Supporting Information

Biophysical Exploration of Protein-Flavonols Recognition: Effects of Molecular Property and Conformational Flexibility

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Time-Resolved Fluorescence. Time-resolved fluorescence was examined with a FLS920 spectrometer (Edinburgh Instruments, UK), using the time-correlated single photon counting system with a hydrogen flash lamp excitation source, in air equilibrated solution at an ambient temperature. The excitation wavelength was 295 nm and the number of counts gathered in the channel of maximum intensity was 10,000. The instrument response function (IRF) was gauged exploiting Ludox to scatter light at the excitation wavelength. The data were analyzed with a nonlinear least-squares iterative method utilizing the Fluorescence Analysis Software Technology, which is a sophisticated software package designed by Edinburgh Photonics for the analysis of fluorescence and phosphorescence decay kinetics, IRF was deconvoluted from the experimental data, and the resolution limit after deconvolution was 0.2 ns. The value of χ^2 (0.9～1.2), the Durbin-Watson parameter (greater than 1.7), as well as a visual inspection of the residuals were used to assess how well the calculated decay fit the data. Average fluorescence lifetime (τ) for multiexponential function fittings were from the following relation:[1-3]

$$I(t) = \sum_i A_i e^{\frac{-t}{\tau_i}} \tag{1}$$

where τ_i are fluorescence lifetimes and A_i are their relative amplitudes, with i variable from 1 to 3.

Circular Dichroism. Far-UV CD spectra were collected with a Jasco-815 spectropolarimeter (Jasco, Japan), equipped with a microcomputer, the instrument was sufficiently purged with 99.9% dry nitrogen gas before starting the apparatus, and then it was calibrated with d-10-camphorsulfonic acid. All of the CD measurements were picked at 298 K with a PFD-425S Peltier temperature controller attached to a water bath with an accuracy of ±0.1 ^{o}C. Each spectrum was scanned with the use of quartz cuvette of 0.2 cm path length and taken at wavelength between 200 and 260 nm with 0.1 nm step resolution and averaged over five scans operated at a speed of 20 nm min^{-1} and response time of 1 s. A reference sample containing buffer and flavonol was subtracted from the CD signal for assay, and the secondary structure was appraised exploiting Jasco Spectra Manager II, which computes the different designations of secondary structures by comparison with raw CD spectra, determined from distinct proteins for which high-quality X-ray diffraction data are available.

Principles of Fluorescence Quenching. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, such as excited state reactions, molecular rearrangements, energy transfer, ground state complex formation, and collisional quenching. Fluorescence quenching is described by the well-known Stern-Volmer equation:[1,4,5]

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \tag{2}$$

In this equation, F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, $[Q]$ is the concentration of quencher, and K_{sv} by linear regression of a plot of F_0/F *versus* $[Q]$. Moreover, the fluorescence intensities were corrected for absorption of the exciting light and reabsorption of the emitted light to decrease the inner filter effect by using the following relationship:[1,6]

$$F_{cor} = F_{obs} \times e^{\frac{A_{ex} + A_{em}}{2}} \tag{3}$$

where F_{cor} and F_{obs} are the fluorescence intensities corrected and observed, respectively, and A_{ex} and A_{em} are the absorption of the systems at the excitation and the emission wavelength, respectively. The fluorescence intensity utilized in this work is the corrected intensity.

Elucidations of Time-Resolved Fluorescence and UV/vis Absorption Spectra. Although fluorescence quenching properties may be distinguished by their different dependence on temperature and viscosity, according to the notion of Lakowicz,[1,7] the measurement of fluorescence lifetimes is the most definitive method to distinguish static and dynamic quenching. Normally, steady-state adducts can be formed between steady-state chromophore group and ligand in static reaction, such procedure shall lower the number of chromophore group, and then give rise to the decrease of fluorescence intensity. In quenching processes, fluorescence lifetime remain unchanged, for static reaction might not affect the excited-state of chromophore group; and furthermore, both the concentrations of chromophore group and the inner filter effects of fluorescence will also not influence fluorescence lifetime. As a result, even though the Stern-Volmer is linear case, the determinations of time-resolved fluorescence lifetime of chromophore groups in enzyme/protein could likewise be employed to thoroughly interpret the innate character of molecular recognition between biological macromolecules and active ligands. This technique has satisfactorily been utilized by some research groups to elucidate the biomolecular recognition of different bioactive substances with certain biopolymers.[8-10] To further dissect the recognition mechanism between protein and flavonol, the representative time-resolved fluorescence of protein at various molar ratios of flavonol in Tris-HCl buffer, pH＝7.4, are scanned and the fluorescence lifetimes and their amplitudes are also collected in Table S1.

Table S1

Fluorescence lifetimes of protein as a function of concentrations of flavonol

Samples	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	A_1	A_2	A_3	τ (ns)	χ^2
Free hemoglobin	0.31	2.33	4.53	0.073	0.32	0.607	3.52	1.07
Hemoglobin＋morin (1：1)	0.24	2.26	4.37	0.067	0.35	0.583	3.35	1.02
Hemoglobin＋morin (1：2)	0.22	2.27	4.46	0.089	0.31	0.601	3.40	1.03

Commonly, time-resolved fluorescence can not only offer exact information regarding the time dependence of the reaction processes, but also supply some vital clues to the surrounding microenvironment of the chromophore groups. As expect, the decay curves could well be fitted to a triexponential function, and the relative fluorescence lifetimes of protein are τ_1＝0.31 ns, τ_2＝2.33 ns and τ_3＝4.53 ns, whereas in the maximum amount of flavonol, the fluorescence lifetimes are τ_1＝0.22 ns, τ_2＝2.27 ns and τ_3＝4.46 ns, respectively. It is worth mentioning that the measured triexponential decay herein reflects the presence of multiple Trp residues in the protein molecule, and these aromatic residues have distinct decay times. This event might mainly be attributed to their diverse microenvironments. Moreover, a forcible explanation of this phenomenon is that the triexponential decay behavior can be chalked up to electronic transition of Trp residues, which shall emerge as dissimilar conformational isomers in the biomacromolecule.[11-13] Owing to steric effects between the side chain of Trp residues and the backbone of polypeptide chain, all rotamers are not tantamount. And the existence of unequal rotamers of amino acid residues has strictly been proved by other methods, e.g. nuclear magnetic resonance.[14-16] Accordingly, we did not try to designate the independent fluorescence lifetime components, in contrast, the average fluorescence lifetime of Trp residues will be utilized to obtain a qualitative analysis.

Apparently, the mean fluorescence lifetime of protein was discovered to be 3.52 ns, which is quite conformed to the former data reported by Szabo et al.[17,18] in a relatively early job. In the meantime, one may note that the average fluorescence lifetime exhibit a faint fluctuation, however, the amplitude is still in the bounds of acceptable level. This may be caused by the charge transfer from the indole ring of Trp residue to the adjacent substituent, which slightly raise in recognition strength of the protein-flavonol and thus leads to a feeble drop in the mean fluorescence lifetime. It is revelatory of a tiny contribution of dynamic behavior as only those ligands that undergo dynamic reaction have been sole contribution in the shrink of the fluorescence lifetime. For this reason the time-resolved fluorescence energy transfer efficiency (E) calculated from fluorescence lifetimes measurements exclusively reveals the energy transfer during the procedure of dynamic manner and is harvested according to the equation: $E=1-\tau/\tau_0$, where τ and τ_0 are the fluorescence lifetime of Trp residue in the presence and absence of natural flavonol, respectively. The sizes of E reckoned from time-resolved fluorescence data are detected to be 4.83% and 3.41%, respectively, in the protein-flavonol adduct at a molar ratios of hemoglobin to flavonol of 1：1 and 1：2. Plainly, the two values are very minute; hence we can take it as read that although energy transfer has done a few operations in this noncovalent recognition, the transfer efficiency is abysmally low, and even negligible. These results accords nicely with previous discussions based on steady-state fluorescence, or, more pertinently, the molecular recognition of flavonol by biomacromolecule is essentially controlled by static process, they formed a noncovalent protein-flavonol

conjugate, and the bioactive flavonol perches on the vicinity of β-37 Trp residue. In such a biomolecular recognition, the conformation of protein has undergone some variations at the same time.

One additional method to discriminate static and dynamic quenching is by cautious scrutinization of the UV/vis absorption spectra of the fluorophore, and ground-state complex formation will frequently result in perturbation of the UV/vis absorption spectrum of the fluorophore. We checked the UV/vis absorption spectra of globular protein in the presence of different concentrations of flavonol (data not shown). Ordinarily, the molecular recognition of a ligand with a biological macromolecule is processed through the ligand molecule target a specific region on such a biopolymer. It is conspicuous that no violent changes can be noticed for the typical absorption peak of hemoglobin at 405 nm accompanying the augment of flavonol concentrations. The strength of absorption peak might merely boost to some extent, and there is no newly characteristic absorption peak appeared simultaneously. These evidences suggest unmistakably that flavonol has chiefly reacted with the amino acid residues of polypeptide chain in protein via noncovalent interactions, while the active ligand has minimal impact on heme contained in the functional center of protein.[19,20] Or the binding cavity of flavonol on biomacromolecule is distantly related to heme, and this point would adequately be certified in the following molecular modeling context. Moreover, there has relatively great distinction of the deviations of UV/vis absorption spectra between the equimolar protein-flavonol and the flavonol and UV/vis absorption spectra of protein, illustrating the molecular

recognition of crude flavonol by biopolymer should be happened in the ground-state. This further testifies that the foregoing argumentations based upon steady-state and time-resolved fluorescence is logically legitimate.

Conformational/Structural Alterations. As noted earlier, the reversible biointeractions of active ligands to biological macromolecules is elementary to almost every aspect of biochemistry and cell biology. Binding events classically do not arise in isolation in biochemistry, and are nearly always coupled to other phenomena, e.g. folding/unfolding, protonation changes and structural transitions. A comprehension of biological recognition requests an estimate of affinity, thermodynamics, and particularly conformational/structural alterations, because the conformations/structures of biomacromolecules correlate closely with the biological functions.[21-23] Currently, circular dichroism (CD) is one of the most important techniques for the quantitative judgment of conformational variations of biopolymers. To inspect the conformational perturbances of model protein by flavonol, CD spectra of globular protein in the presence of different concentrations of flavonol were scanned in Fig. S1, and concurrently, the secondary structure components analyzed based upon original CD data are gathered in Table S2. Visibly, the CD curves of protein revealed two negative peaks in the far-UV CD region at 208 nm and 222 nm, characteristic trait of α-helical structure of globular protein.[24-26] One cogent explanation is that the negative peaks between 208 nm and 209 nm and 222 nm and 223 nm are dominated through both $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of amide groups and are also affected by the geometries of the polypeptide backbones.

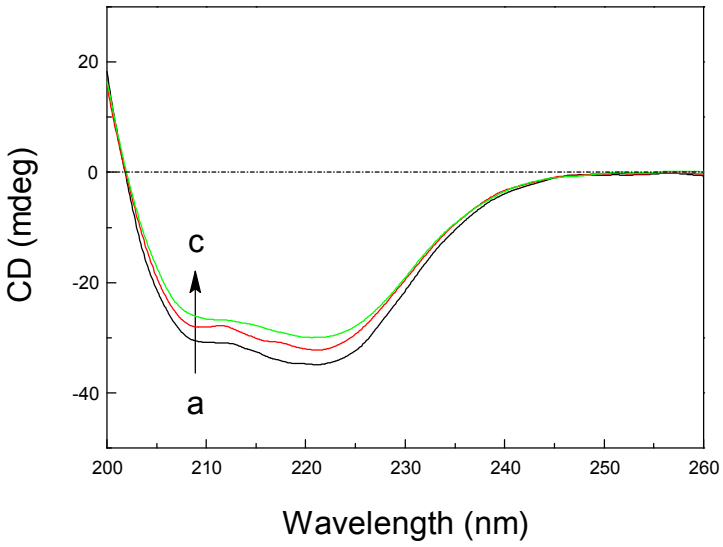

Fig. S1. Far-UV CD spectra of the protein-flavonol adduct at pH＝7.4 and T＝298 K, 5.0 μM protein in the presence of 0 (black), 10 (red) and 20 (green) μM flavonol.

Table S2
Secondary structure components of protein conjugates with flavonol at pH＝7.4 assessed by Jasco Spectra Manager II software

Samples	Secondary structure components (%)			
	α-helix	β-sheet	Turn	Random
Free hemoglobin	78.2	1.9	6.4	13.5
Hemoglobin＋morin (1：2)	71.3	3.4	8.7	16.6
Hemoglobin＋morin (1：4)	67.9	4.2	9.8	18.1

Table S2 exhibits that free protein embraces 78.2% α-helix, 1.9% β-sheet, 6.4% turn and 13.5% random coil; upon binding with flavonol, a reduction of α-helix was noted from 78.2% (free protein) to 67.9% (protein-flavonol), and furthermore, there was an ascension in β-sheet, turn and random coil from 1.9%, 6.4% and 13.5% (free protein) to 4.2%, 9.8% and 18.1% (protein-flavonol) at a molar ratio of protein to flavonol of 1：4. Surely, the decline of α-helix with an enhancement in the β-sheet, turn and random coil affirmed intelligibly that flavonol yielded noncovalent interactions with amino acid residues of the polypeptide chain in protein and enticing the destabilization of the ordered structure of globular protein, e.g. some degree of

protein structural extension after flavonol conjugation.[27,28] Nevertheless, the α-helical structure still occupies a predominant status among these secondary structure elements. As set forth, secondary structure alterations of globular protein evoked by flavonol have been sensitively detected via CD spectra in this attempt. In the spatial structure of protein, the peptide bonds are arranged in highly regularity, and the division situation of energy level transitions of the peptide bonds will be decided by the orientation of permutation. Consequently, both the band location and the absorption intensity of CD spectra produced by proteins or peptides with different secondary structures are often in disagreement. The measurement of the melting curve of protein by utilizing CD approach is also an efficient method for the exploration of conformational disturbances of biomacromolecule in the emergence of ligand. We tested the signal of CD spectra of model protein at wavelength 222 nm within the range of temperature from 298 K to 363 K, and the way proposed by Kapoor et al.[29] was hired to handle the experimental data. It may be perceived that the temperature of thermal denaturation of protein is 342.5 K; upon the addition of flavonol, the temperature of thermal denaturation of protein would be rose to 343.5 K. This fact indicates that the occurrence of flavonol can increase the thermal stability of biopolymer; in other words, the conjugation of flavonol with biomacromolecule is relatively tight, and the globular protein could not easily be denatured through the retroflexion of peptide bonds. Such verdict corresponds with the results of the former UV/vis absorption spectra, and we shall further unveil the conformational/structural changes of model protein exhaustively with the aid of computational chemistry in the

following context.

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