Propagation of Allosteric Changes through the Catalytic–Regulatory Interface of *Escherichia coli* Aspartate Transcarbamylase†

Wei Xu, Margaret A. Pitts, Steven A. Middleton, Kerry S. Kelleher, and Evan R. Kantrowitz*

Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167

Received December 3, 1987; Revised Manuscript Received February 26, 1988

ABSTRACT: Each of two previously isolated strains of *Escherichia coli* containing a single nonsense codon within the *pyrB* gene was suppressed with four different nonsense suppressors. The kinetic analysis using crude extracts of these nonsense-suppressed strains indicated that the mutant aspartate transcarbamylases had altered cooperativity and affinity for aspartate as judged by the substrate concentration at half of the maximal velocity. Both *pyrB* genes were cloned and then sequenced. In both cases, a single base change was identified which converted a glutamine GAC codon into a TAC nonsense codon. Both mutations occurred in the catalytic chain of aspartate transcarbamylase and were identified at positions 108 and 246. The glutamine at position 108 in the wild-type structure is located at the interface between the catalytic and regulatory chains and is involved in a number of interactions with backbone and side chains of the regulatory chain. The glutamine at position 246 in the wild-type structure is located in the 240s loop of the enzyme. Two additional mutant versions of aspartate transcarbamylase were created by site-directed mutagenesis to further investigate the 108-position in the structure, a glutamine to tyrosine substitution at position 108 of the catalytic chain, and an asparagine to glycine change at position 113 of the regulatory chain, a residue which interacts directly with glutamine-108 in the wild-type structure. Both mutant enzymes have reduced affinity for aspartate. However, the Tyr-108 mutant enzyme exhibits a reduced Hill coefficient while the Gly-113 enzyme exhibits an increased Hill coefficient. The response to the allosteric effectors ATP and CTP is also changed for both the mutant enzymes. Analysis indicates that this region of the interface between the catalytic and regulatory chains is critical for the transmission of both homotropic and heterotropic effects between the subunits of the enzyme.

The study of the interrelationship between the structure and function of proteins has made great advances over the last few years with the advent of site-directed mutagenesis (Hutchinson et al., 1978; Gillam & Smith, 1979a,b). The use of this technique allows the replacement of any amino acid within a polypeptide chain with any of the other naturally occurring amino acids in a short period of time. Just a few short years ago, it was extremely difficult to study single amino acid substitutions of a particular protein unless one was fortunate enough to be studying a system in which natural mutations could be isolated easily.

During the period before site-directed mutagenesis was introduced, our laboratory used molecular genetics techniques to isolate a large number of single amino acid substitution mutants of *Escherichia coli* aspartate transcarbamylase (Kantrowitz et al., 1980). *E. coli* aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2), the epitome of a regulatory enzyme, is an excellent candidate for the use of single amino acid substitution mutants to help establish how both homotropic and heterotropic interactions are propagated between the subunits of the enzyme. This enzyme catalyzes the committed step in the biosynthesis of pyrimidines: the reaction between carbamyl phosphate and L-aspartate to form *N*-carbamyl-L-aspartate and inorganic phosphate (Jones et al., 1955; Reichard & Hanshoff, 1956). The 310000 molecular weight enzyme is composed of 12 polypeptide chains of 2 types. The six larger, 33000 molecular weight, chains are grouped together into two sets of trimers (catalytic subunits), while the six smaller, 17000 molecular weight, chains are grouped together into three sets of dimers (regulatory subunits). The holoenzyme[1] shows homotropic cooperative interactions with both substrates (Gerhart & Pardee, 1962; Bethell et al., 1968), is heterotropically inhibited by CTP (Gerhart & Schachman, 1965), the end product of the pyrimidine pathway, and is stimulated by ATP, the end product of the parallel purine pathway (Yates & Pardee, 1956). Treatment of the enzyme with heat (Gerhart & Pardee, 1962) or mercurials causes the dissociation of the enzyme into its constituent subunits (Gerhart & Schachman, 1965). The isolated catalytic subunit is active but does not exhibit homotropic or heterotropic interactions, while the regulatory subunit is inactive but still binds the effectors ATP and CTP. The interface between the catalytic and regulatory subunits is critical for both the homotropic and heterotropic interactions of the enzyme, since the association of the regulatory subunits with the catalytic subunits invokes homotropic cooperative interactions in the enzyme. Furthermore, the heterotropic activation or inhibition of the enzyme occurs upon the binding of the effectors to the regulatory subunits, and the conformational changes induced by this binding are propagated through the subunit interface to the catalytic sites.

Single amino acid substitution mutants of aspartate transcarbamylase have been produced in our laboratory by sup-

† This work was supported by a grant from the National Institutes of Health (GM26237). E.R.K. received a fellowship from the Camille and Henry Dreyfus Foundation.

[1] Abbreviations: holoenzyme, complete aspartate transcarbamylase molecule composed of two catalytic subunits and three regulatory subunits; PALA, *N*-(phosphonoacetyl)-L-aspartate; T and R states, tight and relaxed states of the holoenzyme having low and high affinity, respectively, for the substrate; $[S]_{0.5}$ substrate concentration at half the maximum observed specific activity; Tris, tris(hydroxymethyl)aminomethane; pHMB, *p*-(hydroxymercuri)benzoate.

0006-2960/88/0427-5507$01.50/0

pression of mutagenically introduced nonsense codons within the *E. coli pyrB* gene (Kantrowitz et al., 1980, 1981). This technique ensures that each enzyme will differ from the wild-type enzyme by a single amino acid substitution. By use of bacterial strains that have the nonsense codon at different positions within the *pyrB* gene, a series of enzymes were produced each differing from the next by a single amino acid. Furthermore, by use of different suppressors, the inserted amino acid could be varied.

Kinetic studies on 34 different mutant aspartate transcarbamylases each containing a single amino acid replacement were initially performed in crude extracts (Kantrowitz et al., 1981). The analysis of the data revealed that some of these enzymes had drastically altered homotropic cooperativity. In order to determine the sites of the amino acid substitutions, we next developed HPLC fingerprint techniques (Smith et al., 1986). However, it became obvious that recombinant DNA techniques would be a better approach not only to generate single amino acid substitution mutants but also to further investigate the most important of the nonsense-suppressed mutant enzymes that we had previously created, since only very limited quantities of these mutant enzymes could be isolated in purified form.

Here we report the cloning of two of these *pyrB* nonsense mutants and the location of the nonsense codons as determined by DNA sequencing. One of these mutations is located in the functionally important 240s loop (Middleton & Kantrowitz, 1986) while the other is located at the interface between the catalytic and regulatory subunits of the enzyme. In order to better evaluate the importance of this mutation site and its involvement in the interactions at the interface between the catalytic and regulatory subunits, we constructed one of the nonsense-suppressed mutant enzymes along with an additional mutant enzyme by site-directed mutagenesis.

Experimental Procedures

Materials

ATP, CTP, carbamyl phosphate, *N*-carbamyl-L-aspartate, agar, agarose, ampicillin, L-aspartate, potassium dihydrogen phosphate, *p*-(hydroxymercuri)benzoate, and Tris were purchased from Sigma Chemical Co. The carbamyl phosphate was purified by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C (Gerhart & Pardee, 1962). Enzyme-grade ammonium sulfate was purchased from ICN Biochemicals, and casamino acids were purchased from Difco. Restriction endonucleases were obtained from either U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. T4 DNA ligase, the Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were products of U.S. Biochemicals. NA45 paper used for the isolation of DNA fragments from agarose gels was purchased from Schleicher & Schuell.

The various bacterial strains used in this work are listed in Table I. All bacterial growth was carried out at 37 °C. The plasmids pUC8 and pUC119 as well as the M13 phage M13K07 were obtained from J. Messing.

Methods

Oligonucleotide Synthesis. The oligonucleotides required for the site-directed mutagenesis as well as the sequencing primers were synthesized with an Applied Biosystems 381A DNA synthesizer.

Wild-Type Enzyme Preparation. Wild-type aspartate transcarbamylase was isolated as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104, containing the plasmid pEK2, which carries the entire wild-type *pyrBI* operon. The wild-type enzyme was produced in this fashion since the *pyrB* gene used for mutagenesis was derived from pEK2 (Smith et al., 1986).

Table I: Bacterial Strains Used

strain[a]	sex	genetic markers[b]	source[d]
CA165	Hfr	*thi, relA, lacI, lacZ*$_{oc}$, *supB*	S. Brenner/CGSC 4980
CA167	Hfr	*thi, relA, lacI, lacZ*$_{oc}$, *supC*	S. Brenner/CGSC 5401
EK046	F′	*thi, his, trp, Δilv, mtl, malA, rpsL, ton, tsx, Nal*^{r}, λ$^-$/F′ *his*$^+$, *supD*	this laboratory[c]
EK056	Hfr	*relA, supG, lacZ*$_{am}$, λ$^-$	this laboratory[c]
EK090	F$^-$	*ara, thi, rpsL, ΔpyrB, supC, lacI, lacZ*$_{oc}$	this laboratory[f]
EK110	F$^-$	*thi, pyrB110, his, trp, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK110B	F$^-$	*thi, pyrB110, supB, his, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK110C	F$^-$	*thi, pyrB110, supC, his, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK110D	F′	*thi, pyrB110, his, trp, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$/*F′ his*$^+$, *supD*	this laboratory[c]
EK110G	F$^-$	*thi, pyrB110, supG, his, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK234	F$^-$	*thi, pyrB234, his, trp, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK234B	F$^-$	*thi, pyrB234, supB, his, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK234C	F$^-$	*thi, pyrB234, supC, his, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK234D	F′	*thi, pyrB234, his, trp, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$/F′ *his*$^+$, *supD*	this laboratory[c]
EK234G	F$^-$	*thi, pyrB234, supG, his, galK, mtl, xyl, malA, rpsL*, λ^r, λ$^-$	this laboratory[c]
EK1104	F$^-$	*ara, thi, Δpro-lac, ΔpyrB, pyrF*$^{\pm}$, *rpsL*	this laboratory[e]
HB2151	F′	*ara, thi, Δpro-lac*/F′, *proAB, lacI*^{q}, *lacZ*ΔM15	Carter et al. (1985)
HB2154	F′	*ara, thi, Δpro-lac, mutL*::Tn10(tet^r)/F′ *proAB, LacI*^{q}, *lacZ*ΔM15	Carter et al. (1985)
JM101	F′	Δ(*lac-proAB*), *supE, thi*/F′ *traD, lacI*^{q}, *lacZ*ΔM15, *proAB*	N. E. Biolabs
U39a	F$^-$	*ara, thi, Δpro-lac, ΔpyrB, rpsL*	J. Wild

[a] Cell strains are *E. coli* K12. [b] See Bachmann (1983). [c] See Kantrowitz et al. (1980, 1981). [d] CGSC is the *E. coli* Genetic Stock Center, Yale University. [e] See Nowlan and Kantrowitz (1985). [f] See Smith et al. (1986).

Determination of Protein Concentration. The concentration of pure wild-type holoenzyme was determined by absorbance measurements at 280 nm with an extinction coefficient of 0.59 cm^2/mg (Gerhart & Holoubek, 1967). The protein concentrations of the mutant holoenzymes were determined either by the method of Lowry et al. (1951) or by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976).

Aspartate Transcarbamylase Assay. The transcarbamylase activity was measured at 25 °C by either a colorimetric (Pastra-Landis et al., 1981) or a pH-stat method (Wu &

Hammes, 1973). pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburet. ATP and CTP concentrations were determined by absorbance measurements at 260 nm. All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

Cloning pyrB110 and pyrB234 into pUC8. Chromosomal DNA from EK110 and EK234 was isolated (Schleif & Wensink, 1981) and then cut with *Pst*I and *Sal*I in order to excise a 2.8-kb fragment which contained the entire *pyrBI* operon. In separate reactions, the chromosomal DNA fragments from EK110 or EK234 were mixed with the vector pUC8 which had previously been cut with the same two enzymes. The mixtures were then treated with T4 DNA ligase and used to transform competent EK090 cells, a *supC* strain which carries a deletion in the *pyrB* gene. Selection was accomplished on M9 medium plates containing 0.5% casamino acids and 40 μg of ampicillin/mL. Candidates were selected from these plates and purified prior to isolation of the recombinant plasmid by alkaline lysis (Maniatis et al., 1982). The plasmid isolated from each of the candidates was checked as to overall size and that a fragment of the correct size had been inserted into the vector. Confirmation that the insert had a nonsense mutation within the *pyrB* gene was carried out by parallel transformations into competent EK090 and U39a cells. Both of these strains are Δ*pyrB*, but EK090 also has a *supC* gene. Recombinant plasmids pEK110 and pEK234 transformed both of these strains with high efficiency, as checked by the introduction of the ampicillin resistance gene, but could grow without uracil only in the EK090 background.

Cloning pyrB110 and pyrB234 into M13mp19. In order to sequence the *pyrB* gene and locate the mutation site, the fragments containing the *pyrBI* operon from pEK110 and pEK234 were cloned into M13mp19. This was accomplished by first cutting pEK110, pEK234, and M13mp19 with *Pst*I and *Sal*I. The fragments from the plasmid and the M13mp19 were mixed, and the mixture was treated with T4 DNA ligase overnight at 4 °C followed by transformation into JM101. Clear plaques were picked from YT plates containing 40 μg of ITPG/mL and 40 μg of X-gal/mL and used to inoculate a 2-mL culture of JM101 in 2 × YT medium at an absorbance of 0.1 at 560 nm. Incubation was continued for 7 h at 37 °C. In each case, RF DNA from a number of candidates was isolated by alkaline lysis (Maniatis et al., 1982), and a candidate was selected which exhibited the correct restriction pattern. The recombinant M13 phage containing the *pyrB110* and *pyrB234* genes were called M13pEK110 and M13pEK234, respectively.

Construction of Plasmid pEK54. Previously, we had constructed a plasmid, pEK38, which contained the wild-type *pyrBI* gene inserted into the vector pUC119 (Ladjimi & Kantrowitz, 1988). Sequencing of mutations in this plasmid is simplified because it contains the M13 intergenic region which allows isolation of single-stranded DNA after coinfection with a helper phage such as M13K07. In order to make this plasmid more convenient for inserting mutated fragments of the *pyrB* or *pyrI* genes, a *Bgl*II site located outside the *pyrBI* coding region was removed to form a new plasmid, pEK54, which contains only a single *Bgl*II site within the *pyrI* gene. The extraneous *Bgl*II site of pEK38 was removed by first treating the plasmid with *Bgl*II under conditions which resulted in significant quantities of plasmid with only one *Bgl*II cleavage. After separation of the mixture by agarose gel electrophoresis, the fragments corresponding to linear pEK38 were removed from the gel with NA45 paper. The single-stranded 5′ overhangs were filled in with the Klenow fragment of DNA polymerase, followed by treatment with T4 DNA ligase. After transformation of the mixture into competent U39a, plasmid DNA was isolated from a number of candidates. Confirmation of the construction was determined by restriction enzyme analysis.

Site-Directed Mutagenesis. The introduction of specific base changes in the *pyrBI* operon for the purpose of creating altered versions of aspartate transcarbamylase was accomplished by site-directed mutagenesis as previously described (Ladjimi et al., 1988). After isolation and verification of the mutations by sequencing the M13, a small fragment of the gene was removed with restriction enzymes and inserted into a plasmid which had the corresponding section of the wild-type gene removed.

In the case of the Tyr-108C[2] mutation, a *Pst*I–*Bst*EII fragment of 758 base pairs, containing the desired mutation, was isolated from the purified M13RF after agarose gel electrophoresis, with NA45 paper. In addition, the plasmid pEK38 (Ladjimi & Kantrowitz, 1988) was cut with the same two restriction enzymes, and the larger fragment was isolated in a similar fashion. This fragment, containing the vector pUC119 along with the remainder of the *pyrBI* operon, was combined with the fragment from the mutant M13 RF and treated with T4 DNA ligase. Selection was accomplished after transformation in U39a, a strain which has a deletion in the *pyrBI* region. A plasmid was isolated, pEK52, which carried the desired mutation.

The cloning of the Gly-113R mutation into the plasmid pEK54 was performed with an identical procedure except that *Bgl*II was used to remove a fragment from the M13RF. This fragment was then mixed with pEK54 which had been cut with *Bgl*II and *Bam*HI followed by treatment with T4 DNA ligase. The final plasmid, pEK61, contained the Gly-113R mutation.

Expression and Purification of Mutant Aspartate Transcarbamylases. Overproduction of the mutant versions of aspartate transcarbamylase created by site-directed mutagenesis was accomplished as described by Nowlan and Kantrowitz (1985).

Polyacrylamide Gel Electrophoresis. The purity of the mutant and wild-type aspartate transcarbamylases was verified by nondenaturating polyacrylamide gel electrophoresis (Ornstein, 1964; Davis, 1964).

Data Analysis. The analysis of the steady-state kinetic data was carried out as previously described by Silver et al. (1983). The analysis of the structural data, based on the three-dimensional coordinates of the unliganded (Ke et al., 1984; Kim et al., 1987) and PALA-liganded enzyme (Krause et al., 1985, 1987), was accomplished with the program PS300 FRODO (Department of Biochemistry, Rice University) on an Evans & Sutherland PS390 interfaced to a VAX 11/750.

Reaction of the Wild-Type and Mutant Enzymes with pHMB. The measurements of the rates of reaction of the wild-type and the mutant enzymes with pHMB were performed according to the method of Gerhart and Schachman (1968).

RESULTS

Isolation of EK110 and EK234. Strains EK110[3] and

[2] The suffix C or R has been appended to the residue number to distinguish between the catalytic and regulatory chains of the enzyme, respectively.

[3] The strains EK110 and EK261 have identical patterns of suppression and are believed to contain the nonsense codon at the identical position (Kantrowitz et al., 1981).

Table II: Bacterial Strains Used To Produce Mutant Versions of Aspartate Transcarbamylase

strain designation	suppressor used	amino acid inserted	position[b]	enzyme designation
EK110		none	246C	
EK110B	*supB*	glutamine	246C	Gln-246C
EK110C	*supC*	tyrosine	246C	Tyr-246C
EK110D	*supD*	serine	246C	Ser-246C
EK110G	*supG*	lysine	246C	Lys-246C
EK234		none	108C	
EK234B	*supB*	glutamine	108C	Gln-108C
EK234C	*supC*	tyrosine	108C	Tyr-108C
EK234D	*supD*	serine	108C	Ser-108C
EK234G	*supG*	lysine	108C	Lys-108C
EK1104/pEK52[a]		tyrosine	108C	Tyr-108C
EK1104/pEK61[a]		glycine	113R	Gly-113R

[a] The mutations are carried on a plasmid, and the enzyme is produced with the overproduction system of Nowlan and Kantrowitz (1985). [b] The suffix after the position refers to either the catalytic or the regulatory chains of aspartate transcarbamylase, respectively.

EK234, which have no detectable aspartate transcarbamylase activity, even under derepression conditions, were shown to contain a nonsense codon in the *pyrB* gene by the restoration of aspartate transcarbamylase activity upon introduction of a suppressor gene (Kantrowitz et al., 1981). Since the gene for the catalytic chain (*pyrB*) is upstream from the regulatory chain gene (*pyrI*) (Roof et al., 1982), the original procedure would only select those mutations in the catalytic chain of the enzyme. On the basis of the mutagenic specificity of 2-aminopurine (Drake, 1970), which was used to introduce the mutations, only glutamine or tryptophan codons should be converted to nonsense codons by a single base change. Therefore, it is very likely that the site of the nonsense codon is 1 of the 15 glutamine or 2 tryptophan codons in the catalytic chain of the enzyme (Hoover et al., 1983; Konigsberg & Henderson, 1983).

Suppression of the Nonsense Codons Produces Mutant Aspartate Transcarbamylases with Altered Properties. By suppression of the nonsense codon in strains EK110 and EK234 with a variety of nonsense suppressors, it was possible to produce mutant versions of aspartate transcarbamylase with a single amino acid substitution at the position of the nonsense codon. Strains EK110C and EK234C were constructed by crossing EK110 and EK234, respectively, with the Hfr strain CA167, which introduces *supC* early, as previously described (Silver et al., 1983). Strains EK110B, EK234B, EK110G, and EK234G were constructed in an analogous fashion with Hfr strains CA165 (*supB*) and EK056 (*supG*), respectively, as the suppressor gene donors. EK110D and EK234D were constructed by mating either EK110 or EK234 with EK046 which carries *supD* on an episome (Kantrowitz et al., 1981). Table II summarizes these *pyrB* nonsense-suppressed strains and also indicates the nature of the amino acids introduced.

Initial characterization of these enzymes in crude extracts is shown in Figure 1. The aspartate saturation curves of both EK110B and EK234B are identical with the wild-type enzyme saturation curve (data not shown). The insertion of lysine, tyrosine, and serine at either of these positions causes alterations in substrate affinity and cooperativity. In both cases, the lysine substitution resulted in enzymes with enhanced affinity for aspartate while the introduction of tyrosine caused a decrease in affinity for aspartate. The introduction of serine had a much more dramatic effect on the enzyme isolated from strain EK110D than from EK234D.

Determination of the Sites of Amino Acid Substitutions. To conclusively establish the position of the mutations in

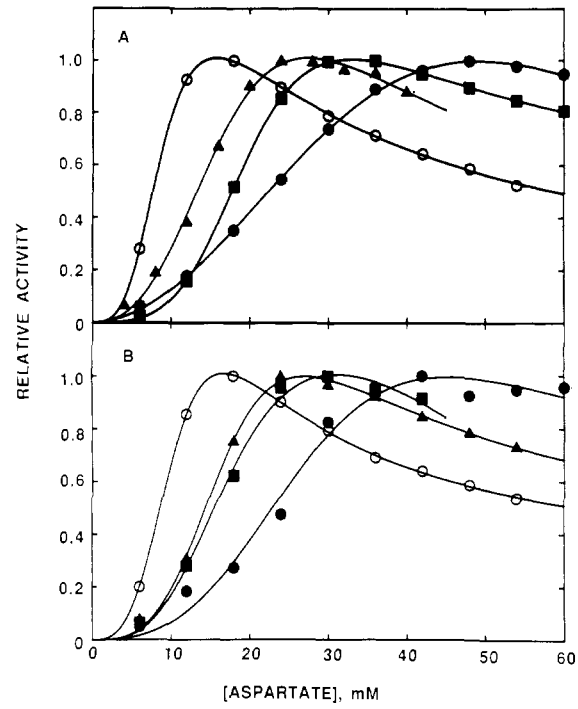

FIGURE 1: Aspartate saturation curves of the aspartate transcarbamylases obtained after suppression of the *pyrB* nonsense mutations of strains EK110 (A) and EK234 (B) from crude extracts. The nonsense codons in the *pyrB* gene of strains EK110 and EK234 were located at amino acid positions 246 and 108, respectively. The nonsense mutations were suppressed with *supB*, which inserts glutamine (▲); *supC*, which inserts tyrosine (●); *supD*, which inserts serine (■); and *supG*, which inserts lysine (○). Enzymatic activity of the enzyme from crude cell extracts was determined by the colorimetric assay in 0.05 M Tris–acetate buffer (pH 8.3) in the presence of 4.8 mM carbamyl phosphate.

EK110 and EK234, the *pyrBI* operon from each strain was first cloned into the vector pUC8 (see Experimental Procedures). Recombinant plasmids pEK110 and pEK234 were isolated which contained the *pyrB110* and *pyrB234* genes from strains EK110 and EK234, respectively. Prior to dideoxy sequencing (Sanger et al., 1977), the *pyrB110* and *pyrB234* genes were transferred into the phage M13mp19, forming M13pEK110 and M13pEK234, respectively.

Dideoxy sequencing (Sanger et al., 1977) was used to determine the location of the nonsense codons in M13pEK110 and M13pEK234 with 5 oligonucleotides as primers which hybridize at approximately 180 base intervals along the *pyrB* gene. In each case, analysis of the sequence data indicated only a single base change in the entire *pyrB* gene. For M13pEK110, the sequence corresponding to amino acid position 246 was changed from CAG to TAG, while for M13pEK234 the sequence corresponding to amino acid position 108 was also changed from CAG to TAG. In both cases, a glutamine codon was converted into an amber codon, which is in agreement with suppression data indicating that both changes corresponded to amber mutations (Kantrowitz et al., 1981).

Site-Directed Mutagenesis. An examination of the X-ray structural data revealed that Gln-246 of the wild-type catalytic chain is part of the 240s loop of the enzyme. This loop undergoes a substantial alteration in position during the allosteric transition (Ke et al., 1984; Kim et al., 1987; Krause et al., 1985, 1987), and its movement has been implicated in the homotropic cooperativity of the enzyme (Middleton & Kantrowitz, 1986). Gln-108 is located at the interface between the catalytic and regulatory subunits $C1/R1^4$ and is involved

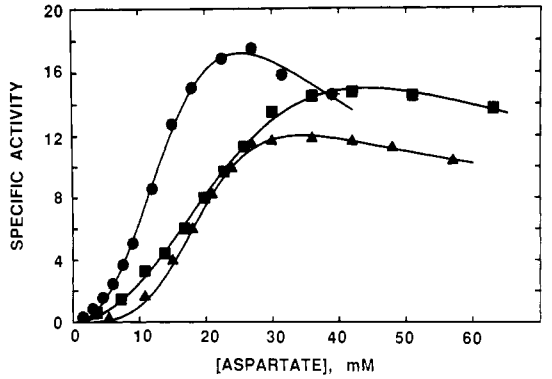

FIGURE 2: Aspartate saturation curves of the wild-type (●), Tyr-108C (■), and Gly-113R (▲) aspartate transcarbamylases. Enzymatic activity was determined at 25 °C by the colorimetric assay in 0.05 M Tris–acetate buffer (pH 8.3) in the presence of 4.8 mM carbamyl phosphate. Specific activity is in units of millimoles per hour per milligram.

in a variety of intersubunit interactions with a number of residues including Asn-113 of the regulatory subunit (Asn-113R).

The initial characterization of the set of enzymes with amino acid substitutions at position 108 revealed that the enzyme with a tyrosine insert (Tyr-108C) exhibited the most significant alterations in both homotropic and heterotropic interactions. In order to study this mutant enzyme in greater detail, we decided to introduce the mutation directly into the DNA by site-directed mutagenesis. In this fashion, large quantities of the pure enzyme could readily be obtained utilizing the overproduction system of Nowlan and Kantrowitz (1985). Therefore, the mutations Gln-108 to tyrosine (Tyr-108C) and Asn-113R to glycine (Gly-113R) were produced by oligonucleotide-directed mutagenesis as described under Experimental Procedures.

Purified Tyr-108C and Gly-113R Mutant Enzymes Have Altered Cooperativity and $[S]_{0.5}$. The aspartate saturation curves of the purified mutant enzymes Tyr-108C and Gly-113R are shown in Figure 2. The Gly-113R and Tyr-108C mutant enzymes exhibit slightly reduced maximal velocity, corresponding to a decrease of 30% and 13%, respectively, when compared to the wild-type enzyme. These small alterations in maximal velocity suggest that the mutations do not substantially alter the ability of these enzymes to catalyze the transcarbamylation reaction. More significant than the changes in maximal velocity, however, are the increased values in the $[S]_{0.5}$ and alterations in the Hill coefficients. The Hill coefficient of the Gly-113R enzyme is 3.3, a substantial increase over the value of 2.2 observed for the wild-type enzyme. On the other hand, the Tyr-108C enzyme exhibits a Hill coefficient of 1.9, somewhat lower than that observed for the wild-type enzyme. For both mutant enzymes, the $[S]_{0.5}$ for aspartate increases to approximately 18 mM compared to approximately 12 mM for the wild-type enzyme, reflecting an altered affinity of these enzymes for aspartate (see Table III).

Influence of the Allosteric Effectors. Comparison of the mutant enzymes was augmented by analysis of the aspartate saturation curves in the absence and presence of a saturating concentration of either the allosteric activator ATP or the allosteric inhibitor CTP. Figure 3 shows the aspartate saturation curves for both the Tyr-108C and Gly-113R mutant enzymes. The maximal velocities of the mutant enzymes in the presence of either ATP or CTP are consistent with the changes observed for the enzyme without effectors (see Table

[4] C or R followed by a number, e.g., C1 or R1, refers to a particular polypeptide chain in aspartate transcarbamylase as specified in Figure 6 of Honzatko et al. (1982).

Table III: Kinetic Parameters for Wild-Type and Mutant Enzymes in the Absence and Presence of ATP and CTP[a]

enzyme	ATP (4.0 mM)	control	CTP (0.3 mM)
	Hill Coefficients[b]		
wild-type	1.3	2.2	2.5
Gly-113R	2.1	3.3	2.9
Tyr-108C	1.4	1.9	2.0
	$[S]_{0.5}$ Values (mM)		
wild-type	5.2	11.8	17.4
Gly-113R	10.5	18.1	21.9
Tyr-108C	10.0	18.8	26.4
	Maximal Velocity[c] ($mmol \cdot h^{-1} \cdot mg^{-1}$)		
wild-type	17.1	17.2	14.2
Gly-113R	13.2	12.0	11.7
Tyr-108C	13.5	15.0	13.3

	Percent Inhibition or Activation at $[S]_{0.5}$	
	ATP[d]	CTP[e]
wild-type	185.7	36.3
Gly-113R	201.7	62.4
Tyr-108C	158.3	49.4

[a] These data are extracted from the aspartate saturation curves (see Figure 3). [b] Hill coefficients were calculated by a nonlinear least-squares procedure employing a modified Hill equation which incoporates substrate inhibition (Pastra-Landis et al., 1978). [c] Maximal observed specific activity. [d] ATP activation is defined as $100 \times (A^{ATP}/A)$ where A is the enzymic activity in the absence of ATP and A^{ATP} is the enzymic activity in the presence of 4.0 mM ATP. [e] Residual activity in the presence of CTP is defined as $100 \times (A^{CTP}/A)$ where A is the enzymic activity in the absence of CTP and A^{CTP} is the enzymic activity in the presence of 0.3 mM CTP.

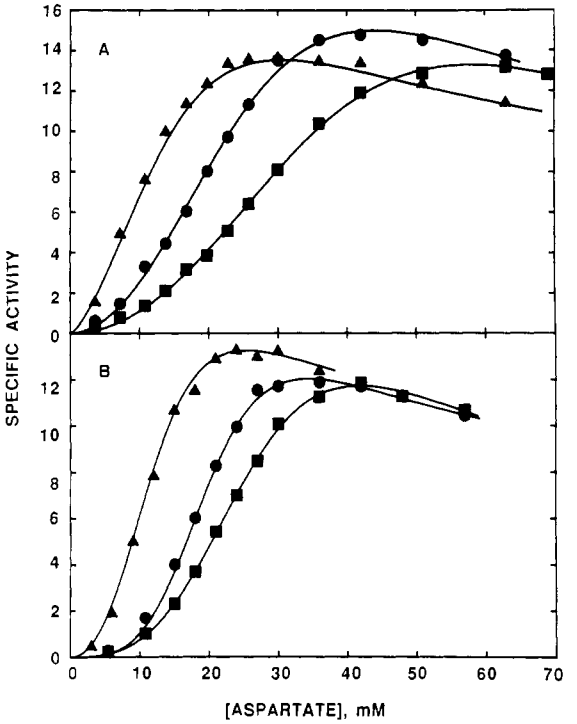

FIGURE 3: Aspartate saturation curves of the Tyr-108C and Gly-113R aspartate transcarbamylases in the absence and presence of the nucleotide effectors: enzymes without allosteric effectors (●); in the presence of 0.3 mM CTP (■); in the presence of 4.0 mM ATP (▲). Colorimetric assays were carried out in 0.05 M Tris–acetate (pH 8.3) at a saturating carbamyl phosphate concentration of 4.8 mM. Specific activity is in units of millimoles per hour per milligram. (A) mutant aspartate transcarbamylase with tyrosine substituted for glutamine at position 108 of the catalytic chain. (B) Mutant aspartate transcarbamylase with glycine substituted for glutamine at position 113 of the regulatory chain.

Table IV: Reaction of pHMB with Wild-Type, Tyr-108C, and Gly-113R Mutant Enzymes[a]

enzyme	ligand	k (M^{-1} s^{-1})	$k_{PALA}/k_{no\ PALA}$
wild-type	none	68	2.9
wild-type	PALA	195	
Gly-113R	none	548	5.3
Gly-113R	PALA	2930	
Tyr-108C	none	117	7.0
Tyr-108C	PALA	818	

[a] The reactions were carried out under pseudo-first-order conditions in 40 mM potassium phosphate buffer, pH 7.0. When PALA was present, it was at a concentration of 50 μM.

III). For the wild-type enzyme and the two mutants, ATP causes a lowering of the $[S]_{0.5}$, while CTP shifts the $[S]_{0.5}$ in the opposite direction (see Table III). The Hill coefficients for the Gly-113R enzyme in the presence of either ATP or CTP are substantially higher than the corresponding values for the wild-type enzyme. Much smaller changes are observed in the Hill coefficients for the Tyr-108C enzyme.

The extent to which ATP activates and CTP inhibits the mutant enzymes was determined by calculating the percentage of activation or inhibition at the $[S]_{0.5}$ of the particular mutant (see Table III). The Tyr-108C enzyme is activated only about 68% as much as the wild-type by ATP, while this enzyme is inhibited 81% of that observed for the wild-type by CTP. For the Gly-113R enzyme, ATP activates the enzyme slightly more than the wild-type, but CTP inhibits this enzyme only about 60% of that observed for the wild-type.

Strength of the C1/R1 Interface. The rate of the reaction of the wild-type enzyme with *p*-(hydroxymercuri)benzoate is substantially increased in the presence of the bisubstrate analogue PALA (Gerhart & Schachman, 1968; Blackburn & Schachman, 1977). Although in the wild-type enzyme this increase in reactivity may correspond to quaternary conformational changes, this may not be the case in mutant versions of the enzyme where increases more likely reflect local alterations in the structure of the enzyme near the reaction site (Ladjimi & Kantrowitz, 1987). Since the reaction of pHMB with the enzyme primarily involves the cysteine residues of the regulatory chain which are located in very close proximity to the C1/R1 interface, the rate of the pHMB reaction may be a good indicator of local structural changes at this interface. Therefore, we measured the rate of the reaction of pHMB with both the Tyr-108C and Gly-113R mutant enzymes. As seen in Table IV, in the presence or absence of PALA the pHMB reaction rate is higher for the mutant enzymes than the corresponding rate for the wild-type enzyme. Furthermore, the substitution at position 113 of the regulatory chain has a much larger effect on the reaction rates than does the mutation at position 108 of the catalytic chain. In the absence of PALA, the Gly-113R enzyme exhibits a reaction rate 8-fold higher than the wild-type, while in the presence of PALA the difference in reaction rate is 15-fold.

To investigate whether the interface itself had been altered, we examined the heat-induced dissociation process. In these experiments, the dissociation (at low ionic strength) of the wild-type and mutant enzymes is followed as a function of temperature (Ladjimi & Kantrowitz, 1987). As seen in Figure 4, the dissociation profiles for the mutant and wild-type enzymes reflect an "all or none" change in agreement with the model proposed by Subramani and Schachman (1982) for the mercurial-promoted dissociation of the wild-type enzyme. These curves can be compared in a more direct fashion by comparing the temperature at which half of the molecules have dissociated (T_D). The T_D for the wild-type enzyme was found

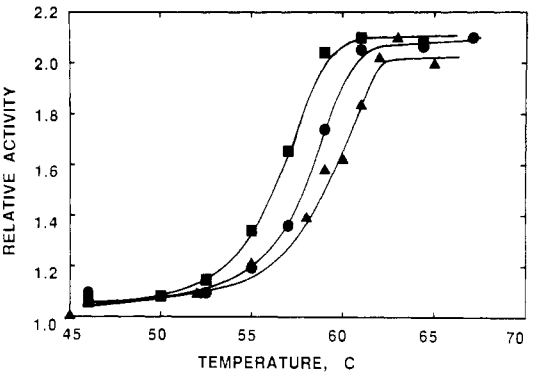

FIGURE 4: Temperature effect on the dissociation of the wild-type and mutant enzymes. The wild-type (●), Tyr-108C (▲), and the Gly-113R (■) enzymes were incubated at the indicated temperature in 5 mM potassium phosphate buffer, pH 7.0. After 2 min an aliquot was removed and quickly chilled on ice followed by determination of aspartate transcarbamylase activity. The increase in activity is due to the higher specific activity of the isolated catalytic subunit as compared to the holoenzyme under the assay conditions (Gerhart & Pardee, 1962).

to be 58.1 °C, which increases to 59.0 °C for the Tyr-108C mutant enzyme and decreases to 56.1 °C for the Gly-113R mutant enzyme. These values have an uncertainty of approximately 0.5 °C, which suggests that there is little if any change for the Tyr-108C mutant but a significant decrease for the Gly-113R mutant enzyme.

Discussion

The results of our previous studies (Kantrowitz et al., 1980, 1981) suggested that the suppression of nonsense codons within the *pyrB* gene would provide a general method for the production of mutant versions of aspartate transcarbamylase with single amino acid substitutions. The drawback to this approach has been the difficulty in obtaining sufficient quantities of the purified mutant enzymes for characterization and determining the locations of the mutations. Our inability to isolate these mutants in quantity results from the very poor suppression of the nonsense codon by the various suppressor genes used, especially in overproducing strains. Although site-directed mutagenesis has generally become the method of choice for generating amino acid substitution mutants, random mutagenesis techniques still provide an advantage in pinpointing sites that have not previously been established as functionally important. By combining the two techniques, it is possible now to create a set of mutant enzymes by random mutagenesis techniques, screen these mutant enzymes in order to select a particularly interesting subset, and then use site-directed mutagenesis to recreate these mutant enzymes and produce them in large quantities for detailed characterization.

Using this approach for aspartate transcarbamylase, we selected two *E. coli* mutation sites in the *pyrB* gene for more detailed characterization on the basis of the kinetic analysis of cell extracts prepared from nonsense-suppressed versions of these strains which indicated that these two sites are important for both the homotropic and heterotropic interactions. The locations of the nonsense mutations in these two strains were determined, after cloning the mutant *pyrB* genes, by Sanger dideoxy sequencing rather than by classical protein chemistry with protein isolated from a nonsense-suppressed version of the strain. The analysis of the sequence data for strains EK110 and EK234 indicated that the nonsense codons were located at positions 246 and 108, respectively, of the catalytic chain of the enzyme. Both of these nonsense codons occurred at glutamine (CAG) codons in the wild-type sequence, in agreement with the expected specificity of the

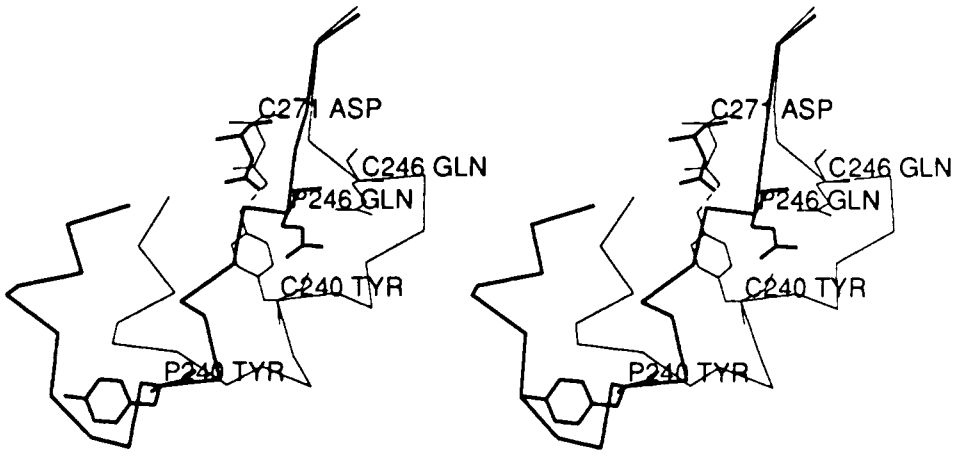

FIGURE 5: Stereoview of the 240s loop of aspartate transcarbamylase in the T (light lines, C prefix on residue numbers) and R (dark lines, P prefix on residue numbers) states. The data for one catalytic chain in the T state (Ke et al., 1984; Kim et al., 1987) and the R state (Krause et al., 1985, 1987) have been superimposed using the program SUPERIMP (R. Honzatko, Iowa State University). In addition to the Glu-246 side chain, the side chains of Asp-271 and Tyr-240 are also shown. In the T state, Asp-271 (C271) forms a link with Tyr-240 (C240) which is completely lost in the R state. The substitution of Phe for Tyr-240 by site-directed mutagenesis (Middleton & Kantrowitz, 1986) results in a destabilization of the T state. The properties of this mutant are similar to those for the Lys-246C mutant enzyme.

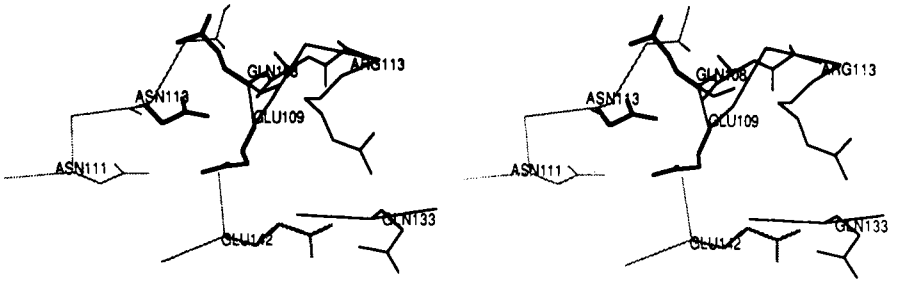

FIGURE 6: Stereoview of the C1/R1 interface region of aspartate transcarbamylase. Residues Asn-111, Asn-113 (bold), and Glu-142 are part of the regulatory chain (light) while Gln-108 (bold), Glu-109 (bold), Arg-113, and Gln-133 are part of the catalytic chain (dark). In previous work, Arg-113C has been replaced by glycine (Ladjimi & Kantrowitz, 1987), and Gln-133 has been replaced by alanine (Robey et al., 1986).

mutagen originally used. In the wild-type structure, position 246 (Gln-246C) is part of the 240s loop of the enzyme which undergoes a very significant alteration in position during the allosteric transition, while position 108 (Gln-108C) is part of the C1/R1 interface which is little altered by the allosteric transition (Ke et al., 1984; Kim et al., 1987; Krause et al., 1985, 1987).

A comparison of the structures of the wild-type enzyme in the absence and presence of PALA indicates that the amide nitrogen of the side chain of Gln-246C moves by 9.0 Å (see Figure 5). However, in neither the unliganded nor the PALA-liganded structures are there any specific interactions between the Gln-246 side chain and other residues of the protein. Since the movement of the 240s loop has been implicated in homotropic cooperativity, alterations at position 246 may simply result in added or diminished stability of this loop. The kinetic data for the serine mutation at position 246 support this conclusion since this rather conservative substitution results in the least alteration in the observed kinetics. The lysine substitution at 246, which exhibits a lower $[S]_{0.5}$, behaves in a similar fashion to the substitution where Tyr-240 is replaced by phenylalanine (Middleton & Kantrowitz, 1986). Both of these substitutions result in a less stable T state, while the tyrosine substitution at position 246 has the opposite effect and actually stabilizes the T state of the enzyme.

The 108 site, in the C1/R1 interface, does not undergo any large conformational changes between the T and R states. However, there are many interactions between Gln-108C and neighboring side chains involving residues from both the catalytic and regulatory chains. Perhaps the most important of these interactions is between Gln-108C and Asn-113 of the regulatory chain. The nonsense-suppressed mutant enzymes at position 108 suggest that this portion of the interface is important in the transmission of homotropic and heterotropic effects between the subunits of the enzyme. To more completely investigate this portion of the C1/R1 interface, a new mutant enzyme was generated with glycine substituted for asparagine at position 113 of the regulatory chain (Gly-113R).

Figure 6 is a stereoview of a portion of the C1/R1 interface of the enzyme, showing the interactions between Asn-113R and both Gln-108C and Glu-109C and the interaction between Glu-142R and both Gln-133C and Arg-113C. Although the replacement of Arg-113C by glycine has little effect on the kinetic properties of the enzyme, this replacement causes a drastic weakening of the stability of the holoenzyme toward dissociation (Ladjimi & Kantrowtiz, 1987). On the other hand, the replacement of Gln-133C by alanine (Ala-133C) causes a significant increase in cooperativity and $[S]_{0.5}$ (Robey et al., 1986). The increase in Hill coefficient that is observed when Gly is substituted for Asn-113R is direct evidence that alterations in the regulatory subunit of the enzyme can cause alterations in cooperativity and substrate affinity at the active site that must be propagated across the subunit boundary from the regulatory to the catalytic subunits.

The rate of the pHMB reaction with the mutant versions of aspartate transcarbamylase has been used to detect alterations in the local environment around the cysteine residues of the regulatory chain and, in a more indirect fashion, the environment around the C1/R1 interface (Ladjimi & Kantrowitz, 1987). Since this interface is very close to the regulatory chain cysteines, alterations at the interface may propagate to the nearby cysteines which would result in changes

in their reactivity toward pHMB. The relationship between alterations at the interface and changes in pHMB reactivity is by no means clear. In the case of the Tyr-108C mutation, any alterations in pHMB reactivity must be due to alterations in the regulatory chain propagated across the C1/R1 interface, again suggesting that this region of the interface is important for transmission of information between the catalytic and regulatory subunits of the enzyme. In the case of the Gly-113R mutation, there is a dramatic increase in the pHMB reaction rate compared to the wild-type. Since this mutation is immediately adjacent to one of the four regulatory chain cysteine residues (Cys-114R), the increase in reactivity may simply be due to enhanced accessibility of the cysteine residues. For both the Tyr-108C and Gly-113R mutant enzymes, PALA induces a larger increase in pHMB reactivity than that observed for the wild-type. In fact, the change for the Tyr-108C enzyme is more than twice that of the wild-type, suggesting that the alterations at these two sites affect the T and R states to different extents.

The region of the C1/R1 interface involving residues Asn-113R, Gln-108C, Arg-113C, and Gln-133C is particularly sensitive to amino acid substitutions; however, depending upon where the substitution is made and which amino acid is inserted, the properties of the resultant mutant enzymes are very different. The mutant versions of aspartate transcarbamylase with the substitutions Gly-113R and Tyr-108C reported here as well as Ala-133C (Robey et al., 1986) all have increased $[S]_{0.5}$ while the Gly-113R and Ala-133C enzymes also have enhanced cooperativity. The Lys-108C mutant exhibits both lower cooperativity and $[S]_{0.5}$, while the Gly-113C mutation causes no alterations in the observed kinetics (Ladjimi & Kantrowitz, 1987). Nevertheless, both the Gly-113R and especially the Gly-113C mutants exhibit much weaker interactions between the subunits as judged by their respective T_D values. These results taken together suggest that this region of the C1/R1 interface is particularly important in the stabilization of the holoenzyme and the transmission of both homotropic and heterotropic effects between the subunits. Furthermore, the data from these mutant enzymes also suggest that the transmission of information between the subunits is a very subtle process and that small disruptions of the interface may cause alterations in some or all of the properties of the enzyme.

The specific activity of the catalytic subunits is reduced substantially when they combine with the regulatory subunits to form the holoenzyme (Gerhart & Pardee, 1962). The C1/R1 interface region investigated in this work may also be involved in the reduction of the specific activity of the holoenzyme relative to that of the isolated catalytic subunit since this region of the interface is quite close to the active site. In particular, Arg-105C which interacts with carbamyl phosphate is very close to Gln-108C, one of the residues involved in this portion of the C1/R1 interface. Therefore, the region of the C1/R1 interface containing residues Asn-113R, Gln-108C, Arg-113C, and Gln-133C not only is involved in transmission of homotropic and heterotropic effects between the subunits of the enzyme but also may be involved in the direct modification of the catalytic activity of the active site as the holoenzyme is formed from the subunits. Additional studies are planned with these mutant enzymes as well as new mutant enzymes which contain substitutions in this area to establish the exact function of the C1/R1 interface and the portion of the 240s loop around Gln-246C.

ACKNOWLEDGMENTS

We thank W. N. Lipscomb for providing the X-ray coordinates and M. M. Ladjimi for his valuable comments on the manuscript.

REFERENCES

Bachmann, B. J. (1983) *Microbiol. Rev. 47*, 180–230.
Bethell, M. R., Smith, K. E., White, J. S., & Jones, M. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1442–1449.
Blackburn, M. N., & Schachman, H. K. (1977) *Biochemistry 16*, 5084–5090.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Carter, P. J., Bedouelle, H., & Winter, G. (1985) *Nucleic Acids Res. 13*, 4431–4443.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
Drake, J. W. (1970) *The Molecular Basis of Mutation*, pp 161–176, Holden-Day, San Francisco.
Gerhart, J. C., & Pardee, A. B. (1962) *J. Biol. Chem. 237*, 891–896.
Gerhart, J. C., & Schachman, H. K. (1965) *Biochemistry 4*, 1054–1062.
Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886–2892.
Gerhart, J. C., & Schachman, H. K. (1968) *Biochemistry 7*, 538–552.
Gillam, S., & Smith, M. (1979a) *Gene 8*, 81–97.
Gillam, S., & Smith, M. (1979b) *Gene 8*, 99–106.
Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Edwards, B. F. P., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C., & Lipscomb, W. N. (1982) *J. Mol. Biol. 160*, 219–263.
Hoover, T. A., Roof, W. D., Foltermann, K. F., O'Donovan, G. A., Bencini, D. A., & Wild, J. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2462–2466.
Hutchinson, C. A., III, Philips, S., Edgell, M. H., Gillam, S., Jahnke, P., & Smith, M. (1978) *J. Biol. Chem. 253*, 6551–6560.
Jones, M. E., Spector, L., & Lipmann, F. (1955) *J. Am. Chem. Soc. 77*, 819–820.
Kantrowitz, E. R., Foote, J., Reed, H. W., & Vensel, L. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3249–3253.
Kantrowitz, E. R., Reed, H. R., Ferraro, R. A., & Daigneault, J. P. (1981) *J. Mol. Biol. 153*, 569–587.
Ke, H.-M., Honzatko, R. B., & Lipscomb, W. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4037–4040.
Kim, K. H., Pan, Z., Honzatko, R. B., Ke, H.-M., & Lipscomb, W. N. (1987) *J. Mol. Biol. 196*, 853–875.
Konigsberg, W. H., & Henderson, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2467–2471.
Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1643–1647.
Krause, K. L., Voltz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527–553.
Ladjimi, M. M., & Kantrowitz, E. R. (1987) *J. Biol. Chem. 262*, 312–318.
Ladjimi, M. M., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 276–283.
Ladjimi, M. M., Middleton, S. A., Kelleher, K. S., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 268–276.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp 368–369, Cold Spring Habor Laboratory, Cold Spring Harbor, NY.
Middleton, S. A., & Kantrowitz, E. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5866–5870.
Nowlan, S. F., & Kantrowitz, E. R. (1985) *J. Biol. Chem. 260*, 14712–14716.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.
Pastra-Landis, S. C., Evans, D. R., & Lipscomb, W. N. (1978) *J. Biol. Chem. 253*, 4624–4630.
Pastra-Landis, S. C., Foote, J., & Kantrowitz, E. R. (1981) *Anal. Biochem. 118*, 358–363.
Reichard, P., & Hanshoff, G. (1956) *Acta Chem. Scand. 10*, 548–566.
Robey, E. A., Wente, S. R., Markby, D. W., Flint, A., Yang, Y. R., & Schachman, H. K. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5934–5938.
Roof, W. D., Foltermann, K. F., & Wild, J. R. (1982) *Mol. Gen. Genet. 187*, 391–400.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schleif, R. F., & Wensink, P. C. (1981) *Practical Methods in Molecular Biology*, Springer-Verlag, New York.
Silver, R. S., Daigneault, J. P., Teague, P. D., & Kantrowitz, E. R. (1983) *J. Mol. Biol. 168*, 729–745.
Smith, K. A., Nowlan, S. F., Middleton, S. A., O'Donovan, C., & Kantrowitz, E. R. (1986) *J. Mol. Biol. 189*, 227–238.
Subranami, S., & Schachman, H. K. (1982) *J. Biol. Chem. 257*, 12219–12223.
Wu, C. W., & Hammes, G. G. (1973) *Biochemistry 12*, 1400–1408.
Yates, R. A., & Pardee, A. B. (1956) *J. Biol. Chem. 221*, 757–770.
Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487–6500.

Enzyme II^{Mtl} of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Identification of the Activity-Linked Cysteine on the Mannitol Carrier†

H. H. Pas and G. T. Robillard*
Department of Physical Chemistry, University of Groningen, Nyenborgh 16, 9747 AG Groningen, The Netherlands
Received November 23, 1987

ABSTRACT: The cysteines of the membrane-bound mannitol-specific enzyme II (EII^{Mtl}) of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system have been labeled with 4-vinylpyridine. After proteolytic breakdown and reversed-phase HPLC, the peptides containing cysteines 110, 384, and 571 could be identified. *N*-Ethylmaleimide (NEM) treatment of the native unphosphorylated enzyme results in incorporation of one NEM label per molecule and loss of enzymatic activity [Roossien, F. F., & Robillard, G. T. (1984) *Biochemistry 23*, 211–215]. NEM treatment and inactivation prevented 4-vinylpyridine incorporation into the Cys-384-containing peptide, identifying this residue as the activity-linked cysteine. Both oxidation and phosphorylation of the native enzyme protected the enzyme against NEM labeling of Cys-384. Positive identification of the activity-linked cysteine was accomplished by inactivation with [^{14}C]iodoacetamide, proteolytic fragmentation, isolation of the peptide, and amino acid sequencing.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] is responsible for uptake of a number of hexoses and hexitols in both Gram-negative and Gram-positive organisms [for a review see Postma and Lengeler (1985)]. The driving force for uptake is phosphoenolpyruvate. During transport over the membrane the substrate becomes phosphorylated. The PTS consists of two general proteins, EI and HPr, which are cytoplasmic, and a sugar-specific EII, which is membrane bound. Some PTS systems, for instance the glucose PTS of *Escherichia coli*, use a fourth protein, $EIII^{Glc}$, which acts between HPr and EII^{Glc}.

Cysteines often play important roles in transport processes in both procaryotic and eucaryotic systems. However, their exact role is uncertain. Localization of the cysteines is a prerequisite for understanding their mechanistic importance. Recently, Menich et al. (1987) showed by site-directed mutagenesis that, of the eight cysteines of the *E. coli* lac permease, Cys-154 is obligatory for lactose/H^+ symport. Robillard and Konings (1981) showed that the activity of the PTS glucose-specific carrier EII^{Glc} was sensitive to oxidants and the redox potential. The activity decreased at potentials greater than −100 mV. Furthermore, the reduced enzyme could be irreversibly inactivated by the thiol reagent *N*-ethylmaleimide (NEM), while the oxidized form was protected. On the basis of these results it was suggested that dithiol–disulfide interchange could play an important role in catalytic activity. Studies on EII^{Fru} from *Rhodopseudomonas sphaeroides* expanded on this concept by demonstrating that, at intermediate redox potentials, turnover of the carrier is accompanied by cycling through the oxidized and reduced states (Lolkema & Robillard, 1986). Roossien and Robillard (1984) quantitated the NEM labeling of the purified mannitol permease EII^{Mtl}. The reduced unphosphorylated enzyme was inhibited by incorporation of NEM at one site per peptide chain. The labeled thiol was named SH_A. Oxidation prevented labeling at this site. One label per peptide chain was also incorporated in the

† This research was supported by the Netherlands Organization for the Advancement of Pure Research (ZWO) through the Netherlands Foundation for Chemical Research (SON).

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I; HPr, histidine-containing phosphocarrier protein; EII^{Mtl}, mannitol-specific enzyme II; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TFA, trifluoroacetic acid; decyl-PEG, decylpoly(ethylene glycol); PEC, *S*-(β-pyrid-4-ylethyl)cysteine; diamide, 1,1′-azobis(*N*,*N*-dimethylformamide); HPLC, high-performance liquid chromatography.

0006-2960/88/0427-5515$01.50/0