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Note

Purification of flavin mononucleotide-dependent and flavin-adenine dinucleotide-dependent reduced nicotinamide-adenine dinucleotide phosphate–cytochrome P-450 reductase by high-performance liquid chromatography on hydroxyapatite

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The mechanism of enzymes that tightly bind low-molecular-weight species is often investigated by removing the prosthetic group and then studying the resulting apoprotein. If there are multiple prosthetic groups, then selective removal can elucidate each group's role in catalysis. We are interested in the membrane-bound reduced nicotinamide-adenine dinucleotide phosphate (NADPH)–cytochrome P-450 reductase (EC 1.6.2.4) that has bound to it one molecule each of flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD)[1]. Studies of approximately 90% FMN-dependent[2,3] and 98% FMN-dependent reductase[4] have contributed significantly to our understanding of the reductase, producing a model for its reaction cycle in which FAD is the sole acceptor of electrons from NADPH and FMN is the sole donor of electrons to cytochrome P-450[3,4].

In order to test and extend the hypotheses concerning the mechanism of electron flow through the reductase, we sought to remove selectively the second flavin, FAD. The tight binding of FAD, and the looser binding of FMN, prevented us from completely removing FAD by mass action. We therefore developed a procedure based upon separating the FAD-dependent reductase from contaminating holoreductase. Here, we demonstrate the ability of a high-performance hydroxyapatite column to separate both the FAD-dependent reductase and the FMN-dependent reductase from the holoreductase.

EXPERIMENTAL

NADPH–cytochrome P-450 reductase was purified from rat liver microsomes using either detergent[5] or steapsin protease[6] solubilization. Protease solubilization removes a small domain responsible for membrane binding and self aggregation[7].

The reductase was assayed by measuring the rate of reduction of cytochrome *c*[5], with modifications necessary to stabilize the aporeductase and increase the sensitivity. A dual-beam assay blanked out the background reduction of cytochrome *c* by dithiothreitol. The enzyme was diluted into 2 ml of the assay mix (0.3 *M* potassium phosphate, 1 m*M* EDTA, 40 μM cytochrome *c*, pH 7.7; filtered and autoclaved prior

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to the addition of cytochrome *c* and placed into two cuvettes. Only the sample cuvette received NADPH, 0.1 m*M*. Absorption at 550 nm was measured with a Cary 210 spectrophotometer (Varian) with a 3.5-nm spectral bandwidth. The sample holder was thermostatted to 22°C. The initial activity was taken to be due to the holoreductase. FMN or FAD was then added to 0.2 μM. The increase in activity was assigned to reconstitution at the appropriate site. The total increase in absorbance was limited to 0.1.

FMN was removed from both the detergent- and protease-solubilized forms of the reductase, with minor modifications[8] of published procedures[2,3]. The steapsin-solubilized reductase became 91% FMN-dependent and the detergent-solubilized reductase became 93% FMN-dependent. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis[9] showed no proteolysis of either form of the FMN-dependent reductase. Neither of the FMN-dependent forms had any FAD dependence.

FAD was removed from the protease-solubilized reductase by dilution into acidic potassium bromide[8]. The enzyme was separated from the perturbing buffer and the released FAD by chromatography on Sephadex G-50. In most preparations, 10 μM FMN was applied to the column immediately prior to the enzyme in order to reconstitute the FMN site, which partially loses its flavin during exposure to potassium bromide. The reductase was then concentrated by diafiltration.

The high-performance hydroxylapatite system (HPHT; Bio-Rad Labs.) consisted of an inert guard column (50 × 4 mm I.D.) and a 100 × 7.8 mm I.D. column packed by a proprietary technique with small crystals of hydroxyapatite. The manufacturer's instructions regarding back-pressure, column storage and buffer preparation were followed, but enzyme samples were generally not pretreated to remove particulates. The low-phosphate buffer (10 μM sodium phosphate, 10% glycerol, 95 μM calcium chloride, 0.05% sodium azide, 1 m*M* dithiothrietol, 0.1 m*M* NADP, pH 7.5) was pumped with a Waters M6000A pump, and the high-phosphate buffer (identical to the low-phosphate buffer, but 150 m*M* in sodium phosphate and 6 μM in calcium chloride with a Waters M45 pump. The HPHT system was equilibrated in the low-phosphate buffer, and the enzyme was applied via a Waters U6K injector with a 2-ml loop. Gradients were controlled with a Waters M660 solvent programmer.

NADP, horse heart cytochrome *c*, sodium cholate, FMN and FAD were from Sigma. NADPH was from Boehringer Mannheim and Renex 690 was from ICI, America.

RESULTS

Our initial aim was to design a system for the removal of FAD from NADPH–cytochrome P-450 reductase. At one point in the development, incubations of the reductase in potassium bromide were adsorbed onto hydroxyapatite in order to concentrate the protein and separate it from the buffer and the released FAD. During desorption, we observed partial resolution of the FAD-dependent activity from the holoreductase. We thereafter pursued hydroxyapatite (high resolution, Calbiochem-Behring; and Bio-Gel HTP, Bio-Rad Labs.) as a means of separating the aporeductase from the holoreductase. The inability of these products, under a variety of conditions, to resolve the species successfully, convinced us to utilize the HPHT system; the high-performance packing surpassed the open columnns (not shown).

The HPHT system resolved the FAD-dependent reductase from the holoreductase (Fig. 1). By discarding the leading edge of the FAD-dependent peak, 90% FAD dependence was achieved with a 63% recovery of the FAD-dependent activity. Individual fractions had as much as 98% FAD dependence.

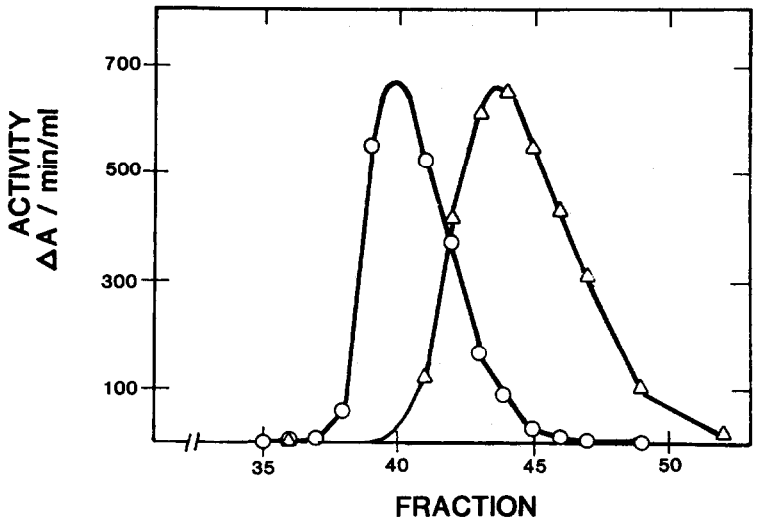

Fig. 1. Resolution of FAD-dependent NADPH–cytochrome P-450 reductase from the holoreductase on high-performance hydroxyapatite. Steapsin protease-solubilized reductase (11 ml), with 6% FMN dependence and 62% FAD dependence, was applied to the HPHT system. The flow-rate was 0.3 ml/min. Immediately after injecting the enzyme (at fraction 8), a 3-min gradient to 70% in the high-phosphate buffer was applied. Immediately thereafter, a 67-min linear gradient to 100% in the high-phosphate buffer was applied. Fractions were collected at 2-min intervals, starting at the beginning of the injections of the reductase. (○) Holoreductase; (△) FAD-dependent reductase.

In early chromatograms, NADP was not present in the buffers. We observed an increase in FMN dependence of both the FAD-dependent and FAD-independent activity. Interestingly, the FMN dependence was higher in the tail of both peaks. Because the bound enzyme was releasing FMN, it was possible that the enrichment in each tail was due only to the longer time that tail fractions were bound to the column, rather than because of chromatographic resolution. However, when NADP was added to the column buffers, virtually no FMN was released during chromatography, but FMN dependence was still present in the tail of both peaks. Thus, the column was separating pre-existing FMN-dependent molecules from FMN-independent molecules. We confirmed that the hydroxyapatite separates the FMN-dependent reductase from the holoreductase by preparing and chromatographing FMN-dependent reductase (Fig. 2).

We next, established the ability of the HPHT system to resolve the detergent--solubilized FMN-dependent reductase from the detergent-solubilized holoreductase. Since the detergent-solubilized reductase self-aggregates[7], we added detergents to the buffers. In 0.2% sodium cholate, the two species nearly coeluted in a broad and late peak (not shown). Better results were obtained with a non-ionic detergent, Renex

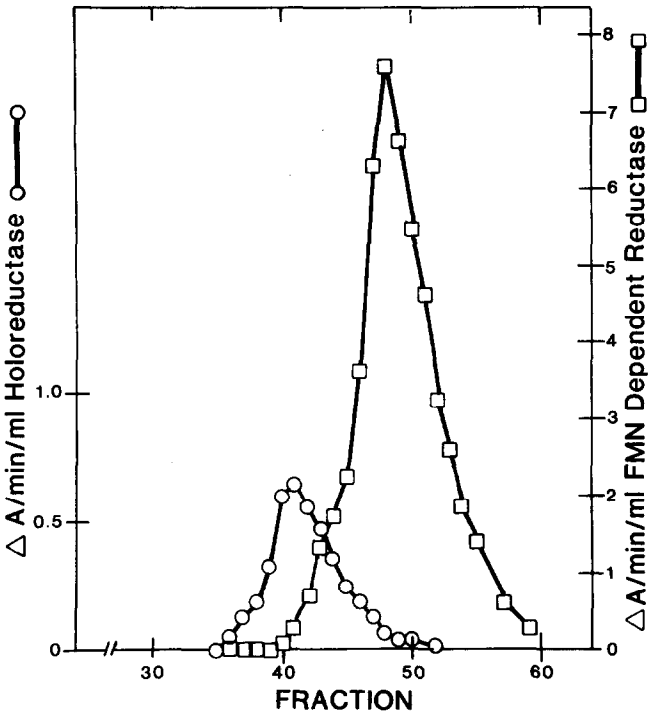

Fig. 2. Resolution of FMN-dependent protease-solubilized NADPH–cytochrome P-450 reductase from the holoreductase on high-performance hydroxyapatite. Steapsin protease-solubilized reductase (230 ml), 91% dependent upon FMN, was applied at 0.4 ml/min. Immediately after the injection, a 3-min gradient to 70% in the high-phosphate buffer was applied. Immediately thereafter, a 50-min linear gradient to 100% in the high-phosphate buffer was applied. Fractions were collected at 1-min intervals. Note the different scale for each species. (○) Holoreductase; (□) FMN-dependent reductase.

690 (not shown). Our best results were obtained with a buffer system containing mixed micelles of 0.2% Renex 690 and 0.5% sodium cholate (Fig. 3).

The degree of flavin dependence after chromatography depends upon the dependence prior to chromatography, the ability of the column to resolve the species, and the recovery. In all cases, the recovery of the holoreductase exceeded 80%, whereas the flavin-dependent species had total recoveries of 50–80%. For the flavin-dependent species, the actual recovery depends upon the desired level of flavin dependence. For example, a pool of the reductase with 96% dependence upon FAD contained 41% of the applied FAD-dependent activity, while the recovery for 90% FAD dependence was 63%.

DISCUSSION

A rapid and simple procedure for the preparation of both FMN- and FAD-dependent NADPH–cytochrome P-450 reductase has been presented. For the FAD-dependent reductase, the HPHT system is the only procedure available to provide a high level of dependence upon FAD. For the FMN-dependent reductase, existing

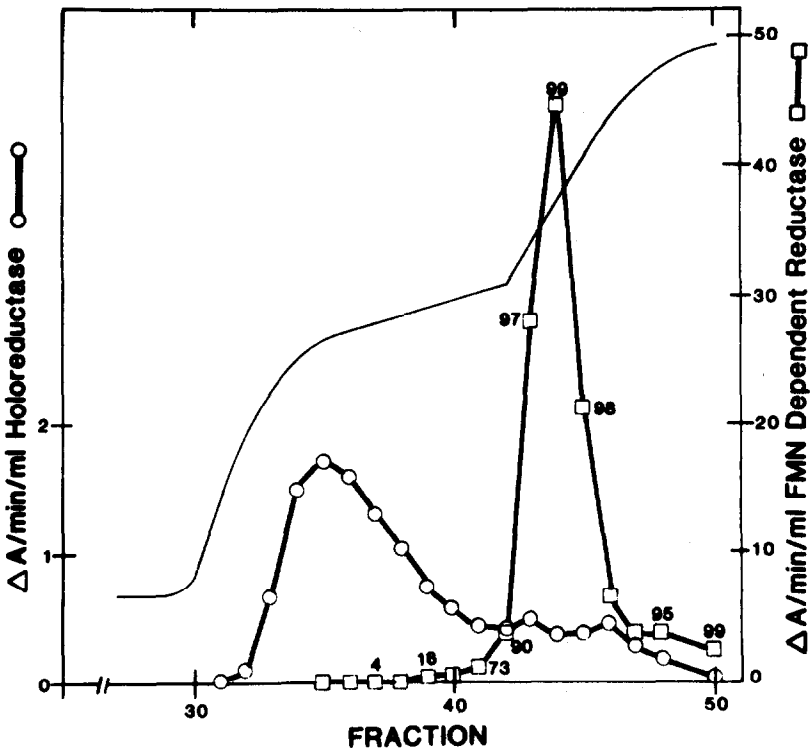

Fig. 3. Resolution of FMN-dependent detergent-solubilized NADPH–cytochrome P-450 reductase from the detergent-solubilized holoreductase on high-performance hydroxyapatite. The detergent-solubilized reductase, 93% dependent upon FMN, was made 0.2% (w/w) in Renex 690 and 0.5% (w/w) in sodium cholate. A 210-μl aliquot was applied at 0.4 ml/min, and 1-min fractions were collected. Immediately after applying the reductase, a 1-min gradient to a 90 mM phosphate buffer (identical to the buffers described in Materials and methods, but 90 mM in sodium phosphate and 11 μM in calcium chloride) was applied. Fourteen minutes later, a second step was applied, to the 150 mM phosphate buffer. All buffers were 0.2% in Renex 690 and 0.5% in sodium cholate. The upper curve shows the conductivity of dilutions of fractions. The percentage dependence upon FMN of some fractions is shown. Note the different scale for each species. (○) Holoreductase; (□) FMN-dependent reductase.

methods provide for 90% dependence[2,3], but higher FMN dependence requires a sophisticated approach in which the released FMN is removed by binding it to apoflavodoxin[4]. The combination of dialysis against potassium bromide and the chromatography described here allows for the rapid and accessible production of highly FMN-deependent NADPH–cytochrome P-450 reductase, either detergent- or protease-solubilized. The procedures described here thus utilize a single column to prepare three distinct species.

By what physical basis does the hydroxyapatite resolve the aporeductase from the holoreductase? The reductase, with a pI of 5.6 (ref. 10), is acidic. Since both FMN and FAD are acidic, the removal of either from the reductase should decrease the acidity of the protein. It was thus surprising that both apoproteins eluted later from the column than did the holoprotein. One explanation is that the hydroxyapatite, which binds both acidic and basic proteins[11], binds a basic surface of the acidic

reductase. The reductase, though, does bind to hydroxyapatite as an acidic species on hydroxyapatite, since calcium chloride (5, 10 and 25 m*M*), which elutes only basic proteins[11], does not elute adsorbed reductase (not shown). Thus, the removal of either acidic flavin results in either apoenzyme behaving as if it were more acidic. This apparent contradiction indicates that the HPHT system does not resolve the species by differences in their total charge. Rather, the crystalline matrix resolves the species by detecting conformational changes induced by the removal of either flavin. The ability of hydroxyapatite to bind species by their net charge, but to resolve them based upon conformational differences, has been extensively utilized in the preparation and analysis of nucleic acids[12]. Similarly, denatured proteins bind more weakly to hydroxyapatite than do native structures[11].

Since the removal of prosthetic groups always confers some conformational change, it is likely that the HPHT system can be utilized to purify other apoproteins. Other situations in which there are few or no differences between the primary structures of two proteins, but in which conformational differences do exist, may also be amenable to purification or characterization by the HPHT system.

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