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非生物体系与蛋白质中金属配位键的单分子力谱研究

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摘要:金属结合蛋白是一类在结构或功能上与金属离子密切相关的蛋白质,在许多生物过程中发挥着关键作用。因此,深入了解金属蛋白中的金属结合位点及其形成的各类金属配位键,不仅有助于解析其功能机制,也为新型蛋白质的设计提供了理论基础。在过去十多年中,基于原子力显微镜的单分子力谱技术(AFM-SMFS)作为一种新兴工具,已被广泛应用于金属蛋白的研究,尤其在金属配体键强度的测量方面展现了独特优势。AFM-SMFS通过沿蛋白主链方向施加拉力,诱导蛋白质二级结构解折叠,并断裂金属簇以及其中的金属配体键,从而实现对其强度的精确测量。AFM-SMFS技术使得不同类型金属配体键强度的测量和比较成为可能,从而揭示其强度规律,例如铁结合蛋白中Fe(III)-配位键的强度趋势。未来,AFM-SMFS有望为揭示蛋白质选择性结合金属离子的机制提供新的视角。

关键词:金属蛋白;原子力显微镜;单分子力谱

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Single-Molecule Force Spectroscopy Studies of Metal-Ligand Coordination Bonds in Abiotic Systems and Proteins

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Abstract: Metalloproteins, characterized by the binding of various metal ions, play a pivotal role in a broad range of biological processes. Understanding the metal-binding sites and the coordination bonds formed between metal ions and protein ligands can not only helpfully analyze their biological functions, but also provide theoretical basis for the designing of novel metalloproteins. In the past decade, atomic force microscopy based single-molecule force spectroscopy (AFM-SMFS) has emerged as a powerful tool for investigating metalloproteins, particularly for quantifying the strength of metal-ligand bonds. By mechanically unfolding proteins along their peptide backbone, AFM-SMFS enables the disruption and measurement of metal clusters and metal-ligand bonds. Recent advancements in enzymatic, site-specific protein conjugation and immobilization techniques for SMFS have significantly enhanced the precision and efficiency of these measurements. These developments have facilitated comparative studies of metal-ligand bond strengths across various metalloproteins, revealing trends such as the robust Fe(III)-ligand bonds found in iron-binding proteins. Looking forward, AFM-SMFS may give some insight into the general principles of the selective binding of proteins to metal ions.

Keywords: metalloproteins; atomic force microscopy; single-molecule force spectroscopy

0 引言

作为一种强大的纳米尺度研究工具,单分子力

谱可以操控单个分子并对其施加机械力进而测量其稳定性^[1-4]。通过逐一研究单个分子,这项技术揭示并量化了分子的机械稳定性及分子内、分子间相互

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作用强度^[5-27]。由于其广泛的力测量范围从数十皮牛到数千皮牛,以及精确的纳米级空间分辨率,以原子力显微镜(Atomic force microscopy, AFM)为基础的单分子力谱(Single-molecule force spectroscopy, SMFS)被广泛用于测量强相互作用或化学键的强度,例如表面或细胞上的蛋白质配体-受体相互作用、金属配位复合物,甚至是共价键^[28-58]。此外,AFM-SMFS技术还被广泛应用于研究生物大分子的构象变化,如多糖、DNA、合成聚合物以及蛋白质等领域^[59-66]。已有多篇综述文章对此进行了总结^[67-79]。金属蛋白中包含多种金属离子和金属配位键,本文将重点介绍AFM-SMFS近期在金属蛋白中的应用。

目前已发现至少三分之一的蛋白质能够结合金属离子,形成种类繁多的金属结合蛋白。这些蛋白质通过结合铁、锌、铜、钙和镍等数十种不同种类的金属离子,不仅稳定了其肽链骨架结构,还可以赋予其独特的功能^[80-82]。通常,金属离子通过与氨基酸侧链中的N、O或S原子结合,形成各种强弱不等的金属配位键。在过去十年间,AFM-SMFS被广泛用于定量分析金属蛋白体系中金属配位键的强度^[83-90]。在对金属蛋白的表征过程中,AFM-SMFS既能通过施加机械力诱导蛋白骨架发生解折叠或者构象变化,又能通过断裂化学键打破金属配位键,进而对金属蛋白的机械稳定性进行全面的定量表征。此外,近年来开发的酶促位点特异性蛋白质连接和

固定技术,使得金属配位键强度的高效、精确测量及其比较成为可能。在计算机模拟和分子动力学模拟的辅助下,AFM-SMFS对金属蛋白的研究正逐步揭示金属配位键强度的一般规律。

本文综述中,我们将首先简要介绍AFM-SMFS的工作原理及其在多蛋白和非生物体系中对含金属化学键的表征,然后主要关注AFM-SMFS在金属蛋白体系中金属配位键的研究。最后,我们还将提及利用AFM-SMFS揭示金属结合对蛋白质稳定性影响的一些实例。

1 AFM-SMFS的基本工作原理

在AFM-SMFS的测量中,目标分子或蛋白质溶液通常以适当浓度沉积在洁净的载玻片基底上(图1a)。由压电陶瓷组件控制的样品台,可带动样品基底实现亚纳米级的精确移动。为了捕获单个分子并测量皮牛顿(10^{-12})级别力,作为核心部件之一的探针会装载在原子力显微镜的扫描头上使用,SMFS使用的探针通常尖锐且柔软,具体表现是半径通常小于30 nm,弹性系数通常在 10^{-1} 到 10^{-2} N/m之间(图1b)。当针尖捕获分子发生微小弯曲时,使用激光系统对此弯曲进行检测和放大。工作时,激光照射到悬臂梁的背面,其弯曲或位移(z)通过分束光电二极管检测器放大并记录下来。如果已知AFM悬臂梁的弹性系数(k),则可以根据胡克定律($F = k \times z$)计算捕获分子的探针所受的力^[91-93]。

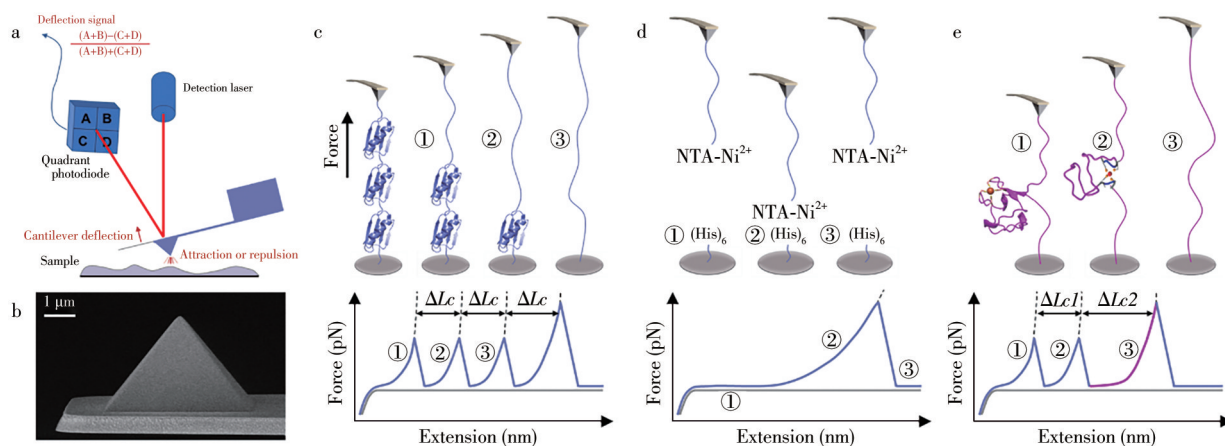


图1 AFM-SMFS工作原理图

Figure 1 Schematic diagram of AFM-SMFS working principle

在进行AFM-SMFS实验时,通过将基底垂直向上移动,使其接触AFM探针,从而在探针与基底之间捕获一个蛋白质分子。随后,基底被垂直向下移动远离探针,分子逐渐被拉伸并受到外力作用,导

致其发生构象变化,例如蛋白质的解折叠或金属配位键的断裂。

例如,图1c展示了通过AFM-SMFS捕获并测量的一个由三个串联结构域组成的多聚蛋白。在机

械力作用下,这三个结构域依次解折叠,每次解折叠在力曲线上对应一个解折叠力峰。由于三个结构域具有相同的结构,每个解折叠力峰均对应相同的轮廓长度增量(ΔLc)。 ΔLc 表示折叠蛋白质结构在完全展开后的延伸长度,可通过以下公式计算:

$$\Delta Lc = N \times 0.36 \text{ nm/aa} - L_0 \quad (1)$$

其中: N 为结构域内的氨基酸数量,0.36 nm为单个氨基酸主链沿轴向的长度(即-N-C-C-键长), L_0 为折叠状态下结构域从氨基端到羧基端的初始长度。这一参数已成为单分子水平表征蛋白质解折叠的明确标志。通过结合多聚蛋白策略及 ΔLc 这一特征标志,过去几十年来,SMFS在蛋白质研究中取得了重要进展,揭示了蛋白质力学性能、折叠与解折叠路径及能量景观的诸多信息^[44,94-98]。

2 AFM-SMFS对非生物体系中化学键强度的测量

自AFM-SMFS发明以来,该技术已被广泛用于探测蛋白质以外的分子间或分子内相互作用以及化学键强度。其中,金属离子与氨基三乙酸-组氨酸标签系统(NTA-Histag)的相互作用是最早通过AFM-SMFS进行定量研究的金属配位键体系之一。NTA-Histag体系利用了金属-配体的配位相互作用,是当前

最广泛应用于蛋白质纯化的标签系统。因此,多支研究团队几乎同时对其力学强度进行了开创性测量^[99-101]。与蛋白质的解折叠研究不同,目标键或相互作用的两端通常分别固定于探针和基底上(图1d)。例如,NTA- Ni^{2+} 和硫醇分别修饰在烷烃链的两端,并且硫醇端与镀金探针通过金硫键连接,His标签则固定在基底上。当针尖与基底接触时, Ni -NTA和HisTag之间形成目标金属复合物。随后,探针相对于基底远离运动,连接在两者之间的分子被拉伸,力逐渐传递至金属复合物并最终导致其断裂。研究测试了多种金属离子(如 Ni^{2+} 、 Co^{2+} 、 Zn^{2+} 和 Cu^{2+}),发现它们的复合物断裂力范围在50~200 pN之间。然而,由于早期固定化方法效率较低,以及单分子事件特征不够明确,对不同金属离子复合物的机械强度和动力学信息进行精确测量和比较仍具有一定挑战性。尽管如此,这些研究充分展示了AFM-SMFS在研究金属-配体键及其多样化金属复合物方面的巨大潜力。

金属-联吡啶复合物是另一种通过SMFS广泛研究的金属-配体键/相互作用体系(图2a),涉及的金属离子包括钌、钼、钨等,进一步展现了该技术对多样化金属复合物的适应性^[102-104]。最近,二茂铁中的Fe-环戊二烯相互作用也被定量分析(图2b),发现从不同方向拉伸时其力学特性存在各向异性^[105-106]。

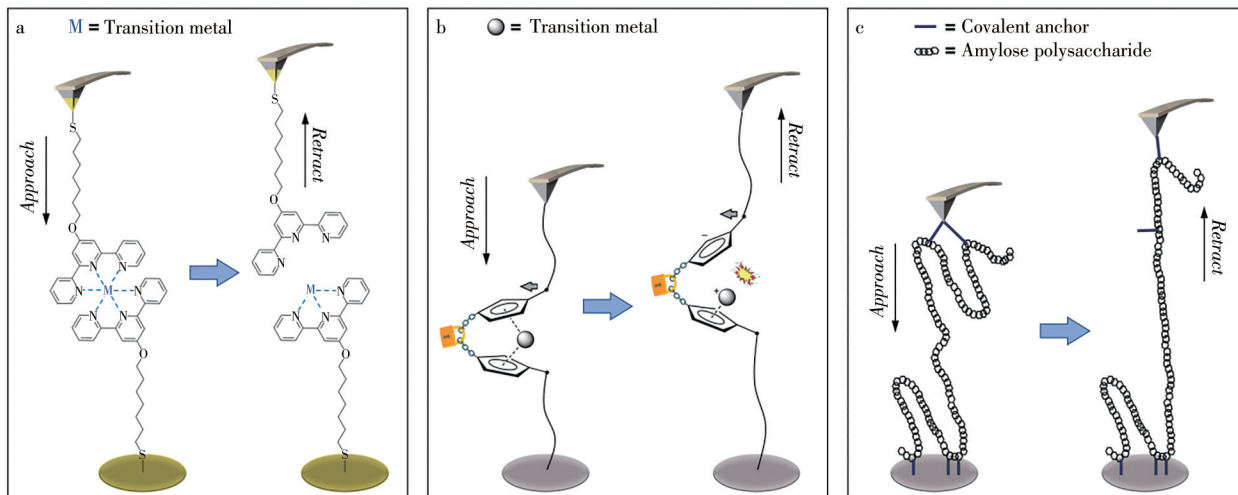


图2 AFM-SMFS表征非生物体系

Figure 2 Abiotic Systems Characterized by AFM-SMFS

Au-S键是另一个被广泛研究的典型例子。镀金基底表面使用氨基硫醇试剂进行氨基化,之后使用碳化二亚胺将直链多糖的侧链与镀金基底表面的氨基共价连接,将直链多糖用作分子连接器完成Au-S键力传递路径的串联(图2c)。1999年研究者首次采用力谱直接测量其强度,研究发现其断裂

力高达约1.4 nN^[52]。后续研究表明,溶液的pH值以及金表面等环境因素也能够调控其稳定性和断裂机制^[107]。

与多聚蛋白的解折叠研究相比,因为缺乏独特的 ΔLc 值作为标志,利用AFM-SMFS研究化学键的强度具有极大的挑战性。然而,在理论模型、计算

模拟和精细实验设计的指导下,这些研究仍然为单分子或单键水平的化学键和相互作用强弱提供了宝贵的知识^[108-130]。

3 AFM-SMFS对蛋白质中金属配位键强度的精准化测量

作为一类特殊的蛋白质亚型,金属蛋白的结构中包含金属结合位点^[131]。在金属蛋白的AFM-SMFS测量中,力不仅会诱导蛋白的二级结构解折叠,同时也会引发金属配位键的解离。金属配位键的解离通常导致先前与之配位的氨基酸残基之间的结构域进一步延展。因此,与非生物体系中金属-配体键断裂的简单事件不同,金属蛋白的测量会产生一个具有独特 ΔLc 的解离力峰,表现出类似蛋白质

解折叠的力学特征(图1e)。

2011年,AFM-SMFS首次表征了一种铁硫蛋白——红素氧还蛋白(Rubredoxin)^[47]。在红素氧还蛋白中,铁离子通过四个来源于半胱氨酸残基的硫原子进行配位,形成 $Fe(SCys)_4$ 金属中心,该中心被37个氨基酸(第5至41号残基)所包裹(图3a)^[132-134]。通过AFM-SMFS的测量,首次对该蛋白中铁-硫配位键及铁金属中心的强度进行了定量分析。结果显示,金属中心的断裂力约为200 pN,表明至少涉及两个铁-硫配位键的断裂。此外,实验还观测到一个长度为13 nm的力峰,与37个氨基酸的轮廓长度吻合。这一结果清晰地证明,在受力条件下蛋白质中的金属配位键确实可以发生解离,并且这一特征可从蛋白质的解折叠信号中明确辨识。

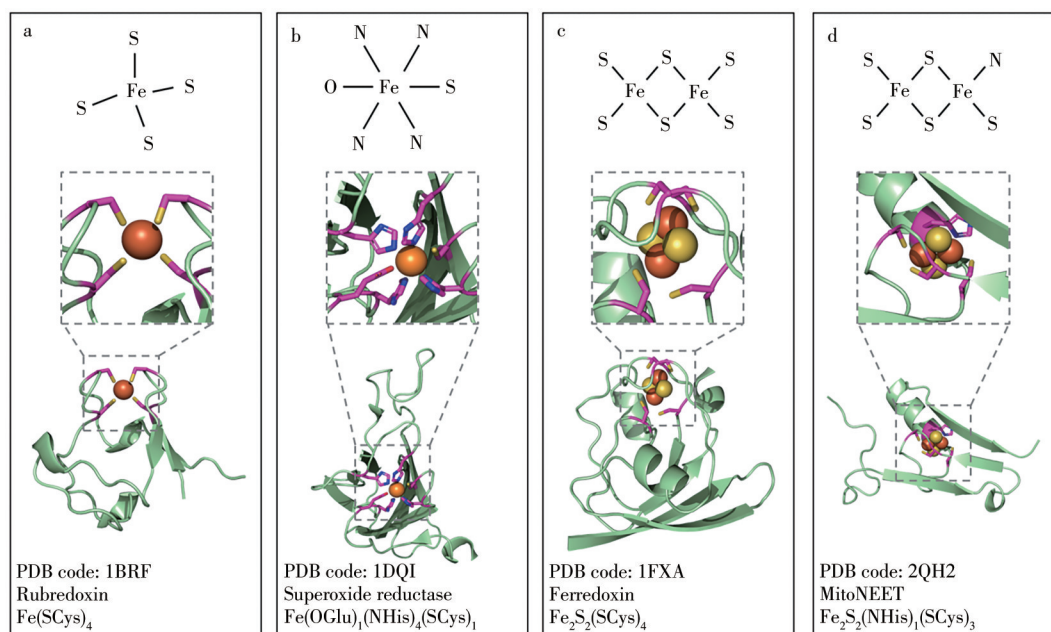


图3 AFM-SMFS表征的天然金属蛋白

Figure 3 Natural iron-binding proteins characterized by AFM-SMFS

另一种单核铁蛋白——超氧化物还原酶(SOR)也通过AFM-SMFS得到了详细研究(图3b)^[85]。其铁原子由四个咪唑氮原子、一个半胱氨酸硫原子和一个谷氨酸羧基氧原子配位,形成了六配位的八面体铁中心 $Fe(His)_4CysGlu$ 。因此,该蛋白包含三种主要的铁-配体键。研究表明, $Fe-O(Glu)$ 键具有较高的机械稳定性,断裂力约为220 pN,而 $Fe-N(His)$ 键较弱,其断裂力低于15 pN。

AFM-SMFS还研究了具有更复杂铁簇的铁硫蛋白,例如含有 $2Fe_2S_2$ 配位中心的铁氧还蛋白(图3c)和具有单一 $Fe-N$ 键的独特mitoNEET蛋白(图3d)。

实验结果显示 $Fe-S$ 键和 $Fe-N$ 键的强度相近^[135-137]。结合这些研究,我们逐渐揭示了铁结合蛋白中 $Fe(III)$ -配体键强度的一般规律,即 $Fe-S \approx Fe-O \gg Fe-N$ 。

除了铁结合蛋白外,其他天然结合蛋白的金属位点强度也得到了研究,例如含铜蛋白(如蓝氧化蛋白和杯红素蛋白)^[88,138-139]、含锌蛋白(如锌指蛋白DnaJ)^[140-141]以及含金蛋白GolB^[142]。这些蛋白的金属位点在AFM-SMFS实验中均表现出具有特征性 ΔLc 的断裂力峰,断裂力范围为50~200 pN。

随着计算机技术和算法的进步,除了天然金属

结合蛋白外,许多从头设计的金属蛋白骨架也得以成功设计和表达,这些设计的序列在自然界中并不存在,主要目的是模拟天然蛋白中的金属结合位点。 α_3 DIV 和 DFsc 是典型代表。在人工智能模型应用于蛋白质设计之前,早期的从头设计金属蛋白骨架多基于经验方法,且骨架通常由 α 螺旋构成^[143]。由于 α 螺旋骨架的解折叠力较低,因此在 AFM-SMFS 表征时,这类 α 螺旋骨架金属蛋白的力峰信号主要来源于金属配位键的断裂^[144]。这使得这些蛋白成为研究金属-配体相互作用的理想模型。

α_3 DIV 是由 DeGrado 教授及其研究团队于 2011 年设计的一种由 73 个氨基酸残基组成的蛋白质,结构由三个反向平行的 α 螺旋束构成^[145-146]。在每个 α 螺旋束上,各有一个半胱氨酸侧链指向疏水核心,分别位于第 18、28 和 67 位残基处。这三个半胱氨酸侧链上的硫原子能够与金属离子形成配位键(图 4a)。AFM-SMFS 表征了 α_3 DIV 与常见金属离子 Zn^{2+} 以及重金属离子 Hg^{2+} 和 Pd^{2+} 的配位行为^[147-149]。结果表明 Zn^{2+} 与两个半胱氨酸的硫原子形成 ZnS_2 配位中心,其断裂力约为 160 pN; Hg^{2+} 和 Pd^{2+} 作为半径更大的重金属离子,在碱性条件下分别与三个半胱氨酸的硫原子形成稳定的 HgS_3 和 PdS_3 配位中心,其最大断裂力分别约为 280 pN 和 200 pN。由于半胱氨酸在碱性条件下具有更强的去质子化倾向, α_3 DIV 的配位中心在碱性条件下表现出更高的稳定性。这种稳定性体现在更高的配位数和更大的断裂力上。围绕 α_3 DIV 的研究进一步揭示了半胱氨酸配位的一般规律,即 $Hg-S > Pd-S > Zn-S$,且碱性条件下的配位稳定性显著高于酸性条件下。

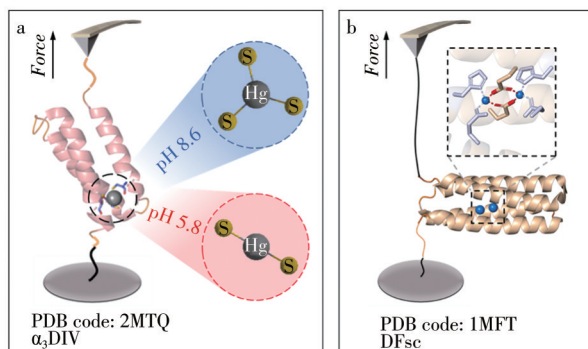


图4 AFM-SMFS表征的从头设计金属蛋白

Figure 4 *De novo*-designed iron-binding proteins characterized by AFM-SMFS

更复杂且更具仿生特性的金属配位中心也已通过人工设计实现。Saven 教授及其研究团队设计了

DFsc (*Due Ferri* single chain), 这是一种单链双铁蛋白,用于模拟天然双核非血红素铁酶的结构和功能^[150]。通过与两个锌离子结合形成惰性替代物, diZn-DFsc 成为 AFM-SMFS 研究双金属配位核心稳定性的理想模型(图 4b)。我们的研究显示, $Zn-O(Glu)$ 键的断裂力范围为 160~200 pN。实验还发现部分解折叠后的双金属核心能够重新极化,从而增强其与剩余配体的相互作用,有效防止双金属配位中心的完全解离。

4 AFM-SMFS 揭示金属结合对蛋白质稳定性的影响

除了测量蛋白质中金属-配体键的强度外,AFM-SMFS 还广泛揭示了金属结合对蛋白质稳定性的深远影响。目前,钙结合蛋白是这一领域的主要研究对象。例如,钙调蛋白(calmodulin)包含两个能结合钙离子的结构域。通过力谱测量发现,钙离子的结合对于钙调蛋白的折叠与功能至关重要(图 5a)^[151]。类似的现象也在其他钙结合蛋白中得以验证,如 M-晶状体蛋白(M-crystallin)^[152-153]、钙黏蛋白(cadherin)^[154]、凝溶胶蛋白(gelsolin)和 SdrG B1 蛋白^[155]。这些蛋白与钙离子的结合显著增强了其机械稳定性(图 5b-d)。此外,镁(Mg^{2+})和锰(Mn^{2+})的结合也被发现可以稳定呼肠孤病毒(reovirus)与 $\beta 1$ 整合素($\beta 1$ integrins)之间的相互作用^[156],而锌结合对视紫红质(rhodopsin)和 α_3 DIV 的稳定性影响同样显著^[157]。

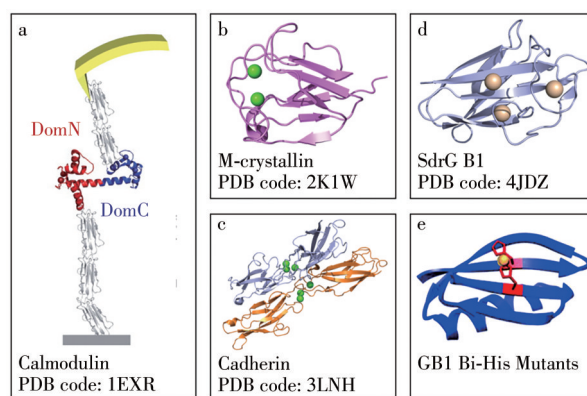


图5 AFM-SMFS表征的金属结合增强蛋白质稳定性的案例^[151]

Figure 5 Representative metal-binding proteins show enhanced mechanical stability revealed by AFM-SMFS^[151]

更进一步研究者们还验证了在蛋白质中引入非天然金属结合位点的可行性,这一策略已经在分子和宏观水平上得到了应用,例如水凝胶的设

计^[107,158-164]。在蛋白GB1中,通过工程化引入一个双组氨酸基序,发现镍离子(Ni^{2+})的结合显著增强了蛋白的机械稳定性,使其力学强度提升100 pN(图5e)。

这些研究表明金属结合不仅显著影响蛋白质的结构和功能,还通过增强机械稳定性提升了其抗力性能。此外,这些方法的应用使SMFS测量更加高效和精准,甚至可以区分金属-配体键强度的微小差异,从而实现其相互之间较为精确的比较。

5 总结与展望

综上所述,金属蛋白作为一种结合了多种金属离子的关键蛋白质亚类,在细胞内外发挥着至关重要的功能。金属离子的配位不仅稳定了蛋白质的三维结构,还在催化、信号传导、电子传递等多方面参与着生命活动的调控。近年来,单分子力谱(SMFS)技术在金属蛋白研究中取得了显著进展,尤其是在金属配位键强度的精确测量和金属离子结合对蛋白质稳定性影响的揭示方面。通过SMFS,研究者能够在原子尺度上对金属蛋白的结构变化、力学特性及金属-配体相互作用进行定量分析,为理解金属蛋白的功能提供了前所未有的视角。

尽管如此,金属蛋白的研究仍面临诸多挑战,特别是在揭示复杂金属配位体系的动力学特征和多层次结构稳定性方面。当前的研究大多集中在天然金属蛋白及其金属-配体相互作用的表征,但如何实现对人工设计的金属蛋白骨架或更复杂蛋白质体系的深入研究,依然是未来的重要课题。此外,随着计算模拟、分子动力学和人工智能等技术的不断进步,SMFS有望与这些技术相结合,为我们提供更加全面和精确的金属蛋白结构与功能关系的描述。

未来,随着蛋白质连接和固定技术的不断创新,SMFS将能够更高效、更精准地研究金属蛋白在不同环境和条件下的行为。这不仅将推动基础生物学研究的突破,也将为新型生物材料的开发、药物设计和疾病机制研究提供重要的实验依据。因此,我们期待SMFS在金属蛋白研究领域,特别是在揭示金属离子结合对蛋白质稳定性及功能的深远影响方面,发挥越来越重要的作用。

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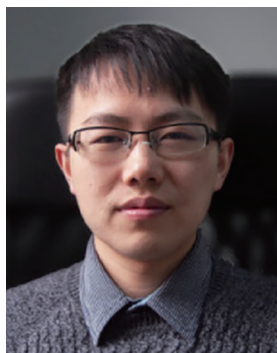
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