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Quantitation of FAD-Dependent Cytochrome P450 Reductase Activity by Photoreduction[1,2]

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NADPH cytochrome P450 reductase binds two flavin cofactors, FMN and FAD, per molecule of reductase. We have developed an assay to quantitate the reduction activity of FMN-bound flavoprotein. This Tris–light assay system takes advantage of the ability of photoactivated flavins to release electrons to acceptors. In turn, electrons derived from Tris buffer restore the flavin to the unexcited, ground state which can again undergo photoactivation to release another electron. FMN-bound reductase, supplied with reducing equivalents from a Tris–light electron generating system, reduces ferricyanide at a rate of 1.8 μmol/min/nmol reductase. Holoreductase in this system is able to catalyze ferricyanide reduction at a rate of 1.6 μmol/min/nmol reductase, while FAD-bound reductase has no activity. The 8-NH$_2$-FAD and 8-OH-FAD analog-reconstituted FMN-bound reductase catalyzes the reduction of ferricyanide at rates of 0.43 and 0.28 μmol/min/nmol reductase, respectively. The riboflavin-reconstituted FMN-bound reductase catalyzes ferricyanide reduction at a rate of 1.1 μmol/min/nmol reductase. FAD or its analogs at the concentrations used to reconstitute enzymatic activity do not support the reduction of ferricyanide in the Tris–light system in the absence of reductase protein. The free flavins, i.e., FMN, 8-OH-FAD, 8-NH$_2$-FAD, and riboflavin, are able to support ferricyanide reduction at a rate of 0.40, 0.52, 0.87, and 0.16 μmol/min/nmol flavin, respectively. This is the first report of an enzymatic assay specific for FMN-bound NADPH cytochrome P450 reductase activity in the absence of its FAD cofactor. Moreover, this report describes the use of an assay procedure based on the provision of reducing equivalents by a Tris–light system which may be useful for other flavin redox enzymes in the absence of reduced pyridine nucleotides or biopterin cofactors. © 1996 Academic Press, Inc.

NADPH cytochrome P450 reductase (reductase[5] or holoreductase) is a 76,500-Da single polypeptide flavoprotein known to bind one molecule each of the flavin cofactors FMN and FAD (1–4). The reductase cDNA has been cloned and sequenced (5) and the FMN (6) and FAD (7) binding domains have been confirmed. The bound flavins transfer reducing equivalents from NADPH to cytochrome P450 which in turn catalyzes the hydroxylation of many drugs, fatty acids, and pesticides (8–10). NADPH donates two electrons (1) to reductase which in turn donates two electrons, one at a time, to P450.

In order to study the redox activity of each flavin, FMN-depleted reductase (11) and FAD-depleted reductase (12) have been prepared from reductase containing both flavins (FMN and FAD) (holoreductase). These studies demonstrated that FAD is the initial electron acceptor from NADPH, accepting two electrons at a time from NADPH and donating them to FMN one at a time (12). The reductase therefore cycles between various oxidized and reduced species through the catalytic cycle (11, 13), representing fluctuations in the state of reduction of the two flavins.

In vitro spectrophotometric assays have been developed to quantitate the ability of holoreductase to reduce several electron acceptors including ferricyanide, cytochrome c, 3-AcPyADP, and dichlorophenolindophenol, using NADPH to supply reducing equivalents. FMN-

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[5] Abbreviations used: 3-AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; P450, cytochrome P450; reductase, NADPH cytochrome P450 reductase.

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depleted reductase has been shown to reduce both ferricyanide and 3-AcPyADP, but is unable to reduce dichlorophenolindophenol and cytochrome c (11). Fragments of the reductase containing only the FAD and NADPH binding domains will also reduce ferricyanide and 3-AcPyADP (7, 14), and accept electrons from NADPH, demonstrating that the FMN binding site of reductase is not required for reduction of these two electron acceptors.

Other familiar assays are unable to assess the electron transfer activity of FAD-depleted reductase (11). Presumably, the FMN which remains bound to the reductase FMN site is unable to accept electrons two at a time from NADPH. To circumvent this reducing equivalent supply problem and establish a functional assay for FMN-reductase, we have developed a Tris–light electron generating system similar to the superoxide generating system (15).

MATERIALS AND METHODS

Flavins were prepared as previously described (12). FAD analogs were the generous gift of Dr. Vincent Massey of the University of Michigan. Aporeductase was purified as previously described (12). Briefly, holoreductase was dialyzed in a KBr buffer to release the FAD and the resulting FAD-depleted reductase was separated from the holoreductase by HPLC using an hydroxyapatite HPLC column. FAD binding fragments were cloned, expressed, and purified as previously described (7). Proteins that were reconstituted with flavins were incubated at room temperature for 30 min in 0.45 μM of the respective flavin, and resolved by G-25 gel filtration chromatography to remove unbound flavin.

Buffer containing 100 mM KP_i, pH 7.5, and 500 μM ferricyanide and the appropriate flavin was placed in cuvettes for photoreduction. Ten microliters of 2 M Tris–HCl, pH 8, was added prior to addition of reductase. Photoreduction was driven by light from three Buchler Instruments (Fort Lee, MI) assemblies, each containing three 24-in.-long 20-W fluorescent bulbs, at an average distance of 30 cm from the samples. Samples were removed from the illumination assembly at various times and placed in a Varian Model 210 Spectrophotometer for less than 30 s at a time, to determine the progress of ferricyanide reduction. Reduction rates were calculated using an extinction coefficient at 420 nm of 1.02 mM^{-1} cm^{-1} (16) at 30°C. Nitroblue tetrazolium reduction was also measured in the same manner in a buffer containing 1 mM NBT and 0.1% Renex detergent. Background reduction activity was established for all samples around buffer only with buffer-only (no protein or flavin) controls containing only phosphate buffer. Tris–HCl or NADPH was added to the controls for the light-mediated or NADPH-mediated assays, receptively.

TABLE 1

Light- and NADPH-Mediated Ferricyanide Reduction

Reducing equivalent source	Reductase activities (µmol ferricyanide/min/nmol reductase)					
	Holo	Apo FMN	Apo FAD	Apo ribo	Apo + 8-NH_2	Apo + 8-OH
NADPH	2.8	0.15	0.0	1.3		
Light	1.57	1.8	0.0	1.1	0.43	0.28

Note. Holo is the FMN-bound reductase reconstituted with FAD, Apo FMN is the FMN-bound reductase, Apo FAD is the FAD-bound reductase; Apo ribo is the riboflavin-reconstituted FMN-bound reductase, and Apo + 8-NH_2 and Apo + 8-OH are the FMN-bound reductase reconstituted with 8-NH_2 FAD and 8-OH FAD, respectively.

RESULTS AND DISCUSSION

In order to characterize further and to quantitate the activity of the FMN-bound (FAD-depleted) reductase, a light reduction assay was developed. The Tris–light electron generating system used for the reduction of flavin was based on the superoxide dismutase assay developed by McCord *et al.* (15). Their assay measured the ability of superoxide dismutase to inhibit the reduction of tetrazolium by superoxide, which is generated by reduced riboflavin. A methionine–light system in a phosphate buffer generates the reducing equivalents required to reduce the riboflavin. Our reductase photoassay is based on this methionine–light-reducing equivalent system, with the elimination of riboflavin, the replacement of the methionine by Tris–HCl in our phosphate buffer, and an addition of detergent, which nearly doubles the catalytic activities.

Light-Mediated vs NADPH-Mediated Flavin-Bound Reductase Activity

As described under Materials and Methods, 1-ml cuvettes containing the appropriate flavin (and reductase) and ferricyanide buffer were placed in the fluorescent light for various time periods. The illumination was interrupted temporarily while the cuvettes were placed in a spectrophotometer to measure the absorption over the course of 50 min. The catalytic ability of reductases containing FAD or FMN or both flavins was assayed using this Tris–light system. Initial velocity rates were calculated from plots of absorbance at 340 nm vs time and are summarized in Table 1. Holoreductase was prepared by reconstituting aporeductase with FMN. Aporeductase refers to FMN-bound (FAD-depleted) reductase. Apo-ribo, Apo + 8-NH_2 and Apo + 8-OH are preparations of aporeductase reconstituted with riboflavin, 8-NH_2-FAD, and 8-OH-FAD, respectively.

The specificity of FMN vs FAD is illustrated by the

activity of holoreductase (1.6 μmol/min/nmol reductase) compared to aporeductase (1.8 μmol/min/nmol reductase) and FAD-reductase (0 μmol/min/nmol reductase). All of the electron flow in holoreductase appears to be present in the apo (FMN flavin-containing only) reductase. Since the FMN-dependent (FAD flavin-containing only) reductase appears not to have any activity in the Tris–light assay we concluded that the Tris–light system reduces FMN in reductase and not FAD. The control assay containing buffer only and control assays developed in the dark showed no activity over the background rates for any of the light-mediated assays (data not shown).

Enzymatic activities of aporeductase reconstituted with flavin analogs were also calculated. Riboflavin was able to reconstitute the FMN site and facilitate approximately 75% (1.1 μmol/min/nmol reductase) of the holoreductase activity. We were not surprised to see this activity since riboflavin was also observed to reconstitute the FMN site of FAD-bound reductase (3) and thereby restore 75% of the holoreductase activity.

The 8-NH and 8-OH FAD analog reconstituted reductases restored 20–25% of the holoreductase activity. This demonstrated that the FAD binding site is somewhat accessible for binding flavins other than FAD. Fragments of reductase containing only the FAD/NADPH site were unable to be reduced by the Tris–light system (data not shown).

The NADPH supported activities for the various reductase preparations are also shown in Table 1 for comparison. NADPH-supported reduction of holoreductase is ~36% higher (2.8 vs 1.6 μmol/min/nmol reductase) than the Tris–light reductase of aporeductase. However, NADPH is practically unable to reduce aporeductase, generating only 10% of the reductase activity generated by the Tris–light system (0.15 vs 1.8 μmol/min/nmol reductase). This assay demonstrates that the Tris–light system is very specific for FAD-depleted reductase versus FMN-depleted reductase and is able to supply the FAD-depleted reductase with reducing equivalents at nearly the same rate as the physiological donor, NADPH.

Free versus Protein-Bound Flavin Activity

Table 2 shows the relative light-mediated activities of five different flavins as bound to reductase and as free flavins in solution. The only flavin present in the FMN-bound reductase is FMN. The other flavins (FAD, riboflavin, and the FAD analog-bound reductases) are bound to reductase that also contains an FMN in the FMN binding site. Therefore, the FAD-bound activity shown is for holoreductase and the activity represents the activity of the total enzyme per nanomole of FAD.

As a free flavin, 8-OH FAD is the most able to reduce ferricyanide (0.87 μmol/min/nmol flavin), followed by 8-NH FAD, FMN, and riboflavin (0.52, 0.40, and 0.16 μmol/min/nmol flavin, respectively). FAD is not active above the control (no flavin) rates. Protein fragments generated from expression of reductase clones containing only the FAD binding site have no activity (data not shown) demonstrating that FAD is not able to reduce ferricyanide in the Tris–light system, but rather relies on NADPH as a source of reducing equivalents.

TABLE 2

Light-Mediated Free- and Protein-bound Flavin Reduction

	Activities of flavins (μmol ferricyanide/min/nmol flavin)				
	FMN	FAD	Ribo	8-NH_2-FAD	8-OH-FAD
Free	0.40	0.0	0.16	0.52	0.87
Bound	1.8	1.6	1.1	0.43	0.28

Note. Ribo is riboflavin. FAD, Ribo, 8-NH_2-FAD, and 8-OH-FAD were incubated with aporeductase (FMN-bound reductase) and therefore contain two flavins/molecule reductase; however, activity is calculated per mole of only the indicated flavin. Free values are for the flavin activities in solution. Bound are the flavin activities of the flavin as bound to reductase.

Once bound to the enzyme, FMN is over four times more active enzymatically per mole of flavin than it was as a free flavin, increasing ferricyanide reduction from 0.40 to 1.8 μmol/min/nmol flavin (Table 2). The FMN-bound reductase is also the most active of all of the flavin-bound reductase preparations in this light assay. Holoreductase (Table 2, FAD, bound, 1.6 μmol/min/nmol flavin) reduces ferricyanide at only 88% of the rate that the FMN-bound reductase does (Table 2, FMN-bound, 1.8 μmol/min/nmol flavin), demonstrating that the Tris–light assay has a high degree of sensitivity for the assay of FMN activity. Riboflavin also greatly increases its activity as bound to the protein, but still manages only a little over 60% of the activity of the FMN-bound reductase. Both of the FAD analogs render the aporeductase less able to transfer electrons to ferricyanide.

The Tris–light system is a sensitive and specific assay to quantitate the relative FMN-associated activity in reductase and is the first enzymatic assay developed for the FAD-depleted reductase. The Tris–light system may also be a useful analytical tool for other flavin-containing enzymes in the absence of their respective pyridine nucleotide or biopterin cofactors.

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