DIFFERENCES IN THE BINDING OF COENZYME TO L-3-HYDROXYACYL-COENZYME A DEHYDROGENASE IN THE CRYSTALLINE STATE AND IN SOLUTION

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Received 14 July 1981

1. Introduction

L-3-Hydroxyacyl coenzyme A dehydrogenase (L-3-hydroxyacyl-CoA:NAD oxidoreductase; EC 1.1.1.35) is a mitochondrial enzyme involved in the beta oxidation of fatty acids. The enzyme, as isolated from pig heart muscle, has M_r 67 000 and contains two identical subunits [1]. The amino acid sequence has recently been completed and appears to have little homology with other dehydrogenases [2]. Like many other dehydrogenases, L-3-hydroxyacyl coenzyme A dehydrogenase (β-HADH) utilizes NAD–NADH as cofactors. However, unlike many other dehydrogenases, the structure of its substrate, L-3-hydroxyacyl coenzyme A, resembles the cofactor, NAD. Structural studies of the enzyme should prove to be of interest in that both the substrate and coenzyme binding sites might be expected to contain binding domains for adenine nucleotide moieties. In addition, the active site of β-HADH must accommodate the varying acyl chain lengths of fatty acids that are metabolized through the beta oxidation cycle. In order to determine the molecular structure of β-HADH, an X-ray crystallographic study is being carried out. From an electron density map at low resolution, the tentative location of the two dimers contained in the asymmetric unit has been determined [3]. In addition, crystallographic studies aimed at locating the binding site of NAD have also been undertaken. Difference Fourier maps between the holo- and apo-forms of the crystalline enzyme yield only 1 major NAD binding site/dimer. This apparent asymmetry in NAD binding to crystalline β-HADH prompted a study of the binding characteristics of coenzyme to β-HADH in solution. This communication reports the comparison of the X-ray and solution studies.

2. Materials and methods

Pig heart L-3-hydroxyacyl-CoA dehydrogenase was prepared as in [4] and crystals suitable for X-ray diffraction studies were grown in the presence of polyethylene glycol-6000 [5]. For solution studies, the crystals were centrifuged, the solvent decanted, and the crystalline precipitate dissolved in 50 mM phosphate buffer (pH 7.0). The enzyme was then passed through a 0.7 × 20 cm Sephadex G-25 column to insure complete removal of the crystallizing medium. The enzyme concentration was determined using a $E^{1\%}_{280} = 4.39$ [6] and subunit M_r 33 500 [2].

NADH (grade III) was obtained from Sigma Chemical Co. (St Louis MO) and was used without further purification. All other chemicals were reagent grade.

Binding measurements were determined by examining changes in the intrinsic protein fluorescence upon binding of NADH. Fluorescence measurements were made on a Spex Fluorolog spectrofluorimeter using an excitation wavelength of 290 nm (bandpass of 3 nm) and observing the emission at 350 nm (bandpass of 40 nm). All measurements were performed at 20°C in a 50 mM potassium phosphate buffer (pH 7.0) which was 1 mM with respect to both EDTA and 2-mercaptoethanol. The enzyme was titrated by the addition of 4 μl quantities of titrant to a constantly stirred 3.0 ml contained in a 1.0 × 1.0 cm quartz cuvette. Fluorescence corrections were made by an identical titration of a buffer blank. These values were subtracted from the sample fluorescence.

The concentration of free NADH was calculated from the following relationship:

$$[\mathrm{NADH}]_{\mathrm{free}} = [\mathrm{NADH}]_{\mathrm{total}} - (\Delta F/\Delta F_{\mathrm{max}})\,[\mathrm{E}]$$

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50 © 1981 Federation of European Biochemical Societies

The variable ΔF in the above equation is the incremental change in protein fluorescence occurring upon each addition of NADH while ΔF_{max} is the total fluorescence increment at NADH saturation of binding sites. The value of ΔF_{max} was determined by linear extrapolation of the double reciprocal plot of $1/[NADH]_{total}$ *vs* $1/\Delta F$.

The details of the crystallographic studies of β-HADH will be published elsewhere [13]. The crystalline form of β-HADH contains two dimers of the enzyme in the asymmetric unit of an orthorhombic cell belonging to the space group $C222_1$. The X-ray studies used a form of the holo-enzyme to prepare 3 heavy atom derivatives which included CH_3HgCl, K_2PtCl_6 and $IrCl_3$. The local symmetry between β-HADH dimers was determined by rotation function analysis and examination of the electron density maps.

3. Results

The binding of NAD to the crystalline form of the enzyme is summarized by the data in fig.1 in which a small portion of the difference electron density map between the holo- and the apo-enzyme is shown. This difference electron density map was calculated and then skewed in sections perpendicular to the presumed local 2-fold rotation axis relating the two dimers. As can be seen in fig.1, only one major peak of electron density is contained in each of the volumes known to be equivalent to a dimer of β-HADH. A third peak of reduced integrated electron density was found in the map and, although it may represent low level substitution at an additional site, in general the peak did not appear to be of sufficient magnitude to accommodate an NAD molecule. The skewing direction of the difference electron density map, shown in fig.1, is based on independent X-ray results such as heavy atom pairing and rotation function studies [3]. Hence the local dyad symmetry visible between the NAD molecules supports the 2-fold symmetry dimer–dimer relationship in the crystalline form of the enzyme.

Although no attempt has yet been made to fit a molecular model of NAD to the major peaks in the difference electron density map, the shape of these peaks is elongated with an estimated distance from one end to another of 12–13 Å. The elongated shape of the electron density is suggestive of the bound

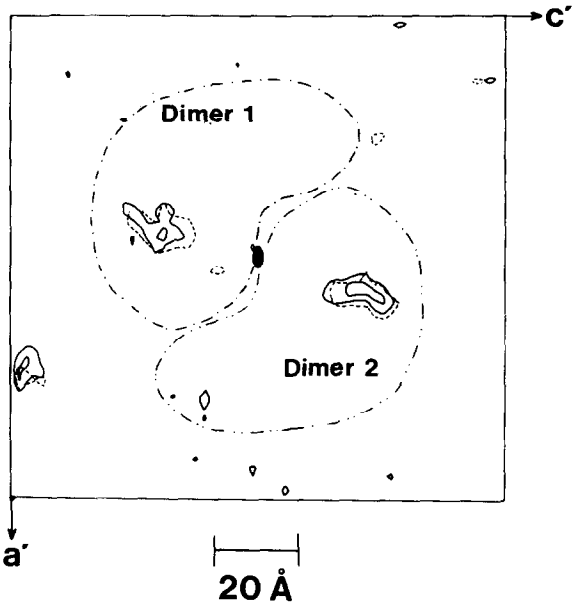

Fig.1. Difference electron density map between holo- and apo-β-HADH. Two sections of the difference electron density map are shown. The coordinate system used in the figure is skewed and translated relative to the crystallographic axes. *a'*, *b'* and *c'* are at roughly 45° with respect to the normal crystallographic axes and the sectioning of the map is perpendicular to *b'* [3,5]. The center of the difference electron density map is indicated by the elliptical symbol and has crystallographic coordinates of 0.359 *a*, 0.643 *b* and 0.193 *c*. This symbols marks the position of the local 2-fold rotation axis relating dimer 1 to dimer 2. Dotted lines are used to denote contour lines on the section farthest from the viewer. The approximate locations of the two dimers are given by the stipled boundaries labeled dimer 1 and dimer 2.

NAD being in an open configuration typically found in other dehydrogenases [7]. In this conformation, the C6 atom of the adenine moiety is separated from the C2 atom of the nicotinamide ring by ~14 Å. Although the amount of NAD bound to β-HADH in

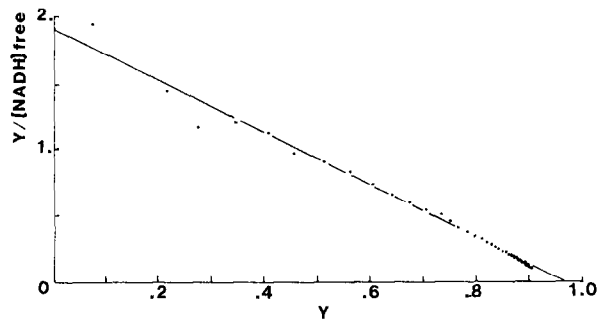

Fig.2. Scatchard plot of the fluorescence titration of β-HADH with NADH. β-HADH sites were 3 μM. *Y* is the fraction of NADH bound.

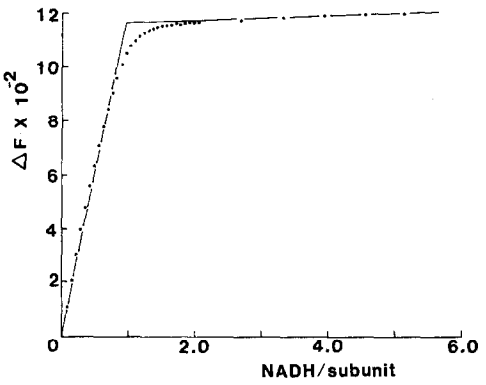

Fig.3. Fluorescence titration of β-HADH with NADH. β-HADH sites were 25 μM. Excitation was at 290 nm and emission measured at 350 nm. Because enzyme site concentration is initially much greater than NADH concentration and NADH binding is tight, no corrections for unbound NADH have been applied to these data.

the crystalline state cannot be precisely determined, only one principal NAD site/dimer appears to be occupied. However, from these results, the possibility of low NAD occupancy at other sites cannot be eliminated. In addition to asymmetry in the coenzyme binding in the crystalline state, the CH_3HgCl derivative of β-HADH contains only one major mercury site/dimer. Since it is known that there is one cysteine/monomer [1,2] two equivalent mercury sites/dimer were anticipated.

In contrast to the crystallographic studies, the fluorescence titration of 3 μM β-HADH with NADH was consistent with simple hyperbolic binding and two equivalent sites/dimer with a binding constant of 0.5 μM. Fig.2 is a Scatchard analysis of the data. The plot is linear throughout the entire range of binding with an extrapolated binding constant of 0.52 μM and a site occupancy of 0.97/subunit (1.94/dimer). A Hill plot of this data is linear with a slope of 1.03 indicating equivalent non-interacting sites. Further support for a stoichiometry of two coenzyme binding sites/dimer in solution is presented in fig.3. At high enzyme concentrations (25 μM sites) the initial slope of the enzyme titration curve extrapolates to 1.92 sites/enzyme dimer.

4. Discussion

While the X-ray data can be interpreted in terms of one tight binding coenzyme site/dimer, the fluorescence data shows that in solution, the enzyme contains two equivalent binding sites for NADH/dimer. Under these conditions, the enzyme exists exclusively as a dimer [1,6]. Therefore the solution studies cannot be caused by dissociation of the enzyme into subunits. The dissociation constant of NADH (0.52 μM) determined in these experiments is similar to, although somewhat lower than, the published values for other dehydrogenases [8].

Asymmetry in crystalline proteins upon binding of coenzymes or other ligands has been reported for other enzymes [9,10]. Generally, the unusual stoichiometry is related to crystalline packing. In this case each of the 4 subunits of β-HADH which are contained in the asymmetric unit is in a different crystalline environment. The steric effects of neighboring molecules presumably prevent equivalent binding at 1/2 of the potential sites.

The crystalline form of β-HADH is of special interest since the two dimers appear to pack in the form of a pseudo tetramer with point group symmetry 222 [3]. Hence the crystalline form of β-HADH may resemble such tetrameric dehydrogenases as glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. However the half-site occupancy observed for NAD and the reactivity of mercurials toward only one cysteine/dimer in the crystalline state of β-HADH is a result of crystal packing arrangements and is not observable when the molecule is in the dimeric state as it exists in solution.

Acknowledgements

The authors wish to acknowledge the excellent technical assistance of Thom Meininger who purified the enzyme and prepared the crystals discussed here. This work was supported by grants from the National Institute of Health (GM07067, AM13332) and the National Science Foundation (PCM921864).

References

[1] Noyes, B. E. and Bradshaw, R. A. (1973) J. Biol. Chem. 248, 3060–3066.
[2] Bitar, K. G., Perez-Aranda, A. and Bradshaw, R. A. (1980) FEBS Lett. 116, 196–198.
[3] Holden, H. M. and Banaszak, L. J. (1981) in preparation.

[4] Glatthaar, B. E., Barbarash, G. R., Noyes, B. E., Banaszak, L. J. and Bradshaw, R. A. (1974) Anal. Biochem. 57, 432–451.
[5] Weininger, M. S. and Banaszak, L. J. (1978) J. Mol. Biol. 119, 443–449.
[6] Noyes, B. E. and Bradshaw, R. A. (1973) J. Biol. Chem. 248, 3052–3059.
[7] Webb, L. E., Hill, E. J. and Banaszak, L. J. (1973) Biochemistry 12, 5101–5109.
[8] Dalziel, K. (1975) The Enzymes (Boyer, P. D. ed) vol. 11, p. 40, Academic Press, New York.
[9] Banaszak, L. J., Birktoft, J. J. and Barry, C. D. (1981) in: Protein–Protein Interactions (Frieden, C. and Nichol, L. W. eds) pp. 31–128, Wiley, New York.
[10] Matthews, B. W. and Bernhard, S. A. (1973) Annu. Rev. Biophys. Bioeng. 2, 257–312.