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Subunit Interactions of Transcarboxylase As Studied by Circular Dichroism†

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ABSTRACT: A change in the secondary structure of transcarboxylase resulting from quaternary interactions is monitored by circular dichroism spectroscopy. The change is traced to interactions among the six polypeptides that make up the $12S_H$ subunit. It is fully reversible and is not a result of the conditions used to dissociate the enzyme. Our new method of analyzing circular dichroism spectra for secondary structure works well for this enzyme and its subunits. Even the odd circular dichroism of the $1.3S_E$ subunit analyzes well. There is an increase of 19% in α helix and a concomitant decrease of 8% in antiparallel β sheet and 7% in random structure on association to form the hexameric $12S_H$ subunit.

Transcarboxylase is a biotin enzyme that occurs in the propionic acid bacteria and catalyzes the transfer of a carbonyl group from methylmalonyl-CoA[1] to pyruvate forming propionyl-CoA and oxaloacetate. A comprehensive review of its structure and properties has been published in 1976 (Wood & Zwolinski, 1976) and a less comprehensive review published in 1979 (Wood, 1979). A general concept of its quaternary structure has been developed by electron microscopy of the enzyme and its subunits. The central $12S_H$ subunit appears to be cylindrical in shape and is made up of six identical polypeptides. There are six outer $5S_E$ dimeric subunits, three at each opposite face of the $12S_H$ subunit, which are attached to it via two biotinyl subunits (see Figure 1).

An interesting property of transcarboxylase is that at neutral pH, the outside subunits dissociate exclusively from one face of the central subunit rather than randomly from both faces (Wrigley et al., 1977) and two biotinyl subunits remain attached to each dissociated $5S_E$ subunit. This combination has been designated the $6S_E$ subunit. Transcarboxylase with three outside subunits on the single face is called 18S transcarboxylase. At about pH 8, the remaining $6S_E$ subunits progressively dissociate from the other face of the $12S_H$ subunit. At pH 9, transcarboxylase dissociates as shown in Figure 1 to the $1.3S_E$ biotinyl subunit, the dimeric $5S_E$ subunit, and the constituent polypeptides of the $12S_H$ subunit that are designated $2.5S_H$. The dimeric $5S_E$ subunit is very stable and thus far has been shown to dissociate to the monomers only by treatment with detergents such as sodium dodecyl sulfate or urea.

Until recently, it was thought that the $12S_H$ subunit dissociated at pH 9 to stable $6S_H$ dimers, but C. Bahler, N. H. Goss, and H. G. Wood (unpublished results) have shown that when strictly anaerobic conditions are maintained during the dissociation, the product is $2.5S_H$ monomers. The formerly observed stable dimer arises from an oxidation resulting in the formation of disulfide bonds between pairs of $2.5S_H$ monomers. In addition, C. Bahler, N. H. Goss, and H. G. Wood (unpublished results) showed that transcarboxylase could be reconstituted from the $1.3S_E$ subunit, the $5S_E$ dimer, and the $2.5S_H$ monomer. On the other hand, the disulfide-linked $6S_H$ dimer is inactive in reconstitution of transcarboxylase, although there is evidence that a native nondisulfide-linked $6S_H$ dimer is formed during association–dissociation of the $12S_H$ subunit. These are not sharply confined to the pHs given above. The dissociation or association is also influenced by protein concentration, by concentration of divalent ions, and by temperature.

The biotinyl subunit contains 123 residues and its sequence has been determined (Maloy et al., 1979). It has recently been shown that attachment of the outside subunits to the central $12S_H$ subunit is via residues 1-23 and another portion is involved in orienting the biotin at the substrate sites (Kumar & Beegen, 1981). The CoA ester sites are on the $12S_H$ subunits, the keto acid sites are on the $5S_E$ subunits (Chuang et al., 1975), and thus the biotinyl subunit serves as carboxyl carrier between the two subunits. It has been shown by photoaffinity labeling that there are two CoA ester sites per polypeptide of the $12S_H$ subunit (Poto & Wood, 1977). Since three outside subunits are each linked by biotinyl peptides at

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[1] Abbreviations: CD, circular dichroism; CoA, coenzyme A; Tris, tris(hydroxymethyl)aminomethane; OD, optical density.

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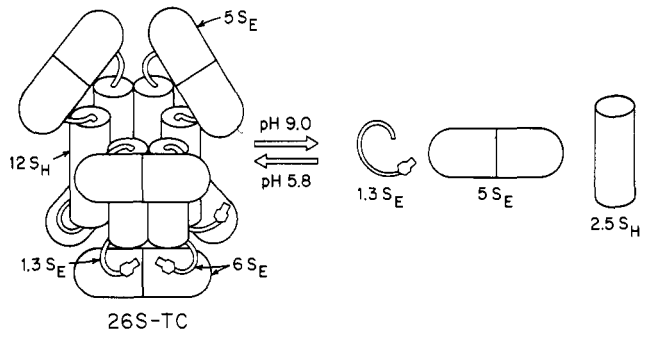

FIGURE 1: Schematic illustration of the quaternary structure of transcarboxylase and the reversible dissociation to its subunits. Further explanation is given in the text.

the two opposite faces of the $12S_H$ subunit, it is apparent that each polypeptide of the $12S_H$ subunit has two binding sites for the $1.3S_E$ subunit. Thus, it is likely there are two homologous sequences in each polypeptide that catalyze similar functions (Zwolinski et al., 1977). There also are 12 keto acid sites, 2 per dimeric $5S_E$ subunit. Clearly, there is a host of protein–protein interactions that occur during the assembly of this enzyme and during catalysis of the reaction.

Circular dichroism (CD) spectroscopy is particularly sensitive to the secondary structure of a protein. Furthermore, it is now possible to analyze such CD spectra for the various types of secondary structure (Hennessey & Johnson, 1981). Since transcarboxylase is a particularly complicated enzyme with many quaternary interactions and the interactions of the subunits are known, it was selected to determine if changes in quaternary interaction are accompanied by changes in secondary structure. The results show a 19% increase in α helix during formation of the hexameric $12S_H$ subunit that is accompanied by a loss of antiparallel β sheet and secondary structure in the "other" category for the $2.5S_H$ monomers.

Materials and Methods

26S Transcarboxylase. The enzyme was isolated with slight modifications of the procedure of Wood et al. (1977). The enzyme had a specific activity of 50.

$12S_H$ Subunit. This subunit was purified by a procedure recently developed by C. Bahler, N. H. Goss, and H. G. Wood (unpublished results), which should be consulted for details. 26S transcarboxylase is placed on a cellulose phosphate column and then 0.05 M phosphate buffer, pH 6.5, followed by 0.05 M Tris–SO_4, pH 8, containing 20% glycerol, is passed through the column removing the $6S_E$ subunits. The $12S_H$ subunit is then eluted with 0.3 M phosphate buffer containing 20% glycerol. When assayed by reconstituting to transcarboxylase with a 12-fold excess of $6S_E$ subunit as described by Wood et al. (1975), the specific activity was 50, based on the protein of the $12S_H$ subunit.

$6S_E$ Subunit. This subunit was isolated by dissociating the 26S transcarboxylase under N_2 in 0.1 M phosphate buffer, pH 7, containing 10^{-4} M dithiothreitol and separating the resulting 18S transcarboxylase from the $6S_E$ subunit by glycerol gradient centrifugation as described by Wood et al. (1977). The specific activity of the $6S_E$ subunit when reconstituted in the presence of a 12-fold excess of $12S_H$ subunit was 40.

$5S_E$ and $1.3S_E$ Subunits. The method of Maloy et al. (1979) was used to isolate these subunits. Transcarboxylase is dissociated at pH 9 and then fractionated with $(NH_4)_2SO_4$. The fraction from 35 to 55% saturation contains both the biotinyl subunit and the $5S_E$ subunit. This fraction is dialyzed vs. 5 mM NH_4HCO_3, pH 9, and applied to a DEAE-cellulose column. The $1.3S_E$ and $5S_E$ subunits are separated with a linear gradient of 8–100 mM NH_4HCO_3.

Purity and Shipment. The purity of the various subunit preparations was demonstrated by disc gel electrophoresis. Furthermore, many samples were analyzed through sedimentation-velocity centrifugation. The enzyme and subunits were packed in dry ice for shipment to Corvallis, OR, from Cleveland, OH.

Preparation of Protein Solutions for CD Analysis. The protein solutions were diluted to approximately 1 mg/mL with 0.1 M phosphate buffer saturated with N_2, pH 5.8, containing 10^{-4} M dithioerythritol. The solution was then dialyzed for 15–20 h at 5 °C against 1 L of the same buffer, with N_2 constantly bubbling through it. The buffer solution was changed twice. Aliquots for both spectroscopic measurements and analysis of protein concentration were removed directly from the dialysis bag for the runs at pH 5.8. The dialysis bag was then purged with N_2 and resealed, and the contents were dialyzed against 1 L of borate buffer saturated with N_2, pH 9.0, containing 10^{-4} M dithioerythritol under the same conditions as described above. Samples were removed directly from the dialysis bag for the runs at pH 9.

Protein Concentration. Protein concentrations were determined with the method described previously (Hennessey & Johnson, 1981). Except for the $1.3S_E$ subunit, the amino acid composition of these proteins is known only from amino acid analysis (Zwolinski et al., 1977) that, due to preparatory hydrolysis, converts asparagine to aspartate and glutamine to glutamate. It is necessary to account for the amine groups given off in this process for correct calculation of protein concentration. Failure to account for this conversion can result in errors of up to 10%. Determination of the asparagine and glutamine composition was approximated with the amino acid analyzer. Free NH_3 of the sample was measured and the base line NH_3 peak subtracted. Calculations via this method produced sufficiently accurate estimates of asparagine or glutamine content for our purposes. Results show 6 mol of Asn or Gln per mol of $1.3S_E$ subunit, 57 mol per mol of $5S_E$ subunit, 256 mol per mol of $12S_H$ subunit, and 672 mol per mol of transcarboxylase. These values were used in subsequent analyses of protein concentration.

Spectroscopy. CD spectra were measured on a vacuum ultraviolet CD spectrophotometer (Johnson, 1971). The instrument was calibrated with (+)-10-camphorsulfonic acid, $\Delta\epsilon = +2.42$ at 290.5 nm (Chen & Yang, 1977). Most measurements were made with 50- or 100-μm path-length cells. The entire experimental procedure was repeated at least twice to confirm results. All spectra were measured at 14 ± 1 °C with either a 10-s time constant and a scanning rate of 2 nm/min or with a 30-s time constant and a scanning rate of 1 nm/min. The spectral slit width was a constant 1.6 nm. CD spectra are presented in units of $\Delta\epsilon$ [L cm^{-1} (mol of amide)$^{-1}$].

Transmission spectra were measured with the same instrument, and extinction coefficients were determined for each protein at 190 nm (Table I). CD spectra were terminated when the total OD of the sample reached 1.0.

Method of CD Analysis. All protein CD spectra, whether experimental or constructed, were analyzed for secondary structure by using the methods described previously (Hennessey & Johnson, 1981).

Results

The CD spectrum of native 26S transcarboxylase at pH 5.8 is given in Figure 2. The spectrum is typical for proteins with minima at 220 and 210 nm and a maximum at 193.5 nm. Transcarboxylase dissociates into its component subunits at

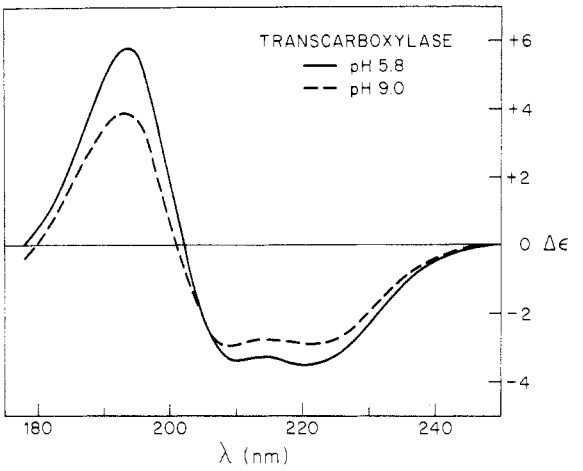

FIGURE 2: CD spectra of intact 26S transcarboxylase at pH 5.8 (–) and of transcarboxylase dissociated into $1.3S_E$, $5S_E$, and $2.5S_H$ subunits at pH 9.0 (---).

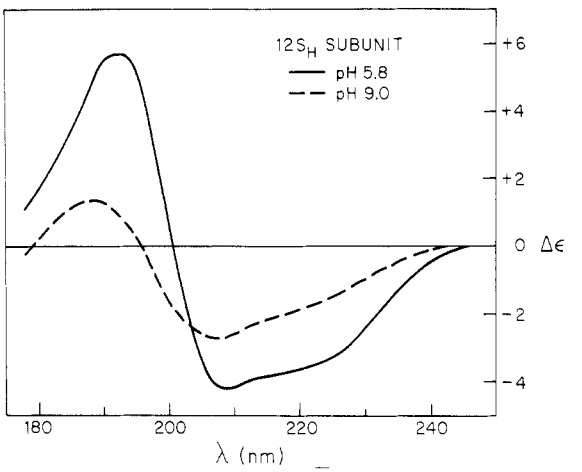

FIGURE 3: CD spectra of the intact $12S_H$ subunit of transcarboxylase at pH 5.8 (–) and of the $12S_H$ subunit dissociated into the component $2.5S_H$ monomers at pH 9.0 (---).

Table I: Extinction Coefficients of Transcarboxylase and Its Subunits

protein or subunit	ϵ_{190} (pH 5.8)	ϵ_{190} (pH 9.0)	ϵ_{275} (pH 5.8)
26S transcarboxylase	10000	10200	40
$1.3S_E$	7700	7300	
$5S_E$	9100	10300	
$6S_E$	9100	8600	
$12S_H$	10300	9800	

pH 9 (Figure 1). The CD spectrum of the dissociated enzyme is also given in Figure 2 and is somewhat less intense, indicating that there has been some change in secondary structure. By use of enzyme that had been dissociated at pH 9, then reconstituted in 0.5 M phosphate, pH 6.5, and then dialyzed into 0.1 M phosphate, pH 5.8, it was shown that the dissociation is fully reversible under strictly anaerobic conditions. The CD of reconstituted 26S transcarboxylase in 0.1 M phosphate buffer at pH 5.8 is identical with that of the original enzyme. Thus, the change in pH did not cause any noticeable irreversible denaturations. Our analyses of the CD spectra are given in Table II. The analysis shows about one-third helical structure for intact 26S transcarboxylase at pH 5.8 (no. 10, Table II). Dissociation to the component subunits at pH 9.0 causes a decrease in the α-helical structure with a concomitant increase in antiparallel β sheet and the other or random category (no. 13, Table II).

Having demonstrated that there is a change in secondary structure on dissociation of the enzyme, we localized this change in the $12S_H$ subunit. The CD spectrum of the $12S_H$ subunit at pH 5.8 and the CD spectrum of this subunit dissociated into $2.5S_H$ monomers at pH 9.0 are given in Figure 3. The dissociation is fully reversible. The $12S_H$ subunit that had been dissociated at pH 9 was reconstituted by precipitation with 30% saturated ammonium sulfate and dialyzed under N_2 against 0.3 M phosphate buffer, pH 6.5, containing 0.03 M cysteine. The CD of reconstituted $12S_H$ in 0.1 M phosphate buffer at pH 5.8 is identical with that of the original $12S_H$ subunit. The significant difference in CD between the dissociated and undissociated $12S_H$ subunit indicates a large change in secondary structure. Qualitatively, it is clear from Figure 3 that there is a loss of α helix. Our analyses in Table II confirm the qualitative expectations. There is a 19% decrease in α-helical structure with an 8% increase in antiparallel β sheet and a 7% increase in the other category for the dissociation of the $12S_H$ subunit into the $2.5S_H$ monomers (no.

Table II: Secondary Structure from Analyzing CD Spectra

				β sheet		β turn					
no.	protein[a]	pH	helix	anti-parallel	parallel	type I	type II	type III	other types	other	total
1	$1.3S_E$ monomer	5.8	0.03	0.22	0.10	0.08	0.04	0.02	0.04	0.42	0.95
2	$1.3S_E$ monomer	9.0	0.03	0.16	0.12	0.07	0.03	0.02	0.03	0.41	0.87
3	$5S_E$ dimer	5.8	0.38	0.06	0.07	0.05	0.03	0.03	0.02	0.30	0.94
4	$5S_E$ dimer	9.0	0.39	0.08	0.06	0.06	0.03	0.03	0.03	0.31	0.99
5	$6S_E$ intact	5.8	0.30	0.11	0.08	0.06	0.03	0.03	0.03	0.35	0.99
6[b]	[0.18]$1.3S_E$ + [0.82]$5S_E$ = $6S_E$	5.8	0.31	0.09	0.08	0.06	0.03	0.03	0.03	0.32	0.93
7[c]	$6S_E \rightarrow (2)1.3S_E + 5S_E$	9.0	0.31	0.12	0.08	0.07	0.04	0.03	0.03	0.36	1.04
8	$12S_H$ intact	5.8	0.38	0.15	0.01	0.07	0.04	0.03	0.02	0.25	0.95
9[c]	$12S_H \rightarrow (6)2.5S_H$	9.0	0.19	0.23	0.00	0.08	0.05	0.03	0.03	0.32	0.93
10	TC	5.8	0.36	0.09	0.07	0.06	0.03	0.03	0.03	0.33	1.00
11[b]	[0.71]$6S_E$ + [0.29]$12S_H$ = TC	5.8	0.33	0.12	0.06	0.06	0.04	0.03	0.03	0.32	0.99
12[b]	[0.13]$1.3S_E$ + [0.58]$5S_E$ + [0.29]$12S_H$ = TC	5.8	0.33	0.11	0.06	0.06	0.03	0.03	0.03	0.30	0.94
13[c]	$TC \rightarrow (12)1.3S_E + (6)5S_E + (6)2.5S_H$	9.0	0.30	0.15	0.06	0.07	0.04	0.03	0.03	0.36	1.04
14[b]	[0.71]$6S_E$ + [0.29]$12S_H$ = TC	9.0	0.27	0.15	0.05	0.07	0.04	0.03	0.03	0.35	1.01

[a] 26S transcarboxylase is abbreviated TC. [b] The values in the brackets indicate the fraction of each measured CD that is summed to give the constructed CD spectrum. [c] The values in parentheses indicate the number of subunits in the dissociation.

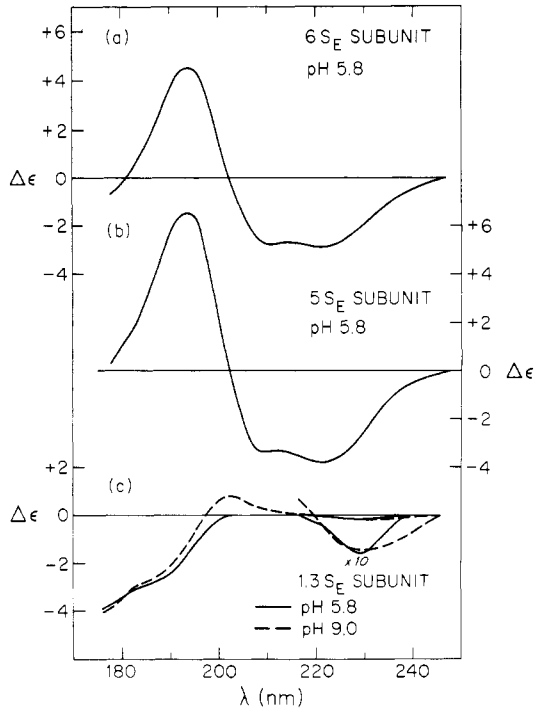

FIGURE 4: CD spectra of (a) the $6S_E$ subunit of transcarboxylase at pH 5.8 (–), (b) the $5S_E$ outer subunit at pH 5.8 (–), and (c) the $1.3S_E$ monomer from the $6S_E$ subunit at pH 5.8 (–) and pH 9.0 (---).

8 compared to no. 9, Table II). This change in secondary structure is consistent with the change seen for the entire 26S enzyme (no. 8 and 9 compared to no. 10 and 13, Table II).

We also determined that the change in secondary structure was dependent upon dissociation, and not upon the change in pH that brings about dissociation. The $12S_H$ subunit dissociates and associates rather slowly in 0.08 M phosphate buffer, pH 8.0, and 10^{-4} M dithioerythritol saturated with N_2. This fact was demonstrated by dialysis of the $12S_H$ subunit against the pH 8 buffer for 21 h and then subjecting the protein solution to ultracentrifugation at 60 000 rpm at 4 °C. Only 20% dissociation occurred after this extended dialysis. Similarly, when the $12S_H$ subunit had been dissociated at pH 9 and then was dialyzed for 3.5 h against the 0.08 M phosphate buffer at pH 8, only a trace of $12S_H$ subunit was observed; the predominant form was the $2.5S_H$ subunit and some $6S_H$ dimer. CD spectra of undissociated or dissociated $12S_H$ subunit at pH 8 are identical with those obtained at pH 5.8 and pH 9, respectively.

In contrast to the $12S_H$ subunit, the other subunits showed no detectable change in secondary structure on dissociation. The CD spectrum of the $6S_E$ subunit of transcarboxylase at pH 5.8 is given in Figure 4a. The CD at pH 9.0 is identical within experimental error and is not shown. Similarly, the CD of the $5S_E$ subunit does not change detectably between pH 5.8 and pH 9.0. The CD spectrum measured for this $5S_E$ dimer at pH 5.8 is shown in Figure 4b. The CD spectrum of the $1.3S_E$ monomer does change slightly with the change in pH as seen in Figure 4c. More interesting than this small change, however, is the unusual CD spectrum. Qualitative features indicate that there must be no α helix. Our method is fairly successful in analyzing even this unusual CD spectrum. We see in no. 1 and 2 of Table II that this monomer features large amounts of other structure, a fair amount of antiparallel and parallel β sheet, and an insignificant amount of α helix.

The CD spectra of the various subunits can be summed and compared with the CD spectra of the quaternary structures. Analyses for some of these constructed CD spectra are given in Table II. Comparison of no. 5 with 6, no. 10 with 11 and 12, and no. 13 with 14 shows that the analyses agree closely.

Discussion

The procedure of analysis for secondary structure is based on 16 different proteins for which the secondary structures were known from X-ray crystallography (Hennessey & Johnson, 1981). From these studies, it was found that there is enough information in the CD spectrum of a protein to predict all types of secondary structure. The error inherent to this method, based on the 16 proteins, yields a correlation coefficient generally greater than 0.90 for the predicted structures of the proteins and the X-ray determinations. For proteins for which X-ray data are unavailable, the reliability of the analysis can be evaluated to some extent by the approximation of the sum of the secondary structures to 1.0. While this conditions does not exclude erroneous analysis, it does serve as an analytical check without introducing artificial constraints. The sums are reasonably close to 1.0 in Table II with the possible exception of no. 2 for the $1.3S_E$ subunit at pH 9.0. Comparison of pH 5.8 and pH 9.0 for the $5S_E$ subunit, which have the same CD spectra within experimental error, gives an idea of the error in the analysis.

The CD spectral analysis will, of course, only apply when the structural characteristics of the protein are well represented in the 16 different proteins used in deriving the analysis. The $1.3S_E$ subunit is not a globular protein (Wood et al., 1975) and is unusual since it does not contain tryptophan. Of its 123 residues only one is tyrosine and only one is phenylalanine (Maloy et al., 1979). This protein also contains biotin. Fall et al. (1976) have shown that biocytin (biotin amide with the ϵ-amino group of lysine) has a positive band at 233 nm. They found in contrast to our results that the biotinyl subunit of acetyl-CoA carboxylase of *Escherichia coli* (M_r 22 500) has a spectrum typical of globular proteins with negative extrema at 222 and 208 nm. The spectrum indicated the presence of significant regions of α helix (Fall et al., 1976). Sutton et al. (1977) have determined the amino acid sequence of a large peptide (M_r 9100) from this biotinyl subunit, and Rylatt et al. (1977) have determined the sequence of small biotinyl peptides from pyruvate carboxylases. There is considerable homology in the sequences of these peptides in the region of the biotin with that of the biotinyl subunit of transcarboxylase (Maloy et al., 1979). From the sequences, it has been calculated by the Chou–Fasman method (Chou & Fasman, 1977) that there should be α-helical regions in each subunit.

Our measurements show there is very little α helix in the $1.3S_E$ subunit, although the Chou–Fasman calculation indicates that about 30% of the sequence has potential for forming α helix (Maloy et al., 1979). Everything else being equal, if the potential α helix were realized, this would cause an increase of about 0.7 $\Delta\epsilon$ unit in the negative bands at 210 and 220 nm and an increase of about 1.5 $\Delta\epsilon$ units in the positive band at 193.5 nm for the $6S_E$ subunit. The measured CD spectrum of intact $6S_E$ at pH 5.8 is virtually identical with the measured CD of $6S_E$ dissociated to $5S_E$ and $1.3S_E$ subunits at pH 9. Thus, there is apparently no formation of the structure predicted by the Chou–Fasman method for the $1.3S_E$ on forming the $6S_E$ subunit although compensating changes in secondary structure cannot be ruled out. However, the Chou–Fasman structure would go undetected with our present methods if it formed on assembly of the whole transcarboxylase. Furthermore, less than 23 residues of the 123 residues of the $1.3S_E$ biotinyl subunit of transcarboxylase are involved in linking together the $12S_H$ and $5S_E$ subunits (Kumar & Beegen, 1981). If formation of α helix were involved in this small portion

Table III: Composition of Transcarboxylase and Its Subunits

component	M_r	AA residues per molecule	fraction of 26S TC[a]
$1.3S_E$	12 000	123	0.130
$5S_E$	120 000	1094[b]	0.576
$6S_E$	144 000	1340	0.706
$12S_H$	360 000	3342[b]	0.294
26S TC	1 224 000	11382	1.000

[a] Transcarboxylase is abbreviated TC. [b] Zwolinski et al. (1977).

during binding, the change in the total α helix of transcarboxylase would not be sufficient to be detected. Likewise, if α-helix formation were involved in the $1.3S_E$ subunit when the carboxyl transfer occurs between the substrate sites on the $5S_E$ and $12S_H$ subunits, it might not occur in the $1.3S_E$ in the static situation when transfer is not occurring (Table III).

It is clear from our results that there is a very significant change in the secondary structure of the $2.5S_H$ monomer when they associate and form the hexameric $12S_H$ subunit. There is a 19% increase in α helix, an 8% decrease in antiparallel β sheet, and 7% decrease in other or random structure (no. 8 compared to no. 9 of Table II). It is interesting that Zwolinski et al. (1977), in considering models for the arrangement of the six polypeptides of the $12S_H$ subunit, suggested that in forming the isologous binding of the $12S_H$ subunit distortion would be necessary. Perhaps the presently observed changes in secondary structure are a reflection of this distortion. However, they also postulated distortion of the $5S_E$ subunit during assembly of the enzyme, and such a change has not been detected by our CD measurements. Again it is possible, though perhaps unlikely, that there are compensating changes in secondary structure between the respective subunits that balance so that the sum of the secondary structures of the complete enzyme does not differ greatly from the sum in the individual subunits.

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Thallium-205 Nuclear Magnetic Resonance Study of the Thallium(I)–Gramicidin A Association in Trifluoroethanol†

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ABSTRACT: Association of the thallous ion with gramicidin in 2,2,2-trifluoroethanol has been investigated by thallium-205 NMR spectroscopy. The data obtained suggest that the gramicidin dimer has two strong binding sites and one or more weak binding sites. Association constants for the strong binding sites were found to have the same value. From the temperature dependence of the strong binding site association constants, values for the association enthalpy and entropy of -2.13 ± 0.12 kcal/mol and $+5.45 \pm 0.04$ eu, respectively, were obtained.

Gramicidin A, a pentadecapeptide isolated from *Bacillus brevis*, is known to form channels which assist the passive transport of cations across membranes (Hladky & Haydon, 1972; Krasne et al., 1971). Significant effort has been directed at the study of ion association of gramicidin A in membrane systems (Myers & Haydon, 1972; Bamberg, & Lauger, 1974; Neher, 1975; Sandblom et al., 1977; McBride & Szabo, 1978; Urry et al., 1979, 1980; Veatch & Darkin, 1980; Venkatachalam & Urry, 1980). Work has also been performed on the solution conformations of gramicidin A (Glickson et al., 1972; Urry et al., 1972, 1975; Fossel et al., 1974; Veatch et al., 1974; Veatch & Blout, 1974; Heitz et al., 1979; Iqbal & Weidekamm, 1980; Sychev et al., 1980) and ion–gramicidin

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