β Structure in Acetoacetate Decarboxylase*

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ABSTRACT: Acetoacetate decarboxylase (acetoacetate carboxylyase, EC 4.1.1.4) was studied by means of optical rotatory dispersion and circular dichroism. In the native state, it shows wavelength characteristics usually attributed to the β form. When it is dissociated by sodium dodecyl sulfate, it probably exists essentially as a mixture of α helix and random coil. The protein shows a distinct fine structure in the aromatic absorption region.

Acetoacetate decarboxylase can be isolated in the crystalline form from *Clostridium acetobutylicum* (Hamilton and Westheimer, 1959; Zerner *et al.*, 1966). Its mechanism of action is well documented and involves the formation of a Schiff base between enzyme and substrate (Westheimer, 1963; Warren *et al.*, 1966). The protein has a molecular weight of about 260,000 and can be readily dissociated by the usual denaturing agents into eight subunits. Besides the usual band arising from the presence of aromatic amino acids, its ultraviolet spectrum shows an absorption band with a maximum at 320 nm, which disappears upon denaturation and does not seem to belong to any coenzyme (Lederer *et al.*, 1966).

Studies of the rotatory properties of the protein have been conducted in order to characterize it more fully. Optical rotatory dispersion and circular dichroism spectra have yielded interesting information concerning its three-dimensional structure.

Experimental Section

Materials. Acetoacetate decarboxylase was prepared according to Zerner *et al.* (1966) and assayed as described in that paper. The various preparations used, once and sometimes twice-recrystallyzed enzyme, had a specific activity ranging between 30 and 50, and a ratio of absorbance at 320 nm to absorbance at 280 nm lying between 0.06 and 0.09. Protein concentrations were determined by using the extinction coefficient $\epsilon_{1\text{ cm}}^{1\text{ mg}}$ 1 at 280 nm (Lederer *et al.*, 1966).

Urea Merck was used without recrystallization. Sodium dodecyl sulfate was purchased from Eastman Chemicals.

Methods. All spectra of the native enzyme were taken in 0.05 M sodium or potassium phosphate buffer solution (pH 5.9). Spectra of denatured decarboxylase were taken in the same buffer to which urea or SDS[1] had been added as indicated in the Results section.

Optical rotatory dispersion studies were conducted with a Cary model 60 spectropolarimeter, at room temperature, using 1-cm cells and various dilutions. Below 220 nm, 1-mm cells were used under a flux of nitrogen.

Results are expressed in the following way. All rotations are expressed in reduced mean residue rotations $[m']$ (eq 1). The refractive indices, n_λ, of water and 8 M

$$[m']_\lambda = \frac{3}{n_\lambda^2 + 2}\frac{MRW}{100}[\alpha]_\lambda \qquad (1)$$

urea solutions were obtained from the tables given by Fasman (1963). The ones for phosphate buffer and solutions containing up to 2×10^{-2} M SDS were taken to be equal to those of water. The mean residue molecular weight, MRW, was computed from the amino acid composition and found to be equal to 116 g (Lederer *et al.*, 1966). The Drude parameter was calculated by conventional methods (Fasman, 1963), and the Moffit parameters a_0 and b_0 by a least-squares computer program lent by Dr. P. Urnes, using a λ_0 value of 212 nm, for wavelengths between 589 and 330 nm.

Circular dichroism spectra were taken with a Jouan Model CD 185 dichrograph, using different concentrations and pathlengths from 2 cm to 0.1 mm. The absorbance in the region of aromatic absorption never exceeded 1.5. Below 220 nm, the cell compartment was flushed with nitrogen. Results are expressed in molecular ellipticity (eq 2) using throughout the same mean

$$[\theta] = 3300 \times (\epsilon_l - \epsilon_r) \qquad (2)$$

residue molecular weight as above.

Results

Optical Rotatory Dispersion. The dispersion of the protein was measured from 589 to 330 nm in buffer (native enzyme), in 8 M urea after about 6-hr incubation and in the presence of 0.998×10^{-2} M SDS after incubation for 1 night. Drude and Moffit parameters derived from this set of data are presented in Table I.

Measurements were also taken from 200 to 330 nm. The full Cotton effects could be obtained for the native

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: SDS, sodium dodecyl sulfate.

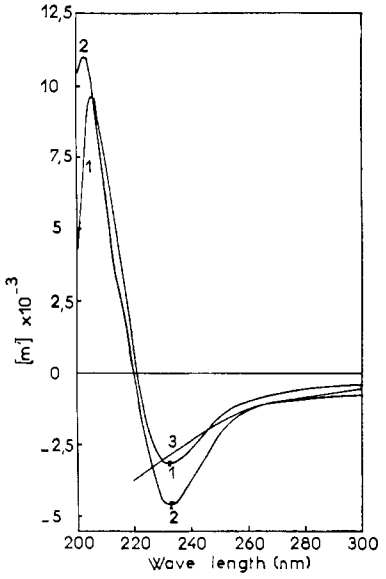

FIGURE 1: Ultraviolet Cotton effects of acetoacetate decarboxylase. The vertical bars represent the limits of variation found in the different measurements. For Figures 1 and 2, curves 1, 2, and 3 represent native protein, protein in SDS, and protein in 8 M urea, respectively.

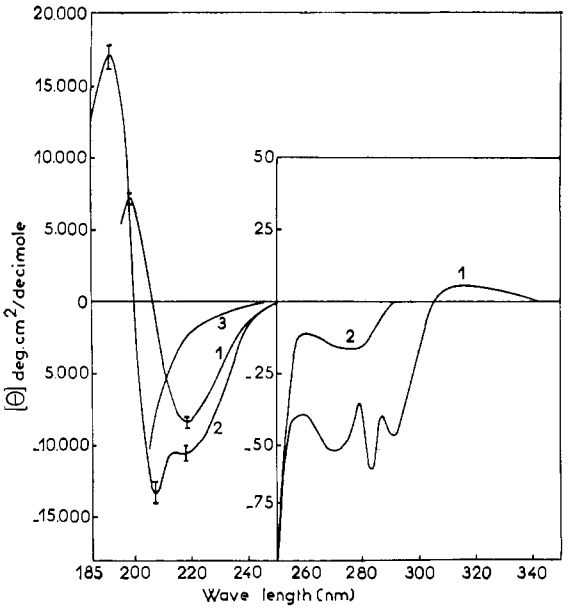

FIGURE 2: Circular dichroism of acetoacetate decarboxylase. The vertical bars represent the limits of variation found in the different measurements.

and SDS-dissociated protein. They are reproduced in Figure 1. It was not possible to reach below 215 nm when 8 M urea solutions were used. The wavelengths of the extrema and crossover point, together with the mean residue rotations at the peak and trough are given in Table II. The 1-nm shift in the position of the minimum and crossover point upon addition of SDS has been consistently observed in the course of the dozen determinations that were made. The region of aromatic absorption was scanned for special Cotton effects, but although a slight change in the slope was perceptible, no fine structure could be measured.

The Circular Dichroism curves of the native and dissociated enzyme are reproduced in Figure 2 and values of molar ellipticities are given in Table III.

A fine structure in the region of aromatic absorption was revealed, consisting of at least three negative dichroic bands with minima at 290, 283, and 270 nm. A weak positive dichroic band could also be observed above 305 nm. Owing to the low signal-to-noise ratio, the figures for the molar ellipticities above 250 nm could all be off by up to 20%; but an error in the absolute values of the ellipticities would not alter the position of the extrema nor change the relative intensities of the bands.

TABLE I: Drude and Moffit Parameters of Acetoacetate Decarboxylase.

Conditions	λ_c (nm)	b_0 (deg)	a_0 (deg)
Phosphate buffer, pH 5.9	240	−82	−312
8 M urea in buffer	214	−19	−385
10^{-2} M SDS in buffer	241	−135	−691

Discussion

From the studies described above, one can gain some insight into the three-dimensional structure of acetoacetate decarboxylase.

In the native state, the enzyme shows optical characteristics of β structure. The indication given by the position of the maximum in the optical rotatory dispersion at 205 nm is corroborated by the circular dichroism spectrum, with a single negative band centered at 219 nm and a positive one at 198–199 mm. Similar positions have been observed for the β form of poly-L-lysine in solution (Sarkar and Doty, 1966; Townend *et al.*, 1966) and for silk fibroin in solution (Iizuka and Yang, 1966).

It would appear that there is very little, if any, α helix in the native decarboxylase. This follows from the fact that the amplitude of the Cotton effect and circular dichroic bands of the helical form of model polymers is much larger than that of the β form (Holzwarth and Doty, 1965; Iizuka and Yang, 1966; Sarkar and Doty, 1966; Townend *et al.*, 1966). The presence of α-helical regions amounting to a nonnegligible fraction of the amount of regions in the β form should displace the position of the extrema to wavelengths intermediate between the positions for the two individual types of structure (Greenfield *et al.*, 1967). It is clear from the figures and Tables II and III that this is not the case. The position of the extrema of the Cotton effects and dichroic bands are those of a β structure. The magnitude of the rotations and the ellipticities indicate the decarboxylase is not entirely in the β form. However, for lack of enough data about relevant reference compounds, it would be hazardous to try to predict the fraction of the protein that has adopted the pleated-sheet conformation.

TABLE II: Far-Ultraviolet Cotton Effects Characteristics.

Conditions	Minimum		Crossover	Maximum	
	λ (nm)	[m'] (deg)	λ (nm)	λ (nm)	[m'] (deg)
Phosphate buffer, pH 5.9	232	−3,100[a]	221	205–206	+9,600
4×10^{-3} to 2×10^{-2} M SDS in buffer	233	−4,600[a]	220	202–203	+10,900

[a] For limits of experimental error, see Figure 1.

TABLE III: Circular Dichroic Characteristics of Acetoacetate Decarboxylase.

Conditions	Negative Band		Positive Band	
	Position (nm)	$[\theta]$[a]	Position (nm)	$[\theta]$[a]
Phosphate buffer, pH 5.9	219	−8,400[b]	198–199	+7,100[b]
$1–2 \times 10^{-2}$ M SDS in buffer	218	−10,500[b]		
	207	−13,400[b]	191–192	+16,800[b]

[a] In (deg cm^2)/dmole. [b] For limits of experimental error, see Figure 2.

To date, very few of the already studied enzyme proteins have been found to show β-structure characteristics in their optical rotatory dispersion and circular dichroism spectra (Chang, 1966; Jirgensons, 1966; Sarkar and Doty, 1966).

The effect of SDS upon the secondary and tertiary structure of polypeptides and proteins has been the subject of some discussion in recent years. Available information now suggests that its effect is not the same for all proteins. It could leave some structures relatively untouched (Bolotina *et al.*, 1967; DiSabato and Kaplan, 1963; Jirgensons, 1966), increase the apparent helical content (Ichishima and Yoshida, 1967; Jirgensons, 1966; Jirgensons *et al.*, 1966; Magar, 1967; Verpoorte and Kay, 1966), decrease it (McCubbin *et al.*, 1966; Velluz and Legrand, 1965), destroy β structures (Troitsky, 1965), or induce β-structure formation (Sarkar and Doty, 1966). This variety of effects is not surprising, considering that SDS is expected to interact mainly with hydrophobic bonds.

Whether they be perceptible through optical rotatory dispersion measurements or not, the changes, brought about by SDS (at or above the critical micelle concentration) in the secondary and tertiary structure of proteins, are sufficient to cause inactivation of all the enzymes submitted to its action, and also their dissociation when they are composed of subunits. The present study does not bring to light any fundamentally new fact about SDS action. But the determination of the circular dichroism spectra of the SDS-treated decarboxylase permits a finer analysis that those of optical rotatory dispersion alone. They allow one to conclude that, at least on acetoacetate decarboxylase, SDS acts in several ways.

The last statement is based on the following observations. First and foremost, the spectra at hand point to the formation of some α helix: in the optical rotatory dispersion, the increase in amplitude of the Cotton effects, the appearance of a shoulder on the long-wavelength limb of the peak, and a displacement of the positive extremum toward shorter wavelengths; in the circular dichroism, the appearance of a second negative dichroic band at 207 nm, the increase in intensity of all the bands, and the shift of the positive maximum from 199 to 192 nm.

Secondly, although the maximum of the Cotton effect at 202–203 nm is at a position intermediate between those expected for α helix and β form, the wavelength characteristics of the circular dichroism curves do not speak in favor of the existence of much pleated-sheet structure.

Considering finally only circular dichroism curves, which are in principle easier to analyze, one notices that the positive band at 192 nm shows an intensity smaller than would be expected relative to that of the negative bands. This suggests the presence of randomly coiled sections in the polypeptide chains, which are known to display a negative band centered around 195 nm (Iizuka and Yang, 1966; Townend *et al.*, 1966). However, the same qualitative conclusion would also hold for the native protein. One may then wonder whether the aperiodical but organized regions in the native structure have remained the same after SDS dissociation, or whether truly random sections exist then.

It is clear that only a computer analysis (Carver *et al.*, 1966; Greenfield *et al.*, 1967) could solve the question of the relative amounts of α helix, β structure, and random form expressed in the spectra shown, and tell whether the three types of structure are enough to account for the observed rotatory properties. Meanwhile, a qualitative conclusion of our study is that the action of SDS upon acetoacetate decarboxylase leads to destruction of probably most of the native β structure and to formation of α helix. The data on hand do not permit to decide whether one derives from the other, or from other regions of the native protein.

Rotatory Properties in the Region of Aromatic Absorption. A number of fine structures of dichroic spectra in the aromatic region have been described in the literature (Beychok, 1966; Beychok *et al.*, 1966; Glazer and Simmons, 1966; Green and Melamed, 1966; Simmons and Glazer, 1967; Velluz and Legrand, 1965). However, no systematic correlation has been yet established between the position of dichroic bands and the nature of the aromatic chromophores which gives rise to their optical activity. It is then hard to deduce anything precise from the sign and position of the dichroic bands displayed by acetoacetate decarboxylase in the 280-nm region. This protein is believed to be devoid of disulfide bridges, and it possesses about 16 tyrosine residues but only 1 tryptophan residue per subunit of mol wt 33,000 g (Lederer *et al.*, 1966). It is highly probable that this tryptophan is one of the optically active aromatic residues; titration studies with *N*-bromosuccinimide revealed that its side chain is unavailable to this reagent in the native protein (F. Lederer, unpublished observations). It then seems likely that the aromatic side chain lies in a region where its rotation is restricted.

It is hardly surprising that the fine structure disappears upon dissociation of the protein by sodium dodecyl sulfate, considering the profound rearrangement its structure has undergone, as has just been discussed. In the new conformation, the tryptophan residue is available to *N*-bromosuccinimide, so that it would appear that the remaining weak dichroic band can be attributed solely to tyrosine residues.

Noticeable is the weak positive dichroic band above 305 nm. It is dissymetrical and would possibly extend further into the ultraviolet region, were it not for the opposing negative dichroic bands in the 280-nm region. Fasman *et al.* (1966) observed in chymotrypsin and chymotrypsinogen a circular dichroic band in the 300-nm region. They suggest it may arise from tryptophan residues. In the case of acetoacetate decarboxylase, the band extends to higher wavelengths and its range corresponds to the unusual ultraviolet absorption band with a maximum at 320 nm. Let us recall that it resists all attempts of purification; it only disappears upon treatment with denaturing agents or enzymatic digestion (Lederer *et al.*, 1966). It is tempting, then, to conclude that this band, for which there seems to be hardly any analogy in the literature, is optically active. The finding tends to strengthen the view that the absorption band can hardly have a trivial origin, and might result from electronic interactions between aromatic side chains, interactions dependent upon the integrity of the tertiary structure.

A final remark has to be made. Neece and Fridovich (1967) reported that when acetoacetate decarboxylase is never exposed to a temperature higher than 4° at any time during the preparation of the enzyme, the material thus obtained contains some "latent" activity, which can be "expressed" in an irreversible fashion upon moderate heating. When the optical rotatory dispersion measurements were started, this property was not yet uncovered, so that at first through ignorance, subsequently on deliberate purpose, no precaution was ever taken to keep the enzyme in the cold at all times. All the measurements described in this paper can thus be considered to have been conducted with completely, or nearly completely, "expressed" decarboxylase.

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Isomerization Reactions of Charcoal-Defatted Bovine Plasma Albumin. The N–F Transition and Acid Expansion*

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ABSTRACT: In view of the possibility that bound lipophilic impurities might make an important contribution to the observed microheterogeneity of bovine plasma albumin (BPA) samples, the character of the population distributions of albumin defatted by the conventional acid procedure and by the newer charcoal procedure has been determined by means of solubility–pH profiles and comparison was made with the nondefatted albumin used as precursor to these preparations. The results suggest that acid defatting leads to an artificial broadening of the population due to two factors, namely (1) partial but incomplete defatting and (2) some unknown irreversible alteration of some of the protein. The charcoal-defatted protein has a much narrower population distribution than either nondefatted or acid-defatted BPA. Improved procedures for determining solubility–pH profiles and for subfractionation of the populations were developed. The most striking property of the charcoal-defatted protein is its lability toward some reaction which results in broadening of the population. This process is most rapid above pH 7 but is appreciable even at the isoionic pH and at more acid pH in deionized solution, but is retarded by adding salt at concentrations as low as 0.03 M. Owing to the more homogeneous population, the acid transitions of charcoal-defatted protein are sharper and better resolved than in previous preparations. The N–F transition and acid expansion can be clearly resolved by optical rotation measurements at the trough of the first Cotton effect, 233 mμ, and by pH difference spectra. Careful measurements have been made of the optical rotatory dispersion properties of charcoal-defatted bovine plasma albumin both at neutral pH and through the range of the acid transitions. Hydrogen ion titration results are presented for the acid range which agree in general with previously published results but the number of titratable carboxyl groups (102) is in better agreement with amino acid composition than the number found earlier (108) for acid-defatted protein, suggesting the possibility that some deamidation may accompany the low pH treatment.

Formation of multiple boundaries in moving-boundary electrophoresis of plasma albumin near pH 4 was interpreted (Aoki and Foster, 1956, 1957a,b) as due to a pH-dependent isomerization reaction (N–F transformation). Resolution of the N and F forms in electrophoresis was explained by the present authors (Sogami and Foster, 1963) as due to microheterogeneity of crystallized plasma albumin. Specifically, we proposed that there exists, in any given albumin sample, a population distribution in terms of the characteristic pH at which various molecules undergo the N–F transformation. The concept of microheterogeneity of plasma albumins was proposed independently (Štokrová and Šponár, 1963) to explain results on thermal denaturation and was further supported by various experimental observations (Foster *et al.*, 1965; Petersen and Foster, 1965a,b).

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