**SUPPORTING INFORMATION**

# N-formyl kynurenine as a marker of high light stress in photosynthesis

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Abbreviations: 2D, two dimensional; B5A, 5-(biotinamido)-pentylamine; CN-PAGE, clear native polyacrylamide electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; MS/MS, tandem mass spectrometry; NFK, *N*-formylkynurenine; PSII, photosystem II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; TFA, trifluoroacetic acid; Tris, tris (hydroxymethyl)aminomethane; TW PSII, Tris washed PSII; UVRR, ultraviolet resonance Raman

## EXPERIMENTAL PROCEDURES

*PSII Derivatization with B5A*: TW PSII membranes (~6 mg of chlorophyll) were derivatized by incubation (Fig. S1, step 2) with 4 mM of a primary amine-biotin conjugate, B5A (Invitrogen, Carlsbad, CA) (Fig. 1B). The chlorophyll concentration was 1 mg/mL, and the incubation was performed for 30 minutes at room temperature in the light (1). As a control, TW PSII samples were also incubated in parallel under identical conditions, except B5A was omitted from the sample solution. B5A binding to PSII was confirmed by Western blot (2) of a urea-SDS-PAGE gel (3) containing derivatized PSII and by detection with an avidin-alkaline phosphatase conjugate (2). B5A-derivatized samples were subjected either to direct *in-situ* trypsin digestion (Fig. S1, step 4B) or 2D electrophoresis, followed by in-gel digestion (Fig. S1, steps 3 and 4C).

*In-situ Trypsin Digestion of PSII*: The samples employed were either intact PSII (Fig. 2), TW PSII (Fig. S1, step 4A), or B5A labeled TW PSII (Fig. S1, step 4B). Labeled TW PSII samples were centrifuged at 108,800 x g for 20 minutes to remove non-covalently bound B5A. The unlabeled TW control was treated similarly. PSII samples were reconstituted to 3 mg/mL in 50 mM HEPES-NaOH, pH 7.5 buffer. For the *in-situ* trypsin digest, 1 µg of porcine trypsin (Sequencing grade, Promega, Madison, WI) in 25 mM ammonium biocarbonate was added per 0.15 µg chloropyll. Samples were incubated for ~20 hours at room temperature under constant agitation. Undigested protein was pelleted by centrifugation at 108,800 x g for 20 minutes. The tryptic peptides released into the supernatant were then lyophilized and stored at -20 ºC. Tryptic cleavage of peptides was confirmed by SDS-PAGE comparison of intact and digested PSII samples (3,4).

*HPLC Purification of NFK-Containing Peptides*: Tryptic peptides were separated by reverse phase chromatography (Fig. S1, steps 5A and B) on a Beckman (Brea, CA) Gold HPLC system equipped with a 125 solvent module, a 168 photodiode array detector (1 cm path length, 2 nm scan interval), and 32 Karat Software, version 7.0. Lyophilized samples were reconstituted in 50 µL of 5% acetonitrile/ 0.1% trifluoroacetic acid (TFA). The samples were filtered with an Acrodisc (Pall, Ann Arbor, MI) 0.2 µm nylon filter and loaded onto an Alltech (Deerfield, IL) Prosphere C18 column (4.6 mm x 250 mm, 300 Å pore size, 5 µm diameter packing). Buffer A was $H_2O$/ 0.1% TFA, and buffer B was acetonitrile/ 0.1% TFA. Peptides were eluted from the column over a 60 minute period with a gradient of 10-60% B. The elution profile was monitored at 350 nm. Column chromatography was performed at room temperature and at a flow rate of 1 mL/ minute. Where appropriate, 1 mL fractions with absorption peaks at 350 nm (Figs. 2 and S2) were lyophilized and stored at -20 ºC. Injection of a trypsin blank (no PSII) gave no significant 350 nm peaks (data not shown).

*2D Electrophoresis and In-Gel Digestion of PSII Proteins*: To ensure that the red-shifted absorption spectra derive from a peptide-derived chromophore, Coomassie dye and bromophenol blue were omitted from the gel purification experiments. B5A-derivatized, TW PSII membranes (~13 mg chlorophyll) were solubilized and separated in the first dimension as previously described (5). These CN-PAGE gels were run like Blue Native gels (6), without Coomassie dye in the buffers. The PSII dimer band deficient in light-harvesting complexes (5) was excised and run in the second dimension by Tricine-SDS-PAGE (Fig. S1, step 3) (7). Bands were visualized with a zinc-imidazole negative stain (8). Protein bands were excised from the second dimension

gels and cut into one $mm^3$ cubes. Residual zinc was removed by incubation with 50 mM EDTA for five minutes. A tryptic digest was generated (Fig. S1, step 4C) with 2.5 µg/ mL of porcine trypsin (Promega, Madison, WI), as described by Rexroth *et al*. (9). Once extracted from the gel, samples were dried completely by lyophilization and either stored at -20 °C or loaded directly onto an avidin affinity column.

*Avidin Affinity Chromatography*: For selective purification of biotinylated peptides (B5A-derivatized), lyophilized peptide samples from *in-situ* or in-gel digests were reconstituted in 100 µL binding buffer (50 mM NaCl, 150 mM HEPES, pH 7.0) and were loaded onto monomeric avidin resin spin columns (10) (Fig. S1, steps 6A & B). Peptides were allowed to bind overnight at room temperature and eluted with a 50% acetonitrile/ 0.1% TFA solution (10). An ELISA-like test (11) was used to quantify eluted peptides by comparison to a biotinylated insulin standard in concentrations ranging from 10 µM to 1 pM. Affinity purified samples were lyophilized and stored at -20 °C.

*Chromatograms of Tryptic Peptides from TW PSII.* Fig. S2 shows a 350 nm chromatogram derived from tryptic digestion of TW PSII. In (B), TW PSII was B5A labeled; in (A), TW PSII was unlabeled. The chromatograms are indistinguishable.

*UVRR of Amino acids, Chlorophyll a, and β-Carotene:* UVRR measurements were conducted by methods previously described (12,13). Spectra of the amino acids (Sigma-Aldrich, St. Louis, MO), histidine (20 mM), phenylalanine (20 mM), tyrosine (1 mM), and tryptophan (1 mM), were conducted in 5 mM HEPES buffer, pH 7.5. The UVRR spectra were collected with a 250 µW, 229 nm beam excitation and a 21 minute exposure time (Fig. S3). Chlorophyll *a* (Sigma-Aldrich) was dissolved in absolute ethanol at a concentration of 0.5 mg/mL. β-carotene (Sigma-Aldrich) was dissolved in a 80:20 acetone:water mixture at a concentration of 1 mg/mL. The UVRR spectra were collected with a 360 µW, 325 nm laser beam for a 30 minute exposure time (Fig. S4).

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**FIGURE LEGENDS**

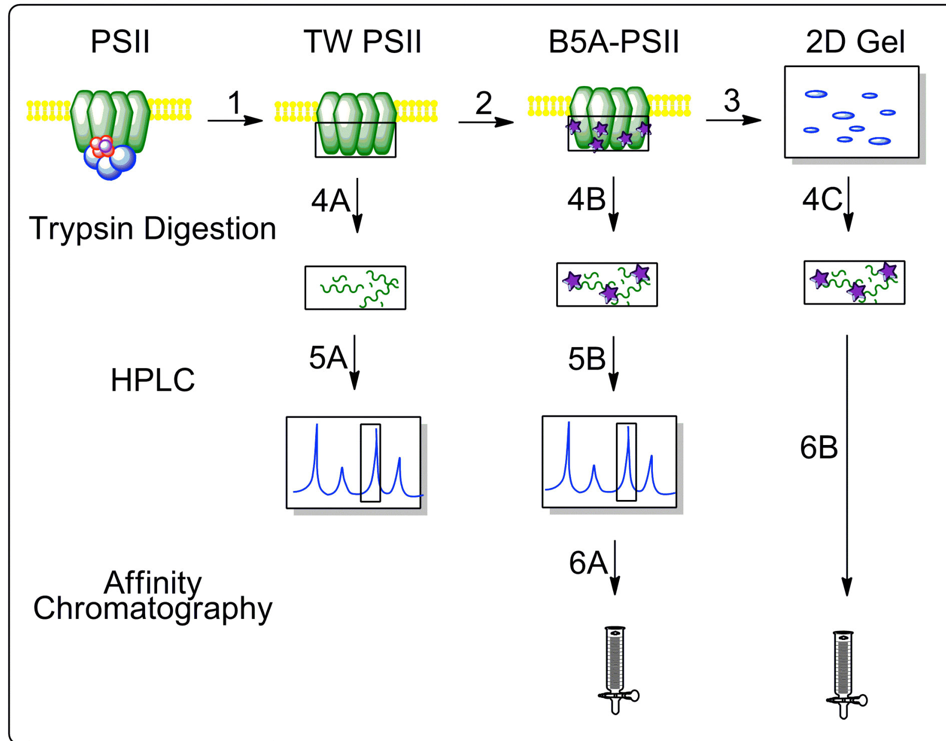
**Figure S1.** Overview of peptide isolation and analysis. ***Peptide isolation by in-situ digestion****:* In *step* 1, intact PSII was Tris-washed (TW) to remove the extrinsic subunits and the $Mn_4Ca$ cluster. The TW PSII was either directly trypsin digested *in-situ* (step 4A) or derivatized with B5A (step 2), and then subjected to digestion (step 4B). Both sets of peptides were separated by HPLC (steps 5A and B). For some experiments, HPLC purified peptides were subjected to avidin affinity chromatography (step 6A). ***Peptide isolation by 2D gel electrophoresis****:* B5A-derivatized peptides were electrophoresized in two dimensions (step 3), in-gel digested, and then extracted from the gel (step 4C). For some experiments, the extracted peptides were subjected to avidin affinity chromatography (step 6B). ***Analysis****:* HPLC-purified peptides were characterized by their optical UV absorption. HPLC and affinity purified peptides were analyzed by MS/MS and UVRR spectroscopy.

**Figure S2.** HPLC separation of peptides derived from TW PSII by *in situ* trypsin digestion. The chromatograms were monitored at 350 nm and show three fractions with the retention times noted. In (A), TW PSII was unlabeled; in (B), TW PSII was B5A-labeled. Chromatograms were displaced by an arbitrary amount on the y axis for comparison. Each y-axis tick mark represents 120 milli-absorbance units.

**Figure S3.** UVRR spectra of amino acids. Spectra of histidine (A), tryptophan (B), tyrosine (C), and phenylalanine (D) in HEPES, pH 7.5 were recorded with a 250 μW, 229 nm probe. The total exposure time was 21 minutes. The spectra were displaced by an arbitrary amount on the y-axis for comparison. Each y-axis tick mark represents 20,000 arbitrary intensity units (A.U.).

**Figure S4.** UVRR of chlorophyll *a* and β-carotene. Spectra of chlorophyll *a* (A) in ethanol (B) and β-carotene (C) in an 80:20 acetone-water mixture (D) were recorded with a 360 μW, 325 nm laser probe. The total exposure time was 30 minutes. The spectra were displaced by an arbitrary amount on the y-axis for comparison. Each y-axis tick mark represents 400,000 arbitrary intensity units (A.U.).

**Figure S1**



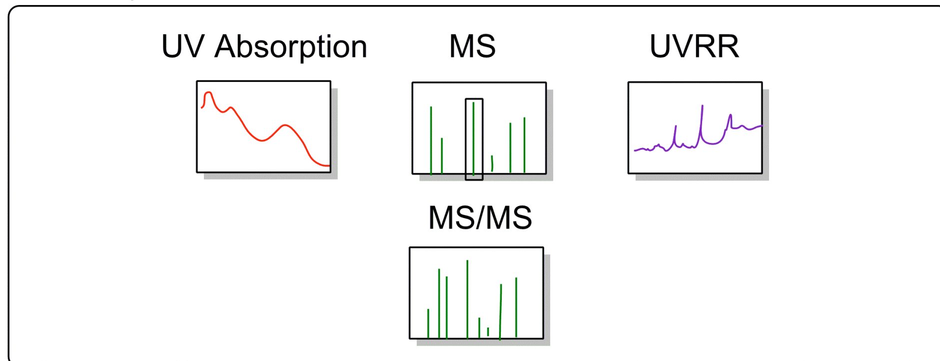



**Figure S2**

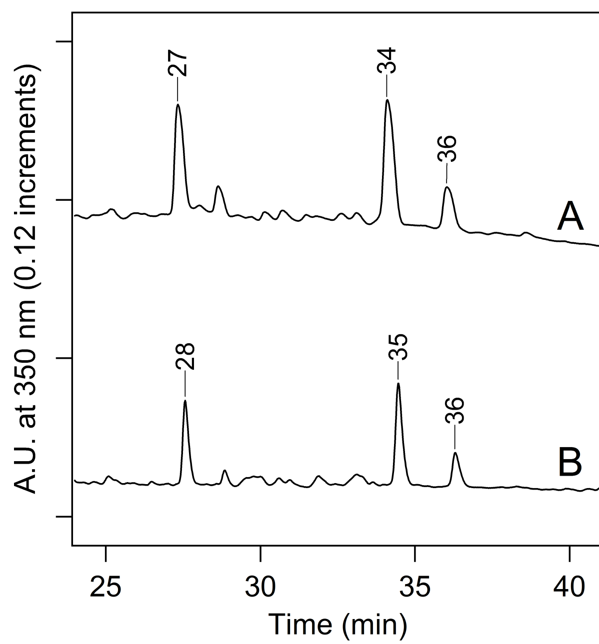


**Figure S3**

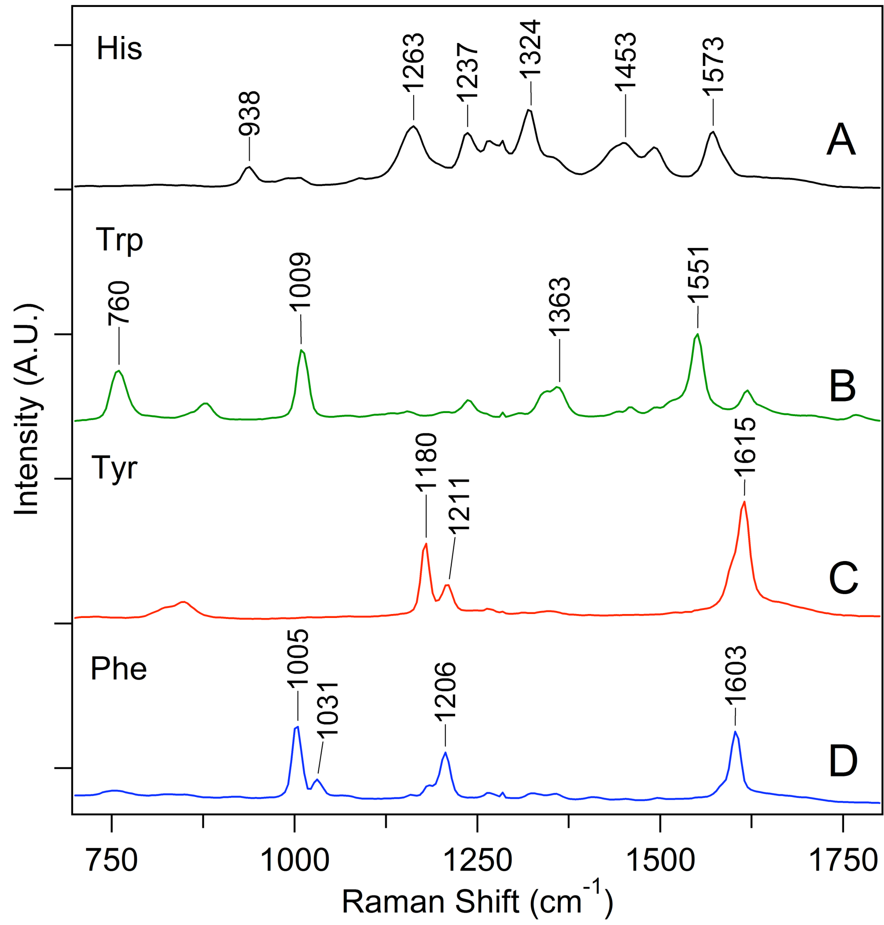


**Figure S4**