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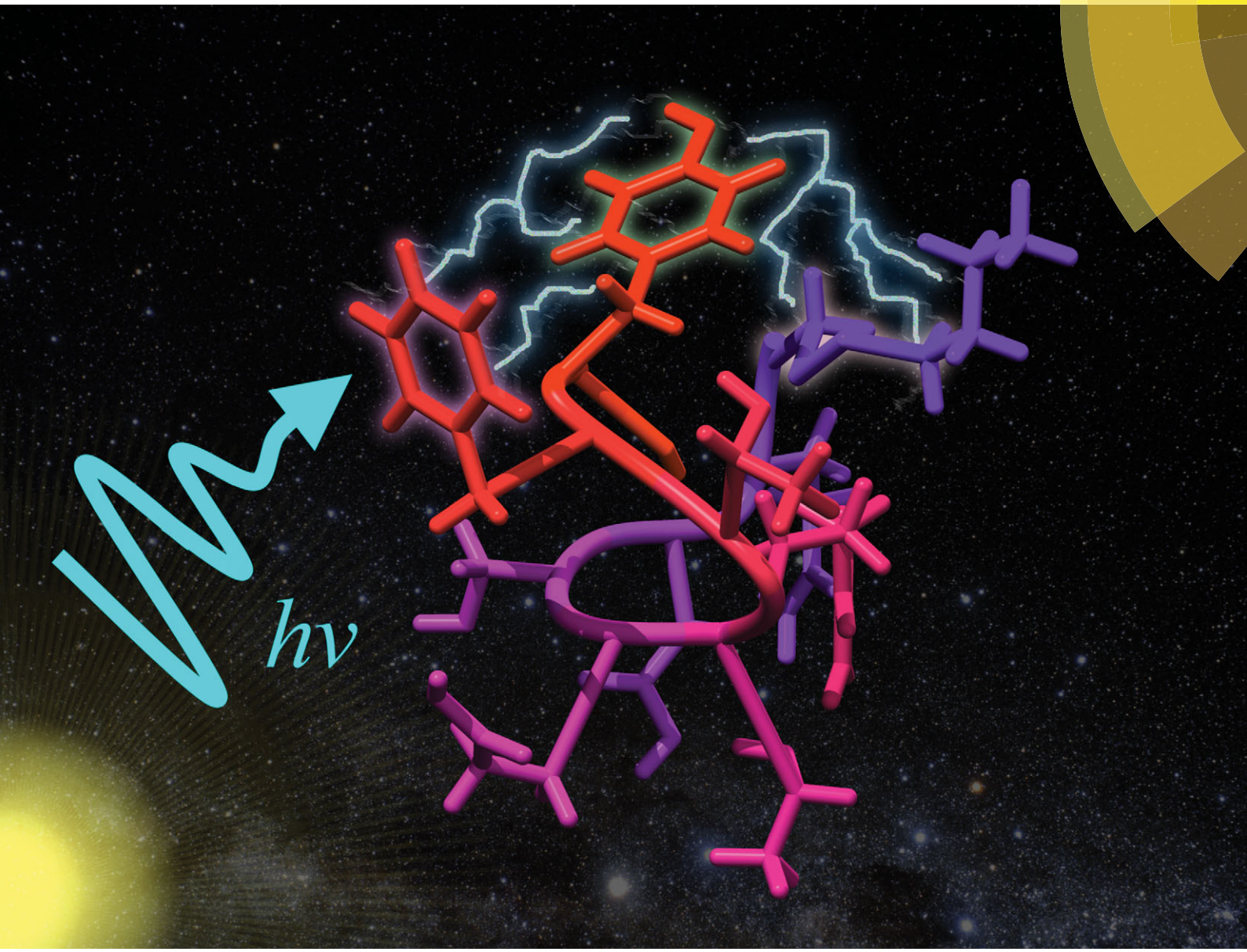

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Two-step energy transfer enables use of phenylalanine in action-EET for distance constraint determination in gaseous biomolecules†

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Two-step energy transfer is potentially useful for exploring macromolecular structure, but it has not been observed previously in the gas-phase. Single step excitation energy transfer (EET) has been recently documented for tyrosine and tryptophan containing peptides, but not for phenylalanine. Herein, we report sequential energy transfer from phenylalanine to tyrosine to a disulfide, resulting in homolytic cleavage of a sulfur–sulfur bond. Interestingly, energy transfer from phenylalanine is only observed in the presence of tyrosine and only occurs within certain distance constraints. Isolated, electronically excited phenylalanine is known to have an extremely long lifetime in the gas phase, potentially suggesting quicker relaxation occurs *via* energy transfer to tyrosine. Alternatively, the direct overlap of states between phenylalanine and disulfide bonds is predicted to be poor, in which case tyrosine would serve to bridge the gap. In either case, the distance constraints imposed by this two-step EET are shown to be useful for evaluation and determination of gaseous biomolecular structure.

Determination of the gas-phase structures of proteins and peptides is a sought-after goal in the field of mass spectrometry (MS).[1–15] One approach relies on energy transfer to reveal distance constraints that can be coupled with simulations to obtain structures. Recently, energy transfer between native residues[16] or between chemically appended groups[17] that results in a diagnostic bond cleavage have been reported, enabling facile distance constraint determination in MS based experiments. Energy transfer from native chromophores to disulfide bonds occurs with a strict distance dependence (less than ~6 Å for Tyr or ~15 Å for Trp). Combined with computational methods, this data can be used to accurately determine the gas-phase structures of proteins and peptides.[18]

Interestingly, our previous investigations did not reveal any energy transfer from phenylalanine, a weak chromophore compared to Tyr and Trp. Herein we present results where Phe is observed to transfer energy to Tyr, which then transfers energy sequentially to a disulfide bond *via* a two-step mechanism. Precedence for sequential energy transfer can be found in Forster Resonance Energy Transfer (FRET) literature. Two-step FRET, in which three or more chromophores participate in sequential energy transfer, has been previously characterized in solution[19–21] and successfully applied in a variety of impactful reports.[22–25]

Experiments in this study began by modifying a cysteine-containing peptide with propylmercaptan (PM) *via* disulfide bond formation. Excitation of a nearby chromophore causes homolytic cleavage of the disulfide and an easily detectable mass shift in the precursor ion. We call this experiment action-EET (excitation energy transfer, a collective term encompassing both FRET and Dexter exchange transfer) in analogy with action spectroscopy.[6] Experiments were conducted in a Thermo LTQ modified with a tuneable OPO laser to excite ions stored in the ion trap. Details of this setup have been described previously.[16] Photons excite the isolated peptide at varying wavelengths while loss of propylmercaptan radical is monitored. The data is compiled to yield an action-EET spectrum. Features of Tyr action spectra are well defined (see below) and make determination of Tyr–disulfide proximity straightforward. The fact that tyrosine must be within ~6 Å of a disulfide to participate in EET makes proximity determination unambiguous in even small peptides.

Fig. 1 (red trace) shows the action-EET spectrum obtained by monitoring loss of propylmercaptan from the peptide CQDSETRTFY, Collagen Binding Fragment (CBF), in the protonated 1+ charge state. The spectrum clearly shows enhanced absorption (relative to that of an isolated disulfide bond) in the 265–285 nm region which is consistent with the spectral features of Tyr (see Fig. 2 and 3, green traces for comparison). However, the two-peak absorption typical for isolated Tyr is dulled significantly. In addition, there is a new feature at 260 nm which has not been found in any previous spectra of Tyr containing peptides. Notably, CBF also contains Phe. To explore the

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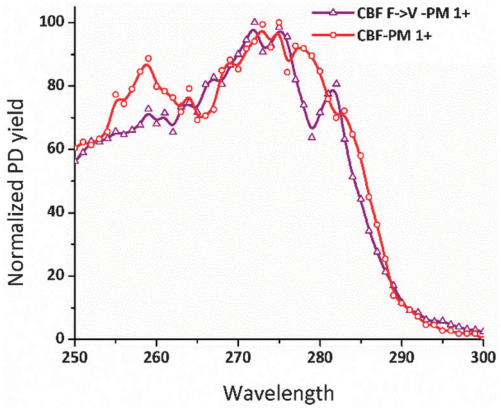

Fig. 1 Action-EET spectra of the CBF peptide (red trace) and a F9V mutant (purple trace) in the 1+ charge state normalized to the highest PD yield in each data set.

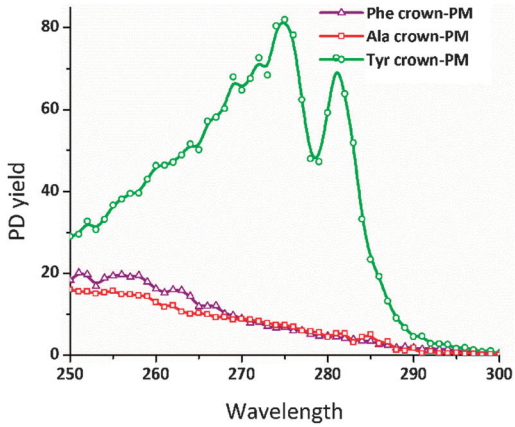

Fig. 2 Action-EET spectra of 18-crown-6-PM noncovalently attached to Ala and Phe.

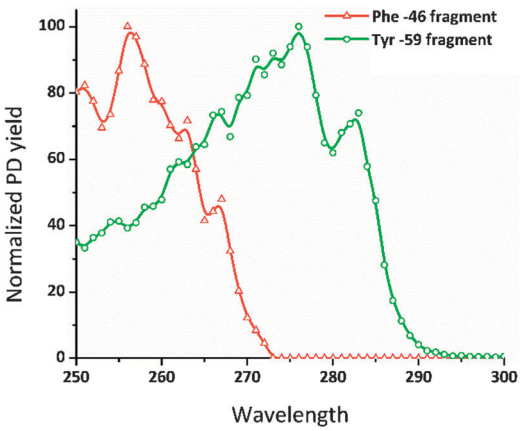

Fig. 3 Traditional action spectra of protonated phenylalanine (orange), and tyrosine (green) showing absorption features from 250–270 nm.

possibility that Phe was responsible for the feature at 260 nm, we synthesized a F9V mutant. The action-EET spectrum for the F9V mutant is shown in Fig. 1 (purple trace). The absorption feature at 260 nm is gone, suggesting that Phe is the originating chromophore for this feature. In addition, the typical two-peak absorption for Tyr is more distinct, suggesting interaction between the chromophores. Examination of the same peptide in the 2+ charge state reveals similar trends to those observed for the 1+ charge state, including a new feature at ~260 nm that disappears in the F9V mutant (see Fig. S1, ESI†).

To explore the single-step EET capabilities of Phe, we compare results for several isolated amino acids. Shown in Fig. 2 are EET spectra for Tyr, Ala, and Phe. Each amino acid was noncovalently attached to a disulfide containing 18-crown-6 based molecule (crown-PM, see ESI† for structure) that places a disulfide bond within close proximity (~4.8 Å) to the side chain. The Tyr spectrum (green trace) exhibits typical features previously observed for Tyr containing peptides. In comparison, the Phe spectrum (purple trace) is featureless and has no obvious peak at ~260 nm, suggesting very minimal energy transfer, if any, occurs. To confirm that there is a lack of significant EET in the Phe spectrum, results obtained with alanine are shown as well. The Phe and Ala spectra are very similar to each other and resemble the absorption spectrum for a disulfide bond, which is weak and featureless in this region.[16]

In order to determine whether Phe absorbs at 260 nm, we obtained a traditional action spectrum for the amino acid (see Fig. 3) Absorption is most abundant at ~256 nm and rapidly declines to zero around 270 nm. This spectrum is consistent with previous spectra obtained in the gas phase.[26] There is a slight shift toward longer wavelength from the absorption maximum to the EET maximum (*i.e.* 256 nm → 260 nm) for Phe. This trend is consistent with the EET behavior of Tyr and Trp. This shift in wavelength may result from stabilization of the excited state due to weak intra-molecular solvation of the chromophores. Solvation effects have been observed to lead to a bathochromic shift in solution when comparing different solvents.[27] This would also account for why there is a small amount of variability in the shift observed for different peptides (shifts are between 2–4 nm).

Taken together, the results in Fig. 1–3 indicate that Phe is capable of EET, but only in the presence Tyr. We hypothesize that the mechanism involves two step energy transfer, *i.e.* from Phe → Tyr → disulfide. Energy transfer from Phe to Tyr is feasible based on absorption/emission spectra. In the gas phase, Phe emits at 281 nm, which overlaps well with the region where Tyr absorbs (see Fig. 3, green trace).[28] Subsequently, energy transfer from Tyr to the disulfide is well-documented.[16]

Why doesn't Phe transfer energy in a one-step process? Recently Zabuga *et al.* reported that Phe has an exceptionally long-lived excited state in the gas phase that arises from a rapid transition to the triplet.[29] Notably, Phe is reported to remain in the excited state in excess of 100 ms whereas Tyr relaxes ~10 times faster. The long lifetime of Phe may allow it to be quenched by collisions with trace molecules in the ion trap. This would explain why Phe is normally dark in our experiments. However, the presence of a nearby Tyr residue may shorten the effective lifetime of Phe by facilitating energy transfer. There is evidence that a shortening of Phe lifetime occurs in the presence of Tyr in solution.[30]

Another explanation is that Phe emission overlaps poorly with the dissociative transition of the disulfide bond (which is predicted by theory to occur at ~342 nm, see ESI† for details). Previous measurements in solution suggest that Phe emission has little or no overlap with 342 nm,[31] and only a minor shift in emission is expected in the gas phase. The lack of overlapping states may prevent EET in the absence of Tyr, which serves to bridge the gap.

Given the consistent trends that exist between all known photochemical properties of Trp, Tyr, and Phe, EET from Phe will likely occur over distances shorter than those observed for Tyr (*i.e.* 6 Å, since the distance dependence for Trp is longer, the trend should yield a shorter distance for Phe relative to Tyr). Based on these considerations, a conservative estimate for the EET cutoff of Phe is $< \sim 6$ Å. We tested this value by examining the peptide YF in a complex with crown-PM. In this experiment significant Phe EET is not observed (see Fig. S2, ESI†). The Phe–Tyr separation cannot exceed ~8 Å for this dipeptide (based on inherent constraints due to allowable bond lengths and rotations). The lowest energy structure for this complex suggests the separation of Tyr and Phe is 7.7 Å (Fig. S3, ESI†). Lack of substantive EET for YF is hence consistent with an energy cut-off inside ~6 Å.

Additionally we investigated the CD36 139–155 peptide (CNLAVAAASHIYQNQFVQ) in the 2+ charge state. The action-EET spectrum showed no presence of Phe features (see Fig. S4, ESI†) but the lowest energy structure for the peptide (Fig. S5, ESI†), which was consistent with Tyr energy transfer, had a Phe-to-Tyr distance of 8.0 Å, further supporting a small distance for Phe energy transfer.

Considering the distance limitations of EET for each donor–acceptor system, the data in Fig. 1 can be interpreted in relation to the structure of the peptide. As demonstrated previously, the Tyr absorption near ~275 nm implies that the disulfide bond is within ~6 Å of the Tyr side chain. Similarly, the feature at ~260 nm suggests the Phe and Tyr side chains are likely within ~6 Å. At room temperature the peptide can be expected to readily undergo sidechain rotations and some degree of conformational flexibility. Initial exploration of the protonated peptide structure (charged at arginine) by simulated annealing did not yield a low energy structure matching these criteria, so distance constraints were used to guide the calculations towards relevant conformational space (computational experiments are described in greater detail in the ESI†). After re-minimization in the absence of distance constraints, the low energy structure shown in Fig. 4 was obtained. This structure is 53 kJ mol^{-1} higher in energy than the global minimum when evaluated by the OPLS 2005 force field. PM6 Semi-empirical calculations yield an energetic difference of only 6 kJ mol^{-1} for the re-optimized structure. From these calculations it is evident that theory predicts multiple structures that are energetically equivalent. Hence the experimental distance constraints obtained through action-EET are highly valuable for identifying the correct conformation. The same simulated annealing approach was used to generate a structure for the 2+ charge state of the peptide (charges on N-terminus, arginine, Fig. 5). In this case, the lowest energy structure is consistent with the distances expected from the action-EET data, with Phe–Tyr and Tyr–Cys distances under ~6 Å.

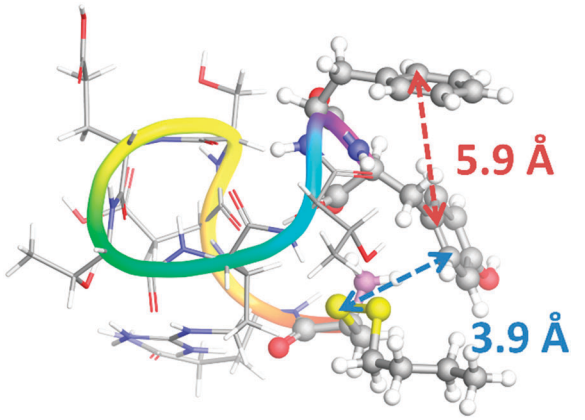

Fig. 4 Lowest energy structure of the CBF 1+ peptide consistent with experimental data. Distances shown are Tyr–disulfide (blue) and Phe–Tyr (red).

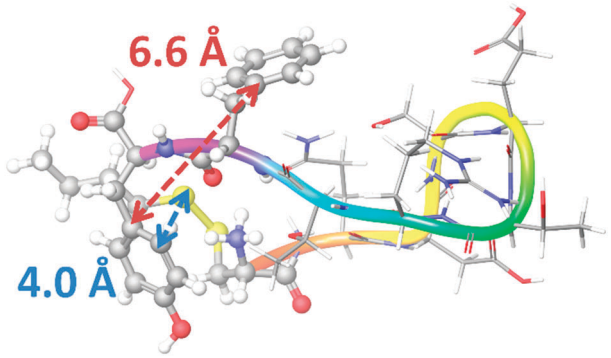

Fig. 5 Lowest energy structure of the CBF 2+ peptide. Distances shown are Tyr–disulfide (blue) and Phe–Tyr (red).

In conclusion, two-step energy transfer is demonstrated in the gas phase for the first time. Although Phe is not useful in single-step action EET experiments, it can be employed in two-step action EET in combination with Tyr. It is likely that other two-step energy partners will be discovered in the future. Furthermore, two-step EET experiments simultaneously reveal two distance constraints that can be used in conjunction with calculations to accurately define peptide structure. These experiments will help guide efforts to delineate the role of mass spectrometry in protein structure determination.

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