FEBS Letters 488 (2001) 196–200 FEBS 24513

Essential role of the N-terminal autoregulatory sequence in the regulation of phenylalanine hydroxylase

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Received 6 November 2000; revised 20 December 2000; accepted 20 December 2000

First published online 5 January 2001

Edited by Hans Eklund

Abstract Phenylalanine hydroxylase (PAH) is activated by its substrate phenylalanine and inhibited by its cofactor tetrahydrobiopterin (BH_4). The crystal structure of PAH revealed that the N-terminal sequence of the enzyme (residues 19–29) partially covered the enzyme active site, and suggested its involvement in regulation. We show that the protein lacking this N-terminal sequence does not require activation by phenylalanine, shows an altered structural response to phenylalanine, and is not inhibited by BH_4. Our data support the model where the N-terminal sequence of PAH acts as an intrasteric autoregulatory sequence, responsible for transmitting the effect of phenylalanine activation to the active site.

Key words: Allosteric regulation; Autoregulatory sequence; Intrasteric regulation; Mutagenesis; Phenylalanine hydroxylase

1. Introduction

Phenylalanine hydroxylase (PAH, EC 1.14.16.1) is a metabolic enzyme that converts phenylalanine to tyrosine using molecular oxygen, enzyme-bound iron, and a 6R-tetrahydrobiopterin (BH_4) cofactor [1–5]. PAH is a member of the aromatic amino acid hydroxylase family, together with tyrosine hydroxylase (TH, EC 1.14.16.2) and tryptophan hydroxylase (TPH, EC 1.14.16.4). TH and TPH are involved in the biosynthesis of the neurotransmitters, L-DOPA (dihydroxyphenylalanine) and serotonin, respectively. The aromatic amino acid hydroxylases share a similar enzyme mechanism and have a common three-domain structure consisting of an N-terminal regulatory domain, a catalytic domain and a C-terminal tetramerisation domain; the highest sequence and structural similarity is found in the catalytic domain [6].

The regulatory domains of the aromatic amino acid hydroxylases are not highly conserved, reflecting the different modes of regulation required by these enzymes. PAH controls the level of phenylalanine, an essential amino acid, which is toxic at pathophysiological levels and is subject to large fluctuations due to dietary intake. Accordingly, mutations in the PAH gene lead to the disease phenylketonuria [7]. PAH is therefore by necessity tightly controlled by a variety of mechanisms, including activation by phenylalanine, inhibition by BH_4 and additional activation by phosphorylation [2]. Activation by the substrate phenylalanine is considered the major regulatory event. Phenylalanine is proposed to bind cooperatively to either an allosteric site, physically distinct from the catalytic site [8], or directly to the active site [9]. Phenylalanine activation leads to large conformational changes as inferred from an increase in tryptophan fluorescence [10], exposure of a hydrophobic surface [8], and a change in susceptibility to proteolysis [11].

The natural cofactor BH_4 acts as a negative regulator. BH_4 has been reported to block phenylalanine activation [12] through binding to a site distinct from the BH_4 catalytic site, and forming an inactive BH_4–PAH complex [13]. The phenylalanine-activated PAH is not inhibited by BH_4 [12]. Synthetic cofactors such as 6-methyl-5,6,7,8-tetrahydropterin (6-$MePH_4$) do not have this inhibitory effect [12]. Finally, phosphorylation by cAMP-dependent protein kinase at Ser-16 [14] is believed to act as a mediator of phenylalanine activation by decreasing the phenylalanine concentration required to activate the enzyme [15].

The only available three-dimensional structure of an aromatic amino acid hydroxylase that includes the regulatory domain is that of a dimeric rat PAH lacking the C-terminal, 24-residue tetramerisation domain (PAH_{1-428}) [16]. The catalytic properties of PAH_{1-428} are indistinguishable from the full-length enzyme [17], and it is similarly regulated by phenylalanine [17], phosphorylation [18] and BH_4 (this work). One of the most interesting features of the structure of the regulatory domain is the location of the N-terminal sequence (amino acids 19–29), which reaches into the active site of the catalytic domain (Fig. 1). This sequence may consequently autoinhibit the enzyme, acting as an intrasteric autoregulatory sequence (IARS) [16,19]. However, the mechanism of the autoinhibition is unclear, as the proposed binding sites for pterin [20,21] and phenylalanine [22] would not be obstructed by the IARS. Residues 1–18, containing the phosphorylatable Ser-16, are mobile in both the phosphorylated and dephosphorylated forms of PAH_{1-428} [16]. Nevertheless, the comparison of the structure of PAH_{1-428} [16] with the structures of the constitutively active fragments lacking the regulatory domain [23,24] shows that there are no significant structural differences in the active site regions, suggesting that the regu-

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Abbreviations: 6-$MePH_4$, 6-methyl-5,6,7,8-tetrahydropterin; BH_4, 6*R*-tetrahydrobiopterin; IARS, intrasteric autoregulatory sequence; PAH, phenylalanine hydroxylase; PAH_{1-428}, PAH fragment comprising residues 1–428; PAH_{30-428}, PAH fragment comprising residues 30–428; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase

0014-5793/01/$20.00
PII: S0014-5793(00)02426-1

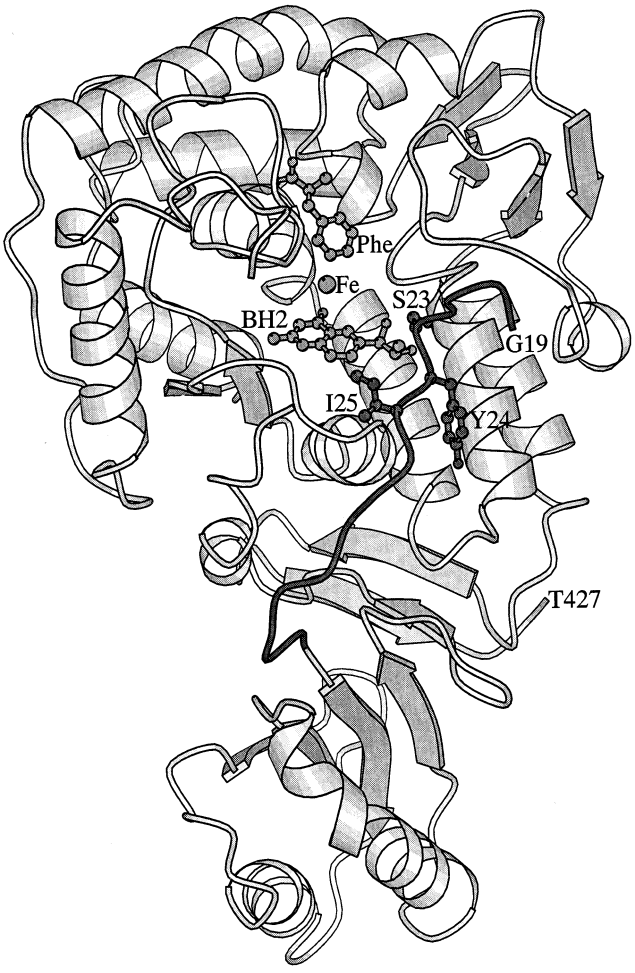

Fig. 1. The interaction of the IARS with the PAH active site. A ribbon diagram of the structure of a PAH monomer (PDB code 1PHZ [16]) is shown with the IARS (Gly-19 to Gln-30) in dark grey, and the side chains of Ser-23, Tyr-24 and Ile-25 interacting with the active site are indicated in a ball-and-stick representation. For orientation purposes only, the proposed positions of the substrate phenylalanine (Phe), and the pterin inhibitor, 7,8-dihydrobiopterin (BH_2), are shown based on the model of Teigen et al. [22].

latory domain must be responsible for the regulation of PAH activity.

To elucidate the role of the IARS in PAH regulation we expressed a PAH protein lacking 29 N-terminal amino acids (PAH_{30-428}) and characterised its regulatory properties. We show that PAH_{30-428} is constitutively active (i.e. it does not require phenylalanine activation), shows an altered structural response to phenylalanine, and is not negatively regulated by BH_4. Our results add functional support to the model previously suggested based on structural data, whereby the N-terminal sequence is responsible for autoinhibition and for transmitting the effect of phenylalanine activation to the active site.

2. Materials and methods

2.1. Protein expression and purification

PAH_{30-428} cDNA was cloned into the pKKT7 plasmid (ATCC, USA [25]). Protein expression was induced at 30°C for 5 h by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. All purification procedures were carried out at 4°C unless otherwise stated. Cells were pelleted, frozen and thawed, resuspended in 0.05 M Tris–HCl (pH 7.25), 10 mM phenylalanine, 0.1 mM EDTA (buffer 1) containing 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.4 μg/ml pepstatin and 0.4 μg/ml leupeptin, and sonicated. The cell extract was centrifuged at $27\,200\times g$ for 20 min. The supernatant was purified by ammonium sulphate precipitation (50% w/v). Following dialysis against buffer 1, the protein was applied to a DE-52 cellulose column and eluted with a linear gradient of 0–0.3 M KCl. The eluted peak was dialysed against 50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) containing 10% glycerol (buffer 2) and applied to a column (5 ml bed volume) of Biogel HTP hydroxyapatite (Bio-Rad) equilibrated with buffer 2. Following a 50 ml wash with buffer 2, the protein was eluted with a linear gradient of 50–350 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) containing 10% glycerol. Fractions containing PAH_{30-428} were pooled and dialysed twice against 2 l of 30 mM Tris–HCl (pH 7.25), 0.15 M KCl and 0.1 mM EDTA. PAH_{1-428} was expressed in insect cells and purified using phenyl-Sepharose chromatography [18].

2.2. Limited proteolytic digestion

PAH was activated, where indicated, by incubation with 10 mM phenylalanine for 10 min at 25°C. Limited proteolytic digestion was carried out by incubating PAH with chymotrypsin at an enzyme:protein ratio of 1:50 at 25°C. Digestion was stopped by the addition of an equal volume of a solution containing 4% (w/v) sodium dodecyl sulphate (SDS), 30% glycerol (v/v) and 10% mercaptoethanol (v/v) in 10 mM Tris–HCl (pH 6.8) and heating at 90°C for 5 min. Samples were then analysed on a 12% polyacrylamide gel containing 0.1% (w/v) SDS (SDS–PAGE).

2.3. Enzyme activity assay

To measure enzyme activity, PAH was added last to a cuvette containing 100 mM K_2HPO_4/KH_2PO_4 (pH 6.8), 65 μg/ml catalase, 2.75 mM phenylalanine, 6 mM dithiothreitol and either 60 μM 6-$MePH_4$ or 90 μM BH_4 (Schirck's Laboratories, Jona, Switzerland), at 20°C. Activity was estimated by measuring the absorbance change at 275 nm over the initial 60 s of the reaction with the activity expressed as nmol of tyrosine produced/min, using an extinction coefficient for tyrosine of 1700. Because PAH_{1-428} and PAH_{30-428} are different protein preparations, the absolute activity values should not be directly compared.

3. Results

3.1. Cloning and expression of the PAH_{30-428} protein

The DNA coding for the PAH_{30-428} protein was expressed in *Escherichia coli*, and purified by ammonium sulphate precipitation, ion exchange chromatography and hydroxyapatite chromatography. The purified PAH_{30-428} protein has the expected MW of 46 kDa as analysed by SDS–PAGE (Fig. 2). N-terminal protein sequencing of purified PAH_{30-428} generated the expected amino acid sequence (data not shown).

3.2. Activation by phenylalanine

The extent of activation by phenylalanine was assayed by adding unactivated PAH or PAH activated by 10 mM phenylalanine to a cuvette containing all the other components sufficient for PAH enzyme activity (Table 1). In the presence of the synthetic cofactor 6-$MePH_4$, phenylalanine activation of PAH_{1-428} resulted in a four-fold increase in k_{cat}; the removal of the N-terminus (PAH_{30-428}) decreased the phenylalanine activation to two-fold. In the presence of the natural cofactor BH_4, phenylalanine activation of PAH_{1-428} expectedly produced a more prominent 12.5-fold increase in k_{cat}. By contrast, phenylalanine activation of PAH_{30-428} had little effect (1.1-fold activation). Thus, in the presence of BH_4 the removal of the N-terminus produced a constitutively active enzyme with little or no requirement for phenylalanine activation.

3.3. Inhibition by BH_4

The effect of the removal of the IARS on BH_4 inhibition

Table 1
Activation of PAH by phenylalanine[a]

Protein	Treatment	Cofactor			
		6-$MePH_4$		BH_4	
		specific activity	fold-activation	specific activity	fold-activation
PAH_{1-428}	activated	589	4.0	176	12.5
PAH_{1-428}	not activated	148	–	14	–
PAH_{30-428}	activated	480	1.9	240	1.1
PAH_{30-428}	not activated	257	–	229	–

[a]PAH (8.6 μg), either activated in the presence of 10 mM phenylalanine for 10 min at 25°C, or not activated, was added last to a cuvette containing the enzyme reaction mixture with 60 μM 6-$MePH_4$ or 90 μM BH_4 as the cofactor. Enzyme specific activity is expressed as nmol tyrosine produced/min/nmol PAH subunit).

was assessed by assaying at BH_4 concentrations below those required for catalysis but sufficient for PAH inhibition, and using 6-$MePH_4$ as the cofactor in the PAH assay (6-$MePH_4$ is not a PAH inhibitor) [13] (Table 2). Enzyme activity of PAH_{1-428}, which under these experimental conditions reflects phenylalanine activation, was significantly decreased (by 64%) in the presence of BH_4. By contrast, no BH_4 inhibition was observed for PAH_{30-428}, lacking the N-terminal IARS.

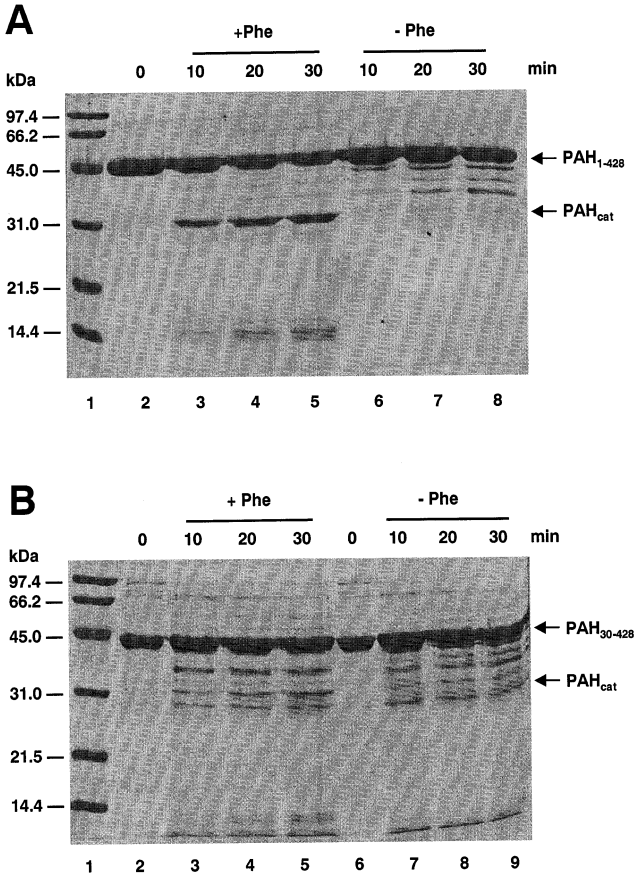

Fig. 2. Limited chymotryptic digestion of phenylalanine-activated PAH. A: Limited chymotryptic digestion (chymotrypsin: PAH_{1-428} = 1:50) of PAH_{1-428}, analysed by SDS–PAGE. Lane 1: Proteins of known molecular weight; lane 2: PAH_{1-428}; lanes 3, 4 and 5: phenylalanine-activated (10 mM phenylalanine added) PAH_{1-428} digested with chymotrypsin for 10, 20 and 30 min, respectively; lanes 6, 7 and 8: unactivated (no phenylalanine added) PAH_{1-428} digested with chymotrypsin for 10, 20 and 30 min, respectively. PAH_{1-428} and the catalytic fragment (PAH_{cat}) are indicated by arrows on the right of the gel. B: Limited chymotryptic digestion of PAH_{30-428}, analysed as in (A). Lane 1: Proteins of known molecular weight; lanes 2 and 6: PAH_{30-428}; lanes 3, 4 and 5: phenylalanine-activated PAH_{30-428} digested with chymotrypsin for 10, 20 and 30 min, respectively; lanes 7, 8 and 9: unactivated PAH_{30-428} digested with chymotrypsin for 10, 20 and 30 min, respectively.

3.4. Limited chymotryptic digestion and hydrophobic interaction chromatography

Upon limited chymotryptic digestion, a 31-kDa fragment is generated from phenylalanine-activated PAH_{1-428}, that has previously been shown [26] to correspond to the catalytic domain of PAH (amino acids 120–424) (Fig. 2A). Unactivated PAH_{1-428} is more resistant to chymotryptic digestion; less catalytic fragment accumulates. In phenylalanine-activated PAH_{30-428}, the generation of the catalytic fragment is slowed down when compared to PAH_{1-428} (Fig. 2B). It is reassuring that only subtle differences are observed in the proteolytic patterns, suggesting no major structural perturbations in the truncated protein.

Previously, both the full-length phenylalanine-activated PAH [8] and the phenylalanine-activated PAH_{1-428} [17] have been shown to bind to phenyl-Sepharose and could subsequently be eluted from the resin by a phenylalanine-free buffer. This behaviour has been postulated to be due to phenylalanine activation of PAH causing conformational changes that expose a hydrophobic surface, which can bind to phenyl-Sepharose [8]. Significantly, phenylalanine-activated PAH_{30-428} did not bind strongly to phenyl-Sepharose and was eluted in the first wash of the column (in a buffer containing 10 mM phenylalanine and 4.8% (v/v) dimethyl formamide).

4. Discussion

The crystal structure of PAH_{1-428} revealed that the N-terminal sequence of the regulatory domain of the enzyme reached into the active site, suggesting it played an autoinhibitory role [16]. However, the mechanism of autoinhibition

Table 2
Inhibition of phenylalanine activation of PAH by BH_4[a]

Protein	Additions	PAH enzyme activity	Inhibition (%)
PAH_{1-428}	none	20.6	–
PAH_{1-428}	2.5 μM BH_4	7.35	64
PAH_{30-428}	none	33.8	–
PAH_{30-428}	2.5 μM BH_4	31.8	6

[a]PAH (8.6 μg), either in the presence or absence of 2.5 μM BH_4, was added last to a cuvette containing the enzyme reaction mixture with 60 μM 6-$MePH_4$ as the cofactor. Enzyme activity is expressed as nmol tyrosine produced/min.

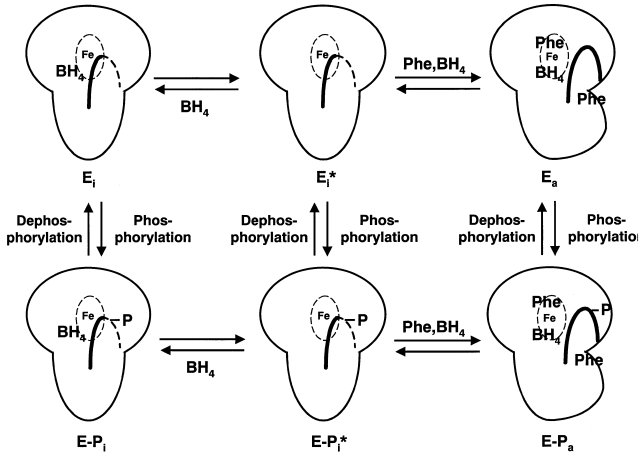

Fig. 3. Schematic diagram of a simplified regulatory pathway of PAH, involving regulation by phenylalanine, BH_4 and phosphorylation. The large object represents a monomer of PAH, with the large protrusion as the catalytic domain and the small protrusion as the regulatory domain. The dashed ellipse with Fe is the active site, and the thick curved line is the IARS. Little is currently known about the locations of phenylalanine and BH_4 binding sites; they are shown in arbitrary locations in the model. E_i depicts the autoinhibited state (IARS obstructs the active site, BH_4 is bound to its regulatory site); E_a depicts the activated state (phenylalanine bound to its activation site, BH_4 ready to bind to its catalytic site); E_i* depicts an intermediate inactive state with no effectors bound to their regulatory sites. Pterin binding to the active site induces localised conformational changes in the catalytic domain and the pterin molecule [20].

was not clear, and no direct functional data were available to support this hypothesis.

Here we show functional evidence supporting the regulatory mechanism based on the structural data, suggesting the 29 amino-terminal residues of PAH are directly responsible for the autoinhibited state of PAH, and that this sequence transmits the effect of phenylalanine activation through to the active site. We deleted the N-terminal 29 amino acids from the dimeric protein PAH_{1-428} and expressed, purified and characterised the regulatory properties of the resulting protein PAH_{30-428}. The dimeric PAH_{1-428} was used as the template to avoid any complications resulting from the use of the full-length protein which exists as a mixture of dimers and tetramers. The regulatory and catalytic properties of PAH_{1-428} are indistinguishable from the full-length protein [17,18].

In contrast to PAH_{1-428} or full-length PAH, the PAH_{30-428} protein was found to be constitutively active; activation by phenylalanine was not required for enzyme activity. Consistent with previous studies on human PAH [27], the effect of phenylalanine was more dramatic when the natural cofactor, BH_4, rather than the synthetic cofactor, 6-$MePH_4$, was used in the PAH assay. In addition, the inhibition of PAH activation by BH_4 was abolished by the removal of the N-terminal 29 amino acids. PAH_{30-428} therefore mimics phenylalanine-activated PAH, which is not inhibited by BH_4 [12]. The changed limited proteolysis pattern generated by chymotrypsin digestion of PAH_{30-428}, and the low affinity of PAH_{30-428} for phenyl-Sepharose suggest that the conformational changes generated by phenylalanine activation of PAH_{1-428} do not occur as efficiently in the constitutively active PAH_{30-428}.

A model for the regulation of PAH enzyme activity by phenylalanine, BH_4 and phosphorylation consistent with the available structural and functional data is shown in Fig. 3. E_i depicts the autoinhibited state where the IARS obstructs the active site and BH_4 is bound to its regulatory site (Fig. 3, left column). Phenylalanine binding to its activation site causes conformational changes, during which the IARS must move away from its position at the active site and BH_4 can bind to its catalytic site, resulting in the activated state E_a (Fig. 3, right column). The middle column in Fig. 3 depicts an intermediate inactive state with no effectors bound to their regulatory sites (E_i*); this is the only state for which structural information is currently available. Phosphorylation (Fig. 3, bottom row) most likely aids the transition from E_i to E_a by facilitating the phenylalanine-induced conformational changes through stabilising the phenylalanine-activated form [16].

The members of the aromatic amino acid hydroxylase family share significant amino acid sequence and structural similarities in their catalytic domains, but their regulatory domains are less conserved. However, the basic folds of the regulatory domains [16] as well as some aspects of the regulatory mechanisms may be conserved among the family members. In TH, catecholamines form an inactive TH complex by binding to the enzyme-bound iron. Phosphorylation of TH at Ser-40 by cAMP-dependent protein kinase decreases the affinity of dopamine for the iron 300-fold [28]. In line with the results reported here for PAH, TH lacking the N-terminal 39 amino acids was found not to be inhibited by catecholamines [29]. Limited proteolysis studies showed that amino acids 33 to 50 on the TH sequence were less sensitive to proteolysis when dopamine was bound to TH, and more sensitive to proteolysis when TH was phosphorylated at Ser-40 [30]. Therefore, similarly to the IARS of PAH, the N-terminal sequence of TH plays an integral role in the regulation of TH activity, acting in concert with catecholamine to form the inactive TH, and phosphorylation produces conformational changes that affect the N-terminus and cause the dissociation of the catecholamine–TH complex, resulting in the active enzyme. By contrast, it has very recently been shown that the deletion of 15 N-terminal residues of TPH does not affect the catalytic properties of that enzyme [31]; the regulatory mechanisms in TPH, however, remain to be elucidated.

Acknowledgements: We thank Professor A. Martinez for the coordinates of the model of the PAH–Phe–BH_2 complex, and Professor B.E. Kemp for critically reading the manuscript. This work was supported by the Australian Research Council and the Wellcome Trust (to B.K.). B.K. is a Wellcome Senior Research Fellow in Medical Science in Australia.

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