D-HYDROXYNITRILE LYASE: INVOLVEMENT OF THE PROSTHETIC FLAVIN ADENINE DINUCLEOTIDE IN ENZYME ACTIVITY

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1. Introduction

D-Hydroxynitrile lyase (mandelonitrile benzaldehyde lyase, EC 4.1.2.10) from bitter almond emulsin was one of the first enzymes known from its activity [1] and, as it turns out, the first flavoprotein described. It catalyzes to splitting of D(+)-benzaldehyde cyanohydrin (D-mandelonitrile) to benzaldehyde and cyanide according to the equation:

$$C_6H_5CH(OH)CN \xrightarrow{H_2O} C_6H_5CHO + HCN$$

Although freely reversible, it is probably used in the plant only in the direction shown, since the formation of nitriles follows a different path [2]. The enzyme was studied by Rosenthaler [3] and eventually purified [4], when it, surprisingly, turned out to be a flavoprotein, containing FAD as the prosthetic group. The holo-enzyme of 74 000 dalton is a single polypeptide with 1 mol FAD rather tightly bound ($K_d \sim 10^{-8}$ M^{-1}; corrected value from fluorometric titration data of the apo-enzyme). The colourless inactive apoprotein, obtained by precipitation with acid ammonium sulfate according to [5], also of 74 000 dalton, denatures at neutral pH with a half time of 20 min (25°C). It is readily reactivated by FAD, but not by FMN, or riboflavin. No change in sedimentation behaviour is observed on binding of the prosthetic group.

As the cofactor is obviously required for a reaction not involving oxidation/reduction of a substrate, the question as to the function of the FAD arises: is it providing groups for the formation of reactive intermediates or is it stabilizing a catalytically active conformation of the protein; is it doing this in the oxidized or in the reduced form, or are both active; is is possible to replace FAD by modified cofactor derivatives or to influence catalysis by substrate and coenzyme analogues?

The following study was undertaken to elucidate the role(s) of the prosthetic group in the enzymatically catalyzed splitting of mandelonitrile and to answer some of the questions indicated.

2. Materials and methods

D-Hydroxynitrile lyase was prepared from defatted bitter almond press residues (Caesar and Loretz, Hilden, FRG) by a slight modification of the original procedure in [4], replacing ethanol by 60% acetone and using concanavalin A–Sepharose in an additional final step to remove most of the accompanying β-glucosidase. The final specific activity of the purified enzyme was 1.26–1.32 × 10^{-6} Kat/mg protein, measured spectrophotometrically at the carbonyl band at 250 nm ($\Delta\epsilon$ = 13 200 M^{-1} cm^{-1}, pH 5.4), using d,l-mandelonitrile (techn., Aldrich-Europe, Düsseldorf) as the substrate. The enzyme is homogeneous on disk- and SDS–gel electrophoresis. Chemicals were of analytical grade (E. Merck, Darmstadt). Deaza-FAD was prepared and purified as in [6]. 5-Deazariboflavin and lumiflavin-3-acetic acid were kindly donated by Professor P. Hemmerich and Dr S. Ghisla, who introduced us into the pertinent techniques.

Anaerobic titrations with dithionite were performed in the outfit described [7]. The thermostatted quartz

cuvette was equipped with a side bulb and a gas lock, closed by a tapered stopper. All solutions were made anaerobic by repeated cycles of evacuating and flushing with argon and finally kept under slight argon pressure. Dithionite solutions were prepared in a special anaerobized vial with a side arm and removal tube from which samples were transferred anaerobically to the cuvette by means of a gas-tight syringe. They were standardized spectrophotometrically using lumiflavin-3-acetic acid ($\Delta\epsilon_{443\ nm}$ = 10 800 M^{-1} cm^{-1}), following the procedure in [7]. The titration spectra were plotted with a Cary Model 14 recording spectrophotometer.

For photoreduction of the enzyme the anaerobic cuvette was placed in a water bath at 10°C and illuminated with a 250 W halogen projection lamp at 15 cm distance.

Activity assays of the enzyme solutions were performed in an anaerobic cuvette with 2 side arms. The assay buffer was 0.1 M sodium acetate, pH 5.4. For the assay of photoreduced hydroxynitrile lyase, enzyme solutions containing EDTA and excess reduced lumiflavin-3-acetic acid as fluorescence indicator and simultaneous oxygen trap were illuminated for 30 min at 25°C. After photoreduction, the end of which was controlled fluorometrically, substrate solution was tipped from one side arm to the enzyme and the $A_{250\,nm}$ increase was followed.

Activity assay of dithionite reduced enzyme was performed in a similar way as described for the titration experiment. Enzyme solutions containing excess dithionite were prepared in the anaerobized vial. Samples were transferred to the anaerobic cuvette containing the substrate, strictly avoiding admission of air.

The assay of boranate reduced material was performed as described for the photoreduction assay. The enzyme solution containing excess of reduced lumiflavin-3-acetic acid was made carefully anaerobic, then mixed with solid $NaBH_4$ from one side arm of the cuvette. The solution was allowed to stand for at least 1.5 h at 0°C to decompose the remaining borohydride completely. After a final evacuation cycle and warming up to room temperature, the reduction of lumiflavin-3-acetic acid was checked fluorometrically and the substrate solution tipped to the enzyme from the side arm.

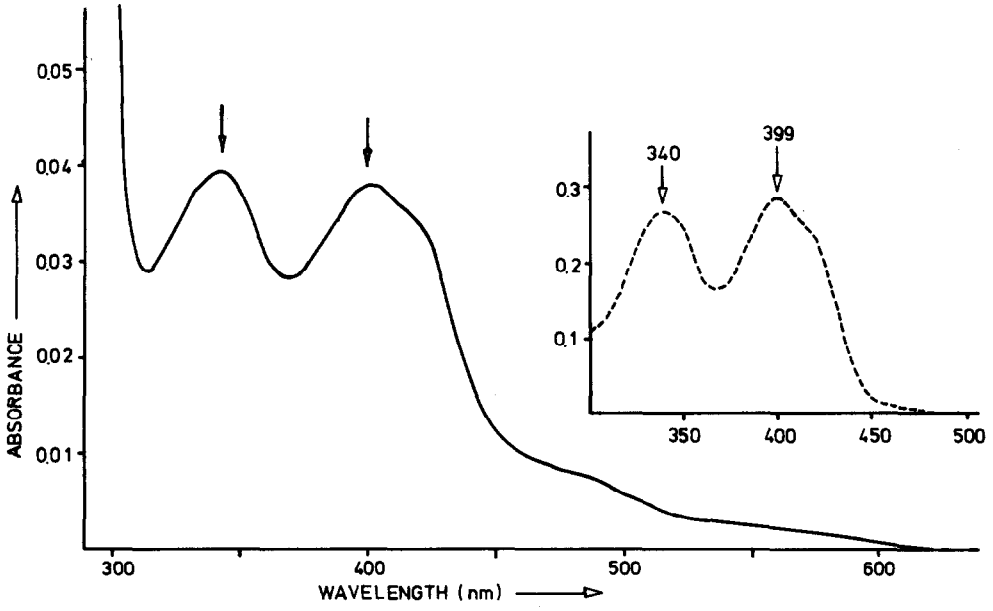

Fig.1. Spectrum of deaza-FAD-apo-hydroxynitrile lyase. 1 ml deaza-FAD (0.14 μmol) in 0.1 M sodium phosphate buffer, pH 7.5, were incubated with 1 ml apoenzyme (28.6 μmol) for 24 h at 4°C, then precipitated with ammonium sulfate, dissolved in the same buffer and desalted by Sephadex fractionation. Protein conc. 7.8 μM. The inset shows the visible spectrum of unbound deaza-FAD in 0.1 M sodium phosphate buffer, pH 7.5. Arrows mark the absorption maxima of both bound and unbound flavin.

3. Results

The enzyme is not bleached by the addition of either mandelonitrile or benzaldehyde and there is no O_2 uptake in the presence of the substrate. On addition of either deaza-FAD or reduced deaza-FAD to the apo-enzyme a deaza-holo-enzyme is formed as evidenced by Sephadex fractionation, but there is no shift in spectra or change in fluorescence (fig.1), and the deaza-enzyme is inactive in our assay system.

We then further investigated the role of the semiquinoid form of the enzyme in the reaction. The flavoprotein semiquinone was prepared according to [8] with the improvements in [9] using EDTA as electron donor and lumiflavin-3-acetic acid as electron transfer catalyst. Spectra were recorded at 5 min intervals. The oxidized enzyme was converted to the semiquinone form within 20 min. Prolonged exposure to light did not result in further reduction. As seen from fig.2, the spectrum after 20 min illumination resembles typically the spectrum of an anionic red semiquinone enzyme with maxima at 355 nm, 395 nm and 485 nm, as already shown [8]. After 3 h air was admitted, and the spectrum returned immediately to the spectrum of untreated enzyme. The inset of fig.2 shows that almost all of the catalytic activity is lost upon photoreduction of the enzyme but is completely restored after reoxidation.

The next series of experiments was designed to indicate whether the fully reduced leuko-enzyme is partner in the cyanohydrin splitting. To titrate the flavoprotein with buffered dithionite (cf. [7]) increasing aliquots of standardized dithionite were mixed with the enzyme solution in the anaerobic cuvette, the spectrum recorded and finally the dithionite solution re-checked with lumiflavin-3-acetic acid as before. The results of an experiment at pH 7.5 is given in fig.3. The spectrum of the enzyme at the end of the titration may represent a mixture of $FADH_2$-enzyme and its bisulfite addition complex. After passing air through the solution its spectrum was identical with that of native holo-enzyme.

On successive additions of dithionite to the flavoprotein, first the spectrum of the solution is changed to that of the semiquinone form, then to that of the leukoenzyme. Plotting the extinction at 460 nm against concentration of titrant from an experiment

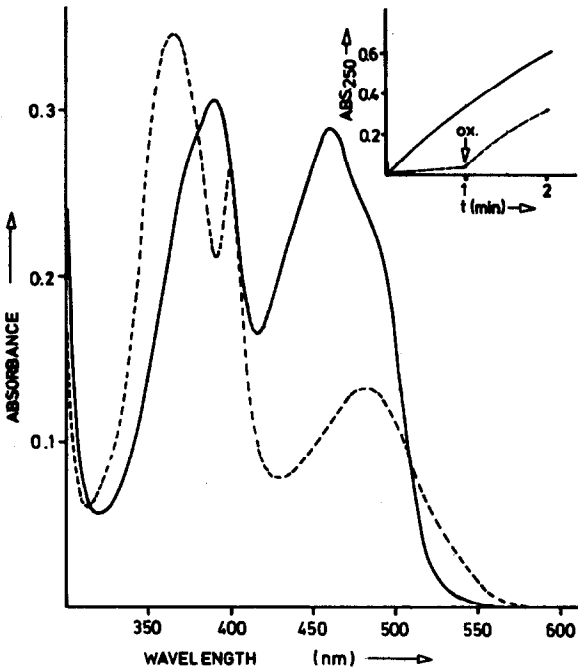

Fig.2. Photoreduction of hydroxynitrile lyase with 50 mM EDTA and 2.25 μM lumiflavin-3-acetic acid. Enzyme: 24 μM in 0.1 M sodium phosphate buffer, pH 7.5; temp. 25°C. The EDTA and lumiflavin-3-acetic acid were illuminated in the side arm of the anaerobic cuvette for 5 min before tipping to the enzyme. (——) Oxidized and reoxidized enzyme; (– –) after photoreduction (20 min). The inset shows the activities of oxidized (——) and semiquinone (– –) enzyme. The arrow indicates time of aeration. Conditions: 1.5 ml enzyme (43 pmol) containing 3 μmol EDTA and 3.4 nmol lumiflavin-3-acetic acid were illuminated and then mixed with 0.5 ml mandelonitrile solution (0.38 μmol).

conducted at pH 5.4 (instead of pH 7.5,), gives the diagram, fig.4, showing two breaks: a slightly perceptible one at 0.5 mol equivalent dithionite (not to be found at pH 7.5), and the definite end point at 1 mol dithionite/mol enzyme, thus confirming the 1 : 1 ratio of FAD to protein in an independent fashion.

The activity of the fully reduced enzyme was checked as described under section 2. As shown in the inset of fig.3 enzyme activity is completely inhibited on reduction with dithionite, but with air (arrow) full activity is restored.

To exclude the possibility that dithionite or the hydrogen sulfite formed thereof on oxidation

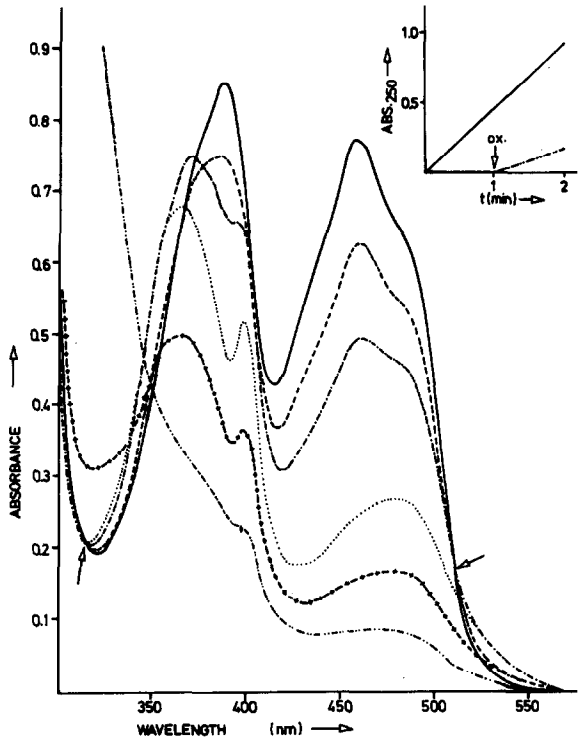

Fig.3. Reduction of hydroxynitrile lyase with sodium dithionite. Conditions: 69.3 μM hydroxynitrile lyase in 0.1 M sodium phosphate buffer, pH 7.5, was titrated with a solution of 2.63 mM sodium dithionite at 25°C. Arrows indicate isosbestic points at 315 nm and 508 nm. (——) oxidized and reoxidized enzyme; (– –) 0.19 mol dithionite/mol enzyme; (– · –) 0.38 mol dithionite/mol enzyme; (· · · ·) 0.76 mol dithionite/mol enzyme; (–+–) 1.32 mol dithionite/mol enzyme and (– ··–) 2.9 mol dithionite/mol enzyme. Curves are not corrected for dilution. The inset shows the activities of oxidized enzyme (——) and of the reduced form (–··–). The arrow indicates time of air admission. Conditions: 100 μl hydroxynitrile lyase–sodium dithionite solution (11 pmol enzyme and 225 nmol dithionite, respectively.) were added to 2 ml substrate solution (mandelonitrile 0.76 mM) in 0.1 M sodium acetate buffer, pH 5.4 and A_{250} recorded.

might interfere with the benzaldehyde handling reaction, the enzyme was reduced with sodium boranate [10] and its spectrum and activity recorded (fig.5).

A comparison with fig.3 demonstrates that, after full reduction, the spectrum resembles qualitatively that of the dithionite reduced enzyme. No activity

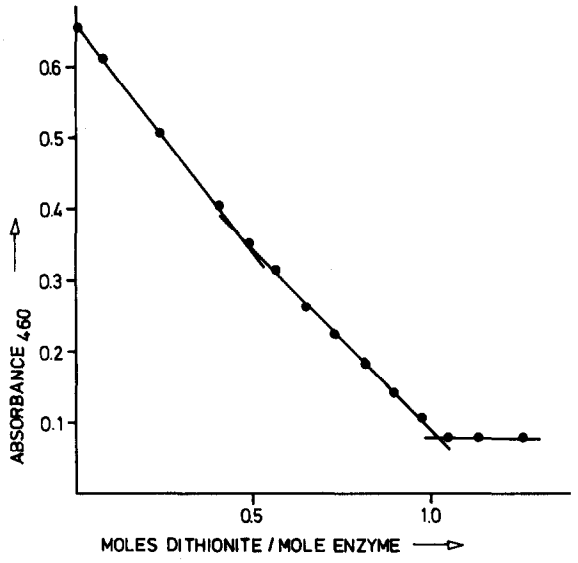

Fig.4. Titration of hydroxynitrile lyase with sodium dithionite at pH 5.4. A_{460} as a function of added dithionite. Conditions: 58.5 μM hydroxynitrile lyase in 0.1 M sodium acetate buffer, pH 5.4, was titrated with a solution of 2.63 mM sodium dithionite at 25°C. Absorbance values are corrected for dilution.

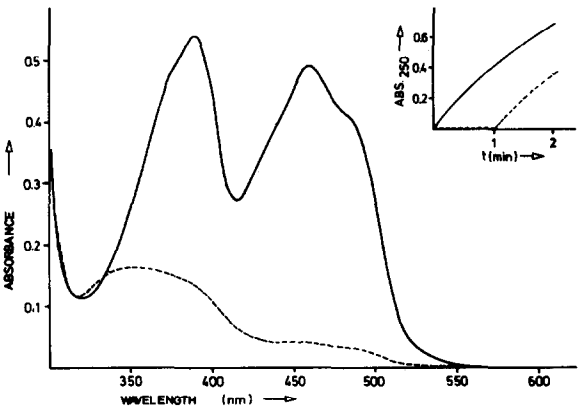

Fig.5. Reduction of hydroxynitrile lyase with sodium boranate. 88 nmol hydroxynitrile lyase in 2 ml 0.1 M sodium phosphate buffer, pH 7.5, were mixed with 26 μmol sodium boranate (solid) at 25°C. (——) oxidized and reoxidized enzyme; (– –) enzyme reduced with boranate. The inset shows activities of oxidized and boranate treated enzyme (——) and (– –) respectively). Conditions: 1.5 ml enzyme solution (43 pmol) containing 5.6 nmol lumiflavin-3-acetic $acid_{red}$ were reduced with 0.9 mg $NaBH_4$ and finally mixed with 0.5 ml substrate solution (0.38 μmol mandelonitrile). Arrow indicates time of air admission.

could be detected after reduction with boranate, but admission of air instantaneously reactivates the enzyme as shown in the inset of fig.5.

4. Discussion

The monomeric hydroxynitrile lyase from bitter almond emulsin contains 1 mol FAD/mol protein tightly bound. The apoenzyme is catalytically inactive; activity can be fully regenerated by addition of the prosthetic factor in its oxidized, not in its reduced form.

The experiments described here indicate that oxidized FAD is definitely involved in enzyme activity, since the enzyme loses its activity:

(i) By replacing FAD by deaza-FAD;
(ii) In the presence of reducing compounds such as dithionite or boranate;
(iii) After photoreduction to the semiquinone enzyme in the presence of EDTA and electron transferring flavin catalysts, such as lumiflavin-3-acetic acid, deaza-riboflavin or reduced deaza-lumiflavin.

The flavin is specifically required for catalysis from the following evidence:

(i) The apo-enzyme obtained by acid ammonium sulfate precipitation is completely inactive;
(ii) The apo-enzyme can be fully reactivated by FAD, but not by FMN or riboflavin in the absence or presence of adenine nucleotides;
(iii) Deaza-FAD, although bound to the enzyme in weak competition with FAD, as shown by binding and spectroscopic studies, does not reactivate the enzyme.

In regard to the function of FAD in the reaction proper, it can be stated that the flavin prosthetic group does not form a spectrophotometrically detectable complex with substrates or undergoes an oxidation/reduction cycle during catalysis, since there is:

(i) No spectral change upon adding either mandelonitrile, benzaldehyde, or HCN to the enzyme;
(ii) No bleaching of the flavin nor any wavelength-shift in flavin fluorescence on addition of substrates. It causes, however, a 50% decrease in flavin fluorescence quench, possibly indicating a slightly better accessibility of the FAD to light excitation, resulting from a conformational change of cofactor binding area.

These observations and experiments strongly suggest a direct participation and not only a by-standing role of flavin cofactor in the catalytic process.

Nitrogen-5 of the isoalloxazin ring-system with its free electron pair may have a function in activating the substrate, resulting in an intermediate flavin-substrate transition state complex although it does not show up on spectrophotometric studies. Such complexes have been proposed [11] in different flavin catalyzed processes. A displacement of nitrogen at position 5 would result in an impeded carbonyl activation or even none at all. However, flavin-N-5 is definitely not the only means of initiating the splitting of hydroxynitriles: *Sorghum vulgare* hydroxynitrile lyase [12] can do without a flavin cofactor. In this case the activating role may be taken over by another base such as a metal cation or an amino acid side group.

Also a more direct involvement of FAD in enzyme action might be visualized: Since the reduced isoalloxazin ring-system has a partly twisted shape, differing from that of its planar oxidized state, a slight perturbation of the substrate binding domain could result, above all, when the cofactor is found close to the active centre. Upon re-oxidation this conformational effect would be abolished, and enzyme activity restored. The prosthetic group is definitely important in maintaining the stable conformation of the enzyme as the great thermal instability of the apo-enzyme shows.

In different respects hydroxynitrile lyase may be set in parallel to glyoxylate carboligase, a TPP-enzyme [13], which also contains FAD [14,15] in an enigmatic function. No evidence was found [16] for a direct participation of the cofactor either; however, the enzyme is inactivated by dithionite and reactivated by oxygen, which might implicate that the activity of this enzyme is controlled in the cell by the oxidation state of its FAD. On binding studies with deaza-FAD and deaza-$FADH_2$ to the apo-enzyme of glyoxylate carboligase, reactivating interaction of the protein with the artificial co-enzyme was found [17]. From this they conclude that flavin oxidation/reduction changes are not involved in the turnover of glyoxylate carboligase.

The strict necessity of an aerobic environment for the catalytic process may indicate a possible regulatory

function of the oxidation state of FAD. In a plant seedling the enzyme would be inactive unless one of the following events takes place:

(i) Germination of the seed;

(ii) Wounding of the germling.

This would establish aerobic conditions and activate the enzyme which then produces the toxic substances HCN and benzaldehyde. This could result in a protection against soil bacteria and other enemies of the growing or hurt seedling, and the large amounts of hydroxynitrile lyase in the seedlings of bitter almonds and other Rosaceae may find a plausible explanation.

Acknowledgements

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