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# Oligomeric structure of the multifunctional protein CAD that initiates pyrimidine biosynthesis in mammalian cells

(carbamoyl-phosphate synthetase/aspartate carbamoyltransferase/dihydroorotase/multifunctional enzymes/oligomeric structure)

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ABSTRACT **The first three steps in mammalian *de novo* pyrimidine biosynthesis are catalyzed by the multifunctional protein designated CAD. Regions of the single 240-kDa polypeptide chain are folded into separate structural domains that have discrete functions. Previous studies suggested that CAD forms predominantly trimers. The trimers are found to be in slow equilibrium with hexamers and higher oligomers composed of multiples of three copies of the CAD polypeptide chain. However, quantitative chemical crosslinking studies of CAD with dimethyl suberimidate were used here to show a progressive conversion of monomer to crosslinked hexamer. High levels of the hexamer accumulate in the reaction mixture, suggesting that the major oligomeric form is hexameric, although residual amounts of smaller oligomers remain present. Larger oligomers may form by association of hexamers and are seen after longer crosslinking times. Sucrose gradient centrifugation shows a 20.8S species to be the slowest sedimenting peak, while the larger species sediments at 27.9 S. Electron microscopic studies of rotary-shadowed preparations of CAD have confirmed that, while small amounts of other oligomeric forms are present, the CAD monomer is primarily associated into cyclic hexamers with an open planar appearance.**

In mammalian cells, the enzymes that catalyze the initial steps of the *de novo* pyrimidine biosynthetic pathway are consolidated in a single 240-kDa polypeptide designated CAD (1). This chimeric protein has the glutamine-dependent carbamoyl-phosphate synthetase, aspartate transcarbamoylase (ATCase) and dihydroorotase (DHOase) activities as well as regulatory sites that bind the allosteric effectors UTP and phosphoribosyl 5′-pyrophosphate. CAD can be cleaved by proteolytic enzymes such as elastase (2) into enzymatically active fragments, suggesting that regions of the polypeptide are folded into separate structural domains that carry the different functions of the molecule. Thus far we have isolated two fully functional domains from proteolytic digests. The ATCase activity is associated exclusively with a 40-kDa fragment called the ATCase domain (3), while a distinct 44-kDa species, the DHOase domain, catalyzes the DHOase reaction (4).

The protein is an oligomer composed of multiple copies of the 240-kDa polypeptide. Trimers, hexamers, nonamers, and higher oligomeric forms have been reported (1). These authors found that the predominant form is the trimer, and that this species is in slow equilibrium with lower concentrations of hexamer and much smaller, but significant amounts, of the larger oligomers. All of these species are composed of multiples of three polypeptides, suggesting that the trimer is the fundamental oligomeric unit. There is evidence (1, 5) that the oligomeric structure is extremely sensitive to proteases and that nicking the polypeptide results in a marked reduction in the molecular weight of the protomer.

We have examined the oligomeric structure of CAD prepared under conditions that eliminate nicking of the polypeptide chain (6, 7). Sedimentation analysis, chemical crosslinking, and electron microscopic studies have shown that CAD forms stable hexamers and that hexamers can associate to form higher oligomeric forms.

## METHODS

**CAD Purification.** CAD was isolated from the overproducing mutant Simian virus 40-transformed Syrian hamster cell line 165-23, which was a gift of George Stark (Imperial Cancer Research Fund Laboratory, London). The cells were grown as described by Kempe *et al.* (8) at the Massachusetts Institute of Technology Cell Culture Center (Cambridge, MA) and removed from the surface by incubation in 0.5 mM EDTA in phosphate-buffered saline at 37°C for 5 min. CAD was isolated by the method of Coleman *et al.* (1) and stored at −70°C at a concentration of 3 mg/ml in 20 mM Tris/50 mM KCl/4 mM glutamine/4 mM aspartate/0.1 mM EDTA/1 mM dithiothreitol/5% glycerol/30% dimethyl sulfoxide, pH 7.4.

**Chemical Crosslinking.** The crosslinking reaction was initiated by the addition of dimethyl suberimidate (Pierce) to CAD in storage buffer to give a final concentration of 0.3 mg of dimethyl suberimidate and 0.2 mg of CAD per ml (1, 9). The pH of the reaction mixture was 9.2. The reaction was carried out at room temperature, and aliquots were removed at timed intervals from 10 to 180 min and overnight. The crosslinking was quenched by the addition of 1 M glycine to a concentration of 0.1 M. The crosslinked species were analyzed by electrophoresis on $NaDodSO_4$/composite 2.2% acrylamide–0.5% agarose tube gels (1, 10) with the Weber and Osborn (11) phosphate buffer system. Electrophoresis was carried out for 7 hr at a constant current of 8 mA per tube. The gels were stained with Coomassie blue and scanned with a Helena Laboratories (Beaumont, TX) Quick Scan densitometer.

**Sucrose Density Centrifugation of CAD.** The buffer exchange method described by Penefsky (12) was used to remove the dimethyl sulfoxide and glycerol from CAD samples used for sucrose gradient centrifugation. The protein at 2.0–2.5 mg/ml (0.20 ml; 0.4–0.5 mg in 0.05 M Tris/0.05 M glycine, pH 8.7) was layered onto 5.0 ml of 5–20% linear sucrose gradients (Schwarz/Mann density gradient grade) in 0.05 M Tris glycine (pH 8.7) and then centrifuged for 0.5–4 hr at 189,000 × *g* in a Beckman L5-65 ultracentrifuge (at 4°C; SW 50.1 rotor). The gradients were then fractionated into 25 0.21-ml aliquots. Each aliquot was assayed for DHOase activity (13), and the concentration of sucrose was determined by refractometry (Bausch and Lomb model Abbe-3L).

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Abbreviations: ATCase, aspartate transcarbamoylase; DHOase, dihydroorotase.

The sedimentation coefficient was calculated by the method of McEwen (14).

**Electron Microscopy.** CAD molecules were prepared for rotary shadowing by the method of Tyler and Branton (15). Immediately before being sprayed, CAD stock solutions (2 mg/ml in storage buffer) were diluted to 20–100 μg/ml in a volatile buffer of 60% glycerol/0.33 M ammonium acetate, pH 7.3, mixed thoroughly, and sprayed at room temperature onto a freshly cleaved mica surface. Sprayed mica pieces were mounted on a rotary stage in an Edwards (Sussex, England) vacuum evaporator and dried by evacuating for 0.5–1 hr at $10^{-5}$ to $10^{-6}$ torr (1 torr = 133.3 Pa). A 6° shadow angle was used for rotary shadowing by evaporation of 0.008-inch (0.002 cm) platinum wire from a heated tungsten filament. The resulting metal replicas were coated at a 90° angle with a supporting film of carbon, floated onto a distilled water surface, and picked up on bare 400-mesh copper grids. Micrographs were recorded with a Philips EM 301 electron microscope operating at 60-kV accelerating voltage. The final magnification in each micrograph was determined by measurement of the 395-Å repeat of negatively stained tropomyosin paracrystals (16) on negatives taken at the same time as the particular micrographs of CAD. Distances on the negatives were measured with a Nikon microcomparator. The average diameter of the hexamers was measured on print enlargements of the electron microscope negatives. The dimensions of the molecules were not corrected for metal thickness, but typically a 10-Å thickness of metal is deposited on the surface (17).

## RESULTS

**Chemical Crosslinking.** The structure of CAD was investigated by chemically crosslinking the protein with dimethyl suberimidate. After quenching aliquots of the reaction mixture at various times by the addition of excess glycine, the samples were analyzed by $NaDodSO_4$ gel electrophoresis on composite polyacrylamide–agarose gels (Fig. 1). A series of discrete species of increasing molecular weight sequentially appeared as crosslinking continued. Six major, regularly spaced bands representing the CAD monomer and each oligomer (dimer, trimer, and so forth) up to and including the hexamer were observed. The linear relationship between the log of the molecular weight, calculated assuming that these species were all multiples of the CAD polypeptide chain, and electrophoretic mobility confirmed this assignment (Fig. 2).

The amount of each crosslinked species on the gel was quantitated by scanning densitometry (Fig. 3). The time course of the crosslinking reaction (Fig. 4) showed that the monomeric form, representing uncrosslinked CAD, rapidly disappeared as the reaction proceeded. After crosslinking proceeded for 1 hr, the only major species observed on the gel was the hexamer. Continued crosslinking produced no heptamer, although during the later stages of crosslinking, a diffuse band of higher molecular weight material began to accumulate. The latter probably resulted from crosslinking between hexamers or hexamers and smaller oligomers. Moreover small, but significant amounts of each oligomer smaller than the hexamer remained even after extensive crosslinking, as seen in the densitometric scans (Fig. 3). Crosslinking kinetics, shown in Fig. 4, indicated that the time-dependent accumulation of each oligomer corresponded to that expected for the sequential crosslinking of a population of molecules that was primarily hexameric but also contained a small fraction of each of the smaller oligomers. The reaction was nearly complete after 2 hr, and the relative amount of each oligomer (see Fig. 8) remained constant throughout the course of the 12-hr reaction, even with a large excess of crosslinking agent. Sixty-eight percent of the protein existed as hexamers.

**Sucrose Gradient Centrifugation of CAD.** The oligomeric structure of CAD was investigated also by sucrose gradient centrifugation. The sedimentation profile obtained after centrifugation for various times (Fig. 5) indicated that CAD was a complex mixture of oligomers. After 30 min a single, somewhat asymmetric peak was observed that gradually was resolved into two or more components as the time of centrifugation increased. The distances of each of the two major species from the center of rotation is plotted against time of centrifugation in Fig. 5 *Inset*. The linear plots indicated that both species were sedimenting at a constant velocity as expected for a noninteracting mixture of oligomers. The more slowly sedimenting species had a sedimentation coefficient $s^0_{20,w} = 20.8$ (centered at fraction 16 in the 4-hr run), whereas the more rapidly sedimenting form (centered at fraction 12) had a $s^0_{20,w} = 27.9$. The latter peak had a large broad shoulder on its leading edge, indicating that even higher oligomeric forms were also present. The slowly sedimenting species was relatively stable because on recentrifugation it sedimented as a single symmetrical species with the same sedimentation coefficient. Chemical crosslinking of this species demonstrated that it was the hexamer.

**Electron Microscopy.** Replicas of CAD molecules, prepared by rotary shadowing with platinum, showed globular protomers, assembled into cyclic structures around a hollow center. The monomer appeared as a slightly flattened ellipsoid with a diameter of ≈100 Å, although the thickness of the platinum coating makes it impossible to discern any substructure. A field of CAD molecules is shown in Fig. 6. While planar hexamers were predominant, other larger and smaller oligomeric structures were also observed. The gallery of images in Fig. 7 shows representative assemblies consisting of two, three, four, five, and six protomers, as well as some larger structures. Twenty electron micrograph fields from three separate experiments were scored for oligomeric size; in all, 2000 assembled structures were counted. Of these

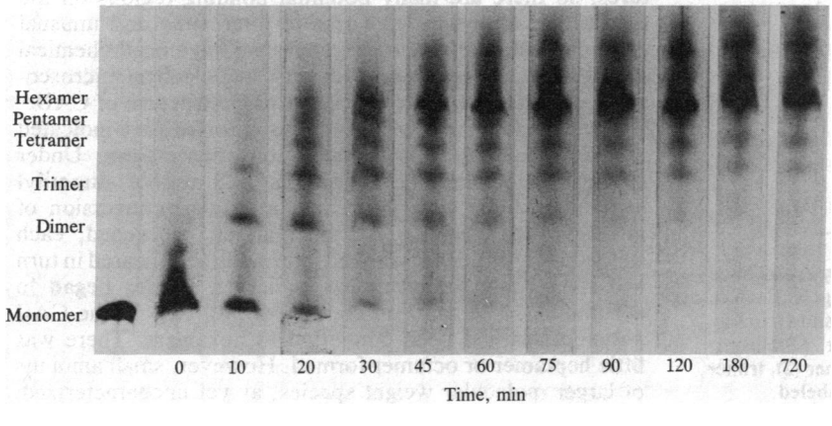


FIG. 1. Polyacrylamide/agarose gel electrophoresis of covalently crosslinked CAD. The crosslinking reaction mixture contained 0.3 mg of dimethyl suberimidate and 0.2 mg of protein per ml (pH 9.2). At the times indicated, aliquots of the reaction mixture were quenched with glycine to 0.1 M. The time dependence of the distribution of crosslinked species was analyzed on composite 2.2% acrylamide–0.5% agarose gels.

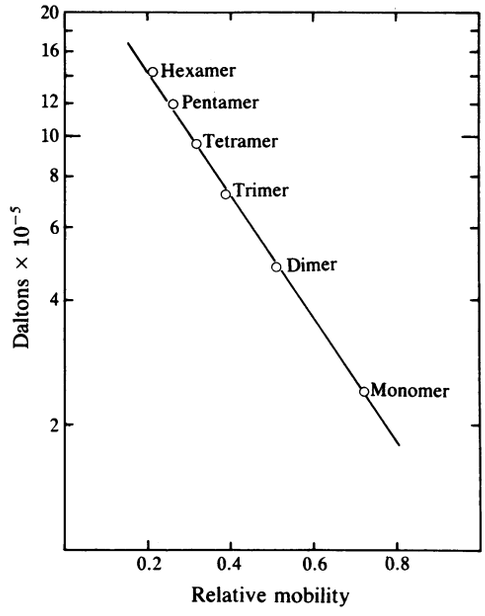


FIG. 2. Semilogarithmic plot of the molecular weight versus mobility of crosslinked CAD. The molecular weights used were based on the assumption that the observed species were all integral multiples of the CAD polypeptide chain.

structures, 10% were polymers of $n > 7$, possibly representing molecules that fell on top of one another. The scoring results are presented in the open bars of Fig. 8, in the form of a histogram showing the percentage of CAD protomers

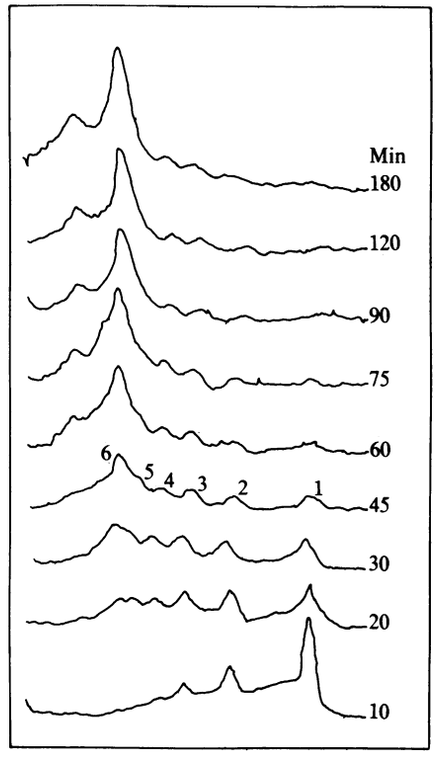


FIG. 3. Densitometric scans of species of chemically crosslinked CAD resolved on composite polyacrylamide–agarose gels. Each of the gels shown representing different incubation times shown in Fig. 1 was scanned on a Helena Quick Scan densitometer. Mobility is from left to right. Peaks representing monomer (1), dimer (2), trimer (3), tetramer (4), pentamer (5), and hexamer (6) are labeled.

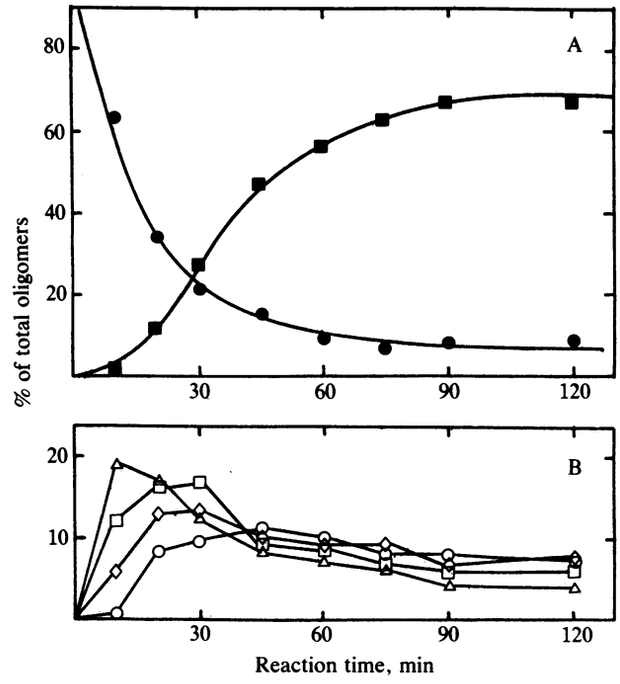


FIG. 4. Determination of the kinetics of crosslinking of CAD by dimethyl suberimidate. The experimental details are described in Figs. 1 and 3. The time dependence of the percentage of each size of oligomer as indicated by the ratio of its peak area to the total area of all observed peaks is shown. (*A*) Percentages of monomer (●) and hexamer (■). (*B*) Percentages of dimer (△), trimer (□), tetramer (◇), and pentamer (○).

found in each oligomeric form. The preponderance of hexamers is evident; 60% of the CAD polypeptide chains were found in hexamers. The histogram also gives a side-by-side comparison of the results obtained by chemical crosslinking and electron microscopy. The proportion of each oligomeric form determined by these two methods was very similar.

## DISCUSSION

Chimeric proteins are comprised of a single type of polypeptide in which contiguous chain segments are autonomously folded into functional domains. These polypeptides often associate to form oligomers. Thus, although each chain contains all of the functional elements of the complex, there may be functional or structural interactions between domains on different polypeptide chains. Previous studies showed that CAD exists as a mixture of oligomers composed of multiples of three copies of the CAD polypeptide chain (1). We have found (3) that the isolated domains, purified from controlled proteolytic digests, also have well-defined subunit structures, so there are many potential bonding regions on the polypeptide that may give rise to interesting and unusual oligomeric structures. In this study we have used chemical crosslinking, sedimentation velocity, and electron microscopy to investigate further the quaternary structure of CAD.

The chemical crosslinking studies reported here indicated that the hexamer was the major oligomeric form. Under moderate crosslinking conditions (0.3 mg of dimethyl suberimidate per ml), there was a gradual conversion of monomer to hexamer. As the reaction proceeded, each successively larger crosslinked intermediate appeared in turn and then began to disappear as larger species began to accumulate. The reaction continued until 68% of the CAD polypeptides had been converted to hexamers. There was little heptamer or octamer formed. However, small amounts of larger molecular weight species, as yet uncharacterized,

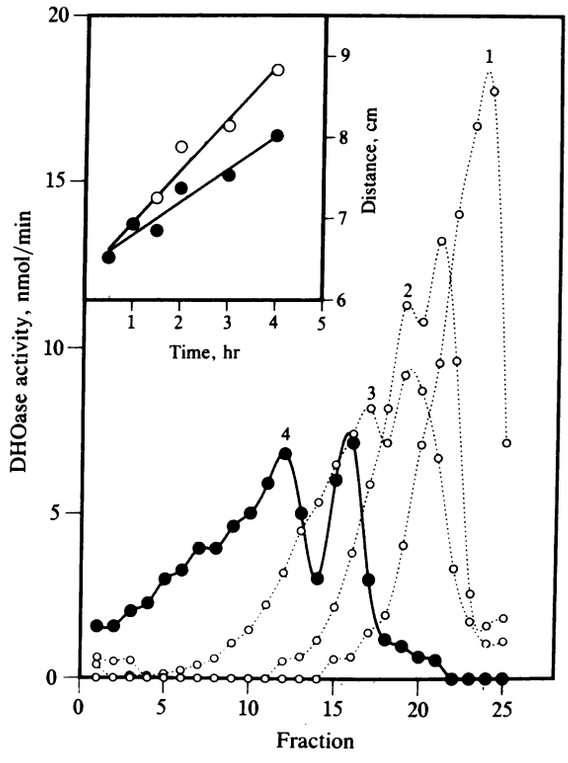


FIG. 5. Sucrose gradient centrifugation of CAD. After equilibration of the protein in 0.05 M Tris/0.05 M glycine, pH 8.7, samples of CAD (0.2 ml; 0.4–0.5 mg of protein) were centrifuged on linear 5–20% sucrose gradients at 189,000 × *g* for 1, 2, and 3 hr (○) and for 4 hr (●). After fractionation, each aliquot was assayed for DHOase activity. At 2 hr, two peaks were resolved. (*Inset*) The distance of each of these two peaks from the center of rotation as a function of time is plotted: ●, 20.8S species; ○, 27.9S species.

began to accumulate at longer crosslinking times, suggesting that the hexamers associated to form larger oligomers. While the trimer was observed during the course of the reaction, it was never a major species as might be expected if significant amounts of the CAD polypeptide were present in this form. The data do not rule out the possibility that the CAD hexamer was a dimer of trimers. However, if the CAD subunits were arranged in this fashion, crosslinking between trimers must have occurred as rapidly as crosslinking within the trimer.

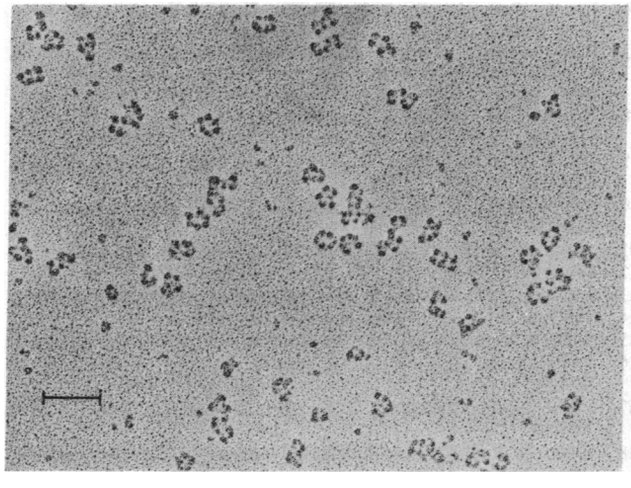

FIG. 6. Electron micrograph of CAD molecules after rotary shadowing with platinum. A field of molecules is shown. (Bar = 1000 Å; ×78,000.)

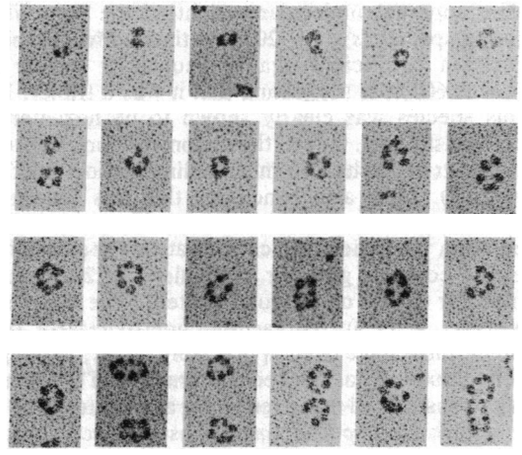

FIG. 7. Electron micrographs of CAD molecules. A gallery of rotary-shadowed CAD molecules is shown, demonstrating the appearance of various sizes of oligomers. (×96,000.)

Even after exhaustive crosslinking, residual amounts of smaller oligomers were always present in the reaction mixture. We suspect that this finding was not the result of incomplete crosslinking but that oligomers smaller than the hexamer were also represented in the population of CAD molecules. If these species were transient intermediates occurring during the crosslinking of hexamers, they should have completely disappeared by the time the concentration of hexamer had reached a plateau. Moreover, crosslinking a mixture of oligomers containing primarily hexamer but also significant amounts of each of the smaller oligomers would be expected to yield progress curves similar to those observed in the kinetic analysis (Fig. 4). This conclusion is also supported by a comparison of the electron microscopy and crosslinking studies (Fig. 8). The good agreement between these two methods suggests that quantitative crosslinking may be a useful tool for the analysis of complex mixtures of oligomers.

The sedimentation velocity experiments gave similar re-

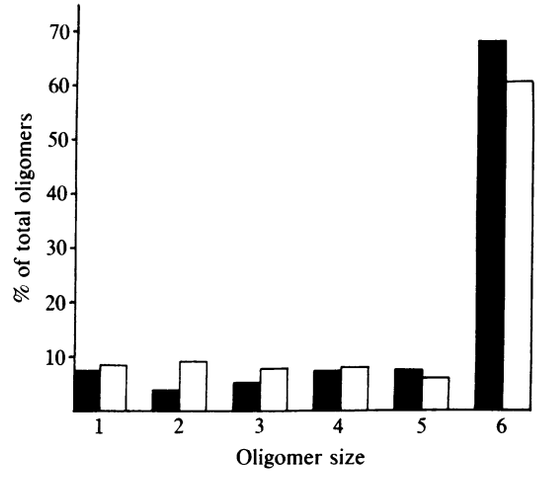


FIG. 8. Histogram correlating the relative amount of CAD found in each oligomeric form as determined by chemical crosslinking and by electron microscopy. The solid bars indicate the percentage of protein found in each oligomeric form after 2–12 hr of crosslinking with dimethyl suberimidate; the open bars indicate the percentage of protomers found in each oligomeric form by electron microscopy. Twenty electron micrographs from three separate experiments were scored for oligomeric size; 2000 assembled structures were evaluated in this analysis.

sults. The apparent molecular weight of the most slowly sedimenting species ($s^0_{20,w}$ = 20.8), estimated from a plot of sedimentation coefficient against molecular weight (not shown), was 600,000, suggesting that it was a trimer. However, this species was clearly shown to be hexameric by chemical crosslinking. One of the oligomeric forms observed by Coleman *et al.* (1) had a similar sedimentation coefficient ($s^0_{20,w}$ = 20.1). They also concluded that this species is a hexamer with a frictional ratio of 1.84. The unusually low sedimentation coefficient indicates that this species did not behave as a compact globular molecule. The 20.8S species accounted for ≈36% of the total protein. The other major species, ($s^0_{20,w}$ = 27.9), representing approximately 28% of the protein, must have been appreciably larger, although its oligomeric structure has not been determined. The remaining protein was associated into even larger aggregates. The more rapidly sedimenting species has not as yet been characterized.

Thus, sucrose gradient centrifugation indicated a greater extent of association of CAD polypeptides than did the crosslinking experiments. One explanation for this apparent difference is that the crosslinking between hexamers in the larger oligomers occurred more slowly than crosslinking between monomers within the hexamer. The observation that a much greater proportion of crosslinked oligomers larger than hexamers was generated when 20-fold higher concentrations of dimethyl suberimidate were used (data not shown) supports this interpretation. However, both methods indicated that hexamers of CAD were prevalent and that trimeric CAD was a minor species.

We cannot as yet account for the discrepancy between our findings and the previous study (1) that showed that CAD trimers predominate, but we suspect that the molecule can potentially assume a variety of different oligomeric forms and that the results obtained depend on the conditions under which the measurements are made. All of the experiments reported here were conducted near pH 9 at a greatly reduced concentration of the stabilizing agents, dimethyl sulfoxide and glycerol. Coleman *et al.* (1) reported that dimethyl sulfoxide and glycerol preferentially stabilize the hexamer at the expense of the trimer. We have evidence that the formation of higher oligomers, larger than hexamers, was promoted by 30% dimethyl sulfoxide/5% glycerol and by reducing the pH to 7.0. Apparently the interactions between hexamers are disrupted by eliminating these cryoprotectants or by elevating the pH.

Electron microscopy provided an alternative view of the quaternary structure of CAD. Rotary-shadowed specimens of intact CAD clearly showed approximately spherical monomers associated, for the most part, into closed ring-like structures. The putative monomers were 90–110 Å in diameter, a size consistent with a compactly folded 200- to 250-kDa globular protein. In agreement with the results obtained in the sedimentation and crosslinking studies, hexamers also predominated in the electron micrographs of CAD. Examination of a large number of molecules showed that a small fraction of CAD was associated with irregular, large oligomers of nine or more polypeptide chains. At this stage of the analysis, it is impossible to determine whether these larger aggregates constituted higher oligomeric forms or simply resulted from chance overlapping of smaller oligomers on the grid. Of the molecules that were associated in clearly discernable oligomeric structures, the fraction of chains associated in $n$-mers, where $n$ = 1–5 and $n$ = 7, ranged from 4–9% of the total. In contrast, hexamers accounted for 60% of the total polypeptide. Moreover, the fraction of hexamers may be even higher, since the majority of pentamers (6% of the total) by and large appeared as open rings, suggesting that these structures were formed by loss of a single monomer. The heptamers (4% of the total) usually appeared as an arrangement of six particles, one of which appeared to be bilobal or larger than the others. The tetramer and all other species constituted a small fraction of the total. Although we cannot with certainty exclude the possibility that small oligomers may be formed by fragmentation of the hexamer during the spraying and drying steps, the fact that these species were also observed in the crosslinking experiment makes this explanation unlikely. Nonetheless it is clear that CAD prepared under these conditions existed predominately as a hexamer composed of identical polypeptide chains.

Multifunctional proteins such as CAD are chimeras coded by large fused genes. These genes are believed to have arisen in the course of evolution by combining genes for preexisting monofunctional proteins. In many cases these ancestral proteins must have had specific oligomeric structures that may well be preserved in the chimera. In this regard the isolated ATCase and DHOase domains of CAD, purified from controlled proteolytic digests (ref. 3 and unpublished observations), have the same subunit structures as their bacterial counterparts. Some but not all of the potential intermolecular binding regions present in the individual domains may be used by the intact multifunctional polypeptide. Consequently rather than forming small compact structures of well-defined size, chimeric proteins may exist in a variety of oligomeric forms, and the structures that they form would be expected to be more open, flexible, and much larger (18). Moreover, the ability of chimeric proteins to assume a variety of polymorphic structures may also have important functional consequences.

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