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Subunit Interactions in the Inducible Arginine Decarboxylase from Escherichia Coli B

Catalina N. Depusoy
Utah State University

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SUBUNIT INTERACTIONS IN THE INDUCIBLE
ARGinine DECARBOXYLASE FROM ESCHerichia CoLi B

by

Catalina N. Depusoy

A thesis submitted in partial fulfillment
of the requirements for the degree
of
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in
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1983
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Catalina N. Depusoy
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The nature of the subunit interactions in the inducible arginine decarboxylase from *Escherichia coli* B is of considerable interest because of the observed differences in the catalytic activities of the dimer and the decamer; the decamer is active and the dimer is inactive. To study these interactions, inactive dimers were prepared by sodium borohydride reduction of the ε-amino--pyridoxal-P Schiff base. Hybrid decamers were then prepared from varying molar ratios of native and reduced dimers. The hybrid decamers were indistinguishable from native decamers as observed in the analytical ultracentrifuge and on acrylamide gel electrophoresis. Kinetic studies indicated that true hybrids were formed rather than mixtures of all-native and all-reduced decamers. Results obtained with the decamers containing 1, 2, 3, or 4 parts in 5 of reduced enzyme showed no significant changes in $K_m$ values from the native decamer. However, the $V_m$ values for these hybrids are greater than predicted from the mole fraction of active dimers. For example, the hybrid containing 20% reduced enzyme
approaches the $V_m$ of the native decamer. These observations suggest that, in the intact molecule, two active sites cooperate catalytically but only one is catalytically active.

(67 pages)
INTRODUCTION

The inducible arginine decarboxylase from *Escherichia coli* B is composed of multiple, apparently identical, subunits, and exists in two aggregation states. The more aggregated form is a decamer with a molecular weight of 820,000. It binds 10 moles of pyridoxal-P per mole of enzyme (Blethen et al., 1968). The decamer dissociates reversibly to five dimers with a molecular weight of 165,000 (Boeker et al., 1969). Both species are readily observable, either independently or simultaneously in the analytical ultracentrifuge. The decamer has a sedimentation coefficient, $s_{20,w}$ of 23.3 S; the dimer is 8.0 S (Boeker & Snell, 1968). The conclusion that the enzyme is composed of 10 identical polypeptide chains is based upon several results: a) acrylamide gel electrophoresis in sodium dodecyl sulfate and mercaptoethanol gave a single band with a molecular weight of 96,000; b) isoelectric focusing of the reassociated dimers gave a single peak with a $pI$ of 4.44; c) peptide maps from tryptic digest gave the expected number for 10 identical polypeptide chains; d) the dissociated species has 1 mole of uniquely reactive sulfhydryl residue per 82,000 g; e) amino and carboxyl terminal residue analysis gave only methionine and alanine, respectively (Boeker et al., 1969); e) the sequence around the pyridoxal-P residue is unique (Boeker et al., 1971). Electron microscopic studies have shown that the decamer has a regular pentagonal structure apparently containing five dimers (Boeker & Snell, 1968). Since the dimer associates in a closed fashion, (dimer to decamer and no further), it appears that the
decamer is a heterologously bonded pentamer of isologously bonded dimers (Boeker et al., 1969).

Conditions are known under which the dimer-decamer transition occurs. Dissociation occurs when the buffer cations are monovalent, the pH is greater than 6.5, and the cation concentration is less than 0.05 M. Low protein concentrations and elevated temperatures also favor dissociation. For reassociation, any one of the following conditions is needed: the presence of divalent cations, a monovalent cation concentration above 0.08 M, or a pH of 6 or less. Reassociation also occurs when the dissociated species are exposed to substrate analogues; the competitive inhibitor, agmatine, and presumably the substrate itself causes reassociation (Boeker & Snell, 1968, Nowak & Boeker, 1981).

The problem of subunit interaction in arginine decarboxylase has arisen because of the observed differences in catalytic activities of the dimer and the decamer (Nowak & Boeker, 1981). A specific activity dependence on protein concentration was observed when the dimer was added to the pH 5.2 assay; the specific activity increases with increasing concentration of the dimer and becomes independent only at high protein concentrations. The increase in specific activity was also found to be time dependent. These observations suggest that the dimer is essentially inactive and does not associate during the assay at very low protein concentration but does associate to an active specie at higher protein concentrations. Presumably, the loss of activity upon dissociation to dimers arises because of lack of
catalytic communication among the active sites. One of these essential residues must be a pyridoxal-P, since it has been shown that the enzyme has an absolute requirement for pyridoxal-P. The apoenzyme, which has been resolved of its cofactor, is inactive and is activated only by the addition of excess pyridoxal-P. In addition, reduction of the Schiff base between pyridoxal-P and the enzyme destroys 98% of the initial activity (Blethen et al., 1968).

The observation that only the decamer is active suggests that the active site involves communication between dimers. Since pyridoxal-P is required, the active site could be composed of a) a pyridoxal-P site on one dimer and another site on a second dimer; or b) two pyridoxal-P sites on separate dimers, where each site is catalytically active and is required in a second capacity as well; or c) two pyridoxal-P sites, one active and the other inactive but required in a second capacity.

To distinguish these three possibilities, kinetic studies of active site-modified hybrids can be carried out. Active site modification can be achieved by reduction of the internal Schiff base between pyridoxal-P and a lysyl residue; loss of activity is complete (Blethen et al., 1968). Hybrids can then be prepared by dissociating the reduced enzyme, recombining it with native dimers in varying molar ratios, and reassociating. For the resulting hybrids, a plot of \( V_m \) versus the mole fraction of active dimer should give, in the first case, \( V_m \) proportional to the mole fraction, in the second case, \( V_m \) less than proportional to the mole fraction, and, in the third case,
$V_m$ greater than proportional. In the first case, the subunits are independently active and the activity follows the mole fraction of active dimer. In the second case, reduction of one of the two pyridoxal-P residue could inactivate both sites; activity would be less than the mole fraction. In the third case, reduction of one of the pyridoxal-P residues need not inactivate the site, since it contains a second, still active, pyridoxal-P; activity will be greater than the mole fraction of active dimers.

In addition, experiments have been undertaken to ensure that the reduced enzyme and the hybrids maintain the same quaternary structure as the native decamer, and that true hybrids were actually formed, rather than the theoretically possible mixtures of all-reduced and all-native decamers.
Subunit Interactions in Multisubunit Enzymes

A great majority of intracellular enzyme have multisubunit structures. In a protein complex, a subunit can be the simplest polypeptide or any separable functional portion of the complex. Usually only one catalytic activity is present in polymeric enzymes. Such enzymes may consist of catalytic and regulatory subunits as in aspartate transcarbamylase (Meighen et al., 1970), identical subunits, as in arginine decarboxylase (Boeker et al., 1969), or non-identical subunits, as in succinyl coenzyme A synthetase (Bridger, 1971). Most oligomers are either dimers or tetramers of identical subunits. When several catalytic activities are present, the subunit enzyme is classified as a multienzyme complex. Fatty acid synthetase is an example of such a complex. Each identical subunit of the fatty acid synthetase dimer has seven different enzyme activities: β-ketoacyl synthetase, β-ketoacyl reductase, β-hydroxyacyl dehydratase, enoyl reductase, acetyl transacylase, malonyl transacylase and thioesterase (Stoops & Wakil, 1981).

Subunit aggregation may lead to an open or a closed oligomer. In an open system, there is continuous aggregation, leading to higher aggregates. In a closed system, aggregation is finite and the subunits form rings. The distinction between open and closed
aggregation arises from the mode of association between subunits. A closed stable dimer, either a native species or the dissociation product of an oligomeric enzyme, is usually isologous; that is, association is head-to-head. The binding domains of each subunit are then saturated. Continued aggregation leading to open structures usually results when the binding domain between subunits are not saturated, as in heterologous or head-to-tail association (Levitzki, 1975). However, a closed heterologous structure can be achieved once the subunit form rings. The smallest such closed structure is a trimer. Trimers, tetramers, pentamers, and hexamers that result from heterologous association have cyclic symmetry, expressed as $360/n$ where $n$ is the number of subunits within the ring. A closed dimer can also be obtained by heterologous association if the relative orientation of the two binding sets are such that steric hindrance prevents further polymerization. The first dimer reported to have heterologous association is creatine kinase (Degani & Degani, 1980).

In isologous association, the binding domain is made up of two identical binding sets which, for a dimer, are related by a two-fold rotational symmetry axis. In heterologous association, there are two different binding sets. An important consequence of heterologous association in open structures is that the sites on identical subunits are no longer equivalent. In the extreme case, half-of-the site's reactivity can result. Half-of-the-sites reactivity is a phenomenon related to negative cooperativity in which out of $n$ potentially identical binding sites in an oligomer only $n/2$ sites react with a
substrate or an inhibitor.

The predominant pattern exhibited by multисubunit enzyme is that the aggregated state is the active one. The most notable advantage of polymerization is that regulation of catalytic activity can occur through the cooperative effects of subunits. Cooperativity is the influence of one subunit on the conformation and activity of other subunits. The original models of cooperativity are based on ligand-induced conformational changes. For example, the effect of positive cooperativity is the enhancement of binding, such that a ligand bound to a subunit increases the affinity of other subunits for subsequent ligands. Conversely, negative cooperativity is a decrease in affinity for subsequent ligands. These original definition of cooperativity adequately describe allosteric control brought about by ligand-induced conformational changes. However, they do not describe the role of subunit interactions in the catalytic mechanism.

The distinction between activity based on aggregation and activity coincident with aggregation depends on whether cooperative or independent activity is exhibited by the subunits upon aggregation. If activity is based on aggregation, the individual subunits are inactive; each subunit can contribute a unique domain to the active site. If activity is simply coincident with aggregation, the subunits independently are either active or potentially active; activation is not due to aggregation. Rather, activation and aggregation occur simultaneously and are sensitive to the same agents. For example, both the monomer and dimer of carbamyl-phosphate synthethase from E.
coli are equally active (Anderson, 1977); in hexokinase, the dimer is ten times more active than the monomer.

Not all oligomeric enzymes exhibit cooperativity; some which do not include mitochondrial malate dehydrogenase (Jurgensen and Harrison, 1982), aldolase (Meighen & Schachman, 1970a), glutamate aspartate transaminase (Boettcher & Martinez-Carrion, 1975) tryptophan synthetase (Hathaway et al., 1969), alkaline phosphatase (Meighen & Yue, 1975), superoxide dismutase (Malinowski & Fridovich, 1979) and hexokinase (Shill & Neet, 1975). Even if a subunit possesses catalytic activity independent of other subunits, aggregation may lead to increased catalytic efficiency. The dimer of yeast hexokinase exhibits a ten fold greater initial velocity than the monomer (Shill & Neet, 1975). X-ray studies suggest that the activator binding site lies between the subunits; binding of ATP and anionic metabolites in this site results in dimerization (Steitz et al., 1977). In some cases, aggregation may not be sufficient for full activation. For example, malate dehydrogenase requires cis/trans isomerization of a proline residue after dimerization for full activity (Wood et al., 1981).

In succinyl coenzyme A synthetase, shared active sites and catalytic cooperativity between the two $\alpha\beta$ dimers have been implicated in the enzyme's requirement for an intact quaternary structure for activity. The enzyme catalyzes the reversible formation of succinyl CoA from succinate and CoA with hydrolysis of ATP; this reaction is part of the citric acid cycle. The overall reaction
proceeds via a three step mechanism in which a phosphorylated enzyme and an enzyme bound succinyl phosphate are the two intermediates. The 3 steps are:

1. \( E + ATP \rightleftharpoons E-P + ADP \)
2. \( E-P + succinate \rightleftharpoons E \cdot succinyl-phosphate \)
3. \( E \cdot succinyl-phosphate + CoA \rightleftharpoons E + succinyl CoA + Pi \)

The active site is believed to be close to the point of contact between the α and β subunits (Bridger, 1981). The first step in the reaction is the formation of a phosphorylated histidine derivative on the α subunit. The β subunit binds succinate and CoA; and the phosphorylated α subunit then transfers the phosphoryl group to the β-subunit-succinate complex. Half-of-the sites reactivity was observed in the formation of the phosphohistidine intermediate; only one of the two identical α subunits could be phosphorylated at any one time. Hybrid enzyme studies and \( ^{31}P \) nuclear magnetic resonance studies of the enzyme and its interaction with substrate indicate that the two active sites cooperate catalytically. The cooperativity involves the release of the product, succinyl CoA, only when ATP is bound at the adjacent subunit. In the \( ^{31}P \) studies, the addition of succinate to the phosphoryl enzyme does not cause the appearance of a succinyl phosphate peak nor a decrease in the phosphorylated enzyme resonance. A succinyl-phosphate peak appears only when ATP is added to the already phosphorylated enzyme, suggesting that succinylphosphate production occurs only when ATP is bound at the other subunit (Bridger, 1981). The first indication that ATP promotes
succinyl-CoA dissociation was obtained by Bild et al. (1980) who demonstrated that the exchange rate between $^{18}O$ and succinate per ATP hydrolyzed increases as the ATP concentration decreases. In order for oxygen atoms to exchange between phosphate and succinate, the phosphorylated enzyme must react with the bound succinyl-CoA with reversal of the last 2 partial reactions in the postulated mechanism. The exchange rate is a measure of the relative residence time of succinyl-CoA; Bild's result indicated that the observed reduction of the exchange rate at high ATP meant that binding of ATP to one subunit of the enzyme promotes release of succinyl-CoA from other subunit. Using hybrid $\alpha\alpha'\beta_2$ where $\alpha'$ is chemically inactivated, the observed rate of exchange was slower. The predicted result, if the $\alpha$ subunits are independent, would be a constant rate of exchange. However, if the subunits are catalytically cooperative with both native and modified $\alpha'$ subunit participating in exchange, the rate would be slower; this was the observed result. Overall, these results indicate catalytic cooperativity in which ATP binding to one subunit promotes a catalytic event at the other subunit (Bridger, 1981).

When the active site of an enzyme is formed from two essential residues on different subunits, the individual subunits are inactive. For example, each identical subunit of the fatty acid synthetase dimer can catalyze all the partial reactions required for palmitate synthesis except for the $\beta$-ketoacyl synthetase reaction. A cysteine-SH in one subunit and a cysteamine-SH from the panthetheine residue of the acyl carrier protein are present at the active site of
the enzyme. Iodoacetamide inactivation studies have indicated that the cysteine-SH is acetylated; acetyl-CoA but not malonyl-CoA protects the enzyme against iodoacetamide inactivation. The pantetheine-SH is believed to be the site where malonyl-CoA binds. Cross-linking with a bifunctional reagent that has alkylating centers about 0.5 nm apart also inactivated the enzyme. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis has shown the appearance of a higher molecular weight species, suggesting that the subunits are cross-linked. Preincubation of the enzyme with iodoacetamide, acetyl-CoA, or malonyl-CoA gave very little of the cross-linked product; this suggests that the bifunctional reagent reacts with the cysteine-SH of one subunit and the pantetheine-SH of the other subunit.

A phenomenon related to half-of-the-sites is negative cooperativity; half-of-the-sites reactivity is considered to be an extreme case of negative cooperativity (Seydoux et al., 1974). Negative cooperativity was first observed in NAD⁺ binding to the apoenzyme of glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (Conway & Koshland, 1968). Glyceraldehyde 3-phosphate dehydrogenase from a wide variety of sources is a tetramer of apparently identical subunits. Equilibrium dialysis of the stable E(NAD⁺)₃ complex with NAD⁺ has demonstrated that the fourth molecule of NAD⁺ is very loosely bound; the calculated dissociation constants, K₁ through K₄ are < 10⁻¹¹ M, < 10⁻⁹ M, < 3 x 10⁻⁷ M, and 2.6 x 10⁻⁵ M, suggesting at least that there are two high affinity and two low
affinity NAD\(^+\) binding sites per tetramer. Controversy still exists as to whether the negative cooperativity in coenzyme binding is a consequence of a preexisting asymmetry or is ligand-induced.

There is no generally applicable mechanism suggesting the circumstances under which enzymes exhibit negative cooperativity. Reasons which have been advanced are a) nonidentical subunits that give rise to different classes of binding sites; b) asymmetric association of identical subunits; c) steric hindrance of ligand binding when two sites are adjacent; d) ligand induced conformational changes that alter the binding capacity of the vacant site (Conway & Koshland, 1968, Levitzki et al., 1971).

Negative cooperativity with one ligand does not imply negative cooperativity with all ligands. For example, both positive and negative cooperativity with other ligands has been observed in CTP synthethase (Levitzki et al., 1971), and in human plasma blood coagulation factor XIIIa (Seelig & Folk, 1980); ligand induced conformational changes has been invoked in both cases.

CTP synthethase catalyzes the amination of uridine triphosphate with glutamine as the amino donor; GTP is an allosteric activator. The binding of 6-diazo-5-oxonorleucine (DON), a glutamine analogue, to half of the subunits abolishes glutamine binding to the other half; negative cooperativity appears to be ligand-induced.

In creatine kinase, asymmetrical association of subunits is implicated as the cause of negative cooperativity; the
non-symmetrical subunit arrangement was detected by chemical modification studies where it was demonstrated that identical subunits react nonidentically with 2, 4-Dinitrophenylthiocyanate. Blocking the reactive thiol in one subunit has no effect on the enzymatic activity of the dimer whereas blocking the same thiol group on the other subunit leads to complete inhibition of the dimer (Degani & Degani, 1980).

Hybridization as a Tool to Study Subunit Interactions

Hybrids occur when two forms of a subunit exist in the oligomer of an enzyme. Lactate dehydrogenase (Markert, 1963) and glutamine synthethase (Ginsburg et al., 1970) have natural hybrids. Hybrids of other enzyme can often be made artificially by dissociating the oligomer into subunits, modifying part of the subunits chemically, and then mixing the two forms under conditions that cause reassociation.

Hybrids can be detected electrophoretically when the modified subunit has a different charge. As an example, succinylation of a lysine residue in glyceraldehyde 3-phosphate dehydrogenase produces a net charge difference such that the hybrid tetramers are electrophoretically separable from the native enzyme (Meighen & Schachman, 1970b). For variants that do not show charge differences, the existence of hybrids can sometimes be deduced from kinetic studies; for example, glutamine synthethase (Ginsburg et al., 1970).

The first hybridization experiment were in fact done on lactate dehydrogenase isozymes using freeze-thaw techniques in the presence of chaotropic anions (Markert, 1963). Isozymes are different proteins,
from the same organism, that catalyze the same chemical reaction. Lactate dehydrogenase has two variants: the H (heart) and M (muscle) types; the names are based on their abundance in these two tissues. The detection of five electrophoretic variants arising from the random hybridization of the H and M types established the tetrameric structure of the enzyme; the different molecular forms expected for a tetramer are $H_4$, $H_3M$, $H_2M_2$, $HM_3$, & $M_4$. The relative amounts of the 5 forms vary in different tissues from the same species; each tissue produces a characteristic ratio of H to M subunits. Lactate dehydrogenase catalyzes the interconversion of pyruvate to lactate in the presence of NAD$^+$. This reaction occurs at the junction between glycolysis and the tricarboxylic acid cycle; the enzyme is believed to be important in the regulation of aerobic and anaerobic metabolism. Since there is no proven allosteric regulation of this enzyme, it is believed that the multiple catalytic forms may by having different affinities for the substrates, play a role in controlling the concentrations of pyruvate and lactate (Kaplan et. al, 1968; Cahn et. al, 1962). For example, the difference in inhibition of the two isozymes by pyruvate is significant. Since the reduction of pyruvate to lactate by the H type is inhibited by low concentrations of pyruvate, it was suggested that rapid accumulation of lactate could not occur in tissue rich in the H type; this would lead to complete oxidation of glucose via the tricarboxylic acid cycle. The M type, on the other hand, is inhibited only at much higher concentrations of pyruvate. Tissues rich in M type would allow the rapid conversion of pyruvate to lactate leading to oxygen debt.
The existence of a natural hybrid involving the enzymatically
catalyzed adenylylation and deamidylation of glutamine synthetase from *E. coli* has been demonstrated from kinetic studies (Ginsburg et al., 1970; Denton & Ginsburg, 1970). Glutamine synthetase is a
highly organized regulatory enzyme that catalyzes the biosynthesis of
 glutamine, an important metabolite necessary for the formation of
proteins and the source of amine groups for many nitrogen containing
compounds. One form of regulation of this enzyme is the covalent
attachment and removal of a 5′ adenylyl group on a tyrosine residue.
This single-site modification dramatically affects the catalytic
properties of the oligomer. Glutamine synthetase is a dodecamer of
identical subunits arranged in two eclipsed hexagonal rings (Valentine
et al., 1968). The extent of adenylylation varies from 0-12 moles AMP
per mole enzyme. Adenylylation changes the metal ion specificity.
The fully adenylylated form is activated only by Mn$^{2+}$ and has no
activity with Mg$^{2+}$. Conversely, the unadenylylated subunit is
activated only by Mg$^{2+}$. Mixtures of unadenylylated dodecamers and
fully adenylylated dodecamers are activated by Mn$^{2+}$ or Mg$^{2+}$ in
proportion to the amount of adenylylated enzyme. However,
preparations of glutamine synthetase obtained by in vitro
adenylylation exhibit a sigmoidal decrease in Mg$^{2+}$ activated
biosynthetic activity and a sigmoidal increase in Mn$^{2+}$ activated
biosynthetic activity as the level of adenylylation increases. The
effects of adenylylation are most marked at the extremes of
adenylylation. It is believed that this change is of physiological
significance in meeting the metabolic needs of the cell. The
non-linear decrease and/or increase support the conclusion that hybrid
form of the enzyme (a dodecamer containing both adenylylated and unadenylylated subunits) does exist; heterologous interactions between dissimilar subunits can happen only in hybrids.

Chemical modification of proteins has broadened the application of hybrids considerably. Hybrids of active and chemically inactivated subunits have been a valuable tool in elucidating the role of subunit interactions in oligomeric enzymes. Site-specific modification was originally used to identify amino acid residues essential for catalysis; the loss of activity was correlated with modification of an amino acid in the active site. Site-specific modifications useful for hybridization can be achieved with side-chain specific modification reagents and active site directed irreversible inhibitors. Irreversible inhibitors do not resemble natural ligands but do possess a reactive group that can form covalent bonds with the enzyme. An example of an active-site directed irreversible modification reaction is NaBH₄ reduction of pyridoxal-P dependent enzymes (Glazer et al., 1975). Hybrids containing native and NaBH₄ reduced subunits have been obtained in glutamate aspartate transaminase (Boettcher & Martinez-Carrion, 1975) and tryptophan synthethase (Hathaway et al., 1969). Side-chain specific modification reagents react with certain functional groups (Glazer et al., 1975). Examples of oligomeric enzymes which have been inactivated by group-specific reagents include: mitochondrial malate dehydrogenase, where an active center histidine residue has been modified by iodoacetamide (Jurgensen & Harrison, 1982); aldolase, by succinylation of a lysine residue (Meighen & Schachman, 1970a); and glyceraldehyde 3-Phosphate dehydrogenase, also by succinylation (Meighen & Schachman, 1970b).
Results obtained from hybrids have established that subunits do make independent contributions to the total activity in some cases where conflicting results concerning site-site interactions had been reported. Enzymes where controversy has been resolved include glutamate aspartate transaminase (Boettcher & Martinez-Carrion, 1975), bovine erythrocyte superoxide dismutase (Malinowski & Fridovich, 1979), alkaline phosphatase (Meighen & Yue, 1975), and malate dehydrogenase (Jurgensen & Harrison, 1982). In each case, the activity was directly proportional to the number of unmodified subunits in the hybrid.

Hybridization has also been useful in elucidating ligand-induced conformational changes in allosteric enzymes. Hybridization studies on aspartate transcarbamylase have demonstrated that the direct effect of ligand binding on one subunit is communicated to the inactive subunit (Yang & Schachman, 1980). Aspartate transcarbamylase is composed of 2 catalytic trimers that are cross-linked noncovalently by 3 regulatory dimers (Meighen et al., 1970). After the introduction of a sensitive spectral probe by nitration of a tyrosine residue with tetranitromethane, hybrids were formed by mixing native and chemically-modified trimers with excess native regulatory subunits. The hybrid exhibited the same allosteric properties as the native enzyme: sigmoidal dependence of activity on substrate concentration and the activation/inhibition pattern caused by ATP/CTP. In fully modified enzyme, the binding of succinate, an active-site ligand, to the trimer causes a decrease in the absorbance of the nitrotyrosyl residue. In the hybrids, succinate binding causes an increase of
absorbance caused by the binding of succinate to the native trimer and modified subunit. In addition, succinate binding to the active catalytic chain of the hybrid causes a decrease in sedimentation coefficient and an increase in reactivity of the sulfhydryl group. These results demonstrate communication between catalytic and regulatory subunits in this allosteric enzyme.
METHODS

Materials

Wild type *E. coli* B was grown under conditions which have been shown to produce high levels of arginine decarboxylase (Boeker et al., 1969). The inducible arginine decarboxylase was purified by the procedure of Boeker, Fisher, and Snell (1969). The final preparation had a specific activity of 400 umoles/min/mg. L-(U-^{14}C)-arginine and Omniflour were purchased from New England Nuclear. Crystalline pyridoxal-P was obtained from Sigma. All other chemicals were reagent grade.

Preparation of the Hybrid Decamer

Arginine decarboxylase is supplemented with enough pyridoxal-P to occupy all the active sites. The amount needed is determined by enzymatic assay with and without added pyridoxal-P. The molar concentration of arginine decarboxylase was calculated from the protein concentration using a molecular weight of 82,000 for the monomer (Boeker et al., 1969). As shown in RESULTS, a 20:1 ratio of NaBH₄ to arginine decarboxylase monomer is then the lowest that gives maximum inactivation. The high ratio apparently results from the instability of NaBH₄ in aqueous solution at pH 5.2. For this same reason, NaBH₄ is added as a solution in 10% ethanol. The reduced enzyme is in 0.2 M sodium acetate, pH 5.2 and has a concentration
between 6.0-7.0 mg/ml.

Native and reduced dimers were prepared by dialysis of native and reduced decamers against two one-liters volume of 10 mM sodium phosphate, pH 7.0. The enzyme was centrifuged at 8,000 g for 10 minutes to remove any precipitate that developed during dialysis. Hybrid decamers were obtained by making mixtures of native and reduced dimers containing 1, 2, 3, or 4 parts in 5 of reduced dimers under conditions that cause reassociation. Reassociation occurs during dialysis of dimers against two one-liter volumes of 0.1 M sodium succinate, pH 5.8. The aggregation states of the dimers and the decamers were checked by sedimentation velocity experiments in the analytical ultracentrifuge as described below.

Activity Measurements

The enzymatic assay measures the production of $^{14}$CO$_2$ from L-arginine, and is based on the method of Morris and Pardee (1965). Calculations of specific activity were based on the percent of substrate converted to product, obtained by comparison to an exhaustion value. The exhaustion value was determined by using concentrated enzyme to convert all the substrate to product. The production of CO$_2$ was found to be linear for at least 25 minutes and up to 75% of exhaustion. In all activity measurements, calculations were done with values for percent exhaustion that are less than 50%. When the percent exhaustion was above 50%, the enzyme was diluted or the assay time was shortened if the enzyme concentration was critical.
Table I. Final Substrate Solution for Standard Activity Assay at pH 5.2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Final Concentration, in 0.25 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium acetate, pH 5.2</td>
<td>0.2 M</td>
</tr>
<tr>
<td>pyridoxal-P</td>
<td>0.02 mg/ml</td>
</tr>
<tr>
<td>L-arginine hydrochloride</td>
<td>0.03 M</td>
</tr>
<tr>
<td>L-(U-14C)-arginine</td>
<td>0.40 µCi/ml</td>
</tr>
</tbody>
</table>

The composition of the final substrate solution for the standard activity assay at pH 5.2 is given in Table 1. A 50 µl enzyme sample was added to 200 µl of an appropriate solution. The enzyme was diluted in 0.2 M sodium acetate, pH 5.2. Protein concentrations were obtained from absorbances at 280 nm using an extinction coefficient of 1.57 for a 1 mg/ml solution (Boeker et al., 1969). For each hybrid, kinetic measurements at different initial substrate concentrations were carried out at pH 5.2 based on the standard assay. A 62 µl solution of enzyme diluted in 0.2 M sodium acetate, pH 5.2, was added to 564 µl of an appropriate substrate solution. To vary the concentration of arginine within series, different volumes of a stock 25 mM arginine solution were diluted to a final concentration of pyridoxal-P of 0.01 M and of L-(U-14C) arginine of 0.12 µCi/ml. The final buffer was 0.2 M sodium acetate, pH 5.2.

Initial rate were estimated from plots of [product] /time versus [product] using the initial rate method of Boeker (1982). $K_m$ and $V_m$ values were obtained from a Hanes plot of [substrate] /velocity versus [substrate].
Analytical Ultracentrifugation of Arginine Decarboxylase

Sedimentation velocity experiments were performed in a Beckman model E analytical ultracentrifuge equipped with Schlieren optics. Double sector cells were used in an An-D rotor at a rotor speed of 58,000 rpm. Measurements of Schleiren pattern photographs were made on a Nikon model 6C Profile Projector. Sedimentation constants were determined to confirm the identity of dimer and decamer. The observed sedimentation coefficients reported for the decamer and the dimer are 23.0 S and 8.0 S (Boeker & Snell, 1968).

Discontinuous Gel Electrophoresis

Proteins were separated on slab gels using Laemmli discontinuous system at pH 8.8, without the addition of SDS (Laemmli, 1970). The separating gel was 7.5% in acrylamide and 3.5% in bis-acrylamide and was made with the Hoeffer SE 600 Vertical Slab Unit. An 18-25 μg protein sample was added in each well. The gels were run at constant voltage of 180 V after an initial voltage of 80 V during which the tracking dye migrates through the stacking gel. The gels were stained with 0.125% Coomassie Blue and destained with 50% methanol and 10% acetic acid.
RESULTS

NaBH₄ Reduction of Arginine Decarboxylase

The effect of varying the concentration of NaBH₄ on the specific activity of arginine decarboxylase after reduction is shown in Table II. A 20:1 ratio of NaBH₄ to arginine decarboxylase monomer is the lowest that causes maximum inactivation. The high ratio needed apparently results from the instability of NaBH₄ in 0.2 M sodium acetate, pH 5.2. For the same reason, NaBH₄ is added as a solution in 10% ethanol. The catalytic behavior of arginine decarboxylase is identical in the presence and absence of 10% ethanol. Control experiments gave a specific activity of 293 μmoles/min/mg with alcohol and 290 μmoles/min/mg without alcohol. Furthermore, the sedimentation coefficient of the native decamer in 10% ethanol is identical to the native enzyme in the absence of ethanol.

Inactivation of arginine decarboxylase by NaBH₄ can be increased by supplementing the enzyme with enough pyridoxal-P to occupy all the active sites (Table III). The amount needed is determined by enzymatic assay with and without pyridoxal-P. The specific activity without added pyridoxal-P is less than the specific activity observed when saturating amounts of pyridoxal-P are included in the assay. This difference represents the unoccupied pyridoxal-P sites on the enzyme. In Table II, unsupplemented arginine decarboxylase has 80% of the activity of the supplemented enzyme. Allowing for dissociation of
Table II. Sodium Borohydride Reduction of Arginine Decarboxylase\textsuperscript{a}

<table>
<thead>
<tr>
<th>[NaBH₄], µM</th>
<th>Specific Activity µmoles CO₂/min/mg</th>
<th>% Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>293</td>
<td>0</td>
</tr>
<tr>
<td>2.6x10⁻¹</td>
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<td>42</td>
<td>86</td>
</tr>
<tr>
<td>2.6x10⁻⁴</td>
<td>36</td>
<td>88</td>
</tr>
<tr>
<td>2.6x10⁻⁵</td>
<td>33</td>
<td>89</td>
</tr>
</tbody>
</table>

\textsuperscript{a}NaBH₄ in 10% ethanol was added to the arginine decarboxylase decamer in 0.2 M sodium acetate, pH 5.2. The arginine decarboxylase subunit concentration was 12 µM. There was no added pyridoxal-P.

Table III. Effect of Pyridoxal-P on NaBH₄ Reduction \textsuperscript{a}

<table>
<thead>
<tr>
<th>[Pyridoxal-P], µM</th>
<th>Specific Activity µmoles CO₂/min/mg</th>
<th>% Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>39</td>
<td>85</td>
</tr>
<tr>
<td>0.1</td>
<td>34</td>
<td>87</td>
</tr>
<tr>
<td>0.3</td>
<td>35</td>
<td>86</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>97</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 50 µl of a 540 µM solution of NaBH₄ in 2 M ethanol were added to a solution of the arginine decarboxylase decamer to give a final concentration of 260 µM NaBH₄. The original arginine decarboxylase had a specific activity of 250 µmoles/min/mg when saturated with pyridoxal-P; assays without added pyridoxal-P showed 80% of this activity. The final concentration of arginine decarboxylase subunit was 12 µM. The buffer was 0.2 M sodium acetate, pH 5.2.
pyridoxal-P during the assay, this is consistent with 85% inactivation of the unsupplemented enzyme. The lowest concentration of added pyridoxal-P that causes maximum inactivation is 3 μM, slightly more than 20% of the monomer concentration.

Properties of the Reduced Arginine Decarboxylase

The association/dissociation behavior of the reduced enzyme as seen in the analytical ultracentrifuge appears to be identical to that of the native enzyme (Figure 1). Dimers were obtained from both the native and reduced enzyme in 10 mM sodium phosphate, pH 7.0. Reassociation of both forms and apparent formation of hybrids took place in 0.1 M sodium succinate, pH 5.8. The calculated sedimentation coefficient of the reduced and the hybrid decamer is 22 S; 7.5 S for the native and reduced dimers. Both these observed values were obtained when the protein concentration was approximately 3 mg/ml. These values agree with the values of 23.0 S and 8.0 S reported for the decamer and the dimer after extrapolation to 0 mg/ml (Boeker & Snell, 1968).

The native and reduced decamers could not be separated by polyacrylamide gel electrophoresis using the Laemmli discontinuous system under non-denaturing conditions (Figure 2, lanes A, B, and C). The hybrids could not be resolved from native and reduced decamers (lanes G, H, I, and J). In addition, both native and reduced dimers are indistinguishable (lanes D, E, and F). These two observations suggest that the active site modification did not disrupt the
quaternary structure interactions of arginine decarboxylase. The similarity between the association/dissociation behavior of the reduced and native enzyme apparently makes NaBH$_4$ reduction an ideal procedure for the preparation of a suitable variant for hybridization.

Hybrid Formation

The dimer of arginine decarboxylase is formed in 0.01 M sodium phosphate, pH 7.0, and reassociates in 0.1 M sodium succinate, pH 5.8 (Boeker & Snell, 1968). Hybrids are evidently formed if appropriate mixtures are allowed to reassociate. In order to draw valid conclusions about possible subunit interactions, it is essential to establish that true hybrids are formed, rather than the theoretically possible mixtures of all-reduced and all-native decamers. Attempts to verify the identity of the hybrid decamers electrophoretically by separating the native, hybrid, and reduced forms, were unsuccessful (Figure 2). Kinetic studies of the reassociation of native and reduced dimers were then carried out to verify hybrid formation.

Previous work by Nowak demonstrated that the dimer is inactive and the decamer is active (Nowak & Boeker, 1981). In Nowak's work, a specific activity dependence on protein concentration was observed when the dimer was added to the pH 5.2 assay. Below a certain concentration, the specific activity increases with increasing concentration of the dimer; it becomes independent only at high protein concentration. This increase in specific activity with protein concentration was also found to be time dependent; the
Figure 1. Analytical ultracentrifugation of arginine decarboxylase. A, native decamer. B, NaBH₄ reduced decamer. C, hybrid decamer containing 3 parts native enzyme. D, native dimer. E, reduced dimer. Pictures are taken from left to right at 4 minutes interval at a rotor speed of 58,000 rpm. The concentration of enzyme in each run are between 3.0 - 3.5 mg/ml. Decamers are in 0.1 M sodium succinate, pH 5.8; dimers are in 10 mM sodium phosphate, pH 7.0.
Figure 2. Vertical polyacrylamide slab gel electrophoresis of arginine decarboxylase. The upper arrow points to the position of the decamer and the lower arrow to the dimer. A, native decamer. B, NaBH₄-reduced decamer. C, mixture of native and NaBH₄-reduced decamer. D, native dimer. E, NaBH₄-reduced dimer. F, mixture of native and NaBH₄-reduced dimer. G, decamer prepared by reassociation of native dimers. H, decamer prepared by reassociation of reduced dimers. I, hybrid decamer containing 4 parts native enzyme. J, hybrid decamer containing 2 parts native enzyme. Polyacrylamide gel electrophoresis was shown by Boeker & Snell (1968) to cause some interconversion of the dimer and the decamer.
specific activity increases with the length of time that the enzyme is incubated in the assay mixture. Nowak's result suggests that the dimer is essentially inactive, and does not associate during the assay at very low protein concentration but does associate, thereby becoming active, at higher protein concentrations.

An experimental modification of Nowak's work was carried out to distinguish formation of hybrids from formation of mixtures of all-native and all-reduced decamers. Reassociation depends on protein concentration and can be measured by the rate of regain of activity. If native dimer associates only with its own kind, adding reduced dimer should not increase the rate of appearance of activity. If hybrids are formed, the rate of appearance of activity should increase when reduced dimer is added.

Figure 3 shows the regain of activity due to reassociation of native and reduced dimers. A sigmoidal curve results from the time dependence of reassociation. A five-fold increase in the protein concentration of native dimer causes an increase in the rate of appearance of activity (●—● and □—□). The curve corresponding to 2.6 µg/ml native dimer is short because the assay ceases to be linear at this protein and substrate concentration. The greatest increase in the rate of appearance of activity occurs when reduced dimers are added to the native dimers; it is apparent that true hybrids are being formed. If mixtures of all-reduced and all-native decamers were being formed, the rate of appearance of activity should correspond to that for 0.52 µg/ml native dimer.
Figure 3. Reassociation of native and reduced dimer at pH 5.2. Dimers were diluted in 10 mM sodium phosphate, pH 7.0. A 50 µl enzyme sample was added to 200 µl substrate solution to give a final concentration of (○) 0.52 µg/ml native dimer; (□) 2.6 µg/ml native dimer; (△) 0.52 µg/ml native dimer and 2.1 µg/ml reduced dimer. The line (D) for 2.6 µg/ml native dimer is short because of assay linearity problems. Inset is a plot of specific activity versus time of the same data. Assay conditions in the 250 µl volume: 1 mM arginine and 0.1 mM pyridoxal-P in 0.1 M sodium acetate, pH 5.2.
Kinetics of the Hybrid Decamers

For each hybrid, \( V_m \) and \( K_m \) were measured by determining the initial velocity at several arginine concentrations. Initial velocities were obtained from the initial rate plot described by Boeker (1982). A sample plot is shown in Figure 4. The Y-axis intercept on this plot is the initial velocity. Initial rate plots were obtained for several arginine concentrations for hybrids containing 1, 2, 3, and 4 parts in 5 of reduced enzymes, the fully reduced decamer, the native decamer, and a mixture of 4 parts native decamer and 1 part reduced decamer.

\( K_m \) and \( V_m \) values were obtained for each hybrid from a Hanes plot of substrate concentration over initial velocity versus the substrate concentration. The results for the native decamer are shown in Figure 5, for the fully reduced decamer in Figure 6, for each hybrid in Figures 7-10, and for a mixture containing 4 parts native decamer and 1 part reduced decamer in Figure 11. On these plots, the X-axis intercept is \(-K_m\); the slope is \(1/V_m\).

Table IV gives the \( K_m \) values obtained for each hybrid and for the mixture containing 4 parts reduced decamer. These values do not appear to show a trend. The values agree with those previously reported: 0.65 mM (Boeker et al., 1969), 1.0 mM (O'Leary & Piazza, 1978) and 0.56 mM (Gale, 1940).
A plot of $V_m$ versus the mole fraction of active dimers is given in Figure 12. Some of the $V_m$ values of the hybrids are greater than the mole fraction of active dimers; the increase is more evident when the proportion of active subunits increases. The hybrid containing 4 parts native enzyme approaches the $V_m$ of the native decamer. In order to verify this apparent departure from the line expected if the subunits are independently active, $V_m$ values for mixtures of reduced and native decamers were obtained. The mixtures were prepared by dialysis of native and reduced decamers against 0.1 M sodium succinate, pH 5.8. The $V_m$ values were then obtained by assaying at saturating arginine concentrations. The results are shown in Figure 13. In addition, both $V_m$ and $K_m$ were determined for a mixture of 4 parts native decamer and 1 part reduced decamer (Figure 11); $V_m$ for this mixture is 80% of that for the identical native decamer.

Unless mixtures are undergoing subunit exchange, the $V_m$ values should be proportional to the mole fraction of active decamer; this is clearly the case in Figure 13. The difference between this result and that for the hybrids further confirms the existence of hybrids. Any deviation from a linear relationship between $V_m$ values and the mole fraction of active dimer can only arise as a result of subunit interactions in the hybrids.
Table IV. $K_m$ Values for the Hybrid Decamer and the Mixture

<table>
<thead>
<tr>
<th>Mole Fraction of Active Dimer</th>
<th>$K_m$ Values</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hybrids</td>
<td>Mixtures</td>
</tr>
<tr>
<td>0.0</td>
<td>0.62</td>
<td>-</td>
</tr>
<tr>
<td>0.2</td>
<td>0.70</td>
<td>-</td>
</tr>
<tr>
<td>0.4</td>
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<td>-</td>
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<tr>
<td>0.6</td>
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</tr>
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<td>0.8</td>
<td>1.00</td>
<td>0.57</td>
</tr>
<tr>
<td>1.0</td>
<td>0.53</td>
<td>0.53</td>
</tr>
</tbody>
</table>
Figure 4. Initial rate plot of the hybrid decamer containing 2 parts native enzyme at 2 mM arginine. The Y-axis intercept is the initial velocity.
Figure 5. Hanes plot for the native decamer after reassociation. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 0.31 µg of enzyme. The line is the least squares fit. The observed $K_m$ is 0.53 mM; $V_m$ is 0.11 µmoles/min.
Figure 6. Hanes plot for the reduced decamer. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 6.2 µg of enzyme. The line is the least squares fit. The observed $K_m$ is 0.62 mM; $V_m$ is 0.019 µmoles/min.
Figure 7. Hanes plot for the hybrid decamer containing 4 parts native enzyme. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 0.12 μg of enzyme. The line is the least squares fit. The observed $K_m$ is 1.0 mM; $V_m$ is 0.045 μmoles/min.
Figure 8. Hanes plot for the hybrid containing 3 parts native enzyme. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 0.31 µg of enzyme. The line is the least squares fit. The observed $K_m$ is 0.55 mM; $V_m$ is 0.081 µmoles/min.
Figure 9. Hanes plot for the hybrid decamer containing 2 parts native enzyme. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 0.31 µg of enzyme. The line is the least square fit. The observed $K_m$ is 0.60 mM; $V_m$ is 0.050 µmoles/min.
Figure 10. Hanes plot for the hybrid containing 1 part native enzyme. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 0.31 µg of enzyme. The line is the least squares fit. The observed $K_m$ is 0.7 mM; $V_m$ is 0.026 µmoles/min.
Figure 11. Hanes plot for a mixture containing 4 parts native decamer and 1 part reduced decamer. Initial velocities were measured in 0.2 M sodium acetate at pH 5.2 using 0.30 µg of enzyme. The line is the least squares fit. The observed $K_m$ is 0.57 mM; $V_m$ is 0.11 µmoles/min.
Figure 12. $V_m$ of the hybrids as a function of the extent of reduction of the active site. The specific activity for each hybrid ($\Delta-\Delta$) was calculated by dividing the $V_m$ obtained from a Hanes plot (Figures 5-10) by the amount of enzyme added. The theoretical specific activity ($\bullet-\bullet$) for complete half-of-the-sites reactivity is given for comparison; this calculation is described in the Discussion.
Figure 13. $V_m$ of mixtures of native and reduced decamers as a function of the mole fraction of active decamer. $V_m$ was obtained from assays at saturating arginine concentrations (0.15 mM) in 0.2 M sodium acetate, pH 5.2.
DISCUSSION

This investigation was undertaken to determine in part the nature of the subunit interactions in the inducible arginine decarboxylase from E. coli B. Previous work has shown that the dimer is inactive and the decamer is active (Nowak & Boeker, 1981). A plausible explanation for the loss of activity upon dissociation to dimers is the loss of catalytic cooperativity among the subunits. This interaction could involve the juxtaposition of two essential residues derived from separate dimers. One of these essential residues must be a pyridoxal-P residue since it has been shown to be required for activity (Blethen et al., 1968). The second residue could be remote from the pyridoxal-P site, or it could be the pyridoxal-P itself, acting in a second capacity.

The three possible sets of subunit interactions that would result in regain of activity upon reassociation are a) the active site is composed of a pyridoxal-P site on one dimer and another site on a second dimer; b) the active site is composed of two pyridoxal-P sites, both active and each simultaneously required in a second role for the activity of the other; c) the active site is composed of two pyridoxal-P sites, only one of which is catalytically active while the other is required in some secondary role that precludes catalytic activity. The first model implies that the pyridoxal-P residue on any one subunit in the decamer will exhibit full enzymatic activity independent of the presence or absence of catalysis on any other subunit. In contrast, the second and third models suggest catalytic
interaction, in which catalysis at one active site is affected by a catalytic residue on an adjacent subunit. However, the second model requires that catalysis at one active site does not happen unless catalysis occurs simultaneously in the adjacent subunit. In order to complete one catalytic cycle, a catalytic event at a neighboring subunit is also required. The third model implies negative interaction in which, out of two active sites, only one is catalytically active at any given time.

These three types of subunit interactions cannot be distinguished using the native decamer. However, kinetic studies on hybrid decamers containing both active and inactive dimers may separate the cases. In the third case, for example, the destruction of the catalytic ability of one of the two pyridoxal-P residues involved in an active site would not necessarily destroy the activity of the site itself, and hybrid decamers containing both modified and native dimers might have activity greater than that expected from the mole fraction of active enzyme. In the second case, reduction of one of the two pyridoxal-P residue could inactivate both sites and activity would be less than predicted from the mole fraction. In the first case, the subunits are independently active and the activity should follow the mole fraction of active dimer.

A theoretical plot of maximum velocity as a function of the mole fraction of active dimer for each model is shown in Figure 14. In order to obtain these curves it is necessary, first, to calculate the actual distribution of hybrid species obtained from a given
Figure 14. Theoretical results of the possible subunit interactions in arginine decarboxylase. The △'s represent half-of-the-sites reactivity; ○'s, independent sites; □'s, 2 active pyridoxal-P residues required for 1 active site.
reconstitution mixture, and second, to determine the number of active sites in each hybrid species. For each reconstitution mixtures, the hypothetical distribution of 0:5, 1:4, 2:3, 3:2, 4:1 and 5:0 (reduced:native) hybrids was calculated using a binomial distribution. For example, a reconstitution mixture containing 3 parts reduced and 2 parts native dimer would be expected to give 1% of 5:0, 7.7% of 1:4, 23% of 3:2, 26% of 1:4, and 7.8% of 0:5 (reduced:native) hybrids.

The configuration of individual active sites must then be considered for each model. If the active sites in arginine decarboxylase are independent, there must be 10 sites; each subunit is identical and the subunits are arranged symmetrically. However, if the sites are catalytically cooperative, there will only be 5 total sites in the decamer. The number of active sites in each hybrid would then vary depending on the model and the placement of inactive dimers. A hybrid containing either 1 or 4 reduced dimmers has only 1 possible configuration. However, a hybrid that contains 2 parts reduced enzyme has two unique configurations: one where the reduced dimers are adjacent and a second where a native dimer separates them (Figure 15). If catalysis requires two active pyridoxal-P residues simultaneously, the first configuration results in a total of two active sites on the decamer while the second configuration results in only one active site. In contrast, if the catalytic sites interact negatively such that only one of the two pyridoxal-P residues need be active, the first configuration predicts 4 active sites and the second predicts 5.
Figure 15. Possible arrangement of reduced dimers in a 2:3 (reduced:native) hybrid, as seen from the top. 
A. The two reduced dimers are adjacent. 
B. A native dimer separates the two reduced dimers. 
In case 1 the active sites are independent; in case 2 they are composed of two active pyridoxal-P residues from separate dimers; and in case 3 they are composed of two pyridoxal-P residues where only one need be active.
Table V. Comparison of Theoretical and Observed Maximum Velocities

<table>
<thead>
<tr>
<th>Mole Fraction of Active Dimer</th>
<th>Observed V^b_m</th>
<th>Theoretical V^a_m</th>
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<tbody>
<tr>
<td></td>
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<tr>
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^a The calculation is described in the text.

^b The calculated standard deviations in % are 1.0, 1.2, 2.5, 3.9, 2.2, and 5.6 arranged in descending order.

The activity predicted (Figure 14) for each reconstitution mixture is then the summation of the fractional activity contributed by all possible configurations taking into consideration the hypothetical distribution of each form.

It is apparent from Figure 12 that the positive interaction model does not apply to arginine decarboxylase. The results summarized in Table V are intermediate between the negative interaction model and the independent sites model. Table V shows the value of V^m_m, along with their standard deviations, calculated by regressions for the data shown in Figures 5-10. This calculation assumes that the experimental error is such that the average error bars in a plot of s_o/v_o versus s_o are of equal size. Although this assumption is not strictly correct
it can be shown to be a reasonable approximation (Cornish-Bowden, 1979). The observed values of \( V_m \) approach the negative interaction model as the mole fraction of active dimer increases from 0.6 to 1.0. However, below a mole fraction of 0.6, the results approach the independent model.

A statistical analysis, using the coefficient of determination, \( r^2 \), was done in an attempt to evaluate which model fits the arginine decarboxylase data better. \( r^2 \) is the amount of variability in the data explained or accounted for by the model and is often used to judge the adequacy of the model. \( r^2 \) is the square of the correlation coefficient, a measure of the linear association between \( y \) and \( x \). An \( r^2 \) value of 0.932 was obtained for the negative interaction model; 0.867 was obtained for the independent model. Since the calculated \( r^2 \) for the two models are not significantly different, it appears that the pyridoxal-P sites of arginine decarboxylase show considerable negative cooperativity but not complete half-of-the-sites reactivity.

The negative interaction model implies that, in the arginine decarboxylase decamer, two pyridoxal-P residues on different dimers are catalytically cooperative, but only one is catalytically active at any given time. Since the dimer is inactive, the two sites are evidently not on the same dimer. This conclusion is strongly supported by the fact that the substrate and substrate analogues can cause reassociation (Boeker & Snell, 1968, Nowak & Boeker, 1981). The interactions between the monomers of the arginine decarboxylase dimer appear to be isologous, or head-to-head (Boeker et al., 1969).
Figure 16. Possible arrangement of pyridoxal-P sites in the arginine decarboxylase decamer given the symmetry described in the text. The active site is composed of two pyridoxal-P sites (labeled PL) that are derived from two complementary halves of two dimers. The shaded areas are the subunit contact regions.
Heterologous interactions can be ruled out since indefinite aggregation of subunits has not been observed. The only symmetrical way of arranging the five dimers would be by heterologous or head-to-tail interactions.

A model for the active site of arginine decarboxylase that is consistent with all this information involves the 2 complementary halves of 2 dimers where the substrate could form a bridge between the dimers. This is illustrated in Figure 16.

Possible mechanisms by which the substrate could bridge the two dimers are shown in Figure 17. In the first, the ionized phosphate interacts electrostatically with the protonated guanido group of the substrate. In the second mechanism, the protonated 5' phosphate could participate in proton transfer to the ketimine Schiff-base formed after decarboxylation. Since the optimum pH of this enzyme is less than 6.0 and the two $pK_a$'s for the phosphate are approximately 2.5 and 6.5, one of the phosphate oxygens should be protonated. A role such as this, where the 5' phosphate group of pyridoxal-P acts as a general acid, general base catalyst has been suggested for glycogen phosphorylase (Sygusch et al., 1977).

Although the roles for the phosphate group shown in Figure 17 were suggested by the negative interaction model, they are supported by coenzyme analogue experiments done by Blethen et al. (1968). Using apoarginine decarboxylase various analogues were tested for their ability to reanimate the apoenzyme. Pyridoxal,
Figure 17. Possible roles for the 5' phosphate group of pyridoxal-P. (A) as an accessory binding site for the substrate and (B) as a proton donor. R is the side chain of arginine.
5-deoxypyridoxal and the analog in which the \(-\text{CH}_2\text{PO}_3^2-\) group is replaced by a \(\text{CH}_2\text{CH}_2\text{COO}^-\) group all fail to activate arginine decarboxylase. It is possible that pyridoxal is inactive because of hemiacetal formation between the \(-\text{CH}_2\text{OH}\) and the aldehyde (Metzler & Snell, 1955). However, since this cannot be true for 5-deoxypyridoxal, it is apparent that the phosphate is required for catalysis. Space filling models of the analog where \(\text{CH}_2\text{CH}_2\text{COOH}\) replaces \(\text{CH}_2\text{PO}_3^2-\) show that the two have the same spatial characteristics. However, the analog is presumably unprotonated at pH 5.2 (\(pK_a\) 4.9 as estimated from propionic acid).

In 1968, Boeker and Snell concluded that the association/dissociation behavior of arginine decarboxylase was most reasonably explained by an ionization of the type \(\text{RH} \rightarrow \text{R}^- + \text{H}^+\) causing the introduction of a new electrostatic repulsion between subunits. Although they suggested that ionization of carboxyl groups might be responsible, it seems that ionization of a phosphate is at least as likely. Dissociation occurs between pH 5.8 and 6.5, very close to the \(pK_a\) of the phosphate.

Taken all together, these arguments support a bifunctional catalytic role for pyridoxal-P in arginine decarboxylase supporting the negative interaction model. The observed half-of-the sites reactivity in the intact arginine decarboxylase may have arisen due to the bifunctional catalytic role of the pyridoxal-P.
SUMMARY

Active-site modified hybrids have been used to elucidate the role played by subunit interactions in the activity of arginine decarboxylase. Reduction with sodium borohydride appears to be specific for the internal Schiff base between pyridoxal-P and the enzyme; the reduced enzyme was virtually inactive. It was suitable for hybridization studies by several criteria: a) it has the same quaternary structure as the native decamer, as determined in the analytical ultracentrifuge and on polyacrylamide gel electrophoresis; b) it can be dissociated and reassociated; c) reassociation experiments indicated that true hybrids were formed, rather than mixtures of all-native and all-reduced decamers.

Kinetic studies of the hybrids showed no significant changes in $K_m$ values. However, the $V_m$ values are greater than predicted if the subunits are independently active. These studies suggest that, in the intact molecule, two active sites cooperate catalytically but only one is catalytically active at any moment.
LITERATURE CITED


