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Autonomous Folding and Coenzyme Binding of the Excised Pyridoxal 5′-Phosphate Binding Domain of Aspartate Aminotransferase from *Escherichia coli*†

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ABSTRACT: The coenzyme (PLP) binding domain (residues 47-329) of the dimeric aspartate aminotransferase from *Escherichia coli* was produced separately by recombinant DNA methods. It folded autonomously both in vivo and in vitro, that is, independently of the native N- and C-terminal extensions that combine to form the small domain of eAAT. The PLP-domain had one binding site for PLP of relatively high affinity involving a covalent bond to the protein. It was monomeric, although the major subunit–subunit interface at the 2-fold symmetry axis remained unchanged. This effect appears to be due mainly to the absence of the N-terminal extension that contains hydrophobic residues, which interact with the PLP-domain of the second subunit in the wild-type dimer. Judged by circular dichroism, fluorescence, and HPLC gel filtration at increasing concentrations of guanidinium chloride, the PLP-domain underwent a three-state unfolding transition ($M' \rightleftarrows M'^* \rightleftarrows U'$) involving a compact intermediate M'^*. This behavior parallels the unfolding of the dissociated native monomer of eAAT.

The three-dimensional structures of many protein monomers consist of two or more compact tertiary substructures or domains that comprise contiguous subregions of the polypeptide chain (Wetlaufer, 1981). Their compact structure implies that they are autonomous units of protein folding, assembly, and sometimes also function. They might have played an important role as modules during protein evolution. However, there is no unequivocal theoretical approach for identifying the boundaries of domains.

The cleavage of an oligopeptide loop at the surface of a native protein can define domain boundaries operationally. If the separated proteolytic fragments either refold autonomously and retain some vestige of function (for example, the Klenow fragment of DNA polymerase; Jacobsen et al., 1974) or reassemble stoichiometrically, it is reasonable to conclude that cleavage occurred in the connector between structural domains. However, even proteins that appear to consist of a single structural domain can be separated proteolytically into autonomously folding and reassembling subdomains (e.g., the α subunit of tryptophan synthase; Hyde et al., 1988; Miles, 1991). Moreover, proteolytic separation into autonomously folding and reassembling domains can occur in loops that are not in the connector defined by protein crystallography (e.g., the β subunit of tryptophan synthase; Högberg-Raibaud & Goldberg, 1977a,b; Hyde et al., 1988).

Recombinant DNA technology is an alternative and more versatile approach for probing the boundaries of putative structural domains. By using the known structure of the protein as a guide, the separation of protein fragments can be performed at the level of the gene, independent of whether the putative connectors are at the surface of the protein or not. Moreover, the subcistrons can be expressed in homogeneous form and in large amounts in transformed microorganisms [e.g., elongation factor Tu (Parmeggiani et al., 1987) and phosphoglycerate kinase (Minard et al., 1989)].

In this work we describe the production and properties of the pyridoxal 5′-phosphate (PLP)[1] binding domain (P-domain) of aspartate aminotransferase from *Escherichia coli* (eAAT). Each of the two identical subunits of eAAT (Figure 1) consists of the P-domain and a second domain formed by its N- and C-terminal extensions. The large P-domain comprises the region from residue 47 to 329 (numbering as for pig cytosolic AAT, cf. Figure 1) and includes all side chains that are important for the binding of PLP (Ford et al., 1980; Jäger et al., 1989). The small domain, which comprises residues 5-46 and 330-409, moves, roughly speaking, as a unit relative to the P-domain during each catalytic cycle (Birchmeyer & Christen, 1971; Jansonius & Vincent, 1987; Picot et al., 1991).

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[1] Abbreviations: eAAT, aspartate aminotransferase from *Escherichia coli* (EC 2.6.1.1); P-domain, genetically isolated coenzyme binding domain of eAAT, comprising residues 47-329; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; DTE, dithioerythritol; GuCl, guanidinium chloride; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; CD, circular dichroism; IPTG, isopropyl thiogalactoside; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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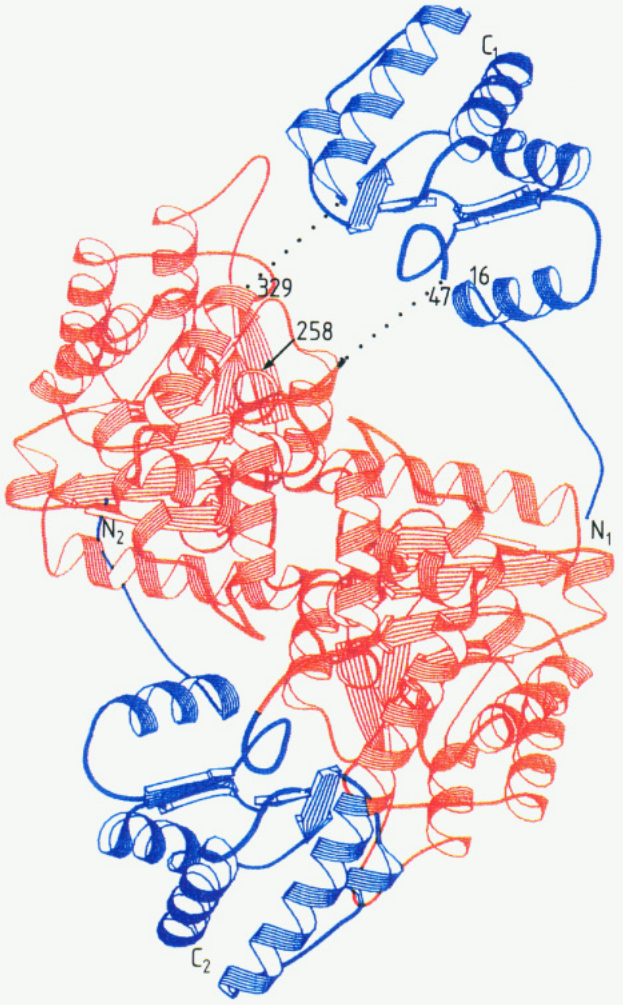

FIGURE 1: Crystal structure of dimeric eAAT in the closed conformation: view along the 2-fold molecular axis. Each subunit is divided into a large pyridoxal 5′-phosphate (PLP) binding domain (red) comprising residues 47–329 and a small domain (blue) comprising residues 5–46 and 330–409. Numbering is as for pig cytosolic AAT. The lysine residue 258 that forms a covalent bond to PLP is indicated by the arrow. The small domain of the upper subunit is translated relatively to the remainder of the protein as indicated by the dotted lines to provide an exploded view of the eAAT monomer. (Courtesy of J. Jäger.)

Thus, the P-domain appears to be a stable substructure.

The P-domain is of general interest for the following reasons. First, the refolding and reassembly of dimeric eAAT occurs with high yield both in vivo and in vitro (Herold & Kirschner, 1990). Moreover, the native monomer of eAAT appears to follow a three-state unfolding transition involving a compact intermediate. The availability of pure P-domain would allow an assessment of its role in the refolding of the denatured monomer of eAAT and its dimerization. It should be possible to probe the correct folding of the P-domain by binding studies with PLP. Second, several members of the AAT family are known to have very similar structures [Smith et al., 1989; Jäger et al., 1989; Kamitori et al., 1990; for review see Christen and Metzler (1985)], which provides compelling evidence that they are evolutionarily related. However, there is also suggestive evidence that other PLP-dependent enzymes, which are not homologous to AAT, possess a P-domain that has the same fold as the P-domain of AAT. Examples are ω-amino acid:pyruvate aminotransferase (Watanabe et al., 1989) and phosphoserine aminotransferase (Stark et al., 1991).

In the following we show that the genetically encoded P-domain can be produced as a folded protein in vivo. It was monomeric under native conditions, bound PLP stoichiometrically and strongly, and followed a reversible three-state unfolding transition when exposed to increasing concentrations of GuCl.

Experimental Procedures

Materials and Buffers. Ultrapure GuCl was purchased from Schwarz-Mann, New York. All other reagents were of analytical grade. Buffers for refolding and reactivation studies, including the 10 mM stock solutions of PLP, were not kept for more than 1 week.

The following buffers were used for the purification procedure: buffer A, 20 mM morpholinoethanesulfonic acid, pH 6.6, and 1 mM EDTA; buffer B, 5 mM sodium phosphate, pH 7.0; buffer C, 100 mM sodium phosphate, pH 7.0; buffer D, 10 mM Hepes, pH 7.4, 5 mM DTE, and 1 mM EDTA. Buffers A–C further contained 10 μM PLP, 1 mM 2-oxoglutarate, and 0.1 mM DTE.

Plasmids, Bacterial Strains, and Growth Conditions. For site-directed mutagenesis, the expression–mutagenesis vector pMa/c5-14 and the host strains WK6 and WK6mutS were obtained from and used as described by Stanssens et al. (1989). The helper phage M13K07 was purchased from Pharmacia. The plasmid pIF100 has been described earlier (Fotheringham et al., 1986). For expression of the two mutants, the vector pDS56/RBII-1 (Certa et al., 1986) with the *E. coli* strain MG204 [*his-23* (*Am*) *proB trpA-605*(*Am*) *lacI3 lacZ118*(*Oc*) *gyrA rpsL aspC*::*kanR tyrB recA*::*Tn10 ilvE*] (gift of I. Fotheringham) was used.

Cells were grown in LB-medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.5; Luria et al., 1960), containing either ampicillin (100 μg/mL), for WK6 and WK6mutS harboring pMa, or chloramphenicol (25 μg/mL), for cells harboring pMc. Both antibiotics were added to the medium for cultivating MG204 harboring variants of either pDS56/RBSII-1, pIF100, or pIF100/aspC.

Site-Directed Mutagenesis. Site-directed mutagenesis in the vector pMa/AAT was performed by the gapped duplex method described by Stanssens et al. (1989). Single-stranded pMc-DNA was isolated after infection with M13K07 (Walker & Gay, 1983).

Oligodeoxynucleotides were synthesized on an Applied Biosystems ABI 380B oligonucleotide synthesizer and purified on Baker 10SPE cartridges. The 28mer 5′-GTAC-CGGGGTTGGATCCGTCTCATCTTT-3′ was synthesized to introduce a *Bam*HI site preceding the codon for amino acid 47 into the *aspC* gene, which was necessary for the construction of the pDS/NT vector (cf. Figure 2).

The 22mer 5′-GCATACGCTGATTAACGCTGGC-3′ was synthesized to introduce both a stop codon and a frameshift mutation following the codon for amino acid 329 (leading to pMc/CT).

Mutants were selected either for the presence of the new *Bam*HI restriction site or by colony hybridization (Grunstein & Wallis, 1979). A further test for the presence of the stop codon in pMc/CT was obtained by performing SDS–PAGE with cell extracts. The presence of a protein band with the expected M_r of 35 000 instead of the band for eAAT of 44 000 confirms the presence of a stop codon at the approximate position. Both mutations were verified additionally by DNA sequencing (Sanger et al., 1977) of the entire coding sequence. All other recombinant DNA techniques followed standard protocols (Maniatis et al., 1982).

Construction of the Coding Region for the PLP-Binding Domain. The short *Eco*RI–*Bgl*II fragment of pIF 100 that carries the entire *aspC* gene was subcloned into the multiple

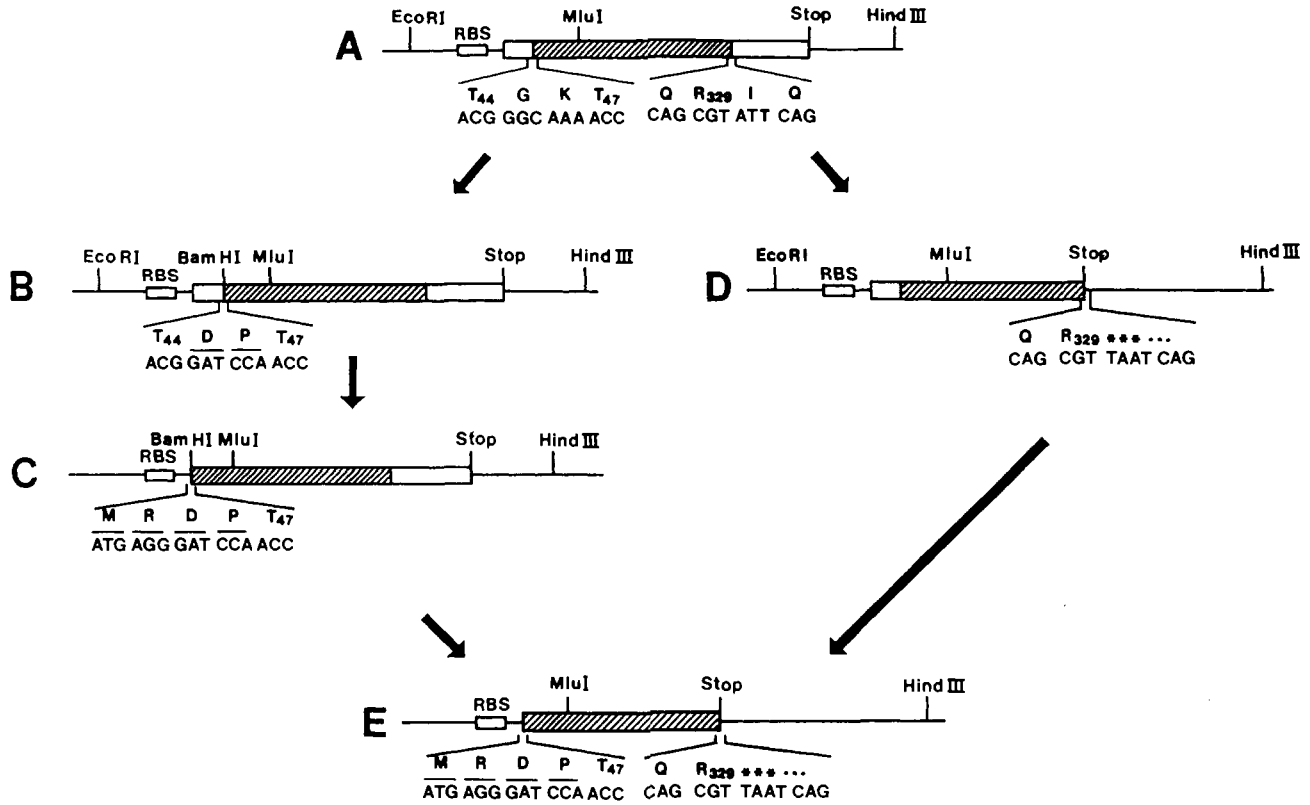

FIGURE 2: Construction of the P-domain at the genetic level. Numbering is as for pig cytosolic AAT. The shaded region corresponds to the P-domain. The novel amino acid residues are underlined. (A) *aspC* subcloned into the pMa5-14 mutagenesis vector (yielding pMa/AAT). (B) Introduction of a *Bam*HI restriction site into pMa/AAT (yielding pMc/NT). (C) Subcloning of the *Bam*HI–*Hin*dIII fragments of pMc/NT into the expression vector pDS56/RBSII-1 (yielding pDS/NT). (D) Introduction of the new stop codon and a frameshift into pMa/AAT (yielding pMc/CT). (E) Religation of the two appropriate *Mlu*I/*Hin*dIII fragments from (C) and (D) (yielding pDS/PLPD).

cloning site of pMa5-14, yielding pMa/AAT (Figure 2A). To create the coding region for the N-terminal deletion mutant, a *Bam*HI restriction site was introduced upstream to the codon for Thr_{47} (pMC/NT), by site-directed mutagenesis (Figure 2B). The *Bam*HI–*Hin*dIII fragment of pMc/NT was then subcloned into the polylinker of the expression vector pDS56/RBSII-1 (yielding pDS/NT, Figure 2C), thus providing the sequence with an in-frame start codon, a ribosome binding site, and the strong Tn25 promoter under control of the *lac* operator. This mutant gene allows the overexpression of an N-terminal deletion mutant in *E. coli*.

A stop codon was introduced 3′-terminal to the codon for R_{329} by site-directed mutagenesis of a second sample of pMa/AAT. At the same time the reading frame was shifted by one nucleotide (pMc/CT, Figure 2D) to avoid an eventual translation readthrough.

The coding region for the PLP-binding domain was constructed from the two plasmids pDS/NT and pMc/CT (Figure 2C,D) as follows: pMc/CT was cleaved with *Mlu*I and *Hin*dIII, and the resulting small 1000-bp fragment carrying the coding region of amino acids 114–329 of eAAT was isolated by agarose gel electrophoresis. From pDs/NT the large 3700-bp *Mlu*I–*Hin*dIII fragment was isolated by similar means. This fragment consists of the main part of the pDS plasmid and carries the coding region for amino acids 47–113 of eAAT. Subcloning of the *Mlu*I–*Hin*dIII fragment of pMc/CT into the large fragment of pDS/NT yielded pDS/PLPD (Figure 2E), encoding the PLP-binding domain in expressible form.

Purification of the P-Domain. The purification procedure for the P-domain was similar to that for eAAT described by Herold and Kirschner (1990). The protein was isolated from *E. coli* MG 204 harboring pDS/PLPD. After the cells were grown at 37 °C to an A_{600} of 0.25, expression was induced by adding IPTG to 1.2 mM. After further incubation for 5 h at 37 °C, the cells were harvested by centrifugation (15000*g* for 30 min) and washed once with buffer A. The yield was about 3.5 g of cells/L of culture.

All further operations were carried out at 4 °C. A 35-g portion of the cells was resuspended in 50 mL of buffer A. After disruption by sonication, the cell debris was removed by centrifugation (16500*g* for 15 min). The DNA and some of the protein was removed by ultracentrifugation (145000*g* for 3 h). The protein solution was then loaded onto a column of DEAE-Sepharose-Cl 6B (2.4 × 32 cm) equilibrated with buffer A. After being washed with 300 mL of the same buffer, the proteins were eluted with 600 mL of a linear gradient of 0–500 mM NaCl in buffer A. The P-domain was eluted at 170 mM NaCl. The fractions absorbing at 425 nm due to the bound PLP were pooled, dialyzed against buffer B, and loaded onto a column of hydroxylapatite (2.4 × 30 cm) (Atkinson et al., 1973) equilibrated with buffer B. The column was washed with 300 mL of buffer B followed by 600 mL of a linear gradient formed with equal volumes of buffers B and C. The protein eluted at 37 mM phosphate. The pooled fractions were dialyzed against buffer D containing 100 μM PLP. After addition of 5% (v/v) glycerol, the protein was concentrated by ultrafiltration with Amicon PM10 filters to a concentration of about 1 mg/mL. The protein was stored at −70 °C after the solution was dripped into liquid nitrogen. In this form the protein was stored without significant loss of PLP binding capacity.

Determination of Protein Concentration. The molar concentrations of wild-type and mutant eAAT were determined by second-derivative absorbance spectroscopy (Levine & Federici, 1982) using a Hewlett-Packard 8452A diode array

spectrophotometer with 1-nm resolution. The molar absorptivities at 280 nm are 50 000 M^{-1} cm^{-1} and 65 300 M^{-1} cm^{-1} for eAAT and P-domain, respectively.

Determination of Molecular Weight. The apparent molecular weight of the mutant was estimated by HPLC gel filtration and analytical ultracentrifugation. HPLC experiments were performed as described previously (Herold & Leistler, 1991). Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge as described previously (Herold & Kirschner, 1990).

Preparation of Apo-P-Domain. After removal of free PLP by dialysis against buffer D, hydroxylammonium chloride was added to a final concentration of 10 mM (De Moss, 1962). The apoprotein was then exhaustively dialyzed against buffer D. The loss of coenzyme was checked by loss of absorbance between 300 and 510 nm. The isolated apo-P-domain was stored at 4 °C for not longer than 1 week.

Coenzyme Binding. (*A*) *Spectrophotometric titration* was performed with a Kontron Uvikon 860 spectrophotometer. Apo-P-domain (1 mL) was titrated at 18 °C in 0.44-cm tandem cuvettes by adding aliquots of PLP stock solution with an Agla microsyringe. The apoprotein in the reference cuvette was titrated with the same aliquot of buffer D for volume compensation. The reference buffer was also titrated with the PLP solution. The absorbance difference spectra were recorded from 300 to 550 nm after incubation for 10 min and analyzed by measuring the positive difference absorbance at 440 nm originating from bound PLP.

(*B*) *Equilibrium Dialysis.* The procedure described by Kirschner et al. (1975) was followed; Sartorius SM 11539-025N membranes that have equilibration times of about 3 h at 18 °C were used. The chambers on opposite sides of the membrane were loaded with 100 μL of apo-P-domain and PLP solution in buffer D, respectively. After equilibration, samples of 50 μL each were withdrawn with a Hamilton syringe and transferred into a microcuvette containing 400 μL of 0.11 M NaOH. The concentrations of bound and free coenzyme were determined spectrophotometrically by using a Hewlett-Packard 8452A diode array spectrophotometer, bandwidth 1 nm. The molar absorptivity of PLP in 0.1 M NaOH is 6600 M^{-1} cm^{-1} at 388 nm (Peterson & Sober, 1954).

(*C*) *Analysis of Coenzyme Binding Data.* The data were fitted to eq 1 by the method of Rosenthal (1967) by means of a nonlinear least-squares procedure (Eberhard, 1990). R

$$R = n_1\left(\frac{[L_{free}]}{[L_{free}] + K_{d,1}}\right) + n_2\left(\frac{[L_{free}]}{[L_{free}] + K_{d,2}}\right) \quad (1)$$

is the binding ratio ($[L_{bound}]/[P_{tot}]$). $[P_{tot}]$ is the total protein concentration, and $[L_{free}]$ is the concentration of free ligand. The number of binding sites is n_i, where the index i designates different independent sites in the same protein. The dissociation constants $K_{d,i}$ are defined by eq 2, where $[P_{free,i}]$ is the

$$K_{d,i} = [P_{free,i}][L_{free}]/[L_{bound}] \quad (2)$$

concentration of free binding sites of class i. In the case of spectrophotometric titration the free ligand concentration $[L_{free}]$ was approximated by eq 3. The absorbance difference

$$[L_{free}] = [L_{tot}] - ((n_1 + n_2)(\Delta A/\Delta A_{max})[P_{tot}]) \quad (3)$$

at saturation (ΔA_{max}) was estimated by extrapolating the fitted saturation curve to large PLP concentrations. The values for n_1 and n_2 were determined by the computer fit procedure denoted above. By this method the titration data fitted equally well to a large range of values for n_1, n_2, $K_{d,1}$, and $K_{d,2}$. For this reason the numbers of binding sites n_1 and n_2 had to be evaluated independently by equilibrium dialysis where $[L_{free}]$ was determined directly.

Reduction of the Schiff Base of Bound PLP. Holo-P-domain (2 mL, 2 mg/mL) was dialyzed against buffer D containing 2 μM PLP to avoid unspecific binding of PLP to other sites, followed by addition of an excess of $NaBH_4$ [20 μL of a freshly prepared 0.5 M solution in 0.05 M NaOH; see Raibaud and Goldberg (1973)]. Foaming was suppressed by centrifugation for 5 min. Subsequently the protein solution was dialyzed three times against buffer D. The reduction of the Schiff base was checked by monitoring the increase of the absorbance at 330 nm.

Determination of the Aldimine pK_a Value. The PLP absorbance difference spectra at different pH values were recorded with a Hewlett-Packard 8452A diode array spectrophotometer, bandwidth 1 nm. Aliquots of 100 μL of an apo-P-domain stock solution (6.5 μM; dialyzed against buffer D, pH 7.5) were supplemented with 880 μL of appropriately adjusted buffer D (such that the mixtures had the predetermined final pH values) and 20 μL of a solution of 4.5 mM PLP in buffer D, pH 7.5. The reference cuvette contained the same mix except for 100 μL of buffer D, pH 7.5, instead of the protein solution. After the samples were incubated for 15 min at 23 ± 2 °C, the absorbance difference spectra were recorded between 300 and 510 nm. Longer incubation times did not lead to any further change of the spectra.

Protein Folding Studies. Reversible unfolding was monitored by fluorescence and HPLC gel filtration (Herold & Leistler, 1991), as well as by circular dichroism measurements. Far-UV circular dichroism spectra were recorded on a Cary Model 60 instrument at 20 °C with cuvettes of 0.1 cm path length at a protein concentration of 0.15 mg/mL in 10 mM Hepes, pH 7.4, 0.1 mM EDTA, and 0.1 mM DTE. Near-UV/vis circular dichroism spectra were recorded on a Cary Model 61 instrument at 20 °C with a cuvette of 0.2 cm path length at protein concentrations of 1.8 mg/mL in buffer D containing 100 μM PLP. The loss of secondary structure of the P-domain induced by GuCl was followed by Θ_{221} in buffer D. Prior to the measurements, the samples were equilibrated for at least 3 h at 20 °C. All measurements were repeated three times. The fluorescence emission spectra were recorded on a SLM 8000 single photon counting recording fluorometer, with a bandwidth of 8 nm on both sides (excitation at 280 nm). The loss of PLP bound to the P-domain was checked by spectroscopic titration with a concentrated solution of GuCl. P-domain (1 mL, 0.8 mg/ml, 24 μM) in buffer D containing 100 μM PLP was titrated at 18 °C in 0.44-cm tandem cuvettes by adding aliquots of an 8 M solution of GuCl. The P-domain in the reference cuvette was titrated with the same aliquots of buffer D for volume compensation. The reference buffer was also titrated with the GuCl solution. The absorbance difference spectra from 300 to 510 nm were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. After the spectra displayed no further changes with time, they were analyzed by measuring the negative difference absorbance at 445 nm.

Results

Strategy for Construction and Expression of the P-Domain. The original suggestion for the demarcation of the P-domain was based on the crystal structure of mitochondrial AAT from chicken (Ford et al. 1980). Since the crystal structure of eAAT had become available recently (J. Jäger, personal communication), that structure was used to analyze the linking regions between the small and large domain in detail.

The hinge for the syncatalytic domain movement runs approximately through residue 16 at the N-terminus of α-helix 1, residue 46 at the N-terminus of α-helix 2, and residue 330 in the middle of the long α-helix 13 (cf. Figure 1). We assume that the hinge residues 46 and 329 define the borders of the P-domain. Because it still retains α-helix 2, which contains most of the residues that form the main intersubunit interface around the 2-fold axis of eAAT (cf. Figure 1), it could still, in principle, dimerize.

The first cut was established between K_{46} and T_{47} as follows. First a *Bam*HI site was introduced into the *aspC* gene (Figure 2A,B). As a result G_{45} was converted to D and K_{46} to P. By subcloning of the *Bam*HI–*Hin*dIII fragment into the expression vector pDS56/RBSII-1, the first 46 residues of eAAT were deleted and two additional residues were introduced at the new N-terminus, namely, M and R (Figure 2C). The new N-terminal sequence was therefore M R D P T_{47} .... Because the side chain of K_{46} is exposed to the solvent, its replacement by P should not affect the stability of the P-domain. It seemed unlikely that the loss of the H-bond of the T_{47} OH group to the main-chain carbonyl of T_{40} in the eAAT structure, which is the only noncovalent interaction between the two domains in this region, would destabilize the P-domain. The two conserved hydrophobic side chains of V_{49} and L_{50} contribute to the intersubunit interface and have contacts with the C-terminal helix of the P-domain. The conformation of their side chains should be indifferent to the cut between K_{46} and T_{47}.

The new C-terminus was introduced behind R_{329} by replacing the codon for I_{330} by a stop codon. A frameshift after the stop codon was introduced additionally in order to avoid readthrough expression of complete eAAT sequences. All side chains following R_{329} are either exposed or involved in contacts within the small domain. According to Thornton and Sibanda (1983), it does not appear fortuitous that the beginning and the end of the P-domain are adjacent in space.

Purification and Molecular Weight Determination. The P-domain was overproduced in *E. coli*. More than 50% of the total protein was found in the cytoplasmic fraction, and it amounted to 10–20% of the total soluble protein. The purification procedure was similar to that used for the isolation of eAAT. The only difference was the omission of the precipitation step with PEG-6000, because the P-domain precipitated even at low PEG concentrations. Moreover the pure P-domain tended to precipitate at a protein concentration of more than 2.0 mg/mL. However, it was possible to stabilize the protein by including 5% (v/v) glycerol in the storage buffer.

After 10-fold purification by chromatography on columns of DEAE-Sepharose-CL 6B and hydroxylapatite, the protein was typically 95% pure as judged by SDS–PAGE (Figure 3). Up to 16 mg of purified protein was obtained from 1 L of culture. Dialyzed solutions of the purified protein were yellow at a pH of 7.4, indicating specific binding of PLP. The P-domain was totally inactive in catalyzing the AAT reaction at concentrations up to 4 μM, nor did it perform the half reaction with L-cysteine sulfinate as substrate.

Gel filtration experiments (Herold & Leistler, 1991) as well as ultracentrifugation studies (data not shown) clearly showed that the P-domain was monomeric up to 40 μM. Sedimentation coefficients were 5.4 S for eAAT and 2.4 S for the P-domain. Equilibrium runs gave an M_r of 33 500 for the P-domain even at a concentration of 40 μM. Therefore the estimated lower limit of the thermodynamic dissociation constant of the hypothetical P-domain dimer is $K_d \geq 400$ μM. This value is 10^5-fold larger than that of the eAAT dimer

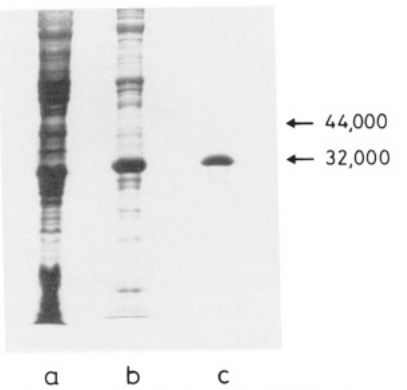

FIGURE 3: Purification of the P-domain. SDS–polyacrylamide gel electrophoresis of the protein at different stages of purification. Lane a, crude extract after induction with IPTG; lane b, DEAE-Sepharose eluate; lane c, hydroxylapatite eluate. Arrows indicate the M_r values of eAAT (44 000) and the P-domain (32 000).

(Herold & Kirschner, 1990). The monomer M_r values calculated from sequence data are 43 573 (apo-eAAT) and 32 367 (apo-P-domain). Thus the P-domain is a monomeric protein under conditions where eAAT is a dimer.

Coenzyme Binding to Apo-P-Domain. The first indication for strong and specific binding of PLP arose from UV/vis absorbance spectroscopy. The binding of PMP was not detectable even in the presence of 10 mM coenzyme. The spectrum at pH 7.4 of PLP bound to the P-domain showed maxima at 340 and 425 nm (Figure 4A), whereas free PLP absorbs at 330 and 388 nm (Peterson & Sober, 1954). Wild-type eAAT has maxima at 360 and 425 nm, corresponding to the unprotonated and protonated forms of the Schiff base of PLP with K_{258} (internal aldimine; Kallen et al., 1985). As a fundamental difference to eAAT, the unprotonated form of the Schiff base of the P-domain predominates at pH 7.4, suggesting an enhanced pK_a value and therefore a different environment of the aldimine double bond. The molar absorptivity at 425 nm and pH 7.4 was 4700 M^{-1} cm^{-1}. The CD spectra of bound PLP (Figure 4B) also reflect the different protonation states in eAAT and the P-domain at pH 7.4. The ellipticity of PLP bound to the P-domain suggests an asymmetric environment of the chromophore that is similar to that in eAAT. This observation indicates that PLP is bound specifically to the apo-P-domain.

Both spectrophotometric titration and equilibrium dialysis data on the binding of PLP to the apo-P-domain were consistent with at least two classes of binding sites. Because the titration data alone did not supply unequivocal information on the free ligand concentration and thus on the binding stoichiometry, we turned to equilibrium dialysis, which determines the values of n_1 and n_2 in eq 1 more reliably. These values were then used as fixed parameters in the fit procedure to determine the dissociation constants $K_{d,1}$ and $K_{d,2}$ from the titration data. As can be seen from the direct plot in Figure 5, reasonable agreement was obtained between titration and equilibrium dialysis data. The two dissociation constants averaged over three experiments differ 250-fold ($K_{d,1} = 2$ μM, $K_{d,2} = 500$ μM). The stoichiometry of binding of the high-affinity binding site ($K_{d,1} = 2$ μM) was about 0.7 binding sites/monomer. The second class of binding sites with $K_{d,2} = 500$ μM and about 2.2 binding sites/monomer must correspond to an unspecific interaction. Wild-type eAAT binds PLP more strongly without significant unspecific binding ($K_d = 0.025$ μM, $n = 0.85$; Köhler, 1990).

PLP Is Bound Covalently to the P-Domain. The holo-P-domain was reduced with $NaBH_4$ to determine whether PLP

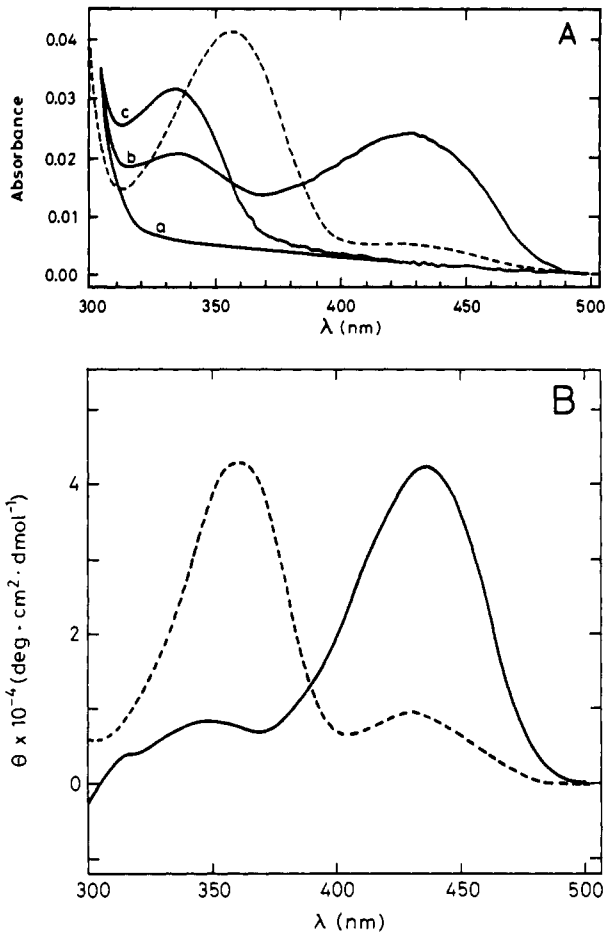

FIGURE 4: Spectroscopic properties of the P-domain and its derivatives. The spectra of holo-eAAT measured under the same conditions are shown for comparison (---). (A) Absorbance spectra at monomer concentrations of 5.3 μM. The buffer was 10 mM Hepes, pH 7.4, 1 mM EDTA, and 5 mM DTE. (a) Apo-P-domain; (b) holo-P-domain in the presence of 10 μM PLP; (c) holo-P-domain after reduction with $NaBH_4$, native, or after unfolding in 6 M GuCl and subsequent refolding by dialysis. (B) Near UV/vis CD spectra, taken at a monomer concentration of 42 μM in the same buffer as in (A) supplemented with 100 μM PLP.

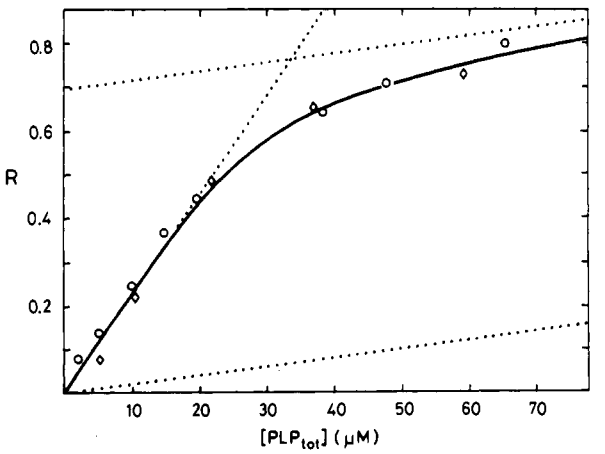

FIGURE 5: Specific and unspecific binding of PLP to the P-domain. The binding ratio $R = [PLP_{bound}]/[P\text{-domain}_{tot}]$ is shown as a function of the total PLP concentration $[PLP_{tot}]$. Data from equilibrium dialysis with 39 μM apo-P-domain (diamonds) and data from spectroscopic titration at 33 μM apo-P-domain, normalized to 39 μM (circles) are shown. The difference molar absorptivity at 440 nm was 1900 M^{-1} cm^{-1}. The solid line is a fitted curve with $K_{d,1} = 2$ μM, $K_{d,2} = 680$ μM, $n_1 = 0.72$, and $n_2 = 2.2$. See text for details. Dotted lines show initial slope, asymptote of the high-affinity binding, and contribution of unspecific binding.

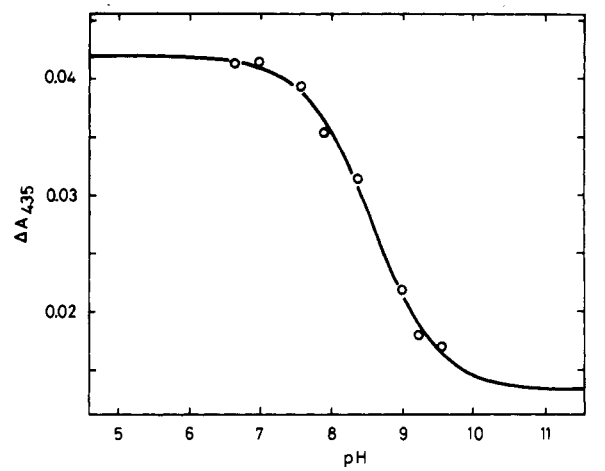

FIGURE 6: pH dependence of the absorbance difference spectra of PLP bound to the P-domain. Aliquots of a stock solution of apo-P-domain were added to buffer at different pH value containing 90 μM PLP. The plot shows the difference absorbance at 435 nm versus the pH value. The data fitted best to a single deprotonation process with a pK_a value of 8.6.

is bound covalently as a Schiff base. As expected, the absorbance maximum at 425 nm was shifted to a single peak at 330 nm (Figure 4A), which is characteristic for ε-(phosphopyridoxyl)lysine. To demonstrate that the reduced PLP is bound covalently to the P-domain, the protein was unfolded in 6 M GuCl for 1 h and dialyzed against buffer D to remove any loosely bound chromophores and to refold the protein. As shown in Figure 4A, the absorbance at 330 nm persisted after dialysis, proving that PLP is bound covalently to the P-domain. To check the reversibility of unfolding of reduced holo-P-domain, CD spectra were measured before unfolding and after refolding. There was no qualitative difference between 210 and 250 nm. The amplitude after refolding of the reduced protein was diminished only by about 10%, which indicates a 90% yield of refolding.

Aldimine pK_a Values. The absorbance spectrum of the internal aldimine in holo-eAAT depends on pH due to the protonation of the aldimine double bond to K_{258}. Because the pK_a value of the Schiff bases of PLP with various amino acids is around 11.7 (Kallen et al., 1985), the pK_a value of eAAT (6.73; Toney & Kirsch, 1987) is therefore a measure for the local environment of the aldimine bond. Figure 6 shows the decrease of difference absorbance at 435 nm of holo-P-domain with increasing pH. The absorbance difference spectra recorded at different pH values showed an isosbestic point at 350 nm. Under the experimental conditions (final protein concentration = 0.65 μM, total PLP concentration = 90 μM), the specific binding site is 98% saturated with the coenzyme at pH 7.4 (cf. Figure 5). The observation of an isosbestic point throughout the titration proves that the degree of saturation remained virtually the same over the investigated range of pH values. The experimental data shown in Figure 6 fitted best to a two-state transition with a pK_a value of 8.6. The difference of 1.9 from the pK_a value of eAAT indicates that the aldimine bond in the P-domain is more exposed to solvent than in eAAT but more shielded than that of the totally exposed bond. An increase of the pK_a of approximately 2 has been predicted for the case of the neutralization of the cationic group that binds to the substrate's α-carboxylic group (Ivanov & Karpeisky, 1969). Indeed, this residue (R_{386}) is missing in the P-domain.

Equilibrium Unfolding. The mechanism of reversible unfolding of the P-domain is of major interest for understanding the reversible dissociation and unfolding of eAAT. After denaturation of apo-P-domain by 6 M GuCl, dialysis at a

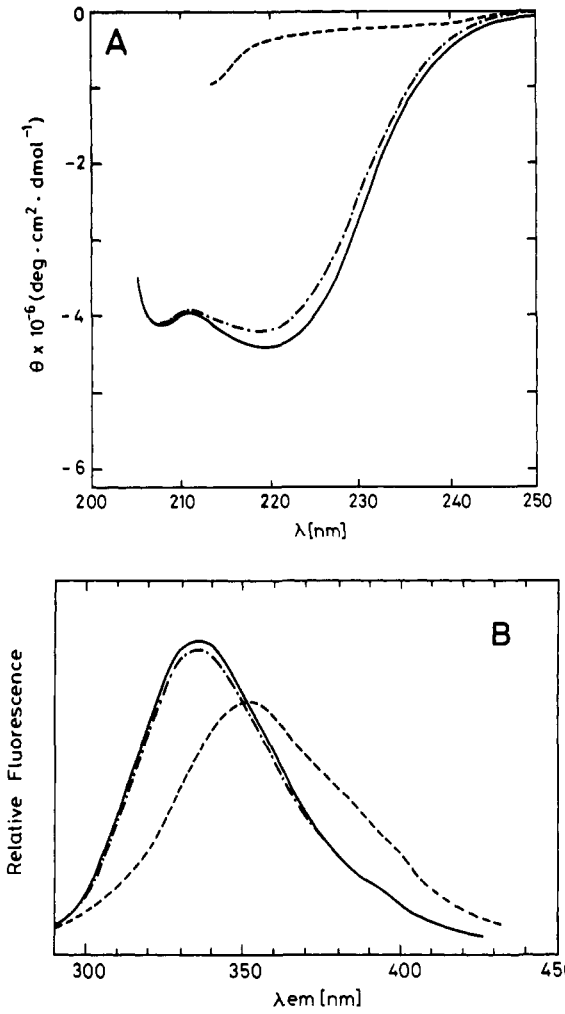

FIGURE 7: Unfolding of the apo-P-domain is fully reversible. Native protein (—); protein unfolded in the presence of 6 M GuCl (---); protein refolded by dialysis (···). (A) Far UV/vis CD spectra of 0.15 mg of protein/mL in 10 mM Hepes buffer, pH 7.4, 0.1 mM EDTA, and 0.1 mM DTE. (B) Fluorescence emission spectra of 0.01 mg of protein/mL in 10 mM Hepes buffer, pH 7.4, 1 mM EDTA, and 5 mM DTE (excitation at 280 nm).

concentration of 0.15 mg of protein/mL yielded folded protein with 90% recovery as judged by CD spectroscopy (Figure 7A). Fluorescence spectra (Figure 7B) confirm the essentially complete reversibility of unfolding of the apo-P-domain.

Equilibrium unfolding and refolding of apo-P-domain monitored by CD spectroscopy showed a cooperative and sigmoidal transition with a midpoint at 1.22 M GuCl (Figure 8). The presence of PLP had no effect on this transition. A more cooperative transition with a midpoint at 1.3 M GuCl was obtained when following the shift of the fluorescence emission maximum upon excitation at 280 nm (Figure 8A). The red shift of the fluorescence emission maximum monitors the exposures of buried tryptophan residues to the solvent (Teipel & Koshland, 1971). Analogous to the increase of fluorescence observed for the M $\rightleftarrows$ M* transition of eAAT (Herold & Kirschner, 1990), we also observed an increase of fluorescence of the apo-P-domain at low concentrations of GuCl. However the relative increase was much smaller (data not shown).

Since the hydrodynamic volume of a globular protein increases during denaturation, it is possible to monitor unfolding by HPLC gel filtration chromatography (Endo et al., 1983). At GuCl concentrations up to 0.3 M GuCl, the elution volume increased (Figure 8A), suggesting an apparent decrease of the hydrodynamic volume of the P-domain (Herold & Leistler,

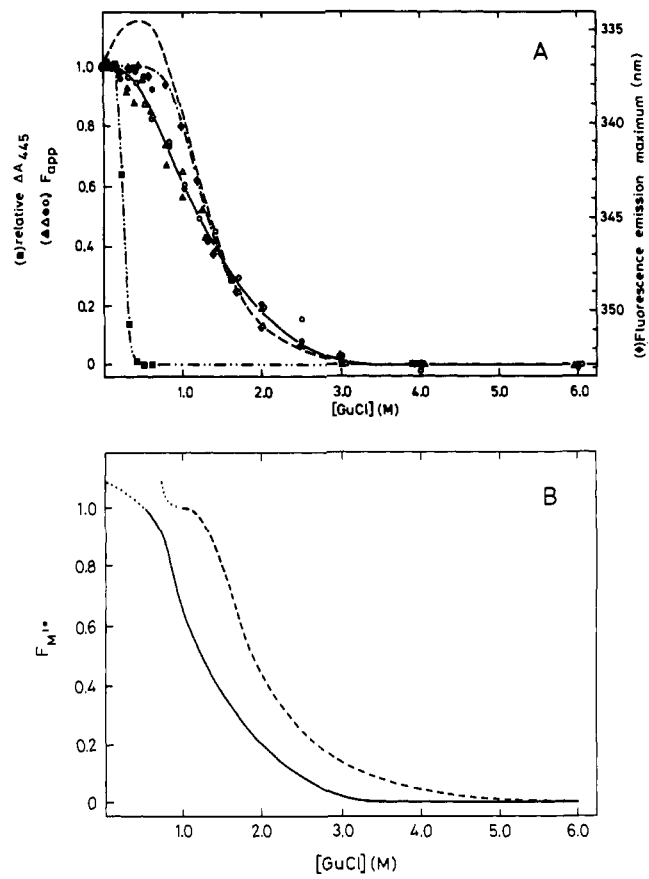

FIGURE 8: Noncoincident unfolding transitions of the P-domain reveal the presence of an intermediate. (A) Unfolding (filled symbols); refolding (empty symbols). Monomer concentrations were 0.8 mg/mL (24 μM) in PLP binding studies, 0.1 mg/mL (3.1 μM) in far-UV CD experiments, and 0.02 mg/mL (0.6 μM) in fluorescence experiments. PLP binding (squares): Spectral changes of holo-P-domain were recorded after titration with small volumes of a concentrated solution of GuCl. See text for details. CD measurements (apoprotein, circles; holoprotein, triangles): The CD curves were normalized by extrapolating the linear change of Θ_{221} above 3.5 M GuCl to 0 M GuCl. The normalized change of the CD signal is the fractional deviation (F_{app}) from this base line. The points were averaged from 2–4 independent experiments. The transition midpoint is at 1.22 M GuCl. Fluorescence emission maximum (diamonds): Excitation at 280 nm. The data are averages from three experiments. The transition midpoint is at 1.3 M GuCl. The transition curve measured by gel filtration (dashed line) is shown for comparison (Herold & Leistler, 1991). (B) Comparison of the M'* $\rightleftarrows$ U' transition monitored by Θ_{221} of the P-domain (—) with the M* $\rightleftarrows$ U transition of eAAT [---, redrawn from Herold and Kirschner (1990)]. The curves were normalized to the concentration of GuCl where the fraction of the respective intermediate is maximal.

1991). The midpoint of the main transition was at 1.3 M GuCl, and the unfolding was complete at about 3.5 M GuCl. Moreover, bound PLP was completely lost in a highly cooperative transition with a midpoint at 0.22 M GuCl, as indicated by the appearance of negative difference absorbance at 445 nm (Figure 8A). This transition monitors the sharp M' $\rightleftarrows$ M'* transition. The noncoincidence of the spectroscopic and the elution volume transitions clearly indicates the existence of a folding intermediate. Because the bound coenzyme is completely lost in the M' $\rightleftarrows$ M'* transition, M'* is the predominant species at 0.6 M GuCl. The M'* $\rightleftarrows$ U' transition can therefore be compared directly to the analogous M* $\rightleftarrows$ U transition of eAAT (Figure 8B). These experiments also demonstrate that HPLC gel filtration is a sensitive method for detecting folding intermediates independently, even if they possess nativelike spectroscopic properties (Shalongo et al., 1989; Ptitsyn et al., 1990).

Discussion

The P-domain produced in *E. coli* was soluble, monomeric, and capable of binding PLP but enzymically inactive. Moreover, the product attained after a cycle of unfolding by GuCl and refolding was indistinguishable from the original protein. These data prove that the P-domain can fold autonomously both in vivo and in vitro. To our knowledge these data show for the first time that an internal domain can fold independently of the native N- and C-terminal extensions and despite severing of an important α-helix.

The inability of the P-domain to catalyze the transamination reaction was not unexpected. Seven substrate-binding residues are missing. Prominent among these are R_{292}*, which neutralizes the distal carboxyl group of the substrate, and R_{386}, which neutralizes the α-carboxyl group. The asterisk denotes contribution of R_{292}* by the second subunit in the shared active site of eAAT.

The P-domain has one binding site for PLP of relatively high affinity ($K_{d,1}$ = 2 μM), involving a covalent bond to the protein. It remains to be determined whether the same residue, namely K_{258}, is involved in binding PLP to the apo-P-domain as to apo-eAAT. Together with the similarity of the absorbance spectra and the existence of ellipticity in the wavelength region where the bound coenzyme absorbs, these data support the notion that the structure of the P-domain is essentially as in eAAT. Nevertheless, the affinity for PLP is 80-fold smaller than observed with the eAAT subunit (K_d = 0.025 μM). Simultaneously, the pK_a value of the internal aldimine has increased by about 2 units. The reasons for these changes are probably, first, the deletion of the N-terminal extension, which contains the residues, 37–39, that shield the aldimine bond from hydrolysis, and second, the loss (due to its monomeric state) of the contact residue Y_{70}* from the other subunit. Support for the latter conclusion comes from the replacement of Y_{70}* by phenylalanine, which led to enhanced coenzyme dissociation rate constants and a decrease of the catalytic efficiency (Toney & Kirsch, 1987). Moreover, inspection of the model of PLP bound to the P-domain reveals extensive solvent exposure of the binding site (J. Jäger, personal communication). Increased coenzyme or substrate dissociation constants of artificially separated domains have been observed previously (Parmeggiani et al., 1987; Minard et al., 1989).

The monomeric state of the P-domain is somewhat unexpected because the major subunit–subunit interface at the 2-fold symmetry axis remains unchanged. Inspection of the eAAT crystal structure reveals that the missing N-terminal extension contains two residues making intimate contacts with the second subunit, namely F_6 and I_9. Additionally, the N-terminal amino group of M_5 (numbering as for pig cytosolic AAT) contributes a salt bridge to E_{249}*. By contrast, the C-terminal extension makes no direct intersubunit contacts in eAAT. These results underline the importance of the hydrophobic contacts of the N-terminal extension for dimerization of eAAT. Sandmeier and Christen (1980) have reported enhanced dissociation of chicken mAAT upon removing the N-terminal residues 1–27 proteolytically. Limited proteolysis during the reconstitution of lactate dehydrogenase removed about 10 N-terminal residues, which led to a dimeric protein that was unable to associate further to tetramers (Girg et al., 1981; Opitz et al., 1987). Nevertheless, we cannot rule out that structural changes at the subunit interface contribute toward weakening the dimer formation of the P-domain.

The protein can be refolded in vitro with a yield of more than 90% up to a concentration of at least 0.5 μM. The equilibrium unfolding of P-domain is at least a three-state process but is less complex than the equilibrium unfolding of eAAT. The dissociation and unfolding of dimeric eAAT can be described formally by the mechanism (Herold & Kirschner, 1990)

$$D \rightleftarrows 2M \rightleftarrows 2M^* \rightleftarrows 2U$$

where D is the native dimer, M the native monomer, M* a compact intermediate, and U the unfolded monomer. The unfolding of the monomeric P-domain (M′) can be explained by an analogous sequence of steps but with the dimer dissociation omitted:

$$M' \rightleftarrows M'^* \rightleftarrows U'$$

where M′* is the compact intermediate and U′ the unfolded state. The comparison of the two unfolding systems is most direct at the level of gel filtration. In both cases the M to M* transition occurs with an increase of elution volume, although in the case of eAAT this transition overlaps the dissociation of the dimer.

Judged by the loss of bound PLP (Figure 8A), the M′ $\rightleftarrows$ M′* transition of the P-domain occurs at 0.22 M GuCl, which is lower by about 0.5 M than the M $\rightleftarrows$ M* transition of eAAT. The latter was monitored by an increase of tryptophan fluorescence [see Figure 2 of Herold and Kirschner (1990)]. Similarly, the normalized M′* $\rightleftarrows$ U′ transition occurs at a concentration of GuCl that is lower by about 0.5 M than the M* $\rightleftarrows$ U transition (Figure 8B). The difference in stability in the M to M* transition is probably due to the additional stabilization of the native structure of M by the small domain that is missing in the P-domain. The shift of the M* to U transition toward higher GuCl concentration could be due to the larger number of hydrophobic residues of the longer polypeptide chain of eAAT that form the core of the compact intermediate (Kuwajima, 1989; Kim & Baldwin, 1990).

Is the isolated P-domain the minimum stable PLP binding structure? Inspection of the eAAT crystal structure reveals that the N- and C-terminal helices of the P-domain (corresponding to helices 2 and 13 of eAAT) are not essential for coenzyme binding. Both helices closely interact with each other but interact more loosely with the remainder of the domain. Thus both helices could probably be eliminated from the P-domain without loss of stability and capacity to bind PLP. The residual domain lacking these helices would be an $\alpha_2(\beta\alpha)_5\beta_2\alpha_2$ protein. These considerations are important with regard to the possible role of the PLP-binding domain in the evolution of PLP-dependent enzymes. Since both ω-amino acid:pyruvate aminotransferase and phosphoserine aminotransferase carry a PLP-binding domain that is topologically very similar to the P-domain of eAAT, it is possible that it has played the role of an intrinsically stable module of structure and partial function in the evolution of several nonhomologous PLP-dependent enzymes.

Acknowledgments

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