

Differential scanning calorimetry as a tool to study protein–ligand interactions

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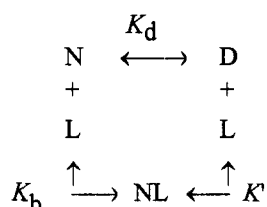
Abstract. Differential scanning calorimetry can be used as an interesting tool to study protein–ligand interactions, with the aid of suitable thermodynamic models. This review analyzes and discusses results recently obtained in our laboratory with regard to the binding equilibrium of 2' and 3' cytidine monophosphate to RNAase A, the interaction of S-peptide with S-protein, the binding of D-glucose to yeast hexokinase and the thermal stability of respective complexes.

INTRODUCTION

Molecular recognition processes are of fundamental importance for life. DNA-binding proteins are responsible for many cell functions, including transcription, replication and restriction (1), and catalysis by enzymes requires their recognition of the substrate. Efforts in many laboratories are currently directed toward achieving a reasonable quantitative understanding of the non-covalent forces that ensure the specificity and efficiency of binding processes. Structural X-ray or NMR investigations provide a direct three-dimensional image of formed complexes (2-5), but are unable to clarify the energetics of recognition processes. Many protein–ligand interactions are known to be accompanied by large changes in entropy and heat capacity (6). Direct thermodynamic measurements allow evaluation of the association constants and the binding enthalpy and entropy changes, and thus have a fundamental role in this field of biophysical sciences (7), but can provide a complete thermodynamic picture only in favourable cases. The product of binding constant times protein concentration, in fact, must be between 10 and 100 (8). Indirect methods are therefore useful for a broad and general analysis of binding processes. Differential scanning calorimetry is one of these methods and is a powerful tool for the study of protein–ligand interactions (9-11). Evaluation of thermodynamic binding parameters is connected to the observation of the denaturation temperature changes and calorimetric profile alterations induced by ligand binding. Thermodynamic models are needed to analyze the experimental data and to obtain quantitative results, as already demonstrated in an insightful paper by Brandts and Lin (11). In this paper, we show that a thermodynamic model based on very simple and reasonable hypotheses can quantitatively analyze the dsc measurements of RNAase A in the presence of subsaturating quantities of 2'CMP and 3'CMP (12), and of S-protein in the presence of S-peptide. Lastly, it is pointed out that a modified version of this model allows precise analysis of the effect of saturating quantities of D-glucose on the denaturation of yeast hexokinase.

THERMODYNAMIC MODEL

We consider a protein that denatures according to a two-state transition and can bind a specific ligand on only one native conformation site. The overall process can be described by the following scheme:



where K_d is the equilibrium constant of the denaturation process, K_b is the equilibrium binding constant and K' is the denaturation constant of the stoichiometric complex NL. However it is easy to show that K' is not an independent parameter, since $K' = K_d / K_b$. Both equilibrium constants are temperature dependent :

$$K_d = \exp -\{(\Delta_d H/R) [(1/T) - (1/T_d)] + (\Delta_d C_p/R) [1 - (T_d/T) + \ln (T/T_d)]\} \quad (1)$$

where $K_d = 1$ for $T = T_d$, and

$$K_b = K_b^\circ \exp -\{(\Delta_b H/R) [(1/T) - (1/T_d)] + (\Delta_b C_p/R) [1 - (T_d/T) + \ln (T/T_d)]\} \quad (2)$$

where $K_b = K_b^\circ$ for $T = T_d$. In these equations T_d is the midpoint denaturation temperature, $\Delta_d H$ and $\Delta_d C_p$ represent the denaturation enthalpy and heat capacity changes, and $\Delta_b H$ and $\Delta_b C_p$ are the binding enthalpy and heat capacity changes. Assuming the native state as reference (11,13), the macroscopic canonical partition function for this system is:

$$Q = 1 + K_b [L] + K_d \quad (3)$$

where $[L]$ represents the free ligand concentration in solution. The excess enthalpy function $\langle \Delta H \rangle$ can be readily obtained by a well-known statistical mechanical relationship:

$$\langle \Delta H \rangle = RT^2 [\partial \ln Q / \partial T]_{p, [L]} \quad (4)$$

When the total ligand concentration is much greater than the protein concentration, the free ligand concentration $[L]$ can be assumed as constant at increasing temperature in a dsc measurement. In this case, the excess heat capacity is simply given by the temperature derivative of $\langle \Delta H \rangle$.

But if the total amount of ligand is lower than the saturation level of the protein binding site, it is necessary to consider the mass balance equation for the ligand to determine the free ligand concentration $[L]$:

$$[L]_{\text{tot}} = [L] + \bar{[L]} [P]_{\text{tot}} \quad (5)$$

where $[L]_{\text{tot}}$ and $[P]_{\text{tot}}$ are the total ligand and protein concentration respectively, and $\bar{[L]}$ represents the binding isotherm.

The free ligand concentration $[L]$ changes with temperature during the denaturation process because the ligand can bind only to the native conformation. Thus in the case of subsaturating amounts of ligand, $[L]$ is a physically significant variable with which to describe the system. Strictly speaking, $\langle \Delta H \rangle = f(T, [L])$ and $[L] = f(T)$, and so:

$$\langle \Delta C_p \rangle = [\partial \langle \Delta H \rangle / \partial T]_{[L]} + [\partial \langle \Delta H \rangle / \partial [L]]_T \cdot (d[L]/dT) \quad (6)$$

The effect of subsaturating amounts of a strong ligand on dsc profiles is dramatic. Two endothermic peaks appear. Their nature has been investigated by Shrake and Ross, starting from their studies of human serum albumin in the presence of fatty acids (14,15). Simulations have shown that the maximum distortion of the dsc peak from the two-state transition profile occurs when the molar ratio $[L]_{\text{tot}}/[P]_{\text{tot}}$ is equal to 0.5 (i.e. when the total ligand concentration is half the concentration of binding sites). Moreover, the complex shape of the dsc profile does not depend on the value of the binding constant alone, but markedly depends on the product of the total protein concentration times the association constant at T_d . This product is a dimensionless parameter which, following Brandts and coworkers (11,16), will be labelled C (i.e. $C \equiv K_b^\circ \cdot [P]_{\text{tot}}$). The thermodynamic parameters characterizing the binding equilibrium of ligand to macromolecule can be determined by means of a simulation-fitting procedure of experimental curves.

RESULTS AND DISCUSSION

I) Analysis of the binding of 2'CMP and 3'CMP to RNAase A

The procedure described can straightforwardly applied in the case of a very tight binding when two distinct endotherms appear in the dsc profile. However, the binding constants of 3'CMP and 2'CMP on RNAase A are not large enough to give rise to appreciable distortions of the calorimetric curve in the conditions usually employed. Indeed for $[P]_{\text{tot}} \approx 0.15$ mM and $K_b^\circ \approx 10^3 \div 10^4$, C is too small to obtain visible effects. It was increased by using higher protein concentrations, i.e. $[P]_{\text{tot}} \approx 2 \div 3$ mM, and marked distortions from the two-state transition were obtained.

Figure 1 compares the experimental profiles of RNAase A alone (curve a) and in the presence of 3'CMP and 2'CMP at molar ratios of 0.50 (curve b) and 0.66 (curve c) respectively, at pH 5.0, acetate buffer solution 0.1M (12).

Figure 2 shows the experimental profiles, at pH 5.5, 0.1M acetate buffer, of RNAase A alone (curve a), and in the presence of 2'CMP, at molar ratio of 0.68 (curve b) and 2'CMP plus 0.1M KCl at molar ratio of 0.60 (curve c). The denaturation processes were always fully reversible according to the reheating criterion (12). The thermal profiles for RNAase A in the presence of 2'CMP clearly show a distortion from a two-state transition curve, due to a marked shoulder, whereas in the presence of 3'CMP, the profile is solely broader than the denaturation peak of RNAase A alone, because 3'CMP is a weaker ligand than 2'CMP.

The binding equilibrium of 3'CMP and 2'CMP to RNAase A was characterized by performing a simulation-fitting procedure of the experimental curves with respect to Eq.6 (Tables 1 and 2). The thermodynamic parameters of denaturation for RNAase A alone, determined through direct measurements, are still fixed. The values of $\Delta_b H$, the binding enthalpy assumed independent of temperature, and C are determined by a non-linear regression algorithm (17). The goodness of the fit convalidates the assumption that there is no heat capacity change associated with the binding process.

3'CMP at pH 5.0 gave: $\Delta_b H = -65 \text{ kJ mol}^{-1}$ and $K_b^\circ = 4670 \text{ M}^{-1}$. Application of the van't Hoff equation to these data gave $K_b = 80700 \text{ M}^{-1}$ at 25°C . The corresponding values for 2'CMP at pH 5.0 were: $\Delta_b H = -65 \text{ kJ mol}^{-1}$, $K_b^\circ = 27100 \text{ M}^{-1}$ and $K_b = 265000 \text{ M}^{-1}$ at 25°C . It is worth noting that the same values of T_d , $\Delta_d H$ and $\Delta_d C_p$ used to perform both simulation fitting procedures, result in a comparable agreement. The results confirm that 2'CMP is a stronger ligand than 3'CMP for RNAase A. Moreover the calculated association constant and binding enthalpy well correspond to the values determined by isothermal calorimetry, either flow or batch (18-21).

Table 1. Values of parameters obtained from simulation-fitting of dsc peaks at pH 5.0. In both cases, the denaturation parameters of RNAase A are: $T_d = 61.3^\circ\text{C}$, $\Delta_d H = 460 \text{ kJ mol}^{-1}$ and $\Delta_d C_p = 7.0 \text{ kJ K}^{-1} \text{ mol}^{-1}$.

	$\Delta_b H$ kJ mol ⁻¹	C kJ mol ⁻¹	σ kJ K ⁻¹ mol ⁻¹
3'CMP*	-65	10	506
2'CMP**	-65	59	540

* 0.1M acetate buffer, $[P]_{\text{tot}} = 2.14 \text{ mM}$,
 $[L]_{\text{tot}} = 1.07 \text{ mM}$.

** 0.1M acetate buffer, $[P]_{\text{tot}} = 2.18 \text{ mM}$,
 $[L]_{\text{tot}} = 1.44 \text{ mM}$.

σ is the standard deviation of the fit.

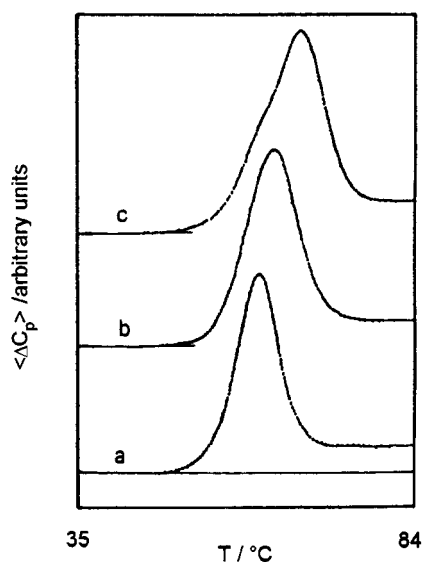


Figure 1. RNAase A complexes melting at pH 5.0.

Table 2. Values of parameters obtained from simulation-fitting of dsc peaks at pH 5.5. In both cases the denaturation parameters of RNAase A are: $T_d = 61.9^\circ\text{C}$, $\Delta_d H = 470 \text{ kJ mol}^{-1}$ and $\Delta_d C_p = 6.5 \text{ kJ K}^{-1} \text{ mol}^{-1}$.

	$\Delta_b H$ kJ mol ⁻¹	C kJ mol ⁻¹	σ kJ K ⁻¹ mol ⁻¹
2'CMP*	-70	59	430
2'CMP**	-85	70	490

* 0.1M acetate buffer, $[P]_{\text{tot}} = 3.34 \text{ mM}$,
 $[L]_{\text{tot}} = 2.27 \text{ mM}$.

** 0.1M acetate buffer, $[P]_{\text{tot}} = 3.34 \text{ mM}$,
 $[L]_{\text{tot}} = 2.00 \text{ mM}$, 0.1M KCl.

σ is the standard deviation of the fit.

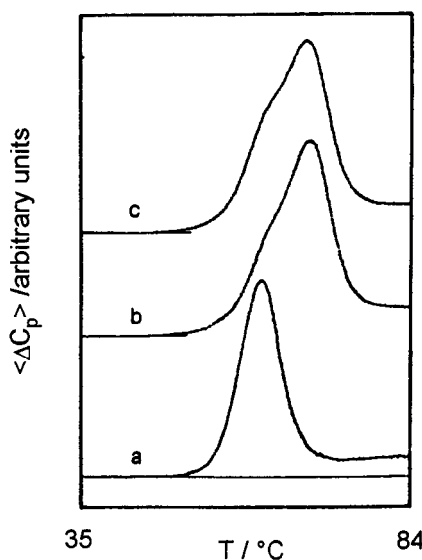


Figure 2. RNAase A complexes melting at pH 5.5.

2'CMP at pH 5.5 gave: $\Delta_b H = -70 \text{ kJ mol}^{-1}$ and $K_b^\circ = 17660 \text{ M}^{-1}$ and then, from the van't Hoff equation, $K_b = 397000 \text{ M}^{-1}$ at 25°C . The figures of 2'CMP at pH 5.5 and 0.1M KCl were: $\Delta_b H = -85 \text{ kJ mol}^{-1}$, $K_b^\circ = 20960 \text{ M}^{-1}$ and $K_b = 930000 \text{ M}^{-1}$ at 25°C . These K_b values are very close to those determined by isothermal titration calorimetry (16,22). Brandts and Lin (11) obtained slightly higher values for K_b° at protein concentrations lower than ours (i.e. in the range 0.56 - 2.88 mM). As these authors showed, K_b° for 2'CMP depends on RNAase A concentration, therefore our results agree very well with their determinations. Analysis of the dsc measurements shows that, at pH 5.5 and in the presence of KCl, the binding of 2'CMP is stronger and more exothermic than in its absence, in line with earlier findings (23). However the mechanism by which KCl enhances the tightness of binding is not clear. One hypothesis is that increases in ionic strength raise the activity coefficients of nucleotides.

II) Analysis of the interaction of S-peptide with S-protein

We have studied the interaction of S-peptide with S-protein (24) at pH 7.0, in 10 mM MOPS buffer and 200 mM NaCl, by means of detailed dsc measurements. The concentration of S-protein was fixed at $2.75 \cdot 10^{-4} \text{ M}$, to reduce the number of independent variables (25). This protein-peptide interaction was found to be strongly specific. Indeed a molar ratio $r = [\text{S-peptide}] / [\text{S-protein}]$ equal or slightly greater than one is sufficient for complete recombination of the two parts, giving rise to the reconstitution of RNAase S. Instead by performing measurements at concentrations of S-peptide subsaturating with respect to S-protein, the dsc profiles are very complex due to the concomitant presence in solution of S-protein, S-peptide and reconstituted RNAase S. In all cases, however, the process is fully reversible according to the reheating criterion. Figure 3 shows two experimental curves for $r = 0.28$ and 0.45 respectively, that emphasize the complexity of calorimetric profile: the height of high temperature peak, representing the denaturation of reconstituted RNAase S, clearly rises when the value of r is increased.

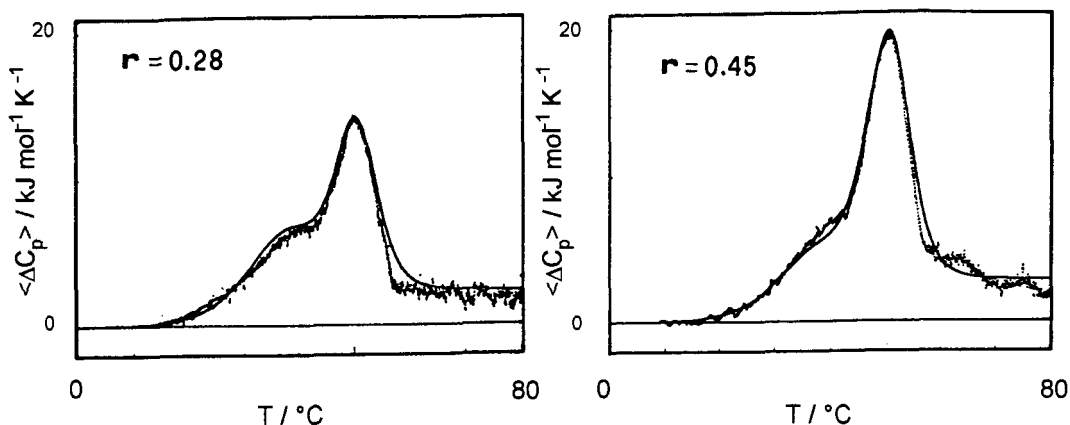


Figure 3. Melting profiles of S-peptide/ S-protein complexes at two molar ratios.

Application of the thermodynamic model to this system was based on two assumptions:

- i) S-protein denatures according to a two-state transition;
- ii) S-peptide is a strong ligand of S-protein, but dissociates during denaturation.

The first assumption allows a quantitative analysis of the experimental measurements, even if it is not strictly correct, because S-protein denaturation does not completely correspond to a two-state transition. The second assumption is supported by our dsc measurements on RNAase S, which unequivocally demonstrate that its two constituents dissociate during unfolding (25). The simulation-fitting procedure with respect to Eq.6 is simultaneously applied to both curves to obtain a more statistically reliable evaluation of thermodynamic parameters. The calculated curves are shown in Figure 3 as continuous lines superimposed on the experimental ones: the agreement is good, considering the peculiarity of the system. The best set of thermodynamic parameters used to calculate both simulated curves is: $T_d = 37.3^\circ\text{C}$, $\Delta_d H = 160 \text{ kJ mol}^{-1}$, $\Delta_d C_p = 1.5 \text{ kJ K}^{-1} \text{ mol}^{-1}$, $\Delta_b H = -170 \text{ kJ mol}^{-1}$, $\Delta_b C_p = -3.0 \text{ kJ K}^{-1} \text{ mol}^{-1}$ and $C = 295$.

The thermodynamic values of S-protein denaturation, calculated on the basis of a two-state transition, are only slightly different from those determined directly by the measurements. The binding of S-peptide is very exothermic and this fact leads one to think that a refolding of S-protein structure also happens, induced by the presence of S-peptide. It is worth noting that the binding enthalpy and heat capacity changes determined from the simulation-fitting procedure are in good agreement with the literature values.

Indeed Sturtevant and co-workers (26,27), determined by means of direct isothermal calorimetric measurements that $\Delta_b H = -168 \text{ kJ mol}^{-1}$ and $\Delta_b C_p = -3.8 \text{ kJ K}^{-1} \text{ mol}^{-1}$ at 25 °C and pH 7.0, in either buffered or unbuffered solutions. It is important to stress the large and negative heat capacity change associated with the binding of S-peptide to S-protein. Firmly established theoretical and experimental results show that this is due to the burial of a large amount of nonpolar accessible surface area, ASA_{np} , from water contact. But in this case it is impossible to make a quantitative correlation between $\Delta_b C_p$ and ΔASA_{np} because the tertiary structure of S-protein is unknown.

From the value of $C = K_b \cdot [P]_{tot} = 295$ and the knowledge of S-protein concentration in solution, $[P]_{tot} = 2.75 \cdot 10^{-4} \text{ M}$, it is possible to derive the binding constant of S-peptide at 37.3 °C. In this manner we obtain $K_b(37.3 \text{ °C}) = 1.1 \cdot 10^6 \text{ M}^{-1}$. By using the van't Hoff equation with determined values of $\Delta_b H$ and $\Delta_b C_p$, we get $K_b(25 \text{ °C}) = 1.2 \cdot 10^7 \text{ M}^{-1}$ and $K_b(15 \text{ °C}) = 6.3 \cdot 10^7 \text{ M}^{-1}$. These values are in agreement with those of Sturtevant and co-workers, who determined $K_b(35 \text{ °C}) = 4.6 \cdot 10^6 \text{ M}^{-1}$ and $K_b(40 \text{ °C}) = 1.0 \cdot 10^6 \text{ M}^{-1}$ at pH 7.0 by means of substrate turn-over measurements (26), exploiting the fact that the complex is catalytically active, whereas S-protein is not. Recently (28), these authors determined by titration calorimetry that $K_b(25 \text{ °C}) = (7.8 \pm 1.5) \cdot 10^6 \text{ M}^{-1}$ and $K_b(15 \text{ °C}) = (6.5 \pm 1.5) \cdot 10^7 \text{ M}^{-1}$ at pH 6.0, 50 mM acetate buffer and 100 mM NaCl, for the binding of S15-peptide (i.e. a truncated version of S-peptide constituted by the first 15 residues). In this respect it is demonstrated that only the first 15 residues are necessary to give a catalytically active complex with S-protein (29). In conclusion, the thermodynamic parameters associated with the binding of S-peptide derived from dsc data are in very good agreement with the literature values obtained with more direct approaches.

III) Effect of glucose on yeast hexokinase denaturation

We have investigated the denaturation process of yeast hexokinase, the isoenzyme B-fraction II of the Kaji preparation (30), at pH 8.5, 5 mM tris-HCl buffer. The dsc profile of the protein alone does not represent a one-step transition because it shows two humps. In the presence of saturating quantities of *D*-glucose, which specifically binds to the enzyme, the dsc profile dramatically changes and a sharp peak appears (Figure 4).

The denaturation temperature markedly increases from 48.2 °C to 53.7 °C, whereas the denaturation enthalpy remain at 660 kJ mol⁻¹. X-ray diffraction studies (31,32) show that hexokinase is composed of two lobes that approach each other when *D*-glucose is bound to give rise to a more compact structure. The dsc results are completely clarified on the basis of the structural information. A slightly different thermodynamic model has been elaborated to analyze the experimental curves (33). This model considers the presence of an intermediate state between the native and denatured conformations and the binding of *D*-glucose to the native state only. The model well reproduces the experimental results, as can be seen from the simulated curves superimposed on the experimental ones in Figure 4.

The thermodynamic parameters used to calculate both curves are: $T_{d,1} = 40.1 \text{ °C}$, $\Delta_d H_1 = 300 \text{ kJ mol}^{-1}$, $T_{d,2} = 48.2 \text{ °C}$, $\Delta_d H_2 = 380 \text{ kJ mol}^{-1}$, $K_b = 15000 \text{ M}^{-1}$ and $\Delta_b H = 0$. Only the *D*-glucose concentration changes, passing from 0 to 40 mM.

The value of the binding constant is in agreement with that obtained by Sturtevant and co-workers (34) by isothermal calorimetry, $K_b = 7000 \pm 600 \text{ M}^{-1}$. Furthermore the fact that the binding enthalpy seems equal to zero is again not in contrast with their results. This figure is unexpected because of the large conformational change associated to the binding, suggesting that compensating effects probably occur.

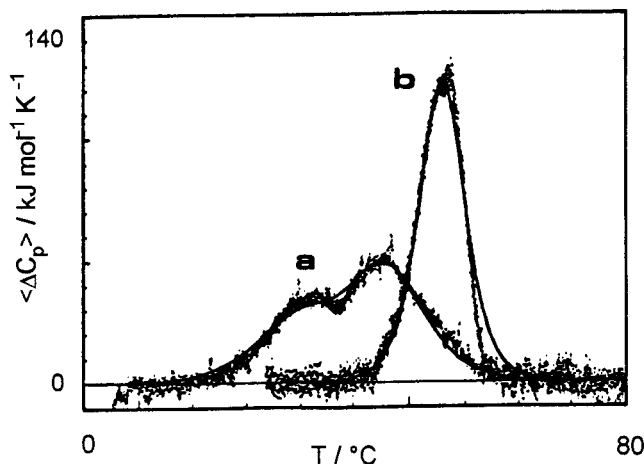


Figure 4. Denaturation of yeast hexokinase alone (curve a) and in the presence of *D*-glucose (curve b).

Therefore the driving force of binding would be entropic, related to the gain of configurational freedom of water molecules. These dsc curves could also be interpreted, on the basis of a simpler model, by recognition of the two domains of hexokinase and their enhanced interaction in the presence of *D*-glucose, as made by Brandts and coworkers for the human serum transferrin in the absence and presence of ferric ions (35). Work is in progress in our laboratory to enlarge the set of investigated experimental conditions and reach more general results.

In conclusion, the determined values of thermodynamic parameters characterizing the binding equilibria are in good agreement with those resulting from other more direct measurements (spectroscopic and isothermal calorimetric techniques). This confirms the power of dsc, with the aid of developed equilibrium thermodynamic description, even for the investigation of binding processes. Finally, it is worth noting that a purely thermodynamic analysis of protein-ligand systems correctly accounts for the experimental results, thus confirming the validity of "thermodynamic hypothesis" to describe the physico-chemical behaviour of aqueous protein solutions (36).

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