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

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Abstract

Proteins search their vast conformational space in order to attain the native fold and bind productively to relevant biological partners. In particular, most proteins must be able to 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 energy usage. This property may be invoked by a physical stimulus (temperature, radiation) or by a chemical ligand, and may occur through mapping of the protein external environment onto a subset of protein conformers. We have therefore stimulated with temperature cycles two partners of an immune complex before and after assembly, and revealed that properties of the external stimulus (period, phase) are also found in characteristics of the protein complex (i.e. periodic variation in the 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 proteins even *in vitro*, for example in cases where phosporilation/dephosphorilation enzymes create cycles of activation/inactivation of the same protein substrate, or when circadian variation in oxygen-carrying ability is promoted, in the absence of transcription-translation, through reversible tetramer-dimer equilibrium of oxyhemoglobin [2, 3]. While this periodic variation in association and disassociation between the same or different 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 predict and anticipate events in its local physical and chemical environment, given the striking competitive value that this anticipative property carries [4]. 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 [5]. 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. 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 [6]. 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.

In contrast to spectroscopic methods used to investigate local protein conformational changes on complex formation, calorimetric methods are ideally suited to reveal the full thermodynamic profile of interaction. We have focused herein on a monoclonal mouse IgG

antibody (MAb k23) relevant for detecting the recombinant antigen, membrane-anchored 19 kDa C-terminal region of the merozoite surface protein 1 ($MSP1_{19}$) from the fever-inducing pathogen *Plasmodium vivax* [7]. We measured with isothermal titration calorimetry (ITC) the thermodynamic parameters of their binding upon cycles of thermal entrainment at 25□ and 37□, prior to complex formation [Supporting Information]. Figure 1 presents thermograms of the antigen-antibody titrations, in the absence (red-colored signal) or presence (blue-colored signal) of the periodic thermal entrainment of 10 minutes cycles at each temperature prior to titrations.

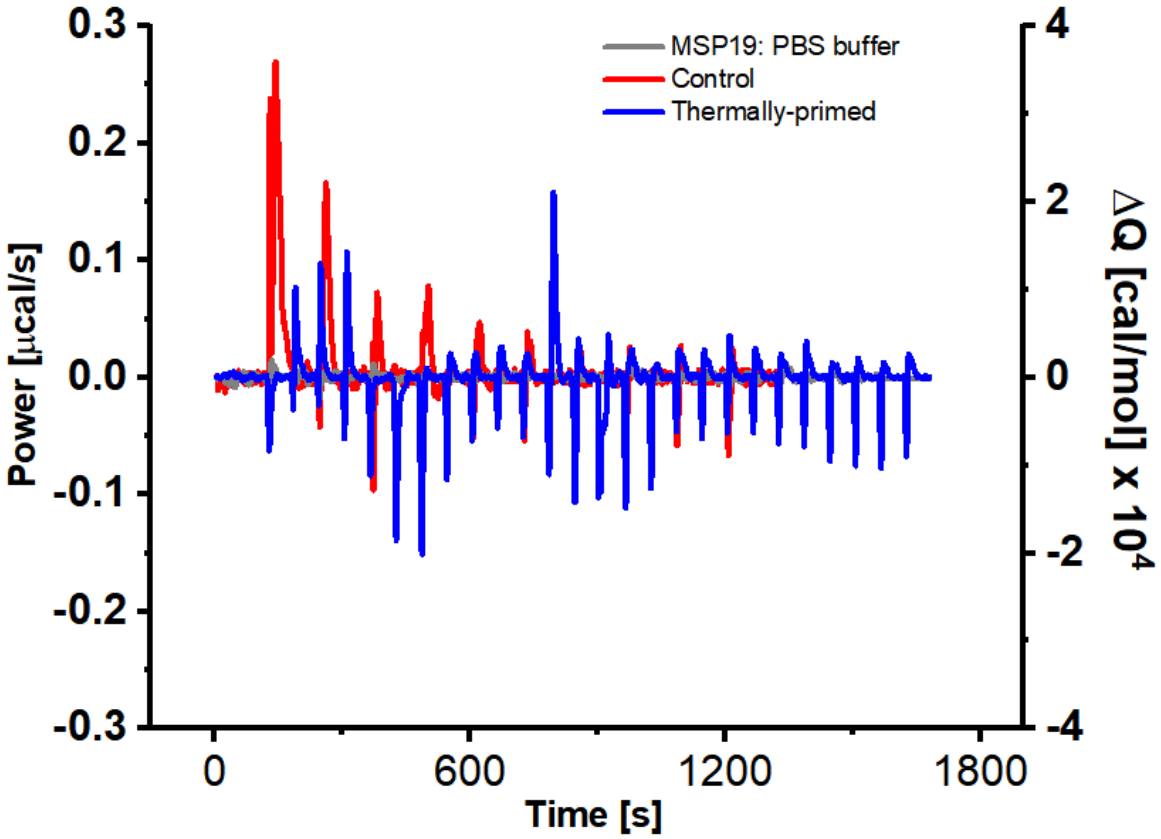

Figure 1. Background-subtracted ITC measurements of malarial $MSP1_{19}$ titrated into $K_2$3 IgG antibody, with (red trace) or without (blue trace) prior periodic thermal priming of each protein partner at 25□ and 37□. Titration of $MSP1_{19}$ into PBS buffer is shown in grey. Measurements performed 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 [8], the thermograms obtained for the $MSP1_{19}$ titrated into IgG $K_2$3 after periodic thermal entrainment revealed a complex 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 600 second periods of prior thermal entrainment, a process indicative of underdamped diffusion caused by transient binding events temperature fluctuations [9]. Importantly, the phase of the oscillation in the binding affinity curve was advanced compared to a fitted harmonic wave [Supporting Information], an observation that may explain a similar phase shift measured *in vitro* with other systems, upon temperature increase but on a lengthier circadian basis [10]. We fitted the thermograms to a single-binding site model with 1:1 stoichiometry, for each individual segment of 600 seconds that corresponds to original entrainment periods, we obtain the following set of thermodynamic parameters (Table 1):

Table 1. Thermodynamic parameters obtained by fitting truncated thermograms

Segment [minutes]	K_D [nM^{-1}]	ΔH [kcal/mol]	T·ΔS [kcal/mol]	ΔG [kcal/mol]	k_{on} [$M^{-1}s^{-1}$] $\cdot 10^6$	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 truncated 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) [11]. This parameter defines the complex stability and thus the effectiveness of interaction between the two protein partners. As with other soluble antibodies, such complex stability must 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 [12]. 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). Interestingly, lowest τ and highest k_{off} were measured in the second segment, mirroring the thermodynamic observations outlined above.

Following immune complex formation, important information as to the binding mechanism and the stability of the complex 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).

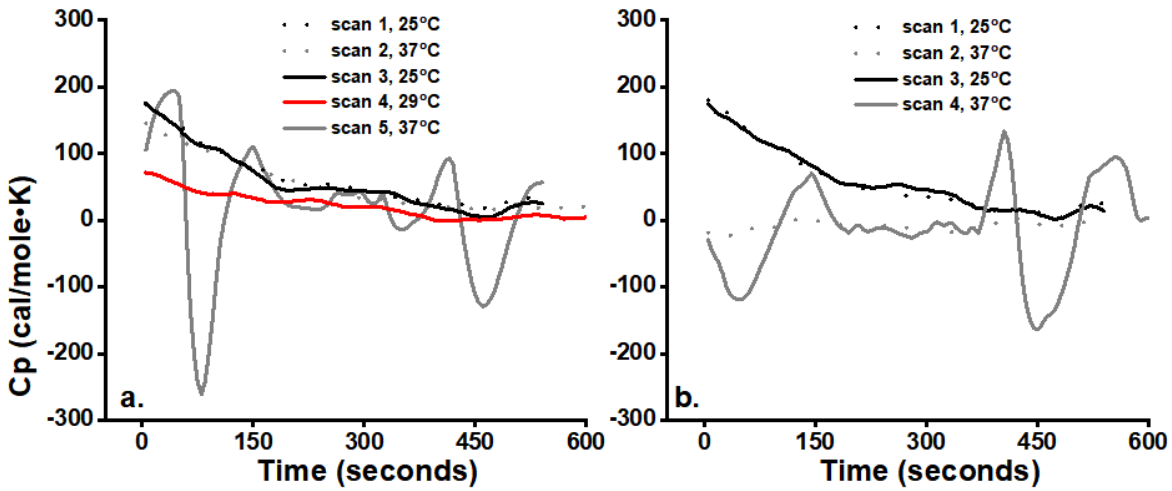

Figure 2. Background-subtracted, normalized DSC scans of equimolar $MSP1_{19}$: IgG $K_2$3 (0.5 mg/ml total protein complex). Reconstitution of the immune complex was achieved *in situ* at 37□ for 30 minutes, prior to temperature ramping. Similar to ITC measurements, following 3 entrainment steps at 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 5 minutes, before 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 (Supporting Information). 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 [13]. Endothermic and exothermic peaks from the DSC scans were modeled separately 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 [seconds]	Peak width [seconds]
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 measured 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 close to the oscillation period measured in ITC. Furthermore, the peak width of the second re-association step of the protein partners is larger than the corresponding first peak width, an increase in the residence time that we also observed in ITC (≈ 1 minute). Importantly, not all DSC curves contained both exothermic peaks, possibly indicating re-association steps that occur during the experimentally inaccessible pre-equilibration periods (Fig S1.a). We have not detected any periodic features in DSC scans after sample removal, storage at 4□ and later measurements without thermal treatment, suggesting the complete loss of initial temperature priming information (Fig S1.b).

The presence of phenomenological molecular memory here described was previously observed in hysteretic enzymes, whereby the protein slowly responds to changes in metabolites [14], binding partners [15] or in pH [16], 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. Importantly, entropy increase across the truncated thermograms may

indicate a causal entropic forcing of the system that maximizes the span of future accessible pathways to stimuli, and thus adaptive behaviour, as theoretically predicted [18]. While protein conformational fluctuations range from femtoseconds to minutes [17], 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 for dissecting *in vitro* the mechanisms of molecular memory.

Supporting Information Available: Experimental procedures, DSC scans and ITC thermograms are included in the supporting information.

Acknowledgments

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References

1. Still S, Sivak DA, Bell AJ, Crooks GE (2012) Thermodynamics of prediction. *Phys. Rev. Lett.* 109: 120604.
2. Tomita J, Nakajima M, Kondo T, Iwasaki H (2005) No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science*. 307: 251-4.
3. O'Neill JS, Reddy AB (2011) Circadian Clocks in Human Red Blood Cells. *Nature*. 469: 498–503.
4. Dodd AN, Salathia N, Hall A, Kévei E, Tóth R, Nagy F, Hibberd JM, Millar AJ, Webb AA (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science*. 309: 630-3.
5. Bennett AF, Dao KM, Lenski RE (1990) Rapid evolution in response to high-temperature selection. *Nature*. 346: 79–81.
6. Sivak DA, Thomson M (2014) Environmental statistics and optimal regulation. *PLOS Comp. Biol.* 10: e1003826.
7. Bargieri DY1, Rosa DS, Braga CJ, Carvalho BO, Costa FT, Espíndola NM, Vaz AJ, Soares IS, Ferreira LC, Rodrigues MM (2008) New malaria vaccine candidates based on the Plasmodium vivax Merozoite Surface Protein-1 and the TLR-5 agonist Salmonella Typhimurium FliC flagellin. *Vaccine*. 26: 6132-42.
8. Pierce MM, Raman CS, Nall BT (1999) Isothermal titration calorimetry of protein-protein interaction. *Methods* 19: 213-221.
9. Bodrova AS, Chechkin AV, Cherstvy AG, Safdari H, Sokolov IM, Metzler R (2016) Underdamped scaled Brownian motion: (non-)existence of the overdamped limit in anomalous diffusion. *Sci. Rep*. 6: 30520.
10. Yoshida Tm Murayama Y, Ito H, Kageyama H, Kondo T (2009) Nonparametric entrainment of the in vitro circadian phosphorylation rhythm of cyanobacterial KaiC by temperature cycle. *Proc. Natl. Acad. Sci.* 106: 1648-53.
11. Tummino PJ, Copeland RA (2008) Residence Time of Receptor−Ligand Complexes and Its Effect on Biological Function. *Biochemistry.* 47: 5481-92.
12. Cho S, Swaminathan CP, Yang J, Kerzic MC, Guan R, Kieke MC, Kranz DM, Mariuzza RA, Sundberg EJ (2005) Structural basis of affinity maturation and intramolecular cooperativity in a protein-protein interaction. *Structure.* 13: 1775-87.

13. Rudolph MG, Linnemann T, Grunewald P, Wittinghofer A, Vetter IR, Herrmann C (2001) Thermodynamics of Ras/effector and Cdc42/effector interactions probed by isothermal titration calorimetry *J. Biol. Chem*. 276: 23914-21.
14. Kroha HK, Panizzi P, Bock PE (2008) Von Willebrand factor-binding protein is a hysteretic conformational activator of prothrombin. *Proc. Natl. Acad. Sci.* 106: 7786-91.
15. Mori T, Williams DR, Byrne MO, Qin X, Egli M, Mchaorab HS, Stewart PL, Johnson CH (2007) Elucidating the ticking of an in vitro circadian clockwork. *PLOS Biology*. 5: e93.
16. Hand SC, Carpenter JF (1986) pH-Induced metabolic transitions in artemia embryos mediated by a novel hysteretic trehalase. *Science*. 232: 1535.
17. Wissner-Gross AD, Freer CE (2013) Causal Entropic Forces. *Phys. Rev. Lett.* 110: 168702.
18. Russel D, Lasker K, Phillips J, Schneideman-Duihovny D, Velázquez-Muriel JA, Sali A (2009) The structural dynamics of macromolecular processes. *Curr. Opin. Cell. Biol.* 21: 97-108.

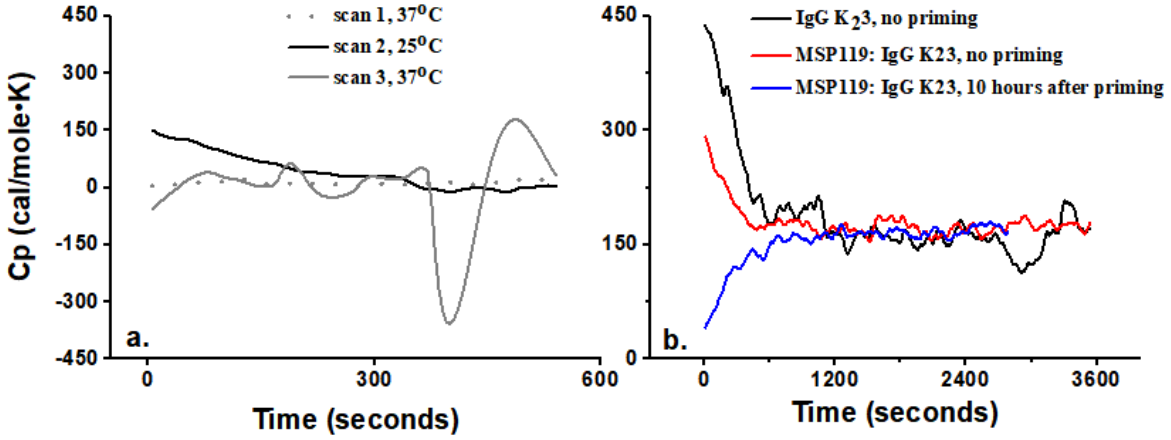

Figure S1. Background-subtracted, normalized single DSC scans at 37□ of a. IgG $K_2$3 (1 mg/ml) and b. $MSP1_{19}$: IgG $K_2$3 in the absence of thermal priming. Single scan $MSP1_{19}$: IgG $K_2$3 complex, 10 hours after thermal priming (c.) Pre-equilibration time is 5 minutes, for all scans, prior to data collection.