Radiation target analysis indicates that phenylalanine hydroxylase in rat liver extracts is a functional monomer

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Abstract The minimal enzymatically functional form of purified rat hepatic phenylalanine hydroxylase (PAH) is a dimer of identical subunits. Radiation target analysis of PAH revealed that the minimal enzymatically active form in crude extracts corresponds to the monomer. The 'negative regulation' properties of the tetrahydrobiopterin cofactor in both crude and pure samples implicates a large multimeric structure, minimally a tetramer of PAH subunits. Preincubation of the samples with phenylalanine prior to irradiation abolished this inhibition component without affecting the minimal functional unit target sizes of the enzyme in both preparations. The characteristics of rat hepatic PAH determined by studies of the purified enzyme in vitro may not completely represent the properties of PAH in vivo.
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Key words: Phenylalanine hydroxylase; Radiation inactivation; Functional size

1. Introduction

Phenylalanine hydroxylase (PAH) catalyzes the conversion of phenylalanine to tyrosine. Defects in PAH result in phenylketonuria in humans which leads to severe mental retardation if untreated. PAH purified from rat liver has a greater than 90% amino acid sequence homology with human PAH [1]. Rat liver PAH has been extensively studied, and has provided most of our knowledge to date of the structure-function relationships of this enzyme.

PAH purified from rat liver is an oligomeric enzyme comprised of subunits identical in primary structure [1]. We have previously shown that the minimal enzymatically active form of purified PAH is a dimer [2–4]; no evidence for an enzymatically active monomer of purified PAH has been found. Radiation inactivation studies [3,4] enabled us to derive a detailed model for the effects of phenylalanine substrate and tetrahydropteridine cofactors on the subunit interactions of PAH purified from rat liver.

While purified rat hepatic PAH has been extensively studied, the structure-function relationships of the enzyme in crude extracts have been only poorly characterized. However, previous studies from our laboratories implied that some differences might be expected between purified PAH and the enzyme in rat liver crude extracts [5,6]. Since the radiation target analysis technique can be applied equally well to the study of structural forms of pure enzymes as well as enzymes in crude extracts, we employed this procedure to compare the functionally active PAH structure in the two environments.

2. Materials and methods

Rat livers were obtained either from newly killed animals or purchased frozen on Dry Ice from PelFreez Biologicals. Livers were stored at −80°C until use. Similar data were obtained with extracts prepared from both sources. All procedures with live animals were carried out in agreement with guidelines established by NIH and by the Canadian Council for Animal Care. Frozen livers were placed in three volumes of 30 mM Tris-HCl (pH 7.6, 4°C) containing 0.2 M KCl and homogenized on ice for 1 min using a Polytron Homogenizer (Brinkman). The homogenates were centrifuged at $30\,000\times g$ for 45 min; the supernatants were recovered and transferred to ampoules at total protein concentrations significantly higher than used previously for purified PAH [3,4]. Samples were quick-frozen on Dry Ice and stored at −80°C. The samples were maintained at −80°C during shipments, irradiated at −135°C, and were rapidly thawed just before use for PAH assay. 6-Methyl-5,6,7,8-tetrahydropterin ($6MPH_4$) and tetrahydrobiopterin (BH_4) were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland).

PAH activity was measured at pH 6.8 in an assay mix that included final concentrations of 0.1 M potassium phosphate (pH 6.8), 100 μg/ml catalase, 12.5 mM glucose 6-phosphate, 21 units glucose 6-phosphate dehydrogenase, 0.26 mM NADH, and excess of partially purified dihydropteridine reductase (Sigma). In assays with the $6MPH_4$ cofactor, 10 mM phenylalanine and 0.25 mM $6MPH_4$ were also provided. In assays with the natural cofactor BH_4, 1 mM phenylalanine and 0.025 mM $(6R)BH_4$ were included. All assays were carried out at 25°C for 30 min; reactions were then quenched by the addition of cold trichloroacetic acid to 7.5% final concentration. Tyrosine formation was measured by the spectrophotometric/fluorometric nitrosonaphthol assay [7].

Methods for irradiation and analysis of radiation data were as previously described [3,4,8].

3. Results

Residual PAH activity in rat liver crude extracts after exposure to radiation was assayed with both the natural cofactor tetrahydrobiopterin (BH_4) and the synthetic cofactor 6-methyltetrahydropterin ($6MPH_4$). The $6MPH_4$-dependent activity of rat liver PAH is relatively unaffected by activating agents and is therefore a reasonable measure of the total phenylalanine hydroxylating activity of a given system. The radiation-induced loss of the $6MPH_4$-dependent PAH activity in crude extracts of rat liver was described by a simple exponential (Fig. 1). These data imply a functional target size of

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Abbreviations: BH_4, tetrahydrobiopterin (6*R*-dihydroxypropyl-L-erythro-5,6,7,8-tetrahydropterin); $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin; PAH, phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1)

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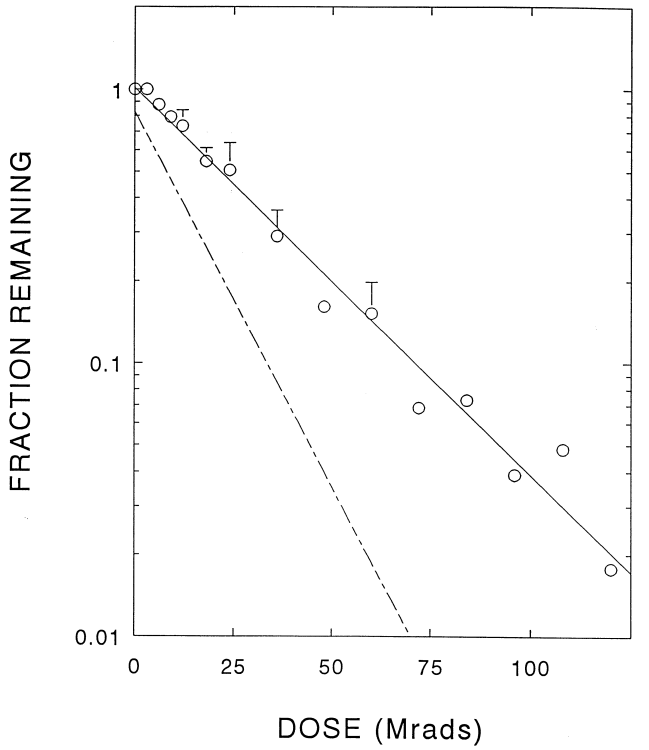


Fig. 1. Surviving activity of PAH in irradiated samples. Crude extracts were assayed with $6MPH_4$ cofactor. Data combined from three independent experiments. Broken line is from data obtained with purified enzymes [3].

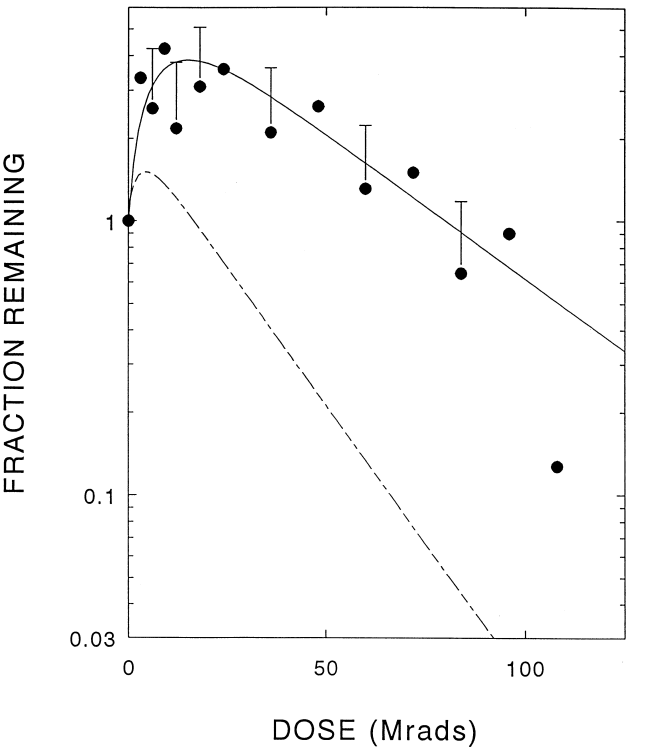


Fig. 2. Surviving activity of PAH in irradiated samples. Crude extracts were assayed with BH_4 cofactor. Data from three independent experiments. Broken line is from data obtained with purified enzymes [3].

63 ± 15 kDa for PAH activity in rat liver, comparable to the size of the PAH subunit [1,9].

The BH_4-dependent PAH activity in crude extracts of rat liver showed a complex response to irradiation (Fig. 2). As was previously observed with pure PAH [3], exposure to low amounts of radiation resulted in increases in BH_4-dependent activity in the extracts, whereas subsequently increasing levels of radiation resulted in a loss of BH_4-dependent PAH activity that was described by a simple exponential function of radiation dose (Fig. 2). The final slope yields the size of the enzymatically active structure (44 ± 3 kDa), whereas the initial rise is believed due to the destruction of a larger inhibitory structure. When the inactivation of BH_4-dependent PAH activity was analyzed as the difference of two exponentials [3,8], the PAH activity in crude extracts of rat liver followed a very different inactivation profile than that of PAH which had been purified from this same source (Fig. 2). The calculated target sizes are given in Table 1.

Preincubation (25°C, 30 min) of the rat liver crude extracts with phenylalanine prior to radiation exposure did not affect the $6MPH_4$-dependent activity of PAH, a result that is similar to our previous observations with the purified enzyme [3,4] (Table 1). However, the BH_4-dependent PAH activity was significantly altered after preincubation with phenylalanine (Fig. 3). The original complex response to radiation exposure was replaced by a simple single exponential decrease in PAH activity, corresponding to a target size equivalent to a single PAH subunit (Table 1).

Table 1
Radiation inactivation target sizes for PAH activity in preparations of crude extracts of rat liver and in preparations of purified enzyme

Sample	Target size (kDa)		
	BH_4 assay		$6MPH_4$ assay
	Small	Large	
No preincubation with phenylalanine			
Crude extract	44 ± 3	435 ± 234	63 ± 15
Pure PAH[a]	96 ± 17	476 ± 206	132 ± 19
Activity ratio (pure/crude)	2.2	1.1	2.1
Preincubated with phenylalanine			
Crude extract	51 ± 15	n.a.	59 ± 14
Pure PAH[b]	123 ± 41	n.a.	122 ± 41
Activity ratio (pure/crude)	2.4		2.1

Values for the crude extracts are the averages ± S.D. of at least three independent experiments.
N.a., not applicable.
[a]Data from Davis et al. [3]; [b]data from Davis et al. [4].

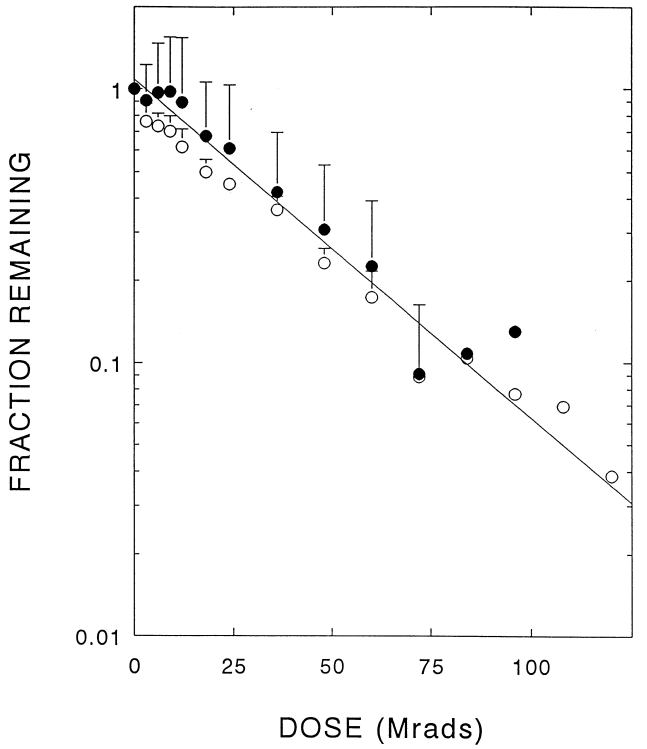


Fig. 3. Surviving activity of PAH in samples preincubated with phenylalanine before irradiation (10 mM, 30 min at 25°C; samples preincubated without phenylalanine served as controls). Crude extracts assayed with $6MPH_4$ (open circles) or BH_4 (closed circles).

4. Discussion

PAH purified from rat liver is an oligomeric enzyme composed of 52 kDa subunits that are identical in primary sequence [1]. Rat PAH possesses one catalytic site per subunit monomer [10–12]; thus, it is conceivable that monomeric PAH might exhibit catalytic activity under appropriate conditions. However, prior studies have shown that the minimum functional unit of the purified enzyme is a dimer; no indication of a functional monomer was found [2–4]. A recent crystal structure of PAH shows that the two monomers of the PAH dimer are intimately entwined at the C-terminus [12]. The dimeric size for PAH activity in purified enzyme preparations, as determined by radiation target analysis, was interpreted to mean that at least two identical polypeptides were required to enable catalysis [3]. Mechanistically this may arise from a requirement for one subunit to stabilize the enzymatically active conformation of a second subunit. The monomeric target size observed in the present study might imply that the PAH monomer in crude extracts does not have this requirement.

However, the 'stabilized conformation' model is not necessarily eliminated. It could be speculated that in a heterogeneous extract, other proteins might substitute and stabilize a PAH monomer. Although radiation would destroy these 'stabilizing' molecules (as well as PAH), these molecules would likely be in large excess relative to PAH. After loss of some of these due to radiation exposure, there would be others to take their place. This of course implies that the 'stabilizing' molecules are freely exchangeable, even after radiation damage.[2] Radiation target analysis of such models has been presented [16]. If these other molecules are not rate-limiting, then the stabilizers will not be detected by radiation inactivation.

Whereas the response of PAH in crude extracts of rat liver to radiation exposure bears a general similarity to that seen with purified enzyme, in every comparison of crude extract and purified samples, a single major difference was seen: the target size of PAH activity in the crude extracts is about 50 kDa, i.e. a monomer, whereas the purified enzyme always yielded a target size of about 100 kDa, which was interpreted to be the homodimer [3,4]. In the purified material, it was shown that although the enzyme activity disappeared as a dimer, the radiation destroyed the individual subunits one at a time. Each primary ionization in a subunit damaged that structure sufficiently that it no longer moved as a monomer on SDS-PAGE. The electrophoretic movement of other subunits was not affected. A single radiation hit must have caused one or more scissions in the polypeptide backbone of a single subunit, but none in any adjacent polypeptides. Lesser effects to the oligomer (damage to amino acid residues, conformational changes) in other subunits is not precluded since these could not be detected by SDS-PAGE.

Incubation of the crude extract with phenylalanine prior to irradiation modifies the radiation response of PAH activity in a manner that parallels the effects noted with the purified enzyme. There is no change in the target size obtained with the $6MPH_4$ assay, the complex response with BH_4 is abolished, and the simple inactivation curve yields the same target size as that observed with $6MPH_4$. In some way, phenylalanine blocks most of the structural modifications caused by BH_4 in both purified [4] and crude samples. However, phenylalanine preincubation did not alter the target size of PAH in the crude extracts; enzyme activity still decayed as a monomer.

Davis et al. [4] proposed a general model for the effects of BH_4 and phenylalanine on the structure/function of purified PAH. In this model, four identical subunits are associated into two dimers, each of which is active enzymatically, but the tetrameric structure is required for BH_4 to act as a negative effector. Phenylalanine can alter the conformation of the PAH enzyme, increasing interactions between the subunits of a dimer and weakening interactions between the dimers in a tetramer; the latter effect prevents BH_4 from decreasing enzymatic activity.

In the crude samples, some features of this model are retained. In both crude and purified material the negative effector role of BH_4 requires a similar-sized structure, possibly a tetramer. However, it is absolutely clear that enzymatic activity is expressed in crude extracts by a single PAH subunit, not by a dimer. The different target sizes for PAH activity found in the crude compared to the purified material (monomer and dimer, respectively) unequivocally shows that purification has in some way modified the structural state of PAH, suggesting a significant alteration of the interaction of the monomers within the PAH tetramer during its purification.

The monomeric target size observed for the crude form of

[2] It was reported [13] that subunits in irradiated protein oligomers did not exchange because the subunits did not dissociate [14,15]. However, this principle cannot be extended to other molecules which may be associated with the protein in crude extracts but are removed during purification.

the enzyme does not require that the individual subunits in the crude enzyme be physically separated. Indeed, the target size for the BH_4 negative effect implies that the crude enzyme is a tetramer or larger. Thus the change in radiation sensitivity observed for the crude form of the enzyme relative to the purified form simply indicates that the monomers in the crude enzyme possess a weaker dimeric character than those in the purified enzyme, i.e. purifying phenylalanine hydroxylase results in the loss of the functional independence of the individual monomers in the tetramer.

It is somewhat surprising that the pretreatment of the crude enzyme with phenylalanine did not measurably increase the dimeric character of the crude enzyme. This further implies that the crude enzyme is significantly different than the purified enzyme.

It was previously shown that certain properties of rat liver PAH were changed during the course of its purification [5,6,17], although the structural changes associated with purification have not been elucidated. Some of the variables that affect the enzymatic activity in crude samples have been identified: dialysis, dilution and phenylalanine stimulate it, whereas BH_4 and certain divalent cations inhibit it [5,6]. It was suggested that PAH activity in crude rat liver extracts was low because the enzyme was complexed with one or more dialyzable inhibitory substances, one of which was a metal and the other may have been BH_4. The present results indicate that the target size of PAH in crude rat liver extracts corresponds roughly to a monomeric species, whereas the target size of the pure enzyme corresponds roughly to a dimeric species. This suggests that in crude samples either the metal or BH_4 (or both) maintain the hydroxylase in a form in which the minimum functional unit is the monomer. Their separation from the enzyme leads to both activation and to stronger interaction between monomers so that the minimum functional unit is now the dimer.

Our investigations of PAH structure and function by radiation inactivation have revealed the molecular mass of the functional enzyme under several different conditions. The data presented in the present report reiterate that caution must be exercised when extending observations made with purified enzymes to speculations about the nature of those enzymes in other states.

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