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Real-time monitoring of conformational transitions of single-molecule histone deacetylase 8 with nanocircuits[†]

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Abstract

Using single-molecule approaches, we directly observed the dynamic interaction between HDAC8 and various ligands as well as conformational interconversions during the catalytic reaction. Statistical analysis identified key kinetic parameters, demonstrating that the enzymatic activity is highly sensitive to both minor variations in the ligand structures and small synthetic molecules.

The dynamics of protein conformation play a major role during protein–protein and protein–ligand interactions, but remain a central question with enormous importance for the design of drugs.^{1–5} For example, multiple conformational subset states of proteins result in significant differences in their affinity, selectivity, and reactivity.⁶ Thus, it is critical to understand the protein dynamics and their roles in ligand recognition, catalysis, and inhibition to advance the relevant design of pharmacologically efficient anti-disease drugs.

Recently, histone deacetylase 8 (HDAC8) has gained considerable attention regarding the design of drugs for a variety of human diseases due to its involvement in epigenetic regulation.^{7–10} The enzyme catalyzes the hydrolysis of acetyl-lysine residues in histone proteins to promote gene repression and silencing. X-ray crystallography and nuclear magnetic resonance studies have revealed that HDAC8 is a highly flexible enzyme, undergoing 5–10 Å conformational rearrangements to adopt and catalyze differently-structured ligands.^{11–13} In addition, several conformational subset states of a HDAC8–ligand complex and an interconversion between the states have been observed in computational modeling studies.¹⁴ However, direct measurements of such dynamic features have not been achieved due to the averaging effects from ensemble-assay measurements and the limited dynamic information from crystallographic methods.

[†]Electronic supplementary information (ESI) available: Additional materials and methods, signal transduction mechanism, fluorescence-based assay measurements, electronic device fabrication and measurements, and the mean normalized variance analysis. See DOI: 10.1039/c6cc09949a

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Here, we apply a novel, electronic single-molecule approach to examine the real-time dynamics of the HDAC8–ligand complex over long periods of time, which are usually limited in single molecule fluorescence resonance energy transfer measurements due to fluorophore bleaching.^{15,16} To systematically monitor the dynamic interaction between HDAC8 and ligands, two configurations of nanocircuits are prepared (Fig. 1). First, we synthesized pyrene-suberoylanilide hydroxamic acid (pSAHA) inhibitors (Fig. S1, ESI[†]) to attach to the sidewalls of single-walled carbon nanotube (SWNT) field-effect transistors (FETs) (Fig. 1a, pSAHA-nanocircuits). The aromatic pyrene moiety adheres to the SWNT sidewall *via* π – π interactions and the SAHA-inhibitor interacts with freely-diffusive HDAC8 enzymes to form a HDAC8–pSAHA complex. As the complex undergoes conformational changes, motions of charge residues associated with the conformational changes induce current fluctuations underlying the SWNT-FET through a charge gating effect, which have been clearly demonstrated in our previous work.^{17,18} Second, individual HDAC8 molecules attached to the SWNT-FETs (Fig. 1b, HDAC8-nanocircuits) using pyrene-iminodiacetate (IDA)-Cu²⁺ linker molecules (Fig. S1, ESI[†]), in which Cu²⁺ ions bind to the N-terminal His-tag of HDAC8. The dynamic motions of the HDAC8–ligand complex formed with freely-diffusive ligands including coumarin-SAHA (Fig. S1, ESI[†], cSAHA), a potent *N*(phenylcarbothiol)benzamide activator¹⁹ (Fig. 1c, hereafter ACT), and a trifluoroacetyl-lysine-methylcoumarin conjugate substrate (Fig. S1, ESI[†], TFAL-AMC) also induce fluctuations in the SWNT-FET current through a similar gating effect. An atomic force microscopy (AFM) image (Fig. 1d) confirmed the presence of a single attached HDAC8 on the SWNT-FET nanocircuits.

Initially, the conformational dynamics of the HDAC8–pSAHA complex were investigated with the pSAHA-nanocircuit. This configuration allows formation of the complex with the same pSAHA-inhibitor and pre-existing conformational states of individual HDAC8 in bulk solution. Fig. 2 depicts typical $I(t)$ signals measured with the pSAHA-nanocircuits. In a buffer solution (25 mM HEPES, 100 mM NaCl, 1 mM TCEP, pH 7.5), the circuit's $I(t)$ signal shows a featureless baseline current state (Fig. 2a). Following the addition of HDAC8 (6 μ M) to the pSAHA-nanocircuit, an additional current state below the baseline current was observed (Fig. 2b). $I(t)$ fluctuated between two current states with a mean amplitude of approximately 4 nA. In addition to HDAC8, the potent activator (ACT, 6 μ M) was added to the same circuit (Fig. 2c). Similar $I(t)$ fluctuations between two states and the fluctuation rate were observed. Fig. 2d illustrates a control measurement from the same circuit performed in the presence of both HDAC8 and excess SAHA-inhibitors (30 μ M) in the buffer solution. Since the freely-diffusive SAHA-inhibitors surrounding HDAC8 immediately bind to HDAC8 in the solution, no SAHA-free HDAC8 is accessible to the pSAHA-nanocircuit,²⁰ resulting in no $I(t)$ fluctuations. The absence of the two-level current fluctuation when no HDAC8 is in the solution or when both HDAC8 and excess SAHA-inhibitors are present confirms that $I(t)$ signals are caused by the HDAC8–pSAHA complex.

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HDAC8 is known to be a highly flexible enzyme during ligand binding and catalysis.^{11,21} For SAHA-inhibitors, the hydroxamate acid moiety chelates the metal ions (Zn^{2+}) in the active site pocket, while the aliphatic chain and the capping moiety interact with the hydrophobic pocket and the protein surface.^{22,23} Crystallographic and computational studies have identified that two loops (L1 and L2) located in the vicinity of the active site pocket entrance are highly malleable to effectively adopt and catalyze structurally different substrates.^{13,14} While SAHA always remains bound to the metal ions, the two loops dynamically interact to stabilize and catalyze the ligand. When the two loops have open conformations, for example, catalysis takes place. During such conformational rearrangement, motions of charged residues on the loops electrostatically gate the SWNT channel current, resulting in two distinguishable current states. Thus, we assign the low- and high-current states of our signals to the dynamic interaction of L1 and L2 loops. The low current state and the open loop conformation of the complex could result from negatively charged tri Asp residues (87–89) in the L2 loop moving away from the SWNT (Fig. S2, ESI[†]).¹⁴

Fig. 3 shows the probability distributions of the duration in the low (τ_{low}) and high (τ_{high}) current states accumulated from 600 s of recordings. All distributions fit simple Poisson distributions, determining single mean values of τ that represent the majority of events (>94%). In the presence of HDAC8 in the buffer solution, the mean values of τ_{low} and τ_{high} were measured to be 1.4 ms and 118 ms, respectively (Fig. 3, blue color). In the additional presence of the activators, the mean value of τ_{low} was increased by a factor of 10, but there were no changes in the mean value of τ_{high} (Fig. 3, red color). The overall turnover rates, $1/(\tau_{\text{low}} + \tau_{\text{high}})$, remain almost identical due to the major contribution of τ_{high} to the rates. The long events (<6%) off from the fit at the tail could be intrinsic to HDAC's conformational variability, which affects the arithmetic average $\langle t \rangle$ values of the entire population. The mean values, overall turnover rates, comparable ensemble rates, and relative energy differences are summarized in Table 1.

To complement the single-molecule observation, we performed fluorescence-assay measurements with HDAC8 and pSAHA (Fig. S3, ESI[†]). The dissociation off-rate (k_{off}) was measured to be 21 s^{-1} . Both a large k_{off} and a relatively short τ_{high} value indicate that pSAHA weakly forms a complex with HDAC8.²² The orientation and static configuration of the pyrene moiety at the end of a SAHA linker are likely less favorable to contact with the active pocket entrance area of HDAC8. However, the τ_{low} values were significantly increased in the presence of the activator. The activator bound to the inside of the active pocket promotes stabilization of the HDAC8–pSAHA complex, inducing longer loop interaction. Furthermore, the Boltzmann statistics in a two-state model allow estimating the relative energy E of one state to another state ($E = k_{\text{B}} T \ln(\langle \tau_{\text{low}} \rangle / \langle \tau_{\text{hi}} \rangle)$). With the activator, the energy difference is reduced by 43%, resulting in a 12-fold increased τ_{low} (Table 1).

To further examine kinetic information regarding the HDAC8–pSAHA complex, the mean normalized variance $r = \sigma^2 / \langle \tau \rangle^2 = 1/n$ of the τ_{low} and τ_{high} is used to assess the number of hidden intermediate steps (n) during the transition between closed and open loop conformations (Table S1, ESI[†]).^{4,24} The variances, τ_{low} and τ_{high} , are slightly greater than 1,

indicating that the loop opening and closing transition follows a single rate-limiting step process with potential reaction pathways for pre-existing conformational subsets of HDAC8 in bulk solution.^{24,25} Thus, the two loops in the complex formation attempt to tighten the weak, non-specific pSAHA inhibitor in the active pocket for the catalytic reaction at the same time.

Next, we reversed the configuration of HDAC8 and SAHA (Fig. 1b) to examine single HDAC8 dynamics with freely-diffusive cSAHA-inhibitors. In the HDAC8-nanocircuit device, the active site pocket of HDAC8 is oriented away from the SWNT, allowing easy access of cSAHA to the pocket entrance areas without a potential interference with SWNTs. The cSAHA has a longer carbon-linker chain than that of pSAHA, which helps cSAHA easily reach to Zn²⁺ ions residing in the deep bottom of the active pocket, and a coumarin moiety at the end of the linker, which gives rise to additional hydrophobic interaction with the active pocket entrance area. In addition, cSAHA has been employed as fluorogenic probes for fluorescence-based binding assays, which permit direct comparisons of the single molecule and ensemble assay results.²⁶

The electronic recordings show similar $I(t)$ fluctuating behaviors to the pSAHA-nanocircuit measurements in the presence of cSAHA (5 μ M) and cSAHA with the activators (20 μ M) (Fig. 4a and b). For quantitative comparison, probability distributions of the τ_{low} and τ_{high} were generated from 600 s recordings (Fig. 4c and d). The distributions reasonably fit single exponential distributions, providing mean τ_{low} and τ_{high} values of 13.2 ms and 1.36 s with cSAHA and 10.3 ms and 1.44 s with cSAHA and the activators. However, a substantial population of longer events (16–21%) in the high-current state resulted in bi-exponential distributions with longer time constants ($\tau_{\text{high}}^{\text{long}}$) of 3.62 s with cSAHA and 4.04 s with cSAHA and the activators.

The τ_{low} value was decreased approximately by 22% with the activator, but the τ_{high} and $\tau_{\text{high}}^{\text{long}}$ were almost identical regardless of the presence of the activators. Accordingly, the relative energy between the two states shows no substantial differences. Instead, the variance analysis resulted in $\eta_{\text{low}} < 1$, indicating two or more rate-limiting steps during the conformational transition of the HDAC8–cSAHA complex. The results suggest that the activator has no significant contribution to the formation of the HDAC8–cSAHA complex. Compared to the pSAHA measurement, τ_{high} values were increased by approximately 10-fold, and accordingly, the overall rates were decreased by 10-fold. As we described above, pSAHA is a weak, non-specific ligand that keeps forming the HDAC8–pSAHA complex, leading to a high frequency of dissociation off-rates and small τ_{low} and τ_{high} values. In contrast, cSAHA has a more favorable structure to form and maintain the HDAC8–cSAHA complex.²⁷ Thus, the longer τ_{high} suggests a longer duration of cSAHA binding to the HDAC8 before the dissociation. Some of the extra-long duration $\tau_{\text{high}}^{\text{long}}$ could result from the large degree of freedom of cSAHA in the active site pocket to become a stable, catalytically-favorable HDAC–cSAHA complex and a prolonged dissociation process of the cSAHA from the complex.

Our previous fluorescence-assay measurements with cSAHA also show supporting results of the long bound duration and the slow dissociation off-rate, k_{off} , of the HDAC8–cSAHA (Table 1).²⁰ The arithmetic average of overall turnover rates is in strong agreement with ensemble measurements, particularly the observation of the bound duration of the HDAC8–cSAHA complex. The agreement suggests that HDAC8 has two major different pre-existing conformations associated with the τ_{high} and $\tau_{\text{high}}^{\text{long}}$, which significantly affects the stability of the complex conformation. One of the major conformations could lead to a longer bound duration ($\tau_{\text{high}}^{\text{long}}$) and its variance value ($r_{\text{high}}^{\text{long}}$) of $\approx 1/2$, suggesting two steps with similar rates before or during the loop opening. An additional step during the conformational transitions might be involved in repositioning or reorienting the ligands within the active site for the hydrolysis. Alternatively, the additional step could result from the conformational transition between two microkinetic states of the HDAC8–ligand complex.¹⁴

Measurements of $I(t)$ fluctuations with SAHA-inhibitors suggest that dynamic conformational transitions associated with the catalytic activity of the enzymes depend on both the structure of inhibitors and the pre-existing conformation of HDAC8. To further support the catalysis-induced $I(t)$ fluctuations corresponding to the loop transition of the complex, we monitored $I(t)$ fluctuations with a HDAC8's cognitive substrate, TFAL-AMC (Fig. S4, ESI[†]). The $I(t)$ recordings revealed a few fluctuations for a long duration measurement (>20 min), indicating a very slow catalytic activity of HDAC8 to the TFAL-AMC substrate. Compared to the SAHA-inhibitors, the loop transition events differ by a factor of 10–100. To complement such slow catalytic activity of HDAC8 to the substrate, fluorescent-assay measurements were performed. Using standard Michaelis–Menten methods, the catalytic turnover rate was measured to be 0.048 s^{-1} (Fig. S5, ESI[†]). This rate is in agreement with the conformational transition rate ($0.072\text{--}0.065 \text{ s}^{-1}$) from the single molecule electronic measurements. Thus, we further conclude that the two-level $I(t)$ fluctuation is caused by the catalysis-induced conformational transition of loops in the complex.

Although both single molecule and ensemble-assay measurements show no significant enhancement of the catalytic turnover rates by the activators, the τ_{low} value was modestly decreased (Table 1). The decreased time spent in the catalysis-ready, open loop conformation resulted in an approximately 29% decrease in the catalytic reaction time. Moreover, the variance of $\tau_{\text{low}} \approx 1$ indicates a simple, transient deacetylation by HDAC8. However, the variance of $\tau_{\text{hi}} \approx 0.64\text{--}0.70$ suggests that multiple steps are involved in the HDAC8–TFAL-AMC complex including conformational transition and dissociation of product steps.¹³ Taken together, the activators minimally and selectively contribute to the HDAC8–TFAL-AMC complex and catalysis.

In conclusion, the electronic readouts directly visualized real-time trajectories of HDAC8–ligand interaction and revealed the number of key rate-limiting steps during the ligand binding and catalysis. Specifically, the following observations provide new insights into the kinetics of the HDAC8–ligand interaction: (1) a substantial conformational rearrangement between two ordered conformational states occurs in the binary complex; (2) the small molecule activator enhances the binding affinity of the weak inhibitor/ligand to the active

site pocket of the enzyme and stabilizes the binary complex; (3) a single rate-limiting step occurs during the closed loop conformation; and (4) two or more steps for either the ligands' reorientation or the product release take place during or after open loop conformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (a) Schematic diagrams of the pSAHA-nanocircuit and (b) the HDAC8-nanocircuit. (c) Chemical structure of the potent activator. (d) An AFM topography image of the single HDAC8 (arrow) attachment on the nanocircuit. The scale bar is 500 nm.

Fig. 2.

Electronic current $I(t)$ fluctuations of the pSAHA-nanocircuit. (a) In the absence of HDAC8 (buffer only), no current fluctuations were observed. The addition of (b) the HDAC8 and (c) HDAC8 with the activators resulted in the $I(t)$ fluctuations between the high-(baseline) and the low-current states corresponding to the conformational transition of the HDAC8-pSAHA complex. (d) A control measurement with excess, freely-diffusive SAHA-inhibitors with HDAC8 in the buffer solution showed no $I(t)$ fluctuations.

Fig. 3. Probability distributions of the duration for two current states: (a) τ_{low} and (b) τ_{high} in the presence and absence of activators. Single exponential fits are shown as solid lines, determining the mean value of τ .

Fig. 4.

The $I(t)$ fluctuations of the HDAC8-nanocircuit monitored with cSAHA-inhibitors in the (a) absence and (b) presence of the activators. Probability distributions of the (c) τ_{low} and (d) τ_{high} in the presence and absence of activators. The τ_{high} distribution shows bi-exponential fits (shown as solid and dotted lines).

Table 1

Kinetic parameters

	Single-molecule					Ensemble			
	$\tau_{\text{low}}(\text{ms})$	$\langle t_{\text{low}} \rangle (\text{ms})$	$\tau_{\text{high}}(\text{s})$	$\langle \tau_{\text{high}} \rangle (\text{s})$	$1/(\tau_{\text{low}} + \tau_{\text{high}}) (\text{s}^{-1})$	$1/(\langle t_{\text{low}} \rangle + \langle t_{\text{high}} \rangle) (\text{s}^{-1})$	$E (\text{kcal mol}^{-1})$	$k_{\text{off}} (\text{s}^{-1})$	$k_{\text{cat}} (\text{s}^{-1})$
HDAC8	1.40 ± 0.0	2.50 ± 5.60	0.118 ± 0.004	0.170 ± 0.176	8.375	5.799	2.66	21	
HDAC8 + ACT	16.4 ± 0.7	22.5 ± 23.5	0.110 ± 0.003	0.152 ± 0.182	7.911	5.731	1.14		
cSAHA	13.2 ± 1.2	13.9 ± 12.9	1.360 ± 0.093	2.500 ± 4.030	0.728	0.398	2.78	0.41–0.77	
cSAHA + ACT	10.3 ± 0.7	11.3 ± 13.6	1.440 ± 0.010	2.440 ± 3.530	0.690	0.408	2.96	0.39	
TFAL-AMC	54.6 ± 3.7	82.0 ± 96.8	13.35 ± 5.140	13.90 ± 3.570	0.075	0.072	3.30		0.048
TFAL-AMC + ACT	38.5 ± 5.7	63.5 ± 107	12.62 ± 1.771	15.30 ± 19.20	0.079	0.065	3.48		0.048