{m2} 8-azaxanthine, (◆) T_{m1} caffeine, (◇) T_{m2} caffeine, (●) T_{m1} azahypoxanthine, (○) T_{m2} azahypoxanthine.

3.5. Interaction with structural analogues of uric acid

8-Azaxanthine is one of the most potent competitive inhibitors of *A. flavus* Uox activity, 8-azahypoxanthine-like hypoxanthine has only a small effect, and caffeine has no effect at all [12]. Evolution of DSC curves versus concentration of 8-azaxanthine are given in Fig. 7. Fig. 8 shows that the change of T_{m1} is a function of their concentration and inhibitory potency. 8-Azaxanthine caused a rapid initial increase of the temperature T_{m1} followed by a slower increase until it reached the T_{m2} value which remained unaffected. At higher concentrations, only one endothermic response was obtained with an enthalpy of 656 kJ mol^{-1} corresponding approximately to the sum of the two previous responses. The initial increase (if any) was low with 8-azahypoxanthine and the second slower effect was still observed. No effect was observed with caffeine.

4. Discussions

Double-peaked transition of proteins are often interpreted as showing the existence within the

molecule of two or more structural domains which unfolded independently.

Based on the consternation that the first endotherm is reversible and on the assumption that our DSC curves are close to the equilibrium value, we can use the Van't Hoff equation. The values of the Van't Hoff enthalpy listed in column 5 (Table 1) were calculated according to $\Delta H_{V'H} = 4RT_{1/2}^2\{C_{ex1/2}\}/\{\Delta H_1\}$. The value 4 corresponds to a simple two state process not involving association or dissociation. $T_{1/2}$ is $t_{1/2} + 273.15$ and $C_{ex1/2}$, the excess apparent heat capacity at $t_{1/2}$. ΔH_1 corresponds to the measured enthalpy of the thermal effect. In the pH range 9.5–7.2, the Van't Hoff enthalpy shows a constant value of 530 ± 40 kJ mol^{-1} close to the value obtained from SEC plot: 512 ± 60 kJ mol^{-1}.

These values were half of the calorimetric enthalpy ΔH_1 related to the tetrameric form N4 (1382 kJ mol^{-1}). The ratio $\Delta H_{V'H}/\Delta H_1$ being different than the unity, we may conclude that there is an intermediate state under the first endothemic response.

The form obtained between the two endothermal effects has a larger hydrodynamic volume than the initial form N4. More experiments are needed to characterize this form which is denatured irreversibly during the second endothermic response.

Concerning the study of interaction of re-Uox with competitive inhibitors, the increase of T_{m1} at constant re-Uox concentrations with an increasing total ligand concentration L_0 takes place with a variation in the extent of ligand binding.

A Van't Hoff plot for the DSC data at various 8-azaxanthine concentrations was performed. The logarithm of the ligand concentration was plotted as a function of T_{m1}^{-1}, the reciprocal of the temperature of the maximum of the thermal effect. According to Fukada et al. [13], for a molar ratio of one molecule of ligand, a value of $\Delta H_{V'H} = 314 \pm 14$ kJ mol^{-1} was found. This value is close to the calorimetric value related to the monomer of the first endotherm in the absence of ligands (326 kJ mol^{-1}). It provides good support for the theory that the process is approximately two-state in character when 8-azaxanthine is present.

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