Purification and characterization of 25-hydroxyvitamin D_3 1α-hydroxylase from rat kidney mitochondria

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Abstract We purified extensively 25-hydroxyvitamin D_3 1α-hydroxylase (calcidiol, NADPH: oxygen oxidoreductase (1-hydroxylating), EC 1.14.13.13) from kidney mitochondria of rachitic rats and disclosed its peculiar properties as a P450. The final preparation was identified as a 55 kDa protein having an intense absorption at 417 nm characteristic of P450. The specific activity was 4.8 nmol/min/mg of protein indicating a 350-fold purification. Specific content of P450 was 1.1 nmol/mg of protein and turnover number was 4.4 min^{-1}.

Key words: 25-Hydroxyvitamin D_3; 1α-Hydroxylase; P450; CHAPS; Rat kidney

1. Introduction

25-Hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase) catalyzes 1α-hydroxylation of 25-hydroxyvitamin D_3 [1]. In 1974, Henry et al. [2] showed evidence that the enzyme was a P450. Although purification of this enzyme has been attempted from mitochondria of the chicken and pig kidney, convincing evidence of even partial purification of the enzyme has been scarce. Owing to the paucity and extreme lability of the enzyme, purification has been considered as a largely intractable problem [3].

So far, purification of the enzyme has not been attempted from the rat because of the inhibitory factor(s) in mammalian tissues [4]. Contrary to these predecessors, we chose the rat kidney as the enzyme source, because we considered that any inhibitor(s) existing in the starting material might be removed during the course of purification. This was proved to be true. In this experiment we purified the enzyme to an extreme extent and elucidated the peculiar properties of the enzyme.

2. Materials and methods

2.1. Materials

Pepstatin and leupeptin were purchased from Wako (Osaka, Japan), CHAPS from Sigma (St. Louis, MO), hydroxyapatite from Bio-Rad (Richmond, CA), octyl-Sepharose CL-4B from Pharmacia (Uppsala, Sweden), TSK-gel DEAE-5PW and Toyopearl HW-40 from Tosoh (Tokyo, Japan), and PEG 6000 from Nacalai (Kyoto, Japan). All other reagents were of the highest grade commercially available. Adrenodoxin and NADPH-adrenodoxin reductase were prepared according to the methods of Suhara et al. and Hiwatashi et al., respectively [5,6]. Vitamin D-deficient rat diets were from Harlan Teklad (Madison, WI).

2.2. Animals

Weanling male rats (Sprague-Dawley strain) were fed the vitamin D-deficient diet containing replete calcium (for 4 weeks) and deplete calcium (for another 2 weeks) according to the method of Paulson and DeLuca [7].

2.3. Preparation of a mitochondrial and nuclear fraction

All buffer solutions used in the following procedure contained 20% glycerol, 1 mM DTT, 1 mM EDTA, and 2 μg/ml each of pepstatin and leupeptin unless otherwise stated. Mitochondria were prepared from twenty rachitic rats according to the method described elsewhere (Eto et al., to be published).

2.4. Enzyme purification

The above preparation was suspended in 100 mM potassium phosphate buffer (pH 7.4) containing the above reagents, to which 10% CHAPS was added dropwise to give a final CHAPS/protein ratio of 1:1 (w/w) under mild stirring on ice. After this, the mixture was kept stirring for another 30 min and then centrifuged at 100 000×*g* for 1 h. The supernatant was fractionated with 50% PEG according to the method described by Coon et al. [8]. A fraction precipitated with 8–15% PEG was re-dissolved in the phosphate buffer (pH 7.4) containing 0.2% CHAPS. The re-dissolved solution was diluted with 20% glycerol containing 1 mM EDTA, 1 mM DTT, and 2 μg/ml each of pepstatin and leupeptin to bring concentration of the phosphate buffer to 40 mM and that of CHAPS to 0.08%. The diluted enzyme solution was then immediately applied to a hydroxyapatite column (2.5×10 cm) that had been equilibrated with 40 mM phosphate buffer containing 0.08% CHAPS (EDTA was omitted). The column was washed with the equilibration buffer. The non-bound fraction that contained enzyme activity was pooled and concentrations of the buffer and CHAPS were increased to 100 mM and 0.2%, respectively, by adding 0.5 M potassium phosphate buffer (pH 7.4) containing 1% CHAPS. Then, the solution (ca. 90 ml in total) was applied to an octyl-Sepharose CL-4B column (1.5×10 cm) equilibrated beforehand with 100 mM phosphate buffer containing 0.2% CHAPS. The column was subsequently washed with the equilibration buffer, 40 mM phosphate buffer containing 0.2% CHAPS, and 20 mM phosphate buffer containing 0.5% CHAPS. Proteins were eluted with the same buffer except CHAPS concentration (1%). The effluents showing the enzyme activity were pooled. The buffer was exchanged to 30 mM Tris-HCl (pH 7.4) containing 0.5% CHAPS using a Toyopearl HW-40 column. A half aliquot of the fraction was applied to a TSK-gel DEAE-5PW column (7.5×150 mm) equilibrated beforehand with the same buffer. The column was washed with the equilibration buffer and proteins were eluted with linear gradients of both NaCl (0–0.1 M, from 0 to 20 min; 0.1–0.3 M, from 20 to 140 min) and CHAPS (0.5–0.8%, from 0 to 20 min; 0.8–1.4%, from 20 to 140 min). The flow rate of the anion exchange column chromatography was 0.4 ml/min, and effluents were monitored at 417 nm and 280 nm.

All the above procedures were carried out in the cold (0–4°C) and any fractions containing enzyme activity were stored at −30°C during intervals between operations.

2.5. Enzyme assay

1α-Hydroxylase was assayed by a reconstitution method using saturating amounts of adrenodoxin and NADPH-adrenodoxin reductase

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Abbreviations: DTT, dithiothreitol; CHAPS, 3[(3-cholamidopropyl)-dimethylammonio]1-propanesulfonate; PEG, polyethylene glycol

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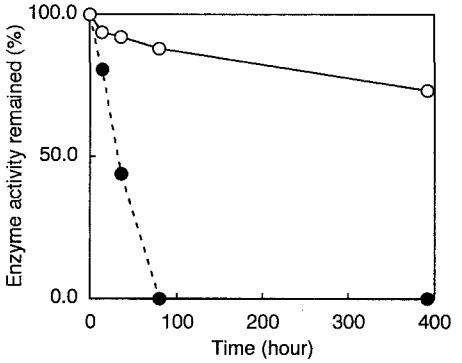

Fig. 1. Stability of 1α-hydroxylase. The 100 000×*g* supernatant of the solubilized suspension was divided into two fractions, of which one was stored at 4°C (●) and the other at −30°C (○). Aliquots of each fraction were subjected to enzyme assay after definite time intervals.

as the electron transferring proteins according to the method of Eto et al. (to be published).

2.6. Other methods

Protein concentration was determined according to the method of Lowry et al. [9] using bovine serum albumin as the standard. Buffer-change and desalting were carried out by gel filtration chromatography using a Toyopearl HW-40 column. P450 concentration was determined from absorption at 417 nm assuming an extinction coefficient of 100 mM^{-1} cm^{-1}. SDS-PAGE was performed according to the method of Laemmli [10].

3. Results

3.1. Enzyme stability

In the early stage of purification, the enzyme seemed to be more labile than other P450s. We therefore tried to find a condition at which the enzyme is stabilized using the 100 000×*g* supernatant of the solubilized mitochondria. As shown in Fig. 1, keeping the 100 000×*g* supernatant in the cold (0–4°C) resulted in rapid loss of the enzyme activity. In contrast, when it was kept at −30°C, the enzyme loss was markedly lessened. The enzyme activity loss was dependent on temperature. A decrease of 30 degrees in temperature lessened the enzyme activity loss to one eighth. The final preparation was more stable standing at −30°C for six months or longer without significant loss of enzyme activity.

3.2. Purification of 25-hydroxyvitamin D_3 1α-hydroxylase

Purification of the enzyme was performed as described in Section 2. Hydroxyapatite column chromatography was carried out under a condition that the enzyme was not adsorbed but eluted in the non-bound fraction. In contrast, many other proteins and nucleic acids were adsorbed to the column, resulting in a significant increase of specific activity at this stage. This method was found to be much better than the conventional one in which PEG was removed before chromatographies. The non-bound fraction was then applied to an octyl-Sepharose column. The column was then washed stepwise with three different buffers. The enzyme was eluted with 20 mM phosphate buffer containing 1% CHAPS. The elution profile is shown in Fig. 2A. As shown in the figure, the enzyme was eluted as a single peak. A half aliquot of the effluents showing the enzyme activity was subjected to HPLC using a DEAE-5PW column. Proteins were eluted with linear gradients of both NaCl and CHAPS (Fig. 2B). The enzyme was eluted as a single peak at an elution volume corresponding to 0.15 M NaCl and 1% CHAPS. This peak coincided with one of the peaks depicted by absorption at 417 nm. The effluents showing enzyme activity were pooled and stored at −30°C.

The final preparation catalyzed 1α-hydroxylation. Specific activity of the partially purified enzyme was 4.8 nmol of product formed/min/mg protein. Specific P450 content was 1.1 nmol/mg of protein and turnover number 4.4 min^{-1}. Table 1 summarizes the results of the purification procedures. Overall purification from the solubilized mitochondria was 350-fold. The purified enzyme was, however, still inhomogeneous judging from the SDS-polyacrylamide gel electrophoretogram, which showed few bands in addition to the one at 55 kDa (data not shown). The 55 kDa protein was found only in the chromatographic fractions that showed the enzyme activity. It was therefore identified as 1α-hydroxylase.

When the method was slightly modified, a preparation

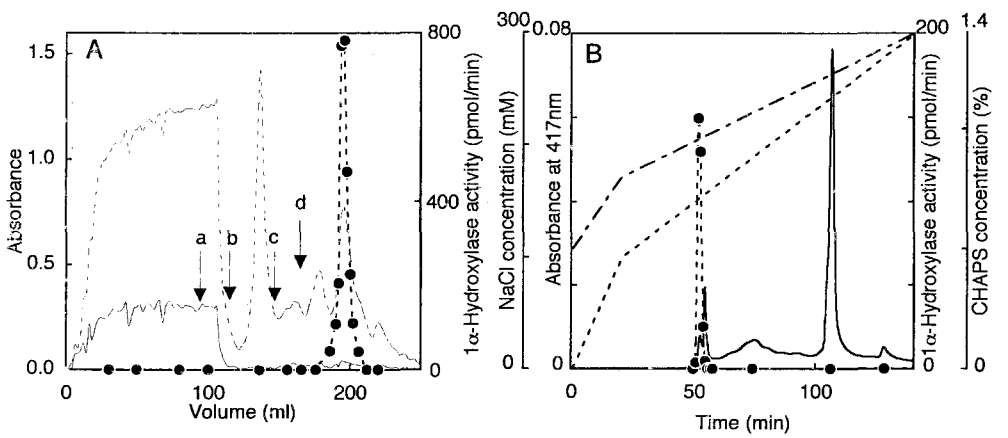

Fig. 2. Elution profile of 1α-hydroxylase of rat kidney mitochondria from an octyl-Sepharose CL-4B (A) and that from an HPLC (B) column. The enzyme solution applied to the octyl-Sepharose column was the pass through fraction from the hydroxyapatite column and that applied to the HPLC column was the eluate showing the enzyme activity from the octyl-Sepharose column. A: Buffers were changed at elution volume indicated by arrows (a, 100 mM KPb and 0.2% CHAPS; b, 40 mM KPb and 0.2% CHAPS; c, 20 mM KPb and 0.5% CHAPS; d, 20 mM KPb and 1% CHAPS). Enzyme was assayed with 20 μl of the effluent. ●, 1α-hydroxylase activity; ——, absorbance at 417 nm; - - -, absorbance at 280 nm. B: The enzyme was assayed using 20 μl of the effluent. ●, 1α-hydroxylase activity; ——, absorbance at 417 nm; — —, NaCl concentration; — - —, CHAPS concentration.

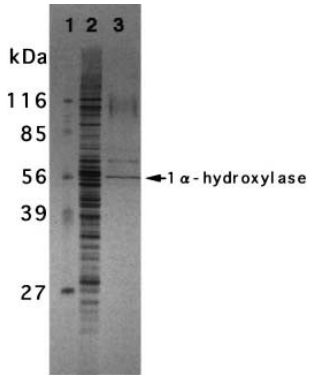

Fig. 3. Electrophoretogram of the solubilized kidney mitochondria of rachitic rats and the partially purified 1α-hydroxylase. Samples underwent electrophoresis on a 12% SDS-polyacrylamide gel and were stained with silver. Lane 1, molecular weight standards (β-galactosidase: M_r 116400; fructose-6-phosphate kinase: M_r 85200; glutamate dehydrogenase: M_r 55600; aldolase: M_r 39200; triosephosphate isomerase: M_r 26600); lane 2, 100000×g supernatant of the solubilized mitochondria; lane 3, the sample obtained by the modified method (see text). The amount of proteins applied were 3.0 μg and 0.6 μg in lanes 2 and 3, respectively.

showing fewer bands in addition to the major one at 55 kDa was obtained (Fig. 3). Modification was as follows: A fraction precipitated between 8–18% (instead of 15%) PEG was subjected to hydroxyapatite chromatography. The effluent showing enzyme activity was then applied to the octyl-Sepharose column. The enzyme was eluted with 20 mM phosphate buffer containing 0.5% CHAPS (without washing the column with equilibration buffer). The enzyme eluted just after the pass through fraction was subjected to anion exchange HPLC.

3.3. *Reconstitution of the enzyme activity*

1α-Hydroxylase activity was reconstituted with the partially purified enzyme, adrenodoxin, NADPH-adrenodoxin reductase, and NADPH. The omission of either the enzyme or the electron transferring proteins resulted in complete loss of activity.

3.4. *Absorption spectrum*

The absorption spectrum of the final preparation is shown in Fig. 4. An intense peak was observed at 417 nm which was recognized to correspond to the γ band of P450. In contrast, this preparation did not show any absorption at 403 nm. The result was at variance with that of Mandel et al. [11] who observed an intense peak at 403 nm in an absorption spectrum of the 1α-hydroxylase purified from the rachitic chicken kidney.

4. Discussion

Recently, we found that an inhibitory factor(s) for 1α-hydroxylase in the rat kidney could be largely removed from the solubilized mitochondria by ultracentrifugation, and established a sensitive assay method of the enzyme (Eto et al., to be published). An attempt to purify the enzyme from kidney mitochondria of rachitic rats employing this new assay method was, however, severely hampered by high lability of the enzyme not experienced before. Enzyme activity disappeared during the course of purification. In due course, we found that the lability was affected by several factors. (1) The solubilizing agent: Because P450 is a membrane-bound enzyme, it should be solubilized by any detergent before purification. It was found that cholate, widely used for solubilizing P450s, was not adequate because it inhibited markedly the enzyme activity. (2) Detergents: It is well known that detergent is required for keeping hydrophobic proteins in the solubilized state during purification. Without detergent, hydrophobic proteins easily adhere to glassware's or column contents. However, none of the detergents usually applied in the purification of many P450s, e.g. Lubrol, Emulgen, was adequate. The detergents inactivated the enzyme almost completely. (3) Some unknown factor(s) deteriorating the enzyme: Although the factor(s) seemed to be somewhat like proteinase, inactivation was scarcely suppressed by increasing the amount of proteinase inhibitors such as pepstatin and leupeptin. (4) Dialysis: Dialysis was found to be fatal to the enzyme activity. In fact, dialysis overnight in the cold destroyed more than 90% of the enzyme activity, though it was not clear whether dialysis itself or keeping the solution in the cold caused deterioration. The fact that the enzyme is affected by these factor(s) seems to go against common sense in P450 purification. The enzyme would not have been purified if one followed the generally established method for purifying P450s. These unusual properties together with the extreme low abundance of the enzyme may be the reasons why the enzyme has not been purified for such a long time since it was found in 1970 [12].

We either solved or evaded these problems as follows: The first problem was already solved in this laboratory by employing CHAPS as a solubilizing agent (Eto et al., to be published). It solubilized mitochondria effectively without inhibiting enzyme activity. The second problem was solved also by using CHAPS, which was effective in keeping the enzyme solubilized. The third problem was circumvented by carrying out all the operations within a short time and keeping any fractions containing the enzyme at −30°C during intervals between operations (e.g. at night and on weekends). The fourth problem was evaded by applying a gel filtration technique using a Toyopearl column instead of dialysis. Thus, we

Table 1
Partial purification of 1α-hydroxylase from kidneys of rachitic rats

Preparation	Total protein (mg)	1α-Hydroxylase		Total P450 (pmol)
		Total activity (pmol/min)	Specific activity (pmol/min/mg)	
Solubilized mitochondria	1460	20300	13.9	
PEG (8–15%)	359	13600	37.9	
Hydroxyapatite	38.5	9060	235	
Octyl-Sepharose	5.60	2540	453	
DEAE-5PW	0.16[a]	767	4790	176[b]

[a]Determined from absorption at 280 nm assuming an extinction coefficient as 1 $(mg/ml)^{-1}$ cm^{-1}.
[b]Specific P450 content calculated from absorbance at 417 nm in Fig. 4 and that at 280 nm was multiplied by the total protein.

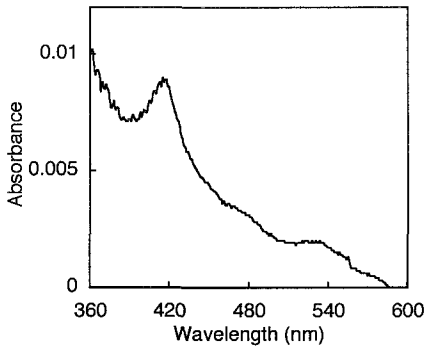

Fig. 4. Absorption spectrum of the oxidized form of the partially purified 1α-hydroxylase. Protein concentration was 8 μg/100 μl in 30 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol.

solved or evaded these problems. Then, purifying 1α-hydroxylase was reduced to a matter of purification of a common P450 though the enzyme is scarce. Finally, the enzyme was purified 350-fold. Judging from SDS-PAGE, however, the final preparation was still inhomogeneous, revealing few bands on the electrophoretogram. Yet, the fact that the protein showing a band at 55 kDa was found only in the chromatographic fractions which had the enzyme activity established that 1α-hydroxylase is a 55 kDa protein. This value is similar to the molecular weight reported for the enzymes of the chicken, rat, and mouse, though their methods were different from ours [13–15]. When purification was slightly modified, a preparation showing better electrophoretogram was obtained. However, the modified method was rather capricious, at times resulting in lower specific activity and yield. Accordingly, this method was not adopted as a standard method. Further attempts to purify the final preparation of either method resulted in considerable enzyme loss.

In conclusion, we have extensively purified 1α-hydroxylase of rat kidney mitochondria and isolated a 55 kDa protein showing the Soret band at 417 nm characteristic of P450 and elucidated the peculiar properties of the enzyme. It is not clear whether the unusually rapid decrease in enzyme activity is related to the regulation of the enzyme. It may also be interesting that in purifying this enzyme several innovations were adopted, some of which might be applicable for purifying other enzymes which resist being purified.

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