The Thermodynamic Evidence of Two Native Conformations Coexisting in Solution For Apoazuirn: a DSC and CD Study*

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Abstract Whether protein could adopt multiple conformations coexisting in solution is disputable. In a previous report, the conformation heterogeneity of apoazurin mutant M121L had been identified. The thermal unfolding of wild type apoazurin from *Pseudomonas aeruginosa* is re-investigated with differential scanning calorimetry (DSC) and circular dichroism (CD) methods. The results show that unfolding in the pH range from 4 to 9 is associated with two heat capacity maxima. The low temperature transitions are reversible at all pH conditions used, while the high temperature transitions are irreversible. The two unfolding transitions were analyzed by the two-interchangeable-conformation model with the fraction for the first transition (N_1) from 64% at pH 4.0 to 55% at pH 9.0. Temperature induced unfolding monitored at 219 nm shows also two separate transitions. The ratio of the signal changes is consistent with the fractions obtained from the corresponding DSC measurements. These results provide further support for the hypothesis that at least two conformations of apoazurin coexists in solution.

Key words thermal unfolding, apoazurin, differential scanning calorimetry, circular dichnoism, protein conformation

Azurin is a member of small blue copper proteins, functioning as an electron-transfer carrier in bacteria. The azurin from *Pseudomonas aeruginosa*, as well as that from *Alcaligenes denitrificans*, is structurally well characterized. This protein consists of one α-helix and eight β-strands packed in a β-sandwich, forming a highly hydrophobic core[1]. Two apoforms of slightly different crystal structure[2] have also been resolved in 0.185 nm resolution. The similarity between the apoform and holoform was believed as the evidence supporting the rack mechanism of metal ion incorporation.

The folding of azurin was also probed using different methods. Winkler *et al*.[3] studied the difference in isothermal unfolding for the holo azurin in both the oxidized and reduced state. They stressed the importance of the copper atom in stabilizing the native conformation. The pressure effect on unfolding of wild type and mutant azurin were also investigated[4]. All results suggest that the folding of azurin can be depicted with a two-state model, although the metal was still coordinated to the unfolded protein[5].

In contrast, the folding mechanism of apoazurin has been studied in less detail[6, 7]. Until recently it was generally accepted that the bimodal unfolding results from intramolecular stability differences of the α-helix and β-sheet domains. However the evidence that under specific conditions this model does not apply for apoazurin mutant M121L, which had been demonstrated in a previous report[8]. The biphasic unfolding induced by urea has been identified as the evidence of two interchangeable conformations coexisting in solution through folding kinetic analysis. In the present report, DSC and CD measurements at a variety of pH values were studied. The results also support the model of at least two conformations of apoazurin coexisting in solution. The two separate transition peaks observed by both DSC and CD are due to unfolding of the pre-existing conformations and each transition can be well described by the assumption of an all-or-none transition model.

1 Materials and methods

1.1 Samples

Recombinant azurin was expressed, purified and prepared as previously described[9]. Its purity was checked by SDS electrophoresis with a single band at molecular mass of 14 ku. The apoprotein was prepared by dialyzing the sample against 100 mmol/L thiourea

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(Sigma) [9] in 50 mmol/L sodium phosphate buffer pH 5.1 for 12 h after it was reduced by 10 mmol/L ascorbate (Merck). The chemical agents of sodium acetate (supper pure) and glycine were from Merck.

1.2 DSC measurements

The temperature dependence of the apparent specific heat capacity of the protein has been determined using a Nano Ⅱ adiabatic differential scanning microcalorimeter from Calorimetry Sciences Corporation. Proper buffer corrections have been made.

To establish protein stabilities, the standard Gibbs energies were calculated for each of the transitions using the stability equations (1) for the first and (3) for the second transition.

$$\triangle G_1^0 = -\frac{\triangle H_1^0(T_m)}{T_{m,1}} \cdot (T-T_{m,1}) + (T-T_{m,1}-T\ln\frac{T}{T_{m,1}}) \cdot \triangle C_{p,1}(T_{m,1}) \quad (1)$$

$$K_1 = \exp(-\frac{\triangle G_1}{RT}) \quad (2)$$

$$\triangle G_2^0 = -\frac{\triangle H_2^0(T_m)}{T_{m,2}} \cdot (T-T_{m,2}) + (T-T_{m,2}-T\ln\frac{T}{T_{m,2}}) \cdot \triangle C_{p,2}(T_{m,2}) \quad (3)$$

$$K_2 = \exp(-\frac{\triangle G_2}{RT}) \quad (4)$$

The temperature dependent transition enthalpy was calculated according to

$$\triangle H_i^0 = \triangle H_i^0(T_{m,i}) + \triangle C_{p,i}(T-T_{m,i}) \quad (5)$$

where $\triangle H_i^0(T_{m,i})$ refers to the standard enthalpy changes at the transition temperature $T_{m,i}$ (i=1,2) for the first and second transition, respectively. The capacity of each transition is assumed to have the following form[10]:

$$C_{p,1} = \frac{(\triangle H_1^0(T))^2}{RT^2} \cdot \frac{K_1}{(1+K_1)^2} + \triangle C_{p,1}\frac{K_1}{1+K_1} + C_{p,1}^{\text{native}} \quad (6)$$

and

$$C_{p,2} = \frac{(\triangle H_2^0(T))^2}{RT^2} \cdot \frac{K_1}{(1+K_1)^2} + \triangle C_{p,2}\frac{K_2}{1+K_2} + C_{p,2}^{\text{native}} \quad (7)$$

With these equations fitting of the experimental DSC data is amenable if one assumes that (1) there are two conformers (N_1 and N_2) of apoazurin in solution, each of which unfolds independently in an all-or-none fashion; (2) the conversion between each conformer is very slow under the experimental conditions used, so that each transition contributes to the observed C_p according to its mole fraction x_i. The sum of x_1 and x_2 is equal to 1. The observed heat capacity can then be written as

$$C_p = x_1 \cdot C_{p,1} + x_2 \cdot C_{p,2} \quad (8)$$

with

$$x_1 + x_2 = 1 \quad (9)$$

The curves are normalized to one mole of apoazurin. Therefore integration of the C_p peaks in the DSC-curve provides an $\triangle H$ value proportional to the molar fraction of the respective conformer.

1.3 CD measurements

CD measurements were performed using a Jobin Yvon CD6 spectropolarimeter. Sample temperature was monitored using a PT100 platnium resistance thermometer immersed in the protein solution. CD spectra were obtained in the range from 185 to 250 nm in 0.1 mm cells employing protein concentrations between 0.9 g/L to 1.89 g/L. Data were collected using the spectrum-scan mode of the CD6 software with the wavelength increment of 0.5 nm or 1 nm and an integration time of 1 s. Unless otherwise stated, spectra were averaged over four scans and corrected for the buffer signal. Temperature scans were performed by scanning continuously from room temperature with a heating rate of 2 K/min. The ellipticity at 219 nm was recorded every 0.5 K with a response time of 5 s. Ellipticity readings were converted into mean residue ellipticity values, $[\Theta]_{\text{MRE}}$, by the formula

$$[\Theta]_{\text{MRE}} = \frac{\Theta \cdot M}{c \cdot d \cdot n_r \cdot 10} \quad (10)$$

where Θ is the measured ellipticity in milidegrees, M the molecular mass of the apoazurin (13 929 u), c the concentration in g/L, d the path-length of the cuvette in cm, and n_r = 128 the number of amino acid residues of azurin.

The fraction of unfolded protein, f_u, of each unfolding transition was calculated respectively using the standard equation

$$f_u = \frac{\theta_N - \theta}{\theta_N - \theta_D} \quad (11)$$

where θ_N and θ_D represent, respectively, the ellipticity values of the native and denatured species at each temperature calculated from the linear extrapolations of the baselines preceding and following the transition regions. Van't Hoff enthalpies, $\triangle H_{vH}$, and T_m values were calculated for the reversible unfolding processes from the CD-transition curves using the standard equation[11]

$$\frac{\partial \ln K}{\partial \frac{1}{T}} = -\frac{\triangle H_{vH}}{R} \quad (12)$$

where R is the gas constant 8.314 $J \cdot K^{-1} \cdot mol^{-1}$, K is defined as $f_u/(1-f_u)$, and $\triangle H_{vH}$ is assumed to be temperature-independent. The T_m values are taken at $\ln K$=0.

2 Results

2.1 Differential Scanning Calorimetry

2.1.1 Reversibility. Figure 1 shows the thermal transition curves of apoazurin with a scan rate of 2K/min and concentration of 0.95 g/L at pH7. Two distinct heat capacity peaks were exhibited, the first transition at $T_{m,1}$=63.9℃ and the second $T_{m,2}$=77.7℃. The unfolding transitions were separated from each other and the C_p curve between the two peaks reaches nearly the bottom of the extrapolated baseline. Reheating of the sample just after the first DSC scan shows that the first transition is recovered almost completely at the same transition temperature whilst the second transition is absent. Similar reversibility was obtained in the pH range from pH 4 to pH 9.

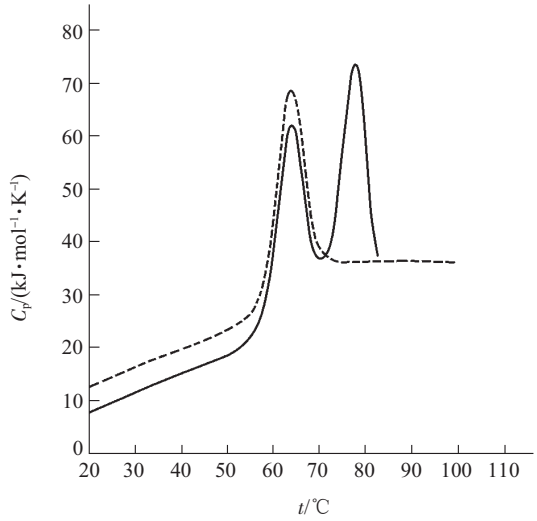

Fig.1 Reversibility studies of the thermal transitions of apoazurin

The solid and dashed line represents, respectively, the first and second scan with the same sample. The scan rate was 2K/min. The buffer was 20 mmol/L sodium phosphate, pH 7.0. Protein concentration: 0.95 g/L.

2.1.2 Thermodynamic parameters of apoazurin derived from DSC. Generally, thermodynamic analysis requires reversibility of the thermal unfolding process. However, under certain kinetic constraints the thermodynamic equilibrium parameters can also be derived from irreversible unfolding reactions as long as certain criteria are fully filled[10]. According to the two native conformations coexistence model [9] the two distinct C_p transition peaks of apoazurin were analyzed by equation from (1) to (9). As an illustration, Figure 2 shows experimental data at pH 5.1 for apoazurin together with the fitted curve. The equilibrium parameters have been summarized in Table 1.

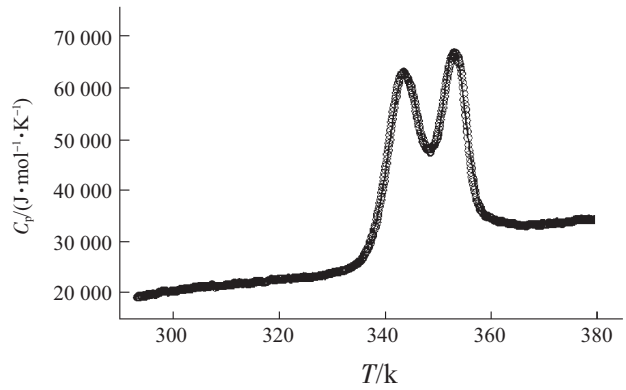

Fig.2 Illustration of the fitting of heat capacity curve of apoazurin at pH 5.1 using equations (1) to (9)

The hollow circle shows the experimental data. The solid line represents the fit calculated on the assumption of two conformations unfolding in two-state manner. Protein concentration: 0.73 g/L; buffer: 20 mmol/L sodium acetate buffer; scan rate of 2 K/min.

Table 1 Thermodynamic parameters of apoazurin from *Pseudomonas aeruginosa* from DSC measurements

pH	Transition	T_m/℃	$\triangle H_{cal}$/(kJ·mol^{-1})	$\triangle H_{fit}$/(kJ·mol^{-1})	x_i	$\triangle H_{cal}/(n_i \triangle H_{fit})$
4.1	1	61.3	230.1	355.5	0.6386	1.0136
	2	72.3	197.3	557.1	0.3614	0.9800
5.1	1	70.5	295.6	476.1	0.6445	0.9636
	2	79.9	218.9	652.6	0.3555	0.9433
5.9	1	69.8	331.0	538.0	0.6046	1.0176
	2	80.1	267.0	667.7	0.3954	1.0124
7.0	1	64.7	288.7	531.2	0.5582	0.9785
	2	77.4	252.9	626.1	0.4418	0.9142
8.1	1	62.3	228.3	531.5	0.5395	0.8546
	2	75.6	222.4	589.0	0.4605	0.8193
9.0	1	63.9	275.7	472.7	0.5505	1.0343
	2	77.7	261.4	600.0	0.4495	0.9692

Numerical integration gives a $\triangle H_{cal}$ value of 296 kJ·mol^{-1} for the low temperature transition ($T_{m,1}$=70.5℃) and 219 kJ·mol^{-1} for the high temperature transition ($T_{m,2}$= 79.9℃). The curve fitting procedure for the low temperature transition yields a van't Hoff enthalpy of $\triangle H_{fit}$=476.1 kJ·mol^{-1} with a mole fraction of x_1=0.6445. The high temperature transition is associated with a van't Hoff enthalpy of $\triangle H_{fit}$=652.6 kJ·mol^{-1} with a mole fraction of x_2= 0.3555. Thus the real cooperative unit, determined by the ratio of $\triangle H_{cal,i}/(x_i \cdot \triangle H_{vH,i})$, is equal to 0.9636 for the low temperature transition, and 0.9433 for the high temperature transition. It is noteworthy that both of these cooperative ratios are close to unity as should be the case for all-or-none transitions. This result is the thermodynamic basis preferring the model of energetically different apoazurin species to that of an intermediate in a sequential unfolding process.

2.1.3 Dependence of the transition curves on pH. Figure 3 also shows DSC scans of apoazurin at other three different pH values. The T_m values of both transitions are pH dependent. For both the transition peaks, the highest T_m is obtained at pH 5.9, with the value of 69.8℃ for the first transition, and of 80.1℃ for the second transition respectively. Increase or decrease pH can both decrease its stability [6]. At pH 8.1, the calculated enthalpy for the first transition is 228.3 kJ·mol^{-1}, with the transition temperature of 62.3℃, in good agreement with published data. However, the transition parameters for the high temperature transition are significantly different from the published data, which may be due to the difference in apoazurin preparation.

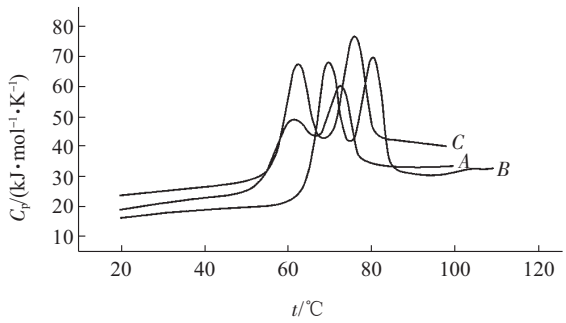

Fig.3 Heat capacity scans of apoazurin at different pH values

A: pH 4.1, 20 mmol/L sodium acetate buffer, B: pH 5.9, 20 mmol/L sodium phosphate buffer, and C: pH 8.1, 20 mmol/L sodium phosphate buffer. The scan-rate was set at 2 K/min in all the cases.

Plotting the change in x_1 versus pH, shows that the fraction of the conformation N_1 is significantly reduced by increasing of pH, while x_2 increases accordingly (Figure 4). At pH 4.0, the fraction of conformation N_1 is 0.64, and the fraction of conformation N_2 is 0.36. At pH 9.0, the fraction of conformation N_1 is 0.55 while the fraction of conformation N_2 is 0.45. The change of conformation fraction induced by pH suggests that the conformation N_1 and N_2 be in pre-equilibrium in solution at room temperature, and their equilibrium can be moved by changing pH.

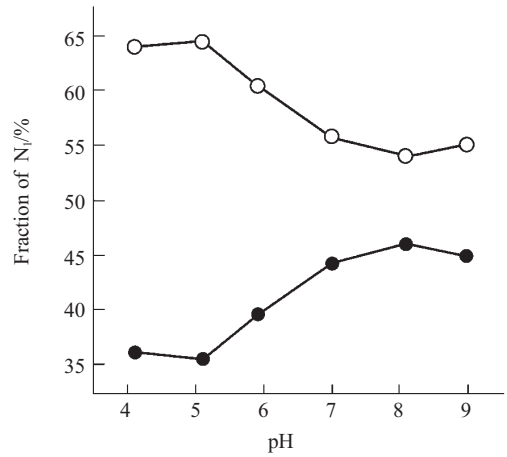

Fig.4 Fraction of each conformation as a function of pH

Open circles refer to the conformation with low transition temperature, and filled circles to the conformation with high transition temperature. The functional form of the heat capacity change is described as a sum of two components ($x_1 \triangle C_{p1}+x_2 \triangle C_{p2}$). For a detailed description, see Materials and methods.

2.2 Circular Dichroism study

The characteristic features of apoazrin CD spectrum are one strong negative peak at 219 nm and a strong positive peak at 195 nm (data not shown). Temperature induced CD transition profiles were also recorded with a scan-rate of 2K/min at 219 nm. Figure 5 shows the CD transition curve of apoazurin at pH 7.0. The significant feature of this measurement is the finding of a two-step transition curve with a plateau region between them. The midpoints of the first and second transition temperatures are shown in Table 2, which are close to those of the DSC measurements. The van't Hoff enthalpy for the low temperature transition (Figure 5b) shown in Table 2 is also close to that obtained from DSC under the same conditions, while that of the high temperature transition does not determined because of its irreversibility. The protein fraction in conformation N_1, calculated by the signal change is 0.54, close to that of 0.5582 obtained from DSC at the same pH (Table 1). These results illustrated that each unfolding transition observed in DSC is correspond to a two-state unfolding in secondary structure.

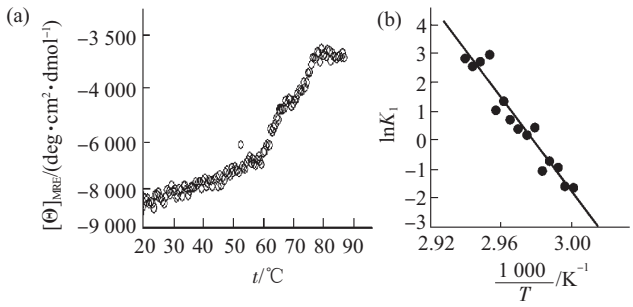

Fig.5 Dependence of mean residue ellipticity on temperature of apoazurin

(a) the measurement made at 219 nm with an apoazurin concentration of 0.95 g/L in 20 mmol/L sodium phosphate buffer, pH 7.0. The sample was prepared by dialysis of the reduced azurin into 100 mmol/L thiourea at pH 5.1. After that the protein was equilibrated with the pH 7.0 buffer. Scan rate is 2K/min. (b) shows the plot of $\ln K_1$ versus $1000/T$ for the first transition. The data up to 70℃ were derived by using equation (11) for the unfolded fraction of conformation N_1 and the formula $K=f_u/(1-f_u)$. The slope (straight line) of curve was obtained by a linear regression analysis and is identical with $-\triangle H_{vH}\cdot(1000R)^{-1}$ according to equation (12). $\triangle H_{vH}$ is assumed to be temperature-independent. The transition temperature, T_m, was calculated from $\ln K_1=0$. The results are summarized in Table 2.

Table 2 Thermodynamic parameters derived from temperature-induced CD measurements

Condition		$\triangle H_{vH}$/(kJ·mol^{-1})	T_m/℃	Fraction
pH 7.0	N_1	662.0±48.0	62.7	0.54
	N_2	undetermined	75.7	0.46

3 Discussion

Generally, the observation of two transitions in a denaturant or temperature induced unfolding process is positive evidence of multistage unfolding of the protein with at least one stable intermediate. However, this conclusion is no longer unambiguous for proteins adopting multiple conformations in their native state. In the case of *P. aeruginosa* apoazurin, as suggested by Engeseth and McMillian [6], there are no spatially isolated domains in the azurin molecule and, therefore, it seems that there is no structural basis for the assignment of the double transition in the DSC profiles to an intermediate characterized by 28 residues being unfolded, which forms preferential helical structure in the native state.

For a single domain protein, there are only three cases for the unfolding cooperative unit, i.e. (1) $\triangle H_{cal}/\triangle H_{vH}=1$, (2) $\triangle H_{cal}/\triangle H_{vH}>1$, and (3) $\triangle H_{cal}/\triangle H_{vH}<1$. For a reversible process, $\triangle H_{cal}/\triangle H_{vH}=1$ has become a widely accepted criterion [12, 13] to identify a reaction fitting a two-state-model. In the case of (2), e.g., the thermal transition of the molten globule of cytochrome c ($\triangle H_{cal}/\triangle H_{vH}$, 1.4~2.0)[14], it suggests that the thermal transition is not as cooperative as a two-state transition. In the case of (3), for irreversible processes, $\triangle H_{cal}/\triangle H_{vH}<1$ can be interpreted as an indication that before the reaction is completed, aggregation occurs, and some energy during the reaction is not released. However, for a reversible process as observed for the first transition of apoazurin at all pH conditions the ratio, $\triangle H_{cal}/\triangle H_{vH}$, smaller than 1 can only be understood as a suggestion that the number of molecules participating in the unfolding process is smaller than the theoretical value. This would be the case if more than one kind of molecules occur in the system. The sum of the $\triangle H_{cal}/\triangle H_{vH}$ for both transitions is equal to 1, suggesting that each transition could be treated as a two-state transition followed by a slow irreversible aggregation, i.e., the Lumry-Eyring model[15]. It is obviously necessary for the coexistence model to be true, that the activation energy for the conversion of the two conformations be high enough to prevent attainment of equilibrium within temperature scan interval. Therefore we have to assume that the two-apoazurin conformers are kinetically trapped. This is a rational explanation for our data.

As discussed in the previous paper, the structural energetic heterogeneity of the system is not due to sample impurity. In this report, the effect of pH on the fraction of x_i, which is equal to $\triangle H_{cal,i}/\triangle H_{vH,i}$, suggests however that the fraction of each conformation could be shifted by environment, such as pH (Figure 4). A similar pH effect on apoazurin has been reported before[16] and was concluded that there are at least two conformations in solution that could be distinguished by time-resolved phosphorescence. The distribution ratio of the two conformations is due to the titration of a group with a pK_a close to 5.6. Comparing the fraction of each conformations obtained here with Hansen's results, it is clear that at low pH, these two sets of data agree very well. However, at high pH, great deviations occur. This difference is understandable, because our measurements probe the overall structure of the macromolecule, while Hansen *et al.* [16] focused on preferentially local conformation. This deviation suggests also that there be other structural elements involved in the two conformations besides the one protonated group.

In summary, *P. aeruginosa* apoazurin has been shown to adopt at least two native conformations in solution. Each species behaves as one regular small globular protein, which unfolds in an all-or-none fashion. There is no stable intermediate in either unfolding pathway. Further studies on the structural

basis of the multiple conformations are in progress.

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去辅基天青蛋白两种天然构象共存的热力学证据 *
——差热温度扫描和圆二色的研究

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摘要 天然态蛋白质能否在溶液中存在多种构象是一个有争议的问题. 在前报道中已经鉴定出绿脓杆菌去辅基天青蛋白突变体 M121L 可以多种构象共存. 用差热扫描量热和圆二色性的方法研究了野生型去辅基天青蛋白的热变性. 结果表明在 pH 从 4.0 到 9.0 的范围内存在着两个摩尔热容最大值. 较低温度下的去折叠反应在所研究 pH 范围内均部分可逆，而较高温度下的去折叠反应均不可逆. 蛋白质去折叠的热容变化双峰用可相互转化的两种构象共存模型进行拟合. 较低温度下能够去折叠的构象在 pH 4.0 时占 64%，在 pH 9.0 时占 55%. 监测热变性过程中圆二色谱在 219 nm 处的信号变化也可以观测到两个独立的去折叠变化. 信号变化的比值与在相同条件下差热扫描法测得的两种构象摩尔比一致. 上述结果进一步支持了前文提出的去辅基天青蛋白在溶液中至少存在着两种构象的设想.

关键词 热变性，去辅基天青蛋白，差热温度扫描，圆二色性，蛋白质构象

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