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Wolfgang Pfleiderer · Hartmut Rokos

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54 University Street,
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Blackwell Science KK
MG Kodemmacho Building
7–10, Kodenmacho Nihonbashi,
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Editors' addresses:
Em. Prof. Dr. Dr. h. c. Wolfgang Pfleiderer
Fakultät für Chemie
Universität Konstanz
Postfach 5560
78434 Konstanz
Germany

Dr. Hartmut Rokos
Robert-Koch-Institut
Abt. Virologie
Nordufer 20
13353 Berlin
Germany

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Mechanistic Studies of 4a-Hydroxytetrahydropterin Dehydratase from *Pseudomonas aeruginosa*

I. Rebrin, J.E. Ayling
Dept. of Pharmacology, University of South Alabama, Mobile, AL, USA
J. Song, R.A. Jensen
Dept. of Microbiology and Cell Science, University of Florida, Gainsville, FL, USA

Introduction

In mammals 4a-hydroxy-tetrahydropterin dehydratase functions in the regeneration of tetrahydrobiopterin, the cofactor product of the three aromatic amino acid hydroxylases. These monooxygenases catalyze the rate limiting steps in the biosynthesis of serotonin and the catecholamines, and in the degradation of excess dietary phenylalanine. A deficiency of the dehydratase leads to a mild form of PKU which is associated with elevated levels of 7-biopterin. The dehydratase is a homotetramer of 103 amino acids per subunit. It is highly conserved, having identical sequence in man and rat, and differing by only one amino acid in mouse. This protein has a second function as DCoH, the dimerization cofactor of the transcription factor HNF1α. DCoH activates HNF1α by forming a heterotetramer consisting of two subunits of DCoH and two subunits of HNF1α (see refs. in 1,2).

Pseudomonas aeruginosa produces a homologue of the mammalian dehydratase exhibiting 33 % amino acid identity (3). This dehydratase (PhhB) is encoded by the middle gene of a three-member operon. The upstream gene, *phhA*, encodes phenylalanine hydroxylase. Expression of *phhB* is translationally coupled to expression of *phhC* which encodes an aromatic aminotransferase. The *phh* operon is positively regulated by a divergently transcribed activator protein, PhhR (4). The operon is induced to maximal expression in the presence of exogenous *L*-phenylalanine or *L*-tyrosine. Each member of the *phh* operon is essential for growth on *L*-phenylalanine as sole source of carbon and energy.

The current study was undertaken to ascertain whether the bacterial protein performs a similar function as the mammalian dehydratase in the regeneration of tetrahydropterin cofactor.

Methods

Expression and purification of enzymes

Pseudomonas aeruginosa dehydratase was cloned and expressed using a T7 expression system. *Escherichia coli* BL21 (DE3) carrying plasmid pJS10 was incubated for 3 h at 30 °C after addition of IPTG to induce T7 RNA polymerase (3,4). Cells were harvested, disrupted by sonication, and the extract centrifuged at 100,000 x g for 1 h. Dehydratase was purified from the supernatant by column chromatofocusing and ion exchange chromatography on DEAE cellulose (5). Rat liver dehydratase was purified as previously published (1).

Results and Discussion

Structure of Pseudomonas aeruginosa dehydratase

P. aeruginosa dehydratase was purified to homogeneity, as determined by SDS–PAGE with coomassie blue staining which showed that all of the protein was in one band with a subunit molecular weight in the range of 13,000 Da. Electrospray mass spectrometry showed that the subunit molecular weight is 13,200 Da, indicating that the N-terminal methionine had been cleaved. This was confirmed by sequence analysis of the N-terminal end which was found to be T-A-L-T.

The oligomeric form of the *P. aeruginosa* enzyme was determined by three different methods. On size exclusion HPLC (Biosep SEC3000, Phenomenex) the native protein appeared to have a molecular weight of 35,000 Da. On the same system, rat liver dehydratase (subunit 11,909 Da) eluted at 45,000 Da. Similar observations were made on native PAGE: rat liver dehydratase migrated with a molecular weight of ~ 48,000 Da, while the *P. aeruginosa* enzyme again appeared to be 35,000 Da. Thirdly, all of the rat enzyme was retained by a nominal 30,000 molecular weight cut-off membrane (Amicon YM30), whereas half of the *P. aeruginosa* enzyme passed through the membrane. These results clearly show that *P. aeruginosa* dehydratase is not a stable homotetramer as is the mammalian enzyme. The behavior of native *P. aeruginosa* enzyme observed in three different systems suggests that either it is an equilibrating mixture between dimer and tetramer, or that it is a dimer with unusual hydrodynamic properties.

Catalytic properties

The ability of the enzyme from *P. aeruginosa* to dehydrate 4a-hydroxy-tetrahydropterins was quantitated with chemically synthesized 6(S)-methyl-4a-hydroxy-tetrahydropterin and 6(S)-propyl-4a-hydroxy-tetrahydropterin as substrates. These compounds were used previously in the characterization of mammalian dehydratase (1), the natural substrate of which is 4a-hydroxy-tetrahydrobiopterin. The natural substrate of the *P. aeruginosa* enzyme is currently unknown. The *P. aeruginosa* enzyme dehydrated both analogs as effectively as the mammalian dehydratase (Table 1). The K_m values determined with the bacterial enzyme are indistinguishable from those measured with the mammalian

Table 1: Effect of pH and Substrate Structure on the Catalytic Properties of the Dehydratase from *Pseudomonas aeruginosa* Compared to the Enzyme from Rat Liver

4a-OH-tetra hydropterin substrate	pH	*Pseudomonas aeruginosa* K_m (μM)	*Pseudomonas aeruginosa* k_{cat} (s^{-1})	Rat Liver K_m (μM)	Rat Liver k_{cat} (s^{-1})
6(S)-propyl-	7.4	1.5	4.5	1.5	11.8
	8.4	1.5	2.6	1.5	5.4
6(S)-methyl-	7.4	2	7.7	2	10
	8.4	2	2.4	2	3.5

Reactions were monitored at 340 nm at pH 7.4 and 10 °C, and contained chemically synthesized stereospecific substrates (2,6), NADH, and dihydropteridine reductase, and an amount of dehydratase which completed the reaction in about 1 min (1).

dehydratase. When pH was changed from 7.4 to 8.4 there was no effect on K_m with either enzyme; a 2–3 fold decrease in k_{cat} was observed with both enzymes. With respect to k_{cat}, at pH 7.4 the bacterial enzyme appears to have a preference for a 6-methyl substituent over 6-propyl, an effect which is not observed with the mammalian dehydratase (Table 1). The *P. aeruginosa* enzyme catalyzes dehydration of the "unnatural" isomer of 6-methyl-4a-hydroxy-tetrahydropterin as does the mammalian dehydratase, and in both cases K_m is higher than with the natural isomer (1,5,7).
The bacterial enzyme is strongly inhibited by the quinoid dihydropterin product of the reaction, as is the mammalian dehydratase (1). The extent of inhibition is similar with both enzymes (Table 2).

Table 2: Product Inhibition of the Dehydratase from *Pseudomonas aeruginosa* Compared to the Enzyme from Rat Liver

Quinoid dihydro-pterin product	*Pseudomonas aeruginosa* K_i (μM)	Rat Liver K_i (μM)
6(S)-propyl-	1	0.7
6(S)-methyl-	2	1.5

Activity was measured as in Table 1 except that NADH and dihydropteridine reductase were omitted.

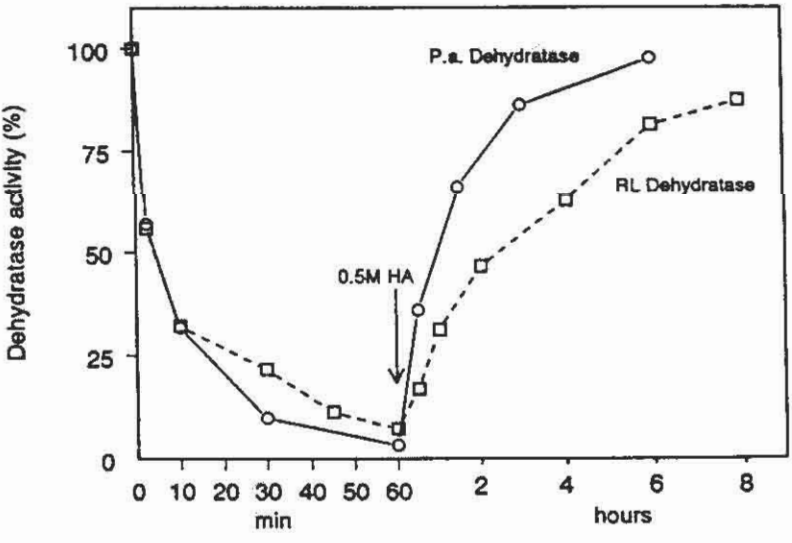

Figure 1: Inactivation of *Pseudomonas aeruginosa* (P.a) and rat liver (RL) dehydratase with DEPC and reactivation with hydroxylamine (HA). Enzymes were incubated with a 20–fold excess of DEPC (1 mM) at 25 °C. After one hour, hydroxylamine (HA), 0.5 M, was added and the mixtures placed on ice. Aliquots were removed at timed intervals as indicated and assayed for catalytic activity.

The reaction of mammalian dehydratase is dependent on reactive histidines at the catalytic site (7). Figure 1 shows that the *P. aeruginosa* enzyme is inactivated by DEPC (diethylpyrocarbonate) to the same extent as rat liver dehydratase. Both enzymes are reactivated by reaction with hydroxylamine (HA), indicating that histidine had specifically been derivatized.

The similarity between the bacterial and mammalian dehydratases with respect to K_m and k_{cat} for 4a-hydroxy-tetrahydropterins, pH dependence of the reaction, inhibition by product, and dependence on reactive histidines, suggests that the two enzymes have the same mechanism and specificity, and that the enzyme plays a similar role in regenerating tetrahydropterin cofactor in bacteria as in mammals.

Acknowledgements

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References

1. REBRIN, I., S.W. BAILEY, S.R. BOERTH, M.D. ARDELL, J.E. AYLING: Catalytic characterization of 4a-hydroxytetrahydropterin dehydratase. Biochemistry 34, 5801-5810 (1995).
2. BAILEY, S.W., S.R. BOERTH, S.B. DILLARD, J.E. AYLING: The mechanism of cofactor regeneration during phenylalanine hydroxylation. In: Ayling, J.E., M.G. Nair and C.M. Baugh (Eds.): Chemistry and Biology of Pteridines and Folates. Plenum Press, N.Y. (1993) 47-54.
3. ZHAO, G., T. XIA, J. SONG, R.A. JENSEN: *Pseudomonas aeruginosa* possesses homologues of mammalian phenylalanine hydroxylase and 4a-carbinolamine dehydratase/DCoH as part of a three-component gene cluster. Proceedings of the National Academy of Sciences, USA 91, 1366-1370 (1994).
4. SONG, J., R.A. JENSEN: PhhR, a divergently transcribed activator of the phenylalanine hydroxylase gene cluster of *Pseudomonas aeruginosa*. Molecular Microbiology 22, 497-507 (1996).
5. REBRIN, I., J. SONG, S.W. BAILEY, R.A. JENSEN, J.E.AYLING: manuscript in preparation.
6. BAILEY, S.W., I. REBRIN, S.R. BOERTH, J.E. AYLING: Synthesis of 4a-hydroxytetrahydropterins and the mechanism of their nonenzymatic dehydration to quinoid dihydropterins. Journal of the American Chemical Society 117, 10203-10211 (1995).
7. AYLING, J.E., I. REBRIN, B. THONY, S.W. BAILEY: Mechanism of dehydration by the bifunctional protein, 4a-hydroxy-tetrahydropterin dehydratase/DCoH. This Proceedings.