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Unraveling Hidden Regulatory Sites in Structurally Homologous Metalloproteases

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Abstract

Monitoring enzymatic activity *in vivo* of individual homologous enzymes such as the matrix metalloproteinases (MMPs) by antagonist molecules is highly desired for defining physiological and pathophysiological pathways. However, the rational design of antagonists targeting enzyme catalytic moieties specific to one of the homologous enzymes often appears to be an extremely difficult task. This is mainly due to the high structural homology at the enzyme active sites shared by members of the protein family. Accordingly, controlling enzymatic activity via alternative allosteric sites has become an attractive proposition for drug design targeting individual homologous enzymes. Yet, the challenge remains to identify such regulatory alternative sites that are often hidden and scattered over different locations on the protein's surface. We have designed branched amphiphilic molecules exhibiting specific inhibitory activity towards individual members of the MMP family. These amphiphilic isomers share the same chemical nature, providing versatile nonspecific binding reactivity that allows to probe hidden regulatory residues on a given protein surface. Using the advantage provided by amphiphilic ligands, here we explore a new approach for determining hidden regulatory sites. This approach includes diverse experimental analysis, such as structural spectroscopic analyses, NMR, and protein crystallography combined with computational prediction of effector binding sites. We demonstrate how our approach works by analyzing members of the MMP family that possess a unique set of such sites. Our work provides a proof of principle for using ligand effectors to unravel hidden regulatory sites specific to members of the structurally homologous MMP family. This approach may be exploited for the design of novel molecular effectors and therapeutic agents affecting protein catalytic function via interactions with structure-specific regulatory sites.

Introduction

Regulating protein function through binding of ligands or effector molecules to sites distal from the active site, known as allostery,[1–3] is one of the most common and powerful means of controlling protein function *in vivo*.[2,3] This type of regulation is frequently observed in metabolic enzymes,[4] although it has been proposed that all proteins are intrinsically allosteric in their nature.[5] In the traditional description of allosteric regulation, the binding of a ligand or effector molecule to an allosteric site induces a conformational change in the protein that alters its function. In recent years, the energy landscape-based, the so-called "new view"[5,6] of allostery has emerged, considering intrinsic protein dynamics as the basis for function and its regulation.[5–11] Accordingly, the existence of a population of conformations suggests the shift in conformational equilibrium towards active/inactive states as a result of the activator/inhibitor binding to specific regulatory sites.[5–11] The advantage of such naturally evolved regulatory mechanisms is the ability to gain high selectivity and specificity for individual members of a

protein family.[12] This is because such regulatory elements display distinct chemistry and higher sequence divergence compared to the conserved active sites shared by the members of the family of structurally homologous enzymes, such as matrix metalloproteinases (MMPs) explored here.[13] Here, we addressed the need for developing experimental and computational approaches for identifying native allosteric regulatory sites hidden within a given protein surface.

Given the importance of allosteric regulation, it has been suggested that druggable regulatory sites can be exploited to interfere with the functions of proteins associated with diseases.[14] The chemical nature of inhibitors targeted at such specific regions or amino acid residues is expected to differ from those targeted to conserved active sites while allowing diverse chemo-type interactions and binding selectivity.[15–18]

Current successful approaches available to identify regulatory sites of individual proteins or enzymes rely on high-throughput screening of large libraries of compounds exhibiting allosteric noncompetitive binding kinetics.[19,20] The rational design of allosteric inhibitors often relies on the mode by which proteins/enzymes interact with their substrates, ligands, and effectors.[19–27] However, this approach is not optimal for identifying hidden regulatory sites controlling protein activity in an allosteric manner.[15] Specifically, the question to be asked is whether structurally homologous proteins exhibit distinct diversity in their regulatory amino acids affecting protein function.

Here, we present a proof of principle for the approach aiming at identifying hidden regulatory sites existing within the family of MMPs, used as a model system (Fig. 1). The MMPs are a family of zinc-dependent endopeptidases consisting of more than 20 structurally homologous members.[21] These enzymes play key roles in many processes spanning from cell proliferation, differentiation, and communication, to pathological states associated with tumor metastasis, inflammation, tissue degeneration, and cell death.[22–24] MMPs share high structural homology in their conserved catalytic machinery containing a zinc ion coordinated to three conserved histidines,[25] while their protein surface potential varies as depicted in Fig. 1 (and in Supplementary Scheme S2). In addition, MMPs are well-established

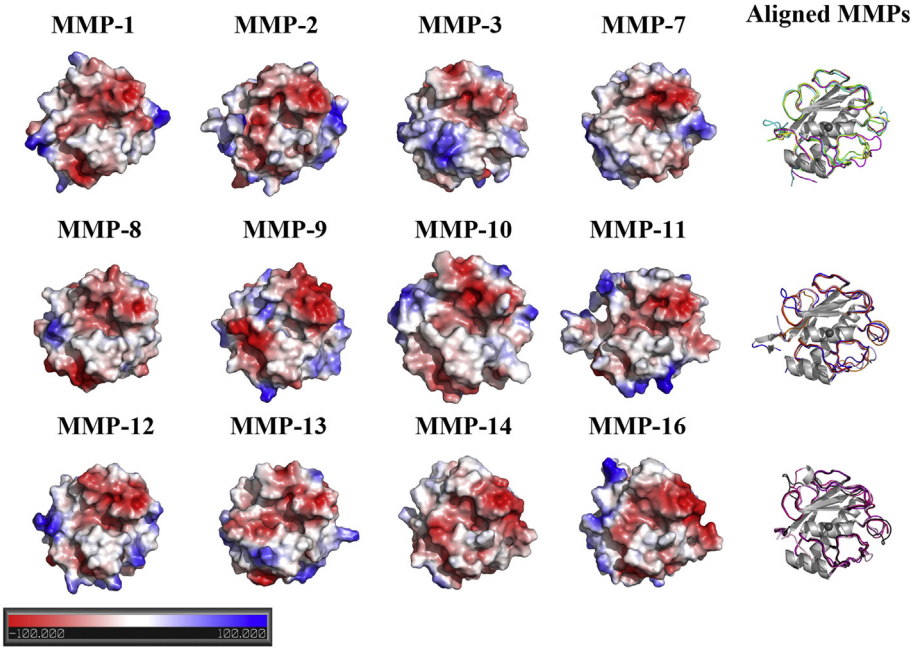

Fig. 1. MMPs possess different surface properties despite their high structural homology. Qualitative calculation of surface electrostatic potential of various catalytic domains of MMPs was done using PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA). Positive potential is shown in blue, whereas negative potential is in red. The following PDB codes were used for the calculation: MMP-1: 2J0T,[61] MMP-2: 1EAK,[62] MMP-3: 2D1O,[63] MMP-7: 1MMP,[64] MMP-8: 2OY4,[39] MMP-9: 2OVX,[65] MMP-10: 1Q3A,[66] MMP-11: 1HV5,[67] MMP-12: 2OXU,[39] MMP-13: 2PJT,[68] MMP-14: 3MA2,[69] and MMP-16: 1RM8.[70] For all MMPs, chain A was taken except for MMP-10 and 13 where chain B was taken. On the right, the structural alignment of MMPs is presented: MMP-1 (Gly105–Asn265) (green), MMP-2 [Tyr110–Asp452, the fibronectin type II domains (Arg222–Tyr395) were omitted] (cyan), MMP-3 (Phe83–Pro253) (magenta), MMP-7 (Tyr100–Lys264) (yellow), MMP-8 (Pro86–Gly242) (red), MMP-9 (Phe110–Tyr243) (orange), MMP-10 (Gly104–Gly263) (light blue), MMP-11 (Met101–Gly262) (blue), MMP-12 (Gly106–Gly263) (purple), MMP-13 (Tyr79–Pro243) (light pink), MMP-14 (Leu117–Gly284) (hot pink), and MMP-16 (Gly124-Pro292) (black). The catalytic zinc is shown as a dark gray sphere. For clarity, we aligned the MMPs in each row by chronological order.