Structure and Arrangement of the Regulatory Subunits in Aspartate Transcarbamylase†

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ABSTRACT: Although it is now known that aspartate transcarbamylase of *Escherichia coli* is composed of six catalytic and six regulatory polypeptide chains and that the catalytic chains are organized as two trimers, there has been little evidence as to the arrangement of the regulatory chains in the intact enzyme. Hence studies were conducted on various types of preparations of the regulatory subunits. Sedimentation velocity and equilibrium studies showed that the mercury-containing regulatory subunits existed in an association–dissociation equilibrium mixture. Most of the protein was found to participate in a monomer–dimer equilibrium with an association constant of 3×10^4 M^{-1}. By contrast the Zn regulatory subunit appeared as a homogeneous dimer of molecular weight 3.4×10^4. Substitution of mercury for zinc did not affect the stability of the dimer but removal of the metal ions by treatment with EDTA produced apo regulatory subunits which existed in a monomer–dimer equilibrium. The addition of cytidine triphosphate caused an increase in the extent of dimerization; moreover, this ligand was bound with greater affinity to Zn regulatory subunits than to apo regulatory subunits. Conclusive evidence for the existence of the regulatory subunits as dimers within the enzyme molecules came from reconstitution experiments with catalytic subunits and cross-linked Zn regulatory subunits. Cross-linking was achieved by reacting the Zn regulatory dimers with dimethyl pimelimidate, and the resulting preparation was fractionated by chromatography on Sephadex G-100 to give a preparation consisting predominantly of cross-linked dimers. Mixing this material with catalytic subunits gave a 50% yield of molecules with the same sedimentation coefficient and electrophoretic mobility as the native enzyme. Analysis of this reconstituted material showed that the regulatory chains existed predominantly as cross-linked dimers. Optical rotatory dispersion (ORD) and circular dichroism (CD) studies on the complex and its subunits showed that the intact enzyme and the catalytic subunits have moderate amounts of organized structure while the regulatory subunits have very little. The Zn and apo regulatory subunits differed substantially in their CD spectra, indicating that the addition of zinc ions caused a change in the conformation of the regulatory polypeptide chains. In addition the ORD and CD spectrum of the enzyme differed from the sum of the subunit contributions, indicating that assembly of the subunits to give the complex is accompanied by conformational changes. A model is proposed for the arrangement of the polypeptide chains in the intact enzyme, based on electron micrographs and other physical chemical evidence. The two catalytic subunits, each a slightly oblate structure having a triangular arrangement of the three polypeptide chains, are superimposed above one another in an eclipsed configuration without being in direct physical contact. The three regulatory dimers, each a V-shaped structure consisting of two cylindrically shaped monomers, extend to the outside of the molecule and serve to interconnect the two catalytic chains displaced 120° in the lower trimer. The location of the zinc ions and their role in the behavior of the enzyme and the regulatory subunits are discussed in the light of this evidence.

The subunit structure of aspartate transcarbamylase of *Escherichia coli* has been the subject of extensive research because of the interesting regulatory properties of the enzyme. Although early studies suggested that this enzyme had a tetrameric structure (Changeux *et al.*, 1967; Hervé and Stark, 1967; Weber, 1968a), it is now known that the enzyme is composed of six catalytic and six regulatory polypeptide chains (Weber, 1968b; Wiley and Lipscomb, 1968; Meighen *et al.*, 1970; Rosenbusch and Weber, 1971a). The catalytic subunit has been shown to be a trimer of polypeptide chains (Meighen *et al.*, 1970; Davies and Stark, 1970; Rosenbusch and Weber, 1971a), and hybridization experiments demonstrated that this trimer is an integral part of aspartate transcarbamylase and that there are two such trimers per enzyme molecule (Meighen *et al.*, 1970).

Uncertainty has existed, however, concerning the physical structure of the regulatory subunit. Early sedimentation equilibrium experiments gave molecular weight values of 3×10^4 for the native regulatory subunit (Gerhart and Schachman, 1965), with the values varying for different preparations, presumably as a result of heterogeneity. In addition, the preparations varied greatly in their ultraviolet absorbance and in the efficiency with which they recombined with catalytic subunit to give intact enzyme. The binding experiments of Changeux *et al.* (1968) suggested that there was one CTP binding site per 2.7×10^4 daltons, and amino-terminal analysis supported a molecular weight of 2.7×10^4 (Hervé and Stark, 1967; Weber, 1968a); but the amino acid sequence of

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the regulatory chain corresponded to a molecular weight of 1.7×10^4 (Weber, 1968b). This latter value for the molecular weight of the regulatory chain was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber, 1968b), carboxy-terminal analysis (Weber, 1968b), and sedimentation equilibrium (Rosenbusch and Weber, 1971a). Recent binding studies have demonstrated one CTP site per 1.7×10^4 daltons (Rosenbusch and Weber, 1971b).

Although the molecular weight of the regulatory polypeptide chain has now been established firmly, uncertainty has remained as to the structure of the regulatory subunit as isolated from aspartate transcarbamylase; moreover, no evidence has appeared concerning the structure of the regulatory subunits within aspartate transcarbamylase molecules. This lack of information has hampered efforts to determine the arrangement of the polypeptide chains in the enzyme. Accordingly we have performed physical chemical studies aimed at determining the structure of the regulatory subunits and the relationship between their structure and the organization of the regulatory chains in aspartate transcarbamylase molecules.

These investigations were aided considerably by the recent development of an improved procedure for preparing regulatory subunits (Nelbach *et al.*, 1972). The regulatory subunits produced by the older method of Gerhart and Holoubek (1967) were shown to contain varying amounts of residual mercury and will be referred to as "mercury-containing regulatory subunits." Exposure of these preparations to zinc chloride yielded homogeneous Zn regulatory subunits which contained 1.0 zinc ion per polypeptide chain, had a reproducible and low extinction coefficient in the ultraviolet region of the spectrum, and showed complete competence in forming aspartate transcarbamylase by reconstitution with catalytic subunits. The zinc ions could be replaced by mercuric ions to give Hg regulatory subunits; alternatively the metal ions were removed by exposure to EDTA to give apo regulatory subunits. The apo regulatory subunits did not combine with catalytic subunits to give aspartate transcarbamylase unless zinc ions were added.

The results presented here show that the polypeptide chains in the Zn regulatory subunit are strongly associated to dimers but exist in a monomer–dimer equilibrium in the mercury-containing protein (Gerhart and Holoubek, 1967) and in the apo derivative. Reconstitution experiments with a Zn regulatory subunit containing covalently cross-linked polypeptide chains indicate that the regulatory dimers are integral structural entities of the aspartate transcarbamylase molecules. These findings, together with the results of earlier work on aspartate transcarbamylase and the catalytic subunit (Meighen *et al.*, 1970; Gerhart, 1970) and recent electron micrographs presented in the preceding paper (Richards and Williams, 1972), provide the basis for a model of the quaternary structure of aspartate transcarbamylase. Further experiments reported here reveal relationships among the presence of zinc ions, the association of regulatory chains to dimers, and the binding of the inhibitor, CTP.

Experimental Section

Materials. Aspartate transcarbamylase was prepared from the mutant strain of *E. coli* described by Gerhart and Holoubek (1967) and purified according to their procedure. Catalytic and regulatory subunits were isolated by two methods. The first was that of Gerhart and Holoubek (1967) involving dissociation of the enzyme by the addition of PMB[1] and the separation of the subunits by chromatography on DEAE-Sephadex A-50. In the second method, the enzyme was dissociated by the addition of neohydrin [1-(3-chloromercuri-2-methoxypropyl)urea], and the subunits were separated by chromatography on DEAE-cellulose (Kirschner, 1971).

The column fractions for each subunit were collected and ME was added to 10 mM. The catalytic subunit was concentrated and then dialyzed as described by Gerhart and Holoubek (1967). The regulatory subunit was treated in a variety of ways to give, respectively, mercury, Zn, metal-free, (apo), and carboxymethyl regulatory subunits.

The mercury-containing regulatory subunit was prepared by the procedure of Gerhart and Holoubek (1967), by collecting the pooled fractions from the DEAE-Sephadex column, precipitating the protein by dialysis against 3.6 M ammonium sulfate–10 mM ME–0.2 mM EDTA at pH 7.0, dissolving the precipitate in 0.04 M phosphate at pH 7.0 containing 10 mM ME and 0.2 mM EDTA, and dialyzing the solution against this buffer.

In the preparation of Zn regulatory subunit, ME and $ZnCl_2$ were added to the pooled fractions from the DEAE-cellulose (or DEAE-Sephadex) column to give final concentrations of 10 mM and 2 mM, respectively. The protein was then precipitated by dialysis against 3.6 M ammonium sulfate–10 mM ME–0.2 mM $ZnCl_2$, at pH 7.0. The collected protein was then dissolved in and dialyzed against 0.04 M K-phosphate–10 mM ME–0.2 mM $ZnCl_2$, at pH 7.0. EDTA was omitted from these solutions.

The Hg regulatory subunit (as opposed to mercury-containing regulatory subunit) was prepared by dialyzing Zn regulatory subunit for 3 days against two changes of 0.05 M imidazole acetate–10 mM ME–1 mM mercuric acetate, at pH 7.0.

Apo regulatory subunit was prepared by adding EDTA to a solution of Zn regulatory subunit to 10 mM, followed by dialysis for several days against daily changes of 0.04 M K-phosphate–10 mM ME–10 mM EDTA at pH 7.0. The concentration of EDTA was reduced to 1 mM in the final dialysis. Deionized water was used in the buffers, and all glassware was soaked in a 1:1 mixture of nitric and sulfuric acids, and then rinsed with deionized water before use. The buffers were flushed with nitrogen to prevent oxidation of sulfhydryl groups.

The carboxymethyl derivative of the regulatory subunit was prepared by reacting the protein (2 mg/ml) with iodoacetamide (tenfold excess over sulfhydryl concentration) for 90 min in 0.3 M Tris-acetate–10 mM ME–0.2 mM $ZnCl_2$ at pH 8.0. Amino acid analysis revealed that the derivative contained 3.8 moles of carboxymethylcysteine per mole of regulatory chains. No zinc binding was detected for this derivative as had been found previously by Rosenbusch and Weber (1971b).

The polypeptide chains of the Zn regulatory subunit were covalently cross-linked by the method of Davies and Stark (1970) through the use of the bifunctional reagent, dimethyl pimelimidate. Protein (4 mg/ml) was allowed to react with 1 mg/ml of [^{14}C]dimethyl pimelimidate (generously supplied by Gregg E. Davies and George R. Stark) for 3 hr at room temperature. The solution contained 0.2 M triethanolamine hydrochloride, 0.2 mM $ZnCl_2$, and 10 mM ME, at pH 8.5. At the end of the reaction the protein was concentrated and dialyzed against the buffer.

[1] Abbreviations used are: PMB, *p*-mercuribenzoate; ME, 2-mercaptoethanol.

The mercurials, PMB and neohydrin (or chlormerodrin), were obtained from K and K laboratories. Each was purified by two precipitations from alkaline solutions by the addition of HCl. CTP, sodium salt, was obtained from P-L Biochemicals (Lot No. 1212). Radioactively labeled CTP was obtained from New England Nuclear (NEC-431 [U-^{14}C]cytidine 5′-triphosphate, tetrasodium salt, Lot No. 488-273). Both samples were checked for contaminating CDP and CMP by paper chromatography in 34% acetic acid–37% 1-butanol–0.3 M NH_4OH–0.08 M EDTA. The radioactive CTP contained about 3% CDP and less than 0.2% CMP, and the unlabeled CTP contained less than 5% of any impurity.

Methods. Optical rotatory dispersion (ORD) and circular dichroism (CD) spectra were obtained on a Cary Model 60 spectropolarimeter with a Model 6003 circular dichroism accessory. Both ORD and CD data were calculated in terms of degrees cm^2 per decimole of residues with a mean residue molecular weight of 112, as determined from the amino acid compositions of aspartate transcarbamylase and its subunits (Weber, 1968b; Changeux and Gerhart, 1968). Corrections of ORD data for the wavelength dependence of the refractive index of the buffers were made by applying the values reported for water (Fasman, 1963).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969) as modified by Davies and Stark (1970), with gels containing 5% acrylamide. After destaining, the gels were frozen and sliced on a homemade gel slicer into 1- or 2-mm slices. The protein was eluted from the gel and assayed for radioactivity by the method of Basch (1968). Cellulose acetate electrophoresis was performed as described previously (Meighen *et al.*, 1970), except that the buffer used was 0.05 M Tris-HCl at pH 8.0 containing 10 mM ME.

The partial specific volumes ($\bar{V}$) of the enzyme and its subunits were calculated from their amino acid compositions (Changeux and Gerhart, 1968; Weber, 1968b) by the method of McMeekin and Marshall (1952), yielding a value of $\bar{V}$ = 0.74 ml/g. Protein concentrations were determined refractometrically in a synthetic boundary cell experiment in the ultracentrifuge, as described by Richards and Schachman (1959). One fringe was assumed to represent a concentration difference of 0.25 mg/ml (Babul and Stellwagen, 1969). All densities were determined pycnometrically. Dialysis of solutions of the regulatory subunit was performed with tubing which had been heated for 20 hr at 90° in an air oven before the customary cleaning process involving boiling in bicarbonate–EDTA solutions (Callanan *et al.*, 1957). Protein hydrolysis was performed with 6 N HCl in evacuated tubes at 110° for 20 hr, and samples were analyzed on a Beckman Model 120B amino acid analyzer. ^{14}C was determined with a Nuclear-Chicago Unilux II scintillation counter. Zinc analyses were performed on a Perkin-Elmer Model 303 atomic absorption spectrometer as described by Nelbach *et al.* (1972).

Sedimentation studies were performed with a Spinco Model E ultracentrifuge equipped with schlieren and Rayleigh optics (Richards *et al.*, 1972) and a split-beam photoelectric scanning absorption optical system (Schachman and Edelstein, 1966). Sedimentation equilibrium experiments were conducted according to the method of Yphantis (1964). In studies on the effect of CTP on the molecular weight of the regulatory subunit, solutions at the same initial protein concentration, with and without CTP (10^{-3} M), were examined simultaneously in cells containing six-channel centerpieces. The temperature was controlled by the use of the refrigeration unit alone since operation of the heating unit intermittently has been found to cause convection.

The sedimentation equilibrium data on native aspartate transcarbamylase and catalytic subunit were analyzed with a computer program MVRHO (Rosenthal, 1971). A different computer program (Teller *et al.*, 1969) was used for analyzing the other experiments. For studies on the association–dissociation behavior of the regulatory subunit the program fit the data of concentration *vs.* radial distance in terms of monomer–dimer, monomer–dimer–trimer, and monomer–dimer–trimer–tetramer equilibria, using a monomer molecular weight 1.7×10^4 for the regulatory chain in the absence of CTP (Weber, 1968b) and a monomer molecular weight of 1.75×10^4, corresponding to a regulatory chain with one molecule of bound CTP, when CTP was present. Equilibrium constants for each fit were determined according to the following scheme: $P + P \rightarrow P_2$, $P_2 + P \rightarrow P_3$, $P_3 + P \rightarrow P_4$, with the appropriate association constants, $K_2 = [P_2]/[P]^2$; $K_3 = [P_3]/[P_2][P]$; $K_4 = [P_4]/[P_3][P]$.

The binding of CTP to the regulatory subunit was evaluated from measurements of the constituent sedimentation coefficient, s, of CTP in mixtures of protein and radioactively labeled CTP. Experiments were performed with a partition cell (Tiselius *et al.*, 1937; Yphantis and Waugh, 1956; Schachman, 1957) and the free CTP concentration was calculated from eq 33 of Steinberg and Schachman (1966). Further details are given elsewhere (Cohlberg, 1972).

Results

Subunit Composition of Aspartate Transcarbamylase. The structure of the enzyme in terms of its constituent subunits and polypeptide chains is summarized in Table I. Column 2 gives the molecular weights of the enzyme and its subunits as determined from high-speed sedimentation equilibrium experiments (Yphantis, 1964). The results for aspartate transcarbamylase, 3.07×10^5, and the catalytic subunit, 1.03×10^5, are in excellent agreement with those reported earlier (Gerhart and Schachman, 1965; Rosenbusch and Weber, 1971a). In contrast the value, 3.37×10^4, for the Zn regulatory subunit is higher than the previously reported values (Gerhart and Schachman, 1965; Rosenbusch and Weber, 1971a). Taken in conjunction with the composition of the enzyme in terms of weight per cent of the two types of subunits, these figures show that an aspartate transcarbamylase molecule contains two catalytic subunits and three regulatory subunits (column 5). With regard to the catalytic subunits, it has been known from hybridization studies (Meighen *et al.*, 1970) that there are two catalytic subunits per enzyme molecule; hence the results presented in Table I provide confirmatory data. Previous evidence regarding the number of regulatory subunits per enzyme molecule has been inconclusive because the molecular weight values obtained for the isolated regulatory subunit were intermediate between the values expected for a single polypeptide chain and for a dimer of such chains. The molecular weights for the catalytic polypeptide chains, 3.2×10^4, and the regulatory chains, 1.72×10^4, are in good agreement with those reported by Rosenbusch and Weber (1971a) and Weber (1968b). It follows that the catalytic subunits contain three polypeptide chains and the regulatory subunits two chains (column 7); hence, there are six catalytic chains and six regulatory chains per the enzyme molecule (column 8). Thus these studies provide support for the hexameric model originally proposed by Weber (1968b) and Wiley and Lipscomb (1968).

TABLE I: Subunit Composition of Aspartate Transcarbamylase.

	Mol Wt $\times 10^{-5}$	% by Wt	Wt/ Enzyme Mol $\times$ 10^{-5}	Subunits/ Enzyme Mol	Mol Wt of Polypeptide Chains $\times 10^{-5}$	Polypeptide Chains/ Subunit	Polypeptide Chains/ Enzyme Mol
Enzyme	3.07 ± 0.03[a]		3.07				
Catalytic subunit	1.03 ± 0.02[a]	68[c]	2.09	2.0	0.32 ± 0.01[d]	3.2	6.4
Regulatory subunit	0.337 ± 0.004[b]	32[c]	0.98	2.9	0.172 ± 0.005[d]	2.0	5.8

[a] By sedimentation equilibrium in 40 mM potassium phosphate–10 mM ME–0.2 mM EDTA, pH 7.0. [b] By sedimentation equilibrium in 40 mM potassium phosphate–10 mM ME–0.2 mM $ZnCl_2$, pH 7.0. For details, see text below. [c] Various figures for the per cent subunit composition have appeared. The earliest value of 63% catalytic subunit and 37% regulatory subunit (Gerhart and Schachman, 1965) was based on the relative areas of the two schlieren boundaries produced upon reaction of the enzyme with PMB, but no attempt was made to correct for the contribution from bound PMB. The estimate of 68% catalytic subunit and 32% regulatory subunit (Gerhart and Schachman, 1965) was obtained by comparison of the apparent protein concentrations of the enzyme before reaction with PMB and of the subunits produced after the PMB reaction, as measured by both schlieren and Rayleigh optics, and making a correction for bound PMB. However, the uncertainty in these results is about ±2%. The estimate of 69% catalytic subunit and 31% regulatory subunit (Changeux and Gerhart, 1968) was based on fitting amino acid analysis data, but the same data are equally consistent with values of 67% catalytic subunit and 33% regulatory subunit, as determined from the molecular weight values presented here. Also, Rosenbusch and Weber (1971a) determined values of 66–68% catalytic subunit and 34–32% regulatory subunit from densitometric scans of polyacrylamide gels produced by electrophoresis of the enzyme in sodium dodecyl sulfate. [d] By sedimentation equilibrium in 7 M guanidine hydrochloride–10 mM ME–0.2 mM EDTA. A partial specific volume of 0.74 ml/g, was assumed in each case, since preferential solvent interactions should be negligible in this solvent (Reisler and Eisenberg, 1969; Syvanen, 1972). The value for the catalytic chains was obtained by extrapolating curves of $1/M_w$ and $1/M_n$ *vs.* concentration to infinite dilution, in order to account for nonideality. This procedure was unnecessary for the regulatory chain, since the plot of ln c *vs.* r^2 was linear.

Physical Properties of the Regulatory Subunit. A. MERCURY-CONTAINING REGULATORY SUBUNIT. Most of these preparations contained high molecular weight aggregates in addition to the principal component, which had a sedimentation coefficient of about 2.5 S. For some preparations, sedimentation velocity patterns obtained with the photoelectric scanner revealed the presence of a small amount of a component with a sedimentation coefficient of 4–6 S in addition to the main component. With other preparations the scanner traces showed a gradual increase in the optical density beyond the principal boundary, which indicated the presence of heterogeneous aggregated species. The aggregates frequently were not detectable in freshly prepared mercury-containing regulatory subunit and, when observed, amounted to only a small fraction of the total protein. When solutions were stored at low temperature (4°) aggregates formed spontaneously. After periods of a few weeks, precipitates formed even under sterile conditions. Sulfhydryl reagents, such as ME at 10 mM or dithiothreitol at 5 mM, retarded aggregate formation. Aggregation was partially, but not completely, reversed by dialysis of aged preparations against fresh buffers containing such sulfhydryl reagents at high concentrations.

As seen in Figure 1 the sedimentation coefficient of the mercury-containing regulatory subunit in dilute solutions decreased sharply with decreasing protein concentration. Such behavior is characteristic of a rapidly reversible associating–dissociating system, as in a monomer–dimer equilibrium (Gilbert, 1955), and was in marked contrast with that observed for aspartate transcarbamylase (Gerhart and Schachman, 1965) and the catalytic subunit (Kirschner and Schachman, 1971). The latter proteins showed a linear decrease in sedimentation coefficient with increasing concentration.

Further evidence that the mercury-containing regulatory subunits existed as an associating–dissociating system came from sedimentation equilibrium experiments. The plots of of ln c *vs.* r^2 showed upward curvature characteristic of heterogeneous or interacting systems. Curve fitting of the experimental data was performed by the computer program in order to determine whether association–dissociation equi-

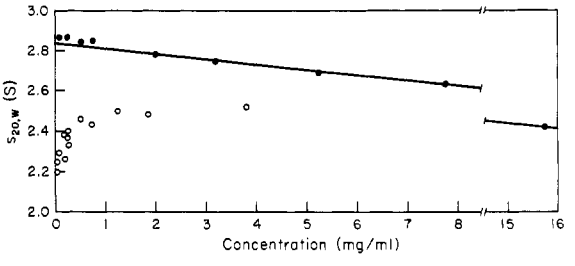

FIGURE 1: Dependence of the sedimentation coefficient of the regulatory subunit on protein concentration. The closed circles (●) represent the Zn regulatory subunit, and the open circles (○) represent the mercury-containing regulatory subunit. Schlieren optics were used for protein concentrations greater than 2 mg/ml, and the photoelectric scanner was used for lower concentrations. Scanner experiments for concentrations greater than 0.3 mg/ml employed light with a wavelength of 280 nm. For lower protein concentrations, light at wavelengths of 222–230 nm was used in conjunction with the multiplexer described by Schachman and Edelstein (1966). The buffer, 0.04 M potassium phosphate at pH 7.0, contained 10 mM ME. For experiments using light at wavelengths of 230 nm and below, the concentration of ME was reduced by dilution of the sample immediately before the experiment. Solutions of the Zn regulatory subunit contained 0.2 mM $ZnCl_2$, and solution of the mercury-containing regulatory subunit contained 0.2 mM EDTA. The protein concentrations were corrected for radial dilution.

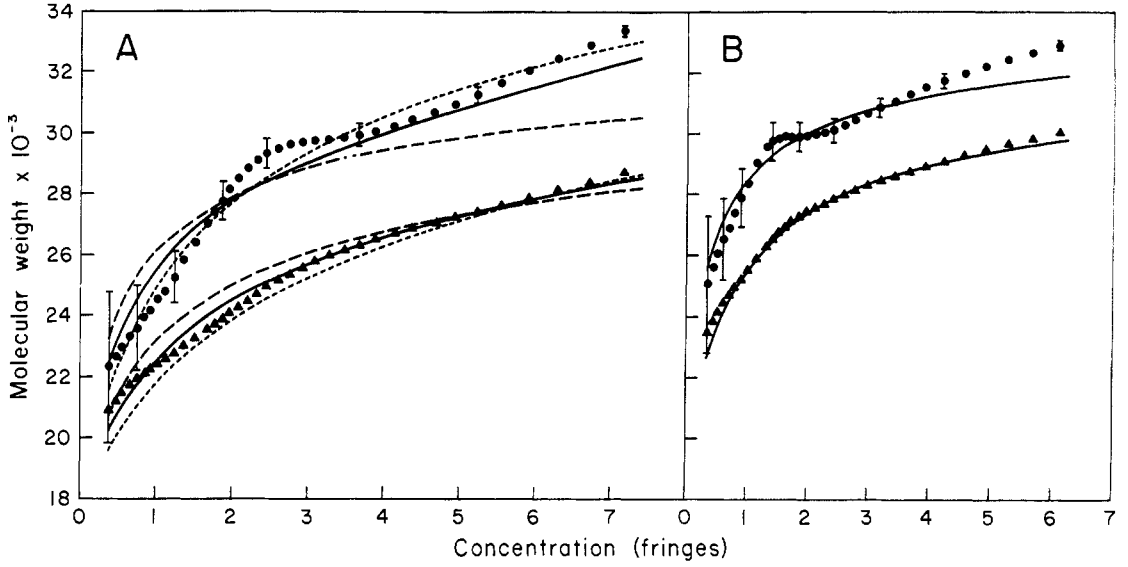

FIGURE 2: Sedimentation equilibrium of the mercury-containing regulatory subunit. Values of the weight-average molecular weight, M_w (●), and the number-average molecular weight, M_n (▲), as determined by the computer program, are plotted *vs.* protein concentration in fringes. The error bars represent probable deviations of the M_w values, as determined by the computer. The molecular weight values for the experiment presented in Figure 2A are shown in A along with theoretical plots of M_w and M_n *vs.* concentration for a monomer–dimer system with an association constant, K_2 of 4.2×10^4 M^{-1} (– – –); for a monomer–dimer–trimer system with a K_2 of 1.9×10^4 M^{-1} and a dimer–trimer association constant, K_3 of 1.1×10^3 M^{-1} (- - - -); and for a system in which 5% of the protein had a molecular weight of 6.8×10^4 and 95% existed in a monomer–dimer equilibrium with a K_2 of 3.2×10^4 M^{-1} (———). The data for an experiment on the same protein preparation in the presence of 1 mM CTP with theoretical curves for a monomer–dimer system with a K_2 of 8.7×10^4 M^{-1} are shown in B.

libria could account satisfactorily for the measured concentration distributions. Figure 2A shows theoretical curves and the experimental results in terms of weight- and number-average molecular weights (M_w and M_n, respectively) as a function of protein concentration (in fringes). For these theoretical calculations, monomer–dimer and monomer–dimer–trimer systems were considered with a molecular weight of 1.7×10^4 for the monomer (Weber, 1968b). Values for the association constants were chosen which gave the best agreement between the theoretical and experimental concentration distributions. These values were 4.2×10^4 M^{-1} for K_2 for the monomer–dimer system and 1.9×10^4 M^{-1} and 1.1×10^3 M^{-1} for K_2 and K_3, respectively, for the monomer–dimer–trimer system.

It is readily seen by comparison of the theoretical curves with the experimental data that the fit for the monomer–dimer system is not satisfactory. In contrast the monomer–dimer–trimer system provided a better fit for the data. Since the sedimentation velocity patterns indicated the presence of aggregates which apparently did not participate in a reversible association–dissociation equilibrium, attempts were made to account for such aggregated species in the sedimentation equilibrium experiments. Thus it was assumed that the initial solution contained 5% by weight of irreversibly aggregated material with a molecular weight of 6.8×10^4. Also shown in Figure 2A are theoretical curves for a system in which 5% of the protein had a molecular weight of 6.8×10^4, and the remainder existed in monomer–dimer equilibrium with a monomer molecular weight of 1.7×10^4 and an association constant of 3.2×10^4 M^{-1}. These theoretical curves also fit the experimental data satisfactorily.

The theoretical calculations presented in Figure 2A demonstrate that, although the experimental results cannot be accounted for solely by a monomer–dimer–equilibrium, satisfactory agreement between theory and experiment can be obtained by considering systems which involve primarily a monomer–dimer equilibrium if it is assumed that there is present, in addition, a weaker reversible association to species heavier than the dimer (as in the monomer–dimer–trimer fit) or a small amount of irreversibly aggregated material heavier than the dimer. Although many other combinations of oligomers could be postulated to account for the data, the results could not be explained solely by the existence of a heterogeneous collection of noninteracting species, since the increase in sedimentation coefficient with concentration clearly indicates that some association–dissociation behavior must be involved. In addition, sedimentation equilibrium experiments performed on a different preparation of mercury-containing regulatory subunit at two different initial protein concentrations gave curves of molecular weight *vs.* concentration which were nearly identical with each other. Such behavior is characteristic of an associating–dissociating system, rather than a heterogeneous mixture of noninteracting components (Roark and Yphantis, 1969).

For our present purposes, estimates of K_2 representing the principal equilibrium between monomers and dimers were obtained by averaging the values of K_2 calculated for the monomer–dimer system and the values of K_2 calculated for the monomer–dimer–trimer system. The uncertainty in the "average" K_2 was estimated as one-half of the difference between the values of K_2 for the two theoretical systems. In effect, the values of K_2 from the monomer–dimer and the monomer–dimer–trimer fits were taken as the upper and lower limits, respectively, of the true K_2. If the monomer–dimer system alone were considered, the computer fit would lead to an erroneously high estimate of K_2; conversely the values of K_2 from the monomer–dimer–trimer fit would probably be too low. It should be recognized that the monomer–dimer–trimer calculation was used merely to allow for the presence of higher aggregates. We do not mean to imply that trimeric species exist or that an association–dissociation equilibrium represented by the constant, K_3, actually exists.

TABLE II: Association Constants for the Regulatory Subunit.

Type	Prepn	Buffer[a]	Temp (°C)	K_2 (M^{-1})	K_2 in the Presence of CTP (M^{-1})
Mercury-containing	18	P	15	$(3 \pm 1) \times 10^4$	$(8 \pm 1) \times 10^4$
Mercury-containing	23	I	10	$(3 \pm 1) \times 10^4$	$(2 \pm 1) \times 10^5$
Mercury-containing	27	I	4	$(4 \pm 1) \times 10^4$	$(2 \pm 1) \times 10^5$
				$(4 \pm 1) \times 10^4$	
			24	$(6 \pm 1) \times 10^3$	$(10 \pm 3) \times 10^3$
				$(5 \pm 2) \times 10^3$	
Apo	28	I	4	$(6 \pm 3) \times 10^4$	$(1 \pm 0.8) \times 10^6$
			14	$(4 \pm 2) \times 10^4$	$(2 \pm 0.8) \times 10^5$
			24	$(1 \pm 0.4) \times 10^4$	$(5 \pm 1) \times 10^4$
Apo	39	P	4	$(8 \pm 1) \times 10^3$	
			21	$(2 \pm 1) \times 10^3$	
Apo	55	P	5	$(5 \pm 0.2) \times 10^4$	
Apo	57	P	4	$(5 \pm 2) \times 10^5$	
				$(3 \pm 0.7) \times 10^5$	
			26	$(2 \pm 0.3) \times 10^5$	
Apo	58	P	3	$(6 \pm 1) \times 10^4$	$(1 \pm 0.6) \times 10^7$
			23	$(1 \pm 0.4) \times 10^4$	$(6 \pm 1) \times 10^4$
Carboxymethyl	60	P	2	$(2 \pm 0.7) \times 10^3$	

[a] P represents 0.04 M potassium phosphate at pH 7.0, and I represents 0.04 M imidazole acetate at pH 7.0. All solutions contained 1 mM ME. The solutions of the various regulatory subunit derivatives also contained the following: mercury-containing, 0.2 mM EDTA; apo, 1 mM EDTA; carboxymethyl, no additions. The protein concentration was between 1 and 2 mg per ml. For preparation no. 27, the first row at each temperature refers to a solution with a protein concentration of 2 mg/ml, the second row to a solution at a concentration of 1 mg/ml. The K_2 values are "average" monomer–dimer association constants obtained by averaging the values of K_2 determined by the computer program for monomer–dimer and monomer–dimer–trimer systems. The columns "K_2 in the presence of CTP" refers to simultaneous experiments on the same preparations at the same initial protein concentration in the presence of 1×10^{-3} M CTP.

The "average" K_2 for the data presented in Figure 2A is 3×10^4 M^{-1} with an uncertainty of about $\pm 1 \times 10^4$ M^{-1}.

Calculations of K_2 for a variety of preparations are summarized in Table II. With one preparation (No. 27) a sevenfold decrease in K_2 was observed when the temperature of the preparation was raised from 4 to 24°.

B. Zn REGULATORY SUBUNIT. Preparations of the subunit stored for long periods of time in buffers containing 10 mM ME and 0.2 mM $ZnCl_2$ showed little aggregation when examined in sedimentation velocity experiments. As seen in Figure 1 the sedimentation coefficient of the Zn regulatory subunit decreased slightly with increasing concentration according to the relationship, $s_{20,w} = 2.84(1 - 0.0091c)$, where c is the concentration in grams per liter and $s_{20,w}$ is in units of svedbergs. This slight negative dependence of $s_{20,w}$ on c is characteristic of noninteracting globular proteins and is in marked contrast with the dependence exhibited by the mercury-containing regulatory subunit (Figure 1). At a concentration of 0.09 g/l., the Zn regulatory subunit had a sedimentation coefficient of 2.87 S while the mercury-containing regulatory subunit had a sedimentation coefficient of only 2.25 S. It thus appears that zinc ions stabilize the dimeric form of the regulatory subunit.

Further evidence regarding the dimeric form of the Zn regulatory subunit was obtained from sedimentation equilibrium experiments; plots of ln c *vs.* r^2 were linear, indicating that the protein was homogeneous. Results from separate experiments, both at 4 and 24°, gave an average molecular weight of 3.38×10^4. This finding, taken in conjunction with the amino acid sequence of the regulatory chains (Weber, 1968b) and other evidence cited above, demonstrates that the Zn regulatory subunit is a dimer.

Storage of the Zn regulatory subunit for extended periods of time in buffers which did not contain zinc ions led to some dissociation of the dimers as indicated by curved plots of ln c *vs.* r^2. The stability of freshly prepared Zn regulatory subunit was not affected by high pH. At pH 8.0 (0.04 M pyrophosphate), pH 9.0 (0.04 M pyrophosphate), and pH 10.0 (0.04 M carbonate) and in the presence of $ZnCl_2$ (1 mM) and ME (10 mM) the plots of ln c *vs.* r^2 were linear with slopes corresponding to a molecular weight of 3.4×10^4 in each case. The protein was insoluble at acidic pH.

C. APO REGULATORY SUBUNIT. This protein had a great tendency to aggregate and only rarely were preparations of the apo regulatory subunit devoid of aggregated material. Aggregation increased rapidly if the solutions were stored at room temperature. Sedimentation equilibrium experiments on the apo regulatory subunit yielded curved plots of ln c *vs.* r^2 and the data were fit satisfactorily in terms of a monomer–dimer equilibrium. Table II shows that the values of K_2 for different preparations at 4° varied widely from a low of 8×10^3 M^{-1} to a high of 4×10^5 M^{-1}. This large variation in association constants probably can be ascribed to differences among the preparations in their zinc content. Preparation No. 57, for example, had a large K_2 and 0.15 zinc atom per polypeptide chain. Also the addition of catalytic subunit to this preparation of apo regulatory subunit led to the formation of a small amount of aspartate transcarbamylase.

TABLE III: CTP Binding to Zn Regulatory and Apo Regulatory Subunits.

Regulatory Subunit	Buffer[a]	Total Concentration (M × 10⁵) Protein[b]	Total Concentration (M × 10⁵) CTP	$s_{20,w}$ (S)	[CTP] (M × 10⁵)	K (M × 10⁴)	Range of K[c] (M × 10⁴)
Zn	P	2.0	2.1	0.88	1.7	0.7	0.5–1.0
Zn	P	5.8	4.2	0.16	2.9	1.0	0.8–1.2
Zn	P	2.9	2.1	0.86	1.7	1.2	0.8–1.6
Zn	P	5.2	5.6	1.30	3.6	0.6	0.5–0.7
Apo	P	6.6	4.2	0.61	3.8	6	4–12
Apo	P	7.5	7.3	0.54	6.9	11	6–50
Apo	P	13.6	11.5	0.71	9.9	7	5–12
Zn	T	1.56	2.1	0.97	1.53	0.3	0.2–0.4
Zn	T	3.1	3.0	1.25	1.9	0.3	0.2–0.4
Apo	T	6.1	5.1	0.56	4.4	3	2–6
Apo	T	22	10.7	0.56	9.3	8	5–13
Apo	T	2.2	19.3	0.88	13.8	4	3–5
Apo + $ZnCl_2$	T	4.1	4.1	0.92	3.1	0.9	0.6–1.3
Apo	T	33	29	0.83	2.2	8	6–10
Apo + $ZnCl_2$	T	4.7	4.2	0.90	3.2	1.1	0.9–1.5

[a] P represents 0.04 M potassium phosphate at pH 7.0, and T represents 0.2 M Tris acetate at pH 8.2. All buffers contained 10 mM ME. Solutions of the Zn regulatory subunit contained 0.2 mM $ZnCl_2$, and solutions of the apo regulatory subunit contained 10 mM EDTA. Sedimentation was performed at 20°. All preparations of the apo regulatory subunit contained less than 0.05 zinc ion per polypeptide chain. [b] In moles of polypeptide chain per liter. [c] The range of K was based on the assumption that the uncertainty in the free CTP concentration was ±5%.

In contrast, preparation No. 39 which had a K_2 of only 8×10^3 M^{-1} was found to contain less than 0.05 zinc atom per polypeptide chain. For those preparations studied at different temperatures it was found generally that the value of K_2 was lower at the higher temperature.

When $ZnCl_2$ was added to a solution of freshly prepared apo regulatory subunit and the solution was dialyzed overnight against buffer containing 0.2 mM $ZnCl_2$ (in the absence of EDTA) the regulatory chains reassociated strongly to form dimers. Sedimentation equilibrium experiments on such material yielded linear plots of ln c *vs.* r^2 with slopes corresponding to a molecular weight of 3.3×10^4. With solutions of apo regulatory subunit which were stored for several days, the addition of $ZnCl_2$ did not lead completely to dimers. Sedimentation equilibrium experiments on such preparations yielded plots of ln c *vs.* r^2 with upward curvature, and the molecular weight values near the meniscus were significantly lower than 3.4×10^4.

D. OTHER DERIVATIVES OF THE REGULATORY SUBUNIT. Since the values of K_2 obtained for the apo regulatory subunit varied widely and showed some correlation with the amount of zinc ions in the solution, studies were performed on a preparation which was devoid of zinc ions. As seen in Table II, carboxymethyl regulatory subunit showed very little tendency to associate. The value for K_2 for carboxymethyl regulatory subunit at 2° was lower than any of the values found for apo regulatory subunit.

For a preparation of Hg regulatory subunit in which the zinc ions had been replaced by mercuric ions (Nelbach *et al.*, 1972) the plot of ln c *vs.* r^2 was linear with a slope corresponding to a molecular weight of 3.6×10^4.

E. EFFECT OF CTP ON THE REGULATORY SUBUNIT. Since the results on the various types of regulatory subunit preparations showed the existence of a monomer–dimer equilibrium with monomer favored in the absence of metal ions and dimers predominating when zinc or mercuric ions were present it was of interest to determine the effect of CTP on this equilibrium. Figure 2B shows the effect of CTP (1 mM) on M_w and M_n for the mercury-containing regulatory subunit. As seen by comparison of Figures 2B and 2A, the addition of CTP caused an increase in the molecular weight of the protein. Curve fitting of the data led to a value of 8×10^4 M^{-1} for K_2 in the presence of CTP as compared to 3×10^4 M^{-1} for the same protein devoid of CTP. As seen in Table II which summarizes data for many preparations, CTP addition always led to an increase in the association constant for both the mercury-containing and apo regulatory subunits. These results indicate that CTP binds more strongly to the dimeric form of the subunit than to the monomers. The effect of CTP seemed to be greater at lower temperatures.

CTP Binding to Regulatory Subunit. Since zinc ions played an important role in stabilizing the enzyme structure (Nelbach *et al.*, 1972) and promoted dimer formation between the regulatory chains it seemed of interest to determine whether zinc ions affected the binding of CTP to the regulatory subunit. Hence measurements were made of the constituent sedimentation coefficient, s_{CTP}, in mixtures containing apo regulatory and Zn regulatory subunits, respectively, and from these values the concentrations of free CTP were evaluated. The resulting values for a number of experiments are presented in Table III along with calculated values of the dissociation constant, K, defined by [CTP][P]/[P–CTP], where [CTP] is the molar concentration of free CTP, [P] is the molar concentration of free protein, and [P–CTP] is the molar concentration of the protein–CTP complex. These calculations of K were based on the assumption that there was one CTP binding site per polypeptide chain (Rosenbusch and Weber, 1971b) and that all sites were equivalent even though some of

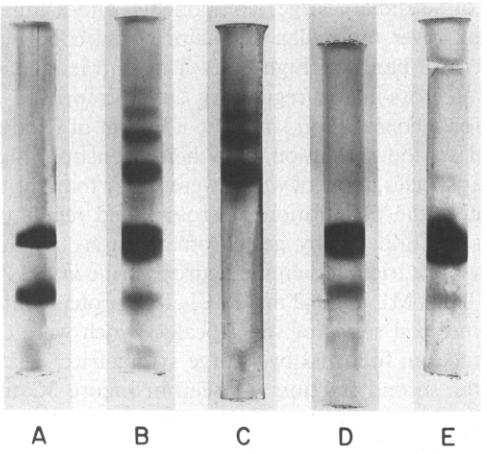

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cross-linked regulatory subunit. Pattern for the chains of the enzyme is shown in A, and that in B was obtained with the unfractionated preparation of cross-linked regulatory subunit. Chromatography of this latter material on Sephadex G-100 yielded a series of small peaks which were pooled to form one fraction, shown in C, and a large symmetrical peak which formed the second fraction, shown in D. The gel in E represents the material obtained from the principal component in the sucrose density gradient centrifugation of the reconstituted enzyme (Figure 5). Electrophoresis was conducted by the method of Weber and Osborn (1969) as modified by Davies and Stark (1970) for 3 hr at 1 mA per gel, wth gels containing 5% acrylamide. Migration was from top to bottom.

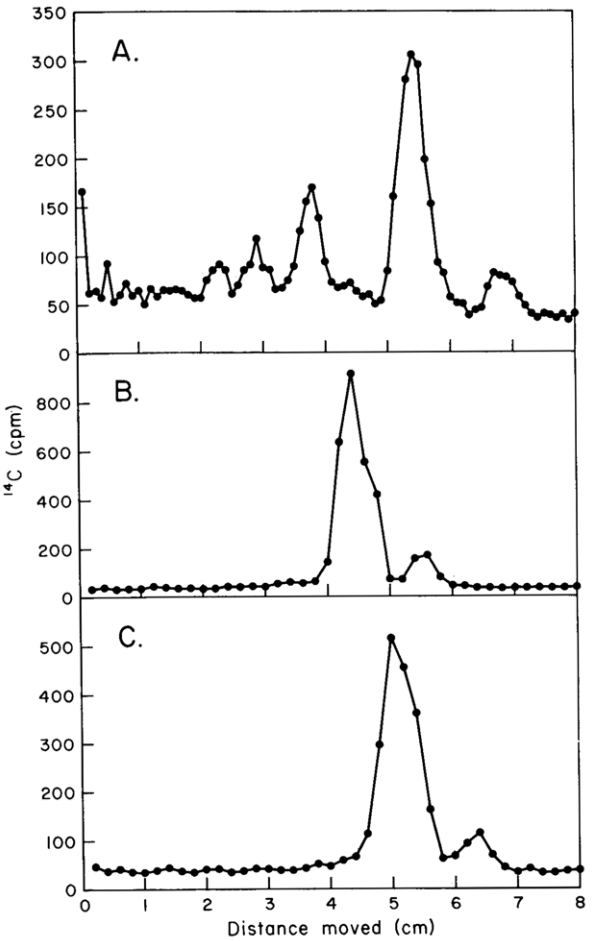

FIGURE 4: Distribution of ^{14}C in sodium dodecyl sulfate–polyacrylamide gels of cross-linked regulatory subunit. Gels were sliced into 1-mm (A) or 2-mm (B and C) sections. The protein was eluted and assayed for ^{14}C by the method of Basch (1968). The pattern shown in A corresponding to the gel in Figure 3B, represents the unfractionated preparation of cross-linked regulatory subunit. Shown in B is the pattern corresponding to the gel in Figure 3D, which represents the main component in the gel chromatography of the cross-linked regulatory subunit on Sephadex G-100. The pattern in C was obtained for the gel in Figure 3E, representing the principal component in the sucrose density gradient centrifugation of reconstituted enzyme (Figure 5). Electrophoresis for the three patterns shown was performed at different times; hence the position of a given protein band differs slightly from one gel to another. The direction of migration was from left to right.

the chains existed as dimers. A molecular weight of 1.7×10^4 was used for calculating protein concentrations (Weber, 1968b).

As seen in Table III, CTP binds more strongly to Zn regulatory subunit than to apo regulatory subunit. In phosphate buffer at pH 7, K was about 1×10^{-4} M for Zn regulatory subunit and 1×10^{-3} M for apo regulatory subunit. Binding of CTP to the regulatory subunit was stronger in Tris buffer at pH 8.2 with K for Zn regulatory subunit *ca.* 3×10^{-5} M and *ca.* 5×10^{-4} M for apo regulatory subunit. These values, though showing that Zn regulatory subunit has a higher affinity for CTP than does apo regulatory subunit, must be taken as approximate since insufficient data were obtained for any set of conditions to warrant construction of binding curves and testing the assumption of equivalence of the sites in dimers.

When apo regulatory subunit was converted to Zn regulatory subunit, most of the CTP binding capacity was restored. This is seen in the last four rows of Table III. Values for K of 4×10^{-4} M and 8×10^{-4} M for two preparations of apo regulatory subunit decreased to 0.9×10^{-4} M and 1.1×10^{-4} M, respectively, when the protein was dialyzed against a solvent containing 0.2 mM $ZnCl_2$ and no EDTA. The CTP binding obtained by the addition of zinc to the apo regulatory subunits was not as strong as observed for preparations of Zn regulatory subunit from which the zinc had not been removed. Nevertheless, this restoration of binding affinity, although incomplete, indicates that the lower affinity of the apo regulatory subunit for CTP was not merely the result of irreversible damage to the CTP binding site.

Reconstitution of the Enzyme from Catalytic Subunits and Covalently Cross-Linked Regulatory Subunits. Although the isolated Zn regulatory subunits existed as stable dimers and the regulatory polypeptide chains in other preparations showed some tendency to form dimers, these findings did not constitute evidence that the dimers were structural entities within the enzyme molecules. The regulatory chains could have been spatially separated from one another in the enzyme molecules, and association of the chains to form dimers could have occurred subsequent to the disruption of the aspartate transcarbamylase molecules upon treatment with mercurials. Hence efforts were made to determine whether the enzyme molecules could be reconstituted from native catalytic subunits and Zn regulatory subunits in which the two polypeptide chains in each dimer were covalently cross-linked to each other. Cross-linking was achieved, as described in the Experimental Section, by allowing the Zn regulatory subunits to react with dimethyl pimelimidate (Davies and Stark, 1970). Fractionation of the modified regulatory subunit preparation was performed on Sephadex G-100 to give a derivative consisting predominantly of cross-linked dimers, and reconstitution of aspartate transcarbamylase like molecules

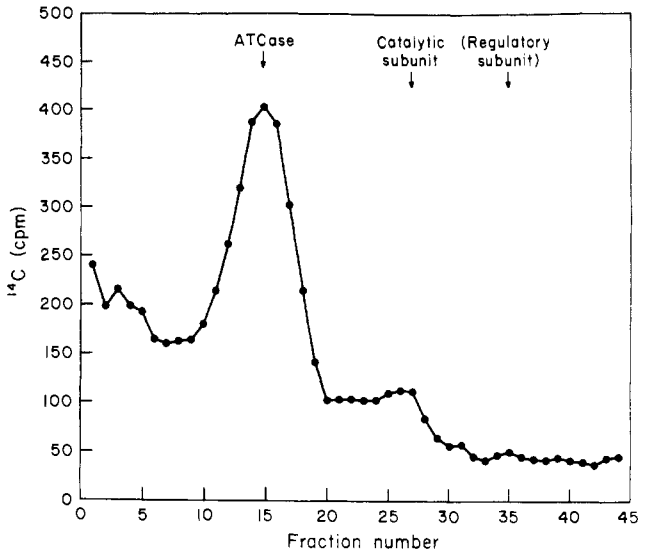

FIGURE 5: Sucrose density gradient centrifugation of reconstituted enzyme. Aliquots of the partially purified preparation of cross-linked regulatory subunit and unmodified catalytic subunit in 0.1 M Tris-HCl at pH 8.0 containing 10 mM ME and 0.2 mM $ZnCl_2$ were mixed to give final concentrations of 2.4 mg/ml of regulatory subunit and 5.3 mg/ml of catalytic subunit. This solution had a specific radioactivity of 2.9×10^6 cpm/ml. An aliquot, 0.2 ml, of this solution was layered onto a 5-ml gradient of 5–20% sucrose, containing 0.1 M Tris-HCl at pH 8.0 and 1 mM ME. Samples containing the enzyme and catalytic subunits, represectively, were applied to two similar gradients. The tubes were spun at 40,000 rpm at 20° for 14 hr in an SW-50 rotor. Fractions containing 5 drops, equal to approximately 0.11 ml, were collected. A 10-μl aliquot of each fraction was assayed for ^{14}C. The arrows at the top indicate the positions of the peaks for the enzyme and the catalytic subunit in the marker gradients and the position expected for the regulatory subunit.

was effected by the addition of catalytic subunits (Gerhart and Schachman, 1965).

Figures 3 and 4 show the steps in the cross-linking and fractionation procedure as followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. An electrophoresis pattern of aspartate transcarbamylase, shown in Figure 3A, provided reference bands of molecular weights 1.7×10^4 and 3.4×10^4, due to regulatory and catalytic chains, respectively. As seen in Figure 3B cross-linking the regulatory chains led to the formation of a major diffuse band with a mobility corresponding to a molecular weight of about 3.4×10^4. Also other bands with lower mobilities, corresponding to molecular weights of 5×10^4, 7×10^4, 1.0×10^5, 1.3×10^5, and 1.6×10^5, were formed. Cross-linking was not complete, as seen by the presence of a minor component having the mobility of individual regulatory chains. Thus the cross-linked preparation contained predominantly cross-linked dimers, small amounts of cross-linked heavier species, and some monomeric polypeptide chains.[2] The species heavier than the dimer arose presumably by cross-linking chains from different dimers. Since the dimethyl pimelimidate was labeled with ^{14}C, the composition of the mixture could be obtained by slicing the gel and measuring the radioactivity in the slices. As shown in Figure 4A, the distribution of ^{14}C in one principal component, a series of subsidiary larger components, and a minor component of lower molecular weight provided confirmation of the pattern of bands in Figure 3B. This unfractionated preparation of cross-linked regulatory subunits proved unsatisfactory for reconstitution since the addition of catalytic subunits led to the formation of a heterogeneous mixture of molecular species, most of which were larger than the enzyme. Accordingly the preparation of cross-linked regulatory subunit was fractionated by gel chromatography on Sephadex G-100 in 0.2 M triethanolamine hydrochloride at pH 8.5, containing 10 mM ME and 0.2 mM $ZnCl_2$. The protein eluted from the column as a series of small peaks, which were collected in one fraction, followed by a large symmetrical peak which formed the second fraction. As seen in Figure 3C the small peaks eluted early from the column represented the heavier species in the cross-linked preparation, while Figure 3D shows that the principal peak consisted predominantly of cross-linked dimers with some monomers[3] and a small amount of a component with a molecular weight of about 7×10^4. Slicing this gel and measuring the ^{14}C in the sections yielded the distribution of radioactivity shown in Figure 4B. About 87% of the total radioactivity was incorporated into protein species which migrated as dimers, with the monomer comprising 11% and the heavier species amounting to about 2%.

When the preparation of partially purified cross-linked regulatory subunit was used along with a slight excess of native catalytic subunit in reconstitution experiments, sedimentation velocity patterns obtained with the photoelectric scanner revealed a major component (60%) with the same sedimentation coefficient, 12 S, as native enzyme. A small amount of regulatory subunit did not combine with the catalytic subunit, and some formed complexes which sedimented more rapidly than the enzyme. In addition, some material with a sedimentation coefficient intermediate between those of the enzyme and the catalytic subunit was present. This mixture of products was subjected to sedimentation in a sucrose density gradient and the resulting pattern of the distribution of radioactivity throughout the tube was very similar to the distribution of optical density obtained with the absorption optical system. As seen in Figure 5, the main component, accounting for roughly half of the radioactivity, had a sedimentation coefficient equal to that of the enzyme. Some of the complexes containing ^{14}C-labeled regulatory subunit sedimented more rapidly than the enzyme, a smaller amount sedimented more slowly than the enzyme, and a very small amount of radioactivity existed as a component which sedimented at a rate corresponding to regulatory subunits.

The material in fractions 12–18 was pooled, precipitated by the addition of ammonium sulfate, and then redissolved, and dialyzed against a Tris buffer (0.1 M at pH 8.0) containing 2 mM ME and 0.2 mM EDTA. Polyacrylamide gel electrophoresis of this fraction in sodium dodecyl sulfate gave the pattern shown in Figure 3E. The bulk of the protein had a mobility corresponding to material of molecular weight 3×10^4, representing catalytic polypeptide chains and covalently cross-linked regulatory dimers. Only a small band corresponding to regulatory polypeptide chains of molecular weight 1.7×10^4 was detected. When this gel was sliced and assayed for radioactivity the pattern in Figure 4C was obtained. Thus the regulatory chains in the reconstituted enzyme existed

[2] All of these species, including the monomer, may have contained intrachain cross-links, and in addition some lysyl residues may have reacted with one reactive group of a pimelimidate molecule whose other reactive group was hydrolyzed by water.

[3] Since the buffer used in the gel chromatography contained $ZnCl_2$, the monomeric regulatory chains associated to form noncovalent dimers which were eluted from the column at the same position as the covalently cross-linked dimers.

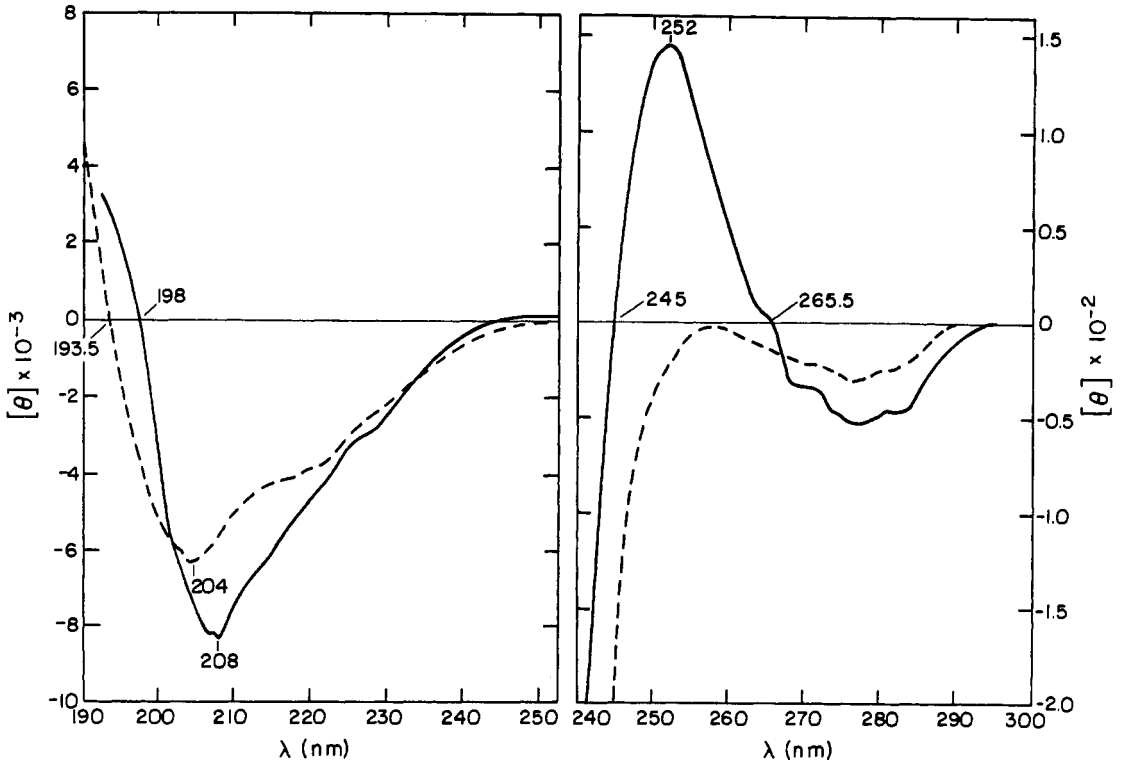

FIGURE 6: Circular dichroism spectra of the zinc and apo regulatory subunits. Spectra of Zn (———) and apo regulatory (----) subunits were determined in 0.04 M phosphate at pH 7.0 containing 0.2 mM $ZnCl_2$ and 1 mM EDTA, respectively, for the two types of subunit. ME was present at 10 mM for measurement of the near-uv spectrum and 1 mM for the corresponding far-uv spectrum. In the near-uv region of the spectrum a cell path length of 1 cm was used with the Zn regulatory subunit present at 3.2 mg/ml and the apo regulatory subunit present at 2.7 mg/ml. The corresponding spectra in the far-uv region were determined in a cell of 0.1-cm path length and a protein concentration of 0.3 mg/ml. Data are expressed in degrees cm^2/decimole of residues based on a mean residue molecular weight of 112.

predominantly (about 89%) as cross-linked dimers. Of the remaining radioactivity about 9% appeared as chains of molecular weight 1.7×10^4 and 2% as material of molecular weight 7×10^4.

This reconstituted enzyme and the partially purified cross-linked regulatory subunit used in the reconstitution were characterized also by electrophoresis on cellulose acetate membranes. The cross-linked regulatory subunit migrated as a diffuse, slowly moving component with a mobility similar to that of ummodified regulatory subunit. The reconstituted enzyme gave a single sharp band which migrated at the same rate as native asparate transcarbamylase.

The results represented in Figures 3–5 show clearly that covalently cross-linked regulatory dimers combine effectively with catalytic subunits to form a complex having the same sedimentation coefficient and electrophoretic mobility as native enzyme.[4] This finding constitutes strong evidence that the regulatory chains in each Zn regulatory dimer remain associated upon incorporation into the enzyme and that the dimers of the regulatory chains are integral structural entities of the enzyme molecules.

Optical Rotatory Dispersion and Circular Dichroism of the Enzyme and Its Subunits. Optical rotatory dispersion studies of the enzyme and the catalytic subunit showed peaks and troughs at 198 and 233 nm, respectively, along with crossovers (zero rotation) at 222 nm. The Moffitt–Yang plots of the data with $\lambda_0 = 212$ nm yielded values of -157 and -170 for b_0. These values of b_0, the magnitudes of the mean residue rotation (18×10^3 and 21×10^3 deg cm^2/dmole at 198 nm), and the positions of the extrema and crossovers are characteristic of proteins with significant helix content (Yang, 1967). By contrast, the Zn regulatory subunit appeared to have much less organized structure as revealed by a trough with a broad minimum at 230–232 nm, a peak at 197 nm with a mean residue rotation of 8.2×10^3 deg cm^2/dmole, a crossover at the unusually low wavelength of 211 nm, and a b_0 of only -45. In addition the ORD of the enzmye and catalytic subunit revealed no detectable anomalous behavior between 250 and 350 nm, whereas distinct Cotton effects were observed in this region for the Zn regulatory subunit.

As shown in Figure 6 the CD spectra of the apo and Zn regulatory subunits reveal contributions from chromophores in the near-ultraviolet region of the spectrum. A series of three overlapping negative ellipticity bands between 265 and 285 nm and a large positive peak at 252 nm were obtained for

[4] The fact that only 50% of the radioactivity was incorporated into complexes with a sedimentation coefficient of 11 S can probably be attributed to the presence in the cross-linked regulatory subunit preparation of cross-linked dimers in which the two polypeptide chains came from different dimers and were cross-linked in a different orientation with respect to each other than in the unmodified Zn regulatory dimers. Although the yield of aspartate transcarbamylase-like molecules was greatly improved by removal of the heavier cross-linked species from the original preparation, the Sephadex fractionation did not remove those cross-linked dimers in which the two chains were oriented abnormally. Such species might have formed complexes with the catalytic subunit smaller than aspartate transcarbamylase which were sterically prevented from aggregating further to form aspartate transcarbamylase-like molecules; alternatively if the orientation of the chains permitted, these species could have formed heavier complexes consisting of aspartate transcarbamylase-like molecules with extra catalytic and regulatory chains attached.

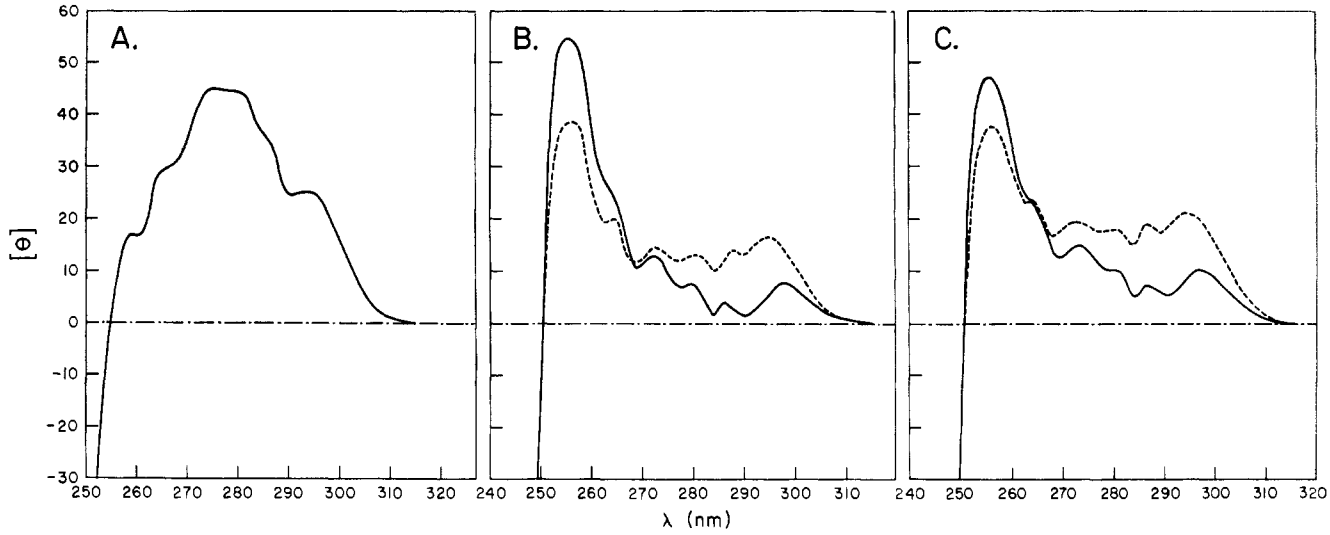

FIGURE 7: Circular dichroism of catalytic subunit, native enzyme, and mixture of subunits. Spectra of catalytic subunit (A), and enzyme (solid line in B) were detrmined in 0.04 M potassium phosphate at pH 7.0 containing 1 mM ME and 0.2 mM EDTA at protein concentrations of 1.7 and 2.0 mg/ml, respectively, in a cell of 1.0-cm path length. The weight-average summation of the CD spectra of the two subunits (dash line in B) was calculated from the catalytic subunit spectrum in A and the Zn regulatory subunit spectrum in Figure 6 by assuming contributions of 67% from the catalytic subunits and 33% from the regulatory subunits. The spectrum represented by the dash line in C, was obtained using a tandem cell containing two compartments of 1.0-cm path length; one contained Zn regulatory subunit at a concentration of 0.60 mg/ml in 0.04 M potassium phosphate at pH 7.0 containing 10 mM ME and 0.2 mM $ZnCl_2$, and the other compartment contained catalytic subunit at a concentration of 1.20 mg/ml in the same buffer, except that the $ZnCl_2$ was replaced by 0.1 mM EDTA. For the spectrum shown by the solid line in C, the two solutions were withdrawn from the cell and mixed, and half the mixture was placed in each compartment. Ellipticities are presented in units of degrees cm^2/decimole of residues, assuming a mean residue molecular weight of 112.

the Zn regulatory subunit. Upon removal of the zinc to give apo regulatory subunit the positive peak at 252 nm disappeared and the negative ellipticity at the longer wavelengths became less prominent.

The CD spectrum of the catalytic subunit showed a series of overlapping positive ellipticity bands between 257 and 295 nm (Figure 7A). Aspartate transcarbamylase, as seen in Figure 7B, gave a large positive band at 255 nm in addition to the small positive ellipticity in the aromatic region of the spectrum. For comparative purposes Figure 7B also shows the weight-average sum of the CD spectra of the catalytic and regulatory subunits (67 and 33%, respectively). This calculated spectrum differs significantly from the experimental curve for the intact enzyme. Similarly Figure 7C shows that the CD spectrum changed markedly when equimolar solutions of Zn regulatory and catalytic subunits were mixed to form reconstituted enzyme. The decrease in ellipticity at higher wavelengths and the increase at 255 nm upon the formation of the enzyme parallel the deviations of the calculated summation spectrum from that of the intact enzyme (Figure 7B).

Discussion

Structure of the Regulatory Subunit. The results of Weber (1968b) and Rosenbusch and Weber (1971a) and those presented in Table I showed that the regulatory polypeptide chains have a molecular weight of 1.7×10^4. This value taken in conjunction with the molecular weight of the enzyme (Gerhart and Schachman, 1965) and its composition in terms of the two types of subunits (Gerhart and Schachman, 1965) indicate that there must be six regulatory polypeptide chains per enzyme molecule (Weber, 1968b; Rosenbusch and Weber, 1971a) as summarized in Table I. However no conclusive evidence has been presented heretofore as to whether these chains occur singly or in groups as has been demonstrated previously for the arrangement of the six catalytic polypeptide chains in the form of two trimers (Meighen *et al.*, 1970). Thus the results in Figure 1 and Table I for the Zn regulatory subunit are of special significance. Since this derivative is a homogeneous species of molecular weight, 3.4×10^4, we conclude that the Zn regulatory subunit is a stable dimer. From this result we can postulate that the six regulatory polypeptide chains exist within the enzyme as three dimeric structures. Further evidence in support of this inference is presented below.

Other types of subunit preparations such as the mercury-containing regulatory subunit and the apo regulatory subunit were found by sedimentation velocity (Figure 1) and sedimentation equilibrium experiments (Figure 2) to exist as an equilibrium system consisting predominantly of monomers and dimers (Table II). The association constants for these equilibria ranged from about 10^4 M^{-1} to 10^5 M^{-1}, depending upon the type of preparation. The Hg regulatory subunit, like the Zn regulatory subunit, had a molecular weight corresponding to a dimer and showed little tendency to dissociate into single polypeptide chains, suggesting that metal substitutions do not affect the integrity of the dimer. In the light of these findings, we can attribute the variable and sometimes confusing results of previous molecular weight determinations to the partial dissociation of the dimers to monomers and differences in the metal content and the amount of aggregate in the older preparations.

Although no dissociation of the Zn regulatory subunits was detected in the sedimentation experiments and these subunits appeared to be stable dimers at pH values from 7 to 10, no special efforts were made to look for dissociation in very dilute solutions. If a monomer–dimer equilibrium existed with an association constant greater than 10^7 M^{-1} it would have escaped detection by the sedimentation methods used here. Evidence for such an equilibrium has come from recent

hybridization experiments with mixtures of Zn regulatory subunit and a succinylated derivative (G. Nagel and H. K. Schachman, unpublished experiments). When such mixtures were stored for 2 min in the presence of $ZnCl_2$ the formation of hybrid material was detected by electrophoresis. Hence some dissociation of the Zn regulatory dimers must have occurred, and the rate constant for dissociation cannot be less than about 10^{-2} sec^{-1}. If the upper limit for the rate constant of association is taken as 10^9 M^{-1} sec^{-1} (Eigen and Hammes, 1963), it follows that the monomer–dimer association constant must be less than 10^{11} M^{-1}. In contrast, mixtures of native and succinylated catalytic subunits can be stored for at least 2 weeks without the formation of hybrid species. Thus we can conclude that, whereas the catalytic trimers show little if any tendency to dissociate into single polypeptide chains, the Zn regulatory subunits must be predominantly dimers in equilibrium with small but finite amounts of monomer and that the association constant is greater than 10^7 M^{-1} and less than 10^{11} M^{-1}.

Evidence for the existence of the regulatory dimers as structural entities of the enzyme molecules was obtained from the cross-linking experiments (Figures 3–5) and from hybridization studies. Covalently cross-linked Zn regulatory dimers combined with catalytic subunits to give enzyme-like molecules in high yield. Also, G. E. Davies and G. R. Stark (1970, personal communication) found that cross-linking native enzyme followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis produced a major band corresponding to a cross-linked regulatory dimer. Finally, G. Nagel and H. K. Schachman (unpublished results) found a four-membered hybrid set after rapid addition of native and succinylated Zn regulatory subunits to a solution of catalytic subunits. This hybridization experiment shows that there are three regulatory combining units in the reconstitution process and that the three regulatory dimers did not dissociate to six single chains in the formation of the enzyme. Thus we can conclude that the regulatory polypeptide chains in the enzyme must exist as dimers. A model such as that proposed by Whitehead (1970) in which the six regulatory polypeptide chains are spatially separated in the enzyme is thus seen to be inconsistent with the experimental observations.

A Model of Aspartate Transcarbamylase. Since the regulatory subunits in the presence of zinc ions exist as stable dimers, show little tendency to aggregate beyond the dimer level, form the enzyme in high yield when mixed with catalytic subunit (Nelbach *et al.*, 1972), and can be reconstituted with catalytic subunits even when the two regulatory chains are covalently cross-linked, we consider these dimers as structural entities within the enzyme. Such stable dimers formed by isologous association of asymmetric polypeptide chains would have a twofold axis of symmetry (Monod *et al.*, 1965). The regions of bonding involve identical sets of residues on the two polypeptide chains and are designated the r:r domains.

The catalytic subunits are stable trimers which show no tendency to dissociate into individual polypeptide chains (Meighen *et al.*, 1970; Rosenbusch and Weber, 1971a) or to associate to larger species (Kirschner and Schachman, 1971). Such closed structures can be formed with all intrasubunit bonding domains satisfied only by a heterologous association among the c:c bonding domains (Monod *et al.*, 1965). A closed trimer of this type would have a threefold axis of symmetry.

We now proceed to examine the arrangement of the six catalytic and six regulatory polypeptide chains within the enzyme in the form of two trimers and three dimers, respectively. This arrangement must be such that the enzyme complex will have both threefold and twofold axes of symmetry as observed in crystallographic studies of the native enzyme (Wiley and Lipscomb, 1968, Wiley *et al.*, 1971). A model of a structure that satisfies these requirements is shown in Figure 8. The dimensions of the structure and many of the details are based on evidence from electron microscopy (Richards and Williams, 1972), which will be discussed below. Each catalytic subunit is represented as a slightly oblate structure having a triangular arrangement of the three asymmetrically shaped polypeptide chains. Arrows on the upper face of the structure indicate the heterologous association of the c:c bonding domains. The arrows pointing toward each other on the side faces of the catalytic chains indicate the isologous relationship of the catalytic trimers to give a structure with twofold axes of symmetry. Each regulatory subunit is shown as a pair of long cylinders oriented to one another at an acute angle; the cylinders represent the two regulatory chains in each dimer, with arrows pointing toward each other to illustrate the isologous association of the r:r bonding domains. The three regulatory dimers serve to interconnect the two catalytic trimers. This association between regulatory and catalytic subunits involves the formation of r:c bonding domains where each of the six regulatory chains comes into contact with one of the catalytic chains.

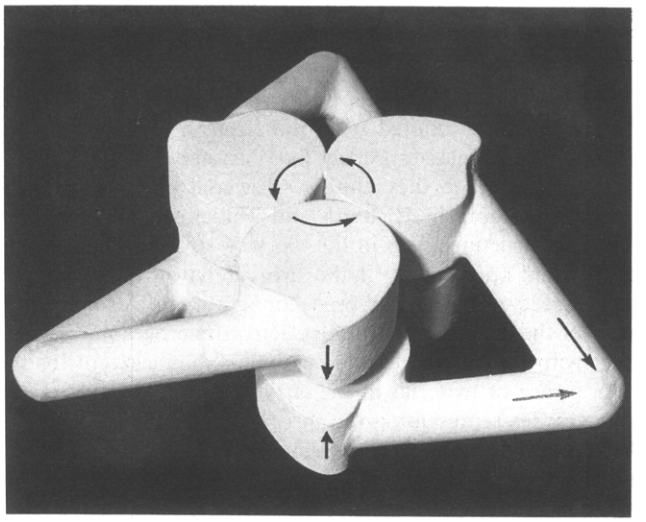

FIGURE 8: Model for arrangement of polypeptide chains in enzyme. Each catalytic subunit is shown as a triangular array of three asymmetrically shaped catalytic polypeptide chains. Arrows on the upper face indicate the heterologous association of the c:c bonding domains; arrows on the side faces of the catalytic chains indicate the isologous relationship of the catalytic trimers. Each regulatory subunit is shown as pair of cylindrical regulatory chains oriented to one another at an acute angle; arrows on the cylinders illustrate the isologous association of the r:r bonding domains.

There are a variety of questions about this proposed model which merit consideration. Are the two catalytic trimers in an eclipsed or staggered arrangement; *i.e.*, do the three polypeptide chains in the lower trimer lie directly beneath the three chains in the upper trimer as in Figure 8 or is the lower trimer rotated with respect to the upper trimer? Are the regulatory subunits sandwiched between the two catalytic subunits, as in the model of Rosenbusch and Weber (1971a), or do they extend to the outside of the molecule, as in Figure 8? Is there direct physical contact between the two catalytic trimers and among the three regulatory dimers? If the two catalytic tri-

mers are in an eclipsed arrangement, does each regulatory dimer link two catalytic chains which are directly under one another or does a regulatory dimer link two catalytic chains which are 120° apart? Tentative answers to most of these questions can be offered from the electron micrographs but additional evidence, especially from crystallographic studies, is required before they can be answered conclusively.

As pointed out by Richards and Williams (1972) the three disk-like structures seen in the top view of the enzyme molecule would appear only if the two catalytic trimers were in an eclipsed or nearly eclipsed configuration. Also, the top view of the enzyme seen in the electron micrographs is identical with the picture of the catalytic subunit except for the appearance of material in the enzyme on the outside of the three circles representing the three catalytic chains. This observation indicates that the regulatory subunits extend to the outside of the molecule rather than being located between the catalytic subunits. Further, the sandwich-like appearance seen in the side view of the enzyme indicates that the two catalytic trimers are spatially separated and hence are connected to each other through the regulatory subunits. The electron micrographs also provide evidence as to the precise arrangement of the regulatory polypeptide chains with respect to the catalytic subunits. The top views of the enzyme shown by Richards and Williams (1972) seem inconsistent with a structure in which each regulatory dimer links two catalytic chains which are directly above one another. The most likely interpretation of the electron micrographs is that shown in Figure 8 in which one regulatory chain in each dimer bonds to one of the upper catalytic chains and the other regulatory chain in that dimer bonds to a lower catalytic chain displaced by 120°. It is interesting to note one feature of the molecule conferred by the twofold symmetry axis and the consequent interrelation of the two catalytic trimers. If the amino acid residues of the catalytic chain which are involved in the r:c bonding domain are located on the left-hand side of each chain in the upper trimer, then the same residues must be located on the right-hand side of each catalytic chain in the lower trimer. The electron micrographs cannot distinguish, of course, between the structure shown in Figure 8 and an alternative structure in which the upper regulatory chain bonds to the right-hand side of a catalytic chain in the upper trimer and the lower regulatory chain bonds to the left-hand side of a catalytic chain in the lower trimer. Thus the regulatory subunits could be arranged like threads on either a right-hand screw or a left-hand screw.[5]

As shown in Figure 8, the two catalytic trimers are not in physical contact and it is assumed that there are no bonding domains between catalytic chains on the upper trimer and catalytic chains on the lower trimer. Similarly the three regulatory subunits are spatially separated so that direct interactions among them would be negligible and there would be no bonding domains between regulatory chains on different dimers. If there were direct attractive interactions between the two catalytic subunits in the enzyme, we might expect the isolated catalytic subunits to form aggregates. Thus far we have no evidence that these subunits aggregate in any specific manner. Similarly, if bonding domains between different regulatory dimers existed in the enzyme we might expect to find oligomers of the isolated Zn regulatory subunits. But the Zn regulatory subunits show little tendency to aggregate. Although the physical–chemical studies on the isolated subunits provide no evidence for bonding interactions between catalytic subunits or among regulatory subunits it is nonetheless possible that alterations in the conformations of the subunits upon the formation of aspartate transcarbamylase molecules, as suggested by the ORD and CD measurements, are sufficient to permit these direct interactions. Thus a model which includes close contacts, especially between catalytic subunits, cannot be ruled out conclusively by the physical chemical evidence, although the sandwich-like side views seen in the electron micrographs make such a model unlikely.

The dimensions of the intact structure and the subunits deduced from the electron micrographs are consistent with the molecular weights and the hydrodynamic properties of the various components. For the catalytic subunit the volume calculated from the molecular weight and partial specific volume is 1.3×10^{-19} cm^3 whereas that computed for three cylinders inscribed in an equilateral triangular prism 90 Å on a side and 40 Å high is 1.5×10^{-19} cm^3. The frictional ratio f/f_0 calculated from the molecular weight and sedimentation coefficient of the catalytic subunit is 1.27, a value in general accord with a slightly swollen, oblate structure of the type shown in Figure 8. For the Zn regulatory subunit the volume determined from the molecular weight and partial specific volume is 4.2×10^{-20} cm^3 whereas that measured from the micrographs for two cylinders 20 Å in diameter and 60 Å long is 3.8×10^{-20} cm^3. Although this agreement is good it should be recognized that the uncertainty in the thickness of the cylinders measured from the electron micrographs can be appreciable and that the correspondence may be fortuitous. The value of f/f_0 for the Zn regulatory subunit is 1.28, a value which is difficult to interpret in terms of the bent arrangement portrayed in Figure 8. Finally, although the model for the enzyme is only slightly anisometric, the large space in the center of the structure between the two catalytic subunits suggests that the molecules are highly solvated, which could account for the rather high value, 1.35, for f/f_0. In this regard it is of interest that the value of β (Scheraga and Mandelkern, 1953) for the enzyme, calculated using the intrinsic viscosity (Gerhart and Schachman, 1965), is 2.11×10^6. Although β

[5] The electron micrographs could be interpreted in terms of another possible but less likely arrangement of the regulatory chains relative to the catalytic chains. In this type of structure each regulatory chain could be visualized as being shaped like a boomerang 20 Å in diameter with two arms, each about 60 Å long. The boomerangs, paired to form dimers, could be arranged in two ways. First, each boomerang could be arranged horizontally, *i.e.*, more or less in the same plane as the catalytic trimers, with the two arms bonding to adjacent catalytic chains in the same trimer. The upper and lower boomerangs would be associated isologously by an r:r domain at their apices. Alternatively the boomerangs could be arranged vertically, *i.e.*, perpendicular to the planes of the catalytic trimers, by bonding to a catalytic chain in the upper trimer and on the same side to a second catalytic chain directly beneath it in the lower trimer. A boomerang bonding to the right-hand sides of two catalytic chains would form an r:r domain at its midpoint by isologous association with a second boomerang which is bonded to the left-hand sides of the two adjacent catalytic chains in the upper and lower trimers. Both of these arrangements would preserve the twofold symmetry and could lead to the top views seen in the electron micrographs. There is reason, aside from aesthetic considerations, to regard these arrangements as unlikely. Both arrangements would require two different types of r:c bonding domains, and each catalytic and regulatory chain would have to possess two binding sets of amino acid residues, one for each type of r:c domain. Hence this type of structure would be much more complex than the one shown in Figure 8, which involves only a single type of r:c bonding domain. These requirements for additional special sites and the absence of evidence for boomerang-shaped regulatory subunits seem sufficient to warrant the conclusion that a model based on them is not likely to be a representation of the true structure of aspartate transcarbamylase. In contrast the model depicted in Figure 8 is much less complicated and contrived; hence we favor it.

values are only slightly sensitive to small changes in axial ratio, the low β value obtained for the enzyme could be taken to indicate that its axial ratio is close to unity. If one assumes that the enzyme is perfectly isometric and that the high values for f/f_0 (1.35) and for the intrinsic viscosity (0.045 dl/g) are due solely to hydration, then one can calculate (Oncley, 1941) that the enzyme is hydrated to the extent of about 1.1 g of water per g of protein. Rough calculations of the amount of water contained in the space between the two catalytic trimers in Figure 8 indicate that such a value for the extent of hydration is not unreasonably large. Thus the structure in Figure 8, a nearly isometric, highly solvated particle, agrees well with the hydrodynamic parameters of the enzyme. It should be noted, however, that the effect of the regulatory "arms" on the hydrodynamic properties of the molecule is difficult to predict and that their presence may also contribute to the high value of f/f_0.

Conformational Differences between the Isolated Subunits and Intact Enzyme. The difference between the CD spectra of the Zn and apo regulatory subunits (Figure 6) suggests that the binding of zinc ions to the regulatory subunits is accompanied by a change in the conformation of the polypeptide chains. Although the precise nature of the positive ellipticity band at 252 nm in the Zn regulatory subunits is not clear, it might be due to tyrosyl or phenylalanyl residues whose environments are altered upon the addition of zinc or to the zinc–mercaptide complex which is thought to be involved in the zinc binding site (Rosenbusch and Weber, 1971b; Nelbach *et al.*, 1972). Since the regulatory subunit contains no tryptophanyl residues, the negative ellipticity bands are probably attributable to the three tyrosyl residues in each regulatory chain. In the far-ultraviolet region of the spectrum the CD of the Zn and apo regulatory subunits differed slightly with both exhibiting relatively shallow minima at about 206 nm and crossovers at about 196 nm; these are features characteristic of proteins with little organized secondary structure (Greenfield and Fasman, 1969).

As shown in Figures 7B and 7C, the CD spectrum of the enzyme is markedly different from the sum of the contributions of the independent subunits. Upon the enzyme formation the CD spectrum may change because of alterations in the local environment of those regions which in the isolated subunits are exposed to solvent and become part of the bonding domains in the intact enzyme. In addition, the assembly of the enzyme may be accompanied by changes in the conformation of the constituent polypeptide chains. The difference between the calculated and measured ellipticity at 255 nm indicates that the enzyme formation leads to a perturbation of the chromophores which give rise to the large positive ellipticity band in the spectrum of the Zn regulatory subunit. However, since the peak at 255 nm is not well resolved from the large negative trough in the far-ultraviolet region, the increase in ellipticity at 255 nm upon subunit aggregation might be due also to decreased negative contributions from the polypeptide chain backbones. The discrepancy in the region of aromatic amino acid residues indicates that perturbations of tryptophanyl side chains of the catalytic subunits and tyrosyl residues of either or both types of subunits occur when the subunits aggregate specifically to form the enzyme. A similar conclusion was reached by Griffin *et al.* (1972)[6] on the basis of reconstitution experiments conducted at pH 8.1. It should be noted that, upon the enzyme formation at the higher pH, the changes in the CD spectrum were similar to those presented here for the wavelengths between 265 and 310 nm; however, no change was observed at the higher pH in the wavelength region from 250 to 265 nm. The CD changes (Figure 7) are supported by ORD studies which revealed a decrease in the magnitude of the 233-nm trough upon the formation of the enzyme from its subunits (Pigiet, 1971).

Location and Role of Zinc in Aspartate Transcarbamylase. For the enzyme to exist as a stable complex of two catalytic trimers and three regulatory dimers, the c:c, r:r, and r:c domains of bonding must each surpass a certain level of stability. As shown by Nelbach *et al.* (1972) zinc ions are essential for the stabilization of the quaternary structure of the enzyme. Hence, the zinc ions could be implicated directly or indirectly at any or all of these bonding domains. However, catalytic subunits lacking zinc have been found to exist as stable, active trimers even at concentrations of 1 μg/ml (M. Springer, Y. Yang, H. K. Schachman, unpublished results). Thus, the stabilization of the enzyme by zinc ions does not involve increases in interchain affinity in the catalytic subunits and we must examine other possibilities for the location and role of the zinc ions. First, they might be located in the r:r domains and their effect might be attributed solely to the enhancement of the strength of these bonding domains. Second, the zinc ions might be situated at the r:c domains where they might influence only the association of the regulatory and catalytic subunits. Third, they might be located at either the r:r or the r:c domains and might strengthen both types of bonding domains. Since the r:r and r:c domains are separated by a large distance in the structure shown in Figure 8, the zinc ions could not participate directly in both types of domains. Hence, if the zinc ions are located at either type of domain, r:r or r:c, their effect on strengthening the other domain would have to be indirect by promoting a conformational change in the regulatory polypeptide chains. Finally, the zinc ions might be located at a region of the regulatory chains separated from both the r:r and r:c domains and influence both types of domains indirectly. At present there is no conclusive evidence as to the location of the zinc ions; as a consequence, considerations of their role must be treated with caution.

Can the stabilizing effect of zinc ions on the quaternary structures of the enzyme be attributed solely to a strengthening of the r:r bonding domains? Is the failure to reconstitute the enzyme from catalytic and apo regulatory subunits (Nelbach *et al.*, 1972) due to weak r:r bonding domains in the absence of zinc ions? As shown in Figure 1 and in the sedimentation equilibrium studies, the strength of the r:r bonding domains is increased markedly by the addition of zinc ions. For the apo regulatory subunits, the monomer–dimer association constant was only about 10^4 M^{-1}. In contrast, this association constant was between 10^7 and 10^{11} M^{-1} for the Zn regulatory subunits. Thus, the addition of zinc ions led to a change in the free energy of association of -4 to -10 kcal/mole. Since there are three such r:r domains in each the enzyme molecule, the considerable increase in affinity at the r:r domains upon the addition of zinc ions could be responsible for the marked enhancement in the stability of the enzyme molecules. Although it is clear that zinc ions lead directly or indirectly to a marked strengthening of the r:r domains, there is as yet no evidence to indicate that this effect is sufficient to account for the stabilization of the enzyme in the presence of zinc ions.

Are the r:c domains moderately strong even in the absence of zinc ions? If so, some association of regulatory chains with

[6] Manuscript in preparation.

catalytic trimers should be observed when apo regulatory subunits are added to catalytic subunits. This type of association has not been detected, but a careful search for the existence of such complexes in a zinc-free medium has not been conducted thus far. Indeed, a small amount of reconstituted enzyme was observed in mixtures of apo regulatory and catalytic subunits (Nelbach *et al.*, 1972), but the presence of a small amount of zinc in either of the preparations could account for the formation of the complex. The bulk of the protein in these mixtures existed as free regulatory and catalytic subunits and no reaction boundary corresponding to intermediate complexes was observed even though the protein concentration was several milligrams per milliliter. It thus appears that the r:c bonding domains in the absence of zinc ions are weak. Upon the addition of zinc ions, however, these domains must be strong since the enzyme shows no tendency to dissociate as low as 3 μg/ml (Schachman and Edelstein, 1966). Similarly, reconstitution is effected from dilute solutions of the subunits when zinc ions are present. These observations, though not warranting any quantitative estimates of the strength of the r:c domains in the absence and presence of zinc ions, provide some indication that zinc ions contribute to a strengthening of these domains.

On the basis of the available evidence, we conclude that the stabilzation of the quaternary structure of the enzyme by zinc ions stems, at least in part, from enhancement of the strength of the bonding forces in the r:r domains. Although crucial information is still lacking, it appears that the zinc ions are implicated also in strengthening the r:c domains. Since one of these effects must involve the indirect participation of the zinc ions, it is of interest to note the differences in CD spectra for the Zn and apo regulatory subunits (Figure 6). It appears as if the zinc ions promote a conformational change in the polypeptide chains. Since the zinc ions in the Zn regulatory subunits are readily removed by the addition of EDTA whereas in the enzyme they neither exchange with $^{65}Zn^{2+}$ nor are accessible for complex formation with the same chelating agent, it could be argued that the zinc ions are buried within the r:c domains. However, the metal ions might be located at the r:r domains and their decreased accessibility in the enzyme (relative to the Zn regulatory subunits) could be the result of conformational changes at the zinc binding sites when the various subunits aggregate. Such changes are suggested by the differences in CD spectra between the enzyme and a mixture of its subunits.

As shown in Table III, the addition of the zinc ions to the apo regulatory subunits leads to an increase in their affinity for CTP amounting to about a tenfold decrease in the apparent dissociation constant both at pH 7 and at pH 8.2. These results differ from those of Rosenbusch and Weber (1971b) who found equal CTP binding to the Zn and apo regulatory subunits. Further work is required to resolve this discrepancy but it should be noted that the experimental techniques, the temperatures of the solutions, and the metal contents of the preparations differed in the two studies. It is uncertain whether the zinc ions participate directly in the binding of CTP since they could lead indirectly to an enhancement of binding by promoting conformational changes in the regulatory chains. It is clear that enhancement of CTP binding by zinc ions, the strengthening of the r:r domains by zinc ions, and the increase in the monomer–dimer association constant upon the addition of CTP, could be explained by the existence of an equilibrium between two conformational states. One form would have a high affinity for both zinc ions and CTP and a strong tendency to dimerize and the other would have a low affinity for both zinc ions and CTP and a weak tendency to form dimers.

The work presented here on the structure, properties, and arrangement of the regulatory subunits in the enzyme are likely to be relevant to the mechanism of the allosteric effects exhibited by the intact enzyme. It is the regulatory subunits which confer allosteric properties on the enzyme, since catalytic subunits alone exhibit neither cooperative kinetics nor CTP inhibition (Gerhart and Schachman, 1965). Thus, the addition of regulatory subunits to catalytic subunits leads to a quaternary structure which changes its conformation in response to substrates and nucleotide effectors. If, as Gerhart (1970) has proposed, the transition between the relaxed and constrained states involves a change in the spatial orientation of the polypeptide chains relative to one another, there might be a movement of the regulatory chains at the r:r domains. Thus, further work should be directed toward assessing the importance of the r:r domains in the conformational changes which are responsible for the homotropic and heterotropic interactions exhibited by the enzyme.

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Added in Proof

The model in Figure 8 is consistent with the electron density map recently described by Wiley *et al.* (1971). Their cork ball model, though having eclipsed catalytic subunits and staggered regulatory subunits, is not as explicit as the wooden model in Figure 8 which has only a single r:c bonding domain between each regulatory and each catalytic chain.

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