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thermochimica acta
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Heat capacity changes in heme protein–ligand interactions

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Received 17 October 2006; received in revised form 25 July 2007; accepted 26 July 2007
Available online 11 August 2007

Abstract

Isothermal titration calorimetry (ITC) has been used to determine thermodynamics of heme protein–ligand interactions with special emphasis of obtaining heat capacity changes (ΔC_p-values) for the reactions. Cyanide and azide have a relative high affinity to metmyoglobin with ΔC_p-values of -175 ± 41 J/K mol and -197 ± 42 J/K mol, respectively, while imidazole to metmyoglobin, and azide and imidazole to cytochrome *c* are low affinity systems with ΔC_p-values of 67 ± 4 J/K mol, 17 ± 4 J/K mol, and 0 ± 8 J/K mol, respectively. The small ΔC_p-values correlate well with there being minor changes is apolar solvent accessible surface areas (ASA_{apolar}). Also, the determination of ΔC_p-values allowed for the parameterization of the reaction entropy changes (ΔS_r). Conformational entropy changes (ΔS_{conf}) were large, and hence, found to be in accordance previous studies describing changes in the heme protein structures upon ligand binding.
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Keywords: Heat capacity changes; Heme protein; Ligand interactions; Conformational changes; ITC

1. Introduction

Heat capacity changes (ΔC_p) in protein–ligand interactions are of great interest because the information that is obtained on the mechanism of ligand binding to proteins [1–10]. Even though traditionally it has been easier to interpret changes in enthalpy (ΔH) and changes in entropy (ΔS) in protein–ligand interactions, both the magnitude and the sign of the ΔC_p-value have been assigned to physical changes within the protein. A traditional interpretation has been that negative ΔC_p-values are associated with changes in hydrophobic hydration and non-polar accessible surface areas [5,9], and that ΔC_p is linked to hydration of a system [1,6,8,11]. Another view is that negative ΔC_p-values can also be obtained when there is cooperative disorder of hydrogen-bonding networks and no evident hydrophobic elements [2,3].

Heme proteins are a large group of proteins with a variety of functions including oxygen binding, electron transfer, substrate oxidation, metal ion storage, ligand sensing, and transport [12]. Two well-studied members of the heme protein family are myoglobin (an oxygen carrier) and cytochrome *c* (a redox protein). In vitro, both myoglobin and cytochrome *c* are in the Fe^{3+} (ferric) state (often referred to as metmyoglobin for myoglobin), and the iron, which has six coordination sites have four of its coordination sites occupied by the heme group. For metmyoglobin, the fifth and sixth coordination sites are occupied by histidine [13] and water molecule [14], respectively. It is the water molecule that can be replaced by small, basic ligands such as azide, cyanide, fluoride, and imdiazole [14–16]. In cytochrome *c*, a histidine and a methionine are in the fifth and sixth coordination sites [17–19], and it is the methionine that can be exchanged [20–23].

We have studied metmyoglobin and cytochrome *c* interactions with azide, cyanide, and imidazole using isothermal titration calorimetry (ITC) with an emphasis of obtaining the ΔC_p of the binding reactions. This can be achieved by determining the temperature dependence change in reaction enthalpy (Eq. (1)):

$$\Delta C_p = \left(\frac{\partial \Delta H_r}{\partial T}\right) \quad (1)$$

By recognizing that the entropy of solvation is close to zero for proteins near 385 K, ΔC_p can be related to solvation entropy change (ΔS_{solv}) of the binding reaction at $t = 25\,^{\circ}C$ as described by (Eq. (2)) [1,6,11,24]

$$\Delta S_{solv} = \Delta C_p \ln\left(\frac{298.15\ K}{385.15\ K}\right) \quad (2)$$

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doi:10.1016/j.tca.2007.07.021

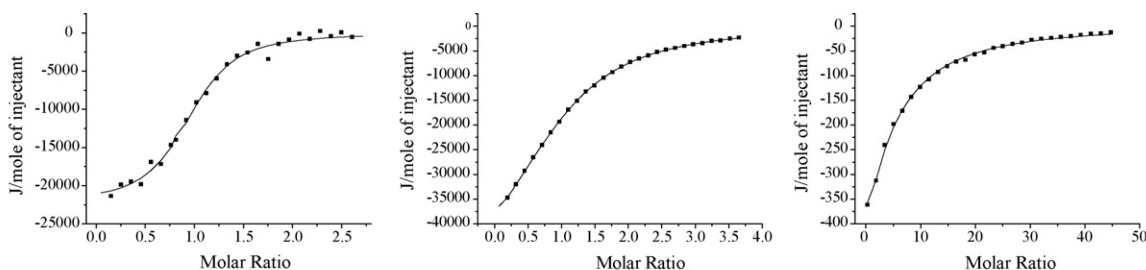

Fig. 1. Theoretical fits with experimental data for the myoglobin binding with cyanide (left), azide (middle), and imidazole (right) at $t = 25\,°C$.

Furthermore, the mixing entropy change (ΔS_{mix}) of the reaction can be calculated as a "cratic" term, a statistical correction that reflects mixing of solute and solvent molecules, and the changes in translational/rotational degrees of freedom (Eq. (3)) [1,25]:

$$\Delta S_{mix} = R \ln\left(\frac{1}{55.5}\right) \quad (3)$$

Finally, the reaction entropy change (ΔS_r) can be viewed as the sum of ΔS_{solv}, ΔS_{mix}, and the conformational entropy change (ΔS_{conf}) (Eq. (4)). ΔS_{conf} details the change in side chain and backbone conformational entropy associated to binding.

$$\Delta S_r = \Delta S_{solv} + \Delta S_{conf} + \Delta S_{mix} \quad (4)$$

Since ΔS_r and ΔS_{solv} are obtained from ITC experiments, ΔS_{mix} is estimated, and ΔS_{conf} can be calculated and depict the conformational changes that takes place in the ligand and heme protein upon binding.

2. Experimental

2.1. Reagents

Sodium azide and sodium cyanide were purchased from Fluka. Imidazole, horse heart cytochrome *c*, and horse heart myoglobin were purchased from Sigma.

2.2. ITC experiments

An isothermal titration calorimeter unit of the VP-ITC system (Microcal, USA) was used with control software, VP Viewer, provided by the manufacturer on a personal computer, Gateway E series, running Windows XP. For all reactions, the heme protein was placed in the calorimeter cell with a volume of 1.4214 mL, and the ligands were placed in syringe. The stirring speed was 260 rad s^{-1}. Following concentrations were used in the ITC experiments: 0.030 mM myoglobin with 1.0 mM cyanide, 20 mM KP_i, pH 7.5; 0.066 mM myoglobin with 3.0 mM azide, 20 mM KP_i, pH 7.5; 0.45 mM myoglobin with 100 mM imidazole, 1 M Tris–HCl, pH 8.5; 0.45 mM cytochrome *c* with 100 mM azide, 1 M Tris–HCl, pH 7.5; 0.45 mM cytochrome *c* with 100 mM imidazole, 1 M Tris–HCl, pH 8.5. For each heme protein–ligand binding reaction, three parallel ITC experiments were performed at $t = 20\,°C$, 25 °C, 30 °C, and 35 °C with the exception for metmyoglobin and cyanide where determined thermodynamic parameters at $t = 20\,°C$ deviated from linearity. For all reactions, the ligand was titrated into sample buffer and sample buffer was titrated into protein solution to check for heat dilution. If there were significant dilution heats, these were subtracted from the protein–ligand titration data. Data were processed and fitted to the single set of identical sites model using Microcal Origin ver.7.0 software.

3. Results and discussion

3.1. Thermodynamic characterization of heme protein–ligand interactions

Fig. 1 depicts the thermograms and the fitting of theoretical data to experimental data of metmyglobin binding to cyanide, azide, and imidazole at $t = 25\,°C$. Thermodynamic parameters are listed in Table 1. Metmyoglobin has a relative high affinity for cyanide ($K_d = 0.0012$ mM) and azide ($K_d = 0.03$ mM). While cyanide binding is favoured both enthalpically ($\Delta H = -21.3 \pm 2.8$ kJ/mol) and entropi-

Table 1
Thermodynamic parameters for binding og cyanide[a], azide[a], and imidazole[b] to metmyoglobin, and azide[c] and imidazole[b] to cytochrome *c* at $t = 25\,°C$

Ligand	K_d (mM)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/K mol)	ΔC_p (J/K mol)
Metmyoglobin					
Cyanide	0.001	−33.5	−21.3 ± 2.8	41	−175 ± 41
Azide	0.034	−25.5	−57.3 ± 2.1	−105	−197 ± 42
Imidazole	2.5	−13.2	−4.4 ± 1.2	30	67 ± 5
Cytochrome *c*					
Azide	1.4	−16.3	−0.3 ± 0.1	54	0 ± 8
Imidazole	1.4	−16.3	−0.7 ± 0.4	52	17 ± 4

[a] 20 mM KPi, pH 7.5.
[b] 1 M Tris–HCl, pH 8.5.
[c] 1 M Tris–HCl, pH 7.5.

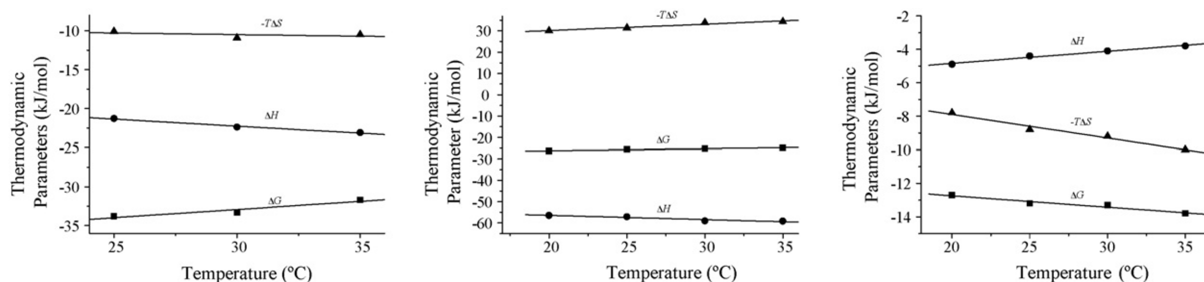

Fig. 2. Temperature dependence of the thermodynamic parameters (ΔG, ΔH and $-T\Delta S$) determined for the binding of myoglobin binding with cyanide (left), azide (middle), and imidazole (right) by ITC. The results are shown as mean from three independent experiments at each temperature. The slope of the linear regression of ΔH vs. temperature yields a $\Delta C_p = -175 \pm 41$ J/K mol for cyanide, -197 ± 42 J/K mol for azide, and 67 ± 5 J/K mol for imidazole.

cally ($\Delta S = 41$ J/K mol), azide binding is enthalpically driven ($\Delta H = -57.3 \pm 2.1$ kJ/mol) and comes with an entropic penalty ($\Delta S = -105$ J/K mol). Imidazole binds less strongly to metmyoglobin ($K_d = 2.5$ mM, $\Delta H = -4.4 \pm 1.2$ kJ/mol, and $\Delta S = 30$ J/K mol) than do cyanide and azide. Cytochrome *c* binding to azide and imidazole is relatively weak ($K_d \approx 2$ mM) and entropically driven ($\Delta S \approx 50$ J/K mol) with enthalpic changes close to zero.

The temperature dependence on ΔH (Fig. 2, Table 1) yields ΔC_p-values of -175 ± 41 J/K mol and -197 ± 42 J/K mol for the binding of cyanide and azide to metmyoglobin, respectively. Imidazole binding to metmyoglobin and azide and imidazole binding to cytochrome *c* are accompanied by small ΔC_p-values (67 ± 5 J/K mol, 17 ± 4 J/K mol, and 0 ± 8 J/K mol, respectively).

3.2. Proton transfer upon binding

The binding reactions of cyanide and azide to metmyoglobin were performed at pH 7.5. At this pH, cyanide will be protonated (pK_a = 9.2). Dou et al. have demonstrated that at such low pH as 7.5, cyanide binds to metmyoglobin as hydrogen cyanide before a proton is transferred from the complex to the solution [26]. This affects the enthalpy change of the reaction as described by the following equation [27]:

$$\Delta H_r = \Delta H_{ind} + nH^+ \cdot \Delta H_{ion} \quad (5)$$

In this equation, ΔH_{ind} is the buffer independent enthalpy change, $n\mathrm{H}^+$ the number of protons taken up or released by the enzyme upon ligand binding, and ΔH_{ion} is the ionization enthalpy of the buffer. To calculate the buffer independent enthalpy change for cyanide binding to metmyoglobin, the ΔH_{ion} of phosphate (5.12 kJ/mol) [28] and the number of transferred protons (−1) can be inserted into Eq. (5). Doing this, a buffer independent enthalpy change of −16.2 kJ/mol is obtained at 298 K. The derived ΔH_{ind} can be used to calculate a ΔS_{ind} (58 cal/K mol) [29]. This is the value that is used in the parameterization of the entropy change (see below). At pH 7.5, azide is already deprotonated upon binding (pK_a = 4.6). This is confirmed by the work of Jacobson et al. where the pK_a for the azide–metmyoglobin complex was found to be 5.5 ± 0.2 [15]. The pK_a (6.95) of imidazole is also much lower than the pH of the buffer (8.5) ensuring that there is no proton transfer upon binding. The same argument can be used for azide and imidazole binding to cytochrome *c*.

3.3. Parameterization of the reaction entropy change

The parameterization of ΔS_r of protein–ligand interactions (Eq. (2)–(4)), enables the obtaining of valuable information with respect to the mechanism of binding. Especially, it allows for the determination of the changes in structure and solvation upon complex formation. Parameterization of ΔS_r for the studied reactions is listed in Table 2. The relative high affinity interactions of metmyoglobin with cyanide and azide are accompanied by very different ΔS_{conf} with cyanide binding being accompanied by a small and a positive value of 46 J/K mol while the value, −121 J/K mol, for azide binding is large and negative. For the cyanide binding, the positive ΔS_{solv} of 45 J/K mol and the positive ΔS_{conf} of 29 J/K mol compensates for the negative ΔS_{mix} while the relatively low ΔS_{solv} of 50 J/K mol is not able to do so for the negative ΔS_{conf} and ΔS_{mix} for azide binding. The low affinity interaction of metmyoglobin with imidazole, and cytochrome *c* with azide and imidazole are driven by relatively large and positive ΔS_{conf} (≈85 cal/K mol). ΔS_{solv} is close to zero because of the low ΔC_p of the reactions.

3.4. Binding to metmyoglobin

Binding of cyanide and imidazole to metmyoglobin are followed by positive ΔS_r while it is negative for azide binding. The parameterization shows that ΔS_{conf} is positive for cyanide binding (46 J/K mol) and imidazole binding (89 J/K mol) and

Table 2
Parameterization of ΔS_r for binding of azide, imidazole, and cyanide to myoglobin, and azide and imidazole to cytochrome *c* at $t = 25\,^{\circ}$C

	Metmyoglobin			Cytochrome *c*	
	Cyanide	Azide	Imidazole	Azide	Imidazole
ΔS_r [a]	58	−105	30	54	52
ΔS_{mix} [b]	−33	−33	−33	−33	−33
ΔS_{solv} [c]	45	50	−17	0	−4
ΔS_{conf}	46	−122	80	87	89

[a] $\Delta S_r = \Delta S_{solv} + \Delta S_{conf} + \Delta S_{mix}$ [1].
[b] $\Delta S_{mix} = R\ln(1/55.5) = -33$ J/K mol [1].
[c] $\Delta S_{solv} = \Delta C_p \ln(T_r/T_{38.515\,\mathrm{K}}) = \Delta C_p \ln(298.15/385.15)$ [1].

one negative (−122 J/K mol) for azide binding. Previous work has shown that both N- and C-terminal regions in myoglobin undergo large conformation changes upon ligand binding [30]. It appears that changes in helix–helix interactions are necessary for ligand binding since helices C, D, E, and F are involved. It is not clear why we observe both positive and negative ΔS_{conf}. Both cyanide and azide binding are followed by positive ΔS_{solv} (i.e. negative ΔC_p). The protein could become more "compact" as a result of the negative ΔS_{conf}, and lose water because of this. The change in solvation entropy for imidazole binding is small.

3.5. Binding to cytochrome c

In order for ligand binding to cytochrome *c*, a methionine needs to be displaced. Binding of azide and imidazole to cytochrome *c* are weak ($K_d = 1.4$ mM). Displacement of methionine with either azide or imidazole is not accompanied by a negative ΔH_r. The parameterization of ΔS_r show that there is positive change in conformation entropy that drives the reaction. This is in accordance with previous work where displacement of the axial methionine with imidazole [31] and cyanide [32] comes with significant conformational changes in both the protein backbone as well as side chains in the distal ligand region. Changes in solvation is not a factor in the binding reactions since $\Delta S_{solv} \approx 0$ in either interactions.

3.6. Changes in heat capacities

ΔC_p-values determined for the studied heme protein–ligand interactions are in general small and negative with the exception of the reaction between metmyoglobin and imidazole where the value is small and positive. The relatively small changes in the heat capacity indicate only minor hydrophobic interactions upon complexation [5,9]. Binding of imidazole inhibitors with various different hydrophobic susbstituents to cytochrome P450, another heme protein, yielded ΔC_p-values as low as −2500 J/K mol [33]. These high and negative values are most likely the result of hydrophobic interactions in the protein upon ligand binding and hydrophobic interactions between the ligand and the active site. Upon inhibitor binding to cytochrome P450, the apolar solvent accessible areas (ASA_{apolar}) were reduced with over 10% [33]. The same difference in ASA_{apolar} between ligand-bound and ligand-free metmyoglobin is only around 0.6% (calculated using GetArea 1.1, Galveston, TX, USA, based on the crystal structures to ligand-bound and ligand-free metmyoglobin [34–36]).

4. Conclusions

Determined ΔC_p-values for heme protein–ligand interactions obtained from this study correlate well with the degree of hydrophobic interactions upon ligand binding. Also, the determination of ΔC_p-values allows for the parameterization of ΔS_r. From this parameterization, ΔS_{conf} are calculated and shown to correlate well with what is observed in other structural works on the same heme protein systems.

Acknowledgment

We are grateful for the funding from the Norwegian Research Council for the ITC instrument (Project 155518/V00).

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