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Competitive ligands facilitate dissociation of the complex of bifunctional inhibitor and protein kinase

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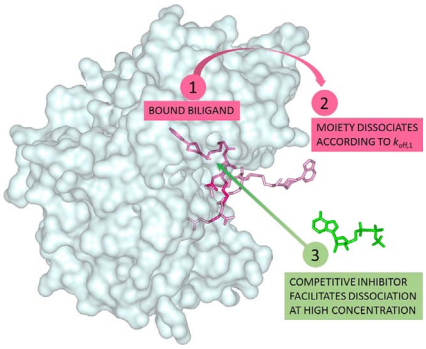
HIGHLIGHTS

- Competitive ligands facilitate (accelerate) the dissociation of the complex of a biligand inhibitor and protein kinase.
- Dissociation of the complex was accelerated by > 50-fold.
- Mechanism of facilitated dissociation of the complex was proposed.
- HTS assay was used for parallel determination of thermodynamic and kinetic parameters.

GRAPHICAL ABSTRACT



ABSTRACT

Dissociation of the complex of a ligand and a protein usually follows the kinetic profile of the first order process and the rate of dissociation is not affected by the presence of competitive ligands. We discovered that dissociation of the complex between a bifunctional ligand and a protein kinase (the catalytic subunit of cAMP-dependent protein kinase), an enzyme possessing 2 different substrate binding sites, was accelerated (facilitated) over 50-fold in the presence of competitive ligands at higher concentrations. Structurally diverse compounds revealed > 10-fold different efficiency for acceleration of dissociation of the complex. These results show that the kinetic behavior of flexible biomolecular complexes possessing two spatially separated contact areas is highly dynamic. This property of biomolecular complexes should be carefully considered for effective application of bifunctional ligands for regulation of activity of target proteins in cells.

1. Introduction

Information regarding the stability and the kinetics of formation and dissociation of complexes is needed for the development of drug candidates. Research in this field has largely been directed to static systems, taking advantage of such methods as X-ray analysis of crystal structures of the ligand:protein complexes, ligand structure-affinity studies, and molecular modelling. Analysis of static systems makes use of well-established techniques to follow the status of interacting molecules and their complexes, whereas the methods used for monitoring the progression of the association process, especially in HTS format are less well acknowledged [1]. However, dynamic changes may uncover

Abbreviations: $k_{off,int}$, intrinsic dissociation rate constant; $k_{off,fac}$, facilitated dissociation rate constant; $k_{off,app}$, apparent dissociation rate constant (in general, mixture of $k_{off,int}$ and $k_{off,fac}$); $k_{a,n}$, association rate constant for individual step; $k_{d,n}$, dissociation rate constant for individual step; τ, residence time (not discriminating apparent or true rate constants); τ_{int}, intrinsic residence time corresponding to $k_{off,int}$; τ_{fac}, facilitated residence time corresponding to $k_{off,fac}$; K_D, equilibrium dissociation constant

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crucial mechanisms that are not disclosed by equilibrium measurements alone [2,3].

In the last decade, the concept of drug-target residence time has come under active debate. The model claims that both the stability and lifetime of a binary drug–target complex (residence time of a drug on a target) are decisive for in vivo pharmacological effect of the drug [4,5]. The thermodynamic stability of the drug:target complex is expressed as the steady-state equilibrium dissociation constant (K_D); the latter quantity corresponds to the ratio of dissociation rate constant (k_{off}) and association rate constant (k_{on}). The residence time of the drug on the target (τ) expresses the lifetime of the complex, corresponding to the reciprocal value of k_{off} ($\tau = 1/k_{off}$). On the one hand, the temporal length of a drug-target interaction determines the timescale of the therapeutic effect after removal of the drug from medium and, on the other hand, prolonged selective inhibition lessens the impact of short-time off-target interactions. The beneficial effects of long residence time of a drug have led to the call for introduction of a kinetics-based lead optimization step into the process of drug development [6].

The request for drugs that dissociate slowly from the complex with its target has, among other approaches, promoted the construction of bifunctional ligands (biligand inhibitors). Biligand inhibitors comprise a tether to link together two functional moieties that simultaneously occupy two spatially separated binding sites of the target protein. Because of the potential for forming more interactions with the target protein, a bifunctional ligand could possess higher inhibitory potency [7] and better selectivity towards the target protein than corresponding monofunctional inhibitors. In many cases prolonged residence time accompanies high inhibitory potency [8–10].

Due to incorporation of two ligands into a single compound, the molecular weight of bifunctional inhibitors tends to surpass the molecular weight limit set by pharmaceutical industry for candidates of oral drugs [11]. However, success with drugs like Navitoclax (MW = 975 Da) and Eribulin (MW = 730 Da) shows that some classical limits (for example, *Lipinski's rule of 5*) set for oral drug candidates are expandable [12,13]. Recent developments highlight bifunctional ligands as promising research tools, especially for targeting physiologically or pathologically important cellular processes that are regulated via protein-protein interactions [14,15].

In this paper we examined the dissociation kinetics of the complex of a bisubstrate enzyme, protein kinase (PK), and its biligand inhibitor comprising two covalently tethered different ligands, each directed to a single substrate binding site of the bisubstrate enzyme. Co-crystal X-ray analysis of stable complexes (K_D values in low nanomolar or sub-nanomolar range) established that both active moieties of the biligand inhibitor are simultaneously bound to appropriate substrate binding sites of the PK [16,17]. Here we used a biligand inhibitor labelled with a fluorescent dye to constitute a photoluminescent probe [ARC-Lum (Fluo) probe] that in complex with the PK possessed photoluminescent signal with long (microsecond-scale) decay time after its excitation with a flash of near-UV radiation [18]. These biligand inhibitors with unique photoluminescent properties were used in the binding assay based on time-gated measurement of luminescence intensity. The free probe possessed no photoluminescence with long decay time and the measured signal intensity was proportional to the concentration of the PK-bound probe. This experimental setup uncovered a previously unknown phenomenon: competing ligands extensively facilitate dissociation rate of the complex of a bifunctional ligand and a protein possessing two closely positioned (but spatially separated) binding sites. Thus this process is fundamentally different from the dissociation of a “classical” ligand:protein complex that is not accelerated in the presence of a competing ligand [19,20].

2. Results and discussion

We studied the dissociation of the bimolecular complex between the biligand inhibitor-derived photoluminescence probe ARC-1063 (Fig. 1B) and catalytic subunit of cAMP-dependent protein kinase (PKAc) (Fig. 1A). An equal total concentration of ARC-1063 and PKAc was used in the equilibrium displacement assay (Fig. S1) for assessment of the excess concentration of the competitive inhibitor at which the complex between ARC-1063 and PKAc would be fully dissociated. The effect of structurally different PKAc ligands (inhibitors and substrates) on dissociation rate was determined (Fig. 1B, C, Fig. S2, Table 1). We discovered that the rate of dissociation of ARC-1063:PKAc complex was dependent on the structure and concentration of a competitive ligand (Fig. 1D).

Depending on the solubility of the ligand and its affinity towards PKAc, each competitive ligand possessed a specific concentration range at which its influence on the rate of dissociation of ARC-1063:PKAc complex could be monitored. For example, ATP (in the presence of Mg^{2+}) could be used at high 10 mM concentration, whereas the lowest concentration of ATP that caused a distinguishable effect on the dissociation kinetics of the probe was 100 μM (Fig. S2D). For comparison, the effect of the high-affinity (K_D = 9 pM) ligand ARC-1416 was tested in the concentration range from 5 mM to 1 nM (Fig. S2A) [16]. Experiments with ATP (Fig. S3) and Mg^{2+} (Fig. S4) were conducted to confirm that the rate facilitation is indeed a result of displacement and not due to secondary effects from high reagent concentration added to the solution.

2.1. Competitor facilitates the dissociation of a bifunctional ligand

We had previously noticed that high concentration of ATP-competitive inhibitor H89 induced an unexpectedly fast dissociation of ARC-type bifunctional probes from PKs in cellular experiments [18,21]. Inconsistent results were seen in biochemical assays as well; that led us to investigate the effect of competitive ligands on the dissociation rate of complexes of biligand inhibitors and the protein kinase. We discovered that the residence time of ARC-1063 on PKAc started to decrease rapidly in the presence of a competitive inhibitor at concentrations exceeding 1 μM of the latter (Fig. 1D). This result pointed to the possibility that the moiety of the bifunctional ligand directed to the ATP-binding site of the PK was moving in and out of the deep hydrophobic pocket with the rate (characterized by $k_{a,1}$ and $k_{d,1}$, respectively, Fig. 2A) similar to that of the ligand formed fully from the dissociated moiety. Therefore, the relatively long residence times ($\tau > 100$ s) characteristic for tight-binding bifunctional molecules result from the favorable re-binding of the ATP-competitive moiety due to existence of the second moiety. The second moiety is continuously bound to the protein and holds the detached moiety in close proximity to its binding site. Increase of the concentration of the competitive compound boosts the probability that the compound occupies the ATP-binding site of the PK within the dissociation-association event of ATP-competitive fragment of the bifunctional ligand. Despite that competitive compound (for example, ATP) can have fast dissociation rate constant, its high concentration allows another molecule to occupy the binding pocket instead of biligand inhibitor. The re-binding of ATP-competitive moiety of the biligand inhibitor is therefore prohibited and subsequent dissociation of the peptide moiety is accelerated, leading to rapid full escape of the bifunctional ligand. For a bifunctional ligand, under completely facilitated conditions this leads to facilitated dissociation rate constants according to the equation $k_{off,fac} = \frac{k_{d,1} \cdot k_{d,2}}{k_{d,1} + k_{d,2}}$ or $k_{off,fac} = \frac{k_{d,3} \cdot k_{d,4}}{k_{d,3} + k_{d,4}}$, depending on the nature of the bifunctional ligand and competitive ligand [22]. The facilitated dissociation rate constant ($k_{off,fac}$) for the complex between a bifunctional ligand and target protein characterizes the rate at infinite concentration of the competitive ligand. This description of the facilitated dissociation is in harmony with the widely acknowledged model of avidity or “forced proximity” of biligands [23] and proposed mechanism of rapid re-binding [24]. Accelerated dissociation of the triple complex of a biligand binder and 2 individual proteins has been briefly described by Mack et al. [25], while Kim et al. have described the facilitated dissociation for protein–protein interaction through an allosteric effect [26]. Here we discovered that a competitive inhibitor was able to