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ノート

Differential Scanning Calorimetric Study of the Thermal Denaturation of Glucoamylase

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The thermal unfolding of *Rhizopus* glucoamylase (EC 3.2.1.3) was studied at pH7 by high-sensitivity differential scanning calorimetry (DSC). The DSC curve showed a single irreversible asymmetric peak having the temperature of maximal excess specific heat, t_p, at around 57°C. The curve was resolved into two components according to the model of independent two-state processes. In the presence of SGI (5-amino-1, 5-dideoxy D-glucopyranose), a sugar inhibitor of the enzyme, t_p increased with increasing concentration of SGI. Analysis of the DSC data of the enzyme-SGI complex suggests two independent domains with dissociation of SGI in the second component.

1. Introduction

Rhizopus glucoamylase (EC 3.2.1.3) is a glycoprotein which hydrolyzes nonreducing end α-1, 4 glucosidic linkages of starch to produce glucose. Many steady state and transient kinetic studies have been done for the binding reaction of the enzyme with its substrates and analogs (see ref. 1 and references sited therein), including SGI (5-amino-1, 5-dideoxy D-glucopyranose), an inhibitor of the enzyme[2]. This note reports the thermal unfolding parameters of glucoamylase and its complex with SGI using high sensitivity differential scanning calorimetry (DSC).

2. Materials and Methods

Glucoamylase from *Rhizopus niveus* was purchased from Seikagaku-Kogyo Co. Ltd. Its concentration was determined spectrophotometrically using the absorption coefficient $A^{1\%}_{280}$ of 16.0cm^{-1} and the molecular weight of 60,000[3]. SGI is a generous gift from Professor Murao of the Kumamoto Institute of Technology. The DASM-4 microcalorimeter[4,5] was used in all the DSC experiments with a scan rate of 1K min^{-1}. For obtaining the thermodynamic parameters describing each DSC curve, the procedures outlined by Sturtevant[5] were followed. Base lines were drawn as outlined by Kitamura and Sturtevant[6]. 50m mol dm^{-3} phosphate buffer, pH7.0 was used throughout, unless otherwise stated.

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3. Results and Discussion

The thermal unfolding of glucoamylase alone was studied at pH7.0 at three different concentrations of the protein. A typical DSC trace is shown in Figure 1 (solid line). A single irreversible endothermic peak was observed. Reversibility was also checked at pH3.9 and at 5.7 using 50m mol dm^{-3} acetate buffer, but no endothermic peak was observed on rescanning after an initial heating. Previous work has shown that even proteins that undergo apparently completely irreversible denaturation can nevertheless closely follow equilibrium thermodynamics[7]. In the following, we have therefore utilized the van't

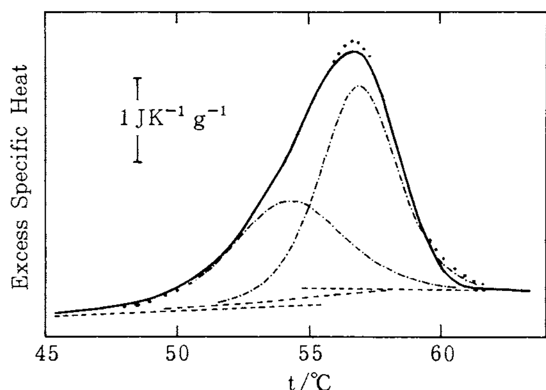


Fig. 1 Typical DSC curve observed with glucoamylase alone and its resolution into two independent two-state curves. (—) Observed curve ; (-・--) component curves ; (・・・) sum of component contributions. Protein concentration, 209μ mol dm^{-3} ; scan rate, 1K min^{-1}. 50m mol dm^{-3} phosphate buffer, pH7.0. The dashed lines are the initial and final base lines, and the dashed curve is the calculated base line, which goes from the initial to the final base line in propotion to the extent of denaturation.

Hoff equation in analyzing the DSC data, as a first approximation. Another justification of this approximation will be discussed later.

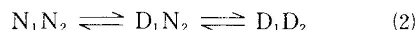
Columns 3 and 4 in Table I summarize the observed values for the denaturation reaction of the protein. There was no dependence of t_p, temperature in degree Celcius at which the excess specific heat reaches its maximal value, c_{max}, on the protein concentration used, indicating that the degree of oligomerization does not change during the denaturation reaction[5]. The denaturational enthalpy Δh_{cal}, the specific enthalpy obtained by evaluating the area of the DSC curve using planimeter, 16.2J g^{-1}, is a usual value for a globular protein.

The highly asymmetric DSC curve indicates that the denaturation process is more complicated than simple two-state. Further indication of this complication is obtained by comparing ΔH_{cal}, the molar enthalpy, with ΔH_{VH}, the van't Hoff enthalpy, for the unfolding reaction. ΔH_{VH} for a simple two-state process can be obtained from the following equation[5];

$$\Delta H_{VH} = 4RT_P^2\ (c_{max}/\Delta h_{cal}) \qquad (1)$$

where $T_P/K = t_P/°C + 273.15$. ΔH_{VH} thus obtained is 611kJ mol^{-1}, whereas ΔH_{cal} is 971kJ mol^{-1}.

All the DSC curves were then resolved into component curves by the curve fitting method according to the model of two endependent domains unfolding in two-state steps:



$$N_1N_2 \rightleftharpoons D_1N_2 \rightleftharpoons D_1D_2 \qquad (2)$$

where N and D denote native and denatured state of component domain 1 and 2*. This assumption may be reasonable, since recent cloning and biochemical study of fungal glucoamylase suggests that the enzyme molecule has two separate functional regions ; a catalytic domain and a starch binding domain[8].

The adjustable parameters for each two-state component are $t_{1/2}$, the temperature at which the unfolding reaction is half completed, ΔH_{cal}, the molar specific enthalpy at $t_{1/2}$, and ΔH_{VH}, the van't Hoff enthalpy. The ratio $\Delta H_{VH}/\Delta H_{cal}$ is assumed to be same for each component and to be independent of temperature.

Table I summarizes the results of the curve resolution, together with those obtained for thermal denaturation in the presence of SGI, which will be described later. The component curves obtained by the resolution procedure are shown in Fig. 1 in dot-dash lines. The ratio $\Delta H_{VH}/\Delta H_{cal}$ is greater than the unity, which indicates the existence of intermolecular cooperation[5]. The value of the ratio, 1.64, suggests that glucoamylase molecules are partially dimerized under the experimental conditions and that the degree of the dimerization is the same before and after the transition[5].

SGI, having the molecular weight of 163, was

*Even if three-component medel is assumed, no essentially better resolution was obtained ; that is, in this model, Δh_{cal} value of one of the three components is very small and the percent value of standard deviation relative to the maximum value of excess specific heat is almost the same as that of the two-component model employed in the text.

Table I : Thermodynamic Parameters for the Thermal Denaturation of Glucoamylase in the Absence and Presence of SGI. pH7.0.

		observed		curve resolution									
				component 1			component 2			SD	ΔC_p [c]	$\Sigma \Delta H_{cal}$ [d]	$\Delta H_{VH}/\Delta H_{cal}$
[protein]	[SGI]	t_p	ΔH_{cal} [a]	$t_{1/2}$	ΔH_{cal}	ΔH_{VH}	$t_{1/2}$	ΔH_{cal}	ΔH_{VH}	(%)[b]	kJ K^{-1} mol^{-1}	kJ mol^{-1}	
μmol dm^{-3}	mmol dm^{-3}	℃	kJ mol^{-1}	℃	kJ mol^{-1}	kJ mol^{-1}	℃	kJ mol^{-1}	kJ mol^{-1}				
50.5	0	56.7	950	54.30	402	661	57.00	552	912	1.84	2.3	954	1.65
94.8	0	56.7	983	54.09	389	636	56.81	565	925	1.74	8.5	954	1.63
209	0	56.7	979	54.06	399	653	56.87	573	937	1.96	8.9	972	1.63
	av.	56.7	971	54.15	397	650	56.89	563	925	1.85	6.5	960	1.64
50.5	0.149	59.2	1046	56.64	452	657	59.36	623	907	2.63	18.6	1075	1.45
50.5	1.49	61.7	1151	59.04	485	636	61.72	703	920	2.81	27.7	1188	1.31
50.5	14.9	63.8	1251	61.06	493	657	63.84	707	937	2.39	30.8	1200	1.33
	av.					(650)			(921)	2.61	(25.7)		

a. Molecular weight × Δh_{cal} obtained by planimeter integration. b. % of c_{max} c. The mean value for the overall ΔC_p d. The sum of ΔH_{cal}'s of the component 1 and 2.

originally isolated from *Streptomyces lavendulae* subsp. *trehalostaticus* No. 2882 as a trehalase inhibitor[9]. SGI binds to glucoamylase with dissociation constant K_d of 24.5μ mol dm^{-3} at pH4.5[2] and 1.9μ mol dm^{-3} at pH7**. These values of K_d are very small for a ligand of glucoamylase[1]. The thermal denaturation of glucoamylase was observed in the presence of SGI. A single asymmetric endothermic peak similar to Fig. 1 was obtained, but the values of t_p and Δh_{cal} were larger.

That $t_{1/2}$ is affected by SGI, as expected on the basis of the van't Hoff equation, shows there is reversible interaction between the enzyme and SGI, as in the case of Taka-amylase A and Ca^{2+} [10]. It is evident that these increases in $t_{1/2}$ and Δh_{cal} are not due to ligand-induced changes in the structure of glucoamylase molecule, since 98.1, 99.87, and 99.99% of the enzyme molecules are saturated by SGI molecules at the SGI concentrations of 0.149, 1.49 and 14.9m mol dm^{-3}, respectively (cf. ref. 11).

**Calculated according to equation 10 using the values of Table II of ref. 2.

All the DSC curves obtained in the presence of SGI were analyzed by supposing that the two-domain model is also valid for this system. Results of the curve resolution are summarized in Table I. As seen in the table, the following points may be discussed :

(1) SGI increases the values of $t_{1/2}$ of the components 1 and 2. Figure 3 shows this effect in van't Hoff plots of $\ln[L]_0$ *vs.* $1/T_{1/2}$, where $[L]_0$ is the initial, or total, concentration of SGI and $T_{1/2}/K = t_{1/2}/°C + 273.15$. In the plot, ΔH_{VH} is obtained from $-nRS$ where n is the number of moles of ligand dissociation per step in the denaturation, R is the gas constant and S is the slope of the plot. Since one SGI molecule is bound to one glucoamylase molecule[2], it follows that n should be 1, and that only one of these slopes in Fig. 2 may be interpreted. Multiplication of the slopes by $-R$ gives 954 and 958kJ mol^{-1} for component 1 and 2, respectively. By comparing these values of van't Hoff enthalpy thus obtained with those obtained from the curve fitting (650 and 921kJ mol^{-1} ; see Table I), it is reasonable to conclude that SGI is bound to component 2. Thus the denaturation

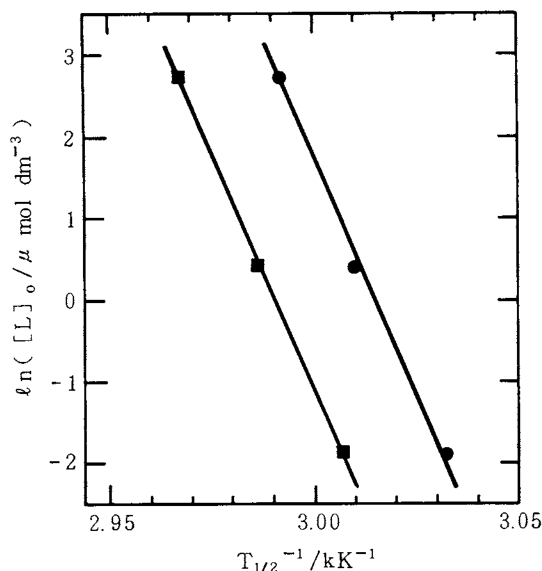
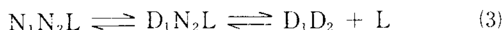


Fig. 2 van't Hoff plots of the logarithm of the total concentration of SGI, $[L]_0$, (in μ mol dm^{-3} unit), vs. $1000/T_{1/2}$ for independent components 1 (●) and 2 (■), obtained by curve resolution.

scheme in the presence of SGI can be written as follows ;



$$N_1N_2L \rightleftharpoons D_1N_2L \rightleftharpoons D_1D_2 + L \qquad (3)$$

where L is SGI. The slope of the line for the component 1 in Fig. 2 might be the result of domain interactions[5]. Considering that SGI is bound to subsite 1 of the enzyme active site[2], where nonreducing end glucose residue of a substrate binds in a productive mode, subsite 1 is located in the component 2.

(2) The ratio $\Delta H_{vH}/\Delta H_{cal}$ approaches to the unity in the presence of SGI, which suggests that the degree of intermolecular interaction decreases in the presence of SGI.

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要 旨

グルコアミラーゼ(EC 3.2.1.3)の熱変性反応を，pH7において，高感度断熱型 DSC を用いて観測した。DSC 曲線は57°C 付近に単一のピークを持ち，非対称であった。反応は見かけ上不可逆であった。測定結果は，タンパク質分子内の2つの独立したドメインを仮定することによりうまく解析することができた。

一方，本酵素の糖質阻害物質である SGI (5-amino-1, 5-dideoxy D-glucopyranose) の共存下ではピーク温度が上昇した。DSC 曲線の解析から，SGI は高い方の変性温度を持つドメインに結合し，そのドメインの変性と共に解離することが示唆された。