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Photochromic Relaxation Kinetics

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A new method for evaluating the rate constants of chemical reactions has been developed based on the differential photoconversion efficiencies of photochromic FRET acceptors incorporated into molecules on both sides of a chemical equilibrium. In the application of Photochromic Relaxation Kinetics (pcRelKin) to a bimolecular binding reaction, one of the two interacting molecules bears a FRET donor and the second a photochromic acceptor. The close proximity required for FRET is achieved only in the complex. After photoconversion, the system exists in a thermodynamically asymmetric state, which relaxes according to first order kinetics. PcRelKin offers unique advantages over classical relaxation kinetics.

Keywords: pcFRET; photochromism; relaxation kinetics

INTRODUCTION

The underlying principle of pcRelKin is that embodied in photochromic FRET (pcFRET) [1–6], a general conceptual and experimental scheme based on a coupled system of a fluorescent donor and a photochromic acceptor. The procedure involves the

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reversible and cyclic spectroscopic depletion of the acceptor, and was initially conceived for the determination of FRET efficiency on a continuous, pixel-by-pixel basis in the microscopy of living cells. However, the modulation of donor fluorescence in pcFRET has implications for a wide range of applications, such as the one illustrated here.

Fluorescence resonance energy transfer (FRET) is a physical process by which energy is transferred non-radiatively from an excited molecular fluorophore (donor) to another chromophore (acceptor) via long-range dipole–dipole coupling [5]. The FRET acceptor need not be fluorescent, but must fulfill the requirement of having an absorption spectrum overlapping the emission spectrum of the donor with the respective transition moments in a favorable, i.e. non-orthogonal, relative orientation. The FRET efficiency between a single donor (D)-acceptor (A) pair varies with the 6th power of the D-A separation and is generally operative over the range of $\sim$1–10 nm, distances corresponding to most biologically significant molecular interactions on and within cells. Applied in the fluorescence microscope, FRET is a highly selective and sensitive tool for resolving details of molecular complexes with a spatial resolution far exceeding that inherent to conventional optical microscopes. In addition to molecular proximity, the photophysical behavior of extrinsic or intrinsic (e.g. genetically expressed) fluorescent entities in biomolecules reflects static and dynamic features of both intra- and intermolecular interactions such as complex formation, conformational transitions, and changes in the microenvironment.

Photochromic FRET (pcFRET) is a member of a subcategory of FRET techniques we have designated as acceptor depletion FRET (adFRET) in a review of FRET imaging techniques [5]. pcFRET provides the required reference state (unperturbed donor) in a local (single pixel) and reversible manner by cyclical light-driven on-off switching of the FRET process. This circumstance is achieved by the use of photochromic compounds as programmable FRET acceptors, exploiting their reversible transformations induced by illumination at appropriate wavelengths, between two structural forms with different absorption properties. In pcFRET, the two probes (donor and acceptor pair) are selected according to the spectroscopic criteria indicated above. The fluorescence emission (degree of quenching) of the donor can be modulated reversibly by systematic photochemical manipulation of the photochromic acceptor. That is, the donor fluorescence is measured in the virtual spectroscopic presence and absence of the acceptor, the latter case corresponding to the reference state of the FRET-unperturbed donor.

PHOTO-STATIONARY STATES AND KINETIC SCHEME FOR PCFRET

The reversible process of photochromic conversion of the acceptor in pcFRET gives rise to a two-state photophysical system. The FRET signal monitors the equilibrium distribution between visible non-absorbing (FRET−) and the visible absorbing (FRET+) forms of the photochromic moiety. In practice it may be difficult to drive the system exclusively into either of these states, and in general one oscillates between two photostationary equilibria dictated by the irradiance and spectral distribution of the two photoconversion light sources. That is, the system is manipulated such as to cycle between a predominantly *off* state and a predominantly *on* state, the term *predominantly* implying a significant but variable/arbitrary extent of conversion lying between the two extreme conditions [3]. Three spectral domains are operative: (i) near-UV: both forms of the acceptor (and generally the donor as well) absorb; (ii) visible domain 1: (+) form of the acceptor absorbs; and (iii) visible domain 2: donor absorbs preferentially. Switching is achieved by alternating the exposure to light of relatively high intensity in the near-UV and visible domain 1. The system is monitored by excitation of the donor at low intensity in visible domain 2 and measurement of its emission in visible spectral domain 1. The corresponding photokinetic scheme is given in Figure 1.

PCRELKIN APPLIED TO SECOND ORDER REACTION

Consider a second order reaction, a biomolecular binding reaction between two partners, one of which carriers a FRET donor and the second a photochromic acceptor (Fig. 2). The close proximity required for FRET is achieved only in the complex, i.e. the product of the reaction.

Assume the system is initially entirely in the *off* state, e.g. corresponding to the open form of a diheteroarylethene acceptor [3]. Application of a suitable (e.g., near-UV or blue) light pulse leads to photoconversion to the FRET *on* or "+" state to a lesser degree for the acceptor in the complex than in the free state, due to the enhancement of the overall rate of the back reaction (k_{+-}^{com}) via FRET-mediated excitation of the acceptor [3,4], Figs. 1 and 2). Because of the creation of two new chemical species (acceptor *on* and complex *on*), the system is in a thermodynamically asymmetric state comprising two parallel reactions, which proceed to redistribute to a new equilibrium in accordance with mass action considerations. Since the chemical rate constants are the same for both reactions, the donor concentration

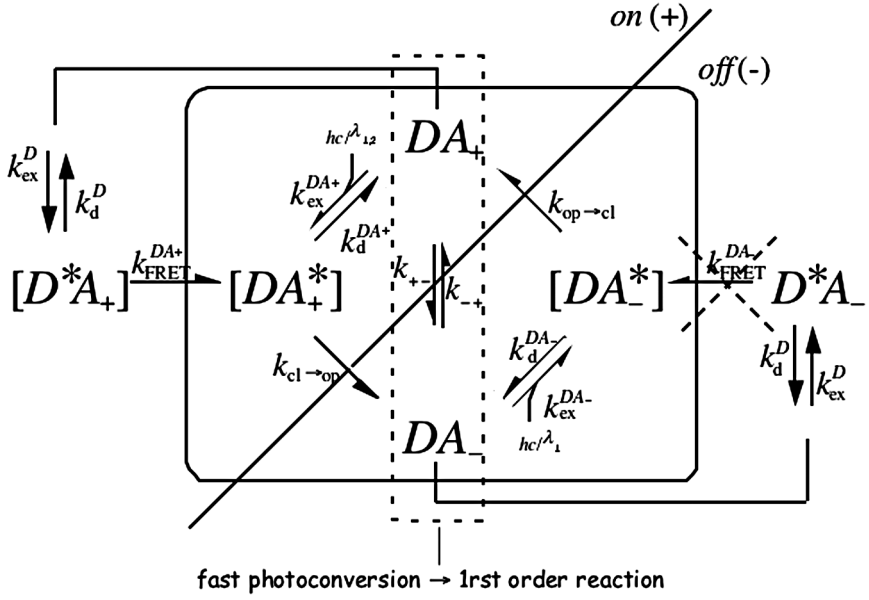

FIGURE 1 pcFRET photophysical-photochromic scheme. The elementary reaction steps reduce to overall transitions (central vertical arrows) between the two ground states of the acceptor (DA_-, DA_+) as a consequence of the very rapid photochromic transitions. The excited states of D and A are denoted by an asterisk. The central region comprises the ring closure and opening reactions of the photochromic acceptor. The external pathways represent the intervention of the donor contributing to the photochromic cycle through coupled excitation and FRET but functioning primarily as a monitor of the photostationary equilibrium state and thus of the FRET process (via low-level excitation in visible spectral domain 2). The photochromic transitions are initiated by excitations in the near-UV (or visible spectral regions (domain 1). Decay rates are indicated by the subscript d; *off* (−), states devoid of significant FRET between donor and acceptor; *on* (+), states potentiating FRET.

remains constant (is "buffered"). Thus, spectroscopic signals arise solely from the redistribution of the two acceptor forms during the relaxation process. As in the case of classical relaxation kinetics (T-jump, P-jump) [7], evaluation of concentration dependencies yields the rate constants for the chemical reaction under study (Fig. 3). The relaxation is first-order regardless of the extent of the photoconversion because of the invariant donor concentration. Thus, the reciprocal relaxation time is given by the expression $\tau^{-1} = k_f$ [donor] $+ k_r$. The simulations generated by direct solution of the relevant differential equations confirm this relationship (Fig. 3).

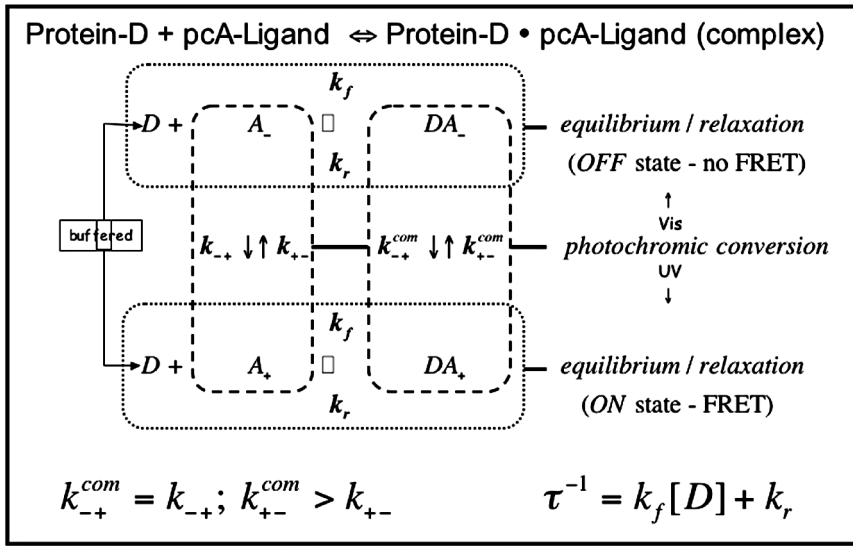

FIGURE 2 pcFRET applied to a bimolecular reaction. See text for explanation.

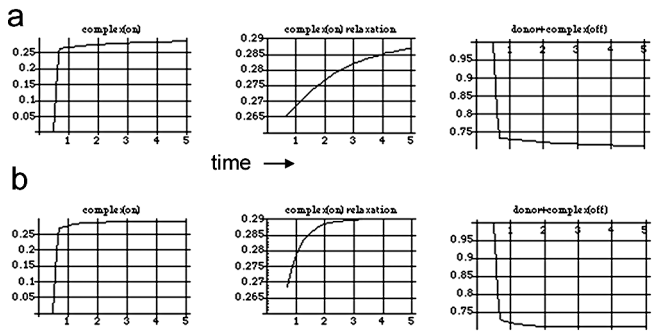

FIGURE 3 Simulations of a biomolecular system relaxing according to the pcRelKin mechanism. The differential equations corresponding to the scheme depicted in Figure 2 were solved numerically by *Mathematica*. In the two simulations, the ratio of k_r/k_f was kept constant but the absolute values were adjusted by a factor of 4 (panel a: $k_f = 0.5$, $k_r = 0.25$; panel b: $k_f = 2$, $k_r = 1$). *Left panels*: The initial light pulse induces the absorbing (open) form of the pc acceptor, which increases further during the relaxation to the final equilibrium state. *Middle panel*s: Relaxation phase expanded. The relaxation rate increases (b vs a) for the larger rate constants in accordance with the expression for the relaxation time (see text). The small changes in amplitude are due to the finite relaxation occurring during the photochromic light pulse (0.2 time units in width). *Right panel*s: Corresponding decrease of the sum of the donor (invariant) and complex *off* concentrations.

CONCLUSIONS

PcRelKin offers numerous and unique advantages as a kinetic technique: isothermal and isobaric transitions; arbitrarily small volumes; first order kinetics even for large displacements due to buffering of the donor-carrying reactant; lack of inherent temporal limitation; high sensitivity and precision due to averaging over many cycles; applicability to high throughput procedures, including those based on micro (nano)arrays; low cost; and ease of implementation. The technique is currently being implemented experimentally and has been submitted for patenting [8].

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