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Purification of near identical *Escherichia coli* aspartate transcarbamoylase holoenzyme hybrids by electroelution method

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SUMMARY

To study the functional cooperativity due to allosteric regulation in *E. coli* aspartate transcarbamoylase, two mutations (Lys60Glu & Lys94Glu) were introduced in the regulatory chain. This was done in order to disable the domain from binding the nucleotide effectors and at the same time would bring sufficient charge change on the surface of the protein molecule to be separated by ion-exchange chromatography. The mutant dimers along with the wild type dimers were used to reconstitute the holoenzyme hybrids. Due to different combinations four different types of hybrids were formed apparently exhibiting similar binding behavior to ion-exchange column. To purify these hybrid enzymes to homogeneity, preparative electrophoresis was successfully employed. The enzymes thus purified were assayed for comparison.

Key words: allosteric, preparative, electroelution, heterotropic, homotropic.

INTRODUCTION

Aspartate transcarbamoylase (ATCase) from *E. coli* (EC 2.1.3.2) is one of the most thoroughly studied enzymes. The holoenzyme is composed of six catalytic chains (Mr. 34,000) associated as two trimeric subunits, and six regulatory chains (Mr. 17,000) associated as three dimeric subunits^{1,2}. The protein exhibits functional cooperativity by allosteric regulation of protein function. It catalyses the carbamylation of the L-aspartate amino group by carbamyl phosphate, which is the first step in the pyrimidine biosynthetic pathway³. The binding of substrate aspartate induces the cooperative transition from the low affinity low activity T-state to the high affinity-high activity R-state⁴. This homotropic cooperativity for the substrate aspartate is heterotropically regulated by ATP, CTP and UTP [4]. The T-R transition in *E. coli* ATCase involves large quaternary alteration notably a 12 Å opening of inter catalytic trimer distance, 5–8 Å rotation of these trimers in opposite direction relative to the C₃ axis and 15 Å global rotation of the regulatory dimers around their pseudo-two fold rota-

tion axes^{5,6}. Apart from this, there are also considerable changes in the tertiary structure including the closure of the active site in each catalytic chain by rigid body movement of the ASP domain towards the CP domain and the displacements of the 240's loop which play a crucial role in the quaternary structural transition by modifying the C₁–C₄ interfaces and removing the C₁–R₄ interfaces⁷. The regulatory polypeptides also contain two discrete structural domains: the amino-terminal allosteric domain (amino acid residues 1–100) and the carboxy-terminal zinc domain (101–153)⁸. The zinc domain provides all the contact residues in the regulatory:catalytic protein interfaces while the allosteric effectors compete for binding at the allosteric binding site⁹. Consequently, the binding of nucleotide effectors at the allosteric site which is nearly 60 Å away¹⁰ must communicate the heterotropic modulation of the catalytic site through the zinc domain. The allosteric modification of the *E. coli* ATCase provides a logical mechanism for regulating the biosynthesis of pyrimidine nucleotides by integrating end product inhibition with activation by the purine ATP. CTP alone inhibits the

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Abbreviations: ATCase, Aspartate transcarbamoylase; ATP, Adenosine Triphosphate; CTP, Cytidine Triphosphate; UTP, Uridine Triphosphate. (Received December 10, 2003, Accepted April 12, 2004, Published June 15, 2004)

enzyme 50–60% and appears to bind tightly to only three of the six allosteric domains³⁾, while UTP alone has no effect¹¹⁾. In the presence of both the end products (CTP + UTP) enzymatic activity is synergistically reduced, causing an inhibition of greater than 95%. On the other hand ATP activates the *E.coli* ATCase 350–400%.

Despite of structural studies of several mutated regulatory chain holoenzymes and hybrid holoenzymes there is no clear cut answer how the allosteric domains signals the modulation of the catalytic domain.

In order to get an insight into these questions two mutations were made in the regulatory chain at the nucleotide binding site so that the mutant regulatory chains would not bind to the nucleotides, consequently, after reconstituting the holoenzyme would not exert any regulatory effect on the catalytic domain. The hybrid holoenzymes thus reconstituted were very much similar in their binding behavior towards ion exchange columns and their separation was extremely difficult. In order to purify them, preparative electroelution method was employed and these holoenzymes were successfully purified to homogeneity.

MATERIALS AND METHODS

Materials

Agarose, ATP, CTP, L-aspartate, N-carbamyl-L-aspartate, potassium dihydrogen phosphate, sodium chloride, glucose, Agar, Ampicilin, Tris and uracil were obtained from Sigma., Q-Sepharose fast flow resin was purchased from Pharmacia. Protein assay dye, sodium dodecyl sulfate, ammonium per sulfate and electrophoresis grade acrylamides were obtained from BIO-RAD. Casamino acids, yeast extracts and tryptone were purchased from Difco. Antipyrine and IPTG were obtained from Fisher. All restriction enzymes, DNA ligase and DNA ladder were purchased from New England BioLab. Plasmid mini-prep and gel extraction kits were purchased from Qiagen. The site directed mutagenesis kit was obtained from Stratagene. The preparative electrophoresis apparatus Model-491 PrepCell was procured from BIO-RAD.

Over expression and purification of ATCase regulatory wild type and mutant subunit

After making the plasmid constructs (K60E & K94E) by site directed mutagenesis following the protocol provided by stratagene, ATCase wild type and mutant regulatory subunits were overexpressed using strain EK1594. The bacteria were cultured at 37°C from overnight mini culture in 2YT media supplemented with glucose, 150 µg of ampicilline. The culture was induced with 1 mM IPTG after 4 h of growth at O.D. value between 0.6–0.7 and allowed to grow for another 4 h. Cells were harvested and resuspended in 0.1 M Tris-HCl, pH 9.2 containing 0.1 mM zinc chloride followed by sonication to lyse the cells. The purification of the regulatory subunit was performed as previ-

ously described¹²⁾. The hybrid holoenzymes were reconstituted by dialyzing the native C-chain trimers with wild type and mutant regulatory chain dimers for overnight at 4°C in 50 mM Tris-CH₃COOH pH 8.3.

Purification of holoenzyme hybrids were carried out after assembling the apparatus as per the instruction in the manual. Three centimeter length 6% polyacrylamide gel was cast in the apparatus and after polymerization the gel was run according to the procedure of Davis et al.¹³⁾. The outlet was connected to BIO-RAD Duoflow chromatographic system to monitor the absorbance of the eluted protein. The run was carefully observed till the dye came out of the gel. The flowthrough containing the dye was discarded. After 2 h of washing the fractions were collected and later analyzed by running PAGE.

RESULTS AND DISCUSSION

The holoenzyme hybrids were reconstituted by dialyzing native C-chain trimers, mutant regulatory dimers and native regulatory dimers in buffer. The model of hybrid formation was depicted in Fig. 1A. The reconstituted pool was checked for hybrid formation. Four different types of protein bands were observed in the gel (Fig. 1B & C). Each band corresponding to a single type of holoenzyme with/without mutant regulatory dimer. Because, the change of charge on the surface of the polypeptide due to mutations was from positive to negative, the mobility of each enzyme increases with the number of mutant regulatory chains dimers. Hence, the holoenzyme with more numbers of mutant dimer has the greater mobility and eluted earlier than others.

The proteins eluted from the preparative PAGE were analyzed in the native 6% PAGE (Fig. 2) and the pure fractions were collected for analysis. The figure showed that the two fractions in the lane 2 and 3 were the partially mutant holoenzymes with two and four mutant regulatory chains respectively. The fractions pooled were found to be much diluted and were concentrated later by Amicon concentrator. The protein concentrations were measured by Bradford method. These pure hybrid holoenzymes were subjected to the test of nucleotide effector influence.

The influence of ATP and CTP on the asymmetric holoenzyme was determined at one-half the [Asp_{0.5}]. This concentration of aspartate was selected since the nucleotide exerts a larger influence on the activity of the enzyme as the aspartate concentration is reduced¹⁴⁾. Based upon these nucleotide saturation curves, the maximal extent of activation or inhibition at infinite nucleotide concentration was determined. As can be seen in Fig. 3, CTP inhibits the enzyme to residual activity 50%, whereas, the hybrids C₆R₄mR₂ and C₆R₂mR₄ are inhibited to 54% and 64% residual activity respectively. The complete regulatory chain (C₆mR₆) mutant holoenzyme had not been inhibited as was expected.

Fig. 1. Reconstitution of ATCase holoenzyme hybrids.

[A], Model for the reconstitution showing the formation of four different hybrids, [B] PAGE, Lane 1, Wt ATCase; 2, Wt C-chain trimer; 3, reconstituted holoenzyme hybrids and excess Wt and mutant R-chains; 4, Wt R-chain dimer; 5, Mutant R-chain dimer; [C], magnified view of the reconstituted hybrids of aspartate transcarbamoylase as migrated in the SDS-PAGE shown in panel [B].

Fig. 2. PAGE of purified holoenzyme hybrids.

Lane 1, Mixture of all holoenzymes; 2, Hybrid $C_6R_4mR_2$; 3, Hybrid $C_6R_2mR_4$. Pure mutant and the native were made after reconstituting with mutant and native regulatory chains respectively.

Fig. 3. Influence of nucleotide CTP on the activity of enzymes.

($\blacktriangle$) C_6R_6 , ($\bullet$) $C_6R_2mR_4$, ($\blacktriangledown$) $C_6R_4mR_2$, ($\blacksquare$) C_6mR_6 . Colorimetric assay was performed at 25°C in 0.05 M Tris-acetate buffer, pH 8.3, at saturating concentration of carbamoyl phosphate (4.8 mM). The aspartate concentration was held constant at $0.5 \times [Asp]_{0.5}$.

Similarly, ATP activates the native holoenzyme to 350% residual activity similar to the reported value¹⁵, whereas, the hybrids $C_6R_4mR_2$ and $C_6R_2mR_4$ are activated by 239% and 152% respectively (Fig. 4). These results showed that the hybrids purified by PAGE have preserved their functionality, and can be used for further biochemical, biophysical and structural analysis.

Fig. 4. Influence of nucleotide ATP on the activity of enzymes.

(■) C₆R₆, (●) C₆R₄mR₂, (▼) C₆R₂mR₄, (▲) C₆mR₆. Colorimetric assay was performed at 25°C in 0.05 M Tris-acetate buffer, pH 8.3, at saturating concentration of carbamoyl phosphate (4.8 mM). The aspartate concentration was held constant at $0.5 \times [\text{Asp}]_{0.5}$.

CONCLUSION

The mutations introduced for structural study in ATCase posed tremendous challenge for the purification of the hybrid proteins by ion-exchange chromatography. The surface charges introduced in order to purify the hybrid enzymes were not sufficient to separate the hybrids from each other. However, the electroelution method employed here was turned out to be a useful tool for large scale preparation of such difficult proteins which are very similar by their size, molecular weight and surface charge distribution. The proteins thus purified were authentic and biologically active, suggesting that their exposure to electric current had neither any effect on their function and probably nor on their structure.

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