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A mutation in the catalytic cistron of aspartate carbamoyltransferase affecting catalysis, regulatory response and holoenzyme assembly

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We describe here a mutation in the gene encoding the catalytic subunit of aspartate carbamoyltransferase (ATCase, *pyrB*) which produces an enzyme retaining catalytic activity as holoenzyme ($2C_3:3R_2$) and catalytic trimer (C_3) but which shows neither cooperative substrate kinetics nor nucleotide effector response. Furthermore, the holoenzyme assembly seems quite fragile in that the enzymatic activity is recovered only partially as holoenzyme following growth of *Escherichia coli* in the presence of zinc. In contrast, the enzyme from wild-type *E. coli* strain E63 is recovered almost entirely in its dodecameric form in identical conditions. The gene encoding the mutant *pyrB* was isolated from a λ*dargI*$^+$*pyrB*$^+$ transducing phage (obtained from N. Glansdorff). This mutation differs from other previously reported mutations affecting the catalytic subunit[1,2] in that significant catalytic activity is retained but homotropic and heterotropic communication is lost[3].

A 6.0-kilobase fragment was isolated from purified λDNA[4], restricted with the endonuclease *Pst*I (Bethesda Research Laboratory)[5], and cloned on to plasmid pBR322 (ref. 6). The recombinant plasmid pPB-h204 (plasmid–pyrimidine-B cistron–holoenzyme-strain 204) was transformed into *E. coli* WR38, which contains a Mu insertion[7] in the chromosomal *pyrB* and does not produce catalytic or regulatory polypeptides as determined by immunoassay with specific antisera (W.D.R., unpublished observations). This plasmid encodes both the catalytic (*pyrB*) and the regulatory (*pyrI*) polypeptides of ATCase. [A mutation in the cistron encoding the regulatory polypeptide is described in the accompanying report by Feller *et al.*[8]. The cistron is designated *pyrI* in agreement with Bachmann (personal communication).] The synthesis of ATCase in the transformed strain, WR38-h204, is repressed by growth in uracil, so that the cloned fragment contains the operator–promoter region as well as both ATCase cistrons. The catalytic activity recovered from this strain is distributed as ~40% holoenzyme with a molecular weight (M_r) of 300,000 and 60% as the catalytic trimer with a M_r of 100,000. Both forms of ATCase from this mutant lack cooperative homotropic kinetics for aspartate, do not respond to the allosteric effectors ATP and CTP, and seem to have altered affinities for aspartate.

Table 1 ATCase holoenzymes from wild-type *E. coli* strain E63 and mutant *E. coli* strain WR38-h204

	Wild-type	Mutant
$[S]_{0.5}$ (mM aspartate)	5.5 mM	2.0–2.5 mM
% Activity + CTP	21%	97%
% Activity + ATP	166%	105%
$\frac{\text{ATCase activity at } p\text{H } 7.0}{\text{ATCase activity at } p\text{H } 8.4}$	3.1	0.51
Shape of velocity–substrate curve	Sigmoidal	Hyperbolic

ATCase assay mixtures of 2.0 ml volume contained 40 mM potassium phosphate, *p*H 7.0, 3.6 mM dilithium carbamoyl phosphate, *p*H 7.0, 5 mM potassium aspartate, *p*H 7.0, for the wild-type and 2.5 mM for the mutant (approximate $[S]_{0.5}$ values), and enzyme (holoenzyme fractions of G-200 eluate, Fig. 2). CTP or ATP was absent from control tubes and present at a concentration of 2 mM when inhibition (CTP) or activation (ATP) was measured. The control activity, that is, the activity in the absence of effector, is set at 100%. ATCase activity was assayed by measuring the amount of carbamoyl aspartate formed in 30 min at 30 °C as previously described[23]. Carbamoyl aspartate production was determined at 466 nm. Specific activity is expressed as nmol carbamoyl aspartate formed per min per mg protein. Specific activity of ATCase was determined in conditions in which carbamoyl aspartate formation was proportional to extract concentration and time. The value obtained for the ratio of ATCase specific activity at *p*H 7.0/ATCase specific activity at *p*H 8.4 may be used as an index for the presence or absence of cooperativity between catalytic sites for aspartate binding according to the methods of Kerbiriou and Hervé[11]. A ratio of <1.0 signifies the absence of cooperativity; one of ≥2.0 reflects cooperative homotropic interactions. Fractions from beneath the holoenzyme peak (Fig. 2) were used to estimate this ratio.

positive homotropic interactions between catalytic sites as evidenced by the sigmoidal dependence of activity on substrate concentrations[9,10], (2) substrate binding is subject to positive heterotropic interactions between catalytic and regulatory sites in the presence of ATP and negative heterotropic interactions with CTP[11–14], and (3) the binding of CTP is subject to negative homotropic interactions between the regulatory sites[15]. Moreover, ATCase is unusual among allosterically regulated enzymes, in that, as with yeast phosphofructokinase[16], the regulatory protein is distinct and can be physically dissociated from the catalytic subunit. The assembly of the enzyme is a cytoplasmic event[17] which produces a dodecamer with six regulatory and six catalytic polypeptides (r_6c_6) associated as two separable catalytic trimers and three regulatory dimers[18,19]. The holoenzyme may be reversibly dissociated by mild treatment with mercurials such as *p*-chloromercuribenzoate[20] or neohydrin[21]. The catalytic subunit (c_3) is insensitive to allosteric effectors, possesses a half-saturation concentration ($[S]_{0.5}$) higher for aspartate than the holoenzyme (8–10 mM compared with 5 mM) and produces a V_{max} that is two- to fourfold higher[9]. The separate regulatory subunit (r_2) has no catalytic activity although ATP and CTP may still be bound[20]. On reassociation of the holoenzyme after removal of the mercurial by zinc replacement dialysis in the presence of dithiothreitol, the original catalytic and regulatory properties of the native enzyme are re-established[22].

ATCase was prepared from *E. coli* wild-type strain E63 and the transformed mutant strain WR38-h204 by the methods of Wild *et al.*[23]. Several pertinent properties of the wild-type and mutant ATCases are compared in Table 1. We must emphasize three points regarding these data. (1) The mutant ATCase is not subject to allosteric regulation by either ATP or CTP at subsaturating concentrations of aspartate (1–5 mM). Minimal effector response was observed when velocity–substrate plots were examined over the range of 0.5 mM to 50 mM aspartate (<5% variation throughout the range). As noted by Gerhart[24], such effects are due only to the direct competition for any phosphate-containing compound. (2) The apparent $[S]_{0.5}$ for aspartate of the mutant enzyme is significantly lower (2.0–2.5 mM) than the wild-type requirements (5.5 mM). (3) The homotropic kinetic responses of the mutant enzyme are dramatically reduced or abolished in the mutant enzyme as shown by the lack of sigmoidal dependence of activity on aspartate concentration (Fig. 1) even when plotted according to Eadie–Hofstee[25] (see insert

ratio of ATCase activity at *p*H 7.0 and 8.4 may be used as an index of the presence or absence of homotropic interactions between catalytic sites as first described by Kerbiriou and Hervé[11]. A ratio of <1.0 signifies the absence of cooperativity and is always found for the catalytic subunits alone, whereas a ratio of ≥2.0 reflects cooperative homotropic interactions[11]. We have verified these observations in similar experiments (K.F.F. unpublished observations). By this criterion, the ATCase from strain WR38-h204 seems to lack cooperativity.

Figure 2 shows an analysis of the M_r values of the ATCases from mutant and wild-type enzymes in the presence of zinc. A single large molecular weight form (~300,000) was observed from the wild-type extract. In contrast, more than half of the enzymatic activity from the mutant extract was observed as a smaller component (M_r ~100,000) which corresponds to the wild-type catalytic trimer. We have shown previously that all native ATCase from the enteric bacteria grown in sufficient zinc (20 μM) studied thus far are recovered entirely as holoenzyme[23]. Only when zinc becomes limiting or there is an assembly defect can the catalytic trimer be recovered from cell-free extracts.

The following evidence suggests that these differences are consequences of an altered catalytic subunit. (1) The catalytic trimer from the mutant enzyme shows increased affinity for aspartate compared with the catalytic trimer ($[S]_{0.5}$ of 5 mM aspartate compared with 8–10 mM) of the wild-type enzyme. (2) The M_r values of the catalytic trimers are indistinguishable, but they form clearly different holoenzymes when reconstituted with wild-type regulatory dimers (Table 2). The holoenzyme

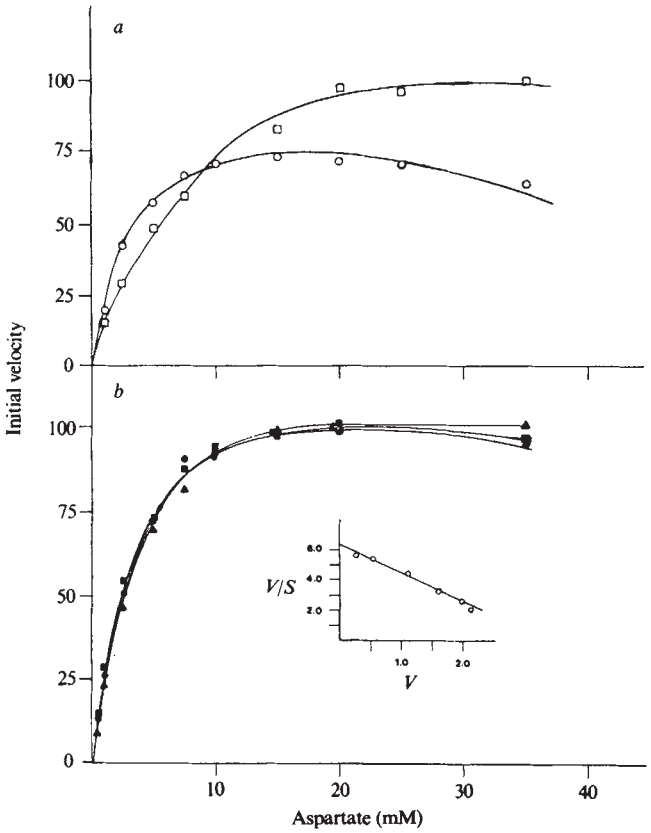

Fig. 1 Aspartate saturation curves for ATCase holoenzyme and its catalytic trimers from *E. coli* mutant strain WR38-h204. Enzyme samples were prepared as reported previously[23]. Assay mixtures were as described in Table 1 legend except that the aspartate concentration was varied from 0.5 mM to 50 mM as indicated on the abscissa. CTP or ATP was absent from control tubes and present at 2 mM when inhibition (CTP) or activation (ATP) was measured. For the above comparisons initial rate values were normalized as described below. *a*, Velocity-substrate plots for aspartate of the ATCase holoenzyme (○) and its catalytic trimer (□) from the mutant. The maximum velocity for the catalytic trimers was set at 100 on the ordinate

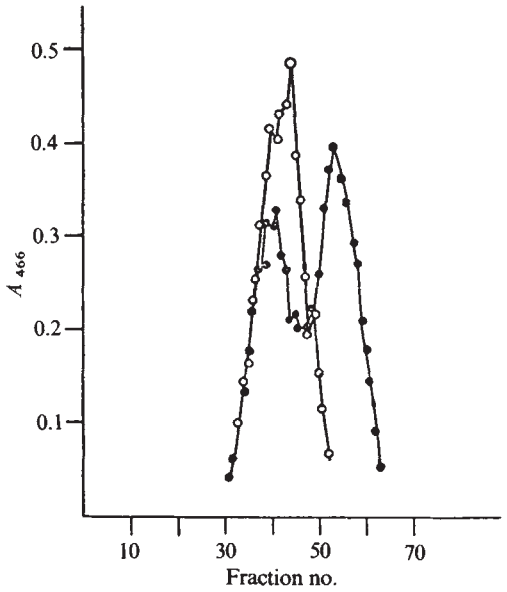

Fig. 2 Analysis of ATCase from *E. coli* wild-type E63 and mutant WR38-h204 by gel filtration on a G-200 Sephadex column; 1 ml, containing ~20 mg protein, of the appropriate cell-free extract was applied to the G-200 column, equilibrated previously with 40 mM potassium phosphate buffer *p*H 7.0, 0.1 mM dithiothreitol and 0.02 mM zinc acetate. The equilibration mixture was also used for elution. Individual fractions (abscissa) were assayed for ATCase by a spot assay. This differed from the usual assay[23] in that the final reaction volume was reduced from 2.0 ml to 0.2 ml and 30–50 mM aspartate was used. The ordinate gives absorbance at 466 nm, the wavelength for monitoring ATCase. A single wild-type peak (○) and two mutant peaks (●) are seen.

formed from wild-type regulatory dimers and mutant catalytic trimers has a M_r of ~300,000, but its regulatory and catalytic properties are those of the original mutant enzyme.

We have shown that a mutation in the *pyrB* gene, encoding the catalytic polypeptide of ATCase, can affect the homotropic and heterotropic interactions of the enzyme while maintaining catalytic activity. The reconstitution experiments with normal regulatory subunits purified from the native enzyme and catalytic subunits from both the mutant and wild-type strains prove that the catalytic polypeptide of the ATCase from strain WR38-h204 is altered. It is surprising that a mutation in the cistron encoding the catalytic polypeptide can affect such a wide range of enzymatic properties without completely disrupting the

Table 2 Catalytic trimers of ATCase from wild-type and mutant *E. coli* strains and their reconstituted holoenzyme derivatives

	Wild-type	Mutant
M_r for c_3	85,000–100,000	85,000–100,000
$[S]_{0.5}$ aspartate for c_3	8–10 mM	5 mM
M_r for holoenzyme	280,000–300,000	280,000–300,000
$[S]_{0.5}$ aspartate for holoenzyme	5.5 mM	2.0 mM
% Activity in 2 mM CTP	20	86–95
% Activity in 2 mM ATP	160	97–105

Reconstitution experiments were performed as follows. Catalytic subunits from the wild-type and mutant strains were mixed individually with excess of wild-type regulatory subunits and concentrated on an Amicon ultrafiltration system (PM-10 membrane) in the presence of 40 mM potassium phosphate buffer, *p*H 7.0, 0.2 mM zinc acetate, 2.0 mM dithiothreitol and 20 μM EDTA. The reconstitution of holoenzyme with both wild-type and mutant catalytic trimers was almost 100%. Effector response was measured exactly as described for Table 1. The effector response was determined at the appropriate $[S]_{0.5}$ for each enzyme (that is 5 mM or 2.5 mM). Molecular weights were estimated using Sephadex G-200 ascending flow column chromatography. Standards used in column calibration were: RNase A, M_r 13,700; chymotrypsinogen A, 25,000; ovalbumin 45,000; aldolase 158,000; ATCase holoenzyme from *E. coli* 300,000; Blue Dextran 2000, 2×10^6. These standard enzymes (5–20 mg of each, except ATCase) were prepared in a 2.0-ml volume of 40 mM potassium phosphate, *p*H 7.0, with 0.02 mM zinc acetate, 0.1 mM dithiothreitol, and applied to the column. The elution profiles of the various proteins were determined by monitoring absorbance at 280 nm. ATCase was used separately to verify the accuracy of the column at higher molecular

catalytic capabilities of the enzyme. This mutation should be compared with that affecting the regulatory polypeptide described by Feller *et al.*[8] in the accompanying report. It is striking that the holoenzyme assembly is apparently deficient in both mutants, one affecting the catalytic sununit and the other the regulatory subunit.

One final observation regarding the catalytic mutation seems pertinent. Examination of the kinetic characteristics (Fig. 1) of the mutant (with or without ATP added) revealed that they approximate the wild-type enzyme in the presence of ATP. Indeed, the high affinity exhibited by the mutant holoenzyme for aspartate suggests that the mutant ATCase may be frozen in its activated R state[26]. The present data suggest that strikingly similar characteristics can exist for two independent mutations in either the catalytic or regulatory cistrons of ATCase.

This research was supported in part by a grant from the National Institute of General Medical Sciences, 1R01 GM29152-01, the US Department of Agriculture (CRGO 59-2485-0-1-463-01), the Robert A. Welch Foundation, and the Texas Agriculture Experiment Station, H-1670 and H-6458.

Received 30 December 1980; accepted 2 June 1981.

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Sequence dependence of the helical repeat of DNA in solution

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Considerable progress has recently been made on the fine structure of DNA. X-ray diffraction studies of crystals of oligonucleotides of defined sequences have already provided several structures at atomic resolution[1–8]. Whereas all the crystals studied so far show bihelical structures with antiparallel chains and Watson–Crick-type base pairing, there is a striking range of structural variability, from the right-handed B-type to the left-handed Z-type helices. In contrast to the well developed methology for crystal structure determination, few methods of high precision and low ambiguity are available for studies of DNA in solution. Here we report the application of the band-shift method previously developed in this laboratory[9] to the

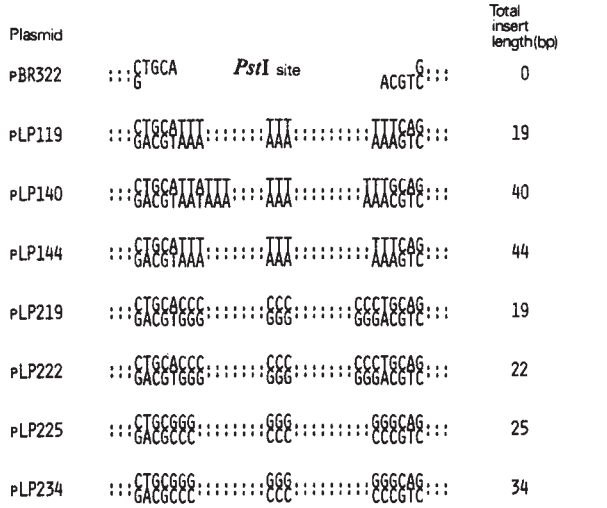

Fig. 1 Sequences of homopolymer tract inserts. Clones were initially screened for insert size by following the restriction endonuclease *Hae*III digest on a 6% polyacrylamide gel. For DNA sequencing, the insert containing the *Alu*I-generated restriction fragment was isolated, end-labelled, and sequenced after strand separation according to the procedures of Maxam and Gilbert[23]. In most cases both strands were sequenced. No changes in plasmid sequence were observed outside the *Pst*I site.

The principle of the band-shift method has been reported elsewhere[9]. Note that the helical repeat, h^0, obtained from the band-shift method is the number of base pairs of the sequence inserted that will increase the average linking number of the DNA in its relaxed state by one. In cases where the insert does not have an intrinsic spatial writhe, h^0 is also identical to the number of base pairs that forms a complete helical turn.

In addition to the absolute magnitudes of the helical repeats of different sequences, the band-shift method gives the relative handedness of the helices. We shall make the generally accepted assumption that a double-stranded DNA of typical sequence is a right-handed helix in solution. The band-shift method then provides directly the handedness of the inserted helical segments as well.

To extend the band-shift method to the determination of the helical repeats of DNAs of defined sequences, families of covalent closed circular DNAs containing inserts of these sequences of known lengths are needed. The method used to insert homopolymer tracts of various lengths into the *Pst*I site of pBR322, a plasmid with known nucleotide sequence[10], involved tailing with terminal transferase and one of the deoxynucleoside triphosphates[11]. On digestion with restriction endonuclease *Bam*HI, preparative gel electrophoresis allowed isolation of the desired fragments. The appropriate pairs of DNA fragments thus obtained were ligated together with T4 polynucleotide ligase and competent *Escherichia coli* cells were transformed. The nucleotide sequences in the regions containing the inserts are given in Fig. 1. In several cases, at the junctions between the plasmid DNA and the homopolymer inserts, a few unexpected base changes occurred. These changes were probably due to a low level of $3' \rightarrow 5'$ exonuclease activity in the calf thymus terminal transferase used in the construction of these plasmids.

An example of the gel electrophoretic patterns of several DNA samples relaxed in identical conditions is shown in Fig. 2*a*; lane 9 illustrates the band pattern observed when a single DNA, that of plasmid pLP222, is present. In lane 8, a second DNA (pLP219) shorter than pLP222 by three GC base pairs (bp) is mixed in. It can be readily seen that the band pattern of the longer plasmids shows a relative upward shift of ~ 0.3 times the interband spacing between topoisomers.

The set of covalently closed DNA samples prepared are all positively supercoiled in electrophoresis conditions[12], thus the faster migrating topoisomers have higher linking numbers than