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Review

Fluorescence tools to investigate riboswitch structural dynamics ☆

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ABSTRACT

Riboswitches are novel regulatory elements that respond to cellular metabolites to control gene expression. They are constituted of highly conserved domains that have evolved to recognize specific metabolites. Such domains, so-called aptamers, are folded into intricate structures to enable metabolite recognition. Over the years, the development of ensemble and single-molecule fluorescence techniques has allowed to probe most of the mechanistic aspects of aptamer folding and ligand binding. In this review, we summarize the current fluorescence toolkit available to study riboswitch structural dynamics. We fist describe those methods based on fluorescent nucleotide analogues, mostly 2-aminopurine (2AP), to investigate short-range conformational changes, including some key steady-state and time-resolved examples that exemplify the versatility of fluorescent analogues as structural probes. The study of long-range structural changes by Förster resonance energy transfer (FRET) is mostly discussed in the context of single-molecule studies, including some recent developments based on the combination of single-molecule FRET techniques with controlled chemical denaturation methods. This article is part of a Special Issue entitled: Riboswitches.

1. Introduction

The last decade has witnessed the discovery of an astonishing variety of non-protein coding RNA sequences that perform a diverse range of functional roles in living organisms. An emerging theme arising from a combination of complementary biochemical, biophysical and crystallographic studies suggests that this complex range of RNA functionalities stems, at least in part, from their ability not only to fold into a specific 3D structure but also to alter its conformation in response to subtle changes in specific cellular signals such as the presence of ions, proteins, small molecules and temperature variations [1,2]. RNA's ability to sense and adapt to the cellular environment as an intrinsic part of its functional role is exemplified by the ever-growing family of metabolite-sensing regulatory mRNAs [1,2]. These RNA elements, so-called riboswitches, are mostly located within the 5′ untranslated region of prokaryotic messenger RNA (mRNA) and usually regulate gene expression in a *cis*-fashion by directly binding to specific cellular cues without protein assistance [1–3]. To understand the regulatory mechanism of riboswitches, it is therefore of primordial importance to study the secondary and tertiary structures of these RNAs, as well as their folding pathway. Crystallography of riboswitch aptamers provided the main source of information for the structure of most riboswitches [1]. However, the RNA needs to be in a highly stable conformation to be crystallized. Because of this limitation, crystallography mainly provided information about riboswitch aptamers bound to their cognate ligand. The examination of aptamer crystals reveals that RNA molecules make extensive contacts with the bound metabolite, mostly resulting in the latter being complete buried within the aptamer. This suggests that the riboswitch needs to adopt an open conformation to allow efficient ligand recognition and that a folding mechanism must occur to allow the formation of a riboswitch–ligand complex. This review will describe different fluorescent techniques that have allowed researchers to examine, at least in part, the folding pathways of various aptamers as a response to ligand binding. These techniques also allowed the observation of the bound ("closed") conformation of aptamer domains and provided a way to identify what conformational changes are involved in ligand binding. Finally, these tools have allowed to understand the dynamics of these RNAs by providing information about folding and unfolding rates as well as direct measurements of the binding constant of aptamers.

2. Fluorescent analogs of adenine and cytosine: spectroscopic properties of 2-aminopurine and pyrrolo-dC

Fluorescent molecules that have the ability to form hydrogen bonds and preserve the self-recognition properties of natural nucleobases have played a pivotal role in our understanding of DNA and RNA structures and how these are altered by environmental conditions including ions, denaturant agents, temperature and small molecules [4,5]. Among these fluorescent nucleobase analogs, 2-aminopurine (2AP) (Fig. 1A)

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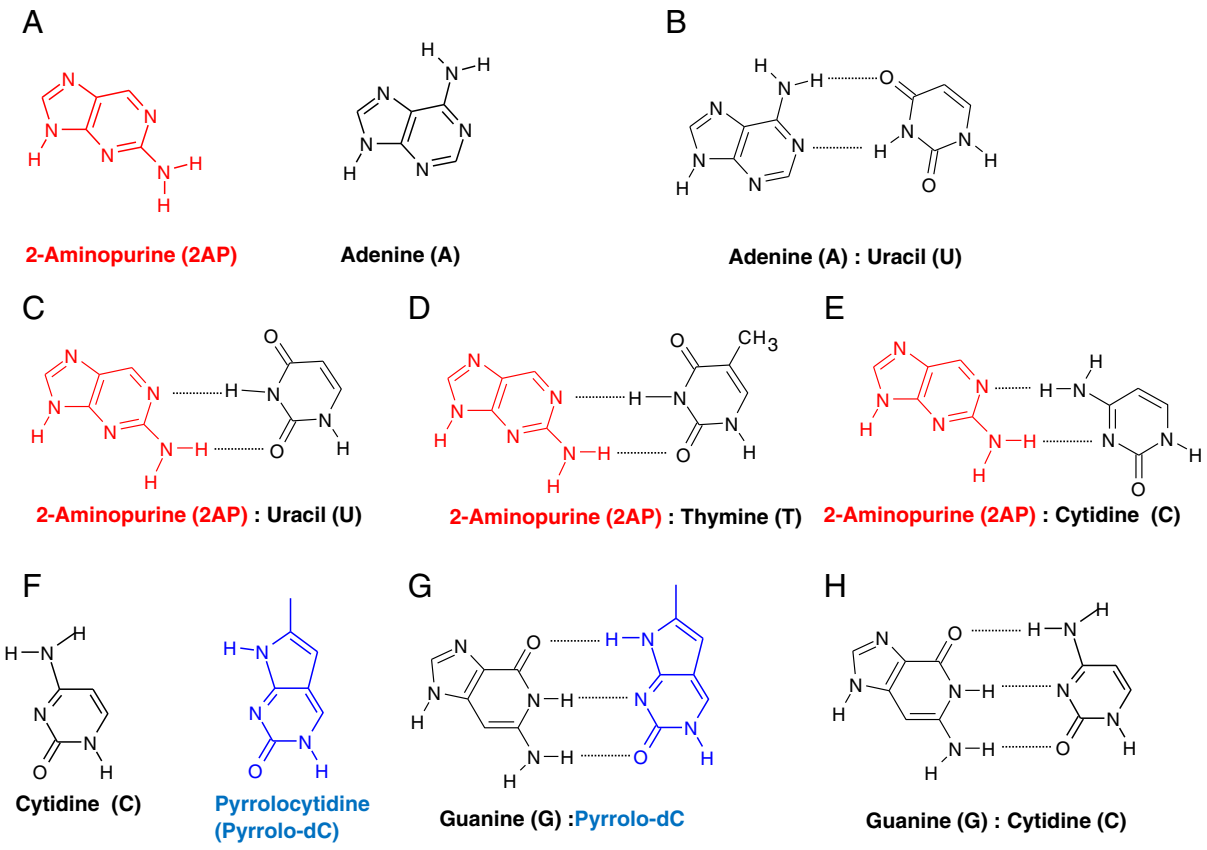

Fig. 1. Chemical structures and summary of base pairing interactions involving nucleotide analogs 2AP and pyrrolo-dC.

has been extensively used to investigate interactions between DNA and RNA regions and to follow their association to other biomolecules including proteins and large ribonucleoprotein complexes [4–9]. 2AP is an analogue of adenine (Fig. 1A) that, like adenine (Fig. 1B), forms stable base pairs with uracil (RNA) and thymine (DNA) (Fig. 1C and D) but also moderately stable base pairs with cytosine in a wobble configuration (DNA or RNA) (Fig. 1E). Although 2AP exhibits a low extinction coefficient ($\sum$ ~ 6000 M^{-1} cm^{-1}), the use of 2AP in fluorescence studies of nucleic acid structure is very attractive because its photophysical properties, in particular its fluorescence quantum yield, lifetime and anisotropy, are extremely sensitive to local changes in the surrounding environment [6–12]. Moreover, the position of the lowest energy absorption band (305 nm) lies outside the absorption band of the nucleic acid structure, thus making it possible to selectively excite 2AP without affecting natural nucleobases. This is an important advantage that enables 2AP to act as a reporter of localized structural changes when compared to other techniques such as circular dichroism and absorption spectroscopy that monitor the entire RNA present in solution [4,5].

The high quantum yield of 2AP free in solution (~0.68) is drastically quenched when incorporated into nucleic acids [4,9,11]. Several spectroscopic and computational studies have investigated the molecular basis of this quenching mechanism as a function of position within the nucleic acid structure, sequence, nature of the adjacent bases and stacking geometries [7–14]. In general, the conformational motion of the stacked bases surrounding the fluorophore is known to be crucial for the quenching mechanism [7–14]. In fact, it has been shown that the emission intensity of 2AP in duplex DNA increases upon cooling and becomes close to that of free 2AP at low temperatures [15]. In contrast, hydrogen bonding and base pairing do not usually affect 2AP fluorescence emission significantly [11,14]. This sensitivity to the stacked micro-environment has made 2AP an ideal probe to investigate DNA and RNA structural changes involving nucleotide base flipping [16,17], nucleic acid interactions [18], local melting [19] and denaturation [20]. 2AP fluorescence has also been extensively applied to study a variety of DNA and RNA processing enzymes [17,21–24] and to elucidate the relationship between folding and catalysis in small nucleolytic RNA enzymes (ribozymes) [25–28]. However, as discussed in the next sections, it is in the study of riboswitch folding and regulatory function where, in recent years, the potential of 2AP substitutions at specific regions of the mRNA sequence has been fully exploited [29–34].

A fluorescent derivative of cytidine, pyrrolocytosine (pyrrolo-dC) (Fig. 1F), was introduced in the late 90s following earlier studies on a fluorescent nucleobase named furano-dT [35,36]. It was found that furano-dT incorporated into an oligonucleotide was converted into pyrrolo-dC after ammonia treatment at the final stage of the solid-phase synthesis [35]. It has been shown that DNA duplexes containing the pyrrolo-dC:G base pair (Fig. 1G) were as stable as unmodified ones (Fig. 1H) [35]. Pyrrolo-dC is commercially available and exhibits its absorption maximum at 350 nm with an extinction coefficient very similar to that of 2AP ($\sum$ ~ 5900 M^{-1} cm^{-1}) and a fluorescence maximum around 473 nm when incorporated into a DNA duplex [36]. The fluorescence quantum yield of the pyrrolo-dC monomer has been reported to be 0.2 and decreases to values of 0.03 in dsDNA and 0.05 in dsRNA [36]. In contrast to 2AP, very little is known about the influence of adjacent bases on the emission properties of pyrrolo-dC. Nevertheless, the use of pyrrolo-dC to investigate DNA and RNA structure continues to grow [13,37]. For example, pyrrolo-dC has been used to investigate the HIV-1 polypurine tract (PPT) using DNA/RNA hybrids [38] and to characterize the mechanism of DNA repair by human alkyltransferase [39]. Furthermore, the combination of 2AP and pyrrolo-dC in a molecular beacon-like fashion to investigate nucleic acid hybridization has already been reported [40]. In the context of metabolite-sensing mRNA sequences, the use of pyrrolo-dC substitutions to report folding and/or ligand binding has been very limited, and to date, only a single study has been reported applying pyrrolo-dC