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Primary Structure of Monkey Osteocalcin†

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ABSTRACT: The complete 49-residue amino acid sequence of osteocalcin from the old world monkey *Macaca fascicularis* has been determined by efficient combination of gas chromatography–mass spectrometry and Edman techniques. This vitamin K dependent protein of bone matrix contains three γ-carboxyglutamic acid residues at positions 17, 21, and 24, as well as a disulfide-bonded loop (23–29). Features of the sequence which apparently are required for the binding of Ca^{2+} have been strongly conserved throughout evolution.

Osteocalcin[1] is an abundant Ca^{2+}-binding protein indigenous to the organic matrix of bone, dentin, and possibly other mineralized tissues (Hauschka et al., 1975; Hauschka & Gallop, 1977; Gallop et al., 1980). This protein contains 47–51 amino acid residues (M_r 5200–5900) depending on the species (Price et al., 1979; Poser et al., 1980; Linde et al., 1980; Carr et al., 1981b). Osteocalcin is distinguished by its content of three γ-carboxyglutamic acid (Gla)[2] residues. Gla appears in the protein as a result of posttranslational, vitamin K dependent carboxylation (Lian & Friedman, 1978) of specific glutamic acid residues at sequence positions 17, 21, and 24 (Price et al., 1979; Poser et al., 1980; Carr et al., 1981b). Biosynthesis and vitamin K dependent carboxylation of osteocalcin occur in bone tissue in organ culture (Lian & Heroux, 1979) and in isolated bone cells in tissue culture (Nishimoto & Price, 1980). Osteocalcin appears coincident with the onset of calcium phosphate mineral deposition in developing embryonic bone and is probably a specific product of cells differentiated with respect to bone formation (Hauschka & Reid, 1978; Hauschka, 1979b). While the function of osteocalcin is not known, this protein exhibits several interesting properties: (1) specific Gla-dependent binding of Ca^{2+} ions (Hauschka & Gallop, 1977; Poser & Price, 1979); (2) Ca^{2+}-induced transition to the α-helical conformation (Hauschka, 1981); (3) tight association with hydroxylapatite and inhibition of brushite → hydroxylapatite metamorphosis in vitro (Hauschka et al., 1975; Hauschka & Gallop, 1977); (4) low-level circulation in blood plasma (Price & Nishimoto, 1980); (5) 1,25-$(OH)_2$-vitamin D_3 stimulation of its biosynthesis (Price & Baukol, 1981); and (6) derivation from higher molecular weight precursor proteins (Lian & Heroux, 1979; Hauschka, 1979b; Nishimoto & Price, 1980). Among osteocalcins of different species, the homology of the Gla positions and the disulfide-bonded loop argues for the functional importance of these conserved regions of the protein. However, numerous structural questions remain, as well as delineation of the calcium binding sites, the role of hydroxyproline in position 9, and the apparent lack of requirement for Gla_{17} in human osteocalcin (Poser et al., 1980).

The protein sequencing technique of gas chromatography–mass spectrometry (GC–MS), recently reviewed by Biemann (1980), has proved advantageous for Gla-containing proteins. Decarboxylation of the intact protein in DCl–D_2O readily converts Gla to γ,γ-dideuterioglutamic acid (Carr, 1980; Hauschka, 1979a; Carr & Biemann, 1980; Carr et al., 1981b), a chemically stable and mass spectrometrically unique derivative of Gla. Following partial enzymatic or acidic hydrolysis and derivatization, the complex mixtures are analyzed (without isolation of individual peptides) by GC–MS (Biemann, 1980; Carr et al., 1981a). Peptide fragments in which Gla residues were present show sequence ions in their mass spectra corresponding to those of glutamic acid, but shifted upward by 2 mass units per Gla (Carr, 1980; Carr & Biemann, 1980; Carr et al., 1981b). Incomplete posttranslational carboxylation at Gla positions may also be determined precisely from the relative abundance of characteristic sequence ions. Chicken bone osteocalcin was the first Gla-containing protein to be sequenced by this technique (Carr, 1980; Carr et al., 1981b), while cow, swordfish, and human osteocalcins have been elucidated by standard Edman methods (Price et al., 1979; Poser et al., 1980). Sequential Edman degradation is complementary to GC–MS because the former provides information on long-range order, amide assignments, His positions, and differentiation of Ile from Leu. This paper presents the complete structure of monkey osteocalcin as determined by GC–MS and Edman methodology.

Materials and Methods

Sacrifice of young adult male cynomolgus monkeys (*Macaca fascicularis*) by sodium pentothal injection was followed by

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[1] Osteocalcin has also been referred to as "bone Gla protein" or "BGP" in the literature (Nishimoto & Price, 1980).

[2] Abbreviations: Gla, γ-carboxyglutamic acid; CmCys, *S*-carboxymethylcysteine; Hyp, 4-hydroxyproline; PTH, phenylthiohydantoin; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; xLeu, Leu or Ile from GC–MS data.

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immediate dissection. Bones were cleaned of adhering soft tissue and frozen in dry ice. Typically 200 g (wet weight) of bone was obtained from a 5-kg specimen. The bone was processed immediately by pulverization in a Waring blender in 500 mL of ice-cold 20 mM Tris-HCl, pH 7.4, containing the protease inhibitors phenylmethanesulfonyl fluoride (30 μM), *p*-(hydroxymercuri)benzoic acid (100 μM), 6-aminocaproic acid (10 mM), and benzamidine (5 mM). Within 30 min at 0 °C, ten repetitions of decanting, resuspension, and rehomogenization, finally employing a Polytron (Brinkman Instruments), reduced the bone to a fine white granular consistency. Lyophilization of the powder was followed by pulverization at −196 °C (Spex mill, Spex Industries, Metuchen, NJ) to a particle size of 74–250 μm, yielding 100 g of dry powder. Osteocalcin was extracted for two 24-h periods by stirring 100 g of bone powder in 1 L of 0.5 M ammonium ethylenediaminetetraacetate, pH 6.1, containing the same protease inhibitors as above. The extracts were clarified (12000*g*, 20 min) and dialyzed in Spectrapor 1 membrane tubing (M_r 6000 cutoff) against water for 2 days, followed by 0.05 M ammonium bicarbonate for 4 days. Following lyophilization, the extracts were chromatographed on Sephadex G-75 and DEAE-cellulose (DE53, Whatman) as previously described (Hauschka & Gallop, 1977; Carr et al., 1981b).

Homogeneous monkey osteocalcin was obtained after being chromatographed 3 consecutive times on DE53. End-group analysis and high-pressure liquid chromatography (Figure 1) served as evidence for homogeneity. The yield of pure protein from a single animal was typically 85 mg. Monkey osteocalcin exhibits an ultraviolet absorption maximum at 276 nm, with $E^{1\%} = 14.3$ for the calcium-free apoprotein in 20 mM Tris-HCl, pH 7.4. Reduction and carboxymethylation with freshly recrystallized iodoacetic acid were performed as described (Carr et al., 1981b), and the protein was rechromatographed on DE53 cellulose, eluting at 0.31 M ammonium bicarbonate, pH 7.75. No *S*-carboxymethylcysteine was formed in the absence of the reducing agent dithiothreitol, confirming the presence of a disulfide bond in osteocalcin. Amino acid analysis was performed with a Beckman 121M analyzer (Hauschka, 1977). No significant variation in composition was observed for acid hydrolysis times ranging from 24 to 72 h. Established methods of hydrolysis in 2 M KOH were employed for quantitation of Gla and Trp (Hauschka, 1977). Half-cystine was determined as *S*-carboxymethylcysteine by using a synthetic standard (Sigma). Phosphate was determined in acid-hydrolyzed protein following Ames (1966). Total neutral sugar was measured by phenol–H_2SO_4 (McKelvy & Lee, 1969) with a D-glucose standard.

HPLC of Monkey Osteocalcin. Osteocalcin was analyzed for homogeneity by reverse-phase HPLC on a μBondapak C_{18} column (3.9 × 300 mm; Waters) with a gradient of 0–45% (v/v) acetonitrile in 0.1% H_3PO_4 (Hauschka, 1979b; Fullmer & Wasserman, 1979; Carr et al., 1981b). Peak detection was by the absorbance at 210 nm. Enzyme digests of osteocalcin were monitored in real time by injection of 2-μg aliquots of substrate. For molecular weight determination by gel filtration (Ui, 1979), 6 M guanidine hydrochloride (Pierce)–60 mM Tris-HCl–5 mM dithiothreitol, pH 8, was pumped at 1 mL/min through tandem TSK columns (3000SW, 7.5 × 700 mm; 2000SW, 7.5 × 1500 mm; Varian). Absorbance was monitored at 278 nm.

Edman Sequencing. Automatic sequenator degradations were performed as described (Carr et al., 1981b), employing a Beckman 890C sequenator. In-line conversion to PTH-amino acids with methanolic HCl (65 °C, 10 min) was

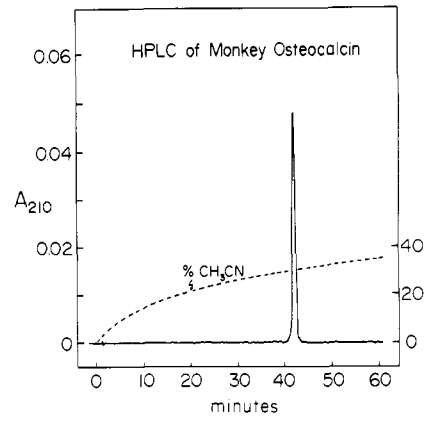

FIGURE 1: HPLC profile of pure monkey osteocalcin. Protein (1 μg) was applied to a C_{18} column operated in the reverse-phase mode with 0.1% H_3PO_4 (see Materials and Methods). Gradient elution resolved osteocalcin as a single sharp peak at 29.5% (v/v) acetonitrile.

achieved with a P-6 autoconverter (Sequemat; Watertown, MA). Reduced, carboxymethylated monkey osteocalcin (100 nmol) was dissolved in trifluoroacetic acid and placed in the cup with 3 mg of Polybrene (Pierce) (Carr et al., 1981b). Methanol–dichloromethane (3:7) was removed under nitrogen, and samples were dissolved in methanol containing 20 nmol/mL PTH-norleucine as internal standard. For complete resolution of the PTH derivatives by HPLC, a 4.6 × 250 mm Zorbax-ODS (Du Pont) column was operated at 54 °C and eluted at 0.9 mL/min with a gradient of 21–65% acetonitrile in 10 mM sodium acetate, pH 5.5. PTH derivatives of Asp, Glu, and CmCys were identified as the methyl esters. Detection at 206 nm showed full-scale peak deflection by 400 pmol of PTH-norleucine.

GC–MS Sequencing. Reduced, carboxymethylated monkey osteocalcin was decarboxylated in a low-pressure atmosphere of DCl under conditions which quantitatively convert Gla to γ,γ-dideuterioglutamic acid (Hauschka, 1979a; Carr & Biemann, 1980; Carr et al., 1981b). After partial enzymatic or acidic hydrolysis, the peptide mixtures were converted to the *N*-trifluorodideuterioethyl *O*-trimethylsilyl polyamino alcohols and injected into the gas chromatograph–mass spectrometer (Carr et al., 1981a). The high volatility, predictable gas chromatographic retention behavior and easily interpretable and predictable mass spectra of these polyamino alcohol derivatives have been thoroughly described elsewhere (Biemann, 1980). Trypsin (TPCK-trypsin, Worthington) digestions were performed at 37 °C, pH 8.5, for 3.5 h; proteinase K (E. Merck) and thermolysin (Calbiochem) digestions were at 55 °C, pH 8.5, for 2.5 and 3.5 h, respectively. An enzyme/substrate ratio of 1/50 (w/w) was used in all cases. Partial acid hydrolysis was performed in vacuo at 110 °C in constant-boiling HCl for 15 min (Carr et al., 1981a).

Results and Discussion

Osteocalcin was readily purified in good yield from bones of *M. fascicularis*. Homogeneity was apparent in HPLC profiles of the protein (Figure 1). The high resolution of this technique is evident from the fact that closely related molecular species such as monkey and cow osteocalcin, differing only by substitution of Tyr_3-Gln_4 (monkey) for Asp_3-His_4 (cow) among 49 total amino acid residues, are readily separated by HPLC, with the cow protein eluting at 27.6% (v/v) acetonitrile compared with 29.5% for monkey. Other criteria of purity include (1) end-group analysis, showing Tyr as the exclusive NH_2-terminal residue, and (2) the integral amino acid analysis data (Table I). Monkey osteocalcin is devoid of Thr, Ser, and Met

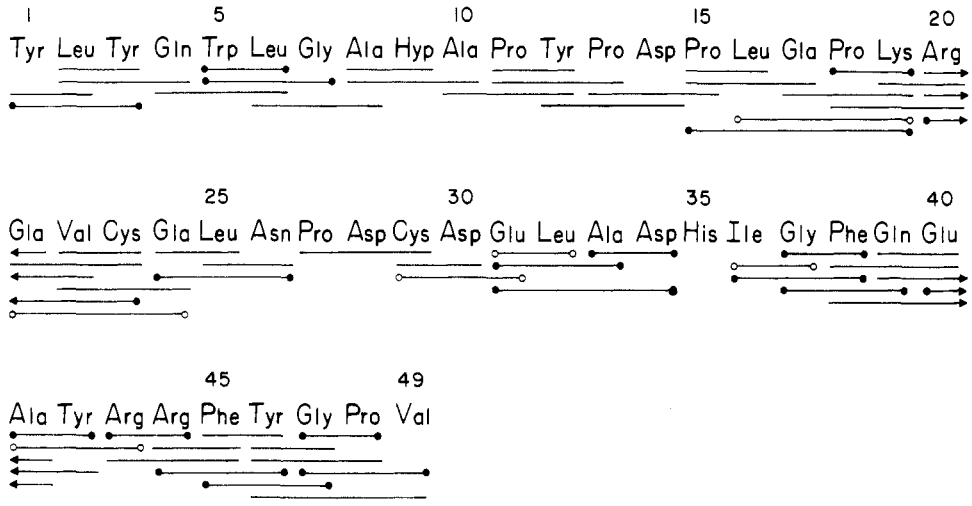

FIGURE 2: Primary structure of monkey osteocalcin showing alignment of sequences identified by GC–MS in partial acid hydrolysates (—), trypsin/thermolysin digests (○), and trypsin/proteinase K digests (●). Peptides in enzymatic digests are only shown if they were not redundant with those identified in partial acid hydrolysates. Residues 1–46 were also identified by sequential Edman degradation.

Table I: Amino Acid Composition of Monkey Osteocalcin

amino acid	res/49 res[a]	amino acid	res/49 res[a]
Gla[b]	2.79 (3)	Val	1.84 (2)
Hyp	0.93 (1)	Met	<0.05 (0)
Asp	5.45 (5)	Ile	0.91 (1)
Thr	<0.05 (0)	Leu	5.09 (5)
Ser	<0.05 (0)	Tyr	4.76 (5)
Glu	4.17 (4)	Phe	1.89 (2)
Pro	6.27 (6)	His	1.18 (1)
Gly	2.99 (3)	Lys	1.23 (1)
Ala	4.01 (4)	Trp[b]	0.92 (1)
half-cystine[c]	1.98 (2)	Arg	2.83 (3)

total amino acid residues	49
mol wt	5889
mean residue weight	120.19

[a] Mean of 16 determinations on eight separate acid hydrolysates and four alkaline hydrolysates. Standard error of the mean was less than ±2% for all amino acids except Gla, Hyp, and Trp (±5%). Integral values confirmed by the sequence appear in parentheses. [b] Determined in alkaline hydrolysates. [c] Determined as *S*-carboxymethylcysteine after acid hydrolysis of reduced, carboxymethylated protein.

and contains insignificant amounts of phosphate (0.036 mol of P/mol of protein) and neutral sugar (0.29% by weight). The Tyr content (14% by weight) accounts for the high $E^{1\%}_{276nm}$ value of 14.3. A molecular weight of 5600 ± 400 was determined for the protein by HPLC gel filtration.

The complete amino acid sequence of monkey osteocalcin (Figure 2) was determined by an efficient combination of GC–MS and Edman methods. We have previously demonstrated that the joint use of these entirely independent and complementary sequencing approaches is more efficient than either method used alone (Biemann, 1980; Herlihy et al., 1981). Automatic Edman degradation was carried out successfully for 46 steps before washout of the COOH-terminal tripeptide. The repetitive cycle yield was 92%, with less than 6% carry-over of residue *n* in cycle *n* + 1 and no "anticipation" at cycle *n* − 1. PTH-Gla expected at positions 17, 21, and 24 was recovered only in low yield, presumably due to poor extraction by chlorobutane (Fernlund & Stenflo, 1979); a small amount of PTH-Glu methyl ester was observed. Positive identification of the Gla positions was achieved by GC–MS (Carr & Biemann, 1980; Carr et al., 1981b). Eleven distinct di- to pentapeptide derivatives with the expected mass shifts resulting from γ,γ-dideuterioglutamic acid at Gla positions were observed as indicated in Figure 2.

Overlapping peptide sequences which were identified by GC–MS are shown in Figure 2. Reassembly of these partial sequences obtained from GC–MS experiments on the derivatized acid and enzymatic hydrolysates was carried out both manually and with the aid of a computer program (Anderegg, 1977). The sequences of four oligopeptides encompassing the regions Tyr_1–Asx_{14}, Pro_{15}–Asx_{26}, Pro_{27}–Asx_{34}, and $xLeu_{36}$–Val_{49} were determined in this way. These sequences account for all amino acids known to be present by composition, with the exception of His_{35}. The continuous sequence Tyr_1–Tyr_{46} obtained by Edman degradation corroborated the GC–MS oligopeptide sequences indicated above and clearly identified His_{35}. Furthermore, the Edman methodology provided assignment of the three amide positions (Gln_4, Asn_{26}, and Gln_{39}) and readily identified Ile rather than Leu at position 36, thereby facilitating tasks which would have required additional experiments had the GC–MS technique been used alone (Carr, 1980; Biemann, 1980; Carr et al., 1981b). HPLC profiles of tryptic digests were compatible with the primary structure shown in Figure 2. The COOH-terminal pentapeptide Phe_{45}-Tyr-Gly-Pro-Val_{49} was quantitatively released within several minutes of digestion, as previously observed for osteocalcin from another species (Carr et al., 1981b). Isolation of this pentapeptide by HPLC yielded the expected integral amino acid composition.

GC–MS and amino acid analysis data indicate that post-translational γ-carboxylation of the Glu residues to Gla at positions 17, 21, and 24 is complete in monkey osteocalcin. No corresponding Glu-containing peptides were observed in the GC–MS data, although they would have been readily detectable at 5–10% abundance (Carr, 1980; Carr et al., 1981b). In contrast to other species, Glu_{17} in human osteocalcin is reportedly not carboxylated (Poser et al., 1980). This is possibly an artifact of the immunologic purification procedure employed for human osteocalcin (Poser et al., 1980) and the increased affinity of decarboxylated osteocalcin for antibody (P. V. Hauschka et al., unpublished experiments), which could have selectively concentrated a minor population of partially carboxylated human osteocalcin. An alternate possibility is that substitution of Arg_{19} in human osteocalcin for Lys_{19} in the monkey and cow proteins may hinder carboxylation of Glu_{17}.

Hydroxyproline occurs at position 9 in three of the six species for which osteocalcin sequence information is available, thus placing this protein squarely in the ranks of collagen, elastin, complement $C1_q$, and acetylcholinesterase as a substrate for prolyl hydroxylase. The osteocalcin sequence is invariably Leu_6-Gly-Ala-Hyp-Ala-Pro_{11}, as observed in monkey, as well as cow (Price et al., 1979) and rat (Linde et al., 1980). This sequence is reminiscent of the Y-position hydroxylation characteristic of vertebrate collagen Gly-X-Y triplets. Presumably the presence or absence of Hyp in osteocalcin reflects the specificity of prolyl hydroxylase. The abundance of proline in this region of osteocalcin (50% of the residues between 9 and 18), and its activity as a substrate for hydroxylation, raises the possibility that collagen-like poly(L-proline) II conformation exists in this domain of osteocalcin.

Monkey osteocalcin shares many structural features with osteocalcins of other species including human (Poser et al., 1980), cow (Price et al., 1979), and chicken (Carr et al., 1981b). There is also some similarity to the relatively primitive swordfish osteocalcin (Price et al., 1979) and to the partial sequence of a Gla-containing protein isolated from rat dentin (Linde et al., 1980). Gla residues at positions 17, 21, and 24, as well as the Cys_{23}-Cys_{29} disulfide bond, are observed in all species. Only two translational substitutions exist between monkey and human osteocalcins [Val_{10} and Arg_{19} in human (Poser et al., 1980)] and between monkey and cow [Asp_3 and His_4 in cow (Price et al., 1979)]. Monkey osteocalcin differs from the chicken protein (Carr et al., 1981b) by 15 substitutions, 1 insertion, and 2 deletions, and from swordfish (Price et al., 1979) by 23 substitutions, 4 insertions, and 2 deletions. Given the evolutionary divergence (Dayhoff, 1972) of old world monkeys from apes and humans (25–30 million years), cow (75 million years), chicken (300 million years), and swordfish (400 million years), the acceptance rate for point mutations in osteocalcin is 11.2 ± 1.4 mutations per 100 links per 100 million years, following the nomenclature of Dayhoff (1972). This is in close agreement with the mutation rates for cytochrome c (11.5) and β-globin (10.7). Interestingly, the NH_2 terminus of osteocalcin exhibits considerable variation, in contrast to the strongly conserved central portion of the molecule which is the locus of the Gla residues and the Ca^{2+} binding sites (Hauschka, 1981).

Osteocalcins of all species show common secondary structural features arguing for functional preservation throughout evolution (Hauschka, 1981). Employing the predictive methods of Chou & Fasman (1978) with the assumption that Gla and Glu have similar probability parameters, two major α-helical domains are predicted in monkey osteocalcin. One of these, the "Gla helix", contains three Gla residues (Pro_{18}–Leu_{25}), while the other region, the "Asp-Glu helix" (Asp_{30}–Ala_{41}) contains four anionic side chains. A highly probable β turn at Asn_{26}–Cys_{29} ($[p_t] = 11.1 \times 10^{-4}$), stabilized by the Cys_{23}–Cys_{29} disulfide bridge, forces the two α-helical segments into opposition. Circular dichroism studies have verified the existence of α-helical conformation in osteocalcin and have further shown that millimolar levels of Ca^{2+} or other specific cations are required to offset electrostatic repulsion if the highly anionic osteocalcin molecule is to achieve its full potential of ~40% α helix (Hauschka, 1981). A striking feature of the α-helical domains is the regular spacing of the charged and hydrophobic amino acids at intervals of three or four residues (e.g., Gla_{17}, Gla_{21}, and Gla_{24}). In conjunction with the 3.6 residues/turn of the Pauling–Corey α helix, this creates amphipathic helices with distinct anionic and hydrophobic surfaces. The α-helix periodicity of 5.4 Å/turn forces the Gla residues into register such that the Ca^{2+} binding sites are most probably 5.4 Å apart (Hauschka, 1981). This is apparently a highly significant structural feature of osteocalcin which may govern its strong interaction with the hydroxylapatite mineral phase of bone and dentin where major Ca^{2+} lattice spacings of 5.45 and 5.84 Å exist (Kay et al., 1964).

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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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