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CIRCULAR DICHROISM SPECTRA OF HYDROGENASE FROM *CLOSTRIDIUM PASTEURIANUM* W5

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SUMMARY

Circular dichroism studies of hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) from *Clostridium pasteurianum* W5 has been investigated in both the far-ultraviolet and visible region. The far-ultraviolet spectra indicate about 11–14 % of α-helix in this non-heme iron sulfur protein. Cotton effects associated with iron of hydrogenase were found at 530 (−), 506 (−), 440 (+), 415 (+), 380 (−), 340 (+) nm in the oxidized state, and at 590 (+), 550 (−), 490 (−), 440 (−), 410 (+), 370 (−), 320 (−) nm in the reduced state. A comparison of these spectral features with those observed in spinach ferredoxin and adrenodoxin is presented.

Biological nitrogen fixation has been the subject of intensive investigation in several laboratories for a number of years. An accessory enzyme involved in nitrogen fixation by the anaerobe, *Clostridium pasteurianum* W5, is hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) which has been shown to reduce ferredoxin when H_2 is the electron donor[1]. Reduced ferredoxin in turn supplies electrons to nitrogenase for reduction of nitrogen to ammonia.

Hydrogenase from *Clostridium pasteurianum* has recently been purified to a state of homogeneity as judged by analytical ultracentrifugation and disc gel electrophoresis[2]. It is a non-heme iron sulfur protein of molecular weight 60000 containing 4.0 iron atoms and an equivalent amount of "acid-labile" sulfide. Other basic chemical and physical features of this enzyme have been described recently by NAKOS AND MORTENSON[3]. These later studies have demonstrated that both the iron and sulfur are intimately involved in the enzymatic process.

Resently, we have begun studies to understand the nature of active site in hydrogenase. This paper describes the circular dichroism studies on this enzyme in both the visible region, where metal-associated optically active bands would be manifested, and the far-ultraviolet region, where bands characteristic of the secondary structure of the enzyme would appear. The oxidized and reduced spectra of the enzyme are also presented.

Hydrogenase was prepared from the crude extract by the procedure of NAKOS AND MORTENSON[2] which combines protamine sulfate fractionation, heat treatment, DEAE-cellulose chromatography and Sephadex gel filtration. All purification steps were undertaken under amaerobic conditions. Cells of *C. pasteurianum* were grown

with N_2 as the sole nitrogen source and cell-free extracts were prepared by the method of MORTENSON[4].

Protein concentration was determined by use of biuret reagent[5]. Iron was determined using *o*-phenanthroline by the method of LOVENBERG *et al.*[6].

Hydrogenase was assayed manometrically[7] by measuring the evolution of H_2 gas in the precence of dithionite and methyl viologen.

The absorption spectra of hydrogenase were recorded with a Cary 15 spectrophotometer using anaerobic cells of 1-cm path length.

Circular dichroism spectra were recorded with a Cary 60 spectropolarimeter equipped with 6001 CD attachment. Molar ellipticity $[\theta]$ was calculated from the relationship:

$$[\theta] = \frac{\theta \times M}{100 \times l \times c}$$

The units of $[\theta]$ are degrees·cm^2 per dmole.

θ is the observed rotation in degrees at concentration c in g/cm^3 using a cell of path length, l, in dm. M is the average residue weight for the ultraviolet region and the molecular weight for the visible region. A residue weight of 110 and molecular weight of 60000 is used in these calculations. Protein concentrations used were in the range of 30–50 μg/ml for ultraviolet region and about 10 mg/ml for visible region. With synthetic poly L-glutamic acid as a reference, % helix can be calculated from the expression[8]:

$$\%\,\alpha\text{-helix} = -100\,([\theta]_{210\,nm} + 2200)/36200 \qquad (1)$$

If poly L-lysine is used as a reference, the following expression has been suggested by GREENFIELD AND FASMAN[9] for calculating the percent α-helix.

$$\%\,\alpha\text{-helix} = \frac{[\theta]_{208\,nm} - 4000}{33000 - 4000} \times 100 \qquad (2)$$

In Fig. 1 is shown the CD spectrum of hydrogenase over the wavelength region 240–200 nm. The spectrum is the average of four sets of measurements at two concentrations. The CD curve is characterized by two negative bands at 222 and 208 nm. The minima observed at 208 and 222 nm are typical of π–π^* and n–π^* α-helical peptide transitions[10]. Both these minima are observed in the far-ultraviolet CD spectrum of hydrogenase. Using either poly L-glutamic acid (Eqn. 1) or poly L-lysine as reference (Eqn. 2), the amount of α-helix present in hydrogenase is calculated to be 14 and 11 %, respectively. The low amount of α-helix is in line with those observed in other non-heme iron sulfur proteins[11].

Fig. 2 presents the visible spectra of hydrogenase in the oxidized and reduced state. Unlike heme proteins, the spectra are poorly resolved. The oxidized spectrum shows a broad shoulder at 420 nm and band at 310 nm. The reduced spectrum does not exhibit any well characterized maximum. Better resolution of the absorption spectra has been achieved with other non-heme iron sulfur proteins[12] at liquid nitrogen temperatures and is desired in this case.

The CD spectra of oxidized and reduced proteins in the visible region are shown in Fig. 3. It is readily seen that CD spectrum of oxidized enzyme shows similarities

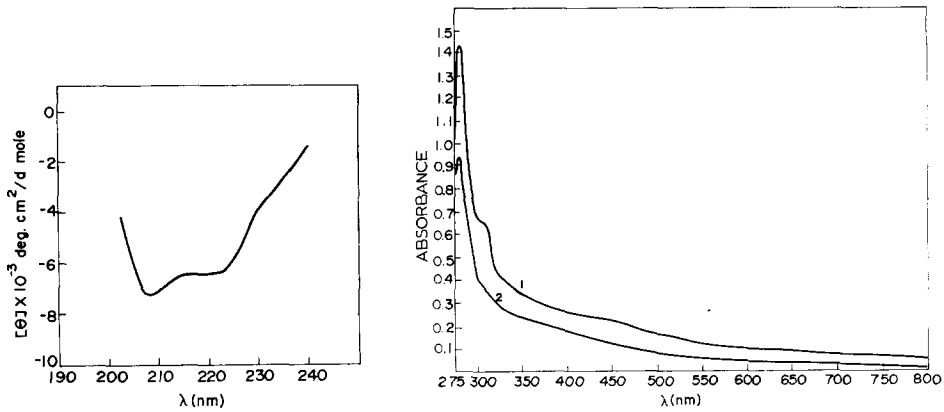

Fig. 1. Far-ultraviolet CD spectra of hydrogenase in 0.01 M phosphate buffer, pH 7.5, temperature 27°.

Fig. 2. Optical spectra of (1) oxidized, and dithionite reduced (2) hydrogenase. (Enzyme concentration was 0.93 mg/ml.) Cell path length 1 cm.

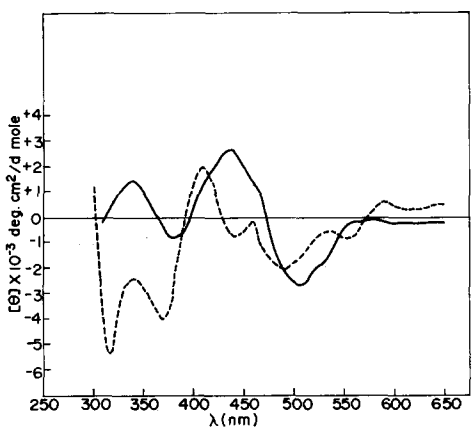

Fig. 3. Visible CD spectra of oxidized (———) and reduced (– – – –) hydrogenase in 0.05 M Tris buffer, pH 8.0, temperature 27°.

to the corresponding spectra observed with spinach ferredoxin and adrenodoxin[12]. Oxidized hydrogenase shows Cotton effects at 530 (−), 506 (−), 440 (+), 415 (+), 380 (−), and 340 (+) nm. Both oxidized adrenodoxin and ferredoxin show bands in the same region which not only are similar in their position but also in sign. However, Cotton effects above 550 nm obtained with adrenodoxin and ferredoxin are not observed with hydrogenase. This may be because the intensity of Cotton effects of hydrogenase are much weaker than the ones present in adrenodoxin and ferredoxin.

On reduction of hydrogenase with dithionite, one can discern transitions at 590 (+), 550 (−), 490 (−), 440 (−), 410 (+), 370 (−), and 320 (−) nm. Again certain similarities can be seen with respect to reduced adrenodoxin and ferredoxin, although some Cotton effects observed with adrenodoxin and ferredoxin are missing from the CD spectra of reduced hydrogenase. A summary of the comparison of CD spectral bands of hydrogenase, adrenodoxin and spinach ferredoxin is in Table I.

TABLE I

COMPARISON OF THE CIRCULAR DICHROISM BANDS OF HYDROGENASE, SPINACH FERREDOXIN AND ADRENODOXIN

Oxidized (nm)			*Reduced (nm)*		
Hydrogenase	*Ferredoxin**	*Adrenodoxin**	*Hydrogenase*	*Ferredoxin**	*Adrenodoxin**
	712 (+)			632 (+)	667 (+)
	588 (+)	632 (+)	590 (+)	588 (+)	592 (+)
530 (−)	551 (−)	549 (+)	550 (−)	538 (+)	538 (−)
506 (−)	500 (−)	500 (−)	490 (−)	515 (−)	506 (−)
440 (+)	446 (+)	463 (+)		473 (−)	467 (−)
	426 (+)	435 (+)	440 (−)	442 (−)	441 (−)
415 (+) sh.	408 (+)	410 (+)	410 (+)	401 (+)	400 (+)
380 (−)	372 (−)	373 (−)	370 (−)	372 (−)	366 (−)
340 (+)	355 (+)	335 (+)	320 (−)	330 (−)	320 (−)
	317 (+)	312 (+)			

* Taken from ref. 12. The positions of various Cotton effects are those required by the computer to fit the observed spectra. Not all bands are actually observed, *e.g.* computer analysis shows that the band at 480 nm observed in both reduced adrenodoxin and spinach ferredoxin is actually composed of three overlapping Cotton effects at 467 to 473, 506 to 515 and 538 nm.

The close resemblance in the CD spectra of hydrogenase, adrenodoxin and ferredoxin suggests that the structure of the metal-ligand chromophore in these proteins are similar. Recent studies on spinach ferredoxin by a variety of spectroscopic and magnetic techniques indicate that the reduced state contains a high spin ferric iron, spin-coupled *via* two bridging sulfides to a high spin ferrous iron (W. R. DUNHAM, G. PALMER, R. H. SANDS and A. J. BEARDEN, personal communication). Moreover, in the reduced state the ferrous iron is thought to be coordinated by four sulfur ligands in distorted tetrahedral coordination. The oxidized state of spinach ferredoxin is indicated from such studies to have two spin-coupled high spin ferric atoms.

In spite of these spectral similarities, there have to be small differences in the structure of the active site to account for the difference in activities associated with these proteins. One major difference between hydrogenase and the other two proteins is that while the latter are two-iron proteins, the former as isolated has four iron atoms. However, it has been shown that two of the four iron atoms in the hydrogenase can be removed by chelating agents without affecting the enzymatic activity[3]. It appears in the light of this observation that only two iron atoms are functional and the other two are either loosely bound to the enzyme as either contaminants or are required for maintaining some structural feature of the enzyme. Another possibility for the similarity of the CD spectra of hydrogenase to the two iron-sulfur proteins is that the former may have a pair of iron atoms on each of its two subunits and these two pairs may be structurally indentical. However, the ease with which only two of the four iron atoms in hydrogenase are lost to the chelating agents makes this possibility highly unlikely.

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