Chapter 3

Cofactor-dependent assembly of the flavoenzyme vanillyl alcohol oxidase.

"The reason that some portraits don't look true to life is that some models make no effort to resemble their pictures."

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Abstract

The oligomerization of the flavoprotein vanillyl alcohol oxidase (VAO) and its site-directed mutant H61T was studied by mass spectrometry. Native VAO has a covalently bound FAD and forms primarily octameric assemblies of 507 kDa. H61T is purified as an FAD free apoprotein and mainly exists as a dimeric species of 126 kDa. Binding of FAD to apo-H61T rapidly restores enzyme activity and induces octamerization, although association of H61T dimers seems not to be crucial for enzyme activity. Reconstitution of H61T with the cofactor analog 5’-ADP also promotes octamerization. FMN on the other hand, interacts with apo-H61T without stimulating dimer association. These results are in line with observations made for several other flavoenzymes, which contain a Rossmann-fold. Members of the VAO flavoprotein family do not contain a Rossmann-fold, but do share two conserved loops that are responsible for binding the pyrophosphate moiety of FAD. Therefore, the observed FAD-induced oligomerization might be general for this family. We speculate that upon FAD binding, small conformational changes in the ADP-binding pocket of the dimeric VAO species are transmitted to the protein surface, promoting oligomerization.
Riboflavin (Vitamin B2) derivatives such as FAD and FMN are essential components of all living organisms serving as cofactors for numerous proteins with diverse functions ranging from electron transport, redox catalysis, oxygen activation and light emission to DNA repair. For most reported flavoproteins the flavin cofactor is noncovalently bound, although ~10% of the human cellular FAD is covalently linked to enzymes like monoamine oxidase and succinate dehydrogenase. Many noncovalent flavoproteins can be reversibly dissociated into their constituents the apoprotein and flavin prosthetic group. Reconstitution of the holoprotein with either artificial, enzymatically modified or isotopically enriched flavin analogues then allows to gain insight into the role of the protein in redox catalysis.

For several flavoenzymes, binding of the FAD cofactor induces subunit association and improves the resistance of the protein to thermal and chemical denaturation. For glucose oxidase, D-amino-acid oxidase and lipoamide dehydrogenase, it was shown that rapid FAD binding to the monomeric apoprotein is followed by relatively slow dimerization and regain of catalytic activity. For bacterial butyryl-CoA dehydrogenase, on the other hand, it was revealed that optimal reconstitution of tetrameric holoenzyme from dimeric apoenzyme and FAD requires the presence of CoA ligands.

Little is known about the role of flavin binding in the assembly of covalent flavoenzymes. Covalent flavinylation mostly occurs via a histidine residue, but enzymes with cysteinyl-flavin, tyrosyl-flavin and threonyl-flavin have been described as well. Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* is a covalent flavoprotein whereby the FAD is bound via a so-called 8α-(N3-histidyl)-FAD linkage. VAO is active with a wide variety of phenolic compounds but the biological function of the enzyme is unclear. Upon growth on 4-methoxymethylphenol, VAO is abundantly expressed in the fungus, which might suggest that the main physiological role of the enzyme is the conversion of this compound into 4-hydroxybenzaldehyde. VAO is primarily a homooctamer of ~0.5 million Da with each monomer subunit being composed of two domains. The larger domain binds the FAD in an extended conformation whereas the isoalloxazine ring of the flavin is covalently attached to the His422 of the cap domain. Sequence comparisons have revealed that VAO belongs to a novel class of widely distributed oxidoreductases sharing a conserved FAD-binding domain.

The functional role of the covalent protein-flavin linkage in VAO has been addressed recently by site-directed mutagenesis. From the properties of His-422 mutants, evidence was obtained that the covalent linkage between His-422 of the apoprotein and FAD is important for
VAO catalysis by raising the redox potential of the flavin. All three mutants H422A, H422C and H422T tightly bound the FAD in a noncovalent mode but the change in redox properties resulted in a marked decrease in the rate of substrate-mediated flavin reduction. The mechanism of flavinylation of VAO was addressed by creation of the design point mutation His-61→Thr\textsuperscript{101}. In this mutant enzyme, the covalent linkage between His-422 and the flavin is not formed whereas the enzyme is still able to bind FAD and perform catalysis. Structural analysis of both apo- and holoforms of H61T indicated that binding of FAD to the apoprotein does not induce major structural rearrangements suggesting that covalent flavinylation is an autocatalytical process in which His-61 is crucially involved in activating His-422. As for the wild type enzyme the apoform of H61T is present as a homooctamer in the crystalline state\textsuperscript{101}. However, preliminary gel-filtration studies have indicated that in solution the mutant enzyme may easily dissociate into dimers.

In this report we use primarily mass spectrometry to probe the influence of cofactor binding on the oligomerization state of VAO. With the development of new ionization techniques such as matrix-assisted laser desorption ionization\textsuperscript{44} and electrospray ionization\textsuperscript{46} and the coupling of these to time-of-flight analyzers, the detectable mass range of macromolecular systems by mass spectrometry has been enhanced extensively\textsuperscript{146,147}. Moreover, the relatively gentle phase transfer from solution- to gas-phase, realized especially by electrospray ionization, has allowed the intact detection by mass spectrometry of larger multiprotein assemblies\textsuperscript{148,150-152}. Several recent examples\textsuperscript{37,73,156,178-180} have revealed that biologically relevant parameters concerning multiprotein assemblies may now be investigated by mass spectrometry and examples include protein complex topology, protein-ligand and protein-protein binding constants and protein complex stability. Biomolecular mass spectrometry combines mass resolution, high sensitivity and an enlarged mass-to-charge (m/z) range and allows the identification of multiple species in a mixture.

In an earlier report\textsuperscript{100}, we showed that the wild type VAO multimer is amenable to detailed investigation by nano-electrospray ionization-mass spectrometry. Here we compare wild type VAO and the single point mutant H61T. In the mutant enzyme, the FAD cofactor is no longer covalently bound and the protein can be prepared in its FAD-free apoform. The apoprotein has a moderate affinity for FAD and 5’-ADP\textsuperscript{101} providing the unique opportunity to address by mass spectrometry the role of cofactor binding in the supramolecular assembly of a covalent flavoprotein.

**Experimental**

**Chemicals.** FAD, FMN and 5’-ADP were obtained from Sigma and used without further purification. Ion-exchange and gel-filtration chromatography resins were from Amersham.
Biosciences. CHT ceramic hydroxyapatite was from BioRad. All other chemicals were from Merck and the purest grade available.

Proteins. Wild type VAO\textsuperscript{130,161} and mutant H61T VAO\textsuperscript{101} were expressed and purified as described previously. Proteins stored in 100 mM potassium phosphate, pH 7.2, were desalted using ultrafiltration units (ultrafree-0.5 Centrifugal Filter Device, Millipore Corporation, Bedford, ENG) with a cut-off of 5 000 Da.

Preparation of apo-VAO. The apoform of H61T VAO was prepared by hydroxyapatite chromatography. 4 mg of H61T VAO in 200 mM potassium phosphate buffer containing 200 \( \mu \)M FAD was applied onto a 1-ml hydroxyapatite column pre-equilibrated in 200 mM phosphate buffer, pH 7.2. Protein-bound FAD was removed by washing the column with 10 column volumes of starting buffer. The apoprotein was eluted from the column with 15 column volumes of 600 mM phosphate buffer, pH 7.2 and concentrated to 4 mg/ml by ultrafiltration (Ultrafree 4, 30 kDa). The FAD content of the apoprotein was < 0.1% as evidenced by absorption spectral analysis. The molar absorption coefficient of apo-H61T VAO was determined at 280 nm by comparison of the absorbance of apoenzyme, holoenzyme and free FAD in 50 mM ammonium acetate, pH 6.8.

Analytical methods. Absorption spectra were recorded on a Hewlett Packard HP 8453 diode array spectrophotometer. Fluorescence spectra were recorded on a Varian Eclipse instrument. All spectrometers were equipped with thermostat cell holders. All spectra were obtained at 25 °C.

Reconstitution kinetics. Reconstitution of holo-H61T from apo-H61T and FAD was performed at 0 and 25°C in 50 mM ammonium acetate, pH 6.8. The apoenzyme and FAD concentrations were 3 and 100 \( \mu \)M, respectively. At time intervals, the reconstitution reaction was monitored by adding an aliquot of the incubation mixture into the assay mixture. The activity of H61T was determined by measuring absorption changes at 296 nm (\( \varepsilon_{296} = 5.5 \text{ mM}^{-1} \text{cm}^{-1} \)) due to the hydroxylation of eugenol into coniferyl alcohol at pH 7.5\textsuperscript{181}.

Fluorescence studies. Protein tryptophan fluorescence emission spectra were recorded from 310 to 560 nm using an excitation wavelength of 295 nm. A spectral bandwidth of 5 nm was used in all fluorescence experiments. Binding of FAD to apo-H61T VAO was monitored by the decrease of protein fluorescence at 340 nm. In the titration studies, after each addition, the sample was incubated for 4 min in the dark before measuring the emission intensity.

Nano-electrospray Mass Spectrometry. For nano-electrospray mass spectrometry experiments, enzyme samples were prepared in aqueous 50 mM ammonium acetate solutions. For
measurements under denaturing conditions, these protein solutions were 1:1 diluted in acetonitrile, containing 1% formic acid. VAO samples were introduced into an LC-T nano-electrospray ionization orthogonal time-of-flight mass spectrometer (Micromass, Manchester, UK) operating in positive ion mode. Source pressure conditions in the LC-T mass spectrometer and nano-electrospray voltages were set for optimal transmission of the larger multimer protein assemblies as described previously. Spraying conditions are as follows: needle voltages 1450-1650 V, cone voltages 45-100 V and source temperature 85°C. In the experiments measuring the protein assemblies, the pressure in the interface region was adjusted by reducing the pumping capacity of the rotary pump by closing the valve. Borosilicate glass capillaries (Kwik-Fil™, World Precision Instruments Inc., Sarasota, FL) were used on a P-97 puller (Sutter Instrument Co., Novato, CA) to prepare the nano-electrospray needles. They were subsequently coated with a thin gold layer (~500 Å) using an Edwards Scancoat six Pirani 501 sputter coater (Edwards High Vacuum International, Crawley, UK).

**Size-exclusion Chromatography.** Size-exclusion chromatography was performed with a Superdex-200 HR 10/30 column connected to an Åkta chromatography system, essentially as described previously. All experiments were performed in 50 mM ammonium acetate, pH 6.8. 3 µM of apo-H61T (or wild type VAO) were incubated at room temperature in the absence or presence of cofactor analogs (30 µM of FAD, 5’-ADP or FMN). At different time intervals, 200 µl aliquots from the incubation mixtures were injected on the column and eluted at a flow rate of 0.7 ml/min. Detection was at 220 nm. Relative abundance of the dimer and octamer species was calculated from the peak areas. To derive the relative abundance of the octamer and dimer, these peak areas were scaled (corrected for the number of subunits) by dividing the area of the octamer by 8 and that of the dimer by 2.

**Results**

**Preparation of apo-VAO.** The noncovalent and relatively weak binding of FAD to H61T VAO allowed a novel gentle procedure of preparing the apoenzyme. By binding the H61T mutant to hydroxyapatite, the FAD could easily and quantitatively be removed by washing the column with 200 mM potassium phosphate buffer, pH 7.5. Subsequently, the apoenzyme was stripped from the column by increasing the buffer concentration to 600 mM. This method of apoenzyme preparation is very simple, can be performed at small and large scale and gives almost quantitative yields. The absorption spectrum of apo-H61T in 50 mM ammonium acetate, pH 6.8, showed a maximum at 280 nm and no significant absorbance in the visible region revealing that the FAD is indeed absent (Fig. 11A). The molar absorption coefficient at 280 nm was $\varepsilon_{280}=140 \pm 1$ mM$^{-1}$cm$^{-1}$. For comparison the absorption spectrum of wild type VAO in 50 mM ammonium acetate, pH 6.8 is also shown in Figure 11A.
Figure 11. Absorption and tryptophan fluorescence properties of wild type VAO and apo-H61 VAO in 50 mM ammonium acetate, pH 6.8. A, absorption spectrum of 3 μM wild type VAO (I) and apo-H61T (II). B, tryptophan fluorescence emission spectra of 3 μM apo-H61T (I), wild type VAO (II) and apo-H61T in presence of 30 μM FAD (III).

Reconstitution studies. In an earlier study, the dissociation constant of the complex between apo-H61T VAO and FAD in 50 mM phosphate buffer, pH 7.5, was estimated to be 1.8 μM. In view of the mass spectrometry studies described below, it was of interest to obtain insight in the kinetic and thermodynamic properties of the apoenzyme-FAD complex in 50 mM ammonium acetate, pH 6.8. Reconstitution of apo-H61T by FAD as monitored by activity measurements was a relatively fast process. When 3 μM apoenzyme were incubated at 25°C with 100 μM FAD, the enzyme activity reached a maximum within 1 min. When excess of FAD (50
µM) was added to the assay mixture, the maximum activity approximated the activity of H61T holoenzyme\textsuperscript{101}. At 0°C, the reconstitution reaction was slower and took about 5 min to complete. Again, under these conditions the activity reached the level of the H61T holoenzyme when the assay was performed in the presence of 50 µM FAD. Figure 11B shows the tryptophan fluorescence emission spectrum of apo-H61T upon excitation at 295 nm. The relative quantum yield of the tryptophan fluorescence of apo-H61T is much higher than that of wild type VAO and holo-H61T (Fig. 11B). Furthermore, the maximum of fluorescence emission of apo-H61T VAO is at 340 nm whereas the emission maxima of wild type VAO and holo-H61T are centered around 325 nm. In line with the data from activity experiments, binding of FAD to apo-H61T was a relatively fast process. When 3 µM apo-H61T were incubated at 25°C with a 10-fold excess of FAD, the fluorescence emission at 340 nm was quenched by about 80% and reached a constant value within 30 s. At 0°C, the same level of fluorescence quenching was observed but the reaction took approximately 5 min to complete.

![Figure 12](image)

**Figure 12.** Tryptophan fluorescence emission of apo-H61T VAO with cofactor analogs was measured in arbitrary units. Upon the addition of FAD to 3 µM apo-H61T in 50 mM ammonium acetate, pH 6.8 (.navigationItem) fluorescence emission was observed at 340 nm upon excitation at 295 nm. Titrations with FMN (▲) and 5'ADP (X) are also presented. As a control, wild type VAO enzyme was titrated with FAD (●).

The dissociation constant of the complex apo-H61T-FAD was determined by fluorescence titration experiments. Titration of 3 µM apo-H61T by FAD resulted in a strong decrease of tryptophan fluorescence emission at 340 nm (Fig. 12). From the titration data, a dissociation
constant $K_d = 0.38 \pm 0.04 \, \mu\text{M}$ and a relative quantum yield $Q = 20 \pm 2\%$ with respect to apo-H61T were estimated for the apoenzyme-FAD complex. This is slightly tighter binding than previously reported\textsuperscript{101}, which might be due to the different buffer system. Titration of apo-H61T with 5’-ADP did not result in a strong fluorescence decrease (Fig. 12). Nevertheless, the fluorescence quenching data support an earlier conclusion\textsuperscript{101} that 5’-ADP binds to apo-H61T with a similar affinity as FAD. When apo-H61T was titrated with FMN, a clear change in protein fluorescence emission was observed (Fig. 12). However, the fluorescence quenching did not reach a constant value suggesting that FMN binds far more weakly to apo-H61T than FAD and 5’-ADP.

**Exact mass determination of wild type VAO and H61T.** Initially, to check the quality of the recombinant VAO proteins the molecular masses of the protein monomers were determined. To that end, the protein of interest was dissolved in a denaturing acidic solution (50% acetonitrile, 1% formic acid). Under these highly acidic conditions, quaternary structures are most often disassembled; proteins tend to denature and the relatively highly charged ions originating from the protein monomers are observed in the mass spectra. From these nano-electrospray mass spectra the mass of the protein monomer can be assessed. From such data the average molecular weight of the wild type VAO monomer was determined to be 63 561 \pm 3 Da. Translation of the gene-sequence published by Benen et al.\textsuperscript{161} leads to a protein mass of 62 915 Da, excluding the covalently bound FAD. When including the mass of FAD, the measured and expected masses are in agreement when it is assumed that the N-terminal methionine has been post-translationally removed. The average molecular mass of the apoform of the VAO H61T variant was determined to be 62 742 \pm 2 Da. The experimental mass difference between the wild type and the apo-VAO H61T mutant is as expected when the single mutation and the covalently bound FAD are taken into account and, again, assuming that the initiator methionine is processed in the apo-VAO H61T mutant. Analysis of tryptic digests by matrix-assisted laser desorption ionization time-of-flight proved indeed the truncation of the methionine in both the wild type and the apo-VAO H61T mutant (data not shown). Additionally, our mass spectrometric data confirmed that no residual covalent FAD was present in the apoprotein\textsuperscript{101}.

**Evaluation of the quaternary structures of VAO assemblies.** In order to establish the stoichiometry of the VAO assemblies, mass spectra of the proteins were recorded whereby the proteins were now nano-electrosprayed from pseudo-physiological solutions, \textit{i.e.} aqueous 50 mM ammonium acetate buffered solutions, pH 6.8. We have shown previously\textsuperscript{40} that, using electrospray time-of-flight mass spectrometry, the detection of large noncovalent assemblies (exhibiting very high $m/z$ values) could be significantly enhanced by optimizing the background pressures in the different regions of the mass spectrometer. In the case of the VAO assemblies studied here, raising the pressure is even indispensable to detect ions originating from the assemblies. In Figure 13A, the nano-electrospray ionization mass spectrum of wild type VAO is
shown, when sprayed from a 50 mM ammonium acetate buffer, pH 6.8, at a protein monomer concentration of 4 μM. As reported previously\textsuperscript{100}, under these conditions, wild type VAO is almost exclusively an octamer. From the crystal structure (inset \textbf{Fig. 13A}) it is well established that the VAO octamer can be described as a tetramer of dimers\textsuperscript{131}. The exact mass of the large octamer assembly turned out to be more difficult to determine. The average mass was 508 700 ± 350 Da. In the mass spectra of the wild type enzyme, only very small ion signals originating from dimeric species were observed (less than 4% of the total ion current).

\textbf{Figure 13.} Nano-electrospray mass spectra of wild type VAO and H61T mutant. \textbf{A}, 4 μM wild type VAO in 50 mM ammonium acetate, pH 6.8. \textbf{B}, 4 μM apo-VAO H61T in 50 mM ammonium acetate, pH 6.8. All experimental conditions were identical.

The nano-electrospray ionization mass spectrum of the apo-VAO H61T mutant, sprayed under exactly the same experimental conditions and protein monomer concentration, is shown in \textbf{Figure 13B}. In sharp contrast to the top spectrum, this mass spectrum is dominated by ions that originate exclusively from the dimer. From the spectrum in \textbf{Figure 13B}, the average mass of the dimer was determined to be 125 575 ± 26 Da. Tetramer and octamer could be detected as well but these ions contributed only with less than 3% to the total ion current. The finding that apo-VAO
H61T prefers almost exclusively a dimeric structure seems to be in disagreement with previous crystallographic data, which revealed that the apo-H61T variant was octameric. However, the experimental conditions whereby the protein crystals were grown are somewhat different from the current conditions (100 mM sodium acetate, pH 5.1 and an enzyme concentration of 12 mg/ml, which roughly corresponds to a protein monomer concentration of 200 µM).

**Cofactor-induced oligomerization of apo-VAO H61T.** In analogy to the spectroscopic studies described above, we prepared for mass spectrometric analyses mixtures of apo-VAO H61T at a monomer concentration of 3 µM with increasing concentrations of FAD. In line with the data depicted in Figure 13B, apo-VAO H61T mass spectrum, Figure 14A, shows a largely dimeric apoprotein. Mass spectra obtained when spraying a solution containing an equimolar concentration of apo-H61T monomer and FAD (both 3 µM) showed several remarkable changes (Fig. 14B). First, originating from the VAO octameric assemblies, ion signals in the m/z ~10 000 Da region appeared. Second, in both m/z ~5 500 and ~10 000 Da regions, binding of one or more FAD molecules to the dimer and the octamer were observed as satellite peaks.

When the concentration of FAD was raised 10-fold to yield saturating conditions, the mass spectrum of the resulting apo-H61T-FAD mixture clearly revealed that the dimer binds primarily two FAD molecules (Fig. 14C). Moreover, even more intense peaks in the m/z ~10 000 Da region are observed in this spectrum. Mass spectra taken from solutions containing apoprotein/cofactor ratios of 1:0, 1:0.5, 1:1, 1:2, 1:4 and 1:10 were all in line with the observations shown in Figure 14 displaying some illustrative examples. Closer examination of the data shown in Figure 14, B and C, revealed that both H61T VAO dimer and octamer showed extensive fine structure, originating from dimeric and octameric species with different amounts of FAD bound molecules. This is remarkable as the FAD molecules have a molecular weight that is only ~0.15% of the mass of the octamer assembly. Thus almost uniquely, mass spectrometry allows the distinction between species containing different amounts of FAD when the instrument mass resolution is sufficiently high and the sample homogeneous. Table II lists the most pronounced measured m/z values for both dimer and octamer ion species depicted in Figure 14B. To calibrate these mass spectra at these high m/z ranges accurately, measurements with separate needles, one containing the sample of interest (3 µM H61T VAO and 3 µM FAD) and one containing an aqueous CsI solution, were performed one after the other, using the CsI clusters as calibrant ions.

Mass resolution (M/Δm) can be determined by measuring the peak width of the ion signals of a single component. For the CsI cluster ions these widths were ~2.5 and 5 Thomson (Th), or Da as the charge is 1, at full width medium height (FWHM or Δm) at m/z 5 000 and 10 000, respectively. For the protein assembly ions the widths in m/z were measured to be 10 Th for the dimer and 8 Th for the octamer. Taking into account the average number of charges these widths
in Th converted to an FWHM of 240 and 430 Da for the dimer and octamer ions, respectively. For these ions the experimentally measured mass resolution \( \frac{M}{\Delta m} \) is therefore 525 and 1 200 for the dimer and octamer ions, respectively. These data show that the instrumental mass resolution is not the limiting factor, neither is the natural width of the isotope envelope of these ions (which is below 50 Da).

![Figure 14](image)

**Figure 14.** Nano-electrospray mass spectra of 3 µM apo-VAO H61T in 50 mM ammonium acetate, pH 6.8 with added FAD at protein/cofactor ratios of A, 1:0, B, 1:1 and C, 1:10.

Additionally, the data in Table II reveal that the measured masses of the dimer complexes are off by ~80 Da, whereas the octamer complexes are off by on average 200 Da (both to higher mass), which is less than 0.05%. The observed peak broadening and the mass deviation observed for the ions of the protein assemblies are most likely due to binding of some ions (such as alkali metal ions) and/or small neutrals to the protein complexes.

It is quite accepted to deconvolute electrospray spectra to produce so-called “zero-charge” mass spectra on a molecular mass scale using maximum entropy analysis.\(^{182}\)
Table II. Ion signals observed in apo-H61T VAO-FAD (1:1) mixture ESI spectrum, displayed in Figure 13B and deduced molecular masses for octameric and dimeric assemblies of the enzyme with different number of cofactors (n).

| m/z (z) | n | Average Mass (Da) | Expected Mass (Da) | Δmass (Da) | Δmass (%) |
|---------|---|-------------------|--------------------|------------|-----------|
| 55 54 53 52 | 4 | 505 341 (±14) | 505 076 | + 265 | 0.05 |
| 9 189 9 359 9 536 9 719 | 5 | 506 065 (±23) | 505 861 | + 204 | 0.04 |
| 9 202 9 373 9 549 9 733 | 6 | 506 809 (±35) | 506 646 | + 163 | 0.03 |

| m/z (z) | n | Average Mass (Da) | Expected Mass (Da) | Δmass (Da) | Δmass (%) |
|---------|---|-------------------|--------------------|------------|-----------|
| 5 023 5 233 5 461 5 709 | 0 | 125 575 (±26) | 125 484 | + 91 | 0.07 |
| 5 053 5 265 5 494 5 744 | 1 | 126 340 (±40) | 126 269 | + 71 | 0.06 |
| 5 086 5 297 5 528 5 780 | 2 | 127 121 (±18) | 127 054 | + 67 | 0.05 |

*a Average mass calculated by averaging the determined mass of each different charge state z of each complex.
Individual peak width is b 8 Th or ~430 Da at FWMH and c 10 Th or ~240 Da at FWMH.

Figure 15. Deconvoluted neutral mass spectrum of the octameric VAO species using maximum entropy analysis. The number of bound cofactors is indicated.

The software maximum entropy is believed to provide probabilistic quantification so that the reliability of features in the spectrum can be ascertained. Because maximum entropy is truthful to the experimental data, the results tend to have improved resolution and signal-to-noise ratio. The deconvoluted spectrum for the octamer ions displayed in Figure 14B is shown in Figure 15. In this deconvoluted mass spectrum the species containing a different number of FAD molecules were indeed well separated by on average 785 Da (mass of one FAD molecule). Therefore, these
neutral masses deconvoluted mass spectra provide a very elegant way to separate distinct quaternary assemblies although care has to be taken in interpreting the results given that the outcome of the deconvolution is dependent on the chosen parameters.

Figure 16. Nano-electrospray mass spectra of 3 µM apo-VAO H61T, in 50 mM ammonium acetate, pH 6.8 with added 5'-ADP at protein/cofactor ratios of A, 1:0, B, 1:1 and C, 1:10.

Figure 16, A-C, shows the nano-electrospray mass spectra of apo-VAO H61T mixed with 5’-ADP in ratios of 1:0, 1:1 and 1:10, respectively. The results obtained are remarkably similar to those found in the case of FAD as noncovalent cofactor. Again, octamerization of apo-H61T is strongly induced by the binding of the 5’-ADP cofactor analog. The mass spectra of the solutions containing 5’-ADP revealed, similarly to the FAD experiments, clear binding of 5’-ADP to the dimeric entities and, less obviously, to the octameric species. The substructure and mass shift observed for the octamer ions provide evidence of 5’-ADP binding but, most likely due to the lower molecular weight of the 5’-ADP moiety, it is more difficult to distinguish octamer ions containing one or more 5’-ADP molecules. Binding of FMN to apo-H61T was studied in a similar manner. Figure 17, A-C, shows the nano-electrospray mass spectra of apo-VAO H61T mixed
with FMN in ratios of 1:0, 1:1 and 1:10, respectively. In sharp contrast to the addition of FAD or 5'-ADP, the presence of FMN hardly induced formation of octamer. Even for higher FMN concentrations (up to a protein/cofactor ratio of 1:25) no octamerization of apo-H61T was observed in the mass spectra. However, it cannot be excluded from our data that FMN binds to the dimeric complex considering the ion signals broadening. Especially in the spectra obtained at a ratio of 1:10 (Fig. 17C), satellite peaks did appear that suggested binding of FMN to the dimer. This observation is in agreement with the observation that FMN induces changes in the tryptophan fluorescence emission of apo-H61T VAO.

Figure 17. Nano-electrospray mass spectra of 3 μM apo-VAO H61T, in 50 mM ammonium acetate, pH 6.8 with added FMN at protein/cofactor ratios of A, 1:0, B, 1:1 and C, 1:10.

In solution protein assembly is often dependent on the ionic strength and/or type of salt\textsuperscript{183}. In all the above reported data the ammonium acetate concentration was 50 mM. To investigate whether buffer salt concentrations affect the dimer/octamer equilibrium, we recorded mass spectra from solutions with ammonium acetate concentrations ranging from 5 mM to 1 000 mM. All data
indicated that the dimer-octamer equilibrium is only marginally shifted towards the octamer at higher salt concentrations (data not shown).

**Figure 18.** Size-exclusion chromatography profiles in 50 mM ammonium acetate, pH 6.8 of I, 3 µM wild type VAO and of apo-H61T in presence of II, 30 µM 5’-ADP and III, 30 µM FMN. Absorbance was measured at 220 nm.

**Table III.** Normalized octamer/dimer ratios observed by size-exclusion chromatography for wild type VAO, apo-H61T and apo-H61T in presence of cofactor analogs.

| Enzyme                  | Octamer    | Dimer     |
|-------------------------|------------|-----------|
| Wild type VAO           | 86 ± 5     | 14 ± 5    |
| Apo-H61T                | 15 ± 5     | 85 ± 5    |
| Apo-H61T + FAD          | 78 ± 10    | 22 ± 10   |
| Apo-H61T + 5’-ADP       | 81 ± 5     | 19 ± 5    |
| Apo-H61T + FMN          | 13 ± 9     | 87 ± 9    |

*Each data point results from three independent experiments.*

**Protein assembly probed by gel-filtration.** In order to compare the mass spectrometric results with data obtained in solution, size-exclusion chromatography was performed on VAO also using ammonium acetate as buffer salt. When 3 µM wild type VAO was incubated for 2 hours at room temperature in 50 mM ammonium acetate, pH 6.8, the enzyme was for ~86% present in its octameric form and for 14% in its dimeric form (Fig. 18; Table III). Under similar experimental conditions, apo-H61T was mainly in the dimeric form (Table III). When apo-H61T was incubated for 2 hours with excess FAD, the octamer/dimer equilibrium clearly shifted towards the octameric form (Table III). A similar shift in the dimer/octamer ratio was observed when apo-H61T was incubated with 5’-ADP (Fig. 18; Table III). In contrast, nearly no effect on the dimer/octamer ratio of H61T was observed when the mutant was incubated with FMN (Fig. 18; Table III). These results show that the size-exclusion chromatography data are in qualitative agreement with the data obtained by mass spectrometry.

Earlier urea unfolding studies indicated that the dimeric form of wild type VAO is nearly as active as the octameric form. The rapid reconstitution of holo-H61T from its constituents apo-H61T and FAD did not allow the discrimination between the activities of dimeric and octameric holo-H61T. Nevertheless, our gel-filtration data suggested that the FAD-induced oligomerization of apo-H61T is a relatively fast process. When 3 µM of apo-H61T was mixed with 30 µM FAD and immediately injected onto the Superdex column, more than 60% of the enzyme eluted in the...
Cofactor-dependent assembly of VAO

octameric form (compared to 10% octamer for 3 µM apo-H61T). This confirms that FAD binding to apo-H61T rapidly shifts the equilibrium between dimeric and octameric species towards the octameric state.

Discussion

In this study we have probed the influence of cofactor binding on the assembly of VAO in different solutions as determined by relative changes in ion intensities in the corresponding mass spectra and for comparison by gel-filtration. Before discussing the results, we believe it is necessary to address some of the factors that may influence the relative ion signal intensities of the various observed protein assemblies. It is now well established that mass spectrometry may be used to detect even weakly bound noncovalent complexes of protein assemblies even when they are very large and high in mass as the ones described here.

We have been able to detect ions in the mass spectra corresponding to the dimeric as well as the octameric assemblies of the protein VAO. Detection of these ions by mass spectrometry depends heavily on the experimental conditions (mass spectrometer source and analyzer conditions such as voltages, pressures and temperature) and the nature of the sprayed solutions (temperature, pH and organic solvent content). We optimized the instrument parameters to optimally detect the octameric species (see “Experimental” and Ref.40). The pressure in the front-end region of the mass spectrometer was specifically raised as far as the pumping system allowed, which led to the best transmission and detection of the octamer ions. In general in mass spectrometry, it has to be assumed that high mass ions will most likely be negatively discriminated. This discrimination occurs during the ionization event, the transmission through the instrument and upon making impact on the detector (usually smaller proteins “fly” better than larger proteins). The higher mass ions attain relatively less charges per entity and have lower velocities through the instrument. Therefore we believe that the relative abundance of the VAO octamer ions in the mass spectra is underestimated when compared with the relative abundance of these species in solution. It is clear that ion abundances observed in mass spectra cannot directly be translated to the species abundances in solution. For this purpose we have optimized the conditions for the high mass ions and kept all experimental conditions constant throughout this work. Trends observed in the mass spectra can then be qualitatively interpreted.

For cofactor-induced stabilization of protein assemblies, the absence of any significant dimeric species in the mass spectra of wild type VAO is a clear indication that the native enzyme is indeed most stable as the octameric assembly of 508.7 kDa (Fig. 13A). Similarly, the absence of any significant octameric species in the mass spectra of apo-VAO H61T suggests that, in solution and at relatively low ionic strength and protein concentration, the apoprotein adopts mainly the
dimeric conformation (**Fig. 13B**). This is in qualitative agreement with the present gel-filtration data but in disagreement with earlier results obtained from X-ray crystallography\(^{101}\). The fact that apo-H61T crystallizes as an octamer might therefore be explained by the high protein concentration and different incubation conditions used. Interestingly, binding of FAD to apo-H61T can be unequivocally demonstrated by our nano-electrospray ionization mass spectrometry approach. In the mass spectra, signals were clearly evident for strong and highly specific FAD-apo-VAO interactions given that the principal dimeric component detected from the FAD-saturated solution is the 2FAD-dimer complex. The mass spectral analysis of the reconstitution of the H61T mutant also indicates that binding of FAD gradually but largely shifts the dimer/octamer equilibrium in favor of the octameric species. Activity and fluorescence studies showed that the reconstitution of apo-VAO H61T with FAD is a fast process. From the full recovery of enzyme activity and the established equilibrium between dimeric and octameric species we conclude that, in analogy to wild type VAO\(^{136}\), the activity of the dimeric form of the holo-H61T mutant must be comparable with the octameric form. However, because octamerization is also relatively fast, the present data do not allow the discrimination between the rate of FAD binding and the rate of octamerization.

Fraaije *et al.*\(^{101}\) reported that 5’-ADP was a strong competitive inhibitor of the H61T mutant enzyme while FMN did not reveal any inhibitory effect. With the observed binding of 5’-ADP to the dimeric form of apo-H61T and the oligomerization provoked by the presence of this cofactor analog, our mass spectral data clearly showed that the adenosine diphosphate moiety of FAD plays an essential role in the formation of the octameric structures. Because the crystal structure of the octameric apo-H61T mutant is highly similar to that of wild type VAO\(^{101}\) and incubation of dimeric apo-H61T with FMN does not stimulate octamerization, we conclude that upon FAD binding, small conformational changes in the ADP-binding pocket of the dimeric species are transmitted to the protein surface, promoting oligomerization. In this respect it is important to note that VAO belongs to a newly recognized family of flavoenzymes\(^{130}\), which lack the well known Rossmann fold for binding the ADP-moiety of FAD\(^{120,139}\). In the Rossmann-fold containing flavoenzymes, ADP and FAD have often similar influence on the oligomerization state of the enzymes whereas FMN has not\(^{174}\). For the members of the VAO family only limited data on cofactor binding are available. Nevertheless it is of interest to note, here, that for all VAO homologues with known structure two conserved loops are responsible for binding the pyrophosphate moiety of the FAD\(^{184,185}\). Furthermore, in heterotrimeric CO dehydrogenase the flavoprotein moiety can only be reconstituted when the apoprotein is bound to the molybdo-iron-sulfur protein moiety\(^{186}\), whereas for heterodimeric *p*-cresol methylhydroxylase noncovalent binding of the FAD (and not FMN) to the flavoprotein subunit triggers binding of the cytochrome subunit, which is necessary for subsequent covalent FAD attachment\(^{177}\).
Chapter 3

Cofactor-dependent assembly of VAO

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