MICROSPECTROPHOTOMETRIC MEASUREMENTS ON SINGLE CRYSTALS OF COENZYME CONTAINING COMPLEXES OF HORSE LIVER ALCOHOL DEHYDROGENASE

Enrico BIGNETTI, Gian Luigi ROSSI and Eila ZEPPEZAUER*

Institute of Molecular Biology, University of Parma, Cornocchio, 43100 Parma, Italy and **Swedish University of Agricultural Sciences, Department of Chemistry, S75007 Uppsala 7, Sweden*

Received 25 January 1979

1. Introduction

In protein crystallography, difference electron density calculations is a widely used method to study ligand binding. Interpretation of difference peaks from data to limited resolution gives the position of the ligand on the protein molecule and the rough shape of the bound substance. However, the detailed chemical nature of the bound molecule often cannot be identified from the difference peaks themselves even at high resolution. For example, structure determinations by difference electron density techniques of dehydrogenase complexes with coenzyme will never solve the question in which oxidation state the bound cofactor is present within the crystal.

The use of electron dense labels on ligands, like Br, Cl, and I substituents, frequently has been used in ligand binding studies in protein crystallography. 8-Br-ADPribose was used in the work with liver alcohol dehydrogenase (LADH) for the identification of the adenine binding pocket [1]. 4-Br-benzaldehyde was introduced as a substrate [2] and the coenzyme analogue 3-I-pyridine adenine dinucleotide was used to demonstrate the binding of an oxidized coenzyme species to the enzyme [3]. Although useful in the X-ray analysis, heavier substituents on ligands might distort the binding mode to the protein. Nonproductive binding can also occur.

Obviously, complementary methods of analysis of single crystals are necessary when one intends to examine and compare protein structures with naturally occurring cofactors or substrates present in different oxidation state. In the work with the flavodoxin semiquinone (radical) structure [4], visible spectra of single crystals were recorded [5] which showed that the semiquinone form predominated in the crystals used for X-ray data collection.

Structure determinations of LADH complexes have accumulated information about coenzyme analogue binding, inhibitor interactions and conformational changes induced by NADH and substrate [6]. Our goal is to describe in structural terms as many as possible of the individual steps in the process going from an aldehyde substrate to an alcohol product. In the search for crystalline NAD^+-containing complexes the need for additional analytical methods arose as crystallization experiments on all types of LADH complexes have been performed in an alcoholic medium, 4-methyl-2,4-pentanediol (MPD). It seemed especially important to check for NADH formation within the single crystals since MPD could act as a substrate during crystallization. Furthermore, in our study of the transient complex between LADH–1,4,5,6-tetra-hydronicotinamide adenine dinucleotide (H_2NADH) and the chromophoric substrate *trans*-4-*N*,*N*-dimethylaminocinnamaldehyde (DACA) [7] we needed reliable tools to measure aldehyde binding within the single crystal.

We report here the use of single crystal microspectrophotometric measurements as a convenient and rapid method to:

(1) Detect NADH present in the lattice;
(2) Follow DACA binding in the active site and
(3) Test substrate conversion in crystals of complexes used for X-ray data collections.

2. Materials and methods

The experimental procedure has been described [8]. The equipment is a Leitz UVMP microscope-photometer, slightly modified to better suit our needs. Single crystals were located within a flow cell on the microscope stage. The upper (mobile) and lower quartz walls of the cell are separated by polyethylene sheets that create a chamber where the crystal can be held in the presence of 25 μl suspending medium. Entrance and exit channels allow replacement of this medium with solvents containing substrates or other reagents within 10–20 s. All the information we wanted from these studies was obtained from spectra using unpolarized light.

Liver alcohol dehydrogenase (the ethanol-active EE-isozyme) was obtained from Boehringer, Mannheim and recrystallized or the enzyme was prepared from fresh horse liver by Dr Åke Åkeson, Karolinska Institute, Stockholm. All chemicals used were of highest available purity. The precipitating agent MPD was purchased from Eastman, lot no. B6B, and double-distilled under purified nitrogen. The coenzyme analogue H_2NADH was synthetized and kindly supplied to us by Leif Wallen, Swedish Univ. of Agricultural Sciences, Uppsala. Dimethylamino-cinnamaldehyde was sublimated prior to use. Enzyme complexes were formed in solution and crystallized at 4°C. Microspectrophotometric measurements were performed at 23°C, which is the optimal temperature for the activity test of LADH in solution.

Crystals were separated from the mother liquor and washed in appropriate buffer plus 30% (v/v) MPD prior to preparation on the microscope stage. No measurable amounts of NADH diffuse out of the crystal during this treatment. Crystals used for

Table 1
Crystalline horse liver alcohol dehydrogenase complexes with coenzyme and substrate used to record spectra and to follow NADH consumption

Complex crystallized from solution	Amount of bound NADH[a]	Activity[b] (conversion of aldehydes)	Crystallization conditions
1. Enzyme–NADH–dimethylsulfoxide[c]	100% saturated	+	[10]
2. Enzyme–NAD^+–4-bromobenzylalcohol	100% saturated	+	[2]
3. Enzyme–NAD^+–trifluoroethanol	100% saturated	+	[2]
4. Enzyme–NAD^+[d]	30% saturated	+[d]	[2]
5. Enzyme–NADH	100% saturated	+	25 mg protein/ml, 1 mM NADH, 10 mM Tes/NH_3[e] (pH 7.5). Slow precipitation with MPD to 30% (v/v) final conc.
6. Enzyme–H_2NADH–dimethylaminocinnamaldehyde	–		10 mg protein/ml, 0.8 mM H_2NADH, 0.4 mM substrate, 10 mM Tris/HCl[e] (pH 9.5). MPD 25% (v/v) final conc.

[a] The estimation is based on the ratio A_{280}/A_{325} using the molar absorption coefficients:

$$\epsilon^{\text{E–NADH}}_{280\text{ nm}} = 2.3 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1};\ \epsilon^{\text{E–NADH}}_{325\text{ nm}} = 5.7 \times 10^3\ \text{M}^{-1}\ \text{cm}^{-1}$$

[b] Activity in single crystals is measured following the disappearance of the absorption band characteristic of the enzyme-bound reduced coenzyme in the presence of aldehyde substrates. Stoichiometric conversion of NADH into NAD^+ was obtained using the pyrazole 'suicide technique' [11]. Medium pyrazole was 2×10^{-2} M

[c] An inhibitory substrate analogue. This complex is used for the high resolution X-ray investigation of the triclinic structure of LADH

[d] To test the presence of NAD^+, in these crystals, ethanol was added as substrate. If, in the crystal, a mixture of complexes with oxidized and reduced coenzyme was present, NADH would be expected to form upon addition of ethanol. However no formation of NADH was detected

[e] *Abbreviations:* Tes, Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid; Tris, Tris(hydroxymethyl)-aminomethane

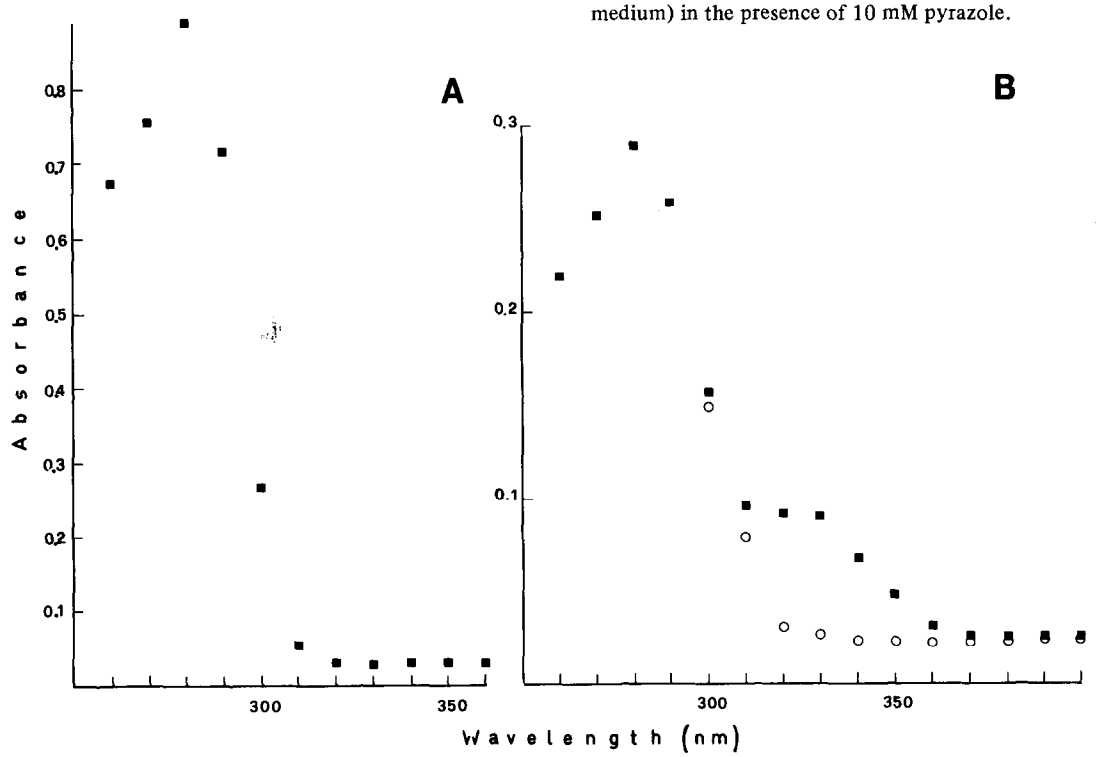
absorption measurements and activity tests were selected either from the same crystallization vial as those crystals used for diffraction experiments or were taken from a parallel experiment. Crystal thickness ranged from 0.08–0.25 mm.

3. Results and discussion

Table 1 summarizes the different types of complexes investigated in this work. Substrate conversion was followed in all types of NAD^+/NADH containing crystals. Freshly prepared as well as 1 year samples were found to be enzymatically active.

Figure 1 compares the absorption spectra of single crystals of apoenzyme [9] (A) and holoenzyme (B). Holoenzyme crystals were grown in the presence of 1 mM NADH (table 1, complex 5). The value of the ratio A_{280}/A_{325} was found to be 4 ∓ 0.4, agreeing with the value expected for the stoichiometry of two NADH molecules bound per LADH dimer. Crystals grown in the presence of 5% (v/v) dimethylsulfoxide (table 1, complex 1) [10] exhibited an identical spectrum which indicates that the mode of binding of coenzyme within the crystal is unaffected by the presence of inhibitor. A demonstration that the crystalline LADH–NADH complex represents a catalytically active species is provided when one of several aldehyde substrates, e.g., acetaldehyde, benzaldehyde, 2-hydroxy-3-nitrobenzaldehyde and DACA, is allowed to react causing the typical LADH–NADH complex absorption band to diminish (fig.2) and eventually disappear. When the pyrazole 'suicide technique' is used [11], i.e., a mixture of aldehyde

Fig.1. Single crystal spectra of LADH. Absorption spectra were obtained as $\log I_0/I$, where I_0 and I are measurements of monochromatic light transmitted by the suspending medium and the crystal, respectively. (A) Spectrum of an apo-LADH crystal in 50 mM Tris/HCl (pH 8.4), 30% MPD (v/v). (B) Spectrum of LADH–NADH crystal (■) suspended in 10 mM Tes/NH_3 buffer (pH 7), 30% MPD (v/v). Spectrum of LADH–NAD^+–pyrazole complex (○) was obtained upon stoichiometric reaction of crystalline LADH–NADH molecules with benzaldehyde (10 mM in the suspending medium) in the presence of 10 mM pyrazole.



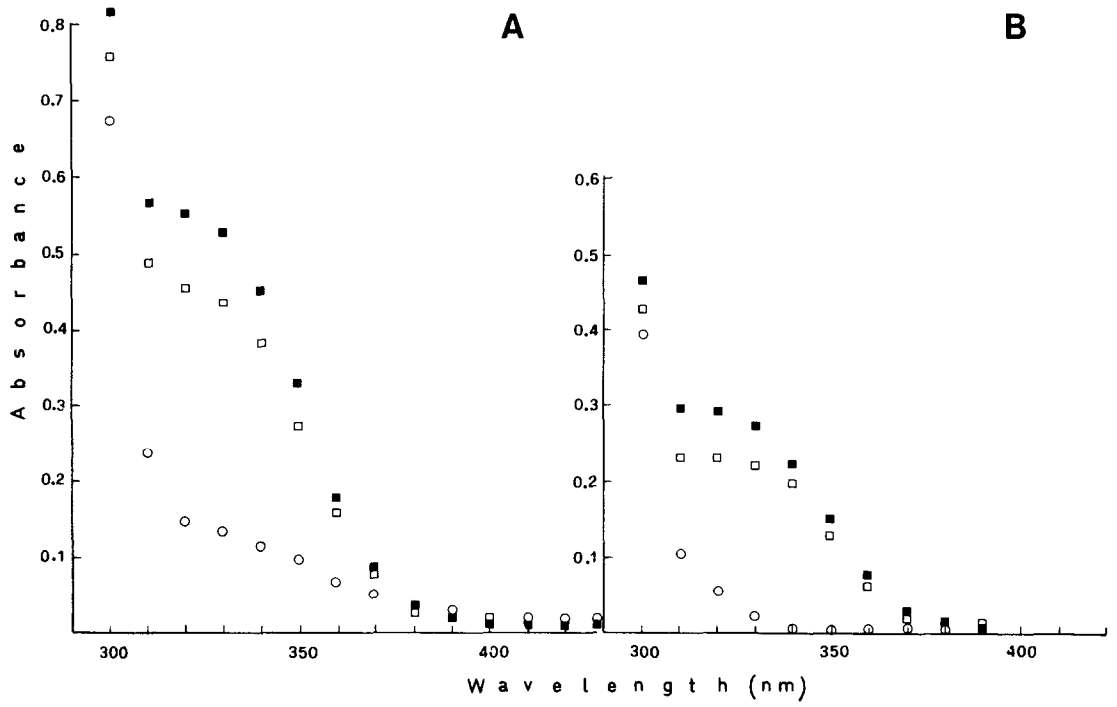


Fig.2. (A) Single crystal spectra of LADH–NADH (■), LADH–NADH/LADH–NAD^+ mixture (○) obtained upon reaction with benzaldehyde, 40 mM in the suspending medium. The third spectrum (□) represents the complex after washing the crystal and suspending it in a solution containing ethanol as substrate (200 mM). The original spectrum of LADH–NADH is essentially recovered within a few minutes. (B) Same as in A with the exception that the spectrum (□) refers to a crystal that has not reacted with ethanol after aldehyde conversion, but has been carefully washed with buffer plus 30% (v/v) MPD and allowed to stand in the cold for a night.

and pyrazole is added to an LADH–NADH crystal, the final spectrum of the crystal appears as in fig.1B (○). This result shows that all the enzyme molecules within the crystal can participate in the oxido–reductive reaction. The ternary complex LADH–NAD^+–pyrazole is iso-morphous with the LADH–NADH–dimethyl-sulfoxide complex and both complexes have been investigated crystallographically. Aldehyde addition to LADH–NADH crystals in the absence of pyrazole, decreased the A_{325} band by variable amounts, depending on the nature and the concentration of the particular aldehyde substrate. If excess ethanol was added to the crystal suspending medium after the reduction of an aldehyde and the spectrum was recorded again (fig.2A) the original intensity of the LADH–NADH absorption band was fully recovered (80–100%). This result indicates that it is possible to generate an equilibrium mixture of enzyme complexes containing oxidized and reduced coenzyme. Furthermore, the result indicates that NAD^+ formed at the active site binds in a catalytically active mode in these crystals.

A finding that might provide an explanation for the results discussed below is presented in fig.2B. If LADH–NADH crystals that have reacted with benzaldehyde to reach equilibrium are washed with buffer and MPD to remove excess aldehyde and the alcohol product and are allowed to stand in the cold for $\geqslant 12$ h, they exhibit the original spectrum characteristic of an LADH–NADH complex. This coenzyme reduction is likely due to methylpentanediol, known to be a 'poor' substrate for LADH and present in the final crystal suspending medium at a concentration of the order of its K_m [2].

The presence of MPD might also be the primary cause of the difficulty to obtain stable NAD^+-con-

taining complexes (table 1, complexes 2–4). An NAD^+-containing complex was sought [2] using 4-bromobenzaldehyde to react with LADH in solution. The complex was crystallized when no traces of NADH were detected in the starting solution. We checked for the spectral properties of crystals from the same batch used for the X-ray analysis and found that reduced coenzyme was present, probably due to the reaction of MPD during crystallization.

Conversely, we showed that a complex, crystallized as LADH–NADH–pyrazole, was in fact spectroscopically identical to LADH–NAD^+–pyrazole, probably because of the presence of aldehyde contaminants in the crystallization medium.

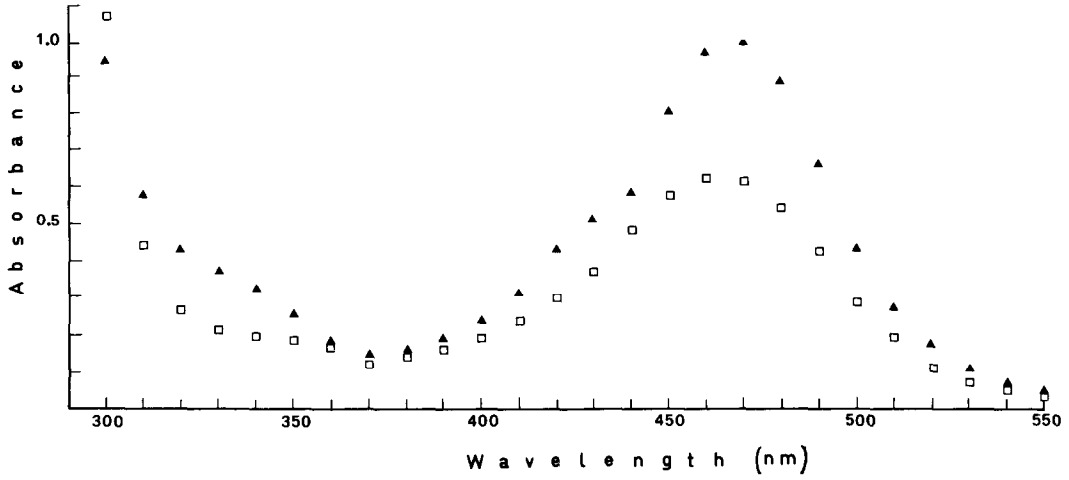
In the above microspectrophotometric measurements, the coenzyme spectrum was the suitable probe for the identification of complexes within the crystal. The chromophoric properties of the aromatic aldehyde DACA provided the means for identifying the transient ternary complex in the crystalline state between enzyme and substrate using the coenzyme analogue H_2NADH (table 1, complex 6). This substrate has been extensively studied in solution [7,12] in the combination with NADH as well as H_2NADH. The formation of the intermediate occurs prior to the oxidation of NADH and is characterized by the development of an A_{max} band at 464 nm. The intermediate formed using H_2NADH is indefinitely stable and the crystals exhibit the same spectrum as the intermediate in solution. Figure 3 compares the spectra of single crystals of the complexes LADH–NADH and LADH–H_2NADH–DACA. The latter complex has been used for the high resolution X-ray analysis of an aldehyde substrate bound to alcohol dehydrogenase (Samama, E. Z., in preparation).

These findings confirm the need for a direct method to recognize the nature of enzyme complexes actually present within a crystal before a crystallographic study should be undertaken. Furthermore microspectrophotometric measurements can be used to explore the conditions from which one can obtain a desired redox state of an enzyme complex in the crystalline state.

Acknowledgements

We are grateful to Professor C.-I. Brändén for his interest in this research and helpful discussions. This work has been supported by the Consiglio Nazionale delle Ricerche and the Ministero per la Pubblica Istruzione, Italy; by the Swedish National Research Council (grant no. 2767) and by an EMBO fellowship to E.Z.

Fig.3. Single crystal spectra of LADH–H_2NADH–DACA complex (□) in 10 mM Tris/HCl buffer (pH 9.5), 30% MPD (v/v) and of LADH–NADH–DACA complex (▲) obtained upon diffusion of substrate (0.4 mM) into crystals of binary enzyme–coenzyme complex suspended in 10 mM bicine buffer (pH 9.5), 30% MPD (v/v).



References

[1] Abdallah, M. A., Biellman, J.-F., Nordström, B. and Brändén, C.-I. (1975) Eur. J. Biochem. 50, 475–481.
[2] Plapp, B. V., Eklund, H. and Brändén, C.-I. (1978) J. Mol. Biol. 122, 23–32.
[3] Samama, J.-P., Zeppezauer, E., Biellman, J.-F. and Brändén, C.-I. (1977) Eur. J. Biochem. 81, 403–409.
[4] Smith, W. W., Burnett, R. M., Darling, G. D. and Ludwig, M. L. (1977) J. Mol. Biol. 177, 195–225.
[5] Eaton, W. W., Hofrichter, J., Makinen, M. W., Anderson, R. D. and Ludwig, M. L. (1975) Biochemistry 14, 2146–2151.
[6] Brändén, C.-I. and Eklund, H. (1978) in: Molecular Interactions and Activity in Proteins, Ciba Foun. Symp. 60 (new ser.) pp. 63–80, Excerpta Medica, Amsterdam.
[7] Dunn, M., Biellman, J.-F. and Branlant, M. (1975) Biochemistry 14, 3176–3182.
[8] Berni, R., Mozzarelli, A., Pellacani, L. and Rossi, G. L. (1977) J. Mol. Biol. 110, 405–415.
[9] Brändén, C.-I., Zeppezauer, E., Söderberg, B.-O., Boiwe, T., Nordström, B., Söderlund, G., Zeppezauer, M., Werner, P.-E. and Akeson, A. (1972) in: Structure and Function of Oxidation Reduction Enzymes (Akeson, A. and Ehrenberg, A. eds) pp. 93–107, Pergamon, Oxford.
[10] Eklund, H. and Brändén, C.-I. (1979) J. Biol. Chem. in press.
[11] McFarland, J. T. and Bernhard, S. A. (1972) Biochemistry 11, 1486–1493.
[12] Dunn, M. F. and Hutchison, J. S. (1973) Biochemistry 12, 4882–4892.