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A circular dichroism study of mitochondrial transhydrogenase from beef heart

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This paper describes a circular dichroism (CD) spectroscopy study of purified proton-pumping nicotinamide nucleotide transhydrogenase from beef heart. The CD spectrum obtained was used to estimate the content of secondary structures of the purified enzyme and suggests the presence of 40–45% α-helical structure and long, possibly membrane-spanning α-helices. The spectrum was essentially unaffected by the absence or presence of transhydrogenase substrates, suggesting that the catalytic and proton-translocating activities of the enzyme occur without major rearrangements at the level of secondary structures.

1. Introduction

The mitochondrial nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) from beef heart catalyzes a hydride ion transfer between NAD(H) and NADP(H) with a concomitant translocation of protons across the mitochondrial inner membrane. In mitochondria, the transhydrogenase catalyzes a forward reaction (reduction of $NADP^+$ by NADH) and a reverse reaction (reduction of NAD^+ by NADPH) linked to an inward and outward translocation of protons, respectively (for reviews, see refs 1–3). The molecular mechanism by which protons are translocated across the membrane is still unknown.

The amino acid sequence of the mitochondrial transhydrogenase has recently been deduced from the corresponding cDNA, revealing a chain length of 1043 amino acids and a molecular weight of 109212 [4]. About 14 possible membrane-spanning helices, deduced from hydropathy plots, were found within a central region (residues 420–850) and C- and N-terminal regions containing the NAD(P)(H)-binding sites which probably are located on the matrix side of the mitochondrial inner membrane [4].

Recent data imply that the two separate nicotinamide nucleotide binding sites constitute part of a less selective nicotinamide nucleotide pocket in the molecule [5]. The minimal functional assembly of active detergent-dispersed and of membrane-bound beef heart transhydrogenase has been found to be a dimer where both subunits are involved in the catalytic activity, possibly according to a half-of-the-sites reactivity mechanism [6]. Varying rates and dissociation constants [1,3,7], thermostability and proteolytic sensitivities [2] of the transhydrogenase, induced by an electrochemical proton gradient and/or NADP(H), may suggest that major conformational changes are induced under these conditions. The recently observed highly NADPH-sensitive phenylarsine

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oxide reactive vicinal dithiol may be of importance in these conformational changes [8]. Indeed, NADP(H) strongly regulates the pK_a of Cys 893 which may constitute part of this dithiol [9].

The present investigation is an attempt to demonstrate conformational changes directly using CD spectroscopy, by estimating the content of secondary structures under different conditions.

2. Materials and methods

2.1. Preparation and assay of transhydrogenase

Nicotinamide nucleotide transhydrogenase was prepared essentially as described previously [10] with the modification that the medium used for wash and elution of the enzyme bound to the calcium phosphate gel was 2 mM sodium phosphate containing 0.5% potassium cholate, and 200 mM potassium phosphate containing 0.5% potassium cholate, respectively.

Transhydrogenase activity was assayed as reduction of $AcPyAD^+$ by NADPH at 375–420 nm using a dual-wavelength Aminco DW-2 spectrophotometer. The assay medium was composed of 80 mM potassium phosphate (pH 6.3), 200 μM NADPH, 200 μM $AcPyAD^+$, 0.025% Triton X-100, and 0.4 mg/ml of lysophosphatidylcholine (egg yolk). Temperature was 30°C. The specific activity of the transhydrogenase was about 7 μmol/min per mg protein, and the homogeneity of the transhydrogenase preparation was ascertained by a 7.5% polyacrylamide gel electrophoresis analysis in the presence of SDS [11]. Staining was accomplished by a silver staining method [12]. Protein concentration of the purified enzyme was determined by a modified Lowry procedure [13]. Prior to the CD analysis the enzyme was dialyzed at 0°C against 500 ml medium containing 5 mM potassium phosphate and 0.03% potassium cholate (pH 7.5) overnight with one change of medium after 4 h.

2.2. CD

CD was measured in a Jasco J-500 spectropolarimeter which was calibrated in linear dichroism mode at a few selected wavelengths using glass tilted plates at various angles [14]. A cell with a diameter of 1 mm was used and the cell compartment was continuously purged with N_2 to avoid O_2 absorption. Temperature was about 20°C. A spectral bandwidth of 2 nm was used and the signals were always checked for stray light. All spectra were averages of at least two scans and five data points were collected per nm using an IBM PC-AT compatible computer. The concentration of transhydrogenase was 0.043 mg/ml, equivalent to 0.394 μM, in a medium composed of 5 mM potassium phosphate and 0.03% potassium cholate (pH 7.5). When present, the concentration of the nicotinamide adenine nucleotide substrates was 20 μM. The total absorption, i.e., of solvent plus solute plus cell, was checked on all samples to ensure enough transmittance to obtain reliable CD signals. All CD data are expressed in differential molar absorptivities ($\epsilon_l - \epsilon_r$) per amino acid residue.

3. Results and discussion

3.1. CD spectra of purified transhydrogenase in the absence of substrates

Fig. 1 shows a CD spectrum of purified, detergent-dispersed transhydrogenase measured as described in section 2. The spectrum was negative at

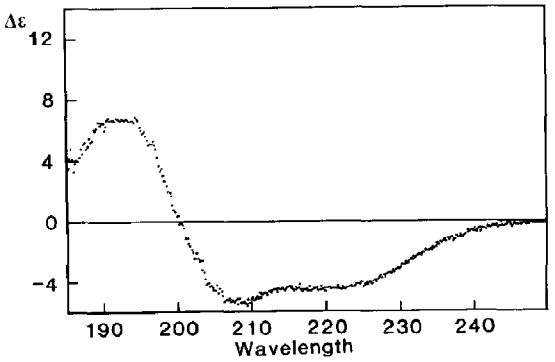

Fig. 1. CD spectrum of transhydrogenase in the absence of substrates. Medium contained 5 mM potassium phosphate and 0.03% potassium cholate (pH 7.5). The spectrum is an average of 14 scans.

long wavelengths with a distinct plateau around 220 nm and a minimum at 209 nm. The CD became positive below 200 nm and a maximum was observed at 194 nm. The overall CD magnitude was large and the ratio between the positive and negative CD maxima was 1.27. These features are characteristic for organized secondary structures, suggesting that the protein was not significantly denatured and that the CD spectrum is likely to be representative for the protein in its native state.

3.2. CD spectra in the presence of substrates

Fig. 2A shows CD spectra of transhydrogenase in the presence of either of the transhydrogenase substrates NADH, NAD^+, NADPH or $NADP^+$. None of these spectra were significantly different from that of pure transhydrogenase and it is evident that binding of a single substrate did not alter the secondary structure significantly. CD spectra of transhydrogenase in the presence of both NAD^+ and NADPH, or of NADH and $NADP^+$, are shown in fig. 2B. These CD spectra were also within experimental error the same as that of pure transhydrogenase.

Since CD sensitively reflects secondary structures, even small changes are expected to be detectable. Binding of nicotinamide nucleotide substrates to the transhydrogenase is an obligatory prerequisite for the enzyme-mediated translocation of protons through a putative proton-conducting channel, and some structural effects upon interaction between the protein and its substrates would therefore be expected. Our finding that the CD spectrum of transhydrogenase was unaltered by either of the four substrates (figs 1 and 2) therefore indicates that this binding does not involve detectable changes in the secondary structure of the protein. That similar results were obtained also in the presence of NAD^+ plus NADPH, or NADH plus $NADP^+$ means of course that all four nicotinamide nucleotides were present at concentrations of about 10 μM, since transhydrogenase under these conditions is catalytically active and rapidly equilibrates the reaction to an equilibrium constant of approximately unity [1,7]. The lack of effect on the CD spectra is, for example, consistent with transhydrogenase being in rapid equilibrium between two states with different CD, but which cancel each other in the resulting overall CD spectrum. However, it appears unlikely that the two hypothetical conformational states would result in CD changes which are opposite and exactly of the same magnitude. These results thus suggest that adduct coordination induces rearrangements of domains, possibly long α-helices, which retain their local secondary structures. Such rearrangements might not affect CD significantly.

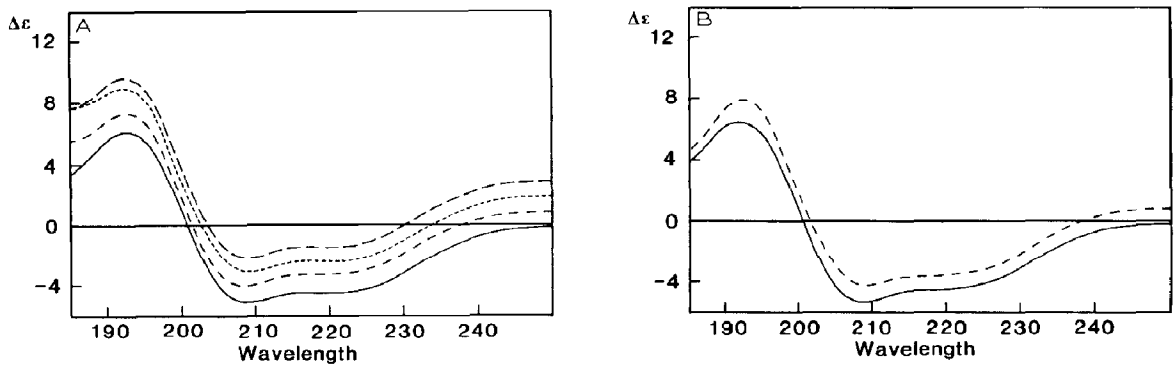

Fig. 2. CD spectra of transhydrogenase in presence of substrates. Additions: (A) NAD^+ (———), NADH, offset + 1 (— — —), $NADP^+$, offset + 2 (- - - - - -) or NADPH, offset + 3 (— — —); (B), $NADP^+$ plus NADH (———) or NADPH plus NAD^+ (— — —). The concentrations of the nicotinamide nucleotides were 20 μM.

3.3. Secondary structure of transhydrogenase

CD, the differential absorption of left- and right-handed circularly polarized light, is for symmetry reasons zero for unperturbed achiral chromophores. For chiral molecules, or symmetric molecules in chiral arrangements or in chiral environments, CD can attain substantial magnitudes. This property makes CD especially useful for characterization of chromophoric arrangements, such as the organization of peptide bonds in proteins [15,16] and nucleic acid bases in DNA [17]. A quantitative interpretation of the observed CD is usually difficult because of the generally limited knowledge about the polarizations of magnetic and electric transition dipoles in the chromophores, and their large dependence on the molecular environment. Although some recent progress has been made in understanding the CD of nucleic acids — CD can be predicted for DNA in various conformations [18] and for dyes associated with DNA [19] — the interpretation of protein CD still remains mainly empirical.

It is well known that polypeptides with unique secondary structures display characteristic CD spectra [20]. However, similar basis spectra of structurally equivalent polypeptides display notable variations and it has been questioned to what extent such CD data represent structural conformations [21]. In order to avoid some of these problems, Saxena and Wetlaufer [22] estimated the spectral CD profiles of α-helix, β-sheet, and random coil by correlating the CD spectra for some proteins to their established secondary structures by X-ray crystallography. The CD spectrum of transhydrogenase cannot, however, be satisfactorily explained by these profiles, since no combination of them can simultaneously account for the minimum at 207 nm and the large positive signal at 194 nm — features that transhydrogenase shares with other proteins such as lysozyme [23], halorhodopsin [24] and ribonucleotide reductase (A. Åberg, B.-M. Sjöberg and M. Kubista, unpublished results). The approach of Saxena and Wetlaufer [22] has been improved by several authors [25–27]. We have chosen to use the method of Hennessy and Johnson [25], utilizing the five basis CD spectra they obtained by decomposing a set of 15 reference protein CD spectra. The advantage of this method is that access to extensive protein CD spectra libraries is not required and further, it does not restrict the sum of secondary structures to one, thereby providing a quality check of the fit.

The CD spectrum of transhydrogenase fitted to the five basis spectra is shown in fig. 3A. From the empirical correlation of the basis spectra and structural components [25], we estimate a content of 47% α-helix, 23% parallel β-sheet, 4% antiparallel β-sheet, 9% β_{I}-turns, 4% β_{II}-turns, −17%

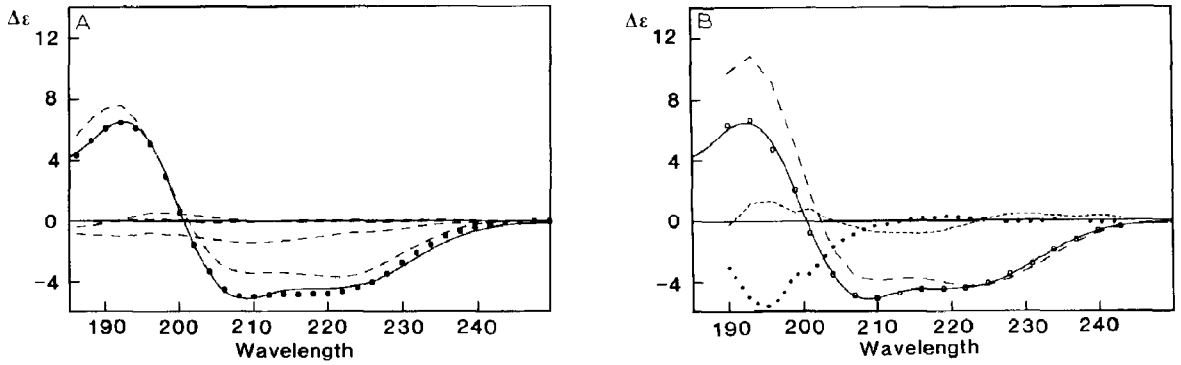

Fig. 3. Fit of the CD spectrum of transhydrogenase to the content of various secondary structures. (A) CD spectrum of transhydrogenase (———) fitted to the five basis CD components (— — —) determined by Hennessey and Johnson [25]. The fit is dominated by two of the basis components the other three being hard to distinguish in the figure; (B) CD spectrum of transhydrogenase (———) fitted to β (- - - - - -), random coil (· · · · · ·) and a length-dependent α-helix (— — —) contribution as suggested by Chen et al. [23]. The fitted spectra are shown with open circles (○).

other β-turns, and 44% other structures, where the numbers denote fractions of pure structures. The fit predicts some 45% α-helical structure, but also a rather large negative occurence of 'other β-turns'. We checked the significance of the estimated parameters by smoothing the data prior to analysis. Although most parameters were essentially unaffected by the choice of smoothing procedure, the estimation of 'other β-turns' displayed large variations. Its predicted negative abundance is therefore not alarming. Further, since information about eight parameters is extracted from only five basis spectra, these eight parameters must be linearly dependent. The relative amounts of secondary structures in a protein are, however, independent and the linear dependence in the analysis is thus imposed by structural similarities between the 15 proteins used in the construction of the basis set. Since transhydrogenase is a membrane protein, and such were not included in the original set, the correlation between the basis spectra and secondary structures may be less valid for transhydrogenase. Also, other factors may affect the protein CD. Chen et al. [23] showed that the contribution from α-helices is expected to depend strongly on their lengths. The CD spectrum in the 185–250 nm region of an ideal α-helix can be resolved into three Gaussian bands centered around 190, 206, and 222 nm representing the $\pi - \pi_{\perp}^*$, $\pi - \pi_{\parallel}^*$, n-π^* transitions, respectively [28]. Chen et al. [23] showed that the CD intensity of the 206 nm band increases relative to the 222 nm band with increasing length of the α-helix. This length dependence is most important for short helices and becomes negligible above about 40 amino acid residues. The unusually large CD amplitude of transhydrogenase around 208 nm is thus indicative of long α-helices. In a different systematic study of protein CD spectra, Manavalan and Johnson [29] correlated the larger CD amplitude of the 206 nm band as compared with that of the 222 nm band with proteins having separate α-helix and β-sheet-rich regions. Although this interpretation does not necessarily exclude a correlation with helical length, it suggests that more factors may affect the relative amplitudes at these bands.

The CD spectrum of transhydrogenase fitted to β-sheet, random coil and a length-dependent α-helical CD component according to Chen et al. [23] is shown in fig. 3B. The best fit was obtained for 30% β, 49% random coil and 41% α-helix, where the latter is described with three Gaussian bands with fitted energy maxima (198, 204, 223 nm), halfwidth (12.5, 8, 8 nm) and length-dependent amplitudes (30 amino acid residues). Although all estimated values are reasonable, the large number of fitted parameters makes the individual values uncertain. We checked especially the parameters for α-helical content and helix length, since they are closely correlated — increasing the parameter for the average helical length slightly decreases the parameter for the total amount of helical structures. Good fits could be obtained for an α-helical content of some 40–45% with an average length of at least about 13–15 residues.

The estimated amounts of secondary structures in both fits sum up to more than unity. This is most likely owing to additional spectral contributions to CD that are erroneously accounted for by the basis spectra used, and consequently limit the accuracy of the analysis. Since essentially only α-structure contributes to protein CD above 220 nm [15,16], where both fits are good (cf. fig. 3), the estimation of its abundance is likely to be accurate. However, the estimations of other structural elements, which also are known to have less conserved CD spectra among various proteins, are rather uncertain and will not be considered.

From the two slightly different approaches to fit the CD spectrum of transhydrogenase, we therefore conclude that the CD is consistent with some 40–45% α-helical structure with a rather long average helical length. The latter could indicate the presence of long α-helices traversing the entire membrane, the orientation of which may be affected by substrate binding. However, single or pairwise binding of nicotinamide adenine dinucleotide adducts does not significantly alter the protein secondary structure.

Acknowledgment

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