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Synthesis, characterization and preliminary crystallographic data of N^6-(6-carbamoylhexyl)-FAD-D-amino-acid oxidase from pig kidney, a semi-synthetic oxidase

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The FAD analogue, N^6-(6-carboxyhexyl)-FAD, carrying a hexanoic acid residue at the N^6 position of the adenine moiety was synthesized. A new semi-synthetic oxidase, N^6-(6-carbamoylhexyl)-FAD-D-amino acid oxidase, was prepared by reacting the succinimido ester of N^6-(6-carboxyhexyl)-FAD with apo-D-amino-acid oxidase from pig kidney in the presence of benzoate. Reaction conditions and methods have been developed for preparing pure semi-synthetic and fully active N^6-(6-carbamoylhexyl)-FAD-D-amino acid oxidase that contains 1 covalently bound FAD analogue/subunit, as verified by redialysis, ultraviolet spectrophotometry, electrospray ionization (ESI)-MS and peptide mapping.

Presumably, the N^6-(6-carbamoylhexyl)-FAD moiety of this semi-synthetic D-amino-acid oxidase (DAAO), selectively bound to Lys163, has a structurally similar position to that of the non-covalently bound FAD of the native holoenzyme, since both DAAO forms show very similar kinetic properties (semi-synthetic DAAO, V_{max}(app) = 17.7 μmol min^{-1} mg^{-1}; K_M(app) = 4.5 mM; native holo-DAAO, V_{max} = 12.2 μmol min^{-1} mg^{-1}; K_M = 1.8 mM). Compared with the native holo-D-amino acid oxidase, this new semi-synthetic N^6-(6-carbamoylhexyl)-FAD-D-amino acid oxidase is a considerably more stable enzyme that shows meso-thermostability and withstands inactivation on dilution. Probably, the lack of dissociation of FAD and, consequently, the absence of the instable apoenzyme are responsible for these phenomena. Preliminary investigations resulted in finding convenient and reproducible crystallization conditions for N^6-(6-carbamoylhexyl)-FAD-D-amino acid oxidase. The single crystals, obtained by the sitting-drop method using ammonium sulfate as precipitant, belong to the tetragonal space group I422 with cell dimensions a = 16.3 nm, c = 13.6 nm. The crystals diffract to 0.3-nm resolution, with two molecules being present in the asymmetric unit, demonstrating the two-subunit quarternary structure of this semi-synthetic D-amino-acid oxidase.

Keywords: semi-synthetic D-amino-acid oxidase.

FAD-dependent D-amino-acid oxidase (DAAO) is a ubiquitously present, enigmatic flavoenzyme that has been conserved through evolution. This may imply an important functional role in physiology, but this role has not been satisfactory elucidated [1].

D-Amino acids, particularly those having hydrophobic side chains, are oxidatively converted by this enzyme to the corresponding 2-keto acids. FAD undergoes a reductive half reaction with concomitant oxidation of the D-amino acid substrate to the 2-imino acid, which then spontaneously hydrolyses to the 2-keto acid and ammonia. The oxidized form of FAD is regenerated by reduction of molecular oxygen to H_2O_2 [2].

The enzymological properties of D-amino-acid oxidases from mammalian sources (present in peroxisomes of kidney, liver and brain cells) and from yeasts (present in microbodies) have been intensively studied, e.g. chemical and physical properties [2], kinetic mechanism [3–5], primary structure [6, 7] and active-site mapping by classic chemical modification of specific amino acid residues [2], by using isoalloxazine modified FAD analogues as structural probes [8, 9] and site-directed mutagenesis [10, 11], based on the available nucleotide sequence of encoding cDNA [12, 13].

DAAO from pig kidney is of analytical interest for the determination of D-amino acids. DAAO from *Rhodotorula gracilis*, showing a high turnover rate for cephalosporin C, is of considerable biotechnological potential as the biocatalyst in the first step of a two-step enzymatic process for the conversion of cephalosporin C to 7-aminocephalosporanic acid, an important intermediate for the synthesis of semi-synthetic cephalosprin antibiotics [14]. Unlike the extensive access to enzymological data for DAAO (the pig kidney enzyme is one of the most thoroughly studied flavoenzymes), no three-dimensional structure has yet been published for this oxidase. It has been reported that crystals of DAAO from pig kidney are difficult to handle for structural investigations by X-ray diffraction analysis, presumably, due to instability of the holoenzyme and concomitant heterogenity with respect to the quaternary structure [15]. Only, predictions concerning the secondary structure of DAAO from pig kidney, based on primary structure and circular dichroism data, point

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Abbreviations. DAAO, D-amino-acid oxidase; LDH, lactate dehydrogenase; GOD, glucose oxidase; MALDI-MS, matrix-assisted laser-desorption-ionization mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast-atom-bombardment mass spectrometry.

Enzymes. D-amino-acid oxidase (EC 1.4.3.3); L-lactate dehydrogenase (EC 1.1.1.27); catalase (EC 1.11.1.6).

to 30% β-structure and 23% α-helix [6]. Its three-dimensional structure is urgently needed for the exact determination of the topology of the active site. A detailed structural characterization of the active site may contribute to the elucidation of the identity of the true natural substrate for DAAO and, consequently, its still unknown physiological role [1]. Furthermore, this will allow the supposed structural similarity with other flavoenzymes, such as parahydroxy-benzoate hydroxylase and glycolate oxidase [2], to be assessed.

DAAO from pig kidney, binding FAD moderately strong (K_d = 0.22 μM) [2], has been chosen as model enzyme for our attempt to prepare an enzymatically active covalent conjugate between a FAD analogue, functionalized at the N6 position of the adenine, and an apo-flavooxidase. Such semi-synthetic oxidases, that catalyse a reaction of interest for biochemical analysis, may prove to be better integrable into biosensor devices than the natural enzymes. Under the stress conditions of biosensor performance, the flavocoenzyme will not be released from the enzyme and the intrinsic stability of the biocatalyst might be increased in the case of the conjugate due to the absence of the less stable apoform and so lead to more robust devices.

Enzymatically active covalent conjugates have not been prepared for flavoenzymes that originally bind the flavocoenzyme non-covalently, although similar conjugates from NAD analogues, functionalized at the N6 position of the adenine, and dehydrogenases have been synthesized and characterized [16–18].

This study reports the synthesis and characterization of an enzymatically fully active semi-synthetic flavoenzyme containing 1 covalently bound FAD analogue/subunit that is coupled at the functionalyzed N6 position of the adenine moiety, as exemplified by N^6-(6-carbamoylhexyl)-FAD-DAAO from pig kidney.

Since this artificial covalent flavinylation approach leads to an easily and reproducibly crystallizable enzyme derivative for N^6-(6-carbamoylhexyl)-FAD-DAAO, this may provide a new strategy for the crystallization of flavoenzymes.

MATERIALS AND METHODS

Materials. All enzymes and coenzymes were from Boehringer with the exception of Lipomod 224 (Biocatalysts), carbonic anhydrase from bovine erythrocytes (Sigma) and FMN (Fluka). 6-Chloro-9-β-D-ribofuranosylpurine and 6-chloro-AMP were from Pharma-Waldhof. Dialysis tubings (molcular mass cutoff of 25000 Da) were from Spectrum Medical Industries, Inc. Amberlite XAD-16 was from Serva. AG 50W-X4 Resin (100–200 mesh) and Bio-Rex 70 Resin (50–100 mesh) were from Bio-Rad. Blue Sepharose CL-6B was from Pharmacia. Servalyte carrier ampholytes were from Serva. All chemicals were of highest purity available from commercial sources and were used without further purification with the following exceptions: phosphorylchloride was freshly distilled before use; dimethyl formamide was distilled from KOH and stored over a molecular sieve of 0.3 nm. Distilled water was always used for preparing aqueous solutions.

Analytical procedures. The progress of the reaction and the purity of all nucleotide and flavin compounds synthesized were checked by HPTLC and HPLC. HPTLC, including the determination of relative mobilities (R_f), was carried out on silica gel 60 F_{254} HPTLC plates (Merck) in 1 M triethylammonium bicarbonate/ethanol (3:7, by vol., pH 7.5). Analytical HPLC was performed at a flow rate of 1.0 ml/min and 35°C on a Nucleosil (120–5 C_{18}, 4 mm×125 mm) reverse-phase column integrated into a Merck Hitachi D-6000 chromatography station. Chromatographic profiles were monitored at 256 nm with a Merck Hitachi L-4200 ultraviolet–visible detector. Spectrophotometry was performed at 35°C using a Hitachi U-3200 spectrophotometer. Spectrophotometric measurements of nucleotide and flavin compounds were carried out in 0.1 M sodium phosphate, pH 7, using molar absorption coefficients of 12500 M^{-1} cm^{-1} at 445 nm for FMN [19], 11300 M^{-1} cm^{-1} at 450 nm for FAD [20] and of 8400 M^{-1} cm^{-1} at 263 nm for 6-chloro-AMP (Fig. 1, I) [21]. The concentrations of apo-DAAO, native holo-DAAO and its benzoate inhibitor complex were determined spectrophotometrically by using respectively the molar absorption coefficients 59388 M^{-1} cm^{-1} at 278 nm, 11300 M^{-1} cm^{-1} at 455 nm and 11300 M^{-1} cm^{-1} at 462.5 nm [22]. The concentration of the semi-synthetic DAAO was determined by assuming the molar absorption coefficient of the native holo-enzyme.

All fractions from free-flow isoelectric focusing were assayed for activity according to Yagi and Ohishi [23], by mixing aliquots (50 μl) of each fraction with a staining solution (150 μl) containing 0.1 M sodium pyrophosphate, pH 8.3, 0.01 M D-phenylalanine, 0.02% (mass/vol.) 2,3,4-triphenyltetrazolium chloride and 0.02% (mass/vol.) phenazine methosulfate and reading the absorbance at 650 nm after 30 min.

The structural assignments of FMN and the analogues of AMP and FAD were based on negative-ion fast-atom-bombardment (FAB)-MS spectra, recorded on a Kratos MS 50 RF mass spectrometer, and on ^{1}H-NMR spectra, obtained in D_2O on a Bruker WM-400 (400.1 MHz) NMR spectrometer. All chemical shifts were measured relative to the sodium salt of 3-(trimethylsilyl)propanesulfonic acid as standard.

Positive-ion electrospray-ionization (ESI)-MS spectra for the several DAAO forms were recorded on a Finnigan TSQ 700 instrument. Before performing ESI-MS, DAAO samples were desalted using Centricon ultrafiltration membranes (molecular mass cutoff of 10000 Da, Amicon) and diluted with methanol/water (1:1, by vol.) containing formic acid (1%) to a final concentration of 10 pmol/μl. Fractions collected from reverse-phase HPLC, were directly used after dilution with the latter mixture to a final concentration of 10 pmol/μl. The ESI-MS spectra were recorded at a flow rate of 1 μl/min and a spray energy of 5.5 kV.

The matrix-assisted laser-desorption (MALDI)-MS spectra for the several DAAO forms were recorded on a Bruker REFLEX-time-of-flight mass spectrometer using sinapinic acid, dissolved in a mixture of acetonitrile/water (4:6, by vol.) containing trifluoroacetic acid (0.06%). Low-molecular-mass impurities and buffers were removed by dialysis in water prior to mass spectrometry. Samples were prepared as described elsewhere [24]. The MALDI-MS spectra were recorded in reflected and in linear mode. The instrument was calibrated with carbonic anhydrase from bovine erythrocytes.

Purification of 5′-FMN from commercial FMN. Crude FMN (520 mg, 1.0 mmol) was dissolved in 8 ml of distilled water and separated on a Merck LiChroprep NH_2 column (Lobar B, 310–25, 40–63 μm), previously equilibrated with 1 mM triethylammonium acetate, pH 5.8. A linear gradient (1236 ml) was applied from equilibration buffer to 1 M triethylammonium acetate, pH 5.8, at a flow rate of 6 ml/min. Two fractions were collected containing a mixture of 3′-FMN and 5′-FMN, and pure 4′-FMN. 5′-FMN was separated from the 3′ isomer on a Merck LiChroprep RP-18 reverse-phase column (Lobar B, 310–25, 40–63 μm) applying a linear gradient (1600 ml) from distilled water to MeOH at a flow rate of 6 ml/min. After evaporation of the solvent, the fractions with 5′-FMN were redissolved in distilled water and lyophylized. 5′-FMN (235 mg, 0.42 mmol), 99% pure by HPLC (isocratic eluent: 0.1 M ammonium formate, pH 3.7, in 20% MeOH) was obtained with 45% yield. The purified 5′-FMN was converted to its tri-*n*-octylammonium salt as described by Hoard and Ott [25]. After dissolving in

100 ml distilled water and passing this solution through a column of AG 50W-X4 (pyridinium resin, 2.5 cm×15 cm), tri-*n*-octylamine (153 mg, 0.43 mmol) was added to the eluate. After evaporation of the solvent, the viscous residue was dried by repeated addition and evaporation of anhydrous pyridine (3×10 ml) followed by addition and evaporation of dimethyl formamide (3×10 ml).

Synthesis of N^6-(6-carboxyhexyl)-FAD. *6-Chloro-AMP.* 6-Chloro-AMP (Fig. 1, II) was prepared by minor modifications of the method, originally described by Guilford et al. [21]. To a stirred solution of 6-chloro-9-β-D-ribofuranosylpurine (Fig. 1, I, 8.4 g, 30 mmol) in triethylphosphate (150 ml) a solution of phosphoryl chloride (4.5 ml, 49 mmol) and water (0.3 ml) in the same solvent (15 ml) was added at −17°C. The addition was repeated three times at intervals of 30 min. The reaction mixture was left at 4°C overnight. The mixture was titrated by adding NaOH (4 M) at 4°C to pH 6.0 until no pH change was observed within 10 min. After extraction of triethylphosphate with diethylether (3×500 ml) and subsequent lyophylization, the residue (48.5 g) contained 6-chloro-AMP and inorganic salts. The residue was divided in three charges of 16.17 g. Each charge was dissolved in ethanol/water (1:9, by vol., 40 ml) and separated at a flow rate of 5 ml/min on an Amberlite XAD-16 column (5 cm×36 cm), previously equilibrated in the same solvent. After evaporation of the solvent, 6-chloro-AMP (6.4 g, 16.8 mmol) was obtained in 60% yield as a white powder, 98% pure by HPLC. The product was shown to be identical with authentic 6-chloro-AMP by HPTLC, HPLC and ultraviolet−visible spectrophotometry.

N^6-(6-Carboxyhexyl)-AMP heptylester (Fig. 1, III). 6-Chloro-AMP (0.56 g, 1.47 mmol) was added to 6-aminocaproic acid heptyl ester (7.0 g, 30.5 mmol) at room temperature. The latter reagent was prepared from 6-aminocaproic acid and 1-heptanol by azeotropic esterification in the presence of *p*-toluenesulfonic acid as described by Braun et al. [26]. The mixture was heated to 80°C and left at this temperature overnight. 6-chloro-AMP was completely dissolved in the amino ester within 1 h. After 4 h almost complete conversion was observed as monitored by HPLC (2.4% 6-chloro-AMP remaining). The reaction mixture was dissolved in isopropanol/water (1:1, by vol., 150 ml) and, after adjusting to pH 3.7 with 1 M HCl, was passed through a Bio-Rex 70 column (hydrogen resin, 2.5 cm×30 cm) previously equilibrated with isopropanol/water (1:1, by vol., pH 3.7). After evaporation of the solvent under reduced pressure, III (0.79 g, 1.4 mmol) was obtained as an oily residue in 95% yield, 97% pure by HPLC. ^{1}H-NMR (CD_3OD) gave δ of 0.92 (t, 3H), 1.32 (t, 10H), 1.47−1.53 (m, 2H), 1.62−1.77 (m, 4H), 2.38 (t, 2H), 3.61 (s, 2H), 4.08 (t, 2H), 4.14 (m, 2H), 4.27 (m, 1H), 4.44 (q, 1 H), 4.72 (t, 1H), 6.12 (d, 1H), 8.26 (s, 1H), 8.51 ppm (s, 1H); calculated for $C_{23}H_{38}N_5O_9P$ $(M)^-$ 559, found $(M-H)^-$ 558. III was converted to its tri-*n*-octylammonium salt by adding tri-*n*-octylamine (520 mg, 1.47 mmol) in dimethylformamide/dioxan (1:1, by vol., 20 ml) and subsequent evaporation of the solvent.

N^6-(6-Carboxyhexyl)-FAD heptylester (Fig. 1, IV). IV was prepared by minor modifications of the method originally described by Michelson [27]. The tri-*n*-octylammonium salt of III (1.28 g, 1.4 mmol) was dissolved in a mixture of dimethylformamide (1.5 ml) and dioxan (10.5 ml). Diphenyl phosphochloridate (0.39 ml, 1.9 mmol) and tri-*n*-butylamine (0.83 ml, 3.5 mmol) were added, the mixture was shaken vigorously, then left at room temperature for 2.5 h. Solvent was removed under reduced pressure. After shaking the residue in dry diethyl ether (50 ml) and removing the solvent, the precipitated gum was dissolved in dioxan (3 ml). The solution was concentrated under reduced pressure to remove traces of ether. A solution of tri-*n*-octylammonium salt of 5′-FMN (2 mmol) in a mixture of dimethylformamide (3 ml) and pyridine (7 ml) was added to the residue. The mixture was shaken vigorously and left at room temperature overnight. After removing the solvent under reduced pressure, the residue was dissolved in water/isopropanol (1:1, by vol., 100 ml) and passed through a AG 50W-X4 column (pyridinium resin, 2.5 cm×15 cm), equilibrated against the latter solvent. After removal of the solvent under reduced pressure, the crude residue was dissolved in a mixture of water (36 ml) and pyridine (4 ml), and applied to a Merck LiChroprep RP-18 reverse-phase column (Lobar B, 310−25, 40−63 µm) integrated into a FPLC System (Pharmacia), equilibrated in 0.1 M ammonium formate, 10% (by vol.) MeOH, pH 3.7. After washing the column with two volumes of this solvent, the residue was purified by gradient elution (equilibration buffer to MeOH, 1.5 l). After evaporation of the solvent under reduced pressure, IV (Fig. 1; 0.70 g, 0.7 mmol) was obtained in 48% yield, 97% pure by HPLC. ^{1}H-NMR (dimethylsulfoxide) gave δ of 0.82 (t, 3H), 1.22 (m, 12H), 1.52 (m, 8H), 2.25 (m, 2H), 2.31 (s, 3H), 2.37 (s, 3H), 3.40−4.94 (m, 13H), 5.91 (d, 1H), 7.77 (s, 1H), 7.88 (s, 1H), 8.16 (s, 1H), 8.44 ppm (s, 1H); calculated for $C_{40}H_{56}N_9O_{17}P_2$ $(M)^-$ 997, found $(M-H)^-$ 996.

N^6-(6-Carboxyhexyl)-FAD (Fig. 1, V). To a clear solution of IV (0.43 g, 0.43 mmol) dissolved in 0.25 M sodium phosphate (400 ml), pH 7.0, Lipomod 224 (0.4 g) was added. After stirring the mixture gently at 35°C for 4 h no IV could be detected by HPTLC (1 M triethylammonium bicarbonate/ethanol 15:85, by vol., pH 7.5, IV, R_f 0.55; V, R_f 0.40). The reaction mixture was concentrated under reduced pressure, filtered and lyophylized. The crude residue was dissolved in water and applied to a Merck LiChroprep RP-18 reverse-phase column (Lobar B, 310−25, 40−63 µm), equilibrated in 0.1 M ammonium formate, pH 3.7. After washing with two column volumes of equilibration buffer, the residue was purified by gradient elution (equilibration buffer to methanol, 4.0 l). After evaporation of the solvent under reduced pressure, V (0.243 g, 0.27 mmol) was obtained in 63% yield, 97% pure by HPLC. ^{1}H-NMR (D_2O) gave δ of 1.16 (m, 2H), 1.25 (m, 2H), 1.42 (m, 2H), 2.12 (t, 2H), 2.23 (s, 3H), 2.32 (s, 3H), 2.88 (m, 2H), 3.91−4.45 (m, 13H), 5.78 (d, 1H), 7.44 (s, 1H), 7.48 (s, 1H), 7.75 (s, 1H), 8.23 (s, 1H), 8.41 ppm (s, 1H); calculated for $C_{33}H_{43}N_9O_{17}P_2$ $(M)^-$ 899, found $(M-H)^-$ 898.

N^6-(6-Carboxyhexyl)-FAD succinimidoester (Fig. 1, VI). VI was prepared with minor modifications as originally described by Bannwarth [28]. V (16 mg, 17.8 µmol) was dissolved in water (200 µl). To this solution was added dimethylformamide (1.8 ml), containing diisopropylethylamine (15 µl, 87.6 µmol) and *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (16 mg, 52.5 µmol). The progress of the reaction was monitored at room temperature by HPTLC. After 1 h, additional diisopropylethylamine (15 µl, 87.6 µmol) and *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (16 mg, 52.5 µmol) were added, to drive the activation reaction to completion. Repeated extraction of the reaction mixture with diethy lether (4×3 ml) and subsequent evaporation of residual solvent under reduced pressure yielded an orange gum, containing VI, pure by HPTLC (1 M triethylammonium bicarbonate/ethanol 15:85, by vol., pH 7.5, VI, R_f 0.50). VI usually was freshly prepared from V before use, since it decomposes slowly, even when stored at −20°C.

Preparation and purification of N^6-(6-carbamoylhexyl)-FAD-DAAO. A yellow suspension of DAAO holoenzyme (17.5 mg/ml, 4.5 µmol) in ammonium sulfate (3.2 M, 10.1 ml), pH 6.5, was extensively dialysed at 4°C against potassium pyrophosphate (100 mM) containing KBr (1 M) and EDTA (1 mM), pH 8.5 (5×0.7 l for 3 days) [29]. The fifth buffer change contained charcoal (0.5%, mass/vol.) to achieve com-

plete removal of the cofactor. The resulting apo-DAAO was dialysed at 4°C against potassium pyrophosphate (100 mM), pH 8.5 (3×0.7 l for 24 h). The concentration of apo-DAAO was set to a final concentration of 2.5 mg/ml by dilution with water. Sodium benzoate was added up to 0.5% (mass/vol.) at 4°C to this apoenzyme solution. VI (16 mg, 17.8 μmol) was dissolved in water (1 ml) and added to the apoenzyme solution (molar ratio, VI/apo-DAAO, 4:1). The pH of the reaction mixture was immediately adjusted to pH 6.5 with sodium acetate (0.5 M, pH 3.5) and incubated in the dark at room temperature for 24 h. The crude semi-synthetic DAAO was precipitated from the reaction mixture by adding solid ammonium sulfate to 50% saturation at 4°C. After centrifugation (20000 *g*, 20 min), the pellet was suspended in potassium pyrophosphate (100 mM, 10 ml), pH 8.3, and dialysed extensively against the buffered KBr-solution (5×0.7 l for 3 days) to remove non-covalently bound VI, *N*-hydroxylamine and benzoate. The protein solution was dialysed in 10 mM potassium pyrophosphate, pH 8.3 (3×0.7 l for 24 h). The dialysate was passed through a Blue Sepharose CL-6B column (2.5 cm×20 cm), equilibrated in the latter buffer, to remove apo-DAAO according to Harbron et al. [30]. The purified semi-synthetic DAAO was concentrated to 10 mg/ml and equilibrated in 10 mM potassium pyrophosphate, 0.2 mM sodium benzoate, pH 8.3, by ultrafiltration with Centriprep-10 (Amicon). The progress of the purification was followed by loading 0.1-ml aliquots of DAAO with 1.2 nmol of protein onto a Vydac PH6PX C4 reverse-phase column (0.4 I. D×15 cm) equilibrated in a mixture of acetonitrile/water (16:84, by vol.) containing trifluoroacetic acid (0.06% by vol.). A linear gradient (45 ml) from equilibration solution to acetonotrile/water (64:36, by vol.), containing trifluoroacetic acid (0.05% by vol.), was applied. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min and the chromatographic profiles were monitored at 215 nm and 360 nm. After the second dialysis step two peaks, corresponding to apo-DAAO (22%, t_R = 31.0 min) and to N^6-(6-carbamoylhexyl)-FAD-DAAO (78%, t_R = 33.1 min) were detected absorbing, respectively, only at 215 nm and at both 215 nm and 360 nm. After affinity chromatography on Blue Sepharose CL-6B, exclusively one peak was detectable absorbing at both 215 nm and 360 nm (t_R = 33.1 min), corresponding to purified N^6-(6-carbamoylhexyl)-FAD-DAAO.

Electrophoresis. SDS/PAGE [31] was performed on commercial 8% to 25% polyacrylamide gels using the Phast System (Pharmacia). The gels were stained by a commercial silver stain kit (Pharmacia). Molecular masses were estimated using standard protein molecular-mass markers (Pharmacia).

Continuous free-flow IEF was carried out with an OCTOPUS free-flow IEF apparatus (Dr Weber GmbH). The enzyme solution (1 ml, 0.375 mg/ml) was diluted with an equal amount of water containing hydroxypropylmethylcellulose (0.4% mass/vol.) and was applied at a flow rate of 0.15 ml/min at the bottom of the electrophoresis unit. Electrophoresis was carried out at 10°C with H_3PO_4 (0.1 M) containing hydroxypropylmethylcellulose (0.2% mass/vol.) at the anode and NaOH (0.05 M) containing hydroxypropylmethylcellulose (0.2% mass/vol.) at the cathode. The pH gradient was achieved by pumping a mixture (40% pH 3–10, 60% pH 5–8, by vol.) of carrier ampholyte (0.5% by vol.) containing hydroxypropylmethylcellulose (0.2%) through the electrophoresis unit at a flow rate of 7 ml/min and constant power (1300 V, 15 mA). The eluate was separated by simultaneously collecting 96 fractions, each fraction being analyzed for activity, pH and absorbance at 450 nm.

Measurement of enzyme activity. The activity of both natural and semi-synthetic DAAO was generally assayed using D-alanine as substrate and oxygen as electron acceptor. Due to the presence of catalase and an excess of hydrogen peroxide a constant saturating oxygen concentration was maintained in the assay mixture. The pyruvate formed was simultaneously converted to L-lactate by L-lactate dehydrogenase (LDH) using NADH as coenzyme. By monitoring the decrease in absorbance at 340 nm the activity of both forms of DAAO was determined at 25°C under the standard conditions (Jürgensen, D., personal communication) 163 mM Tris/HCl, pH 8.3, 37 mM D-alanine, 0.2 mM NADH, 0.01% (by vol.) H_2O_2, 268 U catalase and 23 U LDH in a total volume of 3.15 ml. The reaction was always started by adding 50 μl enzyme stock solution (20 μg protein/ml). Stock solutions were prepared immediately before use by dilution of enzyme solutions of standard concentration (5 mg/ml) by ice-cold water. The initial-rate-assay conditions at one saturating oxygen concentration for determining V_{max}(app) and K_M(app) for semi-synthetic DAAO from a Lineweaver-Burk plot were equal to the standard assay conditions, except that the D-alanine concentration was varied in the range 0.68–57.6 mM. Protein concentrations for both native and semi-synthetic DAAO were based on a molar absorption coefficient for FAD at 450 nm of 11.3 mM^{-1} cm^{-1} at pH 7.0. It was assumed that 1 FAD or FAD analogue molecule was present/subunit.

Analysis of the enzyme stability. The thermostability of the semi-synthetic DAAO was compared with native holo-DAAO by heating aliquots (20 μg protein/ml) for 30 min at 25°C, 35°C, 45°C, 55°C, 65°C and 75°C. The assay of the residual activity was performed under standard assay conditions. The time-dependent influence of dilution on the stability of the native holo-DAAO and semi-synthetic DAAO was determined by diluting the enzyme solutions (from 5 mg to 20 μg protein/ml) in the presence and absence of FAD (3.3 μM) and assaying the residual activity under standard assay conditions, for 10 min in 1-min steps.

The effect of pH on the reaction rate of the native holo-DAAO and semi-synthetic DAAO was determined under standard assay conditions, except that the pH of the assay mixture was varied in the range 6.5–10.5 using sodium phosphate buffer (0.1 M) and sodium borate buffer (0.1 M), from pH 6.5 to 7.5, and from pH 8 to 10.5, respectively.

Enzymatic digestion and peptide mapping. Semi-synthetic DAAO (100 μg, 2.5 nmol) was dissolved in water (50 μl) containing urea (8 M), ammonium bicarbonate (0.4 M) at pH 8.0. To achieve reducing conditions, the solution was incubated for 15 min at 55°C in the presence of dithiothreitol (45 mM, 5 μl). After cooling to room temperature, iodoacetamide (100 mM, 5 μl) was added to completely protect any cysteine residues towards hydrolysis by carboxamidation [33]. After incubation for 15 min at room temperature in the dark and subsequent dilution with water (140 μl), trypsin (5 μg, sequencing grade) was added. This solution was incubated for 18 h at 37°C according to Stone [34]. The progress of the protein digestion was followed by injecting aliquots of digest solution (5 μl) on a Vydac C18 column (4.6 mm×150 mm) and applying a linear gradient (45 ml) of acetonitrile/water (5:95, by vol.) containing trifluoroacetic acid (0.06%) to acetonitrile/water (1:1, by vol.) containig trifluoroacetic acid (0.06%). The final digest was chromatografied by injection of digest solution (100 μl) on the Vydac C18 column (4.6 mm×150 mm) and applying the latter gradient. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min and stored at −22°C. The chromatographic profiles were monitored at 256 nm and 360 nm to detect any peptide-coupled FAD analogue absorbing at the latter wavelength.

Characterization of the peptide with the covalently bound FAD analogue V. Amino acid analysis of the peptide with the covalently coupled FAD analogue V was performed after hydrolysis in 6 M HCl (gas phase) at 160°C for 75 min with an amino acid analyzer (model 420 A/H, Applied Biosys-

tems). Automated Edman degradation was performed with a gas-phase sequencer (Applied Biosystems Model 470) on line with a phenylthiohydantoin analyzer (Model 120A). The amino acid sequence of the peptide with the covalently coupled FAD analogue V was determined by directly analysing/run 30% of the material from the reverse-phase chromatography step. Identification of the labeled amino acid residue was achieved by alignment of the obtained peptide sequence with the published amino acid sequence of DAAO from pig kidney [6].

Crystallization of N^6-(6-carbamoylhexyl)-FAD-DAAO. A computer-controlled autodiluter (Beckman, Mannheim, Germany) was used to find suitable crystallization conditions. All crystallization experiments were performed with crystallization plates using the sitting-drop method [35]. To remove minor contaminations prior to crystallization, N^6-(6-carbamoylhexyl)-FAD-DAAO (89 mg, 2.21 μmol) was adsorbed onto a DEAE-Sepharose FF column (2.5 cm×20 cm), equilibrated in Tris buffer (10 mM) containing KCl (125 mM) and sodium benzoate (0.2 mM), pH 8. Elution was carried out by using a linear increase of the KCl concentration from 125 mM to 600 mM in 400 ml. Fractions eluting at KCl concentrations in the range 350−450 mM were pooled and equilibrated in 10 mM potassium pyrophosphate with 0.2 mM sodium benzoate, pH 8.3, by ultrafiltration with Centriprep-10 (Amicon). From the resulting stock solution (6 ml), containing the benzoate complex of the semi-synthetic DAAO (10 mg/ml), drops of 10 μl were mixed with drops of 10 μl 1-*O*-*n*-octyl β-D-glucopyranoside dissolved in water (2%, mass/vol.) in the crystallization cones of the crystallization plate. The resulting solutions (20 μl) were mixed with aliquots (10 μl) of the precipitant solutions (1 ml), previously pipetted into the reservoirs of the crystallization plates by the autodiluter. Initial crystallization conditions were established by statistical screening methods and a systematic variation of the precipitant concentration and the pH, with ammonium sulfate as precipitant. The concentration of ammonium sulfate was varied in the range 0.5−1.0 M in increments of 0.1 M. The variation in pH was from pH 4.5 to 8 in increments of 0.25, using 0.02 M citrate/phosphate with the required pH. This screen yielded crystals with ammonium sulfate at pH 5.75−6.25. The optimal conditions were 0.7 M ammonium sulfate and 0.02 M citrate/phosphate at pH 6.0. The crystallization plates were always sealed with transparent tape and stored at 25°C.

X-ray investigation. For X-ray analysis, crystals were mounted in glass capillaries with a drop of mother liquor. For space-group determination test data were collected at the BW6 beamline of the DESY synchrotron using a MAR imaging plate detector. Data were collected with monochromatized radiation (λ = 0.1 nm) at a crystal to detector distance of 180 nm with a scan angle of 1.0° at 10°C. Exposure times varied between 30 s and 2 min. The data were processed and indexed with DENZO [36]. The packing densities were calculated from the refined lattice constants using the calculated mean molecular mass of the semi-synthetic DAAO of 40217 Da.

RESULTS AND DISCUSSION

Synthesis. In previous reports, adenine-modified FAD analogues have been synthesised by two different methods, either by specifically modifying the adenine moiety of intact FAD, or by coupling modified AMP derivatives to FMN. The coupling reaction was originally carried out with AMP-imidazolides, prepared from imidazol and trichloracetonitrile acting as phosphate-activating reagents [37]. An improved version of this approach using diphenyl phosphochloridate [27] has been shown to be superior with respect to the reaction yield, even in the case of modified AMP derivatives [38, 39]. The alternative route of specifically modifying FAD at the N1 position of the adenine ring by nucleophilic displacement followed by Dimroth rearrangement to prepare N^6-functionalized FAD has been described by several authors [40, 41, 18]. Although the latter method seems to be more straightforward than the coupling approach, this method is disadvantageous, if the modification should lead to controlling the spacer length by -CH_2- increments. Since the nitrogen at the N1 position of FAD is a weak nucleophile, a few special highly electrophilic compounds are available for the displacement reaction, e.g. the alkylating heterocyclic reagents propiolactone, ethyleneimine, epoxides that limit the variation of the spacer length [18, 41]. Furthermore, the usual reaction conditions for the Dimroth rearrangement are too alkaline, potentially leading to undesirable side reactions with respect to FAD or derivatives, e.g. splitting of the N10-C bond between the isoalloxazine and the ribitol moiety. For the preparation of N^6-functionalized FAD derivatives with a spaced terminal carboxyl group, the original coupling strategy of Cramer et al. [37] was reinvestigated.

Preliminary attempts to synthesise 6-chloro-FAD from II and 5′-FMN led to complex reaction mixtures, and 6-chloro-FAD could not be obtained. The approach of first synthesizing N^6-(carboxyalkyl)-AMP from 6-chloro-AMP by nucleophilic displacement with aminoalkyl carboxylic acids and then coupling the carboxylated AMP to 5′-FMN was not successful. Although N^6-(carboxyalkyl)-AMP can be synthesized without complications, the dominating cross-reactivity of the free carboxyl group that interferes with the diphosphate coupling could not be controlled, independently of the phosphate-activation method. To avoid this cross-reactivity a new strategy was developed for synthesizing N^6-(carboxyalkyl)-FAD derivatives, as exemplified for N^6-(6-carboxyhexyl)-FAD (V).

First, 6-chloro-AMP (II) was prepared by phosphorylation of 6-chloro-adenosine (I) under standard reaction conditions in 60% yield. The formation of the heptylester N^6-(6-carboxyhexyl)-AMP (III) was achieved almost quantitatively (95% yield) by heating 6-chloro-AMP (II) without solvent in a 15-fold molar excess of the aminocaproic acid heptylester. The protected AMP derivative III was coupled to 5′-FMN by diphosphate coupling, as we expected that the heptylester of N^6-(6-carboxyhexyl)-FAD (IV) might now be synthesized without serious complications and that the protected carboxyl group might be deprotected enzymatically by a lipase. For this purpose III was converted to the corresponding diphenyl pyrophosphate that was not isolated due to its high reactivity. The diphenyl pyrophosphate of III was then allowed to react at room temperature with the tri-*n*-octylammonium salt of FMN without complications and the resulting heptylester of N^6-(6-carboxyhexyl)-FAD (IV) was isolated by reverse-phase chromatography with 48% yield. Attempts to hydrolyse the heptyl ester of IV using traditional chemical means failed due to simultaneous hydrolysis of the pyrophosphate moiety. As for similar cases [26], the deprotection of IV to V was investigated by lipase-catalyzed hydrolysis. Of the 17 lipases investigated only the commercial lipase Lipomod 224 could conveniently catalyze the specific hydrolysis reaction to complete conversion for both the heptylester of N^6-(6-carboxyhexyl)-AMP (III) and of N^6-(6-carboxyhexyl)-FAD (IV) both in approximately 65% yield. After purification by reverse-phase chromatography, the FAD derivative (V) was quantitatively converted to its succinimido ester (VI) by using *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate as activating reagent. *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate [28], with its uronium ion as leaving group, is a considerably more convenient activating reagent for carboxylic groups than the usual dicyclohexylcarbodiimide and

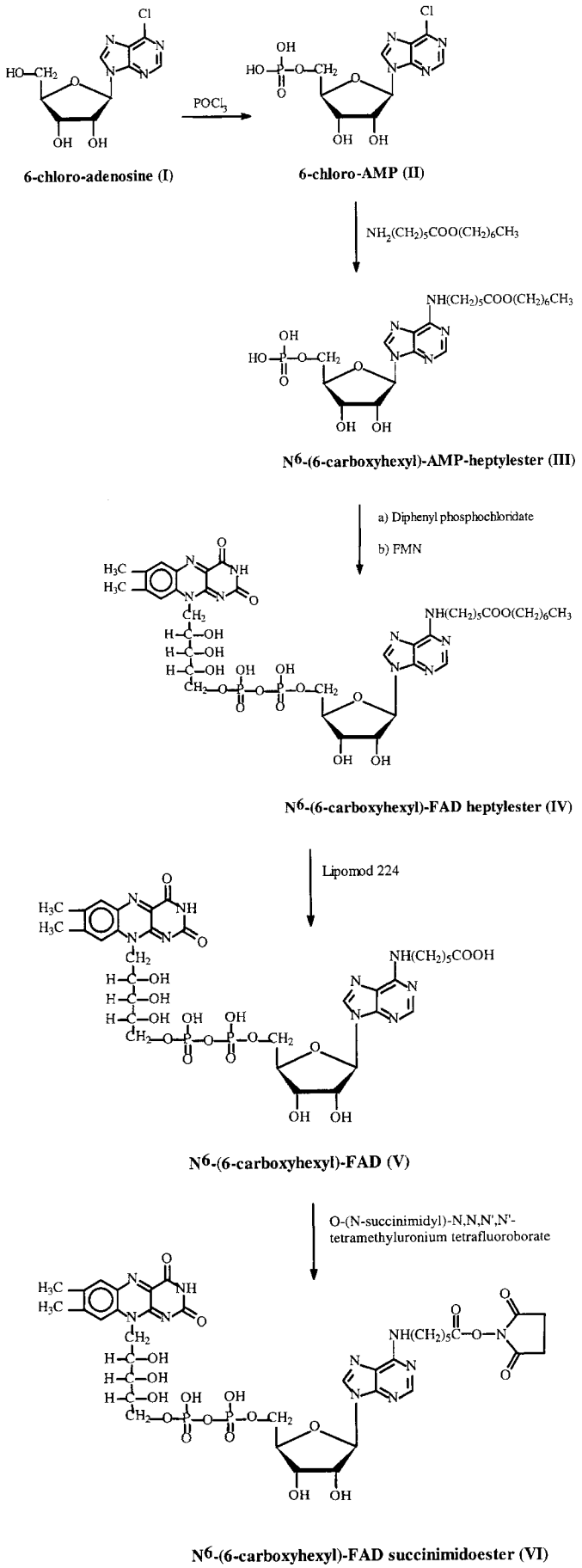

Fig. 1. Reaction scheme for the synthesis of N^6-(6-carboxyhexyl)-FAD succinimido ester (VI).

N-hydroxysuccinimide [43]. The synthesis of VI is summarized in Fig. 1.

By means of this route a convenient synthesis strategy is now available for the preparation of a homologous series of N^6-(carboxyalkyl)-FAD analogues or their activated derivatives in overall yields of 17–20%.

Preparation and purification of N^6-(6-carbamoylhexyl)-FAD-DAAO. The moderate binding of FAD in the original holo-DAAO may contribute to the intrinsic instability of the enzyme and its heterogeneity with respect to the quarternary structure. Presumably, these are the main factors that complicate the preparation of suitable crystals for structural studies. Since the structure of DAAO is not known, a rational design of an enzymatically active covalent conjugate between a N^6-functionalized FAD analogue and the apoform of DAAO was not possible.

To prepare such a conjugate, a suitable spacer length between the N6-position of the adenine and the carboxyl group reacting, for example, with the ε-amino group of a lysine of the protein backbone, is crucial. The covalently bound FAD analogue should have a similar spatial orientation as the non-covalently bound FAD in the native holoenzyme. The location of the covalent linkage between the FAD analogue and the lysine should not have a too strongly deactivating effect, either structurally oriented or by blocking the access of the substrate to the isoalloxazine moiety of the coupled FAD analogue. Finally, the reaction conditions should be optimized carefully to achieve a highly specific modification at a single target lysine residue. Considering the known three-dimensional structure of *p*-hydroxybenzoate hydroxylase, supposedly highly similar to DAAO [44] and glucose oxidase [45], it was expected that the N^6 position of the adenine of FAD would be suitable for the introduction of a functionalized spacer. This choice was supported by our recent findings, that it is possible to prepare enzymatically active non-covalent conjugates of glucose oxidase and N^6-functionalized FAD derivatives [46]. For the carboxylated FAD derivative V with a spacer length of approximately 1.1 nm it was speculated, that this FAD analogue might be applicable for covalent incorporation by way of such an ε-amino group without seriously disturbing the native structure of DAAO.

This method was based on extrapolating the results of trial and error coupling experiments, varying the reaction conditions with respect to pH, temperature and molar ratio of VI and apoenzyme. A reproducible method was developed to prepare semisynthetic DAAO with 1 FAD analogue/subunit. Since apo-DAAO tends to precipitate at pH values below 8, VI was added to the apoenzyme at pH 8.5 and 4 °C, before the reaction mixture was adjusted to pH 6.5 and room temperature. Since succinimido esters selectively react with primary amino groups in exceptionally high yields and are remarkably resistant towards hydrolysis in the optimal pH range 6–6.5, this step was found to be crucial [47]. Also, the presence of benzoate that binds only strongly to the holoenzyme and enhances the binding of FAD [48] was required to minimize the excess of VI, when preforming the complex between apo-DAAO and VI prior to adjusting the optimal reaction conditions for the covalent coupling. Further investigations on the coupling procedure by testing molar ratios of VI/apoenzyme subunit in the range 2–32:1 resulted in the finding, that a 4:1 ratio is optimal for simultanously obtaining high yields of conjugates carrying 1 FAD analogue/subunit and suppressing unspecific binding of VI to apo-DAAO. This molar ratio of 4:1 is still low in comparison with native FAD that requires at least a 2:1 molar ratio for complete saturation of apo-DAAO [49]. After separating reagents and non-covalently bound VI from the reaction mixture by ammonium sulfate precipitation and dialysis, the preparation usually contains residual

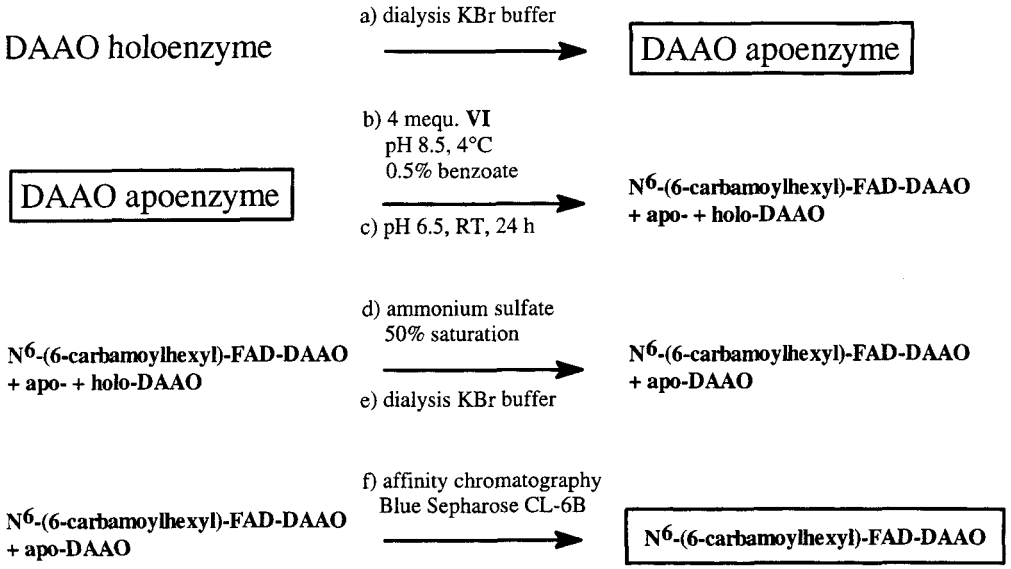

Fig. 2. Preparation of the N^6-(6-carbamoylhexyl)-FAD-DAAO from N^6-(6-carboxyhexyl)-FAD succinimido ester (VI) and apo-DAAO (pig kidney).

apo-DAAO (always approximately 20% as was determined by reverse-phase chromatography). By introducing an additional affinity-chromatography step on Blue-Sepharose CL-6B the quantitative separation of the apo-DAAO from semi-synthetic DAAO could be achieved in a way analogous to the routine method for separating native apo-DAAO from holo-DAAO [30]. The preparation and purification of semi-synthetic DAAO is outlined in Fig. 2.

The KBr-dialysis method was chosen for preparing the apoenzyme and for removing any non-covalently bound VI after the coupling reaction, since this method is known to procceed almost quantitatively in the case of DAAO [49]. Following the procedure depicted in Fig. 2, coupling yields in the range of 50–54% were determined for the diverse preparations, assuming the molar absorption coefficients of the benzoate complexes of the semi-synthetic DAAO and the native holoenzyme are identical according to [22]. By repeating the dialysis in the presence of KBr for a sample of the purified semi-synthetic DAAO, it was observed that no free FAD analogue was present, indicating that all V was really covalently bound.

As shown in Fig. 3, the purified semi-synthetic DAAO (curve I) could be converted to its fully reduced form (curve II) by addition of its substrate D-alanine under anaerobic conditions according to [22]. This is the first proof that 1 FAD analogue V/subunit is present. The visible part of the spectrum (curve I) was found to be identical with the spectrum of the benzoate complex of the native enzyme displaying a characteristic absorption maximum at 462.5 nm. Any unspecifically coupled V would remain oxidized under these conditions leaving absorbance in the 450 nm range. As described by Curti et al. [29] the ratio of the absorbance for the purified holoenzyme-benzoate complex at 274 nm to that at 462 nm is 9.5. As expected, the corresponding ratio of 10.7 for the pure benzoate complex of the semi-synthetic DAAO with 1 FAD analogue/subunit was higher, since the molar absorbance around 267 nm for N^6-modified-adenine-containing coenzyme derivatives is generally higher than that of the native coenzymes [18]. Using MALDI-MS, the purification of semi-synthetic DAAO from the reaction mixture could be followed very conveniently by monitoring qualitatively molecular heterogeneity [50]. For example, it could be demonstrated that the affinity chromatography step on Cibacron-Blue-Sepharose resulted in pure semi-synthetic DAAO. As expected,

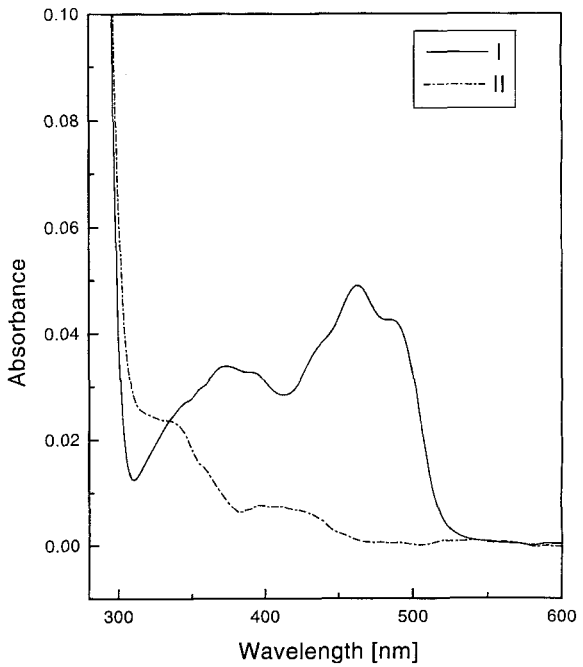

Fig. 3. Ultraviolet–visible absorption spectra of N^6-(6-carbamoylhexyl)-FAD-DAAO in the oxidised (I) and fully reduced state (II). The semi-synthetic DAAO was reduced at 25°C within 3 min under nitrogen in a closed cuvette containing 163 mM Tris/HCl, pH 8.3, 50 mM D-alanine.

no significant difference with respect to molecular mass between the native and semi-synthetic DAAO was observed from the SDS/PAGE pattern. The determination of the mean mass of the purified semi-synthetic DAAO was carried out by ESI-MS using native holo-DAAO as control probe. Under ESI-MS conditions complete dissociation of FAD from native holo-DAAO is observed. Its $[M + H]^+$ peak maximum of 39338 is only 2 mass units higher than the expected 39336 of the apoenzyme, calculated from the sequence [6]. The mass of the semi-synthetic DAAO of 40224 deviates from the maximum of the apoenzyme by 886. This mass increment is 7 masses higher than the calculated 40217 of the semi-synthetic DAAO. Nevertheless, ESI-

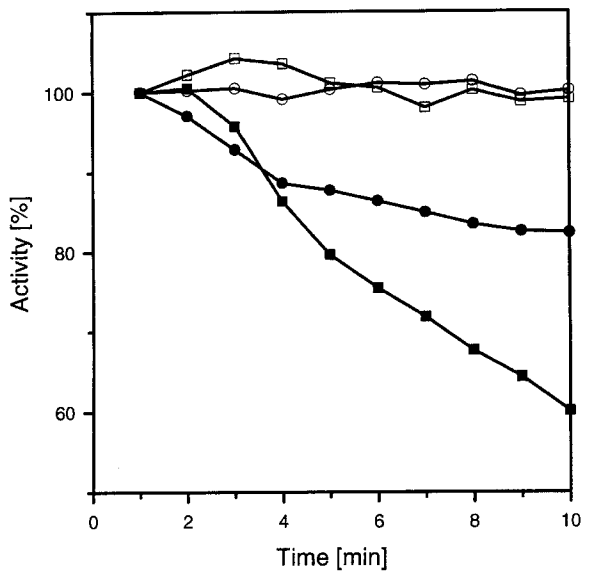

Fig. 4. Progress of the inactivation of N^6-(6-carbamoylhexyl)-FAD-DAAO compared with native holo-DAAO upon dilution in the presence and absence of exogenous FAD. (○) Semi-synthetic DAAO with exogenous FAD; (□) native holo-DAAO with exogenous FAD; (●) semi-synthetic DAAO without exogenous FAD; (■) native holo-DAAO without exogenous FAD. For assay conditions see Materials and Methods.

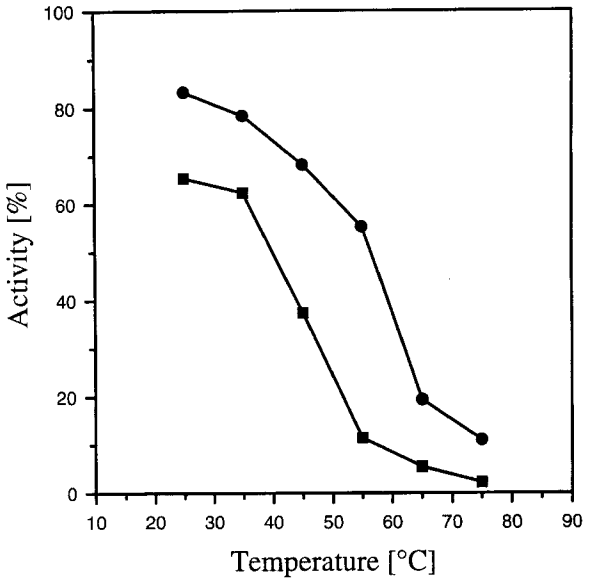

Fig. 5. Progress of inactivation of N^6-(6-carbamoylhexyl)-FAD-DAAO and of native holo-DAAO at different temperatures. (●) Semi-synthetic DAAO; (■) native holo-DAAO. For assay conditions see Materials and Methods.

MS together with ultraviolet-visible spectrophotometry clearly proves, that conditions have been found for the successfull preparation of N^6-(6-carbamoylhexyl)-FAD-DAAO with 1 FAD analogue/subunit.

Identification of the modified amino acid residue. To determine at which amino acid residue the carboxylic group of V is covalently linked to N^6-(6-carbamoylhexyl)-FAD-DAAO, peptide mapping was carried out after digestion with trypsin. Reverse-phase chromatography led to 29 peak fractions with only one showing absorbance at both 215 nm and 360 nm. After isolation and characterization by amino acid and sequence analysis, the peptide of this fraction showed the sequence KVESFEEVAR. The only side-chain of this peptide, susceptible to chemical modification by VI, is the ε-amino group of the side chain of lysine. Since the sequence of this peptide was in agreement with the 163−172 segment of the published sequence of DAAO from pig kidney [6], V can only be specifically attached to Lys163. The presence of the covalently bound FAD analogue in the peptide K163VESFEEVAR was further verified by MALDI-MS, since for the major peak a mass-to-charge ratio of 2084 was determined, which is very near the calculated mean mass (2092 Da) of the peptide K163VESFEEVAR with covalently bound V, considering the limitations of the MALDI-MS technique. The mean mass of the second minor peak differs from that of the main peak by 440 Da, indicating the presence of a modified peptide fragment that had lost the FMN moiety.

Summarizing, the results from redialysis, ultraviolet−visible spectrophotometry, ESI-MS and peptide mapping in combination with MALDI-MS prove that the functionalized FAD analogue V is covalently attached to Lys163 of semi-synthetic N^6-(6-carbamoylhexyl)-FAD-DAAO with 1 molecule V/subunit.

Enzymological characterisation of N^6-(6-carbamoylhexyl)-FAD-DAAO. Since it is known that native apo-DAAO from pig kidney is less stable than native holo-DAAO [22], the behavior of N^6-(6-carbamoylhexyl)-FAD-DAAO, under stress conditions with emphasis on exposure to dilution and heat, has been investigated in comparison with native holo-DAAO. Furthermore, the kinetic parameters K_M(app) and V_{max}(app) of the semi-synthetic DAAO and K_M and V_{max} of native holo-DAAO have been compared.

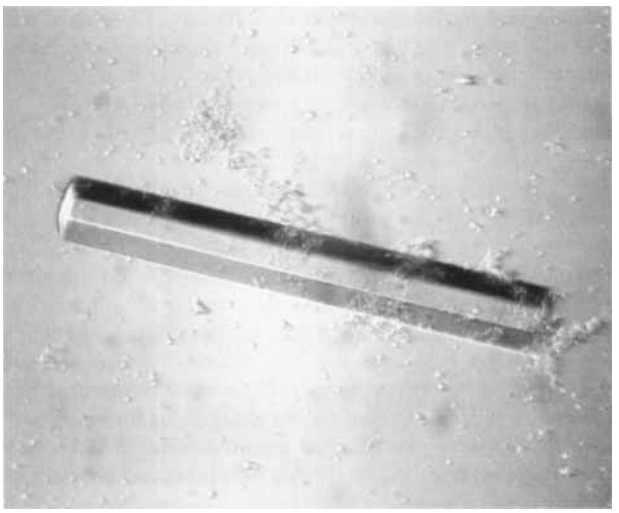

Fig. 6. Crystal of N^6-(6-carbamoylhexyl)-FAD-DAAO (pig kidney). The dimensions are 0.8×0.08×0.08 mm.

Fig. 4 shows the time dependent residual activities of the semi-synthetic DAAO after dilution with and without exogenous FAD compared with native holo-DAAO. The patterns of the curves show that the presence of exogenous FAD has a similar protecting effect for both DAAO forms. This protective effect for native DAAO has been explained by Dixon and Kleppe as a shift of the equilibrium between holoenzyme and apoenzyme to the stable holoenzyme [51]. A similar explanation may hold for the semi-synthetic DAAO insofar as the FAD moiety may also leave the binding site due to the flexibility of the linkage between the FAD moiety and the enzyme. Nevertheless, covalent binding of V to apo-DAAO leads to a significant stabilization of DAAO, presumably, due to a faster rebinding of this linked FAD analogue.

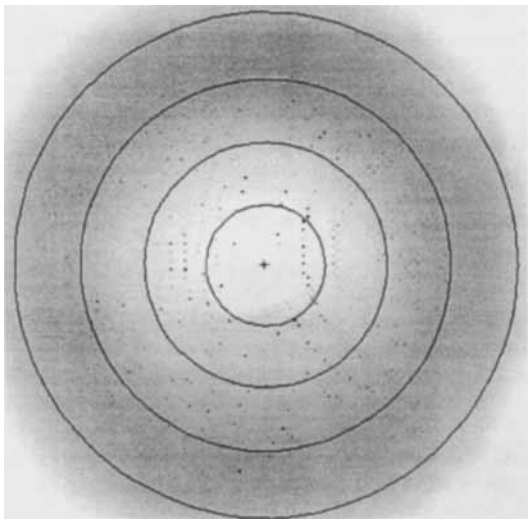

Fig. 7. Preliminary X-ray diffraction pattern of N^6-(6-carbamoylhexyl)-FAD-DAAO (pig kidney). Rings at 0.85, 0.43, 0.28 nm.

In Fig. 5 the results of the thermostability study for N^6-(6-carbamoylhexyl)-FAD-DAAO compared with native holo-DAAO have been summarized. It could be shown that the semi-synthetic DAAO can be converted to a meso-thermostable enzyme by the specific modification. Presumably, the responsible factor is the full coenzyme stabilization due to the absence of apo-DAAO, although a slight change of the protein structure that favours thermotolerance due to modification of Lys163 cannot be discounted. These results may indicate a new approach for stabilizing flavoenzymes that bind originally their flavocoenzyme non-covalently. This stabilization strategy may lead to flavoproteins that are better exploitable for use in biotechnology.

The pH dependency of the reaction rates is identical for both native holo-DAAO and N^6-(6-carbamoylhexyl)-FAD-DAAO. The decreased isoelectric point of the semi-synthetic DAAO (4.7) compared to that of the native holoenzyme (6.2) results from the modification of the free amino residue of Lys163, leaving only 11 free lysine residues.

The specific activity of pure semi-synthetic DAAO (17 U/mg) was found to be 78% of the specific activity of native holo-DAAO (21.7 U/mg) under standard assay conditions. Comparing the measured V_{max}(app) and K_M(app) values from the Lineweaver-Burk plot for the semi-synthetic DAAO (respectively 17.7 μmol min^{-1} mg^{-1} and 4.5×10^{-3} M) to the published V_{max} and K_M values for the native holo-DAAO (respectively, 12.2 μmol min^{-1} mg^{-1} and 1.8×10^{-3} M) [52], leads to the conclusion that the FAD analogue V linked to the side chain of Lys163 must have a similar structural orientation in the protein as FAD in native holo-DAAO. The access of the substrate D-alanine to the isoalloxazine moiety of covalently coupled V in the active site is not obstructed.

Crystallization and preliminary crystallographic study. The benzoate complex of the holoenzyme of DAAO from pig kidney has been found to crystallize in orthorhombic and in trigonal prisms [15]. In both cases the protein was found to be highly associated [15]. However, for N^6-(6-carbamoylhexyl)-FAD-DAAO from pig kidney conditions have been found for its easy and reproducible crystallization by the sitting-drop vapor-diffusion technique [35]. Long, plated crystals appeared after 4–7 days and grew to dimension of $0.8\times0.08\times0.08$ mm in approximately 3 weeks (Fig. 6).

The crystals diffract at least to 0.3 nm. Indexing of several rotation images (Fig. 7) with DENZO [36] consistently indicated a centered tetragonal lattice, space group I422, with cell dimensions $a = 16.3$ nm, $c = 13.6$ nm. Assuming an asymetric unit content of two monomers this yields a packing density of 0.29 pm^3/Da, corresponding to a solvent content of 58%. The crystals are suitable for detailed crystallographic analysis, since their asymetric unit is small and they express high molecular symmetry.

Further native data collection and a search for a suitable heavy-atom derivative is in progress with the aim of solving the structure of semi-synthetic N^6-(6-carbamoylhexyl)-FAD-DAAO. Since the artificial flavinylation approach leads for N^6-(6-carbamoylhexyl)-FAD-DAAO to an easy and reproducible crystallization procedure, this may point to a new strategy for the crystallization of other flavoenzymes.

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