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The role of double covalent flavin binding in chito-oligosaccharide oxidase from *Fusarium graminearum*

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ChitO (chito-oligosaccharide oxidase) from *Fusarium graminearum* catalyses the regioselective oxidation of N-acetylated oligosaccharides. The enzyme harbours an FAD cofactor that is covalently attached to His^{94} and Cys^{154}. The functional role of this unusual bi-covalent flavin–protein linkage was studied by site-directed mutagenesis. The double mutant (H94A/C154A) was not expressed, which suggests that a covalent flavin–protein bond is needed for protein stability. The single mutants H94A and C154A were expressed as FAD-containing enzymes in which one of the covalent FAD–protein bonds was disrupted relative to the wild-type enzyme. Both mutants were poorly active, as the k_{cat} decreased (8.3- and 3-fold respectively) and the K_m increased drastically (34- and 75-fold respectively) when using GlcNac as the substrate. Pre-steady-state analysis revealed that the rate of reduction in the mutant enzymes is decreased by 3 orders of magnitude when compared with wild-type ChitO ($k_{red} = 750\ s^{-1}$) and thereby limits the turnover rate. Spectroelectrochemical titrations revealed that wild-type ChitO exhibits a relatively high redox potential (+131 mV) and the C154A mutant displays a lower potential (+70 mV), while the H94A mutant displays a relatively high potential of approximately +164 mV. The results show that a high redox potential is not the only prerequisite to ensure efficient catalysis and that removal of either of the covalent bonds may perturb the geometry of the Michaelis complex. Besides tuning the redox properties, the bi-covalent binding of the FAD cofactor in ChitO is essential for a catalytically competent conformation of the active site.

Key words: bi-covalent FAD, oligosaccharides, oxidase, pre-steady-state kinetics, redox potential.

INTRODUCTION

ChitO (chito-oligosaccharide oxidase) is a recently discovered enzyme from *Fusarium graminearum* that catalyses the oxidation of C1-hydroxyl moieties on carbohydrates [1]. The enzyme preferably acts on chito-oligosaccharides, which results in the formation of the corresponding lactone that spontaneously hydrolyses to form the chito-oligosaccharide acid. For catalysis ChitO employs a covalent FAD cofactor. ChitO shares homology with several other carbohydrate oxidases, e.g. GOOX (gluco-oligosaccharide oxidase), alditol oxidase, lactose oxidase and hexose oxidase [2–5]. All these carbohydrate oxidases belong to the so-called VAO (vanillyl-alcohol oxidase) family [6]. Members of this family share a similar protein structure consisting of two domains. One domain, the FAD-binding domain, binds the adenine part of the FAD cofactor, whereas the second domain, the cap domain, covers the isoalloxazine moiety of the flavin cofactor and in this way forms the major part of the active site. Most of the VAO-type flavoproteins, including the above-mentioned carbohydrate oxidases, bind FAD in a covalent fashion. Several explanations have been proposed for the occurrence of this covalent flavin–protein bond. It has been shown that the covalent linkage can have a significant effect on (i) the redox behaviour of the flavin cofactor by increasing the redox potential to a great extent and (ii) the tertiary structure of the enzyme by increasing its stability [7,8]. Other reported rationales for covalent flavinylation are, for example, facilitating electron transfer in the case of the tyrosyl–FAD linkage in *p*-cresol methylhydroxylase [9], preventing dissociation of the cofactor [10] and preventing suicide inactivation at the C6 position [11]. However, the finding that there are enzymes which catalyse the same reaction with comparable catalytic and kinetic properties, but differ in the mode of FAD binding, shows that the raison d'être of covalent flavinylation can be more complex. This is nicely illustrated by cholesterol oxidase type II compared with type I, which contain covalent FAD and non-covalent FAD respectively [8].

Recently it was discovered that the covalently bound FAD, which is present in GOOX, is tethered to two amino acid residues (Figure 1A) [12]. Since this discovery, a similar double covalent anchoring of FAD has been recognized in other oxidases such as hexose oxidase, BBE (berberine bridge enzyme) and AknOx (aclacinomycin oxidoreductase) [13–15]. In these cases, the enzyme contains an 8α-histidyl-6-*S*-cysteinyl-FAD bound cofactor (Figure 1). In view of the previous suggestions on the role of covalent flavinylation, the finding of bi-covalently linked FAD raises the question of what the functional role of covalent anchoring via two covalent bonds is. Clearly, one covalent linkage already assures binding of FAD. For BBE it has been shown that the cysteinyl–FAD linkage influences the redox potential [16]. This has been directly linked to the drastic decrease in the rate of conversion using the substrate (*S*)-reticuline.

In this paper, we have analysed the role of the bi-covalently linked FAD in ChitO by mutating the residues involved and studying the effect on the kinetic behaviour and redox potential. The results show that both covalent bonds are crucial for efficient catalysis. As well as influencing the redox properties of the flavin cofactor, the double covalent linkage also appears to play a crucial role in cofactor positioning in the active site.

Abbreviations used: AknOx, aclacinomycin oxidoreductase; BBE, berberine bridge enzyme; ChitO, chito-oligosaccharide oxidase; EMT, enzyme-monitored turnover; GOOX, gluco-oligosaccharide oxidase; MBP, maltose-binding protein; NCBI, National Center for Biotechnology Information; OTTLE, optically transparent thin-layer electrode; SHE, standard hydrogen electrode; VAO, vanillyl-alcohol oxidase.

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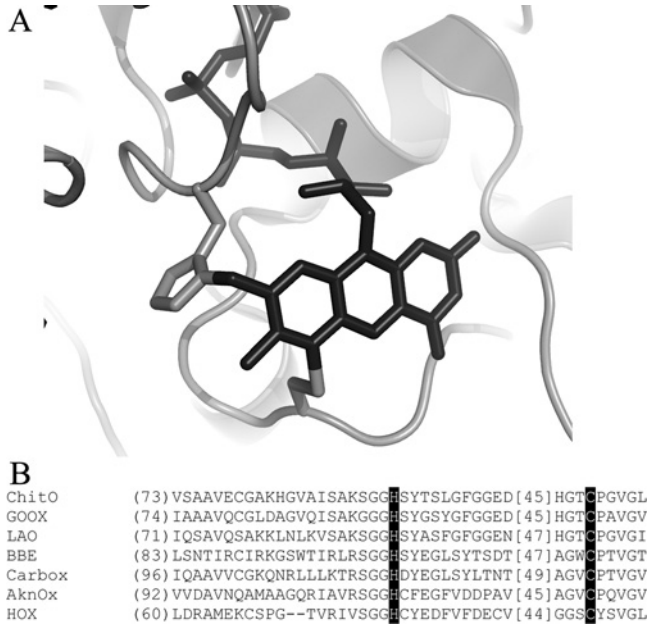

Figure 1 FAD cofactors in oxidases

(**A**) The 8α-histidyl-6-*S*-cysteinyl-FAD cofactor in GOOX. The FAD cofactor is shown as black sticks, the linking cysteine and histidine residues are highlighted in grey sticks and the protein backbone is shown in a grey cartoon representation. The Figure was prepared using the 1ZR6 PDB file and the PyMol software (http://www.pymol.org). (**B**) Multiple sequence alignment of ChitO and other VAO-type oxidases which (putatively) contain a bi-covalently linked FAD cofactor. The regions surrounding the conserved histidine and cysteine residues involved in covalent FAD binding are shown. The following amino acid sequences were used: ChitO (XP_391174) from *F. graminearum*, GOOX (AAS79317) from *A. strictum*, lactose oxidase (LAO) from *Microdochium nivale*, BBE (AAC39358) from *Eschscholzia californica*, carbox (AAL77103) from *Helianthus annuus*, AknOx (ABI15166) from *Streptomyces galilaeus* and hexose oxidase (HOX; AAB49376) from *Chondrus crispus*. The amino acid residues which are expected to be involved in covalent FAD binding are highlighted in black. The numbers in front of every sequence refer to the amino acid numbers; the numbers in between the sequences refer to the number of amino acids that are between the two segments.

EXPERIMENTAL

Chemicals and restriction enzymes

Restriction enzymes were obtained from Roche and New England Biolabs. *Escherichia coli* ORIGAMI was obtained from Novagen, while the TOPO TA cloning kit for sequencing and the pBAD/*Myc*-His A vector were obtained from Invitrogen. VAO (EC 1.1.3.38) was a gift from Dr. W.J.H. van Berkel (Wageningen University, The Netherlands). All other chemicals were of analytical grade.

Structure modelling and sequence analysis

Putative double covalent flavoproteins were identified by performing PSI-BLAST (position-specific iterative BLAST) searches using the GOOX sequence in the non-redundant genome databases of the NCBI (National Center for Biotechnology Information). A structure model of ChitO was obtained using the CPHmodels 2.0 server (http://www.cbs.dtu.dk/services/CPHmodels). The ClustalW tool (http://www.ebi.ac.uk/clustalw) was used to produce multiple sequence alignments of ChitO and the identified homologues to indicate the residues involved in covalent FAD binding.

Cloning and expression of ChitO

The cloning and expression procedures have been published before, but will be mentioned briefly here. The *chitO* gene was amplified from a *F. graminearum* cDNA library and cloned into a pBAD-MBP (pBAD-maltose binding protein) vector [1]. For this the following primers were used: m-ChitO_fw (5′-TTCTAGAATGGTCCCGACCAAGCGCGAA-3′, with the XbaI site underlined) and m-ChitO_rv (5′-GTCTAGAA*CTA*AG-CCTTAGGCTTAATAGACTGA-3′, with the XbaI site underlined and the stop codon italicized). The pBAD-MBP-*chitO* construct was obtained by ligating the *chitO* gene into an XbaI-digested pBAD-MBP vector. For expression of m-ChitO, *E. coli* ORIGAMI was transformed with pBAD-MBP-*chitO* and cultured in LB (Luria–Bertani) medium containing 50 μg/ml ampicillin, 15 μg/ml kanamycin, 12.5 μg/ml tetracycline and 0.4 % (w/v) L-arabinose for 3 days at 17 °C.

Site-directed mutagenesis

The H94A, C154A and H94A/C154A mutants were prepared using the QuikChange site-directed mutagenesis kit from Stratagene. The primers used for this are: H94A_fw, 5′-GCC-AAGAGTGGCGGCGCCAGCTATACCTCTCTAGG; H94A_rv, 5′-CCTAGAGAGGTATAGCTGGCGCCGCCACTCTTGGC; C154A_fw, 5′-GCTCTAGCTCATGGAACGGCCCCTGGAGT-CGGCCTCG; and C154A_rv, 5′-CGAGGCCGACTCCAGG-GGCCGTTCCATGAGCTAGAGC. The sites of mutation are underlined.

Purification of m-ChitO wild-type and mutants

For wild-type and mutant m-ChitO purification, a total culture volume of 3.3 litres was harvested and cells were pelleted by centrifugation at 4 °C, after which the cells were resuspended in KP_i buffer (50 mM potassium phosphate buffer, pH 7.6). The cells were disrupted by sonicating for 15 min at 0 °C. The cell extract obtained in this way was centrifuged and the supernatant was filtered through a sterile 0.2 μm filter and loaded on to a 200 ml DEAE Sepharose Fast Flow (Pharmacia, anion exchange) column using an ÄKTA Prime purification system. The column was washed with KP_i buffer and the protein was eluted during this wash step, as the binding of this protein under the described conditions to the DEAE column was weak. Yellow fractions containing m-ChitO were concentrated using the Amicon concentrator (Millipore) fitted with a YM30 filter and stored at -80 °C in 1 ml aliquots. Samples containing m-ChitO with covalently bound FAD were detected by incubating SDS/PAGE gels with 5 % (v/v) acetic acid for 5 min and observing fluorescence with a UV-bench (transilluminator UST20M8K, Biostep, Germany). The same SDS/PAGE gels were subsequently stained with Coomassie Brilliant Blue.

Analytical methods

All experiments were conducted with m-ChitO, the MBP-ChitO fusion protein, in KP_i buffer at 25 °C. The absorbance spectrum of purified m-ChitO was recorded between 300 and 600 nm before and after addition of 0.1 % (w/v) SDS. The absorption coefficient and concentration of purified m-ChitO were determined using the absorption coefficient of free FAD ($\varepsilon_{450} = 11.3\ mM^{-1} \cdot cm^{-1}$) for the C154A mutant and the absorption coefficient for 6-*S*-cysteinyl–bound FMN ($\varepsilon_{445} = 11.6\ mM^{-1} \cdot cm^{-1}$) for wild-type and H94A m-ChitO [17]. An FAD molecule with 8α-histidyl and 6-*S*-cysteinyl modifications has not yet been synthesized. On the basis of the spectral analysis of a related bi-covalent flavoprotein, BBE, it was decided to use 6-*S*-cysteinyl-bound FMN as the most accurate reference compound [14,17].

To measure oxidase activity and to determine steady-state kinetic parameters, the production of H_2O_2 by m-ChitO was coupled to a horseradish peroxidase-mediated oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid. The resulting pink to purple coloured product which is formed was measured at 515 nm ($\varepsilon_{515} = 26\ mM^{-1} \cdot cm^{-1}$) [18]. The reaction mixture contained KP_i buffer, 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 5 units of horseradish peroxidase and 16 nM m-ChitO.

The pH optimum of m-ChitO was determined using the coupled assay as described above. Instead of the KP_i buffer, a Britton–Robinson buffer was employed which covered the range of pH 4.5 to 9.4 (0.2 M boric acid, 0.2 M acetic acid and 0.2 M phosphoric acid adjusted to the desired pH with 2 M NaOH) [19]. Activity was measured using 30 mM GlcNAc as substrate.

Stopped-flow kinetic measurements

In all cases the terms reductive and oxidative half-reaction relate to the flavin redox state. Enzyme monitored turnover experiments were conducted by aerobically mixing equal volumes of approximately 6–14 μM wild-type m-ChitO or mutant m-ChitO in KP_i buffer and 100 mM GlcNAc in KP_i buffer. The change in absorbance was followed at 450 nm for 10 s in the case of wild-type m-ChitO and for 200 s at 445 nm and 447 nm in the case of the H94A and C154A mutants respectively.

Pre-steady-state kinetic measurements were performed with GlcNac as substrate using an Applied Photophysics stopped-flow apparatus with diode array and photo-multiplier detection. For anoxic measurements the samples contained 1.0 mM 4-ethylphenol, were flushed with N_2 for 10 min and were subsequently supplemented with 100 nM VAO to remove any remaining O_2. The reductive half-reaction was followed using diode-array detection to observe spectral changes and with photo-multiplier detection to determine the observed rates. For this, equal volumes of 6–20 μM m-ChitO and varying concentrations of GlcNAc were mixed at 25 °C in KP_i buffer. Results were collected using the XScan software package with an oversampling option (Applied Photophysics) and the absorbance between 200 and 700 nm was recorded. The reaction was followed for 1 s and a scan was taken every 2.5 ms, yielding 400 spectra per reaction. Raw data were exported and deconvoluted with Pro-K software (Applied Photophysics) to identify the number of kinetic processes present and to determine a suitable wavelength at which to perform reduction and reoxidation measurements with single wavelength detection. Single wavelength experiments were conducted by following the change in absorbance at 450 nm and yielded the observed rates for every observed kinetic event. The traces were fitted to a double exponential function (eqn 1) where $A(t)$ exhibits the absorbance change in time and t is the time in s, while k_{obs1} and k_{obs2} are the observed rate constants for the first and second kinetic events respectively, A and B are the corresponding amplitudes and C is the final observed value for absorbance. The presented observed rates represent the average of 4–8 measurements with a standard deviation of approx. 15 %.

$$A(t) = A \times e^{(-k_{obs1}t)} + B \times e^{(-k_{obs2}t)} + C \quad (1)$$

Reoxidation kinetics of m-ChitO were studied with single wavelength detection at 450 nm. First, reduction of 8 μM m-ChitO was established by removing oxygen as described above and subsequently adding 60 μM O_2-free GlcNAc. Disappearance of the yellow colour indicated reduction of m-ChitO. Equal volumes of 8 μM reduced m-ChitO and KP_i buffer with 0.25 mM or 1.25 mM O_2 were mixed at 25 °C. The increase in absorbance was measured at 440 nm and the reaction was followed for 1 s collecting raw data using SX18MV software with oversample option. The absorbance against time traces were fitted to a single exponential function (eqn 2):

$$A(t) = -A \times e^{(-k_{obs3}t)} + C \quad (2)$$

Redox potential measurements

For redox potential measurements an OTTLE (optically transparent thin-layer electrode) cell was used. A modified EPR cell with a 0.5 mm path length was used to construct the OTTLE cell, which was subsequently fitted with a Pt gauze working electrode from Aldrich (52 mesh), a Pt wire counter electrode and a home made Ag/AgCl reference electrode. The reference electrode was calibrated against Methylene Blue [+ 11 mV compared with the SHE (standard hydrogen electrode)] and was found to be + 264 ± 2 mV compared with SHE. All measurements were performed in KP_i buffer with 200 mM KCl at 25 ± 1 °C. The following mediators were used at 10 μM: Methyl Viologen, 2-hydroxy-1,4-naphthoquinone, Methylene Blue, 1,2-naphthoquinone, 1,4-naphthoquinone, phenazine ethosulphate and Variamine Blue. The samples and OTTLE cell were made anaerobic by flushing with argon. The spectroelectrochemical titrations were conducted using a μAutolab III potentiostat from Eco Chemie in combination with a Perkin Elmer UV/vis spectrophotometer. The potential of the working electrode was decreased by 10–30 mV until the enzyme was fully reduced, and subsequently increased until fully reoxidized. After every change in potential, spectra were collected every 10 min until no further spectral changes were observed. The change in absorbance at a convenient wavelength was plotted against the potential of the working electrode and fitted using modified Nernst equations (eqn 3 for one 2-electron redox couple and eqn 4 for two 1-electron couples) [20]. In these equations, E is the applied potential by the potentiostat, E_1 is the oxidized/reduced couple midpoint potential, E_2 is the oxidized/semiquinone couple midpoint potential, E_3 is the semiquinone/reduced couple midpoint potential, a represents the oxidized species absorbance, b represents the semiquinone species absorbance and c represents the reduced species absorbance.

$$A(E) = \frac{a + c \times 10^{\left(\frac{E_1 - E}{29.5}\right)}}{1 + 10^{\left(\frac{E_1 - E}{29.5}\right)}} \quad (3)$$

$$A(E) = \frac{a \times 10^{\left(\frac{E - E_2}{59}\right)} + b + c \times 10^{\left(\frac{E_3 - E}{59}\right)}}{1 + 10^{\left(\frac{E - E_2}{59}\right)} + 10^{\left(\frac{E_3 - E}{59}\right)}} \quad (4)$$

RESULTS

Sequence and structural model analysis

Previously the *chitO* gene was identified in the genome of *F. graminearum* and cloned as an MBP (*malE*) fusion into the pBAD(NdeI)-myc/HisA expression vector [1]. On the amino acid level ChitO shares 45 % sequence identity with the recently reported GOOX from *Acremonium strictum*, which contains an 8α-*N*1-histidyl and 6-*S*-cysteinyl bound FAD cofactor [12]. Comparative sequence analysis of ChitO, GOOX and other closely related oxidases show that the responsible histidine and cysteine residues are also present in ChitO, His^{94} and Cys^{154}. This indicates that ChitO also contains a bi-covalently linked FAD cofactor (Figure 1B). Using the CPH-model server, a structural model of ChitO was made based on the structure of GOOX from *A. strictum*. This model revealed that the conserved

histidine and cysteine residues of ChitO are indeed located at equivalent positions as compared with the corresponding residues in GOOX. This strongly suggests that these residues are involved in covalently linking the flavin cofactor to the enzyme.

Expression, purification and spectral analysis

Wild-type m-ChitO was expressed and purified by anion-exchange chromatography. From a 1 litre culture broth, approx. 40 mg of m-ChitO was purified and analysed using SDS/PAGE for the presence of a fluorescent protein band to confirm covalent FAD incorporation (Supplementary Figure S1 at http://www.BiochemJ.org/bj/413/bj4130175add.htm). The respective protein band was found to be fluorescent at pH 4, indicative of the presence of a covalently bound flavin cofactor. Purified wild-type m-ChitO runs at about 95 kDa upon SDS/PAGE, which agrees nicely with the calculated mass of 94857 Da (including FAD and 43.3 kDa for MBP). The spectrum of wild-type m-ChitO has absorbance maxima at 449 nm and 385 nm. Upon unfolding the protein with 0.1 % SDS, the higher energy maximum surprisingly disappears and only one maximum at 440 nm remains (Figure 2A). This feature has also been reported for BBE [14]. ESI–MS (electrospray ionization MS) analysis under native and denaturing conditions revealed that m-ChitO is a monomeric protein that contains one covalent FAD molecule (94870 ± 24 Da and 94870 ± 16 Da for native and denaturing conditions respectively).

To test whether m-ChitO has the flavin cofactor bound to the proposed amino acid residues, the H94A, C154A and H94A/C154A mutants were prepared. From a 1 litre culture broth equal amounts of H94A and C154A m-ChitO could be purified as compared with wild-type m-ChitO. Mutants of m-ChitO were expressed and purified by anion-exchange chromatography. The wild-type and mutants were subsequently examined for the effects of removing covalent bonds on the kinetic and redox properties of m-ChitO. The presence of fluorescent protein bands upon irradiating SDS/PAGE gels with UV light confirmed covalent FAD incorporation in both mutants (Supplementary Figure S1). Purified H94A and C154A m-ChitO run at approx. 95 kDa upon SDS/PAGE, which agrees well with the calculated masses of 94792 Da and 94827 Da respectively (including FAD and MBP). For the H94A/C154A double mutant, no expression was observed, indicating that the inability to form a covalent FAD–protein linkage drastically influences the expression level.

The unusual spectral behaviour observed with wild-type m-ChitO is also observed with the H94A mutant, which in the native state has absorbance maxima at 438 nm and 393 nm, and when unfolded with 0.1 % SDS shows only one broad absorbance maximum at approximately 430 nm (Figure 2B). The disappearance of the higher energy maximum in wild-type and H94A mutant m-ChitO seems to be linked to the presence of a 6-*S*-cysteinyl-FAD bond. This becomes clearer when the spectra of the native and unfolded C154A mutant are inspected (Figure 2C). This mutant shows similar spectral properties as compared with other covalent flavoproteins in which the FAD cofactor is covalently attached to a histidine residue [3,21]. In the native state this mutant shows absorbance maxima at 444 nm and 365 nm, but also the unfolded protein shows two absorbance maxima (448 nm and 354 nm). The spectrum is comparable with the wild-type m-ChitO spectrum, except that in the C154A mutant the higher energy maximum is broader and exhibits a higher absorbance. The observation that the higher energy maximum in the C154A mutant is shifted hypsochromically with respect to the corresponding band in free FAD is indicative of the presence of an 8α-histidyl-FAD [22].

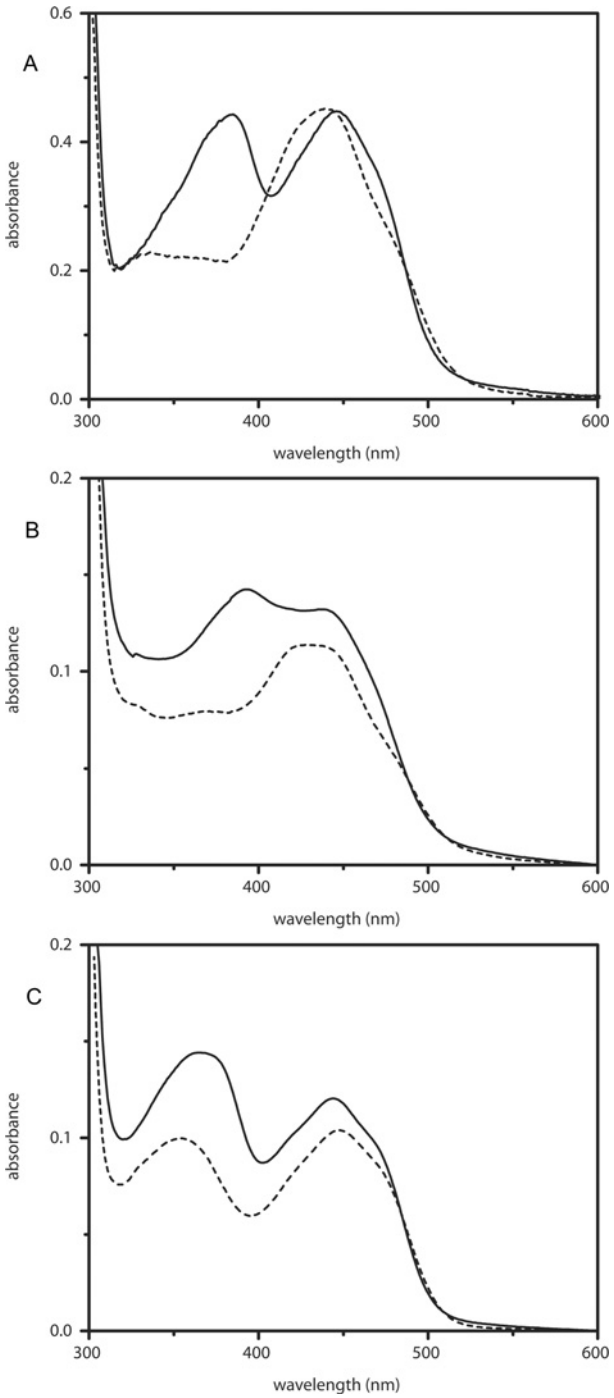

Figure 2 Flavin spectra of native (solid line) and unfolded (broken line) enzymes

Native enzyme preparations were unfolded upon treatment with 0.1 % SDS. The following spectra are shown: (**A**) wild-type m-ChitO (38.8 μM), (**B**) H94A m-ChitO (9.5 μM) and (**C**) C154A m-ChitO (9.2 μM).

Steady-state kinetic analysis

A pH optimum for GlcNAc oxidase activity was observed at pH 7–8. Therefore, all subsequent analyses were performed at pH 7.6. The influence of the mutations on the kinetic behaviour of m-ChitO was analysed by determining the k_{cat}, K_m and k_{cat}/K_m values for GlcNAc, lactose, maltose and glucose using wild-type m-ChitO and the H94A and C154A mutants. Removing one of the

Table 1 Kinetic parameters for wild-type m-ChitO and the H94A and C154A mutants

The dashes indicate experiments in which no activity was found; ND, could not be determined; WT, wild-type.

		GlcNac	Lactose	Maltose	Glucose
WT	k_{cat} (s^{-1})	6.36 ± 0.06	4.6 ± 0.1	7.6 ± 0.6	4.0 ± 0.1
	K_m (mM)	6.3 ± 0.3	192 ± 9	332 ± 52	752 ± 36
	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	**$(1.0 \pm 0.1) \cdot 10^3$**	**24 ± 2**	**23 ± 5**	**5.3 ± 0.4**
H94A	k_{cat} (s^{-1})	0.77 ± 0.05	ND	ND	-
	K_m (mM)	213 ± 27	ND	ND	-
	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	**3.6 ± 0.7**	**$(42 \pm 1) \cdot 10^{-3}$**	**$(54 \pm 1) \cdot 10^{-3}$**	-
C154A	k_{cat} (s^{-1})	2.1 ± 0.2	ND	ND	-
	K_m (mM)	475 ± 73	ND	ND	-
	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	**4 ± 1**	**$(121 \pm 4) \cdot 10^{-3}$**	**$(9.1 \pm 0.2) \cdot 10^{-3}$**	-

covalent links between FAD and the enzyme has a dramatic effect on both the k_{cat} and K_m values (Table 1). For GlcNAc this results in a decrease in k_{cat} by a factor of 8.3 or 3 for the H94A or C154A mutants respectively. The effect on the K_m value is much larger, as the K_m increases from 6.3 mM for the wild-type m-ChitO to 213 mM for the H94A mutant (34-fold increase) and 475 mM for the C154A mutant (75-fold increase). Similar effects on the k_{cat}/K_m values were seen for maltose and lactose. The H94A mutant exhibits a 570-fold decrease in k_{cat}/K_m for lactose and a 425-fold decrease in k_{cat}/K_m for maltose. The C154A mutant shows a 200-fold decrease in k_{cat}/K_m for lactose and a 2500-fold decrease in k_{cat}/K_m for maltose. In the case of glucose, which was only converted at a low catalytic efficiency by the wild-type enzyme, no detectable conversion was observed with the mutant enzymes. The large difference in catalytic efficiency between GlcNAc and glucose is caused by the presence of the N-acetyl moiety at the C2 position in GlcNAc which is recognized by Glu^{268} in the active site [1].

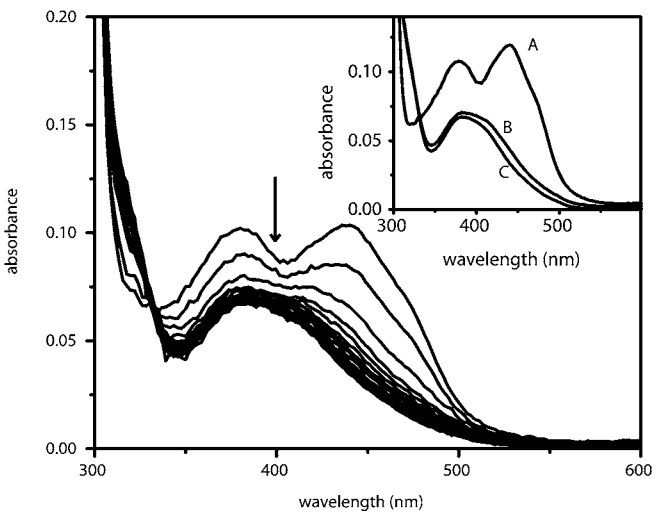

Figure 3 Flavin spectra obtained upon anaerobic reduction of wild-type m-ChitO with 50 mM GlcNAc

The spectra are displayed with 7.5 ms time intervals. The inset shows the deconvoluted spectra A, B and C created with Pro-K software using the model A→B→C, arrows indicating decreasing absorbance.

Stopped-flow kinetic analysis

Stopped-flow kinetic experiments were performed to analyse specific kinetic steps in the catalytic cycle of m-ChitO. This should reveal specific effects of disrupting the individual covalent FAD–protein bonds on the kinetic behaviour of ChitO. First, the redox state during steady-state catalysis was studied by performing EMT (enzyme-monitored turnover) experiments in the presence of molecular oxygen. For this, the flavin absorbance was followed during turnover of GlcNAc. As GlcNAc was present in excess (100 mM), depletion of molecular oxygen was the limiting factor for the reaction. For wild-type m-ChitO, steady-state was reached within 100 ms after mixing with substrate and lasted for about 200 ms. The wild-type enzyme was found to be in an approx. 10 % oxidized state during steady-state catalysis, indicating that the rate for the reductive half-reaction is relatively high when compared with the rate for the oxidative half-reaction. EMT experiments were also conducted for the C154A and H94A mutants to examine the effect of removing each individual covalent FAD bond on the redox state during steady-state catalysis. Both mutants reached steady-state within 100 ms, and this lasted for approx. 50 s in case of the C154A mutant and 150 s in case of the H94A mutant. The shorter steady-state phase of the C154A mutant corresponds to the higher k_{cat} ($2\ s^{-1}$) when compared with the k_{cat} of the H94A mutant for GlcNAc ($0.8\ s^{-1}$). Both mutants were found to be in an approx. 95 % oxidized state during steady-state catalysis. This suggests that removing one covalent bond between the FAD co-factor and the enzyme has a drastic effect on the rate of reduction.

The reductive half-reaction was studied in more detail by performing pre-steady-state kinetic experiments. For this, the enzyme was mixed with varying concentrations of GlcNac under anoxic conditions. The reductive half-reaction displayed two phases: the first phase, which represents reduction of the flavin, exhibits the largest change in absorbance, and the second phase, which exhibits minor changes in absorbance, may reflect product release (Figure 3 and inset). Unexpectedly, the observed rate of the first phase did not follow a substrate concentration-dependent hyperbolic trend. Instead, the data could be fitted with a function consisting of a combination of a hyperbolic equation and the Hill equation to include possible allosteric effects (see the Discussion section) (Figure 4 and eqn 5). In this equation h stands for the Hill constant, k_4 is the upper observed rate limit for the hyperbolic equation, S is the substrate concentration, K' is the concentration of substrate at ½ k_4, k_5 is the upper observed rate limit for the Hill equation and K'' is a constant comprised of the intrinsic dissociation constant and interaction factors. The Hill constant was shown to be 4.3 ± 0.1, and for k_4 and k_5 values of $148 \pm 134\ s^{-1}$ and $605 \pm 113\ s^{-1}$ were determined respectively. For K' and K'', values of 27 ± 46 mM and 95 ± 3 mM were determined respectively. The second phase displayed a similar behaviour, and a maximum observed rate of $80\ s^{-1}$ was found.

$$k_{obs} = \frac{k_4 \times S}{K' + S} + \frac{k_5 \times S^h}{(K'')^h + S^h} \quad (5)$$

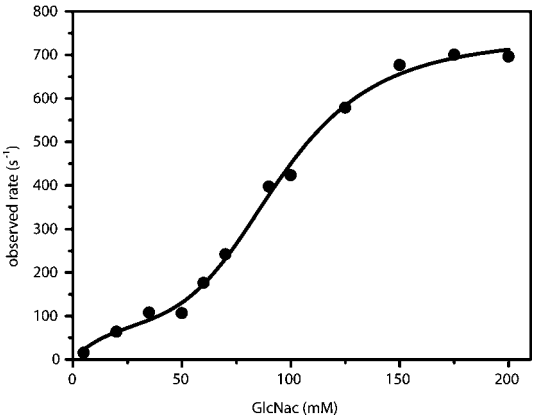

Figure 4 Observed rate of the first phase of the reductive half-reaction of wild-type m-ChitO (20 μM) plotted against the GlcNAc concentration

The results for wild-type m-ChitO reduction were fitted with a modified sigmoidal function (eqn 5).

Table 2 Redox potentials for wild-type m-ChitO and the H94A and C154A mutants

Reduction and oxidation midpoint potentials are indicated for the oxidized/semiquinone (ox/sq) and semiquinone/reduced (sq/red) couples. The average of these midpoint potentials yields the midpoint potential for the ox/red couple ($E_{m,red}$ and $E_{m,ox}$). Subsequent averaging of these midpoint potentials then yields the redox midpoint potential (E_m). All values are presented in mV compared with the SHE. -, not observed.

		Wild-type	H94A	C154A
Reduction	ox/sq	$+139 \pm 6$	-	$+102 \pm 8$
	sq/red	$+114 \pm 7$	-	$+26 \pm 10$
	$E_{m,red}$	**$+126 \pm 5$**	**$+164 \pm 10$**	**$+64 \pm 7$**
Oxidation	red/sq	$+109 \pm 11$	-	$+46 \pm 7$
	sq/ox	$+162 \pm 10$	-	$+103 \pm 6$
	$E_{m,ox}$	**$+135 \pm 8$**	-	**$+75 \pm 5$**
E_m		**$+131 \pm 5$**	**$+164 \pm 10$**	**$+70 \pm 5$**

For the m-ChitO mutants, the observed rate of reduction measured at 175 mM GlcNac was drastically decreased from $> 700\ s^{-1}$ in the case of wild-type m-ChitO to $1\ s^{-1}$ in the case of both mutants. This again confirms that for both mutants the extremely slow reduction rate prevents effective catalysis.

The oxidative half-reaction was followed by mixing reduced enzyme with varying concentrations of molecular oxygen. To determine the reoxidation rate constant of reduced m-ChitO with molecular oxygen, m-ChitO was first anaerobically reduced with 1.5 equivalents of GlcNAc. The reoxidation of fully reduced m-ChitO was followed with diode-array detection and was found to be a monophasic process. Reduced m-ChitO was rapidly reoxidized with varying concentrations of molecular oxygen. From the linear relation between the observed rate and O_2 concentration, a bimolecular rate constant (k_{ox}) of $2.1 \times 10^5\ M^{-1} \cdot s^{-1}$ was calculated. This is a typical rate constant found for oxidases, indicating that m-ChitO is a true oxidase [23].

Redox potentials

Redox potentials of wild-type m-ChitO and both mutants were measured using an OTTLE cell (Table 2; also see Supplementary Figures S2, S3 and S4 at http://www.BiochemJ.org/bj/413/bj4130175add.htm). With wild-type m-ChitO, some formation of the anionic semiquinone was observed during reduction and reoxidation. Midpoint potentials for the reductive and oxidative half-reaction ($E_{m,red}$ and $E_{m,ox}$) were obtained by fitting the data with eqn 4. Taking together, the midpoint potentials for the reductive and oxidative half-reaction yield a redox midpoint potential (E_m) of $+131 \pm 5$ mV for the oxidized/reduced couple of wild-type m-ChitO [$E_m = (E_{m,red} + E_{m,ox})/2$]. With the C154A m-ChitO mutant, some formation of the anionic semiquinone was also observed during reduction and reoxidation. A midpoint potential of $+70 \pm 5$ mV for the oxidized/reduced couple of the C154A m-ChitO mutant was found, which is significantly lower when compared with wild-type enzyme. During the reduction of the H94A m-ChitO mutant, no radical formation was observed and the data could be fitted with eqn 3. Reduction of this mutant required relatively long incubation times of up to 2 h per selected potential. Combined with the tendency of this mutant enzyme to precipitate upon long incubation times, a slight distortion of the collected data was observed (see the Supplementary Figures). Nevertheless, we were able to calculate a midpoint redox potential for the reductive half-reaction of $+164 \pm 10$ mV. The oxidative half-reaction could not be determined because of precipitation of the H94A m-ChitO mutant upon reduction in the OTTLE cell.

DISCUSSION

In this paper we report a study on the unusual bi-covalent binding of the FAD cofactor in chito-oligosaccharide oxidase from *F. graminearum*. The gene of this enzyme was previously cloned and expressed after being discovered as a result of a search for carbohydrate oxidases with a different substrate scope as compared with the known carbohydrate oxidases [1]. Sequence analysis revealed that ChitO shares 45 % sequence identity with GOOX from *A. strictum*, which contains an 8α-*N*1-histidyl- and 6-*S*-cysteinyl-bound FAD cofactor. By sequence comparison of ChitO and GOOX and preparing a model structure of ChitO, it was shown that the responsible histidine and cysteine residues are also present in ChitO (His^{94} and Cys^{154}). This strongly suggests that ChitO contains a bi-covalently linked FAD cofactor as has also been reported for proteins such as hexose oxidase, BBE and AknOx [13–15]. On the basis of the multiple sequence alignment and inspection of the GOOX structure, we have identified a conserved sequence motif that contains the conserved histidine and cysteine residues which are involved in covalent FAD binding: $GGHX_{55-65}GXCXVG$ (Figure 1). A PHI-BLAST (pattern hit initiated BLAST) search in the NCBI database using this sequence motif reveals that there are over 100 putative bi-covalent flavoproteins present in the VAO family including, for example, Δ^1-tetrahydrocannabinolic acid synthase from *Cannabis sativa* and carbohydrate oxidase from *Helianthus annuus* [24,25].

Wild-type and mutants of m-ChitO involving the residues covalently binding to FAD (H94A and C154A) were prepared, expressed and purified. In contrast with the BBE histidine mutants [16], we were able to express and purify the H94A mutant, enabling a detailed study on the spectral, kinetic and redox properties of both the C154A and H94A mutants. Expression of wild-type m-ChitO and the mutants was boosted by fusion to the C-terminus of MBP and using *E. coli* ORIGAMI as the expression host. The latter expression host was proven successful as it allows oxidative disulfide bridge formation. Our ChitO model indeed indicates that there is at least one disulfide bridge present in the ChitO structure. Additionally, the usage of *E. coli* ORIGAMI may also promote covalent linking of the FAD cofactor as formation of the covalent bonds is expected to be an autocatalytic oxidative process. The wild-type m-ChitO and both single mutants contained covalently linked FAD as was observed by fluorescence

analysis of SDS/PAGE gels. This indicates that each covalent bond can be formed independently from the other. For the double mutant H94A/C154A, no expression and also no fluorescence signal upon SDS/PAGE analysis was observed. This suggests that by removing the ability to link the FAD cofactor covalently, the protein is probably not able to fold into a stable form and bind FAD in a non-covalent manner and may be subjected to proteolysis. This means that at least one covalent bond to the FAD cofactor is necessary for structural reasons. The same effect has been shown for, amongst others, HDNO (6-hydroxy-D-nicotine oxidase) [26], cholesterol oxidase [8] and alditol oxidase [3].

Spectral analysis of wild-type m-ChitO and both single mutants showed unusual behaviour as compared with what is normally found for covalent flavoproteins. Upon unfolding of wild-type m-ChitO and the H94A mutant, the higher energy peak disappeared and the spectrum of the unfolded proteins showed only one absorbance maximum at 440 and 430 nm respectively. The same observation has been made with the wild-type BBE [14]. Next to this, the typical spectra of unfolded wild-type and H94A m-ChitO, that show one absorbance maximum, are comparable with what is found for histamine dehydrogenase, which contains a 6-*S*-cysteinyl-FMN, and the H79K mutant of hexose oxidase that contains 6-*S*-cysteinyl–FAD [13,27]. This confirms that, in the case of m-ChitO, the unusual spectral properties are also caused by the 6-*S*-cysteinyl–FAD link. The C154A mutant shows normal spectral characteristics upon unfolding. In this case the flavin cofactor is linked to His^{94}, and the spectrum of the unfolded m-ChitO C154A mutant closely resembles the spectrum of free FAD.

For all tested substrates, the effect of removing one of the covalent FAD–protein bonds resulted in a drastic decrease in catalytic efficiency. Enzyme-monitored turnover experiments also revealed that the redox state during steady-state catalysis is affected by removing one of the covalent FAD–protein bonds. For both mutants the flavin cofactor was found to be in a 95 % oxidized state during steady-state catalysis (against a 10 % oxidized state for wild-type m-ChitO). This indicates that the reductive half-reaction is relatively slow compared with the oxidative half-reaction. To verify this, pre-steady-state experiments were conducted by analysing the reductive and oxidative half-reactions separately for wild-type m-ChitO. Normally one would expect to observe a hyperbolic relation between the measured reduction rate and the concentration of substrate because of two consecutive kinetic events: substrate binding and flavin reduction. However, a clear sigmoidal relationship was observed, suggesting that the rate of reduction is allosterically influenced by increasing concentrations of GlcNAc. In a previous report we showed that m-ChitO is not only active with GlcNAc [1]. In fact, the oxidase is more efficient in terms of k_{cat}/K_m with GlcNAc polymers such as N,N′-diacetylchitobiose and N,N′,N″-triacetylchitotriose. This effect is mainly caused by a relatively low K_m for these chito-oligosaccharides. Furthermore, by analysing the steady-state kinetics of an m-ChitO mutant (Q268R ChitO), it was confirmed that m-ChitO is able to recognize more than one GlcNAc moiety in a chito-oligosaccharide. Therefore, it is tempting to assume that, at relatively high GlcNAc concentrations, a second GlcNAc residue binds near the active site in such a way that it enhances correct positioning of the first GlcNAc residue in front of the FAD cofactor. Such a binding event would explain the observed allosteric behaviour. From the structure of GOOX it is clear that the −2 glucosyl moiety of 5-amino-5-deoxy-cellobiono-1,5-lactam has one direct hydrogen bridge with the protein and five water-mediated hydrogen bonds [12]. This supports the idea that binding of a second GlcNAc residue can improve the binding of the first GlcNAc residue which is to be oxidized and is in line with the Hill constant of 4.3. The maximum observed rate of reduction was found to be unusually high (750 s^{-1}). The oxidative half-reaction showed one phase leading from fully reduced to fully oxidized FAD. The determined reoxidation rate constant for wild-type m-ChitO is 2.1×10^5 $M^{-1} \cdot s^{-1}$, which is a typical value found for flavoprotein oxidases [3,23,28]. Since the reduction and reoxidation rates are much higher than the measured k_{cat} value for wild-type ChitO, some other, perhaps conformational, step(s) dictates the rate of catalysis. For the mutants, the reduction rates were found to be very low (1–2 s^{-1}) and thereby determine the rate of catalysis. Next to this, up to a concentration of 250 mM GlcNAc, no sigmoidal behaviour was observed with both mutants (H94A and C154A) during the reductive half-reaction. The rationale for this lack of sigmoidal behaviour may be the increased K_m that the mutants exhibit with the measured substrates. This would suggest that both covalent FAD–protein links are necessary for a correct positioning of the flavin cofactor and/or the substrate.

For wild-type m-ChitO and the C154A mutant similar redox potentials (+131 and +70 mV, respectively) were found as compared with the values reported for the wild-type BBE and the analogous C166A mutant (+132 and +53 mV, respectively) [16]. Analogous to what has been proposed for BBE, the relatively low redox potential of the C154A ChitO mutant could provide a reasonable explanation for the observed slow substrate-mediated flavin reduction and the lowered k_{cat}. For BBE, a midpoint potential for a His^{104} mutant could not be measured because of low expression levels, according to the authors [16]. Fortunately, we were able to express and purify the H94A mutant of m-ChitO and determine the redox potential for the reductive redox titration. Although the redox titration experiment with the H94A mutant was troublesome, we were able to show that the midpoint potential for this mutant (+164 mV) is even higher than was found for wild-type m-ChitO (+131 mV). The high redox potential of H94A m-ChitO indicates that the histidyl-FAD link is not required for attaining a high redox potential. The increase in K_m values with all substrates measured for the H94A mutant suggests that the 8α-histidyl-FAD linkage is necessary for fixation of the FAD cofactor in order to establish productive substrate binding. For the cysteinyl-FAD link it is tempting to assume that this link is primarily important for increasing the redox potential and thereby facilitating catalysis. Nevertheless, the C154A mutant shows a decrease in k_{cat} as well as an increase in K_m with all tested substrates, indicating that removing the cysteine link drastically affects both the rate of catalysis and the affinity for the substrates. In contrast with what has been published for BBE, in the case of m-ChitO it cannot be concluded that the cysteinyl-FAD bond is only present to increase the redox potential. It appears that both covalent bonds to the FAD cofactor are essential for correct positioning of the flavin cofactor and/or formation of a productive Michaelis complex, while the cysteinyl-FAD bond also contributes to the extremely high redox potential.

The role of the bi-covalent FAD linkage in m-ChitO appears to be ambivalent. The covalent anchoring increases the redox potential and thereby boosts the oxidative power of the cofactor. Furthermore, it also appears to be important for positioning of the FAD cofactor in a catalytically competent orientation to facilitate substrate binding and oxidation. The latter effect may be related to the following. When comparing mono-covalent and bi-covalent flavoprotein structures, a striking difference is observed. Substrate access to active sites of histidyl-FAD-containing enzymes typically involves passage through a narrow tunnel. This becomes clear when inspecting e.g. the structures of VAO and cytokinin dehydrogenase: in both cases the FAD is buried well within the protein (Figure 5). Contrarily, in the structures of GOOX and AknOx, the isoalloxazine ring of the FAD cofactor

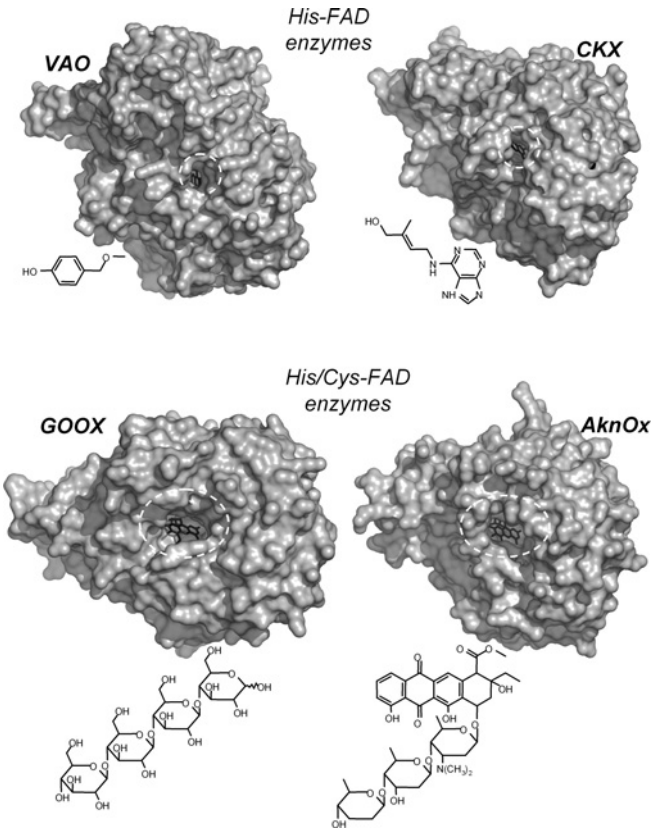

Figure 5 Protein surface representations of His-FAD-containing flavoproteins and bi-covalent Cys/His-FAD-containing flavoproteins

The His-FAD containing flavoproteins VAO and CKX (cytokinin dehydrogenase) have a far less accessible active site than the bi-covalent Cys/His-FAD-containing flavoproteins GOOX and AknOx. The FAD cofactor is shown in black sticks and the corresponding substrate for each enzyme is indicated. The Figure was prepared using the PyMol software (http://www.pymol.org) using the 1AHU (VAO), 1W10 (CKX), 1ZR6 (GOOX) and 2IPI (AknOx) PDB files.

is fully exposed to solvent, due to an extremely open active site (Figure 5). The easily accessible active sites facilitate binding and oxidation of the relatively bulky GOOX, AknOx and ChitO substrates. Such open active site architecture, needed to accommodate bulky substrates, precludes extensive interactions between the protein and the isoalloxazine ring of the cofactor. Therefore, bi-covalent binding might have evolved to ensure proper positioning of FAD where the protein-mediated positioning of the cofactor is not sufficient. This resembles the trend observed for covalent haem binding in cytochromes: for these covalent cofactor-containing proteins, the rationale behind covalent coupling appears also to be ensuring effective anchoring of the cofactor [29]. Solving the crystal structure of wild-type m-ChitO and both single mutants could give insight into the positioning of the FAD cofactor. Furthermore, the ability to express and purify the H94A mutant enables further research on the remarkably high redox potential. The role of covalent flavinylation is versatile and it appears that, even for structurally related proteins, the reasons behind evolving towards covalent flavin-containing enzymes can vary.

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