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The molecular mechanism of heme loss from oxidized soluble guanylate cyclase induced by conformational change

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ABSTRACT

Heme oxidation and loss of soluble guanylate cyclase (sGC) is thought to be an important contributor to the development of cardiovascular diseases. Nevertheless, it remains unknown why the heme loses readily in oxidized sGC. In the current study, the conformational change of sGC upon heme oxidation by ODQ was studied based on the fluorescence resonance energy transfer (FRET) between the heme and a fluorophore fluorescein arsenical helix binder (FlAsH-EDT_2) labeled at different domains of sGC β1. This study provides an opportunity to monitor the domain movement of sGC relative to the heme. The results indicated that heme oxidation by ODQ in truncated sCC induced the heme-associated αF helix moving away from the heme, the Per/Arnt/Sim domain (PAS) domain moving closer to the heme, but led the helical domain going further from the heme. We proposed that the synergistic effect of these conformational changes of the discrete region upon heme oxidation forces the heme pocket open, and subsequent heme loss readily. Furthermore, the kinetic studies suggested that the heme oxidation was a fast process and the conformational change was a relatively slow process. The kinetics of heme loss from oxidized sGC was monitored by a new method based on the heme group de-quenching the fluorescence of FlAsH-EDT_2.

1. Introduction

Soluble guanylate cyclase (sGC) is a primary nitric oxide (NO)receptor in mammalian NO-sGC-cGMP signaling pathway [1,2]. Binding of NO to the ferrous heme cofactor of sGC initiates the NO-dependent signaling cascade, and stimulates the cyclase activity by catalyzing the conversion of guanosine 5′-triphosphate (GTP) to 3′, 5′-cyclic guanosine monophosphate (cGMP). cGMP, as a second messenger, regulates the downstream physiological process, such as vasodilation, platelet aggregation and neurotransmission [3,4]. Impaired NO-sGC-cGMP signaling has been implicated in various cardiovascular diseases such as arterial hypertension, neurotransmission, and heart failure [5,6].

sGC is a heme-containing α/β heterodimeric enzyme and the most extensively studied isoform is α1β1 heterodimer. The prosthetic heme group is non-covalently bound to the β1 subunit *via* the His105. Each subunit contains four domains: an N-terminal heme-NO/O_2-binding (H-NOX) domain, a Per/Arnt/Sim (PAS) domain, a helical (CC) domain and a C-terminal catalytic domain (Fig. 1). Although the structure of eukaryotic sGC holo-enzyme remains unknown, the crystal structures of truncated eukaryotic sGC domains (PAS, CC, and catalytic domains) and H-NOX domains from prokaryotic sGC have been reported [7–10]. The high-order domain architecture of sGC also has been revealed from single-particle EM, indicating that sGC is assembled from two rigid modules: the catalytic domain and the clustered PAS and H-NOX domain flexibly connected by a parallel helical domain [11].

NO activates sGC through binding to the ferrous heme of H-NOX domain leading to the rupture of the Fe^{2+}–N (H105) coordination bond [12,13]. The mechanism of the NO-induced sGC activation also has been proposed by diverse approaches [14–17]. Haase et al. have revealed closed proximity between N and C-termini of sGC using fluorescent fusion protein based on fluorescence resonance energy transfer (FRET), supporting that the direct interaction between the H-NOX domain and the catalytic domain represses sGC activity [14]. Recently, Underbakke et al. have revealed the domains' motion in several discrete region upon NO binding using HDX-MS [16], suggesting that the NO-induced conformational changes involve in the heme-associated helix

Abbreviations: sGC, soluble guanylate cyclase; NO, nitric oxide; FRET, fluorescence resonance energy transfer; H-NOX domain, heme-NO/O_2-binding domain; PAS domain, Per/Arnt/Sim domain; CC, helical domain; *Ns* H-NOX, the H-NOX domain of *Nostoc sp*; FlAsH-EDT_2, fluorescein arsenical helix binder; GTP, guanosine 5′-triphosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; DEA/NO, diethylammonium (Z)-1-(N, N-diethylamino) diazen-1-ium-1, 2-diolate; EPR, electron paramagnetic resonance; ODQ, 1H-(1, 2, 4) oxadiazolo (4, 3-a) quinoxalin-1-one; TCEP, Tris (2-CarboxyEthyl) Phosphine; Ni-NTA, Nickel-nitrilotriacetic acid.

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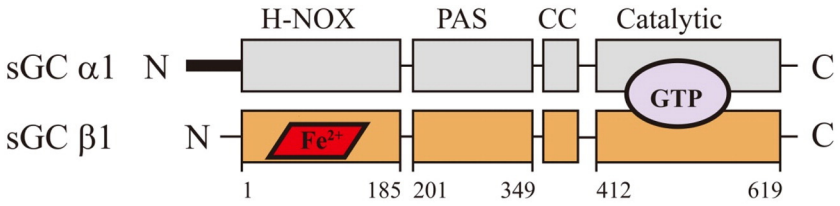

Fig. 1. sGC domain organization of sGC α1 (gray) and β1 (tan) subunit. Each subunit contains four domains. The H-NOX domain of β1 subunit contains heme cofactor, shown in red.

surface, an H-NOX-associated surface of the PAS domain, the PAS-helical domain linker, the helical domain and the active site of the catalytic domain. These domain interactions also have been observed previously [18]. A FRET study based on FRET between endogenous tryptophan and substrate analog 2′-Mant-3′-dGTP has recently provided a unique opportunity to directly detect the movement of the functional domain relative to the substrate binding catalytic region upon NO binding, which also supports the mechanism proposed by Underbakke et al. [17]. In general, it is an allosteric pathway transmitting the NO-signaling from the H-NOX domain to the active site catalytic domain.

The ferrous heme group plays a key role in sGC-mediated NO-signaling activation and stabilization of sGC. And NO is a poor ligand for ferric sGC and can't activate sGC [19,20]. Previous studies have shown that sGC is prone to heme loss during isolation, particularly after oxidation [21]. This also occurs *in vivo*, as the reactive oxygen species (ROS) can oxidize heme group of sGC under conditions of oxidative stress, leading to the heme dissociation from the oxidized enzyme and the generation of NO-insensitive sGC in the diseased tissue [22]. The levels of oxidized or heme-free sGC are increased in certain cardiovascular diseases [23,24]. Heme-free sGC is also prone to ubiquitin-mediated degradation [24,25]. In addition, the oxidation of sGC heme is thought to be an important risk factor to the development of cardiovascular diseases [26,27]. Thus, based on the oxidized or heme-free sGC, heme-independent sGC activators which can activate oxidized or heme-free sGC by acting as the heme group, such as cinaciguat (BAY 58-2667), are discovered [26–28]. Furthermore, Fritz et al. have found that the ferrous heme in sGC is very stable and resistant to heme loss both in the absence and presence of NO, but the ferric heme in sGC more readily loses its heme as observed using spectroelectrochemical titration and heme transfer experiments [29]. Surmeli et al. also have demonstrated that oxidized sGC loses heme more readily than the ferrous sGC and the activator cinaciguat activates sGC involving facilitation of heme loss from ferric sGC and subsequent replacement of heme in the heme pocket [30]. However, it has not been clearly why oxidized sGC loses its heme more readily compared to reduced sGC, and the molecular mechanism of heme dissociation from sGC is not understood clearly. To this end, we herein investigated the molecular mechanism of sGC heme oxidation and loss, based on the energy transfer between the heme and the fluorescein arsenical helix binder (FlAsH-EDT_2) labeled at different domains of the sGC β1 subunit.

As an effective probe to monitor the protein conformational change, protein tertiary and quaternary structure formation *in vitro* and *in vivo* [31–35], FlAsH-EDT_2 can bind specifically to a small tetracysteine (TC: CCPGCC) with rather small size, and can be introduced by mutagenesis. The emission spectrum of FlAsH-EDT_2 has a good overlap with α and β absorbance bands of sGC heme group [22,36–38]. Thus, the heme group can effectively quench the fluorescence of FlAsH-EDT_2, which can be used to study the conformational change upon sGC heme oxidation by ODQ. The fluorophore FlAsH-EDT_2 was also used to study the heme loss of sGC *in vivo* [22].

In the present work, we investigated systemically conformational change of sGC β1 upon heme oxidation by ODQ based on the energy transfer between heme and the fluorophore FlAsH-EDT_2. The fluorophore FlAsH-EDT_2 was labeled at different domains of sGC β1. The treatment of sGC by ODQ resulted in fast oxidation of the ferrous truncated sGC β1 to the ferric state, and subsequently initiated the slow conformational change of the truncated sGC β1. The synergistic effect of these conformational changes of the discrete region induced by heme oxidation contributed to the heme loss.

2. Materials and methods

2.1. Materials

1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ) and diethylammonium (Z)-1-(N, N-diethylamino) diazen-1-ium-1, 2-diolate (DEA/NO) were purchased from Cayman Chemical Company. Fluorescein arsenical helix binder (FlAsH-EDT_2) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). KOD-Plus-Mutagenesis Kit was purchased from TOYOBO (Osaka, Japan). Hemin and aminolevulinic acid (5-ALA) were purchased from Sigma. Nickel nitrilotriacetic acid (Ni-NTA) resin and Sephadex G-25 resin were purchased from QIAGEN (Chatsworth, CA, USA). DEAE Sepharose™ Fast Flow was purchased from GE Healthcare Bio-Science. The Superdex™ 200 Hiload 16/60 gel filtration column was purchased from Pharmacia. All other reagents were of analytic grade.

2.2. Mutagenesis

FlAsH-EDT_2 binds tightly to a small tetracysteine (TC: CCPGCC) motif, which can be readily introduced into different positions of protein by mutagenesis. In this work, TC motif was chosen to be introduced into the C-terminus of the truncated sGC [sGC β1(1–195)-^{196}TC^{201} and sGC β1(1–385)-^{386}TC^{391}]. In addition, the residues of 243–248 in the PAS domain were replaced by TC motif through three mutations L243C, Q244C and N247C [sGC β1(1–385)-^{243}TC^{248} and sGC β1(1–619)-^{243}TC^{248}]. The truncated wild type human sGC β1(1–195) and sGC β1(1–385), which were constructed previously by us, were used for site-directed mutagenesis [39]. Full-length human sGC β1(1–619) was first constructed into the vector pBAC-1, and then TC motif was introduced into the human sGC β1(1–619) through site-directed mutagenesis. Mutagenesis was performed using KOD-Plus-Mutagenesis Kit according to the manufacturer's protocol. The sequences of the primers are shown in Table S1. All mutations were confirmed by DNA sequencing.

2.3. Expression and purification of sGC

Expression and purification of human sGC β1(1–195)-^{196}TC^{201}, sGC β1(1–385), sGC β1(1–385)-^{243}TC^{248} and sGC β1(1–385)-^{386}TC^{391} were performed as described previously with the following modifications [2,39,40]. All purification processes were in glove-box under high pure nitrogen. In brief, plasmids were transformed into *Rosetta* (*DE3*) cells. Cultures were grown to an OD_{600} of 0.6–0.8 and cooled down to 27 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM, and aminolevulinic acid (5-ALA) was added to 1 mM. Cultures were grown overnight for 12–16 h and harvested by centrifugation. The cell pellets were frozen and stored at −80 °C. Frozen cell pellets were re-suspended in lysis buffer A [50 mM Na-Pi, 300 mM NaCl, 10 mM dithionite, 5 mM β-mercaptoethanol (β-Me), 5% glycerol,