

Identifying and Quantitating FAD and FMN in Simple and in Iron-Sulfur-Containing Flavoproteins

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1. Introduction

The number of known flavin-containing proteins is steadily increasing thanks to the combination of several factors. Among them the following may be of particular interest: (1) increasing power of separation techniques and of molecular biology tools for overproduction of various proteins, and (2) recognition of putative flavin-dependent proteins through analysis of amino acid sequences deduced from those of (putative) genes discovered through genome-sequencing projects. Particularly interesting is the fact that novel flavin-dependent proteins, which play roles different from electron transport or redox catalysis are being discovered (e.g., gene transcription regulation as in the case of NifL, (1)). On the other hand, the picture can be complicated by the fact that the same protein may harbor one or more flavin nucleotides plus one or more additional cofactors (2). Among such nonflavin centers iron-sulfur clusters are common.

Essential steps for the characterization of flavin-containing enzymes are, (1) the recognition of the presence of a flavin cofactor; (2) the identification of the flavin cofactor(s), and (3) the determination of the stoichiometry of the bound flavin cofactor(s). Several methods are available in the literature in order to achieve such goals, and each flavinologist is indeed familiar with one or more of them (3–6). We present here three different procedures for both the identification and the quantitation of protein-bound flavin cofactors: a fluorimetric method, a spectrophotometric method, and a high-performance liquid chromatography (HPLC)-based method. These procedures, in our experience, yield reliable qualitative and quantitative information, while requiring limited

amounts of material, minimum sample preparation, and common laboratory equipment.

2. Materials

1. Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) used to construct standard curves are from Sigma Chemical Co. (St. Louis, MO). Stock solutions (5 mM) are prepared in MilliQ (Millipore, Bedford, MA) water and stored at -20°C . Individual batches of flavin nucleotides should be checked for the presence of interfering impurities. If required, FAD and FMN can be purified by the HPLC method described in this chapter. The concentrations of FAD and FMN solutions are determined spectrophotometrically, by recording the absorbance spectrum of 25–100 μM solutions in buffer at pH 7–8. The following extinction coefficients are used:

Flavin	λ_{max}	$\epsilon(\text{M}^{-1}\text{cm}^{-1})$	Reference
FAD	450	11,300	7
FMN	446	12,200	8
FAD or FMN	473	9200	This work

2. Sodium dodecyl sulfate (SDS) is from Sigma. A 10% (w/v) solution is prepared in MilliQ water and stored at room temperature. Snake venom phosphodiesterase (PDE) (3 mU/ μL) is from Boehringer-Mannheim (Mannheim, Germany), and was stored at 5°C .
3. Enzymes used to test the methods presented are prepared in our laboratories according to standard procedures.
 - a. The G298A mutant of glutamate synthase (GltS) β subunit is prepared with a modification of the protocol used to prepare the wild-type recombinant GltS β subunit (P. Morandi, B. Valzasina, and M. A. Vanoni, unpublished data, *see ref. 9*), and is stored in 25 mM HEPES/KOH buffer, pH 7.5, 10% glycerol at -80°C .
 - b. The recombinant GltS α subunit is prepared as described in (10) and stored in 25 mM PIPES/KOH buffer, pH 7.5, 10% glycerol, 0.1 mM dithiothreitol (DTT).
 - c. The recombinant GltS holoenzyme is prepared using a procedure similar to that used for the preparation of GltS from *Azospirillum* cells (H. Stabile, and M. A. Vanoni, unpublished data, *see ref. 11*). It is stored in 25 mM HEPES/KOH, pH 7.5, 10% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM 2-oxoglutarate and 1 mM DTT.
 - d. Recombinant spinach leaf ferredoxin-nicotinamide-adenine-dinucleotide phosphate (NADP)⁺-reductase (FNR) is prepared as described in (12).
 - e. Maize root FNR is produced by heterologous expression in *E. coli* using a modified form of the expression plasmid described in (13), and purified using a procedure similar to that used for the spinach FNR, replacing anion-exchange with hydrophobic-interaction chromatography on Phenyl-Sepharose (Pharmacia, Uppsala, Sweden) (A. Aliverti et al., manuscript in preparation). Fd/FNR chimeric protein was purified as described (14).

4. Protein concentration is determined using the method of Bradford (**15**), the Amresco Protein Assay Reagent (Amresco, Inc., Solon, OH), and bovine serum albumin as the standard protein. One to 3 μg protein are used for each assay.
5. 10 mM HEPES/NaOH, pH 7.5.
6. 10 mM Tris/HCl, pH 7.5–8 (at 25°C).
7. 10 mM HEPES/KOH, pH 7.5, or 50 mM Tris-HCl, pH 7.6 (at 25°C). All buffers (items 5–7) are freshly made in MilliQ water (Millipore) and filtered through 0.2 μm sterile filters.
8. Protein samples are used directly for analyses or after gel filtration through Sephadex G25 (medium) columns. PD10, prepacked disposable columns from Pharmacia are used. All samples are either centrifuged in a microfuge in the cold (top speed, 10 min) or filtered through 0.45 μm filter cartridges or 0.1 μm centrifugal filters (Ultrafree-MC filter units, Millipore) to remove even faint turbidity.
9. Emission spectra are recorded with a Jasco FP-777 spectrophotofluorimeter (Jasco, Inc., Easton, MD) at 20°C.

Fluorimeter settings are as follows:

λ_{ex} :	450 nm	Excitation slit	5 nm
λ_{em} :	480–600 nm	Emission slit	5 nm
Gain:	High		
Scan speed	100 nm/min		
Response:	1 sec	Data interval	0.5 nm

Instrument zeroing conditions:

Emission shutter: closed	λ_{ex} : 450 nm	λ_{em} : 480–600 nm
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10. Absorbance spectra are recorded with Hewlett-Packard 8453 diode-array (Hewlett-Packard, Palo Alto, CA), Cary 219 (Varian, Palo Alto, CA) or Uvikon 810 (Kontron Elektronik, Eching, Germany) spectrophotometers at 20 or 25°C, with identical results.
11. HPLC analyses are carried out using a $\mu\text{Bondapack C-18}$ silica column (Waters Associates, Milford, MA) connected to a Waters 600E HPLC system equipped with a UK6 manual injector and controlled through the Millenium software (Waters) run on a PC. A 2-mL sample loop is used, and the column is not thermostated. Eluate absorbance is monitored continuously using an on-line 486 detector (Waters) set at 264 nm. Solvents are 5 mM ammonium acetate, pH 6.5, and methanol (HPLC grade). Both solvents are filtered through 0.22 μm filters and degassed by continuous helium bubbling.

3. Methods

3.1. Fluorimetric Identification of the Flavin Cofactor as FAD or FMN

The method of Forti and Sturani (**16**) is used. It exploits the fact that fluorescence of a FMN solution is 10-fold higher than that of an FAD solution of the same concentration, and that phosphodiesterase (PDE) catalyses the conver-

sion of FAD into FMN and adenosine monophosphate (AMP). Thus, the fluorescence intensity of a flavin-containing solution is measured before and after addition of PDE. No increase of fluorescence is interpreted as due to absence of FAD in the solution; a 10-fold increase of fluorescence allows the identification of the flavin present as FAD, an intermediate increase of fluorescence allows the calculation of the relative concentration of FAD and FMN in the starting solution. Several modifications of the following method are possible, depending on the quantity of enzyme and on the equipment available. This method can also be employed to test the purity of the FAD stock solution used.

3.1.1. Calibration and Linearity Test of the Assay

It is important to determine the range of linearity of the instrument by measuring the emission spectrum of solutions containing increasing concentrations of FAD or FMN in the same buffer, in the same cuvet, and under the same settings that will be used for the actual experiment.

1. A 0.25–0.3 mM solution of FAD or FMN is prepared in water, or in the buffer that will be used for the enzyme solution. Its concentration is determined by measuring the absorbance spectrum (*see Subheading 2.1.*).
2. One microliter aliquots are added to a cuvet containing 2 mL buffer. Alternatively, a 200 μ L cuvet can be used (*see Note 1*).
3. After mixing, emission spectra are recorded.
4. The emission intensity at 524 nm, corrected for buffer emission value and dilution (F_{524}), is plotted as a function of flavin concentration.
5. The initial linear part of the curve sets the interval of fluorescence values that will yield reliable results. From the slope of the line $F_{524} = f_{\text{FAD}}[\text{FAD}]$ or $F_{524} = f_{\text{FMN}}[\text{FMN}]$, the values of intensity of light emitted at 524 nm by a 1 μ M solution of FAD (f_{FAD}) or FMN (f_{FMN}) are also calculated.

3.1.2. Fluorimetric Analysis of the Cofactor(s) Released From the Flavoprotein Solution

1. A 5–10 μ M solution of enzyme is prepared by gel filtration through a Sephadex G25 (Pharmacia) column equilibrated with either 10 mM HEPES/NaOH buffer, pH 7.5 or 10 mM Tris/HCl buffer, pH 7.5.
2. The absorbance spectrum is recorded, and the protein and activity content of the sample is measured.
3. The emission spectrum of 2 mL buffer is measured in a 3 mL glass cuvet to be used as a blank in subsequent measurements.
4. A 100 μ L aliquot of the enzyme solution is added to the fluorimeter cuvet.
5. After mixing, the emission spectrum of the solution is recorded.
6. The sample is recovered with a Pasteur pipet, transferred to microfuge tubes, wrapped in aluminum foil, and incubated for 10 min at 100°C.

7. After being cooled on ice, microfuge tubes are centrifuged at 4°C for 10 min at 13,000 rpm (14,500g).
8. The supernatant is recovered and transferred directly into the fluorimeter cuvet.
9. The emission spectrum of this solution is measured as before.
10. 2 μ L (6 mU) of the PDE solution are added.
11. The emission spectrum of the solution is recorded at different times after mixing, until no further changes are observed.
12. Both the emission and excitation shutter of the instrument are closed between measurements to avoid photodegradation of the flavin sample. Within 5 min the maximum increase of emission at 524 nm is obtained. After 20–30 min, emission tends to decrease (*see Note 2*).
13. Depending on the results obtained, measurements are repeated using (a) a different sample dilution (e.g., 5- or 50-fold dilution), (b) a different incubation time at 100°C (5, 15, or 20 min), and (c) a different sample denaturation method (*see Subheading 3.1.3.*). If limited by the amount of sample available, 200 μ L cuvetts may be used.

3.1.3. Alternative Protein Denaturation Methods

1. The protein stock solution (5–10 μ M) can be denatured by incubation at 100°C for 10 min in the dark, and denatured protein can be removed by centrifugation at 13,000 rpm (14,500g) for 10 min in a microfuge in the cold. The supernatant is transferred to a clean microfuge tube, kept on ice in the dark and aliquots can be diluted 10–50-fold for fluorimetric analyses as described in 3.1.2.
2. The protein stock solution (5–10 μ M) can be denatured by incubation at room temperature in the presence of 0.2% SDS (*see Subheading 3.2.1.*). Aliquots of such sample can be diluted 10–20-fold for fluorescence analyses described above. Although 0.2% SDS does not allow PDE-catalyzed conversion of FAD into FMN (*see Table 1*), we observed that experiments carried out in the presence of 0.02% SDS gave results essentially indistinguishable from those obtained in the absence of SDS.

3.1.4. Fluorimetric Data Analysis

As already reported, if the protein contains only FMN, no fluorescence change is observed upon PDE treatment. Conversely, if the protein contains only FAD a 10-fold fluorescence increase is expected. For proteins containing both flavin cofactors, the molar ratio (r) between FAD and FMN can be calculated from the fluorescence increase after PDE treatment using Eq. 4, which is based on Eqs. 1–3.

$$F_o = f_{\text{FAD}} \cdot [\text{FAD}] + f_{\text{FMN}}[\text{FMN}] \quad (1)$$

$$F_{\text{fin}} = f_{\text{FMN}} ([\text{FMN}] + [\text{FAD}]) \quad (2)$$

$$f_{\text{FAD}} = 0.1f_{\text{FMN}} \quad (3)$$

$$r = (10 \times (F_{\text{fin}}/F_o) - 10) / (10 - (F_{\text{fin}}/F_o)) \quad (4)$$

Table 1
Fluorimetric Identification of FAD and FMN in GltS Holoenzyme and in Its Isolated Subunits

Sample	Concentration ^a (μM)	Buffer	Pretreatment	Denaturation method	F _o	F _{fin}	F _{fin} /F _o	Notes
FAD	0.60	A + 0.1 mM MgSO ₄	None	None	20.63	206.3	10	Chromatographically purified FAD
FAD	0.26	A + 0.1 mM MgSO ₄	None	None	39.06	202	5.17	Commercial FAD
GltS β^b	0.781	B + 0.1 mM MgSO ₄	Gel filtration	10 min, 100°C	56.6	611.8	10.81	
GltS β^b	0.443	A + 0.1 mM MgSO ₄	Gel filtration	10 min, 100°C	37.6	399.1	10.6	
GltS β^b	0.762	A	Gel filtration	0.2% SDS to 7.62 μM β subunit stock solution	80.3	777.8	9.72	SDS concentration in fluorimeter cuvet was 0.024%
GltS β^b	0.381	A	Gel filtration	0.2% SDS to 7.62 μM β subunit stock solution	40.22	374.4	9.3	SDS concentration in fluorimeter cuvet was 0.012%
GltS β^b	0.381	A + 0.1 mM MgSO ₄	Gel filtration	0.2% SDS to 7.62 μM β subunit stock solution	40.84	410.5	10.5	SDS concentration in fluorimeter cuvet was 0.012%
G298A-GltS β^c	9.56	A	Gel filtration	0.2% SDS	32.7	69.94	—	PDE activity inhibited by 0.2% SDS

G298A-GltS β^b	0.212	A + 0.1 mM MgSO ₄	Gel filtration	10 min, 100°C	21.35	169	7.916	The enzyme was not shielded from light during heat denatura
G298A-GltS β^c	0.429	A + 0.1 mM MgSO ₄	Gel filtration	10 min, 100°C	32.26	316	9.79	The sample was shielded from light during heat denatura
GltS α^c	0.46	A	Gel filtration	10 min, 100°C	93.3 ^d	91.5 ^d	0.98	
rGltS ^{c,e}	4.81	C	None	10 min, 100°C	356.3	645.2	1.81	
rGltS ^{c,e}	0.534	C	None	10 min, 100°C	39.65	82.47	2.07	same sample as above, diluted 9-fold

^aProtein concentration was determined by the method of Bradford (15).

^bIn 3 mL cuvet containing 2220 μ L sample.

^cIn 200 μ L cuvet with 200 μ L sample volume.

^dExcitation light was at 440 nm.

^eIn 25 mM Hepes/KOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM 2-oxoglutarate, 10% glycerol,
Buffers were A, 10 mM Tris/HCl, pH 7.5; B, 10 mM HEPES/NaOH, pH 7.5; C, 10 mM HEPES/KOH, pH 7.5.

where F_0 = fluorescence of the released flavin solution; F_{fin} = fluorescence after reaction with PDE; f_{FAD} = fluorescence of a 1 μM solution of FAD; and f_{FMN} = fluorescence of a 1 μM solution of FMN. Fluorescence refers to the intensity of light emitted at 524 nm on excitation with 450 nm light.

In the case of the GltS β subunit or of its G298A mutant, from the changes of emission intensity of the solutions (**Table 1**) it can be concluded that the enzyme contains FAD as the flavin cofactor. The results are not influenced by the presence of 0.1 mM MgSO_4 , which was called for in the original method (**16**), nor by the incubation time at 100°C. Instead, we found it important to protect the sample from light throughout the treatment. As shown in **Table 1**, no fluorescence changes were obtained on incubation of the flavin cofactor released from a sample of GltS α subunit indicating that the protein contains FMN as the flavin cofactor beside a 3Fe/4S cluster (**10**). Fluorescence changes on addition of PDE to the flavins released from GltS holoenzyme are more complex. The increase of fluorescence is about twofold, consistent with the presence of approximately equimolar amounts of FAD and FMN in the GltS holoenzyme (**Table 1**). A more precise estimate of the flavin stoichiometry is obtained by HPLC analysis of the released flavins (see **Subheading 3.3.**).

The method can be used to calculate the FAD and/or FMN concentration of the solution under analysis provided the intensity of emission at 524 nm as a function of FAD or FMN concentration has been determined experimentally. Under our conditions, using GltS, the emission intensity of solutions containing between 1 and 8 μM FMN was measured in the 200 μL cuvet, so that it was determined that a 1 μM FMN solution yields a fluorescence intensity of 76.4. Assuming that the GltS protomer contains one FAD and one FMN, from the fluorescence after the PDE treatment, an enzyme concentration of 4.8 μM could be calculated in agreement with the determination made from protein assays (4.8 μM).

3.2. Spectrophotometric Determination of the Flavin Content, Identification of the Flavin Cofactor as FAD or FMN, and Calculation of Extinction Coefficients

3.2.1. SDS Treatment of Simple Flavoproteins

1. The absorbance spectrum of the protein solution (5–10 μM , 1 mL) in 10 mM HEPES/NaOH buffer, pH 7.5, or 10 mM Tris-HCl buffer, pH 7.5–8, is recorded.
2. A 20 μL aliquot of a fresh 10% SDS solution is added (see **Note 3**).
3. After mixing, spectra are recorded until no further changes are observed. Typically, within 5–10 min conversion of the spectrum of the bound flavin into that of free FAD or FMN is observed.
4. Direct comparison of equimolar solutions of FAD and FMN reveals that at 473 nm the solutions exhibit a similar extinction coefficient (9200 $\text{M}^{-1}\text{cm}^{-1}$).

Thus, the following conclusions can be drawn from the comparison of the spectrum of the native protein with that obtained after SDS addition:

- a. From the position of the absorbance maximum at 450 vs 446 nm, or of the absence of a well-defined peak between 446–450 nm the presence of FAD, FMN or of a mixture of the two in the released flavin solution can be inferred.
- b. Once the type of flavin present is identified, its concentration can be precisely estimated using $\epsilon_{450} = 11,300 \text{ M}^{-1}\text{cm}^{-1}$ for FAD and $\epsilon_{446} = 12,200 \text{ M}^{-1}\text{cm}^{-1}$ for FMN. In case of doubt, or of other information, or if a mixture of FAD and FMN may be present, the total (FAD + FMN) concentration can be calculated from the absorbance at 473 nm, using $9200 \text{ M}^{-1}\text{cm}^{-1}$ as the extinction coefficient.
- c. Once the concentration of the flavin is known, both the flavin/protein molar ratio and the extinction coefficient of the bound flavin can be calculated.

3.2.2. Heat Denaturation of Simple and Complex Flavoproteins

SDS-treatment sometimes fails to denature proteins, in particular those containing iron-sulfur centers as in our case (i.e., GltS, the GltS α subunit, the chimeric Fd/FNR fusion protein). An effective method to destroy the protein's iron-sulfur clusters, while preserving the flavin cofactors, is heat treatment in the dark.

1. The spectrum of the protein solution (5–10 μM , 1 mL) is recorded.
2. The sample is transferred to a microfuge tube, and incubated in a boiling water bath for 10 min.
3. After cooling, the denatured protein is removed by centrifugation (10 min at 13,000 rpm (14,500g) in the cold in a microfuge).
4. The supernatant is recovered, and the absorbance spectrum is recorded (*see Note 4*).
5. If centrifugation is not sufficient to remove denatured protein the supernatant can be either filtered through 0.2 μm filters, or using centrifugal filters.
6. Again the identification of the flavin can be attempted from the position of the absorbance maximum (450 nm for FAD as opposed to 446 nm for FMN).
7. Flavin quantitation can be carried out as described above (*see Subheading 3.2.1.*), allowing determination of stoichiometry and extinction coefficient of the bound flavin.

3.2.3. Spectrophotometric Identification of FAD and FMN by PDE Treatment Following Heat Denaturation

The differences of the absorbance spectra of FAD and FMN can be exploited to carry out the identification of FAD and FMN using the PDE treatment, directly in the spectrophotometer cuvet.

1. The spectrum of a sample containing 5–10 μM flavin (1 mL) is recorded.
2. A 2 μL aliquot of PDE is then added, and spectra are recorded at different times after PDE addition.

3. Using a FAD-containing solution, absorbance changes consistent with conversion of FAD into FMN (+ AMP) are observed with an isosbestic point at 473 nm, and they are complete within 10 min. As expected, no changes are observed when a FMN-containing solution is treated with PDE.

3.2.4. Quantitation of Total Flavin Content in Iron-Sulfur Flavoproteins

Determination of the non-heme iron content of iron-sulfur containing proteins is often affected by the precision of the protein concentration determination. In order to avoid this problem with GltS, a method was designed (17) that allowed us to measure non-heme iron concentration and total flavin concentration on the same sample derived from heat denaturation, under acidic conditions, of GltS or GltS α subunit solutions.

1. 0.8 mL Samples of GltS or GltS α subunit (5–10 μ M) that have been gel filtered in 10 mM HEPES/KOH buffer, pH 7.5 or 10 mM Tris/HCl buffer, pH 7.5–8, are prepared.
2. The absorbance spectrum of the solution is recorded, and the solution is transferred into a microfuge tube. One-tenth microliter of 100% trichloroacetic acid (TCA) and 0.1 mL 75 mM ascorbic acid are added.
3. After being mixed, the sample is incubated in a boiling water bath for 5–10 min.
4. The combination of acid and heat treatment results in hydrolysis of FAD present into FMN.
5. Inclusion of ascorbic acid helps in maintaining Fe in the +2 form required for iron concentration determination.
6. After being cooled, denatured protein is removed by centrifugation for 10 min at 13,000 rpm (14,500g) in a microfuge in the cold.
7. The absorbance spectrum of the supernatant is measured, and the total flavin concentration is determined using 11.1 mM⁻¹cm⁻¹ as the extinction coefficient of FMN at 446 nm, under acidic conditions (8).
8. The total flavin concentration can be used as an estimate of the GltS concentration, assuming that 1 FAD and 1 FMN are present per enzyme protomer, or that 1 FMN is present per GltS α subunit.
9. Enzyme concentration determined through protein assays and through the total flavin content never differed by more than 20% (Table 2). The supernatant from the heat denaturation step under acidic conditions can be directly used for the determination of the iron content of the preparation (17).

3.3. HPLC Identification and Quantification of FAD and FMN

Several methods for the separation of flavins by reverse phase chromatography have been published. We propose a modification of the method of Light et al. (18,19) which uses 5 mM ammonium acetate, pH 6.5, and methanol as the solvent system. Thus, it allows both to separate FAD and FMN on an analytical scale, and to obtain homogeneous preparations of FAD or FMN from commercially available compounds. When used on a preparative scale, the solvent

Table 2
Spectrophotometric Quantitation of FAD and FMN Extracted from Several Flavin-Containing Enzymes

Enzyme	Concentration ^a (μM)	Pretreatment	Buffer	Denaturation	Native λ_{\max} , A@ λ_{\max}	Denatured λ_{\max} , A@ λ_{\max}	Flavin concentration (μM)	Stoichiometry	ϵ ($M^{-1}cm^{-1}$)
GltS β subunit	16.56	Gel filtration	C	0.2% SDS	454, 0.157	450, 0.152	(FAD), 13.4	0.81	11,698
GltS β subunit	8.85	Gel filtration	B	0.2% SDS	454, 0.083	450, 0.085	(FAD), 7.5	0.85	11,080
G273A GltS β subunit	8.8	None	A	10 min, 100°C	452, 0.099	450, 0.086	(FAD), 7.6	0.86	12,955
G273A GltS β subunit	4.3	Gel filtration	B	0.2% SDS	452, 0.049	450, 0.048	(FAD), 4.3	0.99	11,448
GltS α subunit	2.3	Gel filtration	B	10 min, 100°C	440, 0.046	446, 0.029	(FMN), 2.4	1.04	19,200
rGltS	4.8	Gel filtration	D	10 min, 100°C	444, 0.300	448, 0.115	(FAD + FMN)		
						473, 0.09 ^c	9.8 ^c	2.04	61,200
rGltS	3.35	Gel filtration	B	10 min, 100°C ^b	444, 0.210	446, 0.07 ^d	6.2 ^d	1.85	67,742

^aEnzyme concentration was determined by the Bradford method (15).

^bIn the presence of 10% TCA, 7.5 mM ascorbic acid, and light.

^cThe extinction coefficient at the isosbestic point for FAD and FMN ($9200 M^{-1}cm^{-1}$) was used.

^dThe extinction coefficient of FMN under acidic conditions is used ($11,100 M^{-1}cm^{-1}$, 8).

A, 25 mM HEPES/KOH, pH 7.5, 10% glycerol, 1 mM EDTA; B, 10 mM Tris/HCl, pH 7.5; C, 10 mM HEPES/NaOH, pH 7.5; D, 10 mM HEPES/KOH, pH 7.5.

can be easily removed by lyophilization. The eluate can be monitored continuously by absorbance or fluorescence, depending on the online detection system available. We routinely monitor the absorbance of the eluate at 264 nm, where the absorbance of FAD and FMN solutions are maximal. Greater sensitivity could be obtained by measuring the eluate absorbance at shorter wavelengths, but quantitation may be affected by baseline absorbance changes due to varying eluent composition.

1. A 2 mL sample loop is mounted on the UK-6 injector and varying sample volumes (50–250 μ L) are injected onto the column using microsyringes.
2. The column is equilibrated at a flow rate of 1 mL/min for 30 min with 85% solvent A (5 mM ammonium acetate buffer, pH 6.5) and 15% methanol.
3. After sample injection the column is washed with 85% solvent A and 15% methanol for 5 min.
4. The concentration of methanol is increased linearly from 15–75% in 20 min and from 75–100% in 5 min.
5. Methanol concentration is kept constant at 100% for 10 min and lowered to 15% in 5 min.

3.3.1. Construction of the Calibration Curve

1. A calibration curve is constructed by injecting 50 μ L aliquots of solutions containing known amounts of FAD and FMN (40–800 pmol each).
2. Under these conditions, FAD elutes as a sharp peak at 9.5 min, and FMN as a broader peak at 18 min.
3. Integration of the areas corresponding to the peaks obtained by injecting known amounts of FAD and FMN allows the construction of standard curves.
4. Under these conditions, peak position and quantitation is independent from sample volume (50–250 μ L of a 1 μ M solution of FAD and FMN). Moreover, incubation of FAD and FMN solutions at 100°C for up to 20 min does not lead to any detectable change of the chromatogram, provided the samples are kept in the dark. Furthermore, freezing of such solutions for up to one week also does not lead to any detectable change. On the contrary, exposure of FAD and FMN solutions to light, to acidic conditions (10% TCA) or to both acidic conditions (10% TCA) and heat (10 min at 100°C) leads to conversion of FAD into FMN, and (presumably) AMP, which is not retained by the column.

3.3.2. Sample Preparation and Analysis of the Released Cofactors

1. Flavoprotein samples are diluted to a final concentration of 5–20 μ M.
2. Protein solutions are transferred to microfuge tubes and incubated at 100°C for 10 min.
3. The denatured protein is removed by centrifugation, and the supernatant is filtered.
4. The absorbance spectrum of each solution is measured, and 50, 100, and 150 μ L aliquots are injected onto the HPLC.
5. To test the method, samples of GltS holoenzyme, GltS α subunit, spinach leaf FNR, maize root FNR, and the chimeric Fd/FNR protein are analyzed. As shown in **Table 3**, the chromatographic analysis reproducibly yields results consistent

Table 3
HPLC Quantification of the Flavin Content of Several Flavoproteins

Experiment	Enzyme	Concentration ^a (μ M)	Buffer ^b	Denaturation method	Enzyme (pmol)	FAD (pmol)	FMN (pmol)	FAD/ subunit	FMN/ subunit
1	rGltS	5.8	A	100°C, 10 min	290	247	248	0.85	0.85
					580	471	507	0.81	0.87
2	GltS α	5.3	B	100°C, 10 min	265	None	275	—	1.04
					530	None	520	—	0.98
3	Leaf-FNR	5.50	C	100°C, 10 min	275	262	None	0.95	—
					550	527	None	0.96	—
4	Root-FNR	4.86	C	100°C, 10 min	243	230	None	0.95	—
					486	469	None	0.97	—
5	Fd/FNR	5.52	C	100°C, 10 min	276	289	None	1.05	—
					552	588	None	1.07	—

^aConcentration determined with the Bradford method (15).

^bBuffers used were: A, 25 mM Hepes/KOH, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM 2-oxoglutarate, 1 mM DTT; B, 25 mM Pipes/KOH, pH 7.5, 10% glycerol, 1 mM EDTA, 0.1 mM DTT; C, 50 mM Tris-HCl, pH 7.4.

with the known flavin content of the enzymes, as determined previously and by using the alternative methods presented above (see **Subheadings 3.1.** and **3.2.**).

4. Notes

1. When 200 μL cuvetts are used, care must be taken to transfer the samples with automatic pipets, and to start from at least 250 μL samples. Mixing must also be carried out with automatic pipets. We discourage the use of microsyringes when the sample contains PDE, as the enzyme is difficult to wash away and may contaminate samples that will be handled later on.
2. We recommend soaking cuvetts that have come in contact with PDE in a solution containing 3 M HCl in ethanol and extensively rinsing with MilliQ water (Millipore) for cleaning and effective removal of PDE traces.
3. In these experiments, it should be kept in mind that due to the limited solubility of potassium dodecyl sulfate, the protein should be transferred into a potassium-free buffer.
4. Comparison of the spectrum of the flavin released by the SDS treatment and that released by heat denaturation sometimes yields different results: up to 20% flavin loss during heat denaturation has been observed, presumably due to trapping of some of the flavin in the protein pellet. Thus, it is recommended that the experiment is repeated using protein solutions of different concentrations.

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