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Shared active sites in oligomeric enzymes: Model studies with defective mutants of aspartate transcarbamoylase produced by site-directed mutagenesis

(catalytic site/*in vitro* complementation/interchain hybrid)

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ABSTRACT **Many oligomeric enzymes are functional only in the assembled form, and it is often difficult to determine unambiguously why monomers are inactive. In some cases individual monomers cannot fold into stable correct ("native") conformations without contributions from interchain interactions. For other oligomers, catalysis requires the contributions of amino acid residues at the interface between adjacent polypeptide chains, and monomers are inactive because they cannot form complete active sites. A test for the presence of shared sites was devised that is based on the formation of active hybrid oligomers from appropriate inactive parental mutants produced by site-directed mutagenesis. This approach was applied in a study of the catalytic trimer of aspartate transcarbamoylase (aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli*, using three mutants, in which Ser-52 was replaced by His, Lys-84 by Gln, or His-134 by Ala. Hybrid trimers formed from the virtually inactive Ser and Lys mutants were 10^5 more active than the parental proteins, and the specific activities of each hybrid were about 33% that of the wild-type trimer, as expected for the scheme based on shared sites. Hybrids from the His and Lys mutants had comparable specific activities. Moreover, one hybrid with ≈33% activity had one high-affinity binding site for a bisubstrate analog as compared to about three for wild-type trimer. As a further test, hybrids were also formed from wild-type and double-mutant (Lys-84→Gln and His-134→Ala) trimers. The hybrid containing two chains with the double mutation and one wild-type chain had very little activity, and that composed of one double mutant and two wild-type chains had 32% the specific activity of wild-type trimers. This negative complementation experiment is in quantitative accord with the scheme based on shared sites at or near the interfaces between adjacent chains. The techniques used to demonstrate shared active sites in the catalytic subunits of aspartate transcarbamoylase can be applied generally to various types of oligomers (dimers, tetramers, etc.) to determine whether the participation of amino acid residues from adjoining chains is essential for forming active sites in oligomeric enzymes.**

Many proteins require the association of subunits into oligomers to achieve a functional form, and interactions between these subunits have been found to modulate diverse physiological properties. Such interactions and the effect of ligands on them are responsible for the regulatory properties of tetrameric hemoglobin, most notably cooperative oxygenation and the Bohr effect (1). The association of two dissimilar monomers in lactose synthase results in an altered substrate specificity for the galactosyltransferase subunit (2). In aspartate transcarbamoylase the association of catalytic and regulatory subunits leads to a regulatory enzyme exhibiting cooperativity and sensitivity to activators and inhibitors (3). The monomer of aspartate aminotransferase possesses no intrinsic catalytic activity until it self-associates, producing a fully active dimer (4).

Despite numerous examples of oligomeric enzymes, in few cases is it explicitly understood how subunit interactions mediate the observed catalytic advantages. Formation of a functional enzyme from inactive monomer subunits (e.g., lactate dehydrogenase) could be due to several factors. In one model, a conformationally active state within a monomer may be obtained only upon forming the oligomer. Alternatively, the active site may be at the interface between adjoining polypeptide chains in the oligomer. In such enzymes, monomers are intrinsically inactive even if they have the proper conformation, because catalysis requires the joint participation of amino acid residues from adjacent polypeptide chains. How can one test directly for a shared active site? We present here an extension of the hybridization approach originally described by Robey and Schachman (5). The rationale for our experiments is based on two types of experiments. In one, hybrid molecules formed from two inactive parental proteins have greatly increased specific activities if the defects in the two oligomers are on opposite sides of the interface between the chains. The formation of active hybrids results from the assembly of a wild-type interface. In the second type of experiment, hybrids are formed from the wild-type enzyme and an oligomer defective on both interfaces, resulting in a loss of specific activity.

The catalytic (C) trimer of aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase; carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli* provides a well-defined model system for studying the interactions between polypeptide chains and for studying the necessity of oligomerization for enzyme activity. ATCase, which is composed of six catalytic chains organized as two trimers and six regulatory chains arranged as three dimers, catalyzes the first committed step in pyrimidine biosynthesis through the formation of *N*-carbamoyl-L-aspartate from L-aspartate and carbamoyl phosphate. The fully active C trimer, which displays Michaelis–Menten kinetics, can be easily prepared from the allosteric holoenzyme by treatment with mercurials (3). Isolated C trimers are readily dissociated into folded, inactive monomers upon treatment with NaSCN, and subsequent reconstitution studies have shown that the regeneration of enzyme activity is coincident with formation of the C trimer (6). Crystallographic studies of liganded and unliganded ATCase (7, 8) and hybridization experiments with C subunits (5) have led to the

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Abbreviations: ATCase, aspartate transcarbamoylase; C trimer, catalytic trimer; C_{xxx}, C trimer with identity of mutated residue in the polypeptide chain designated by X subscript; subscript L, Lys-84 replaced by Gln; subscript H, His-134 replaced by Ala; subscript S, Ser-52 replaced by His; subscript D, double mutant with Lys-84 replaced by Gln and His-134 replaced by Ala; subscript W, wild-type polypeptide chain; PALA, *N*-(phosphonoacetyl)-L-aspartate.

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view that the active sites in the C subunits of ATCase involve residues from adjoining chains. The hybridization experiments presented here, with defective proteins produced by site-directed mutagenesis, provide convincing evidence for shared sites and present a general approach for exploring the unique and obligatory interactions at interfaces within homo- and heterooligomers.

EXPERIMENTAL APPROACH

The rationale for demonstrating the presence of a shared functional site is illustrated schematically for a trimer in Fig. 1. This approach is equally applicable for dimers, tetramers, and other oligomers, as long as the activity is dependent upon the proper juxtaposition of amino acid residues on each side of the interface. A functional active site requires both a △ and a □ in close proximity and in the correct orientation at or near the interface between adjacent chains. In a wild-type trimer there are three active sites, in which △ and □ represent individual amino acid residues essential for activity. Mutations leading to defective proteins are indicated for one amino acid residue by ▲, and for the other, by ■. If the two mutant trimers are incubated under conditions that promote exchange of polypeptide chains, a binomial distribution (1:3:3:1) of four different trimeric species will result. As shown in Fig. 1 *Upper*, the hybrid set will be composed of the two parental trimers with no functional sites plus two different hybrids each containing one active site. According to this scheme each of the purified hybrid trimers would have 33% the specific activity of the wild-type trimer if the active sites are shared and if hybrids from proper defective mutants can be produced.

Although a high specific activity for each of the hybrids constitutes strong evidence for shared active sites, it is also possible that the formation of active species is attributable to conformational corrections within one or more of the chains in the hybrid oligomers. Aside from obvious controls of constructing hybrids from the wild-type trimer and each of the defective mutants to determine whether additional activity is generated, there is another set of experiments, illustrated in Fig. 1 *Lower*, aimed at providing more convincing evidence for shared sites. In this negative complementation experiment, hybrids are formed between wild-type trimers and those containing both mutations (△→▲ and □→■) in each chain. The hybrid set contains two new species along with the parental trimers. One hybrid, composed of two wild-type chains and one double-mutant chain, has only one active site and 33% the specific activity of the wild-type trimer. The other hybrid has no activity even though it contains one wild-type chain. In effect the two double-mutant chains destroy the capacity of the wild-type chain to function, by not providing the proper amino acid residues at the interface.

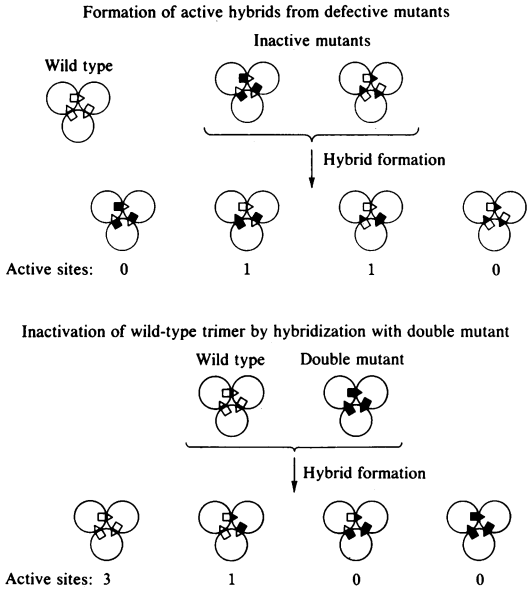

FIG. 1. Schematic view of hybridization technique for demonstrating shared active sites. Large circles correspond to individual subunits, with △ and □ representing unmodified essential amino acid residues. Mutated residues are represented by the corresponding ▲ and ■. (*Upper*) Formation of active hybrids from different defective mutants. (*Lower*) Loss of activity of wild-type enzyme in hybrids with a double mutant. For both schemes, the number of functional active sites, △ and □, per trimer is given. With equal proportions of parental trimers and random interchain exchange, a binomial distribution (1:3:3:1) will be formed.

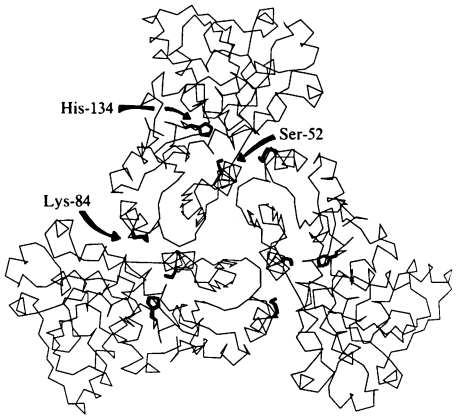

FIG. 2. α-Carbon backbone structure of a C trimer in unliganded ATCase (8). The view is from the center of the ATCase molecule, looking down the 3-fold axis of symmetry. The light line is the α-carbon trace of each monomer chain. The side chains of Lys-84, Ser-52, and His-134 are represented by dark lines.

Little is known about the identity and role of the amino acid residues implicated in the catalytic mechanism of ATCase. Nonetheless, site-directed mutagenesis experiments have identified three residues whose replacement leads to a drastic reduction in enzyme activity (9). Two of the residues, Ser-52 and His-134, as seen in Fig. 2 [based on the crystallographic studies on unliganded ATCase (8)], are close to each other in the folded chain and are located near the interface to the adjoining chain, which contains Lys-84.† When Ser-52 is replaced by Phe by random mutagenesis (10) or by His by site-directed mutagenesis (Y. R. Yang and H.K.S., unpublished data), virtually all enzyme activity is lost. The replacement of His-134 by Ala leads to a drastic reduction in activity (to 6% of wild-type enzyme) and a 6-fold increase in K_m. When Lys-84 is replaced by Gln or by Arg through site-directed mutagenesis, the resulting enzyme exhibits negligible activity (9). Thus, the mutations at Ser-52 and His-134 can be considered as equivalent to □→■ and the substitution at Lys-84 is designated by △→▲. In recent x-ray diffraction studies (7), all three of these residues have been implicated in binding the bisubstrate analog *N*-(phosphonoacetyl)-L-aspartate (PALA) to ATCase.

MATERIALS AND METHODS

The various mutant forms of ATCase were expressed from plasmids derived from pPYRB11 (11) in *Salmonella typhimurium* strain TR4574, which has a large deletion (12) removing the genes *pyrB* and *pyrI*, which encode the catalytic and regulatory chains of ATCase, respectively. This strain also contains the *pyrH700* allele, which causes high expres-

†The positions of these residues in *N*-(phosphonoacetyl)-L-aspartate (PALA)-liganded ATCase are known (7), but the coordinates are not generally available.

sion from the *pyrBI* promoter (13). Plasmid pPYRB768 leads to the synthesis of mutant ATCase with Lys-84 in the catalytic chain replaced by Gln; the C trimer isolated from the holoenzyme is designated C_{LLL}. Likewise, pPYRB771 contains the mutation responsible for replacing His-134 by Ala, and the isolated subunit is designated C_{HHH}. Construction of these strains has been described (9). pPYRB776 contains two amino acid alterations, Lys-84 to Gln and His-134 to Ala, in each catalytic chain, and the C trimer with the double mutation is designated C_{DDD}. pPYRB776 was constructed by subcloning pPYRB768 and pPYRB771 together via the unique *Bss*HII and *Eco*RI restriction sites. pPYRB774 was produced by oligonucleotide-directed mutagenesis (14) and contains the replacement of Ser-52 by His in the catalytic chain; the mutant subunit, designated C_{SSS}, was kindly provided by Y. R. Yang of this laboratory. Wild-type ATCase was expressed in *E. coli* strain TR4363 containing pPYRB3 (15) and was purified by the method of Gerhart and Holoubek (16). C trimers from wild-type ATCase (C_{WWW}) and the mutants were purified and characterized as described by Robey *et al.* (9).

The formation of active species from inactive C trimers was achieved by mixing equal amounts of the respective mutant trimers (final concentration, 2 mg/ml) at 4°C in 50 mM Tris acetate, pH 8.3/0.2 mM EDTA/2 mM 2-mercaptoethanol containing either 10 mM sodium pyrophosphate or 0.8 M urea to promote interchain exchange. Aliquots were withdrawn at various times and diluted 1:1000 into 40 mM potassium phosphate (pH 7.0) containing 10 mM aspartate and 4 mM carbamoyl phosphate. The formation of carbamoylaspartate at 30°C was measured by the increase in absorbance at 210 nm in a Cary model 118 double-beam spectrophotometer (17). The same procedure was used for monitoring the loss of activity when wild-type trimers were incubated with the double-mutant trimer, C_{DDD}.

Individual hybrid trimers were prepared and purified by the procedure of Gibbons and Schachman (18). In separate experiments, C_{LLL}, C_{SSS}, and C_{DDD} were reversibly acylated with 3,4,5,6-tetrahydrophthalic anhydride to introduce charged groups and to facilitate the subsequent chromatographic separation of the various species in the hybrid sets. Purified and deacylated trimers were assayed as described in Table 1. Ultraviolet difference spectra for the measurement of PALA binding to purified hybrids were obtained with a Cary model 118 double-beam spectrophotometer as described by Collins and Stark (19).

RESULTS

Formation of Active Hybrids from Defective Mutants. The regeneration of enzymatically active species from inactive parental mutants was demonstrated with C_{LLL} and C_{SSS}, each of which had a specific activity about 10^{-4} that of C_{WWW}. Incubating these mutants, with specific activities (V_{max}) of 0.003 and 0.001 μmol·hr^{-1}·μg^{-1} (compared to 34 μmol·hr^{-1}·μg^{-1} for C_{WWW}), in the presence of urea led to a progressive increase in activity approaching a limiting value greater than 10^5 that of the initial solution.‡ The absence of urea in the incubation buffer resulted in a minimal activity increase, reflecting virtually no interchain exchange. The final value after a 120-hr incubation of the mixture in the urea solution corresponded to 21% that of an equal amount of pure C_{WWW}. If there had been a random rearrangement of polypeptide chains (Fig. 1), to form a four-membered hybrid set, and each hybrid contained one active site per trimer, the activity of the mixture would have been 25% that of C_{WWW}.

Since the large increase in activity provided preliminary evidence that active hybrids were formed during the incubation of the inactive mutants in the urea solution, we purified the individual hybrids by the procedure described in ref. 18. Fig. 3 shows the gel electrophoresis pattern of C_{LLL} and acylated C_{SSS} prior to mixing, along with that of the four-membered hybrid set obtained after 90 hr of incubation. The patterns for the individual trimers purified by ion-exchange chromatography are shown in the four right-hand lanes; they correspond to C_{LLL}, C_{LLS}, C_{LSS}, and C_{SSS} (in these species the chains containing His in place of Ser-52 are acylated).

Enzyme assays were performed on all four deacylated species (Table 1). For both C_{LLL} and C_{SSS} isolated from the hybrid set, the values of V_{max} were the same as those measured initially for the two mutants. In contrast, the hybrids, C_{LLS} and C_{LSS}, had specific activities almost 10^5 greater than those of the parental mutants. These two hybrids had values expected for trimers containing one active site: respectively, 34% and 32% the activity of C_{WWW} (Table 1).

Analogous experiments were performed with C_{LLL} and C_{HHH}. The latter mutant (9) has about 6% the specific activity of C_{WWW} and its K_m for aspartate is much higher, 45 mM compared to 7 mM. When these two mutants were incubated in 10 mM sodium pyrophosphate solution there was a 110-fold increase in enzyme activity within 7 hr. The final activity was 20% that of an equal amount of C_{WWW}, in good agreement with the expected 25% for a binomial distribution with each of the hybrid trimers containing one active site. When sodium pyrophosphate was omitted from the incubation mixture, there was little change in activity during the 7-hr period, thereby indicating that each mutant trimer showed little tendency to dissociate into individual polypeptide chains in the absence of sodium pyrophosphate.

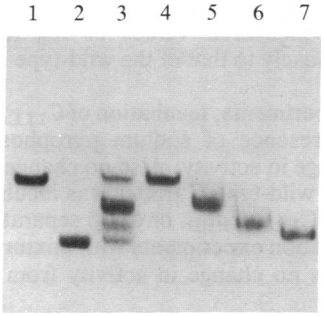

FIG. 3. Electrophoretic analysis of hybrid set formed from the inactive mutants C_{LLL} and C_{SSS}. C_{SSS} was reversibly acylated, therefore increasing its net negative charge per chain. Patterns for the parental trimers, C_{LLL} and acylated C_{SSS}, are shown in lanes 1 and 2. The hybrid set (lane 3) was formed by incubating 20 mg each of C_{LLL} and acylated C_{SSS} in a final volume of 8 ml at 4°C in 50 mM Tris acetate, pH 8.3/0.2 mM EDTA/2 mM 2-mercaptoethanol/0.8 M urea for 90 hr. Based upon their differing number of acylated subunits (and therefore overall net charge), the individual trimers were separated from the hybrid set by ion-exchange (DEAE-Sephadex A-50) chromatography at room temperature with a gradient of 0.3 to 0.9 M KCl in 50 mM Tris·HCl, pH 7.5/0.2 mM EDTA/2 mM 2-mercaptoethanol. Purified trimer species with acyl groups on the C_S chains are shown in lanes 4–7 (C_{LLL}, C_{LLS}, C_{LSS}, and C_{SSS}, respectively). Electrophoresis was performed in 7.5% acrylamide/0.2% bisacrylamide gel, using a Tris/glycine buffer system at pH 8.9 (22). Migration is downward with the origin at the top. Deacylation was achieved by dialysis for 24 hr at room temperature in 40 mM potassium phosphate, pH 6.1/0.2 mM EDTA/2 mM 2-mercaptoethanol.

‡With some trimers (C_{LLL}, C_{HHH}, and C_{WWW}), interchain exchange occurred readily in dilute (10 mM) sodium pyrophosphate. However, this reagent was ineffective in promoting hybrid formation with C_{SSS}, apparently because of inadequate binding; hence, 0.8 M urea was used for experiments with C_{SSS}.

Table 1. Specific activity of purified hybrids

Trimer	K_m (aspartate), mM	V_{max}, μmol·hr^{-1}·μg^{-1}	% activity relative to C_{WWW}
		Experiment 1	
C_{WWW}	7.2	34	100
C_{LLL}	*	0.003	0.008
C_{LLS}	4.7	11.6	34
C_{LSS}	7.9	10.8	32
C_{SSS}	*	0.001	0.003
		Experiment 2	
C_{LLL}	*	0.003	0.008
C_{LLH}	5.6	11.1	33
C_{LHH}	7.4	13.5	39
C_{HHH}	45	2.0	6
		Experiment 3	
C_{WWW}	7.2	34	100
C_{WWD}	5.6	10.7	32
C_{WDD}	7.6	1.2	3.6
C_{DDD}	*	0.0005	0.001

Trimers were purified as described in *Materials and Methods* and the legend to Fig. 3. Enzyme assays at various aspartate concentrations were conducted at 30°C in 50 mM imidazole acetate, pH 7.0/0.2 mM EDTA/4.8 mM carbamoyl phosphate, using the method of Prescott and Jones (20) as modified by Pastra-Landis *et al.* (21). Data were fit to the Michaelis–Menten equation.

*Because of the low values of V_{max}, accurate determinations of K_m were not possible.

As in the experiments with C_{LLL} and C_{SSS}, the hybrid set was formed from a mixture of acylated C_{LLL} plus unmodified C_{HHH} in a 20 mM sodium pyrophosphate solution. The electrophoresis pattern showed four species, which were readily separated on DEAE-Sephadex A-50 to yield the individual trimers. After deacylation of the purified components, the individual species (C_{LLL}, C_{LLH}, C_{LHH}, and C_{HHH}) were assayed for enzyme activity (Table 1). The two parental mutants had virtually the same values of K_m and V_{max} as those measured previously (9), but the two hybrids, C_{LLH} and C_{LHH}, had specific activities close to 33% that of C_{WWW}. Moreover, the measured values of K_m for the hybrids corresponded closely to that of the wild-type subunit and not that of C_{HHH}.

In control experiments, incubation of C_{LLL}, C_{HHH}, or C_{SSS} alone in the presence of sodium pyrophosphate or urea yielded no change in activity. Also no change in activity was observed when wild-type C trimer was incubated with each of the mutants (C_{LLL}, C_{HHH}, or C_{SSS}) separately. Moreover, in similar incubation experiments with mixtures of C_{HHH} and C_{SSS}, there was no change in activity from that measured initially.

Inactivation of Wild-Type Enzyme in Hybrids Containing Chains with Double Mutation. According to the model in Fig. 1 *Lower*, a mutant protein having defective functional residues (▲ and ■) at each of its interfaces with other polypeptide chains in the oligomer would cause a loss in activity of wild-type chains in hybrids. This negative complementation was tested by incubating C_{WWW} and C_{DDD} in a 10 mM sodium pyrophosphate solution. Aliquots were removed at various times for assays, and the enzyme activity was found to decrease progressively over 15 hr to a final value corresponding to 23% that of an equal amount of wild-type C trimers. This result is in excellent agreement with the expected 25% based on a random redistribution of chains yielding one-eighth of the population with three active sites per trimer and three-eighths of the trimers containing one active site. A similar incubation of C_{WWW} and C_{DDD} in the absence of sodium pyrophosphate resulted in a negligible decrease in activity over 75 hr.

As in the experiments described with the pairs of single mutants, C_{DDD} was acylated with 3,4,5,6-tetrahydrophthalic anhydride and incubated for 20 hr with an equal amount of C_{WWW} in 20 mM sodium pyrophosphate. The four species in the hybrid set were separated by ion-exchange chromatography and, following deacylation, the purified trimers were assayed for enzyme activity (Table 1). The specific activities (V_{max}) of C_{WWW} and C_{DDD} recovered from the hybridization experiment were the same as those of the parental proteins. The hybrid C_{WWD} had 32% the specific activity of wild-type trimers even though it contained two wild-type chains. Much less activity (3.6% that of C_{WWW}) was observed for the hybrid, C_{WDD}, containing one wild-type chain.

Neither C_{WWW} nor C_{DDD} changed in activity when incubated alone in solutions containing 10 mM sodium pyrophosphate. Also, the incubation of any of the single mutant trimers (C_{LLL}, C_{HHH}, or C_{SSS}) with C_{DDD} showed no detectable changes in enzyme activity.

Spectral Titration of PALA Binding to C_{LSS} Hybrid. The binding of PALA to wild-type C trimer (or to intact ATCase) causes a perturbation of the ultraviolet absorption spectrum of the protein, which is readily observed by difference spectroscopy (19). Moreover, the affinity of the C trimer for PALA is so great that Collins and Stark (19) were able to use difference spectroscopy for titrating the number of PALA binding sites. The inactive mutant C_{LLL} apparently binds PALA, although weakly and nonstoichiometrically compared to wild type as indicated by the spectral perturbation. In contrast, C_{SSS} does not exhibit any spectral shift upon the addition of PALA, due presumably to the inability of PALA to bind because of the replacement of the small serine side chain by the more bulky histidine residue. Hence, in the C_{LSS} hybrid there would be three types of interfaces. One, containing Lys-84 (△) adjacent to Ser-52 (□), would constitute a "wild-type" active site and bind PALA strongly. The second interface, in which Gln-84 (▲) is adjacent to His-52 (■), would not be functional catalytically and almost certainly not bind PALA. At the third interface, Lys-84 (△) would be adjacent to His-52 (■), yielding an inactive site that probably would not bind PALA. Based on these considerations, we performed a spectral titration of C_{LSS} with PALA. As a control, analogous difference spectra were measured for C_{WWW}. The results (Fig. 4) show that C_{WWW} titrates at ≈2.7 PALA molecules per trimer and C_{LSS} at 1 PALA molecule per trimer. Moreover,

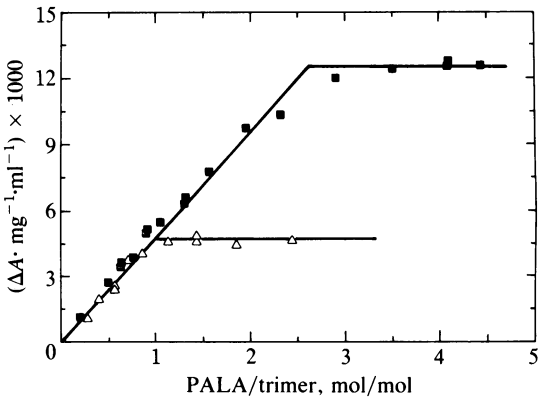

FIG. 4. Spectral PALA titration of C_{WWW} and C_{LSS}. Data are plotted as 1000 times the change in absorbance ($\Delta A_{289} - \Delta A_{285}$) per mg of protein per ml versus the molar ratio of PALA to C trimer. The effect of binding the bisubstrate analog on the absorption spectra, from 320 nm to 250 nm, of the various C-trimer species was measured directly as difference spectra (19). The addition of PALA to one cell was compensated for by the addition of an equal volume of buffer (40 mM potassium phosphate, pH 7.0/0.2 mM EDTA/2 mM 2-mercaptoethanol) to the reference cell. Initial protein concentrations were 2.12 mg/ml for C_{WWW} (■) and 1.87 mg/ml for C_{LSS} (△).

the maximal amplitude of the difference spectrum for C_{LSS} was 36% that observed for C_{WWW}.§

DISCUSSION

The results for the formation of active hybrid trimers from the defective parental mutants are in excellent agreement with those expected for the scheme (Fig. 1) with shared active sites at the interfaces between adjacent chains. Both the activities of the unfractionated hybrid set formed from the two pairs of mutants (C_{LLL}/C_{SSS} and C_{LLL}/C_{HHH}) and the specific activities of the purified hybrids are in quantitative accord with the view that the hybrid trimers contain one active site.

The finding that each hybrid had about 33% the specific activity of the wild-type trimer could be interpreted as the result of conformational corrections rendering each monomer partially active, rather than the formation of one wild-type interface and active site. But a variety of observations provide a strong argument against an interpretation based on conformational corrections. First, hybrids formed from C_{LLL} and either C_{SSS} or C_{HHH} have virtually identical specific activities. Second, activation requires interchain exchange; when the two mutational alterations are in the same polypeptide chain as in C_{DDD}, the trimer is inactive. Third, hybridization of any of the defective mutants (C_{LLL}, C_{SSS}, or C_{HHH}) with either C_{WWW} or C_{DDD} does not cause any change in activity relative to the initial mixture. Fourth, the spectral titration of C_{LSS} with PALA shows that the perturbation in the spectrum is complete upon the addition of one ligand molecule per trimer and that the amplitude between peak and trough in the difference spectrum corresponds closely (36%) to the value expected for a trimer with only one binding site. Fifth, and perhaps the most convincing evidence favoring the shared site model and invalidating an interpretation based on conformational corrections, is the loss in activity when hybrids are formed from C_{WWW} and C_{DDD}.

The results from both the positive and the negative complementation experiments provide an explanation for the observations that "folded" monomers are inactive and that enzyme activity is generated only upon the association of the chains into oligomers (6). In effect, each folded chain has a split active site with the two potentially competent regions separated on different surfaces. Each surface requires an adjoining or complementary one to form a shared active site. Thus, the combination of C_{HHH} and C_{SSS} does not produce an active species, because each defect is in the same side of the interface between the chains. Those mutational alterations (His-134→Ala and Ser-52→His) are designated in Fig. 1 by ■. But each of these mutants has a competent region on the other surface, containing Lys-84 and designated by △; therefore, when they are incorporated into hybrids with C_{LLL}, active species are formed from the competent region of C_{HHH} or C_{SSS} and the competent region of C_{LLL}. It follows, also, that a wild-type chain with two potentially competent interfaces loses activity when it is incorporated into a hybrid with chains containing two defective interfaces because of the double mutation.

The mutants used for these experiments were selected because of their low catalytic activity (9) and the known location of the individual amino acid residues, determined from crystallographic studies of ATCase in the T (less active) state (8). As yet, little is known about the roles of Ser-52, Lys-84, and His-134 in binding and catalysis, and the formation of active hybrids may not be unique for the mutants described here. Other combinations may suffice as well if the defects in the individual chains are located on opposite sides of the interfaces between the polypeptide chains.

These studies provide a structural basis for one type of *in vivo* interallelic complementation experiment in strains containing two alleles each of which produces an inactive gene product (23, 24). They also present a general method for demonstrating shared active sites in oligomeric enzymes.

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§A spectral titration was also performed with the purified C_{LLS} hybrid, which had two putative PALA sites, one of high affinity due to the presumed wild-type interface with Lys-84 (△) adjacent to Ser-52 (□) and another of lower affinity at the interface between Gln-84 (▲) and Ser-52 (□). A biphasic curve indicating two classes of PALA binding sites was observed, with strong binding to 1 PALA molecule per trimer followed by weaker binding to 1.8 PALA molecules per trimer. The maximal change in absorbance was 71% that observed with C_{WWW}. An unequivocal demonstration that the hybrids contain only one "wild-type" site must await the availability of an alternative mutant to C_{LLL} that does not bind PALA.