Epidermal H$_2$O$_2$ Accumulation Alters Tetrahydrobiopterin (6BH$_4$) Recycling in Vitiligo: Identification of a General Mechanism in Regulation of All 6BH$_4$-Dependent Processes?


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It has been shown in vivo that patients with the depigmentation disorder vitiligo accumulate hydrogen peroxide (H$_2$O$_2$) accompanied by low catalase levels and high concentrations of 6- and 7-biotin in their epidermis. Earlier it was demonstrated that epidermal 4a-OH-tetrahydrobiopterin dehydratase, an important enzyme in the recycling process of 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH$_4$), has extremely low activities in these patients concomitant with a build-up of the abiogenic 7-isomer (7BH$_4$), leading to competitive inhibition of epidermal phenylalanine hydroxylase. A topical substitution for the impaired epidermal catalase with a pseudocatalase effectively removes epidermal H$_2$O$_2$, yielding a recovery of epidermal 4a-OH-tetrahydrobiopterin dehydratase activities and physiologic 7BH$_4$ levels in association with successful repigmentation demonstrating recovery of the 6BH$_4$ recycling process.

Examination of recombinant enzyme activities, together with 4a-OH-tetrahydrobiopterin dehydratase expression in the epidermis of untreated patients, identifies H$_2$O$_2$-induced inactivation of this enzyme. These results are in agreement with analysis of genomic DNA from these patients yielding only wild-type sequences for 4a-OH-tetrahydrobiopterin dehydratase and therefore ruling out the previously suspected involvement of this gene. Furthermore, our data show for the first time direct H$_2$O$_2$ inactivation of the important 6BH$_4$ recycling process. Based on this observation, we suggest that H$_2$O$_2$ derived from various sources could be a general mechanism in the regulation of all 6BH$_4$-dependent processes. Key words: hydrogen peroxide/4a-OH-tetrahydrobiopterin dehydratase/6- and 7-tetrahydrobiopterin/vitiligo. J Invest Dermatol 116:167–174, 2001

Patients affected with the depigmentation disorder vitiligo express a characteristic fluorescence in their white skin patches upon Wood’s light (351nm) examination (Schallreuter et al, 1994a, b). In this context, it was demonstrated that this phenomenon originates from the accumulation of oxidized pterins (Schallreuter et al, 1994a, b). In the presence of molecular oxygen 6(R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH$_4$) is the immediate electron donor for the hydroxylation of the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan (Kaufman, 1970; Huang et al, 1994b). This enzyme is of major importance in the recycling process of 6BH$_4$ (Schallreuter et al, 1994a, b). It has also been demonstrated that 6BH$_4$ can regulate tyrosinase (EC 1.14.18.1) by uncompetitive inhibition (Wood et al, 1995).

Figure 1 presents a scheme for the de novo synthesis/recycling of 6BH$_4$ and its link to melanocytes and keratinocytes. In this context, it was realized that patients with vitiligo show an increased epidermal de novo synthesis and recycling of this cofactor in association with barely detectable 4a-OH-tetrahydrobiopterin dehydratase (DH; EC 4.2.1.96) activities (synonyms: pterin 4 alpha carbinolamine dehydratase (PCD), dimerization cofactor of hepatocyte nuclear factor 1 a (DCoH)) (Schallreuter et al, 1994a; 1994b). This enzyme is of major importance in the recycling process of 6BH$_4$ (Kaufman, 1970; Huang et al, 1973; Ayling et al, 1997) (Fig 1). Low activities of DH can lead to the accumulation of the abiogenic 7-isomer (7BH$_4$) (Curtius et al, 1990; Davis et al, 1991; Adler et al, 1992), which is a potent competitor of the natural cofactor 6BH$_4$, hence severely affecting phenylalanine hydroxylase (PAH; EC 1.14.16.1) activities (Davis et al, 1992). Decreased PAH activities were indeed demonstrated in the skin of patients with vitiligo (Schallreuter et al, 1994a, b). One consequence of low
epidermal PAH activities in these patients is an increase in epidermal L-phenylalanine levels (Schallreuter et al, 1994a, b; , 1998), which has been confirmed by in vivo Fourier transform Raman spectroscopy (FT-Raman spectroscopy). This amino acid resolves as a well-defined peak at 1004 cm⁻¹ (Schallreuter et al, 1998). Both the epidermal L-phenylalanine and 7BH₄ build-up, together with decreased PAH and DH activities, lead to the generation of hydrogen peroxide (H₂O₂) in the skin due to an increased short circuit in the recycling process of 6BH₄ (Davis et al, 1992; Schallreuter et al, 1999) (Fig 1). To date several other impaired metabolic steps have been identified in this disorder yielding an accumulation of H₂O₂ in the epidermal compartment (Schallreuter et al, 1996; 1999; Maresca et al, 1997). On the one hand, it was only recently that in vivo noninvasive FT-Raman measurements on the skin of this patient group confirmed high concentrations of H₂O₂ (Schallreuter et al, 1999). On the other hand, accumulation of H₂O₂ can deactivate catalase (Aronoff, 1965). In this context, low catalase levels, as well as low glutathione peroxidase levels, have been identified in patients with vitiligo (Schallreuter et al, 1991; Maresca et al, 1997; Beazley et al, 1999). Moreover, these results are in agreement with various degrees of vacuolation/lipid peroxidation in epidermal cells of vitiligo skin biopsies described by many groups (Moellmann et al, 1982; Bhawan and Bhutani, 1983; Gokhale and Mehta, 1983; Tobin et al, 2000). H₂O₂ accumulation can oxidize 6BH₄ as well as 7BH₄ (Armarego et al, 1983; Armarego, 1984; Schallreuter et al, 1999) and 6-biopterin is cytotoxic to melanocytes (Schallreuter et al, 1994c). Here, it is noteworthy that both oxidation products can explain the characteristic fluorescence of the depigmented/white skin in these patients (Schallreuter et al, 1994a, b). This fundamental scientific observation led to the development of a pseudocatalase as a
substitution for the impaired H₂O₂ degrading systems (Schallreuter et al, 1995, 1999). The topical application of this narrow band (311 nm) ultraviolet B (UVB) activated complex yielded four fundamental clinical results: (i) the arrest of an active depigmentation process in 95% of the patients (Schallreuter et al, 1995, 1999); (ii) the recovery of epidermal cells from intracellular vacuolation (Schallreuter et al, 1999; Tobin et al, 2000); (iii) a successful repigmentation in 60%-65% of all patients treated so far (n > 700); and (iv) initiation of repigmentation independent of the duration of the disease (Schallreuter et al, 1995, 1999). Until now, however, it remained unknown whether H₂O₂ accumulation could directly affect pterin homeostasis in the epidermis. We therefore embarked on a detailed analysis of the 6BH₄ de novo synthesis and recycling with special emphasis on DH enzyme activity and on DH protein expression.

Our results identified restored epidermal DH activities in association with normal 7BH₄ levels in patients treated with a topical pseudocatalase. This observation was accompanied by a significant decrease/complete loss of epidermal H₂O₂ confirmed in vivo by FT-Raman spectroscopy. The validity of direct H₂O₂ deactivation of DH has been proven by experiments with pure recombinant enzyme, as well as with immunohistochemical analysis of skin sections from treated and untreated patients. Computer modeling identified a structural change of the enzyme active site of DH after H₂O₂ oxidation of the cysteine and tryptophan residues. All of the above observations were supported in patients with vitiligo by an examination of genomic DH to rule out a suspected point mutation of this important enzyme. Only wild-type sequences were detected in the DH coding region in all cases studied. The observations from this study indicate a general mechanism for H₂O₂-impaired 6BH₄ recycling in this disorder.
MATERIALS AND METHODS

Patients Two independent patient groups and ten healthy age and sex matched controls were selected for this investigation. Both studies were approved by the local Ethics Committee and patient consent forms were obtained from each patient and control prior to the experiments. All patients and controls had photo skin type III ( Fitzpatrick classification) ( Fitzpatrick et al, 1971). The patients expressed the common type vitiligo vulgaris (mean age 50.9 ± 6.8 years; range from 19 to 66 years), and the mean duration of the disease at the point of investigation was 18 ± 4.5 years. All patients were otherwise healthy and did not take any additional medication or cosmetics. The first group included 13 patients for the analysis of pterins and enzyme activities before and after 6 mo treatment with a UVB-activated pseudocatalase ( Schallreuter, 1999; Schallreuter et al, 1999). The second group included 15 patients for the in vivo FT-Raman measurements of epidermal H2O2 before treatment and 13 d after treatment with a UVB-activated pseudocatalase.

Cell cultures Human epidermal melanocytes and keratinocytes from normal healthy probands and patients with vitiligo were established from suction blister material in MCDB 153 medium using the method of Wille et al (1984).

Epidermal cell extracts and protein determination Cell extracts were produced from suction blister roofs using the method of Künstl (1968). Protein content of each sample was determined employing the method of Kihl and Bernlohr (1977).

Analysis of epidermal pterins Total biotin content was determined in epidermal cell extracts obtained from suction blister material prior to any treatment and 6 mo after treatment with pseudocatalase after acidic iodine oxidation using reverse phase high performance liquid chromatography (HPLC) following the method from Ziegler and Hüttner (1992). The concentration of pterin was calculated on the basis of protein content (Kalb and Bernlohr, 1977).

PAH activities in epidermal cell extracts PAH activities were measured in the same epidermal cell extracts as were used for analysis of pterins using the method of Abita et al (1987; Schallreuter et al, 1994a). Enzyme activities were determined per mg protein per min.

DH activities in epidermal cell extracts Enzyme activities were determined in the same epidermal cell extracts as above in a coupled assay according to Schallreuter et al (1994a).

DH mRNA expression in normal and vitiliginous melanocytes and keratinocytes Total mRNA was extracted from cells prior to the fourth passage using Ambion Totally RNA isolation kit (AMS Biotechnology, Oxfordshire, U.K.) based on the guanidium isothiocyanate method. cDNA was synthesized using the reverse transcription system (Promega, Southampton, U.K.) and oligo DT primers, incubated at 42°C for 1 h, followed by a 5 min denaturation step at 95°C. Polymerase chain reaction (PCR) assays were conducted as described previously (Schallreuter et al, 1998).

All primers were obtained from Genoysis Biotechnologies, Europe Ltd, Pampisford, U.K., and were designed based upon the mRNA sequences of Genbank accession numbers: DH (LI4539), 5-GGCCATCTCTAACG- CAGTGTCT-3' and 5'-AGTTCGATACCCCTTCCG-3'; β-actin (M10277), 5'-ATCTGAGGGAGAGTCCAATTCAATGAGTCCG-3' and 5'-CGTCACTACTGCTTGTGCTATCCACTGTCGC-3'. Primers were added to final concentrations of 1.0 μM and 0.4 μM for DH and β-actin, respectively. DH reactions contained 10% dimethyl sulphoxide and all assays were subjected to incubations of 95°C for 5 min followed by 35 cycles of 95°C 1 min, 55°C 1 min, 72°C 1 min, and a final extension period of 72°C 11 min. PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining.

Immunohistochemistry Full-skin biopsies were obtained under local anesthesia from healthy controls of photo skin type III (n = 8) and from lesional white and uninvolved skin of patients with vitiligo (n = 10) with the same photo skin type. Samples were frozen in liquid nitrogen after cryoprotection. Seven-micron cryosections were allowed to air dry and were fixed in acetone for 10 min. Briefly, sections were incubated for 20 min with a biotinylated secondary antibody followed by peroxidase labeled adriamycin (Dako, Carpintina, CA). AEC was applied as chromogen (large volume Dako AEC substrate system, under microscopic control with a Leica DM IRB inverted microscope (Wetzlar, Germany) until the precipitate staining was visible. For embedding, glycerol mounting medium (Sigma-Aldrich, Dorset, U.K.) was used. For negative controls the primary antibody was omitted.

Transformation and purification of rat recombinant DH His-tagged rat recombinant DH was transformed into E. coli BL21 (DE3) cells by using a PACYC 9d kanamycin-resistant vector according to the method of Ficner et al (1995). DH was overexpressed with 2 × 10-13 M isopropyl-β-D-thiogalactopranoside. Cells were harvested after 4 h and DH was partially purified by elution from DEAE-sephrose (1.5 × 10 cm) using a 100 ml gradient of 0.05–0.5 M NaCl in Tris buffer 0.05 M pH 8.0. DH fractions were then added to a Hi-Trap His-tagged affinity column (5.0 ml) loaded with Ni2+ and eluted by using a 100 ml gradient of 0.005–1 M imidazole buffer pH 8.0. DH purity was confirmed by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis. DH migrates as a single band at 14 kDa (data not shown). Note that the sequences of rat and human DH are identical (Ficner et al, 1995).

DH mutation analysis in nine patients with vitiligo Genomic DNA was isolated of full-skin sheets of full-skin biopsies and from blood leukocytes following standard procedures (Sambrook et al, 1989). Mutation analysis was performed by exon-specific amplification and direct DNA sequencing of the PCR products. The corresponding primer sequences for PCR and DNA sequencing have been published elsewhere (Thöny et al, 1998).

In vivo detection of H2O2 in the epidermis using FT-Raman spectroscopy FT-Raman spectra were produced with a Bruker RFS 100/S spectrometer equipped with a liquid nitrogen cooled germanium detector. Sample excitation was accomplished using an Nd3+:YAG laser operating at 1064 nm. The laser power was 400 mW. Each spectrum was accumulated from the wrist area over 5 min with 300 scans and a resolution of 4 cm-1. Total H2O2 was assigned as a well-defined peak at 875 cm-1 based on the O–O stretch (Schallreuter et al, 1999a). Levels are expressed in arbitrary units. In vitro experiments show that millimolar concentrations are required to follow a well-resolved peak at 875 cm-1 (Schallreuter et al, 1999a).

Molecular modeling based on the crystal structure of DH All molecular models were based on the crystal structure of PCD/DCoH (Protein Databank accession number 1DCP) ( Cronk et al, 1996). For studies of the active site, a molecular model of the natural substrate 4a-OH-BH4 was produced with the HyperchemTM suite of programs (Hypercube, Canada). Geometry optimization was obtained using the MM+ forcefield (Allinger, 1977). Both stereo isomers of 4a-OH-BH4 were modeled and superimposed on the same location as the product analog. In addition, selected residues were oxidized to mimic the effects of exposure to H2O2. All protein structures were optimized with the AMBER forcefield using a Polak-Ribiere conjugate gradient on a Pentium II personal computer running at 300 MHz with 64 Mb of memory ( Weiner et al, 1986). Structures were visualized employing Chemics Chime version 2.03 (MDL Information Systems, San Leandro, CA).

Statistical analysis Statistical analysis was based on the Student’s paired t test on SPSS for Windows.

RESULTS

Presence of millimolar H2O2 concentrations in the epidermis of patients with vitiligo and its removal by a pseudocatalase The results of in vivo FT-Raman spectroscopy confirmed millimolar H2O2 accumulation in 15 untreated patients with active, progressive vitiligo. H2O2 was successfully removed after 13 d treatment with a topical application of a pseudocatalase (Fig 2).

Recovery of epidermal DH activities and 7BH4 levels We therefore determined both DH activities as well as 7BH4 levels in epidermal suction blister material obtained from patients before and 6 mo after initiation of the treatment. The results of all treated patients showed a restoration of DH activities concomitant with normal levels of 7BH4 (Figs 3-4). This observation clearly evidence...
indicates the recovery of the recycling process for 6BH4 after removal of excessive H2O2 in the epidermis of these patients. Enzyme activities and pterin levels, however, are determined on the basis of protein content. Therefore, this value is influenced by the thickness of the stratum corneum, which does not express any enzyme activities. Highest activities are always found in proliferating cells (i.e., the basal layer) with a significant reduction after differentiation (Schallreuter et al, 1994a; 1994b). Note that after 6 mo treatment the stratum corneum is always thicker and therefore total DH activities probably fully recover. This assumption is supported by the full recovery of 7BH4 production in these patients. Here it should be noted that significant 7BH4 levels are only produced when DH is absent or inhibited (Curtius et al, 1988; Davis et al, 1991).

Recovery of DH protein in the epidermis after H2O2 removal In addition to the recovery of DH enzyme activities, we documented normal expression of DH immunoreactivity using a polyclonal antibody F 3862 against DH. Figure 5 demonstrates a significantly higher expression of DH in the basal layer of the epidermis in both patients and controls. This observation is in agreement with upregulated proliferation in these cells concomitant with autocrine 6BH4 production/recycling/regulation and catecholamine biosynthesis (Schallreuter et al, 1992; Schallreuter, 1997). Untreated patients show a much lower expression of DH protein compared with treated patients and healthy controls of the same photo skin type (Fitzpatrick classification) (Fig 5a–c). These results demonstrate a decrease in protein levels of the enzyme in the untreated patients in association with epidermal accumulation of H2O2 (Fig 2). As the expression of DH mRNA from epidermal melanocytes and keratinocytes (Fig 6) appeared to be unaffected compared with control cells, it can be suggested that the decrease in the DH protein as shown in Fig 5(b) could be directly initiated by H2O2 due to protein instability.

Figure 2. In vivo FT-Raman analysis of epidermal H2O2. In vivo H2O2 levels in white skin of patients with vitiligo (n = 15) compared with healthy controls (n = 15) were obtained before and after 13 d of treatment with pseudocatalase. These data prove the complete reduction of epidermal H2O2 after treatment with pseudocatalase (p < 0.0001 ± SEM).

Figure 3. Epidermal 7BH4 levels before and after 6 mo treatment with pseudocatalase compared with healthy controls. The results demonstrate the recovery of pathologic to normal/physiologic levels of 7BH4 in patients after removal of H2O2 with pseudocatalase (n = 10) (error bars show standard error of the mean) (p < 0.0001 ± SEM) (normal = nonlesional, vitiligo = lesional).

Figure 4. DH enzyme activities in epidermal cell extracts before and after treatment with pseudocatalase. Recovery of DH activities after 6 mo treatment with pseudocatalase (n = 6) compared with untreated patients (n = 7) and healthy controls (n = 6) (error bars show standard deviations). These data confirm the restoration of enzyme activities in the 6BH4 recycling process in vitiligo after successful removal of H2O2 (●, nonlesional skin; □, lesional skin) (p < 0.001 ± SEM).
Inactivation of recombinant DH enzyme by H$_2$O$_2$. To further substantiate this finding, we incubated purified recombinant DH with H$_2$O$_2$ to follow its direct effect on the enzyme activity. We utilized a coupled assay for this purpose as DH can stimulate PAH activity (Kaufman, 1970; Huang et al., 1973; Ayling et al., 1997). Figure 7 demonstrates DH-stimulated PAH activity with a 7-fold increase in the initial rate ($V_0$). This result is in agreement with Kaufman’s earlier report (Kaufman, 1970). After preincubation of DH with H$_2$O$_2$ for 10 min prior to the PAH assay, however, there was a time-dependent significant decrease of enzyme activity, confirming a H$_2$O$_2$-induced deactivation (Fig 7).

DH active site alteration by H$_2$O$_2$. Molecular modeling of the natural substrate 4a-OH-BH$_4$ in the active site of DH supports previous observations that His$^{79}$ and His$^{61}$ are responsible for the catalytic activity of the S- and R-isomers of the substrate (Ayling et al., 1997). Oxidation of the Cys$^{81}$ by H$_2$O$_2$, however, affects the orientation of both of these histidine residues, implicating the loss of the hydrogen bond from the imidazole ring of His$^{79}$ to the hydroxyl group of the S-isomer in position 4a on the pterin molecule. By contrast, the hydrogen bond from His$^{61}$ to the hydroxyl group of the R-isomer remains unaffected. Oxidation of Trp$^{81}$ substantially moves both histidine residues and breaks all possible hydrogen bonds with the pterin, resulting in an inactive binding site for the substrate (Fig 8a, b). To our knowledge, this observation identifies for the first time the direct deactivation of DH by H$_2$O$_2$. Our results exclude the earlier proposed involvement of Trp$^{81}$ (Ficner et al., 1995).

**DH is not mutated in vitiligo** Originally low epidermal DH activities implicated the possibility of a leaky mutation in the DH gene (Schallreuter et al., 1994a). Therefore, the direct effect of H$_2$O$_2$ on the enzyme described above was further supported by an investigation of the DH gene to rule out possible point mutations or mosaics. In all cases studied, we found only wild-type sequences for the DH coding region (data not shown). Based on these results we conclude that low DH activities in vitiligo are not caused by a mutant DH.

**DISCUSSION**
Over the past the importance of 6BH$_4$ homeostasis in health and disease has been the subject of numerous reports (Smith, 1974; Blau et al., 1996; Kaufman, 1997). Previous studies established the presence of a full autocrine 6BH$_4$ synthesis/ recycling/regulation in keratinocytes and melanocytes of the human epidermis (Schallreuter et al., 1994a; 1994b). Patients with the depigmentation disorder vitiligo exhibit a severely impaired epidermal 6BH$_4$ homeostasis together with an accumulation of H$_2$O$_2$ in this compartment (Schallreuter et al., 1994a, b, 1999). So far it has remained elusive whether the recycling of 6BH$_4$ (Fig 1) with low DH activities in association with high levels of the abiogenic 7BH$_4$ could be caused by a possible mutation of the DH gene or by deactivation of the enzyme itself in these patients (Schallreuter et al., 1994a, b). The identification of accumulated 7BH$_4$ is a rather rare event in pterin metabolism (Curtius et al., 1988, 1990; Davis et al., 1991; Adler et al., 1992). To date, it still remains obscure whether this 7-isomer has any physiologic function. 7BH$_4$ accumulation has only been described so far in two disorders, in mild variants of phenylketonuria (PKU) (Curtius et al., 1988; Dhoondt et al., 1988; Citron et al., 1993; Ayling et al., 2000) and in vitiligo (Schallreuter et al., 1994a, b). There is one fundamental difference. Patients affected by the former PKU variants express a transient mild hyperphenylalaninaemia and mutations in the DH gene (e.g., from T $\rightarrow$ C causing a substitution of Cys$^{81}$ by Arg$^{81}$). Patients with vitiligo show no hyperphenylalaninaemia (n $\approx$ 321) (Schallreuter et al., 1998; Schallreuter, unpublished results). The results of this study clearly rule out a mutant DH in vitiligo. The removal of epidermal H$_2$O$_2$ in patients with vitiligo yields normal levels of DH and PAH (Figs 3, 4). This observation strongly suggests direct involvement of H$_2$O$_2$ on this enzyme activity. The immunohistochemical approach in this investigation confirmed the presence of DH in the epidermis of healthy controls and untreated/treated patients, predominantly in the basal layer. Our data support restoration of the protein after removal of H$_2$O$_2$ by pseudocatalase (Fig 5). In addition, we were able to prove direct deactivation of the enzyme by H$_2$O$_2$ with recombinant DH and PAH (Fig 7). In this context, it is noteworthy that the enzyme DH functions as a tetramer of identical subunits, whereas its dimers serve for the dimerization of the transcription factor hepatocyte nuclear factor 1 (Ficner et al., 1995; Kim and Burley, 1995). Studies employing X-ray crystallography elucidated a binding site for the substrate analog 7,8 dihydrobiopterin on the enzyme (Ficner et al., 1995). From these studies, it was proposed that the analog/substrate would bind between the...
subunits of the tetramer. Accordingly, Cys\textsuperscript{81} and Trp\textsuperscript{65} were implicated in the vicinity of the catalytic histidine residues in the binding site (Ficner \textit{et al.}, 1995). As both amino acids would be susceptible to oxidation via H\textsubscript{2}O\textsubscript{2}, we suspected that this mechanism could account for low DH activities as observed in vitiligo. Therefore, we examined the entire amino acid sequence of DH, which offers only four possibilities for H\textsubscript{2}O\textsubscript{2} oxidation, i.e., Trp\textsuperscript{24}, Trp\textsuperscript{65}, Cys\textsuperscript{81}, Met\textsuperscript{102}. Computer modeling using the natural substrate 4a-OH-BH\textsubscript{4} clearly identifies the oxidation of Cys\textsuperscript{81}, which severely affects the structure of the active site for the binding of the S-isomer, whereas the oxidation of Cys\textsuperscript{81} in the binding of the R-isomer does not alter the enzyme. However, the oxidation of Trp\textsuperscript{24} changes the binding site for both isomers (Fig 8a, b). By contrast, Trp\textsuperscript{65} and the methionine residue are located in hydrophobic areas of the protein and therefore do not affect the overall structure of the enzyme.

Figure 6. DH \textit{in vitro} mRNA expression in human epidermal melanocytes and keratinocytes from normal healthy and vitiliginous probands. Qualitative agarose gel electrophoretic analysis of PCR assays for DH mRNA expression, standardized to β-actin. The order of the samples on the gel is, from left to right, 100 bp ladder, normal healthy keratinocyte, normal healthy melanocyte, lesional vitiliginous melanocyte, lesional vitiliginous keratinocyte, negative control (omission of template cDNA). This lower expression of melanocyte mRNA can be explained by the observation that melanins can inhibit the PCR (Eckhart \textit{et al.}, 2000).

Figure 7. The effect of H\textsubscript{2}O\textsubscript{2} on the activation of PAH by DH. The production of \textsuperscript{14}C L-tyrosine from \textsuperscript{14}C L-phenylalanine was followed after 35 min by PAH activity alone (●). The initial rate (\(V_0\)) of PAH activity was stimulated 7-fold by the addition of recombinant DH (1:1) (●). In order to examine the direct influences of H\textsubscript{2}O\textsubscript{2} on DH coupling to PAH, rDH was preincubated with 10\,\mu M of H\textsubscript{2}O\textsubscript{2} and the activation assay was repeated showing a time-dependent deactivation of this coupled system (●). Note that, at the incubation time of 10 min, the initial rate of DH activity (\(V_0\)) is only reduced by 40\%. After 10 min, however, the active coupling is terminated, proving the total loss of DH activity by H\textsubscript{2}O\textsubscript{2}.

Figure 8. Molecular modeling of the DH active site before and after oxidation with H\textsubscript{2}O\textsubscript{2}. (a) Binding of the pterin S-isomer. (b) Binding of the pterin R-isomer. The substrate 4a-\textsubscript{OH-BH}_4 is shown in yellow. The backbone of the active site of the enzyme is demonstrated in green. The effect of the oxidation of the Cys\textsuperscript{81} residue in the enzyme is presented in red and shows a conformational change only for the S-isomer (a) whereas the R-isomer does not affect the enzyme structure (b). The reaction of H\textsubscript{2}O\textsubscript{2} with Trp\textsuperscript{24} changes the binding of both isomers (white backbones). Oxidation of this residue by H\textsubscript{2}O\textsubscript{2} results in the movement of the active site imidazole rings of His\textsuperscript{61} and His\textsuperscript{79}.
To our knowledge, this paper identifies for the first time direct deactivation of the major 6BH4 recycling enzyme, DH, by H2O2, both in vivo by utilizing the biologic model of the deactivation disorder vitiligo and in vitro by employing recombinant enzymes. In addition, as 6BH4 is an extremely ubiquitous cofactor involved in several important metabolic pathways, our observation could indicate a general mechanism for regulation of the 6BH4 recycling process via H2O2. In this context, it has recently been shown that H2O2 induces activation of T cells by a novel cholinergic T cell receptor (Ruttut et al., 1999). As both cell types hold the capacity for 6BH4 synthesis and recycling, our results presented in this study could suggest an H2O2/6BH4-mediated control of the immune response. Current studies in our laboratory are under way to further elucidate this interesting hypothesis. Furthermore, it would be of great interest to follow the influence of H2O2 on the de novo synthesis of 6BH4 in vitiligo.

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REFERENCES


Smith I: A typical phenylketonuria accompanied by a severe progressive neurological illness unresponsive to dietary treatment. Arch Dis Child 49:245, 1974

Thöny B, Neuhöser F, Kierat L, et al.: Hyperphenylalaninaemia with high levels of 7-biotin is associated with mutations in the PCBD gene encoding the


