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# Distribution of 4a-hydroxytetrahydropterin dehydratase in rat tissues

## Comparison with the aromatic amino acid hydroxylases

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A 4a-carbinolamine intermediate is generated stoichiometrically during the tetrahydrobiopterin-dependent phenylalanine hydroxylation reaction catalyzed by phenylalanine hydroxylase. The dehydration of the carbinolamine is catalyzed by the enzyme, 4a-hydroxytetrahydropterin dehydratase. We have now examined the distribution of the dehydratase activity in various rat tissues by activity measurements and by immunoblot analysis to explore the possibility that the dehydratase may also play a role in tyrosine and tryptophan hydroxylation. The only two tissues that express relatively high dehydratase activity are liver and kidney, which are also the only two tissues that express phenylalanine hydroxylase activity. The dehydratase activity was generally very low in those tissues which contain high levels of tyrosine and tryptophan hydroxylase activity, except for the pineal gland. These results suggest that the dehydratase may not play an important role in the regulation of the synthesis of those neurotransmitters which are derived from the hydroxylated aromatic amino acids.

Phenylalanine; Hyperphenylalaninemia; Tetrahydrobiopterin; 7-Biopterin; Phenylalanine hydroxylase; 4a-Carbinolamine dehydratase; 4a-Hydroxy tetrahydrobiopterin

## 1. INTRODUCTION

Phenylalanine hydroxylase catalyzes the tetrahydrobiopterin ($BH_4$)-dependent conversion of phenylalanine to tyrosine, the rate-limiting step in the catabolism of phenylalanine to carbon dioxide and water [1] (equation (1)).

$$\text{Phenylalanine} + O_2 + BH_4 \rightarrow \text{Tyrosine} + BH_3OH \quad (1)$$

The tetrahydropterin cofactor is stoichiometrically oxidized to a carbinolamine, 4a-hydroxytetrahydrobiopterin ($BH_3OH$), and must be regenerated to function catalytically [2]. Two additional enzymes and a reduced pyridine nucleotide play a role in this regeneration: 4a-carbinolamine dehydratase, which catalyzes the dehydration of $BH_3OH$ to quinonoid dihydrobiopterin, and an NADH- dependent dihydropteridine reductase, which catalyzes the reduction of quinonoid dihydrobiopterin back to $BH_4$. Little is known about the dehydratase beyond its mechanism of action, which was elucidated in studies carried out with the pure rat liver enzyme [2,3]. The tissue distribution has not been reported. The other aromatic amino acid hydroxylases, tyrosine and tryptophan hydroxylase, have a high degree of homology with phenylalanine hyroxylase and catalyze similar $BH_4$-dependent hydroxylation reactions [1]. To date, however, there have been no studies on the role of the 4a-carbinolamine dehydratase in the regeneration of the cofactor after the hydroxylation of tyrosine and tryptophan.

We have now determined the distribution of the dehydratase in various rat tissues, including different areas of the brain. The results of these studies may have a bearing on the potential role of the dehydratase in the regulation of tyrosine and tryptophan hydroxylase activities.

*Abbreviations:* $BH_4$, 6-[dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin (tetrahydrobiopterin); $BH_3OH$, 4a-hyd oxytetrahydrobiopterin (4a-carbinolamine).

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## 2. MATERIALS AND METHODS

L-Phenylalanine, L-tyrosine, NADH, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) were purchased from Sigma. L-[U-$^{14}$C]phenylalanine (17.1 GBq/mmol) was purchased from Amersham. Beef liver catalase was from Boehringer-Mannheim. Sheep liver dihydropteridine reductase was purified through the calcium phosphate gel step as described [4]. Phenylalanine hydroxylase was purified from rat liver and activity measured as previously described [5]. (6R)-$BH_4$ was obtained from B. Schirks' Laboratory (Jona, Switzerland). 4a-Carbinolamine dehydratase was purified to homogeneity from rat liver [6] and its activity determined by minor modifications of the method described by Huang et al. [6] which measures the stimulation of the $BH_4$-dependent rate of phenylalanine hydroxylation under conditions where the rate is limited by the breakdown of the carbinolamine intermediate. The endogenous hydroxylase activity in crude extracts was measured for each tissue sample and the appropriate corrections were made.

Polyclonal antibodies were raised in rabbits by subcutaneous injections of pure dehydratase in complete Freund's adjuvant. Four injections were made at 9–10 day intervals. Six weeks after the last boost, a final injection was administered. 40 μg of either pure dehydratase or gel slices containing the purified protein were used as antigens. The antibody was purified from serum by precipitation with 0.66 vol. of saturated ammonium sulfate followed by affinity chromatography on a dehydratase-AminoLink column (Pierce).

## 3. RESULTS AND DISCUSSION

The 4a-carbinolamine dehydratase was originally detected as a contaminant in several partially purified liver enzyme preparations on the basis of its ability to stimulate the $BH_4$-dependent activity of phenylalanine hydroxylase [7,8]. The stimulator protein was subsequently purified to homogeneity from rat liver and its stimulating activity was shown to be due to the catalysis of the dehydration of the 4a-carbinolamine intermediate generated during the $BH_4$-dependent hydroxylation reaction [2,3,9]. The dehydratase activity was also shown to be present in human liver [6] but its distribution in other tissues was not examined at that time. Since the other aromatic amino acid hydroxylases, tyrosine hydroxylase and tryptophan hydroxylase, catalyze $BH_4$-dependent hydroxylation reactions which presumably also generate the same 4a-carbinolamine intermediate, it might be expected that the dehydratase could play the same role in the formation of quinonoid dihydrobiopterin with these two hydroxylases as it does with phenylalanine hydroxylase. However, the possible stimulating effect of the dehydratase on in vitro activity of either tryptophan or tyrosine hydroxylase has not yet been reported.

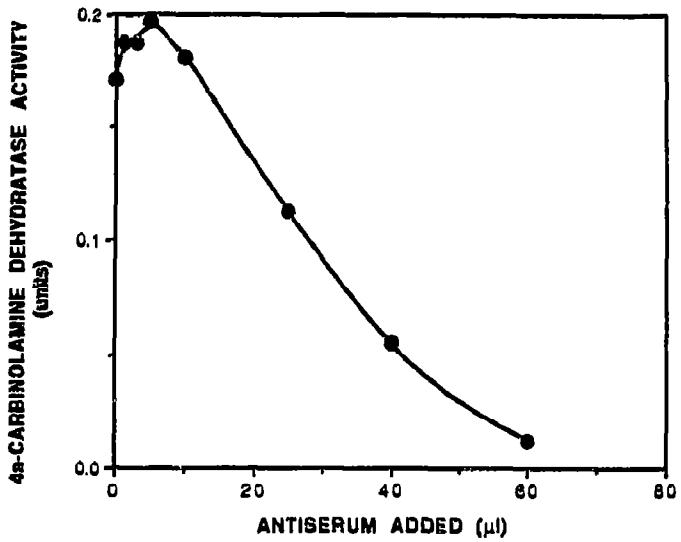


Fig. 1. Immunotitration of 4a-carbinolamine dehydratase activity in rat liver extracts. Rat liver extracts (25 μl) were incubated overnight at 4°C with increasing volumes of rabbit antiserum. Control rabbit antiserum was added to maintain the total volume of rabbit serum at 100 μl. The samples were assayed by the continuous spectrophotometric assay [6].

Table I

The 4a-carbinolamine dehydratase activity in various rat tissues

| Tissue | Relative dehydratase activity |
|---|---|
| Liver | 100 |
| Kidney | 32.0 |
| Pineal | 14.4 |
| Pituitary | 6.3 |
| Hypothalamus | 4.0 |
| Adrenal medulla | 2.6 |
| Pancreas | 2.4 |
| Cerebellum | 2.1 |
| Whole brain | 2.0 |
| Striatum | 1.9 |
| Mid-brain | 1.7 |
| Cortex | 1.6 |
| Heart | 1.5 |
| Spleen | 1.4 |
| Lungs | 1.4 |
| Medulla-pons | 1.3 |
| Hippocampus | 1.3 |
| Ovaries | 0.6 |
| Adrenal cortex | 0.4 |

The dehydratase activity in each rat tissue extract (1 g/4 ml) was determined by measurement of the stimulation of the phenylalanine hydroxylase-dependent conversion of [$^{14}$C]phenylalanine to [$^{14}$C]tyrosine. This assay contained 60 mM Tris–HCl (pH 8.5), 0.1 mg/ml catalase, 0.256 mM NADH, 1.0 mM phenylalanine (0.2 GBq/mmol), 10 mM glucose 6-phosphate, an excess of both glucose 6-phosphate dehydrogenase and dihydropteridine reductase, phenylalaninie hydroxylase (1.4 μmol of tyrosine per min), the indicated extract, and 5 μM (6R)-$BH_4$. The results are expressed relative to the amount of activity found in rat liver and are on a wet weight basis. The dehydratase activity in rat liver was 174 U per g. One U of activity is defined as the amount that stimulates the formation of 1 additional μmol of tyrosine over the amount formed in the absence of stimulator activity in 30 min.

One approach to investigate a potential in vivo role for the dehydratase in the hydroxylation of tyrosine or tryptophan is to determine the distribution of the dehydratase activity in various rat tissues and compare it with the known distribution of aromatic amino acid hydroxylase activities. As expected, the greatest dehydratase activity was found in rat liver and kidney, the only two tissues that contain phenylalanine hydroxylase activity [1,10] (Table I). We also re-confirmed the presence of dehydratase activity in human liver and determined that it contains 30% of the activity found in rat liver (data not shown). Interestingly, rat adrenal gland and rat striatum, tissues with the highest levels of tyrosine hydroxylase activity [11], have very low levels of dehydratase activity. In contrast, the pineal gland, which contains a large amount of tryptophan hydroxylase activity [1], has nearly 50% as much dehydratase activity as is present in the kidney. However, midbrain regions which have similar tryptophan hydroxylase activities as the pineal, were found to be nearly devoid of dehydratase activity. In contrast, significant amounts of dehydratase activity were also found in the pituitary

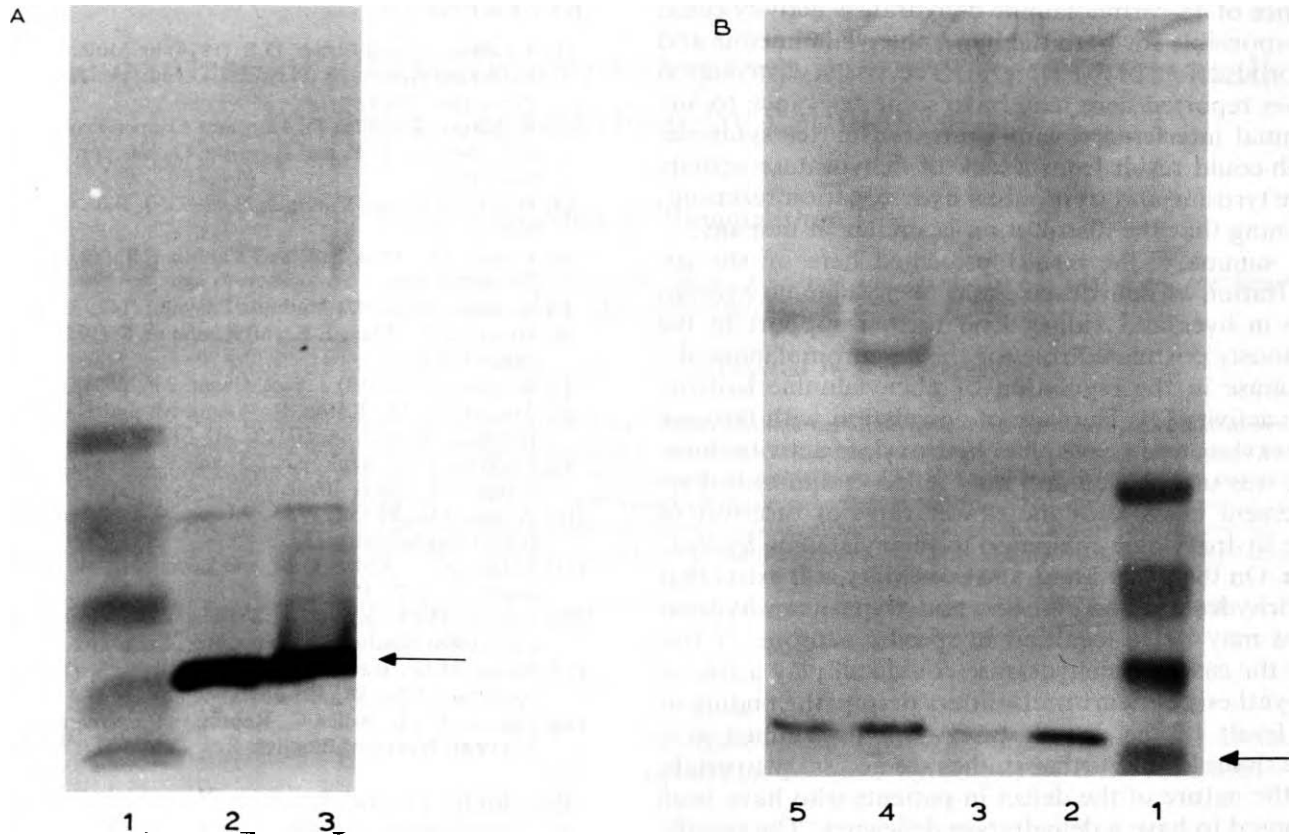


Fig. 2. Immunoblot analyses of 4a-carbinolamine dehydratase in rat tissues and human liver. SDS-PAGE was performed with pre-cast 10% gels (Novex) in a mini-gel apparatus (Novex). Antibody-positive bands were visualized after transblotting by reaction with alkaline phosphatase-conjugated goat anti-rabbit Fc (Promega Biotec) according to the manufacturers directions. (A) lane 1, pre-stained standards (Integrated Separations Systems) of molecular weights (kDa, from top to bottom) 20.4, 17.0, 14.4, 8.2, 6.2; lane 2, 1.9 μg of pure dehydratase; lane 3, 5 μl of rat liver extract. (B) lane 1, molecular weight standards; lane 2, 0.4 μg of pure dehydratase; lane 3, 2.5 μl of human liver extract; lane 4, 2.5 μl of rat kidney extract; lane 5, 5.0 μl of rat pineal extract. The arrows indicate the position of the 12.3 kDa subunit of the pure dehydratase. All tissues were extracted at a weight-to-volume ratio of 1:3.

gland and in the hypothalamus, two areas of the brain which are relatively low in hydroxylase activity. In fact, dehydratase activity above background levels could be detected in all tissues that were examined. However, it must be stressed that because of the known rapid spontaneous and non-specific catalytic dehydration of the carbinolamine [2], the very small amounts of activity detected in some rat tissues may not be solely due to the catalytic activity of the 4a-carbinolamine dehydratase itself.

In order to further probe this question, an antibody was prepared against pure rat liver carbinolamine dehydratase and used as a specific inhibitor. This antibody was effective at completely inhibiting the dehydratase activity in crude rat liver extracts as shown in Fig. 1, suggesting that the dehydratase is responsible for all of the measured activity in rat liver. Furthermore, the antiserum inhibited the dehydratase activity of kidney, pineal, pituitary, and hypothalamus extracts (data not shown). Other tissues with very low activity were not tested.

Nitrocellulose blots of pure rat liver dehydratase and of rat liver extracts were probed with affinity-purified anti-dehydratase antibody as shown in Fig. 2A. A major positive band was found which corresponded with the Coomassie blue-stained peptide at 12.3 kDa of the pure dehydratase (not shown). This molecular weight is in excellent agreement with the molecular weight of the subunit determined previously [6]. As shown in Fig. 2B, extracts from human liver, rat kidney, and rat pineal gland also contain detectable amounts of anti-dehydratase antibody positive material at 12.3 kDa. There were correspondingly fainter bands with the same molecular weight detectable in pituitary as well as in adrenal medulla extracts, in agreement with the results of the direct activity measurements shown in Table I. We could not detect stimulating activity or Western-positive material in primary human fibroblasts or in human blood cells, limiting the usefulness of this technique for routine genetic screening purposes.

Recently, a new form of hyperphenylalaninemia has been described which is characterized by an increased excretion of the 7 isomer of biopterin [12]. Our laboratory [13], as well as others [14], has suggested that an

absence of 4a-carbinolamine dehydratase activity could be responsible for both the hyperphenylalaninemia and the production of 7-biopterin. The tissue distribution studies reported here may have some relevance to any potential interference with neurotransmitter syntheses which could result from a lack of dehydratase activity in the tyrosine and tryptophan hydroxylation reactions, assuming that the distribution is similar in humans.

In summary, the results presented here on the co-localization of dehydratase and phenylalanine hydroxylase in liver and kidney lend further support to the previously postulated role for the 4a-carbinolamine dehydratase in the regulation of phenylalanine hydroxylase activity [3]. The lack of correlation with tyrosine hydroxylase and tryptophan hydroxylase activity, however, was unexpected and may reflect a diminished requirement because of the slower rates of turnover of these hydroxylases compared to phenylalanine hydroxylase. On the other hand, the possibility still exists that the dehydratase and tyrosine and tryptophan hydroxylases may be co-localized in specific neurons. If this were the case, the dehydratase could still play a role in the synthesis of neurotransmitters despite the finding of low levels of the dehydratase when determined at a gross tissue level. Further studies are necessary to establish the nature of the defect in patients who have been proposed to have a dehydratase deficiency. The specific antibody that was generated for these studies should be useful to obtain cDNA for further human genetic screening and confirmation of diagnoses.

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