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Studies on transcriptional regulation of GTP cyclohydrolase I and catalytic mechanism of sepiapterin reductase

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Tetrahydrobiopterin (BH4) is an essential cofactor in catecholamine and nitric oxide biosynthesis, serving as the electron-transfer intermediate for aromatic amino acid hydroxylase (tyrosine hydroxylase TH, tryptophan hydroxylase TPH, phenylalanine hydroxylase PAH) and nitric oxide synthase (NOS). The *de novo* synthesis of BH4 is accomplished by a three-step reaction catalyzed by GTP cyclohydrolase I (GCH), 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase (SPR). In catecholamine synthesis reactions, BH4 is converted to *quinonoid* BH2 and recycled back to BH4 by *quinonoid*-dihydropteridine reductase (DPR); while in NOS reaction, there is no net consumption of BH4, since it is regenerated *via* FAD or FMN within the reaction complex.

In the thesis, two aspects regarding BH4 biosynthesis are discussed, focusing on GCH transcriptional regulation and SPR catalytic mechanism, respectively.

GCH is the first as well as the rate limiting step in *de novo* BH4 synthesis, in which GTP is converted to dihydroneopterin triphosphate (NH2TP). The activity of GCH is regulated mainly at transcription level, so it is of significance to study the transcriptional regulation mechanism of GCH, in order to understand how the biosynthesis of BH4 is regulated. The transcription of GCH has been shown to be upregulated by various stimuli, including cAMP, NGF, NO, estrogen, lipopolysaccharides (LPS), and TNF-α.

The promoter region of mammalian GCH gene has been extensively studied, in which several regulatory *cis*-elements were identified in response to stimuli *via* cAMP pathway. However, in immune activated GCH transcription, the regulatory mechanism remained to be uncovered. With luciferase reporter assay, we found that a 5.2 kb promoter region did not respond to inflammatory stimuli like LPS or TNF-α/IFN-γ, indicating other additional cis-

elements are involved in immune activated GCH transcription. Efforts were made to search for the responding elements downstream of the transcription starting site. An enhancer element locating in intron 1 spanning around 300-bp region was identified, which is involved in LPS activated GCH transcription in a position and orientation independent manner. Two highly conserved fragments were selected as the candidate targets which trans-activators act on. With electrophoretic mobility shift assay, we observed specific nuclear factors binding to these regions. Further analysis using consensus oligonucleotides as well as antibody revealed that C/EBP-β and Ets family proteins bind with these sequences. The significance of these binding motifs was shown with site-directed mutagenesis experiment, in which reporter plasmids carrying disrupted motifs showed significantly attenuated response to LPS stimulation. Finally, the involvement of C/EBP-β in LPS induced GTPCH transcription *in vivo* was shown by chromatin immunoprecipitation as well as siRNA experiments.

In the second part, catalytic mechanism of SPR is described. SPR belongs to the short-chain dehydrogenase/reductase (SDR) family, a very large superfamily in which members show low sequence identities but high structural similarities. Most SDRs consist of at least two domains, one for coenzyme NAD(H) or NADP(H) binding while the other recognizes specific substrate. SPR catalyzes the last step of BH4 *de novo* synthesis, converting 6-pyruvoyl tetrahydropterin (PPH4) into 6-(1',2'-dihydroxypropyl)-tetrahydropterin (commonly known as tetrahydrobiopterin, BH4). Not only SPR, other aldose reductases are also shown to be involved in this process, however, those reductases can only catalyze the 2'-oxo reduction, forming a 6-lactoyl tetrahydropterin, which is further reduced to BH4 by SPR. Thus SPR is the absolutely required enzyme for BH4 biosynthesis. SPR was also found to be capable to reduce sepiapterin [6-(1'-oxo-2'-hydroxypropyl)-7,8-dihydropterin] into 6-(1',2'-dihydroxypropyl)-7,8-dihydropterin (commonly known as 7,8-dihydrobiopterin, BH2), which can be reduced by dihydrofolate reductase (DHFR) back to BH4 – which is called the salvage pathway of BH4 anabolism.

It is widely accepted that the reduction by SPR on the two carbonyl group of the pyruvoyl side chain of PPH4 does not occur simultaneously, probably involving a sterospecific

isomerization process. That is to say, SPR probably possesses a preference to either the 1’-oxo or the 2’-oxo group. The crystal structure of murine SPR has been resolved, showing the binding mode of pterins and SPR inhibitors to the enzyme, but the catalytic mechanisms still remains to be uncovered. In our study, we collected biochemical evidence for understanding the catalytic mechanism of SPR. First, site-directed mutations were prepared toward two key residues, S157 and Y170 in human SPR, which are supposed to be involved in electron transfer during the reaction, and mutant proteins were expressed and purified. Next, the biochemical features of these mutants (hSPR-S157D and hSPR-Y170V) were studied, in comparison with hSPR-WT. We perfomed BH4 biosynthesis as well as sepiapterin reduction *in vitro*, and the products were examined by HPLC. It was shown that hSPR-Y170V could reduce the 1’-carbonyl group of PPH4, to produce 6-(1’-hydroxy-2’-oxopropyl)-PH4, but it could not reduce that of 6-(1’-oxo-2-hydroxypropyl)-PH4. The hSPR-S157D mutant showed no reducing activity. The isomerase activity or SPR, to isomerize the 1’-carbonyl group of sepiapterin or 6-(1’-oxo-2’-hydroxypropyl)-PH4 to 2’-position, was also examined. The data showed neither hSRP-Y170V nor hSPR-S157D possessed the isomerase activity. All these data suggested that the S157 residue could mediate the reduction of 1’-carbonyl group of PH4, but the complete reduction from PH4 to BH4 requires both S157 and Y170 residues, although the function of Y170 requires further examination, since the mutation S157D might have detected substrate binding.