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On the Mechanism of Pterin-4a-carbinolamine Dehydratase. Synthesis of New Substrate Analogues and Interaction with the Enzyme.

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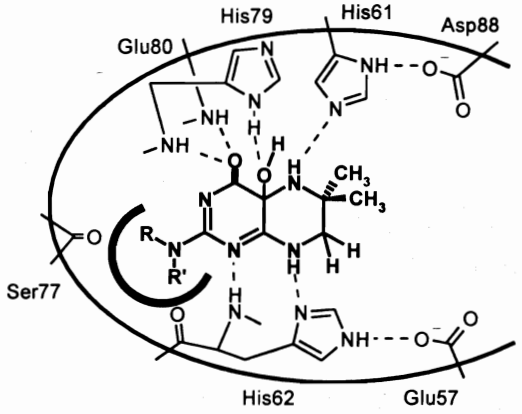
Introduction

Pterin-4a-carbinolamine dehydratase (PCD) is a bifunctional mammalian enzyme that acts, on the one hand, as dimerization cofactor of the hepatocyte nuclear factor 1-α (1). On the other hand it takes part in the metabolism of tetrahydrobiopterin, the natural cofactor of the aromatic amino acid hydroxylases. PCD catalyzes the dehydration of 4a-OH-tetrahydrobiopterin (carbinolamine) to quinonoid 7,8-dihydrobiopterin (2). Recently, the active site of human PCD has been located and a reaction mechanism for the dehydration has been proposed (3,4). From these studies it appears that in PCD the mode of recognition of the pterin is different from that encountered in other enzymes using pterins or folates as substrates (5,6). To understand the interaction in detail, analogues of the carbinolamine have been synthesized which are substituted in the pyrimidine ring position C(2)-NH_2, the moiety which appears to interact with the protein backbone in PCD (Fig.1). Some chemical features of these analogues and their interaction with PCD are reported.

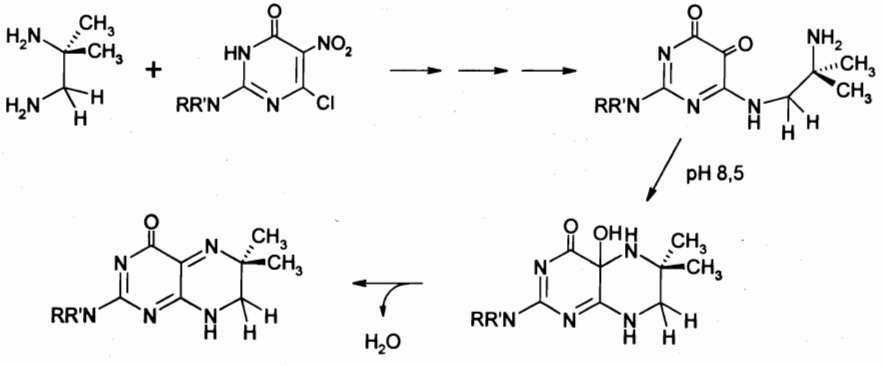
Figure 1: Schematic representation of the assumed interactions of pterin-4a-carbinolamine with protein functional groups at the active center of PCD. (---) denotes H-bridges. R and R' = H or CH_3.



Materials and Methods

The synthetic path of quinonoid 2-dimethylamino- (2-Me_2N-PH_2) and 2-methylamino-6,6-dimethyl-7,8-dihydropterin (2-MeNH-PH_2) is illustrated in Scheme 1. The pyrimidines were prepared starting from 2-substituted-6-chloro-5-nitro-pyrimidones(4). Condensation to the quinonoid dihydropterins were achieved according to Bailey and Ayling (7) with slight modifications. To follow the dehydration of 4a-carbinolamine (CA) the synthesis was modified according to (8). PCD was expressed in *E.coli* and purified to homogeneity as detailed in (3). Enzymatic activity was measured in an assay procedure according to (3). Interactions of the quinonoid dihydropterins with PCD were measured fluorimetrically as detailed in (8).

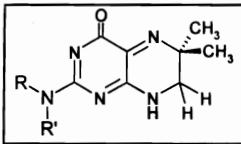
Scheme 1: Synthetic pathway for the preparation of carbinolamines and of quinonoid pterins. R and R' = H or CH_3.



Results

2-Me_2N-CA and 2-MeNH-CA (Scheme 1) decay to the quinonoid form in a mode analogous to that reported for the pterin unsubstituted at pos. C(2)- NH_2. Hereby a shift of the λ_{max} of 2-Me_2N-CA (275 nm) to that of the product (λ_{max} ~340 nm) occurs. The absorption spectrum of 2-MeNH-CA with its two λ_{max} at 245 and 290 nm is similar to that of the 2-unsubstituted CA. In the product, however, the longer wavelength λ_{max} is shifted to ~320 nm. The uncatalyzed conversion to the quinonoid product proceeds with 2-Me_2N-CA with a rate of ~0.02 s^{-1} and with 2-MeNH-CA with ~0.013 s^{-1} at pH 8.5. The pH dependence of the rate of decay reflects in both cases a pK ~8.7. It should be pointed out, that with C(2)-NH_2 unsubstituted CA's, maximal stability is at pH 8.5, the rate of decay increasing both on the acidic and alkaline sides (8,9). Addition of human wild-type PCD (up to 4 μM) has no effect on the dehydration rate. Similarly the fluorescence of the protein is not quenched upon addition of the quinonoid product in contrast to what was obtained with several pterins which bind to PCD (8). From this it is concluded that substitution of C(2)-NH_2 of the pterin interfers with recognition by the protein.

Table 1: Selected Properties of 2-Amino-substituted Quinonoid 6,6-Dimethyl-7,8-dihydropterins. (* no intermediate CA can be observed directly, the rate probably reflects that of ring closure to form the CA).

	absorption λ_{max} [nm]	fluorescence λ_{max} emission (exc.: 280 nm)	rate of formation (s^{-1})	apparent pK	binding to enzyme K_d (μM)
R = R' = H	242, 306	474	0.02		1.0
R = H, R'= CH_3	245, 318	485	0.013	8.7	> 50
R = R' = CH_3	247, 341	493	0.02 *	8.7	> 50

Discussion

The present results establish that the mode of recognition of CA´s by PCD is very stringent with respect to the properties of the C(2)-NH_2 function of the pyrimidine moiety. Indeed replacement of a single hydrogen with -CH_3 is sufficient to abolish activity and binding of the quinonoid product. This contrasts with the tolerance towards modifications at the substituent in position C(6), which does not affect activity or binding drastically (8). Our results are in good agreement with the three-dimensional structure of the complex of PCD with the product analogue 7,8-dihydrobiopterin (4). In comparison with the mode of recognition of the pyrimidine in e.g. dihydrofolate reductase or 6-pyruvoyl-tetrahydropterin synthase (5,6) in which recognition is mediated by an aspartate or glutamate residue, in the present case the C(2)-NH_2-group interacts with the backbone of Ser77 and His62 of the protein. The chemical behaviour of C(2)-NH_2-modified pterins is also of interest in that it indicates that the ring closure to form the CA, and the dehydration of the latter are strongly influenced by the electronic properties of the substituents at this position and that +I groups inhibit ring closure, and probably facilitate elimination.

References

1. MENDEL, D.B., P.A. KHAVARI, P.B. CONLEY, M.K. GRAVES, L.P. HANSEN, A. ADMON, G.R. CRABTREE: Characterization of a cofactor that regulates dimerization of a mammalian homeodomain protein. Science 254 (1991) 1762-1767
2. KAUFMAN, S.: A protein that stimulates rat liver phenylalanine hydroxylase. J. Biol. Chem. 245 (1970) 4751-4759
3. KÖSTER, S., G. STIER, R. FICNER, M. HÖLZER, H.-CH. CURTIUS, D. SUCK, S. GHISLA: Location of the active site and proposed catalytic mechanism of pterin-4a-carbinolamine dehydratase. Eur. J. Biochem. 241 (1996) 858-864
4. CRONK, J.D., J.A. ENDRIZZI, T. ALBER: High-resolution structures of the bifunctional enzyme and transcriptional coactivator DCoH and its complex with a product analogue. Protein Science 5 (1996) 1963-1972
5. BOLIN, J.T., D.J. FILMAN, D.A. MATTHEWS, R.C. HAMLIN, J. KRAUT: Crystal structure of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 Å resolution. J. Biol. Chem. 257 (1982) 13650-13662
6. BÜRGISSER, D.M., B. THÖNY, U. REDWEIK, D. HESS, C.W. HEIZMANN, R. HUBER, H. NAR: 6-Pyruvoyl tetrahydropterin synthase, an enzyme with a novel type of active site involving zinc binding and an intersubunit catalytic triad motif; site-directed mutagenesis of the metal binding site and modelling of substrate binding. J. Mol. Biol. 253 (1995) 358-369
7. BAILEY, S.W., J.E. AYLING: 6,6-Dimethylpterins: Stable Quinoid Dihydropterin Substrate for Dihydropteridine Reductase and Tetrahydropterin Cofactor for Phenylalanine Hydroxylase. Biochemistry 22 (1983) 1790-1797
8. KÖSTER, S., B. THÖNY, P. MACHEROUX, H.-CH. CURTIUS, C.W. HEIZMANN, W. PFLEIDERER, S. GHISLA: Human pterin-4a-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor-1α. Characterization and kinetic analysis of wild-type and mutant enzymes. Eur. J. Biochem. 231 (1995) 414-423
9. 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, C.M. Baugh (Eds.): Chemistry and Biology of Pteridines and Folates. Plenum Press, N.Y. (1993) 47-54