Hysteresis in the thermal relaxation dynamics of an immune complex as basis for molecular memory

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Abstract

The mechanisms by which a protein searches its vast conformational space in order to attain its native fold in physiological times and to bind productively to relevant biological partners are not well understood. In particular, most proteins must be able alternate between at least one active conformational state and back to an inactive conformer, especially for the macromolecules that perform work and need to optimize ATP usage. This property may be invoked by a physical stimulus (temperature, radiation) or a chemical ligand. We have stimulated with temperature cycles two partners of an immune complex and revealed that properties of the external stimulus (period, phase) are also found in characteristics of the protein complex (i.e. periodic variation in its binding affinity). These results may be important for delineating *in vitro* the bases of molecular memory.

All biological molecular machines must construct an internal model of prior interactions with relevant stimuli, in order to predict and adequately respond to a changing environment [1]. This adaptive capacity is manifested by single proteins even *in vitro,* for example in cases where phosporilation/dephosphorilation enzymes create cycles of activation/inactivation of the same protein substrate [2]. While this periodic variation in association and disassociation between the three protein partners generates a corresponding periodic variation in their activity, it has not been established how the system reacts if the external stimulus is removed after a few entrainment cycles. This is essential for assessing the ability of a biological molecule to adapt and anticipate events in its local physical and chemical environment. In particular, we asked whether we can induce in protein partners a periodic change in their binding affinity in response to periodic temperature changes *in vitro*. Underlying this sensitivity of biological systems to temperature is the impact that changes in the thermal energy of the environment has on biochemical and thus on physiological processes, especially in organisms subject to ample temperature fluctuations. We experimentally investigate the theoretical prediction that due to the finite memory of these macromolecules, a selection of the protein conformational states must occur in order to avoid or optimize the energetic cost of erasing irrelevant information caused by stochastic environmental fluctuations [3]. Furthermore, we demonstrate that following application of temperature waveforms, the memory of these periodic external events is manifested in the absence of the original stimulus and may form the basis of a molecular predictive capacity.

Since most of the biochemical activities of a cell are mediated by the transient formation of complexes between macromolecules and their ligands, any given binary complex must have a finite useful lifetime. The functional consequences of complex formation are therefore transient and must be tightly regulated in terms of timing of initiation, duration of action, and amplitude of action. In contrast to spectroscopic methods used to investigate local protein conformational changes on complex formation, calorimetric methods are ideally suited due to absence of reporter labels needed to probe the conformational state of the proteins under investigation. We have focused herein on a malarial monoclonal IgG antibody (MAb k23) relevant for detecting the fever-inducing pathogen *P. vivax,* and its recombinant antigen, the 19 kDa C-terminal region of the merozoite surface protein 1

($MSP1_{19}$) [4]. We measured *in vitro* by calorimetric techniques the thermodynamic parameters of their binding upon cycles of thermal entrainment at 25□ and 37□, prior to or after complex formation. Isothermal titration calorimetry (ITC) was used to assess the binding affinity changes in proteins during their complex formation (Figure 1), in the absence (red-colored thermogram) or presence (blue-colored signal) of the periodic thermal entrainment of 10 minutes cycles at each indicated temperature prior to titrations.

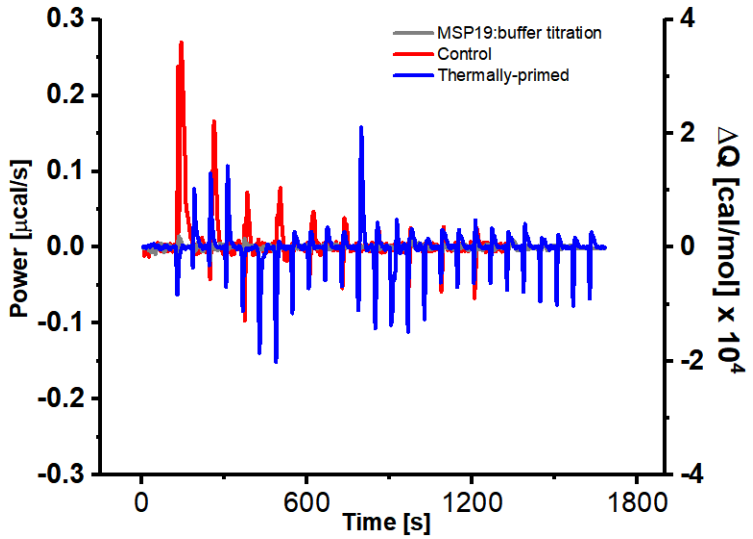

Figure 1. Background-subtracted ITC measurements of malaria $MSP1_{19}$ titrated into K_23 IgG antibody, with (red trace) or without (blue trace) prior periodic thermal priming of each protein partner at 25□ and 37□. Titration of $MSP1_{19}$ into PBS buffer is shown in grey. Measurements performed at 37□, after 3 cycles at each indicated temperature, last ramp up from 25□, before start of titrations. Pre-equilibration time 15 minutes.

In contrast to the expected endothermic reaction described by a sigmoid curve [4], the thermograms obtained for the $MSP1_{19}$ titrated into K_23 IgG after periodic thermal entrainment revealed a sinusoidal pattern composed of both endothermic and exothermic peaks. The presence of intermediate peaks allowed for the determination of the period of oscillations at 430 ± 30 seconds, ≈ 25% less than the 10 minute periods of prior thermal entrainment, a process indicative of underdamped diffusion caused by temperature fluctuations [5]. Importantly, the phase of the oscillation in the binding affinity curve was advanced compared to a fitted harmonic wave [SI], an observation that may explain a similar phase shift measured *in vitro* with other systems, upon temperature increase but on a lengthier circadian basis [6]. By fitting the thermograms to a single-binding site model

with 1:1 stoichiometry, for individual segments of 600 seconds that correspond to original entrainment periods, we obtain the following set of thermodynamic parameters (Table 1):

Table 1. Thermodynamic parameters obtained by fitting truncated thermograms

Segment [min]	K_D [nM^{-1}]	ΔH [kcal/mol]	T·ΔS [kcal/mol]	ΔG [kcal/mol]	k_{on} [$M^{-1}s^{-1}$] ·10^6	k_{off} [s^{-1}]	τ [s]
0´-10´	28.4 ± 4.7	-9.8 ± 2.6	9.1 ± 2.3	-0.6 ± 0.3	1.2 ± 0.3	0.034 ± 0.005	29.4
10´-20´	9.8 ± 2.6	-6.7 ± 2.1	4.9 ± 1.2	-3.2 ± 0.9	2.8 ± 0.5	0.28 ± 0.3	3.5
20´-30´	39.4 ± 4.4	-33.8 ± 4.8	37.3 ± 3.7	-3.5 ± 0.8	0.4 ± 0.11	0.016 ± 0.003	62.5
Control	25 ± 3.4	17.2 ± 3.1	28 ± 2.5	-10.8 ± 0.6	0.29 ± 0.05	0.007 ± 0.002	142

An important characteristic of the oscillations in binding parameters is the reversal of the enthalpy sign compared to control titrations, suggestive of net favorable binding for each thermogram. The decrease in entropy in the second segment corresponds to the time frame of entrainment at 25□ for either complex partner, prior to titrations. The increase in entropy in the third segment is significant, suggesting extensive ordering at the binding interface and a conformational selection for each protein partner that presents the most favorable binding interface and thus the highest binding affinity. Because the affinity alone of a ligand for its binding partner is not a sufficient condition to measure the effectiveness and duration of biological action, the lifetime and stability of the binary receptor-ligand complex was also calculated (parameter τ) [6]. As with other soluble antibodies, such stability must thus be optimized, for instance in cases where antigen binding to B-cell receptors occurs through a two-step, induced-fit process prior to endocytosis, requiring sufficiently large residence times for efficient antigen processing [7]. Kinetically, we measure the highest binding affinity in the third segment of the thermograms, accompanied by the lowest k_{off} and thus the largest complex residence time ($\tau \approx 1$ minute). This parameter directly defines the complex stability and thus the effectiveness of interaction between the two protein partners. Interestingly, lowest τ and highest k_{off} was measured in the second segment, mirroring the thermodynamic observations outlined above.

Following immune complex formation, important information as to the binding mechanism can be obtained using Differential scanning calorimetry (DSC) in isothermal mode, since,

unlike ITC, the thermal entrainment cycles and corresponding variations in heat capacity (Cp) are measured *in situ* (Figure 2).

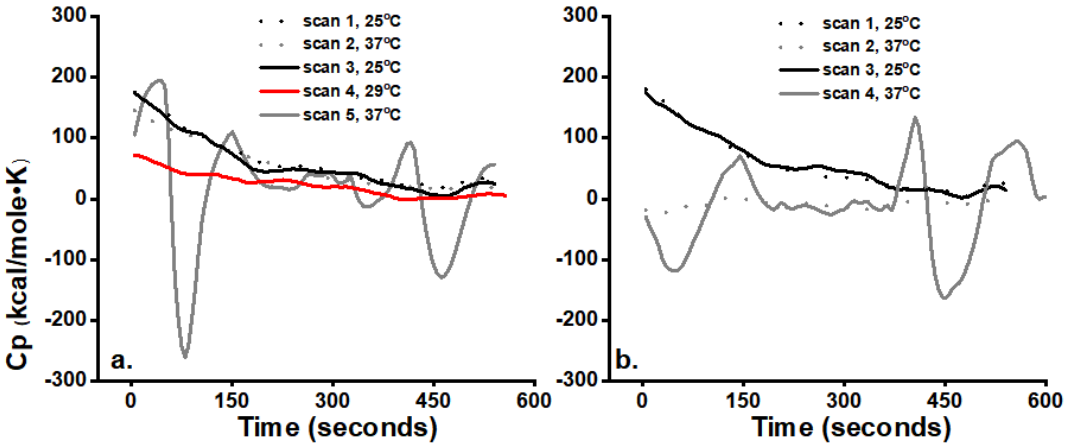

Figure 2. Background-subtracted, normalized DSC scans of equimolar $MSP1_{19}$:$K_2$3 (0.5 mg/ml total protein complex concentration). Reconstitution of the immune complex was achieved *in situ* at 37□ for 30 minutes, prior to temperature ramping. Similar to ITC measurements, following 3 entrainment steps at 25□ and 37□ for 10 minutes each, data was collected from scan 1, as indicated in fig. 2.a and 2.b. Pre-equilibration time is 10 minutes, prior to each data scan collection.

Most DSC curves start with an endothermic effect, indicating that a process had started before it could be monitored, a time limitation imposed by the necessary equilibration period for heating/cooling the samples. We measured disruptions of the immune complex accompanied by an increase in heat capacity (positive Cp), followed by a subsequent decrease indicating complex reformation, as observed with other systems [8]. Endothermic and exothermic peaks from the DSC scans were modeled with a bigaussian function, whose fitting parameters are shown in Table 2:

Table 2. Fitting parameters for the averaged DSC scans

Peak	Peak height [kcal/mole·K]	Peak center [sec]	Peak width [sec]
1	176 ± 44	53 ± 12	27 ± 12
2	-309 + 21	110 ± 31	33 ± 14
3	79 ± 17	139 ± 22	31 ± 13
4	82 ± 23	435 ± 29	52 ± 18
5	-173 ± 31	470 ± 34	34 ± 12

Similar to ITC measurements, we measure autonomous fluctuations in heat capacity changes of the immune complex, characterized by marked decrease in peak amplitudes

with time and by phase shifts, as measured from the position of exothermic peak centers. We calculate a period of 390 ± 20 seconds for averaged DSC scans, based on the peak centres of the exothermic oscillations, a value closed to the oscillation period measured in ITC. Importantly, the peak width of the second re-association step of the protein partners is larger than the corresponding first peak width, an increase close the residence time we also observed in ITC (≈ 1 minute).

The presence of phenomenological molecular memory here described was previously observed in hysteretic enzymes, whereby the protein slowly responds to changes in metabolites [9], binding partners [10] or in pH [11], thus providing a time-dependent buffering capacity to stochastic oscillations of signalling partners or physical stimuli. Hysteretic behaviour can also be present when the ligand uncouples from its protein partner at a sufficiently slow rate. Necessarily, free energy barriers must separate the distinct internal memory states of a system, absent which it will randomly fluctuate from one memory state to another under the influence of thermal fluctuations. However, the periodic optimization in the thermodynamic and kinetic binding parameters we observed, coupled to the increase in k_{off} rates, suggests that a subpopulation of protein conformers is still present for a specific stimulus, even after the system has been subjected to a new and distinct signaling event. While protein conformational fluctuations range from femtoseconds to minutes [12], the potential to retain or revisit specific conformational states upon periodic external stimulation has not been previously explored. Whether this property can be optimized, by example through reversibly creating binary and ternary complexes of competing ligands, will be important in dissecting *in vitro* the mechanisms of molecular memory.

Supporting Information Available: Experimental procedures, raw ITC thermograms and fitting procedures.

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