Journal of Thermal Analysis and Calorimetry, Vol. 65 (2001) 737–743

SOLUBILIZATION AND HYDROLYSIS OF OVOTRANSFERRIN. SOLUBILIZATION OF α-CHYMOTRYPSIN
Enthalpy changes for three processes

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(Received in April 10, 2001)

Abstract

At 298.15 K, the solubilization of hen ovotransferrin at buffered pH 7.8 (0.08 M Tris·HCl buffer, containing 0.1 M $CaCl_2$) and the solubilization of α-chymotrypsin (from bovine pancreas) at non-buffered pH 3.0 (0.001 M HCl) both resulted in large exothermic reactions, being the apparent ΔHs –2485 in the first case and –780.1 kJ mol^{-1} in the second case, respectively. By contrast, the complete hydrolysis of ovotransferrin (pH 7.8) achieved by using α-chymotrypsin (pH 3.0) gave an endothermic reaction with ΔH=+31.84 kJ mol^{-1}.

Keywords: α-chymotrypsin, heat of hydrolysis, isothermal, ovotransferrin, quasi-adiabatic

Introduction

Transferrins comprise a group of single-chain glycoproteins (approx. 80 kDa) found in the blood plasma of vertebrates down to the hem lymph of chordates [1, 2]. These glycoproteins include mainly serum transferrin from many species, lactotransferrin from mammalian milk and ovotransferrin from avian egg white. All types of transferrins possess two similar lobes, N- and C-terminal, each with a single binding site for Fe(III) [3]. The main function associated with these glycoproteins is the transport of iron to dividing cells and in some cases a bacteriostatic action has been also evidenced as for lactotransferrin [3].

Hen ovotransferrin, the molecular mass of which is 77 770 Da [4], consists of 686 amino acid residues, 61 of which are aromatic, and a tetra-antennary carbohydrate side-chain (glycan) attached to asparagine-473 located at C-lobe [4, 5].

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1418–2874/2001/ $ 5.00
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The only calorimetric studies reported for this protein concern mainly the binding of ferric ions and interaction between binding site, both in the whole molecule and in the corresponding N- and C-terminal half-molecules [6].

Chymotrypsin (EC. 3.4.21.1) is a serine endopeptidase, specifically hydrolyzing peptide bonds at the C-terminus of aromatic amino acids such as tyrosine, phenylalanine and tryptophan [7]. The enzyme from bovine pancreas, with a molecular mass of 25 666 Da [8], has been extensively studied [9–11] and it is stable for days in solution at non-buffered pH 3.0 and for years as a dry powder when stored refrigerated. Although a plenty of thermodynamic studies have been reported for such an enzyme [12–16], at the moment no calorimetric data are available, concerning the heat of protein hydrolysis where chymotrypsin acts as a protease.

Thus, in order to get further accurate knowledge of the thermodynamics properties of hen ovotransferrin and chymotrypsin in its α-form (from bovine pancreas), in the present paper we report the heats of solubilization of both proteins and then the heat of hydrolysis when ovotransferrin is treated with α-chymotrypsin.

Moreover, although our main interest was devoted to the determination of heat of hydrolysis, for such a determination, in our experimental design, it was necessary to measure the heats of solubilization of both ovotransferrin and α-chymotrypsin, each protein in its specific buffer.

Eventually, in our experimental conditions large enthalpy values accounting for an exothermic reaction were obtained when both proteins were separately solubilized, but an endothermic reaction was detected when ovotransferrin hydrolysis occurred in the presence of α-chymotrypsin.

Experimental

Hen ovotransferrin (OTf) was purified to homogeneity according to Williams [17]. α-Chymotrypsin (α-CT) type II from bovine pancreas (53 units mg^{-1} of protein), 3× crystallized from 4× crystallized chymotrypsinogen; essentially salt-free was from Sigma Chemical Co. (St. Louis, MO, USA). Both proteins were separately dialyzed extensively against MilliQ grade water and, when analyzed by SDS-PAGE, both gave a single band corresponding to their specific molecular mass (about 80 kDa for OTf and around 25 kDa for α-CT), indicating that both protein preparations were pure (data not shown).

In the first experimental phase, after calibration, weighed amounts of Tris buffer (0.08 M Tris·HCl, pH 7.8 containing 0.1 M $CaCl_2$) were loaded in the reaction vessel of a Tronac 450-458 isoperibol calorimeter which works in quasi-adiabatic conditions; then, pre-determinated amounts of lyophilized OTf were added by means of glass bulbs. The solution reaction that was recorded in this case was the following:

$$OTf_{(s)} = OTf_{(aq)}$$

In the second experimental phase, after calibration, weighed amounts of 0.001 M HCl solution were loaded in the calorimeter reaction vessel; then, pre-determinated

amounts of lyophilized α-CT were added by means of glass bulbs. In this case the monitored solution reaction was the following:

$$\alpha\text{-}CT_{(s)}=\alpha\text{-}CT_{(aq)}$$

In the third experimental phase, after calibration, weighed amounts of OTf in Tris buffer (0.08 M Tris·HCl, pH 7.8 containing 0.1 M $CaCl_2$) were loaded in the calorimeter reaction vessel; then, pre-determinated and appropriate amounts of α-CT in 0.001 M HCl were added by means of a calibrated micro-titration buret. In this last case the solution reaction under study was the following:

$$OTf_{(aq)}+\alpha\text{-}CT_{(aq)}+H_2O_{(aq)}=oligopeptides_{(aq)}+\alpha\text{-}CT_{(aq)}$$

In all three cases, thermal equilibrium was usually obtained within 20 min after charging the apparatus at around 298.15 K, and then about 10 min was allowed for the reaction to reach completion after mixing the reagents.

The temperature changes in the vessel were measured by using a thermistor and a Fluka model 8810A digital multimeter, and both reaction and cooling curves were

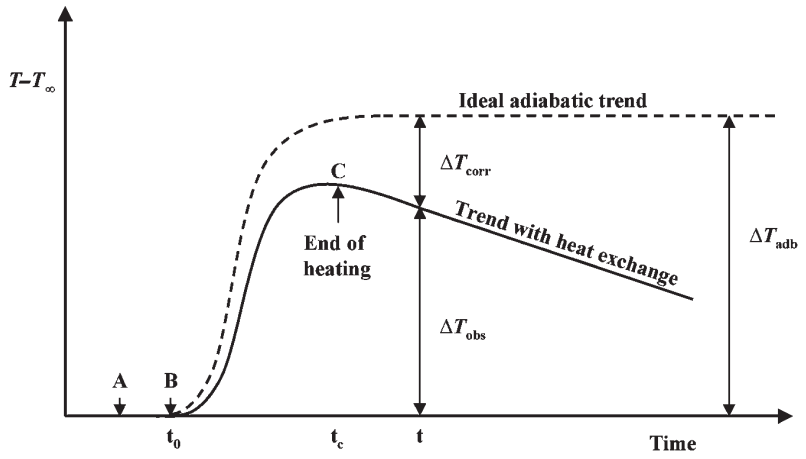

Fig. 1 Temperature-time curves for exothermal real and ideal adiabatic exothermal reactions

recorded by means of an Olivetti M24 computer and a Hewlett Packard HP3396A integrator. Computer programs (in BASIC) containing calculation methods for the determination of solubilization and hydrolysis heats and reaction in isothermal and quasi-adiabatic calorimeters were used [18–20]. In particular, the method of heats calculation was founded on the hypothesis that the calorimeter really exhibited adiabatic behaviour throughout the reaction, but in the real trend the adiabatic value temperature is given by the following reaction (Fig. 1).

$$\Delta T_{adb}=\Delta T_{obs}+\Delta T_{corr} \quad (1)$$

The ΔT_{corr} can be written as [16]

$$\Delta T_{corr}=\int_{t_0}^{t}(T_t-T_\infty)dt \tag{2}$$

Then, since after the end of the calibration or the chemical reaction ΔT_{adb} would have been equal to zero, from Eq. (1) one can obtain

$$\Delta T_{obs}=-\Delta T_{corr} \tag{3}$$

and again

$$T_0-T_\infty=-K/C\int_{t_\infty}^{t}(T-T_\infty)dt \tag{4}$$

where K/C represents the instantaneous power and the mole number of the reaction curve.

Equation (4) provides a differential equation, which solved with the initial condition $T_{t_0}=T_0$ gives the solution

$$T-T_\infty=(T_0-T_\infty)e^{-K/C(t-t_0)} \tag{5}$$

The term K/C can be obtained as

$$K/C=-\ln(T-T_\infty/T_0-T_\infty)/(t-t_0) \tag{6}$$

Lastly, for the reaction curve, two steps were necessary:

a) calculation of the corrective temperature term

$$\Delta T=K/C\int_{t_0}^{t}(T_t-T_\infty)dt \tag{7}$$

b) calculation of the heat capacity by means of the expression

$$C=P_{cal}/dT/dt+K/Cx(T-T_\infty) \tag{8}$$

where P_{cal} is the instantaneous power which is measured by reading the calibration current and the potential drop of calibration heater resistance (for any further detail about program description and equations applied [20]).

The heats of hydrolysis reported in Table 1 were obtained subtracting to the changes of heats, the heat of dilution (–2 kJ mol^{-1}) corresponding to the addition of 0.001 M HCl to OTf (0.3 mg mL^{-1}) in Tris buffer solution (0.08 M Tris·HCl, pH 7.8 containing 0.1 M $CaCl_2$).

Last, protonization thermal effects of OTf, α-CT, and buffer after hydrolysis reaction were not considered because it was beyond the aim of the present study.

Table 1 Change in enthalpy at 298.15 K on: A) solubilization of OTf (considering a molecular mass of 77 770 Da [4]); B) solubilization of α-CT (considering a molecular mass of 25 666 Da [8]); C) hydrolysis of OTf by α-CT

Solvent/protein solution/g	Protein/ $g \cdot 10^2$	ΔH/ $J \cdot 10^3$	ΔH/ $J\ g^{-1}$	ΔH/ $kJ\ mol^{-1}$	Average[a] (Std)[a]
A) Tris buffer, pH 7.8	OTf[b]				
50	1.4590	–464.3	–31.82	–2475	
50	1.8091	–578.7	–31.99	–2488	–2485 (±8)
50	1.3582	–435.4	–32.06	–2493	
B) 0.001 M HCl	α-CT[b]				
50	1.2512	–370.9	–29.65	–761.0	
50	1.1760	–359.4	–30.56	–784.4	–780 (±14)
50	1.1481	–355.6	–30.97	–795.0	
C) OTf[c]	α-CT[d]				
50	0.0620	+6.159[e]	+0.410[e]	+31.93[e]	
50	0.0619	+6.139[e]	+0.409[e]	+31.83[e]	+31.84[e] (±0.07)
50	0.0618	+6.126[e]	+0.408[e]	+31.76[e]	

[a]Referred to molar enthalpy (fifth column)
[b]Proteins added in their lyophilized form
[c]OTf concentration was 0.3 $mg \cdot mL^{-1}$ in Tris buffer (0.08 M Tris·HCl, pH 7.8 containing 0.1 M $CaCl_2$)
[d]α-CT concentration was 0.25 $mg \cdot mL^{-1}$ in 0.001 M HCl
[e]Values related to the amount of OTf

Results and discussion

Lyophilized OTf (from hen egg white) and α-CT (from bovine pancreas) were separately dissolved in two different aqueous solutions and the heats of solubilization were calculated by processing the corresponding thermal curves with a specific computer program. Subsequently, an aqueous solution of α-CT (2.5 g; α-CT=0.25 mg mL^{-1} in 0.001 M HCl) to an OTf buffered solution (50 g; OTf=0.3 mg mL^{-1} in 0.08 M Tris·HCl, pH 7.8 containing 0.1 M $CaCl_2$) was added and the heat of hydrolysis measured after processing the thermal curve.

When the proteins were separately dissolved in their specific aqueous solutions an exothermic reaction was recorded in both cases. In particular, an associated ΔH value of –2485 kJ mol^{-1} was obtained for OTf (considering a molecular mass of 77 770 Da [4]) and –780.1 kJ mol^{-1} for α-CT (considering a molecular mass of 25 666 Da [8]) (Table 1).

When OTf was subjected to the hydrolytic action of α-CT an endothermic reaction occurred being the ΔH value around +31.84 kJ mol^{-1} (Table 1). This value was corrected for the heat measured when 0.001 M HCl (2.5 g) was added to 50 g of Tris buffer solution with OTf at 0.3 mg mL^{-1}; in this case an exothermic reaction with a ΔH value of about –2 kJ mol^{-1} was obtained (data not shown). Interestingly, the standard deviation corresponding to the hydrolysis heat was lower than the standard devi-

ations related to the heats of solubilization (0.069 *vs.* 7.594 for OTf solubilization and 14.31 for α-CT solubilization) indicating a great reproducibility and congruity for the hydrolysis experiments (Table 1).

The aqueous solutions where the two proteins were separately dissolved, were chosen according to a standard procedure set up by Hummel [21]. In this procedure, which permits to determine the intrinsic α-CT activity, the assay is conducted at 25°C and the substrate to be hydrolyzed is dissolved in 0.08 M Tris·HCl buffer, pH 7.8 containing 0.1 M $CaCl_2$ whereas the hydrolytic enzyme is dissolved in 0.001 M HCl, in fact at pH 3.0 α-CT shows its greatest stability [21].

Considering the temperature we have chosen to measure all the heats here reported, there are two main reasons. The first one is that as stated above, the enzymatic activity of α-CT is usually recorded and measured at 25°C (298.15 K), the second reason is that we have also tried to measure the heat of hydrolysis at 37°C but our protease was very unstable in such conditions, in fact, the corresponding thermal curves obtained were not suitable to be processed by our computer program (data not shown).

Moreover, to be sure that the reaction hydrolysis was completed in a very short time (<30 s), 33 units of α-CT (corresponding to 0.620 mg) were added to about $2 \cdot 10^{-7}$ mol of OTf. In fact, since by standard definition, one unit of α-CT at 25°C will hydrolyze 1.0 μmole of substrate per min at pH 7.8, in our experimental conditions, the C-terminus of the 61 aromatic amino acid residues present in OTf [4], could be completely hydrolyzed by α-CT in less than 22 s (producing 62 different oligopeptides). In effect, as judged by SDS-PAGE analysis, OTf resulted to be completely hydrolyzed in our conditions (data not shown).

Then, in this paper, besides some thermodynamics properties of two proteic macromolecules, we report the heat of hydrolysis when a protease such as α-CT (from bovine pancreas) acts upon a single-chain glycoprotein such as hen OTf. In all the other known studies where α-CT is reported to catalyze hydrolysis reactions, the substrates used are very small molecules compared to hen OTf [12–16]. Then, our study is the first one where a biomacromolecule such as OTf is utilized as substrate for the action of α-CT. For that, due to the lack of any scientific literature that may exist in this area of research, attempts to integrate the present results have been in vain.

Of course, it would also be very interesting to know how the enthalpy of hydrolysis varies with the pH or with the temperature or with the buffer, but these types of studies are beyond the aim of the present paper.

Conversely, at the moment studies are in progress to ascertain if other forms of chymotrypsin, i.e. β-, γ- and δ-chymotrypsin, from the same source, behave differently from the α-form.

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This work was supported by funds from M.U.R.S.T. (Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Rome, Italy).

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