差示扫描微量热法研究蛋白质及其相关体系进展

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2016-04-27 收稿, 2016-05-20 修回, 2016-05-23 接受, 2016-09-20 网络版发表

国家自然科学基金(21273130)资助

摘要 蛋白质是一类重要的生物大分子, 其结构与性质得到了广泛的研究. 差示扫描微量热法(DSC)可以获得蛋白质的热容(C_p)与温度的关系, 对我们认识蛋白质的性质有重要的帮助作用. 同时, DSC研究蛋白质相关体系新方法也在不断涌现. 本文介绍了差示扫描微量热法在研究蛋白质解折叠机理、蛋白质稳定性以及复杂蛋白质体系等几个方面的进展.

关键词 差示扫描微量热, 蛋白质, 解折叠, 蛋白质稳定性, 淀粉样纤维, 疾病诊断

作为一种常见的研究生物体系分子热性质的方法, 差示扫描量热法(DSC)具有方便、快速、无化学干扰等优点, 在生命科学、材料学、制药学等领域有着重要的应用^[1~4]. 按照仪器灵敏度的大小, DSC又可以分为普通DSC与微量热DSC, 后者与前者相比灵敏度更高, 微量热DSC可以测得微瓦量级的热信号, 而普通DSC只能测得毫瓦量级的热信号. 相比之下, 微量热DSC可以测出更微弱的热效应, 所需的样品量更少, 对于生物样品的量热实验更加适合, 因此在生物量热上具有更加广泛的应用.

差示扫描微量热仪的工作原理是通过同时加热或冷却参比池与样品池中的缓冲液或者样品, 测量两者维持相同温度所需的热量差, 通过计算直接获得样品的表观热容(C_p)与温度的关系^[5]. 对于蛋白质体系而言, 我们还可以通过微量热DSC获取其热变性的温度(T_d)以及其变性的焓值(ΔH). 甚至, 通过设计微量热DSC实验, 我们可以获取蛋白质的绝对热容^[6,7].

当然, DSC能够做的远远不止这些, 其潜在的应用还有待开发. 下面介绍近十年来DSC在研究蛋白

质方面的若干进展与应用.

1 差示扫描微量热法研究蛋白质解折叠机理

蛋白质实现其生物功能需要特定的结构. 通常来说, 天然态蛋白质具有四级层次的结构, 分别是一级结构(肽链的序列)、二级结构(肽链有序折叠形成的有规则的重复结构单元, 如 α -螺旋、 β -折叠、 β -转角与无规卷曲)、三级结构(在二级结构的基础上, 多肽链进一步折叠卷曲形成复杂的球状分子结构)与四级结构(具有独立三级结构的多肽链通过非共价键相互连接而成的聚合体结构). 维系蛋白质二级与高级结构的主要作用包括氢键、疏水作用、二硫键、配位键等作用. 在外界条件发生变化的时候, 这些作用会受到影响, 天然态蛋白质的结构也会随之发生变化, 主要表现为解折叠与聚集. 解折叠(unfolding)指的是蛋白质二级及高级有序结构解开变为无序结构的过程, 通常伴随着蛋白质功能的丧失, 故又被称作变性(denaturation). 常见的引起蛋白质变性的因素有温度、pH以及变性剂等.

引用格式: 孙海源, 邓耿, 周瑜, 等. 差示扫描微量热法研究蛋白质及其相关体系进展. 科学通报, 2016, 61: 3091~3099

Sun H Y, Deng G, Zhou Y, et al. Recent progress in protein and protein-related systems using DSC techniques (in Chinese). Chin Sci Bull, 2016, 61: 3091~3099, doi: 10.1360/N972016-00531

蛋白质的解折叠机理受到了广泛的研究. 通常情况下, 蛋白质的解折叠机理可以大体上分为二态变性与非二态变性, 前者指的是蛋白质在解折叠的过程中仅仅存在天然态与解折叠态, 整个过程是协同性的; 后者指的是蛋白质在解折叠的过程中存在一个或者多个热力学稳定中间态^[8,9].

人们后来还发现了一种新的蛋白质变性模式: downhill 变性^[10,11]. 这种变性过程正如其名字一样, 天然态与解折叠态之间不存在能垒或者能垒很小, 导致其从初始的天然态不断发生微小的解折叠过程, 积少成多, 最终形成解折叠态. 这种变性过程可以看作是具有很多个中间态, 因此在广义上也属于非二态变性过程. 但是downhill变性与传统的非二态变性过程相比, 前者的变性路径中能垒很低甚至不存在能垒, 与后者具有较大的能垒形成了鲜明的对比, 因此两者具有截然不同的特点.

蛋白质的折叠与解折叠机理对于我们理解蛋白质的功能有着重要的意义, 因此受到了广泛的关注. 人们发展出多种方法来研究蛋白质的折叠与解折叠机理, 我国在这方面也有很多相关工作, 如赵新生课题组^[12]将单分子探测与微流控技术结合起来研究蛋白质的折叠过程, 发现不同结构域的折叠动力学是存在差异的; 高毅勤课题组^[13]发展了分子动力学模拟研究蛋白质折叠的方法, 提出了温度积分抽样法, 被公认是最好的取样方法之一. 传统的量热技术在这方面也占有重要的一席之地. 近10年来, DSC技术在研究蛋白质解折叠机理方面取得了一系列进展, 本文将按照蛋白质解折叠的机理分别对其进行讨论和分析.

传统的二态与非二态变性机理的分辨主要是依靠van't Hoff分析^[14,15]. 该方法的本质是将蛋白质变性过程按照二态平衡假设进行分析, 得出蛋白质变性的van't Hoff焓, 与实验中积分得到的焓值进行对比. 若两者的比值为1, 则说明蛋白质发生的变性过程为二态变性过程, 否则为非二态变性过程. 然而, 该分析方法要求蛋白质的变性过程可逆且不受聚集等因素的影响, 而蛋白质变性过程的可逆性通常会受到多种因素的影响, 比如囊泡对蛋白质的静电吸附作用有可能会使得蛋白质变性后不再发生复性^[16]. 另外, 由于DSC基线的选取对积分面积有很大的影响, 也会影响到最终的判断. 这些因素导致了van't Hoff分析方法在现实的应用方面受到了极大的限制.

此外, 蛋白质的解折叠机理也不是一成不变的, 会受到外界环境的影响^[17~19].

本课题组^[20]发展了一种新的判断蛋白质变性机理的方法, 称为“中止-恒温法”. 该方法的主要思路如下: 一般来说, 蛋白质变性是一个较慢的过程; 对于二态蛋白质而言, 由于蛋白质变性过程仅存在两种状态, 那么在不同的温度下进行恒温, 根据恒温时间的不同, 蛋白质只会以不同比例的两种状态(天然态与解折叠态)存在; 再次升温, 蛋白质的变性温度不会发生变化, 而仅仅会发生峰面积的改变; 假如在足够高温以上恒温足够长时间, 蛋白质将会完全变性. 对于非二态蛋白质而言, 不同的“态”有不同的解折叠温度; 在不同温度下进行恒温, 只有较低解折叠温度的蛋白结构发生变化, 再次升温不仅仅会发生峰面积的改变, 更会导致蛋白质变性温度的改变. 对于鸡蛋清溶菌酶(二态蛋白质)与牛血清白蛋白(非二态蛋白质), 本课题组^[21]使用中止-恒温法进行了研究, 结果如图1所示, 完全符合预测.

按照蛋白质解折叠能量图景中能垒的大小, 蛋白质可以分为3类: 能垒较大(远大于 RT , 即气体常数与温度的乘积)的蛋白质(如二态变性的蛋白质)、临界能垒(通常小于或者近似等于 RT , marginal barrier)蛋白质以及无能垒(global downhill)蛋白质. 三者的解折叠能量图景如图2内插图所示, 同时给出了相应的DSC结果^[21].

对于global downhill类蛋白质与marginal barrier蛋白质而言, 由于其能垒较低或者不存在能垒, 按照化学平衡的观点, 从蛋白质变性过程为二态平衡或者多态平衡或者某一步为决速步等不同的动力学假设出发, 解出其动力学方程并结合DSC结果进行分析的方法^[22~24]已经不再适用, 需要新的模型来对其数据进行分析.

Muñoz和Sanchez-Ruiz^[25]将朗道相变理论应用于蛋白质变性过程, 发展了使用DSC法分析downhill类蛋白解折叠能量图景的方法, 该方法被称为“可变量垒模型(variable barrier model)法”. 他们将蛋白质解折叠的焓变作为标志蛋白质解折叠的序参量, 引入2个参数 α 与 β 分别来描述蛋白质的能量分布情况与能垒高度, 建立状态方程并求解, 最终获得蛋白质热容与温度的关系. 通过变换 α 与 β 2个参数的数值, 对DSC数据进行拟合, 拟合所得最优解对应的参数即代表蛋白质解折叠过程中的能量分布情况与能垒大

图1 (网络版彩色)BSA(a)和lysozyme (b)在不同中止温度恒温不同时间后第2次升温DSC结果,最上面的线是天然态蛋白质变性过程结果^[20]
Figure 1 (Color online) DSC results of the second heating scans of BSA (a) and lysozyme (b) after they were heated to predesignated temperature, incubated for different period of time, and then cooled to 25°C. The fullscan curves at the top are the complete denaturation results of the proteins^[20]

小。据此,他们分析了埃希氏大肠杆菌硫氧还蛋白(thioredoxin from *Escherichia coli*,典型的二态蛋白)与埃希氏大肠杆菌 α -酮戊二酸脱氢酶多酶复合物外周亚基连接域(peripheral subunit binding domain from *Escherichia coli*'s 2-oxoglutarate dehydrogenase multienzyme complex,简称为BBL蛋白,典型的downhill蛋白),按照他们给出的模型对BBL蛋白的确给出了较好的拟合结果。他们还进一步完善了这个方法^[26],通过详细的计算与讨论得出了该理论在能垒高度不大于10 kJ($\sim 4RT$)能给出较精确的结果,对于能垒高度更大的蛋白质其可信度需要其他实验证据的佐证。同时,他们还分析了15种折叠速率已知蛋白质的DSC结果。虽然这15种蛋白质结构各异且折叠速率存在量级上的差异,但是通过分析,他们发现这些蛋白质的折叠速率与其能垒高度和特征温度的比值呈相关关系,如图3所示。这说明,对于这些蛋白质而言,其折叠速率与蛋白质的结构、大小关系不大,主要取决于其折叠路径能垒的高低。

需要指出的一点是,对于已知为global downhill机理的蛋白质或marginal barrier机理的蛋白质,应用“可变量垒模型”分析得到的解折叠能量图景是可信的。但是反过来,该方法在判断蛋白质是否为downhill类蛋白质方面并不一定可信。Farber等人^[27]使用DSC法与NMR法分析了前B细胞转录因子的同源区域(human pre-B cell transcription factor-homedomain,简称PBX-HD),得出的结论是不一致的。使用DSC方

法给出的结论为该蛋白质解折叠为downhill机理,而使用NMR方法给出的结论为该蛋白质解折叠为two-state机理。这说明该模型对于预测蛋白质变性机理方面还存在一些不完善之处。

关于使用DSC研究蛋白质解折叠机理的相关工作还有很多,鉴于其核心思想要么将蛋白质变性视作化学平衡过程处理,要么视作相变过程进行处理,不同之处仅仅在于化学平衡过程中间态的多少或者相变过程采取的是朗道相变模型还是平均场模型或者Ising模型,其基本思路与我们上面讨论的基本一致,故在此不再赘述,对此感兴趣的读者可以阅读相关文献[28~36]。

2 差示扫描微量热法研究蛋白质稳定性

差示扫描微量热在研究蛋白质稳定性方面也取得了一些新的进展。一般来说,蛋白质的稳定性与蛋白质变性温度的高低相关,变性温度越高标志着蛋白质的稳定性越好。但是,更加准确的说法是,蛋白质的稳定性与蛋白质的变性吉布斯自由能相关。蛋白质的变性吉布斯自由能可以通过计算获得^[37],计算的过程中需要知道蛋白质变性温度、蛋白质变性焓变以及蛋白质变性前后热容差值。通常情况下,我们可以通过DSC实验获得上面的3个热力学参数,进而计算出蛋白质的变性吉布斯自由能与温度的关系,获得蛋白质稳定性曲线(protein stability curve)。但是在一些情况下,DSC技术并不能给出或者准确给出上

图2 (网络版彩色)二态、临界能垒与无能垒3种蛋白质DSC曲线与能量图示意图。A, B, C三种蛋白质在变性中点对应的能垒高度分别为18, 2与0 kJ^[21]

Figure 2 (Color online) Simulated DSC thermograms for proteins with different folding barriers. (a) Two-state-like (midpoint barrier of ~18 kJ/mol); (b) marginal barrier (midpoint barrier of ~2 kJ/mol), and (c) global downhill (no barrier at midpoint). Insets show the free energy curves at the midpoint temperatures^[21]

述3个参数, 导致蛋白质稳定性曲线难以获得。最近, Schön和Freire^[38]将DSC与等温条件下化学变性(isothermal chemical denaturation, ICD)方法相结合, 提出了解决这个问题的可行方法。他们分别讨论了3种情况下的处理方式: 蛋白质变性过程可逆但是变性前后热容差值难以通过DSC直接获得的情况; 蛋

图3 (网络版彩色)15种不同蛋白质在298 K折叠速率与能垒高度和特征温度比值的相关关系^[26]

Figure 3 (Color online) Correlation between refolding rate k at 298 K and the ratio of barrier height (β) and the characteristic temperature (T_0)^[26]

白质热变性非可逆导致不能使用化学平衡处理的情况; 蛋白质变性温度不处于仪器可测范围内的情况。对于第1种情况, 他们首先通过ICD获得蛋白质在特定条件下的变性自由能, 然后根据此自由能计算获得在该条件下的热容差值, 再近似认为蛋白质热容差值不随温度而发生改变, 进一步结合DSC获得的焓变与变性温度两个热力学参数, 计算蛋白质变性吉布斯自由能与温度的关系, 从而获得蛋白质稳定性曲线。对于第2种情况, 他们采用了间接的方式来估算蛋白质的变性吉布斯自由能。对于某些蛋白质而言, 虽然在中性或者碱性的溶液环境中, 其热变性不可逆, 但是在较大的pH范围内其化学变性以及在酸性溶液环境下的热变性均可逆, 可以利用这个特点进行分析。采用ICD可以获得较大pH范围内(如pH 2~8)蛋白质的变性吉布斯自由能, 而DSC数据可以给出其在pH较低时热变性可逆的蛋白质的变性吉布斯自由能。鸡蛋清溶菌酶在酸性环境下热变性可逆, 可以直接计算获得蛋白质在酸性环境下的稳定性曲线, 但是其在中性或者碱性环境下热变性不可逆, 不能直接通过DSC获得蛋白质在中性或者碱性条件下的稳定性曲线。但是该蛋白由盐酸胍引起的化学变性在3种pH情况下均可逆, 符合该方法对于蛋白质性质的要求。他们对其进行了分析, 发现两者得到的自由能结果在低pH下能够很好地吻合, 据此推测在较高pH情况下的变性吉布斯自由能也应该可信, 进而对蛋白质稳定性作出估计。对于第3种情况, 蛋白质的稳定性可以通过ICD实验给出, 只不过需要用到多种

变性剂。总之, ICD与DSC能够起到互相辅助的作用, 对于我们全面认识蛋白质稳定性意义重大。

此外, 有研究表明凡是能够提高蛋白质稳定性的因素如合适的pH、离子强度或者添加剂都能够增加蛋白质成功结晶的可能性^[39,40]。这是因为蛋白质的晶体状态一般与蛋白质天然态相似, 在平衡热力学的概念中, 物质总是具有形成最稳定状态的倾向, 所以提高蛋白质稳定性意味着蛋白质天然态能量与变态能量差值的增加, 很可能意味着天然态自由能的降低, 有利于蛋白质形成稳定的与天然态类似的晶体。DSC可以获取蛋白质的稳定性信息, 在蛋白质结晶条件优化上面起着重要的作用, 这方面的应用已有相关的文献[41]报道。

3 差示扫描微量热法研究复杂蛋白质体系

淀粉样纤维指的是某些蛋白质在特定的条件下形成的富含 β -折叠结构的纤维状蛋白质聚集体, 通常与某些疾病相关, 如阿兹海默症、帕金森症等^[42~45]。常见的能够形成淀粉样纤维的蛋白质(或者多肽)有很多, 比如 $A\beta$ 肽、Tau蛋白、朊蛋白、 α -突触核蛋白、 β 2-微球蛋白、前胰岛淀粉样多肽、溶菌酶等^[46,47]。

Sasahara等人^[48~52]详细地研究了 β 2-微球蛋白与鸡蛋清溶菌酶两种蛋白质形成淀粉样纤维的过程。他们发现, 将蛋白质在特定的条件下预处理后, 在DSC加热的过程中会生成淀粉样纤维, 该过程为放热过程。对于不同处理条件下形成的淀粉样纤维过程, 其结果也有较大的差异。Morel等人^[53]研究了突变血影蛋白(N47A)SH3域的淀粉样纤维的热变性性质, 发现对于该蛋白而言, 其淀粉样纤维在加热的过程中会出现1个吸热峰, 该峰出现的温度比天然态蛋白变性的温度要高约35℃。另外, 他们还发现淀粉样蛋白吸热峰出现的温度点对淀粉样纤维的制备方法不敏感, 与 β 2-微球蛋白和鸡蛋清溶菌酶两种蛋白质形成淀粉样纤维有较大的差异。这说明对于不同的蛋白质而言, 其形成淀粉样纤维的过程与淀粉样纤维的性质均会有较大的差异, 不可一概而论。

Garbett等人^[54,55]发展了使用DSC技术研究血浆样品的方法。人体的血浆是一种复杂的混合物, 包含1000种以上的蛋白质与小分子。一方面, 他们使用DSC测得了正常人类的血浆DSC热谱(测15个健康人体的血浆取平均); 另一方面, 他们根据生化分析的结

果将血浆中存在的主要的蛋白质DSC热谱按照其含量进行加权平均, 获得血浆的计算DSC热谱。两者进行对比后发现吻合情况很好, 说明这些蛋白质在血浆中基本不相互影响其热性质。相应的结果如图4所示。

Garbett等人^[55~62]充分发展了利用DSC技术进行疾病诊断的方法。他们发现, 对于不同的疾病患者, 其血浆DSC热谱也有所差别, 可以作为“fingerprint”用来判断疾病的类型。另外, 其他的复杂生物样品如脑脊液等也被人研究^[63~66], 同样也可以用于疾病的诊断。

4 结论

差示扫描微量热法可以用于获取样品热容随温

图4 (网络版彩色)人体血浆DSC谱图。(a)实线代表血浆中16种主要蛋白质的DSC热谱, 虚线代表16种蛋白质DSC热谱的加权平均值; (b)黑线代表15名健康人体血浆的平均DSC热谱, 阴影区域代表在该温度点的偏差^[54]

Figure 4 (Color online) DSC thermograms of human plasma. (a) Calculated thermogram (dashed line) obtained from the sum of the weighted contributions of the 16 most abundant plasma proteins (solid lines); (b) DSC thermogram of normal plasma. The solid line is the average of 15 thermograms of plasma samples from 15 individuals. The shaded area is the standard deviation^[54]

度变化的信息,因此在蛋白质相关体系的研究中起着不可替代的作用;同时,使用差示扫描微量热法研究蛋白质相关体系的新方法也层出不穷.本文总结了差示扫描微量热法在研究蛋白质解折叠机理、蛋白质稳定性以及复杂蛋白质体系性质等几个方面的进展.一方面,通过建立合适的蛋白质解折叠模型,差示扫描微量热法在判断蛋白质解折叠机理、获取蛋白质解折叠能量图景方面取得了重要的进步;另一方面,采用与等温化学变性方法相结合,DSC在研究蛋

白质热稳定方面也取得了新的进展,能够获得单独使用DSC技术不能够获得的热力学参数,另外,使用DSC研究蛋白质的稳定性也为蛋白质结晶条件的优化提供了新的视角.此外,对于淀粉样纤维与血浆、脑脊液等复杂生物样品的研究也拓展了差示扫描微量热法的研究范围,为我们研究并理解蛋白质相关疾病病变机理与疾病诊断打开了新的大门.总之,我们相信,随着人们认知水平的提升,传统的微量热技术也会在将来发挥越来越重要的作用.

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Recent progress in protein and protein-related systems using DSC techniques

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As one family of the most important biomolecules, proteins have gained more and more attention since the day they were discovered and the trend is still growing. Even so, many problems are emerging in this field and thus new techniques and ideas are required for solving these problems. In this regard, differential scanning calorimetry (DSC) is a conventional but useful method to obtain the relationship between heat capacity and temperature of proteins. In the past decade, new ideas have been applied to DSC and new methods have been developed. This is reflected in this minireview. First, important progresses have been made on protein unfolding or folding mechanisms. A new method called “interruption-incubation protocol” was developed to judge whether a protein unfolds in a two-state mechanism or not. Compared to the conventional van’t Hoff analysis method, the new approach breaks the limitations of old method on the requirement of protein properties, namely the reversibility of protein folding and the equilibrium assumption during the unfolding process. Also, this method avoids the inaccuracies of enthalpy change for protein unfolding caused by improper baseline determination. Thus, the “interruption-incubation protocol” has a lower possibility of misjudgement and turns out to be an effective way for analysing “two-state or non-two state” proteins. In addition, by combining DSC with phase transition models instead of chemical equilibrium assumption, several theories were proposed which can provide a rough landscape of protein folding or unfolding process. Among these theories, the “variable barrier model” is an interesting one, which can directly give the barrier heights in protein folding as long as the barrier heights are smaller than $\sim 4RT$. Second, a new method was proposed to derive protein stability curve directly by combining DSC with isothermal chemical denaturation (ICD) method. This method targets those “problematic” proteins, when the denaturation process is not perfect reversible. Three cases were discussed, namely the unreliability of protein ΔC_p determination, the irreversibility of protein thermal denaturation and the extreme stability of specific proteins. For the first case, a more reliable protein temperature-dependent ΔC_p was obtained by a combination of DSC and ICD method, which was used for further calculation of ΔG of the unfolding process. For the second case, the unfolding process of some proteins under low pH is reversible and protein stability result from DSC was highly consistent with that obtained by ICD method. Then, protein stability under other conditions such as high pH, where the denaturation would be irreversible, could be estimated by ICD method. For the third case, a combination of several denaturants were used to obtain the protein stability curve. In addition, DSC can also be applied to optimize conditions for protein crystallization. Third, the application scope of DSC is expanded to complex protein-related systems such as amyloid fibers or even biological samples like blood plasma. For amyloid fibers, DSC can provide useful information of their thermodynamic properties which can lead us to a better understanding of amyloid fiber formation; for biological samples, DSC thermograms can be used as “fingerprints” for clinical diagnosis since the features of DSC thermograms for healthy and sick individuals are different. To summarize, DSC is a powerful tool to obtain protein properties and will play a more important role in the future.

differential scanning calorimetry, protein, unfolding, protein stability, amyloid fibrils, clinical diagnosis

doi: 10.1360/N972016-00531