

## Activation/deactivation of acetylcholinesterase by H<sub>2</sub>O<sub>2</sub>: more evidence for oxidative stress in vitiligo

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### Abstract

Previously it has been demonstrated that the human epidermis synthesises and degrades acetylcholine and expresses both muscarinic and nicotinic receptors. These cholinergic systems have been implicated in the development of the epidermal calcium gradient and differentiation in normal healthy skin. In vitiligo severe oxidative stress occurs in the epidermis of these patients with accumulation of H<sub>2</sub>O<sub>2</sub> in the 10<sup>-3</sup> M range together with a decrease in catalase expression/activity due to deactivation of the enzyme active site. It was also shown that the entire recycling of the essential cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin via pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) is affected by H<sub>2</sub>O<sub>2</sub> oxidation of Trp/Met residues in the enzyme structure leading to deactivation of these proteins. Using fluorescence immunohistochemistry we now show that epidermal H<sub>2</sub>O<sub>2</sub> in vitiligo patients yields also almost absent epidermal acetylcholinesterase (AChE). A kinetic analysis using pure recombinant human AChE revealed that low concentrations of H<sub>2</sub>O<sub>2</sub> (10<sup>-6</sup> M) activate this enzyme by increasing the V<sub>max</sub> > 2-fold, meanwhile high concentrations of H<sub>2</sub>O<sub>2</sub> (10<sup>-3</sup> M) inhibit the enzyme with a significant decrease in V<sub>max</sub>. This result was confirmed by fluorescence excitation spectroscopy following the Trp fluorescence at λ<sub>max</sub> 280 nm. Molecular modelling based on the established 3D structure of human AChE supported that H<sub>2</sub>O<sub>2</sub>-mediated oxidation of Trp<sup>432</sup>, Trp<sup>435</sup>, and Met<sup>436</sup> moves and disorients the active site His<sup>440</sup> of the enzyme, leading to deactivation of the protein. To our knowledge these results identified for the first time H<sub>2</sub>O<sub>2</sub> regulation of AChE. Moreover, it was shown that H<sub>2</sub>O<sub>2</sub>-mediated oxidation of AChE contributes significantly to the well-established oxidative stress in vitiligo.

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The skin presents with the size of 1.8 m<sup>2</sup> one of the largest organs of the human body where a plethora of fine-tuned mechanisms keep this outermost layer in homeostasis. Nowadays there is increasing evidence that oxidative stress via hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can severely affect this epidermal balance [1–6]. Over the past several detailed studies on the depigmentation disorder vitiligo provided continuing evidence for the important role of H<sub>2</sub>O<sub>2</sub> in cellular function/dysfunction [1–6]. In this context it has been shown in vivo that the epidermis

of patients with vitiligo accumulates up to 10<sup>-3</sup> M levels of H<sub>2</sub>O<sub>2</sub> [1,2,7]. These concentrations lead to deactivation of catalase [8] as well as to deactivation of pterin-4a-carbinolamine dehydratase (PCD, EC 4.2.1.96) and dihydropteridine reductase (DHPR, EC 1.6.99.7) [2,9]. Both latter enzymes are of major importance in the recycling of the essential cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (6BH<sub>4</sub>) [10]. A detailed kinetic analysis showed activation of DHPR by H<sub>2</sub>O<sub>2</sub> up to 35 μM, whereas higher concentrations inhibited enzyme activities [9]. Computer modelling supported oxidation of Trp and Met residues in the active site of DHPR as well as PCD [2,9]. These results together with enzyme kinetics favoured inhibition by deactivation of the

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protein due to structural alteration. These data were indeed strengthened by *in vitro* FT-Raman- and fluorescence excitation spectroscopy [11]. Moreover, these results gained more support through *in vivo* FT-Raman spectroscopy following the  $\text{H}_2\text{O}_2$  signal via the O–O stretch at  $875\text{ cm}^{-1}$  before and after removal of  $\text{H}_2\text{O}_2$  with a pseudocatalase PC-KUS on the skin of patients with vitiligo [1,7].

In this context it was demonstrated that removal of  $\text{H}_2\text{O}_2$  in the  $10^{-3}\text{ M}$  range coincides with recovery of the entire epidermal  $6\text{BH}_4$  recycling process via PCD and DHPR as well as catalase activities in association with repigmentation of affected individuals [1,2,7,9]. Moreover, it was shown that due to the presence of  $10^{-3}\text{ M}$   $\text{H}_2\text{O}_2$  in the epidermis of those patients, DHPR activities were also decreased in full blood, whereas after topical application of pseudocatalase-PC-KUS even systemic enzyme activities returned to normal [9]. These results indicated for the first time the transfer of epidermal  $\text{H}_2\text{O}_2$  to the vascular system. In 1989 Iyengar showed for the first time low epidermal acetylcholinesterase (AChE, EC 3.1.1.7) in patients with vitiligo [12]. Further support of a possible cholinergic involvement in vitiligo stemmed from *in vivo* studies showing decreased sweating in the depigmented epidermis of these patients [13]. We therefore asked the question whether AChE enzyme activities as well as protein expression are possibly affected by  $\text{H}_2\text{O}_2$  using the clinical model vitiligo together with enzyme kinetics and fluorescence excitation spectroscopy with recombinant human (rh) AChE as well as computer modelling.

In this report we provide evidence *in situ* and *in vitro* that AChE is deactivated by  $\text{H}_2\text{O}_2$  in the range of  $10^{-3}\text{ M}$  concentrations, whereas the enzyme is activated by  $\text{H}_2\text{O}_2$  in the  $10^{-6}\text{ M}$  range. Detailed kinetics using rh AChE, fluorescence excitation spectroscopy, and computer modelling support protein inhibition due to deactivation of the enzyme active site via oxidation of the Trp<sup>432</sup>, Trp<sup>435</sup>, and Met<sup>436</sup> residues by  $\text{H}_2\text{O}_2$  whereas the Trp in position 84 in the binding site is only weakly affected. These *in vitro* results are supported in patients with vitiligo, where epidermal AChE protein expression recovers after decreasing  $\text{H}_2\text{O}_2$  to  $10^{-6}\text{ M}$  with pseudocatalase PC-KUS. The recovery and upregulation of AChE protein expression coincides with recovery of catalase protein expression and repigmentation of these patients. In conclusion, our results uncovered a novel mechanism for the regulation of AChE activities by  $\text{H}_2\text{O}_2$  and provide further evidence for  $\text{H}_2\text{O}_2$ -oxidative stress in the depigmentation process of vitiligo.

## Materials and methods

**Human tissue biopsies.** Human full skin biopsies ( $\varnothing 3\text{ mm}$ ) were taken under local anaesthesia from sun unexposed areas of healthy volunteers, skin phenotype III ( $n = 4$ ). (Fitzpatrick classification) [14]

and from untreated and treated patients with vitiligo ( $n = 4$ ). The samples were embedded into O.C.T. compound (Sakura, Eastbourne, UK), immediately snap-frozen in liquid nitrogen, and stored at  $-86^\circ\text{C}$  until further use. Sections were cut in  $5\text{ }\mu\text{m}$  thickness and placed on poly-L-lysine coated glass slides (Sigma–Aldrich, St. Louis, USA). This study was approved by the local Ethics committee.

**Removal of epidermal  $\text{H}_2\text{O}_2$  in patients with vitiligo by treatment with low dose narrowband UVB-activated pseudocatalase PC-KUS.** Patients were treated twice daily with a topical application of narrowband UVB-activated pseudocatalase PC-KUS for the removal of  $\text{H}_2\text{O}_2$  as reported in detail previously by Schallreuter et al. [1,2]. This protocol was approved by the Local Ethics Committee.

***In situ* and *in vitro* double immunofluorescence studies.** Cryo cut sections ( $5\text{ }\mu\text{m}$ ) of normal human full skin biopsies were fixed in acetone for 15 min at  $-20^\circ\text{C}$ . Sections were blocked with 10% normal donkey serum (NDS). In order to demonstrate the presence of AChE and catalase we used a goat monoclonal anti-human AChE antibody (dilution 1:2500 in 1% NDS, Abcam, Cambridge, UK) together with fluorescein isothiocyanate (FITC)-labelled donkey anti-goat (dilution 1:100, Jacksons Immunoresearch Laboratories, PA, USA). For demonstration of catalase we utilised a mouse polyclonal anti-human catalase antibody (clone HAS-11, dilution 1:25 in 1% NDS, Sigma, Poole Dorset, UK) together with FITC-labelled donkey anti-mouse (dilution 1:100, Jacksons Immunoresearch Laboratories, PA, USA). Subsequently, sections were incubated overnight at  $4^\circ\text{C}$  with the primary antibody followed by incubation over 90 min with the FITC-labelled secondary antibody. Immunoreactivity was viewed and captured using a Leica DM-IRB inverted microscope coupled to a digital camera together with Neotech “Image Grabber PCI” imaging software. In order to compare the intensity of the protein expression, all stainings were carried out in one set. To validate these data all images were taken under the same microscope settings.

**AChE enzyme assay.** Recombinant human AChE (rh AChE, Sigma, Dorset, UK) was used for the  $\text{H}_2\text{O}_2$  activation/deactivation experiments. One unit of enzyme hydrolysed  $1.0\text{ }\mu\text{mol}$  of Ach per minute at  $37^\circ\text{C}$  at pH 8.0 [15]. Enzyme activities were measured with fluorescence spectroscopy (Perkin–Elmer fluorescence spectrophotometer 1000) using the excitation at  $560\text{ nm}$  with emission at  $590\text{ nm}$ . Time-dependent hydrolysis of Ach to acetate and choline was based on the Amplex Red Ach/AChE assay kit (A-12217, Molecular Probes, UK). Quantitative analysis of Ach was accomplished by calibration of reaction rates over the range  $0\text{--}100\text{ }\mu\text{M}$  Ach (Sigma, Dorset, UK).  $\text{H}_2\text{O}_2$  activation/deactivation of AChE was followed after preincubating standard aliquots of enzyme with  $\text{H}_2\text{O}_2$  for 60 min at RT over a concentration range of  $0\text{--}40 \times 10^{-6}\text{ M}$  (activation) and  $0\text{--}40 \times 10^{-3}\text{ M}$  (deactivation). Separation of AChE from excess  $\text{H}_2\text{O}_2$  was achieved by G-25 Sephadex gel filtration (Pharmacia, Uppsala, Sweden).

**Fluorescence spectroscopic analysis of human rh AChE in the absence and presence of  $\text{H}_2\text{O}_2$  ( $2 \times 10^{-3}\text{ M}$ ).** For the *in vitro* fluorescence measurements we used the SkinScan instrument (Jobin Yvon-Horiba, Stanmore, UK) equipped with a xenon lamp as light source and subtractive monochromators (reflection gratings, 1200 grooves/mm) for both, excitation and emission. For the excitation spectra of the 13 identified Trp residues in AChE [16], the fluorescence intensity was followed at the fixed emission wavelength of  $340\text{ nm}$  with varying excitation wavelengths ranging from  $250$  to  $325\text{ nm}$ ,  $15\text{ nm}$  below the emission wavelength to avoid the Rayleigh scatter peak. The spectra were corrected for the spectral output (wavelength and voltage changes) of the xenon lamp using the reference photodiode. The enzyme was exposed to  $2 \times 10^{-3}\text{ M}$   $\text{H}_2\text{O}_2$  at pH 7.0 over time (0–18 h).

**Hyperchem molecular modelling of AChE.** Molecular modelling of AChE was accomplished based on the 3D-structure obtained from X-ray crystallographic analysis [16]. Trp and Met residues, predicted to be oxidised by  $10^{-3}\text{ M}$   $\text{H}_2\text{O}_2$ , were introduced into the structure sequence near to the active site His<sup>440</sup> and for Trp<sup>84</sup> in the Ach binding site using Hyperchem software. The structure was then minimised in water and compared to native AChE by Deep View analysis.

## Results

### *In situ* expression of AchE and catalase in normal human epidermis and in patients with vitiligo is affected by H<sub>2</sub>O<sub>2</sub> at 10<sup>-3</sup> M concentrations

Immunohistochemical examination of normal human epidermal sections *in situ* confirmed the expression of AchE (Fig. 1A) [12,17,18]. AchE is present in a perinuclear distribution throughout the entire epidermis, but not restricted to the basal layer as reported previously (Fig. 1A) [17,18]. Examinations of the untreated depigmented epidermis of patients with vitiligo revealed a significant decrease in expression of AchE (Fig. 1B) compared to skin phototype matched healthy controls (Fig. 1A). Repigmenting patients treated with PC-KUS showed a strong increase in AchE throughout the epidermis (Fig. 1C). These results supported and extended the observation reported earlier by Iyengar [12]. However, the mechanism for decreased AchE protein expression escaped explanation at that time.

Since catalase levels and activities have been shown to be significantly decreased in both the epidermis and in melanocytes in vitiligo together with 10<sup>-3</sup> M concentrations of H<sub>2</sub>O<sub>2</sub>, we decided to use this protein as positive control for epidermal H<sub>2</sub>O<sub>2</sub> stress [5,6]. Fig. 2 shows the expression of catalase in epidermal sections of

patients with vitiligo before (Fig. 2B) and after removal of epidermal H<sub>2</sub>O<sub>2</sub> with PC-KUS (Fig. 2C) compared to a matched skin phototype control (Fig. 2A). Catalase is expressed throughout the epidermis, but is almost absent from the depigmented skin in vitiligo (Fig. 2B). This result is in agreement with deactivated catalase by 10<sup>-3</sup> M H<sub>2</sub>O<sub>2</sub> and by significantly lower epidermal enzyme levels as reported earlier [6,8]. After removal of H<sub>2</sub>O<sub>2</sub> with pseudocatalase PC-KUS, epidermal catalase levels increase significantly compared to control values (Fig. 2C). Taken together these immunohistochemical studies show that H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress in 10<sup>-3</sup> M range as observed in vitiligo decreases the protein expression of both AchE and catalase. However, after decreasing H<sub>2</sub>O<sub>2</sub> from 10<sup>-3</sup> to 10<sup>-6</sup> M concentrations, AchE as well as catalase protein expression is induced.

### *Activation/inhibition of human recombinant AchE by H<sub>2</sub>O<sub>2</sub>*

In order to test a possible contribution of H<sub>2</sub>O<sub>2</sub> to the decrease of epidermal AchE protein expression, we used purified human enzyme, preincubated for 1 h with H<sub>2</sub>O<sub>2</sub>, over a concentration range of 0–40 × 10<sup>-6</sup> M as well as over a range of 0–40 × 10<sup>-3</sup> M. These experiments were designed to ascertain the influence of low and high concentrations of H<sub>2</sub>O<sub>2</sub> on AchE activities. Figs. 3A and

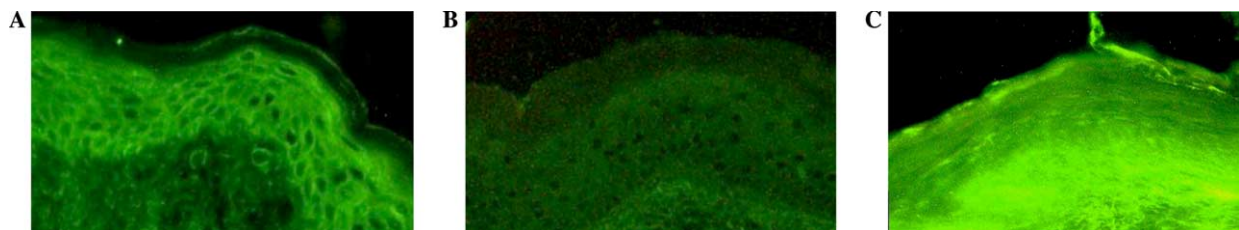


Fig. 1. *In situ* epidermal expression of AchE in the lesional skin of vitiligo before and after H<sub>2</sub>O<sub>2</sub>-removal with PC-KUS (magnification 200×). (A) Normal healthy control skin phototype III (Fitzpatrick classification). (B) Lesional skin with low enzyme expression before treatment with PC-KUS. (C) Lesional skin after treatment with PC-KUS. Note the upregulation of AchE protein expression after decrease of H<sub>2</sub>O<sub>2</sub>.

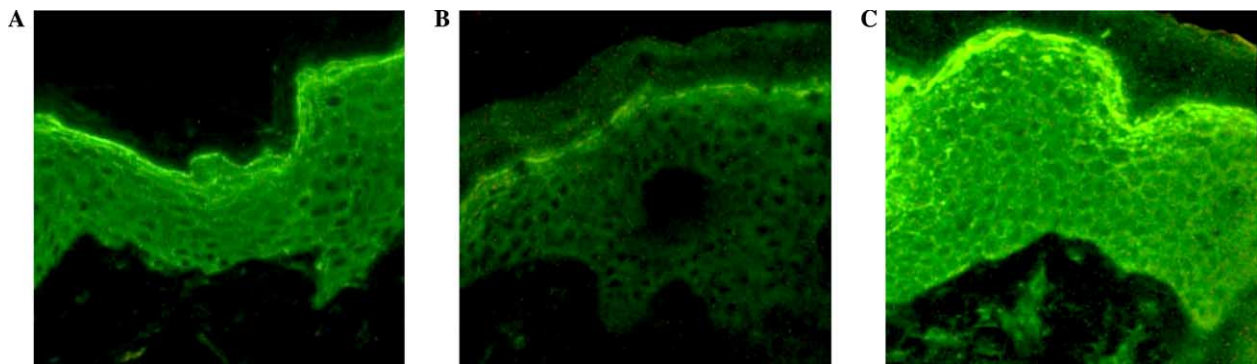


Fig. 2. *In situ* expression and localisation of epidermal catalase in lesional skin of vitiligo before and after H<sub>2</sub>O<sub>2</sub> removal with PC-KUS (magnification 200×). (A) Normal healthy control skin phototype III (Fitzpatrick classification). (B) Lesional skin before treatment with PC-KUS. (C) Lesional skin after treatment with PC-KUS. After H<sub>2</sub>O<sub>2</sub> removal the protein expression is upregulated.

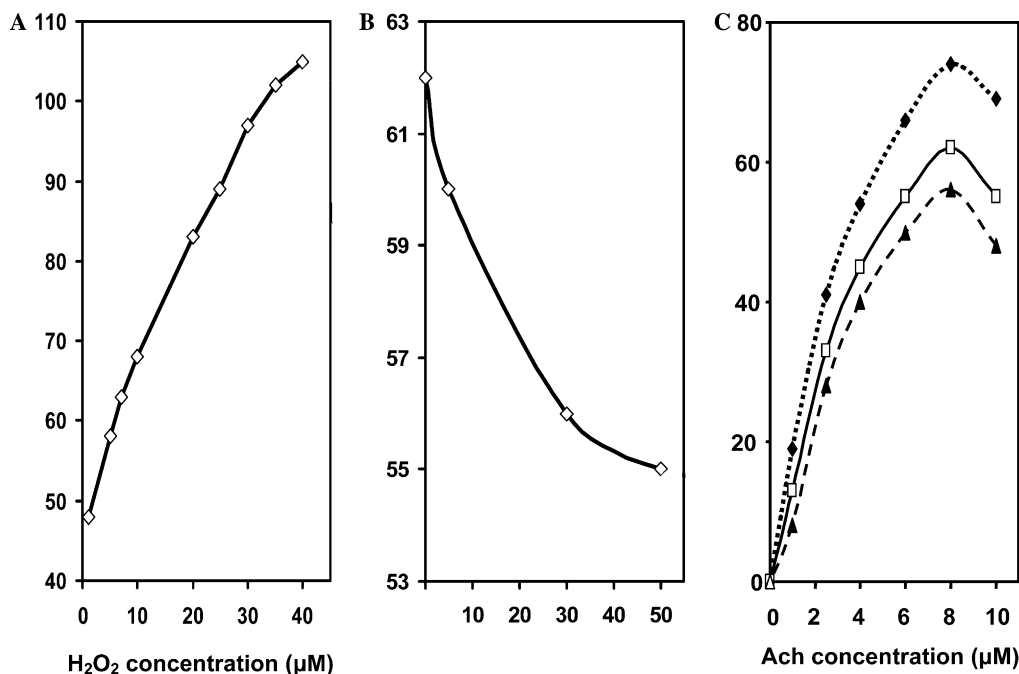


Fig. 3. Activation/inhibition of rh AchE by different concentrations of H<sub>2</sub>O<sub>2</sub>. The enzyme was pre-incubated with different concentrations of H<sub>2</sub>O<sub>2</sub> for 60 min prior to measuring the reaction rates/5 min as outlined in methods. (A) Rates for rh AchE/5 min over a concentration range of H<sub>2</sub>O<sub>2</sub> from 0 to 40 × 10<sup>-6</sup> M yielding a 2.5-fold increase in V<sub>max</sub>. (B) Rates over a concentration range of H<sub>2</sub>O<sub>2</sub> from 0 to 40 × 10<sup>-3</sup> M. (C) V vs S plots for the activation and inhibition of rh AchE by H<sub>2</sub>O<sub>2</sub>: (a) control (□-□), (b) activation with 40 × 10<sup>-6</sup> M H<sub>2</sub>O<sub>2</sub> (◆-◆), and (c) inhibition with 40 × 10<sup>-3</sup> M H<sub>2</sub>O<sub>2</sub> (▲-▲).

B presents the results. Low concentrations of H<sub>2</sub>O<sub>2</sub> (10<sup>-6</sup> M) activate AchE increasing the V<sub>max</sub> > 2 fold over control values in the absence of any H<sub>2</sub>O<sub>2</sub> (Fig. 3A). High concentrations of H<sub>2</sub>O<sub>2</sub> (10<sup>-3</sup> M) inhibit AchE in a concentration-dependent manner (Fig. 3B). Since the V<sub>max</sub> decreases, this result favours enzyme inhibition due to oxidative deactivation rather than non-competitive or un-competitive inhibition. Fig. 3C shows the V vs S plot for both activation and inhibition of AchE demonstrating no change in the K<sub>m</sub>. These results indicate classical activation and deactivation kinetics without a significant influence on the rate of Ach-binding. Taken together this observation favours H<sub>2</sub>O<sub>2</sub>-mediated structural alteration of the AchE active site, rather than the binding site.

#### Fluorescence excitation spectroscopy of Trp residues in rh AchE proves partial oxidation of the total 13 Trp residues in the enzyme

The fluorescence excitation maximum of Trp in AchE at a λ<sub>max</sub> 280 nm shows a strong signal as expected. After incubation of the enzyme with 2 × 10<sup>-3</sup> M H<sub>2</sub>O<sub>2</sub> over time the peak decreases, indicative of H<sub>2</sub>O<sub>2</sub>-mediated oxidation (Fig. 4). However, even after 16 h incubation a signal is still present. This result supports that some Trp residues are in a protected environment.

#### Hyperchem molecular modelling and Deep View analysis of H<sub>2</sub>O<sub>2</sub>-deactivated AchE

To substantiate the predicted H<sub>2</sub>O<sub>2</sub>-mediated structural alteration of AchE, we utilised computer modelling and Deep View analysis.

Based on the X-ray crystal structure of AchE, both, the active site and the binding site for Ach, were examined before and after the oxidation of the key residues Trp and Met by H<sub>2</sub>O<sub>2</sub> [16]. Fig. 5A shows the structure of the active site of AchE presenting a typical serine esterase. In this context it was established earlier that His<sup>440</sup> is critical for acid/base catalysis by interacting with the essential Ser<sup>200</sup> and the carbonyl group of the Ach, whereas Glu<sup>327</sup> stabilizes His<sup>440</sup> in the active site [16]. Only four or five residues away from His<sup>440</sup> are Met<sup>436</sup> and Trp<sup>435</sup>. The oxidation of Trp<sup>432</sup>, Trp<sup>435</sup>, and Met<sup>436</sup> to 5-OH-Trp<sup>432/435</sup> and methionine sulphoxide, respectively, causes a conformational change moving His<sup>440</sup> away from the active site together with disorientation of the imidazole group (Fig. 5B). This result is in agreement with the kinetic analysis presented in Fig. 3C.

Next we studied the enzyme binding site. The binding site for the quaternary ammonium group of Ach has been established as Trp<sup>84</sup> and Phe<sup>330</sup> [16]. In the model oxidation of Trp<sup>84</sup> to 5-OH-Trp<sup>84</sup> disrupts this binding domain (Fig. 5C). However, the kinetic analysis revealed

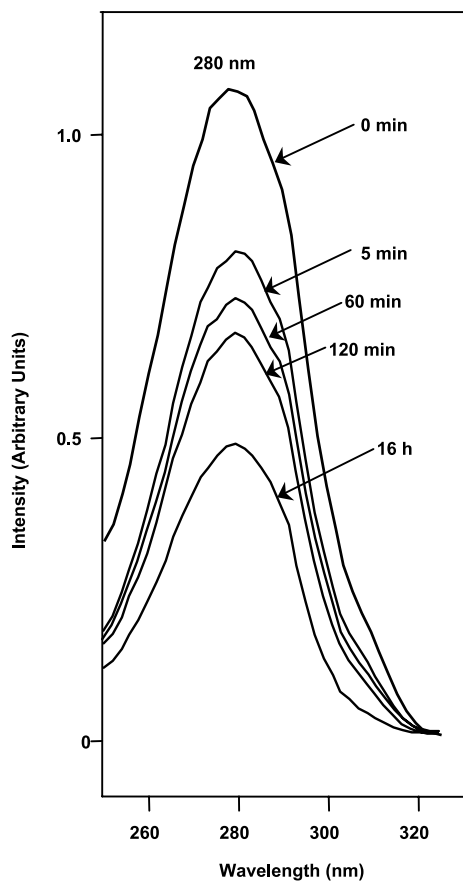


Fig. 4. Fluorescence excitation spectra of the Trp residues in rh AChE at a  $\lambda_{\text{max}}$  280 nm before and after oxidation with  $2 \times 10^{-3}$  M  $\text{H}_2\text{O}_2$ . The decrease of the peak over time is in agreement with partial  $\text{H}_2\text{O}_2$ -mediated oxidation of the Trp residues in AChE.

under the experimental conditions employed herein that there is only a very small change in the rate for  $\text{H}_2\text{O}_2$  oxidised AChE compared to AChE alone, i.e.,  $K_m$   $24 \times 10^{-6}$  M compared to  $K_m$   $23 \times 10^{-6}$  M, respectively. This result would suggest that Trp<sup>84</sup> is not readily available for rapid oxidation by  $10^{-3}$  M  $\text{H}_2\text{O}_2$ . This assumption is supported by the kinetics of the V vs S plot (Fig. 3C) and by the fluorescence excitation spectroscopy yielding partial oxidation of the Trp residues in AChE.

## Discussion

To date there is overwhelming evidence for systemic and epidermal oxidative stress via  $\text{H}_2\text{O}_2$  in the depigmentation disorder vitiligo [1,5,19–21]. Detailed *in vitro* and *in vivo* studies on various metabolic mechanisms have provided a new understanding of physiological and pathophysiological events in this disease process, despite that the precise step in the cascade for initiation of this peculiar disorder remains still unknown. However, recently the  $\text{H}_2\text{O}_2$ -mediated oxidation/deactivation of

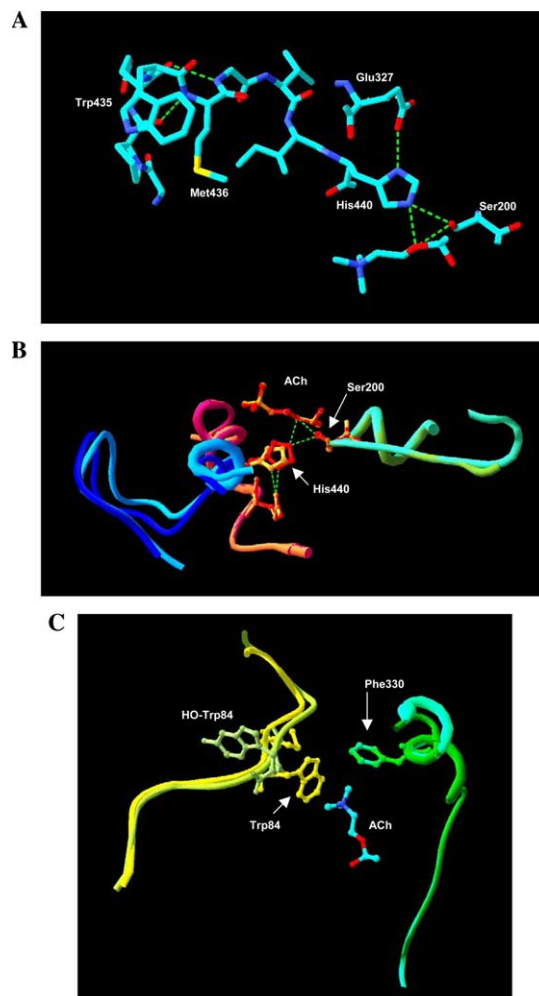


Fig. 5. Hyperchem molecular modelling of the active site of AChE before and after deactivation by  $\text{H}_2\text{O}_2$ . (A) Deep View analysis of the active site of AChE showing the important residues His<sup>440</sup>, Ser<sup>200</sup>, and Glu<sup>327</sup>. H-bond interaction between N<sub>3</sub> of His<sup>440</sup> and the carbonyl group of ACh together with the OH-group of Ser<sup>200</sup> is indicated. The Glu<sup>327</sup> carboxylate group orientates His<sup>440</sup> by binding of N<sub>1</sub>. Five residues away from His<sup>440</sup> is Trp<sup>435</sup> bonded to Met<sup>436</sup>. Both residues are  $\text{H}_2\text{O}_2$  sensitive targets (C-backbone/blue, N/dark blue, O/red, and S/yellow). (B) Orientation and distance of the His<sup>440</sup> imidazole ring after oxidation of Trp<sup>432</sup>, Trp<sup>435</sup>, and Met<sup>436</sup> by  $\text{H}_2\text{O}_2$  (oxidised enzyme (yellow) compared to control unoxidised enzyme (red)) revealing evidence for  $\text{H}_2\text{O}_2$  deactivation of the enzyme active site. (C)  $\text{H}_2\text{O}_2$  influences in the model also Trp<sup>84</sup> (yellow) to 5-OH Trp<sup>84</sup> (light green) in the ACh binding site of AChE, whereas Phe<sup>330</sup> (dark green) remains unaffected. However, this predicted  $\text{H}_2\text{O}_2$  mediated Trp<sup>84</sup> oxidation in the binding site could not be confirmed under the experimental conditions used in this study.

important enzymes added a new reaction mechanism to the regulation of enzyme activity [2,8,9]. The accumulation of  $10^{-3}$  M  $\text{H}_2\text{O}_2$  directly deactivates catalase as well as the  $6\text{BH}_4$  recycling enzymes PCD and DHPR [2,8,9]. In this context it has also been shown that decreased PCD enzyme activity as well as protein expression are based on the oxidation of Trp<sup>24</sup> to 5-OH-Trp<sup>24</sup> leading to a conformational change of the active site His<sup>61</sup> and His<sup>79</sup> residues [2]. Oxidation of Met<sup>146</sup> and

Met<sup>151</sup> in the short helix of DHPR yields a structural change of the enzyme preventing the binding of the cofactor NADH [9]. Consequently, the uncoupled 6BH<sub>4</sub> recycling leads to the non-enzymatic accumulation of the 7-isomer, 7BH<sub>4</sub>, which in turn inhibits phenylalanine hydroxylase (PAH, EC 1.14.16.1) as well as the key enzyme for melanogenesis, i.e., tyrosinase (EC 1.14.8.1) [22–25].

In the report presented herein we uncovered a new mechanism in the regulation of AchE by H<sub>2</sub>O<sub>2</sub>. Based on in situ immunofluorescence we show decreased epidermal protein expression in untreated patients with vitiligo together with H<sub>2</sub>O<sub>2</sub> concentration in 10<sup>-3</sup> M range, meanwhile after removal of epidermal H<sub>2</sub>O<sub>2</sub> with pseudocatalase PC-KUS protein expression strongly increases. This result is in agreement with earlier in vivo FT-Raman spectroscopy demonstrating the removal of 10<sup>-3</sup> M H<sub>2</sub>O<sub>2</sub> from the epidermis as well as with the recovery of catalase after H<sub>2</sub>O<sub>2</sub> removal (Fig. 2C) [1,3,26]. However, due to the detection limit of this method it is impossible to determine epidermal H<sub>2</sub>O<sub>2</sub> concentrations in the 10<sup>-6</sup> M range. The loss of AchE protein expression can be best explained based on conformational changes of the enzyme via 10<sup>-3</sup> M H<sub>2</sub>O<sub>2</sub> oxidation. This assumption is supported by detailed enzyme kinetics using rh AchE (Figs. 3A–C), fluorescence excitation spectroscopy (Fig. 4), and computer modelling (Figs. 5A–C). Moreover, the results of the computer modelling demonstrated that oxidation of Trp<sup>432</sup>, Trp<sup>435</sup>, and Met<sup>436</sup> causes a major disruption of the His<sup>440</sup> active site. This hypothetical result is backed up by the enzyme kinetics. The computer also predicted oxidation of Trp<sup>84</sup> in the binding site of AchE, but neither the enzyme kinetics nor the fluorescence excitation spectroscopy analysis supports this oxidation under the experimental conditions used in this study. It is tempting to speculate that this residue is more protected due to the hydrophobic environment.

In summary, one lesson can be learned. H<sub>2</sub>O<sub>2</sub> plays a pivotal role in physiology and pathophysiology. In high concentrations (10<sup>-3</sup> M) it can be deleterious—but in the physiological range (10<sup>-6</sup> M) it is a very important signalling molecule.

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