Volume 308, number 1, 26–29 FEBS 11391 August 1992

Coenzyme binding of a folding intermediate of aspartate aminotransferase detected by HPLC fluorescence measurements

Marzell Herold and Bernd Leistler*

Hewlett-Packard GmbH, Waldbronn Analytical Division, PO Box 1280, W-7517 Waldbronn 2, Germany

Received 5 June 1992

Equilibrium dissociation and unfolding of dimeric aspartate aminotransferase from *Escherichia coli* proceeds via two compact monomeric intermediates which have similar hydrodynamic volumes but different fluorescence properties. We probed binding of the coenzyme pyridoxal 5'-phosphate to these intermediates by coupling fluorescence detection to size-exclusion HPLC. This procedure gave additionally an internal conformational probe of the unfolding transitions of the enzyme. It was shown that the first intermediate, M, is able to bind the coenzyme, whereas the second intermediate, M*, is not. It is likely that M is the correctly folded monomer of the protein.

Folding intermediate; Aspartate aminotransferase; HPLC

1. INTRODUCTION

There is good evidence that in vitro re-folding and re-association of oligomeric proteins generally proceeds via conformational states intermediate between those of the native and the fully denatured protein [1]. Due to autonomous folding of protein domains, intermediates of multi-domain proteins might contain one folded domain, whereas the rest of the protein is unfolded [2]. Moreover, intermediates with properties described by the 'molten globule' model [3,4], or of a 'collapsed state' [5] appear to be common amongst most proteins.

Aspartate aminotransferase from *Escherichia coli* (eAAT) is a homodimer with a relative molecular mass of 87,146. The coenzyme pyridoxal 5'-phosphate (PLP) is bound covalently via a Schiff base to K258 of its coenzyme binding domain. This domain (275 amino acids) makes up the largest part of the protein monomer (396 amino acids). The guanidinium chloride (GuCl)-dependent equilibrium mechanism of dissociation and unfolding of eAAT [6,7] and of the excised coenzyme binding domain (P-domain) [8] have been studied in detail. The formal pathway of folding is represented in the following scheme:

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

where D is the native dimer, M and M* are globular monomeric folding intermediates, and U is the unfolded chain. M* has many properties of the molten globule state, whereas it was proposed that M is the folded monomer of eAAT [6,9]. Furthermore, the studies of the P-domain [8] suggested that M contains the correctly folded coenzyme binding domain.

While M* has been characterized in much detail, few data could be achieved for M by direct measurements, because M accumulates only at very low protein concentrations. Coenzyme binding is an important measure for the structural integrity of these folding intermediates. As a molten globule type of folding intermediate, M* is unable to bind PLP despite its compactness and its high degree of secondary structure [9]. However, no data on PLP binding to M have been achieved so far.

We investigated coenzyme binding to the equilibrium folding intermediates of eAAT using size-exclusion chromatography (SEC) on an HPLC system equipped with on-line fluorescence detection. PLP binding to eAAT can be probed by the large quenching of the protein fluorescence by bound PLP. Dissociation and unfolding of eAAT have already been monitored by earlier SEC-HPLC studies [7]. Our approach couples the great sensitivity of fluorescence detection to simultaneous monitoring of the change of the Stokes' radius by SEC. Thus, change of binding capacity and conformational stability upon unfolding could be tested in a single experiment, and the large margin of error of two

Abbreviations: eAAT, aspartate aminotransferase (EC 2.6.1.1) from *Escherichia coli*; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; GuCl, guanidinium chloride; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethansulphonic acid; P-domain, excised coenzyme binding domain of eAAT; PLP, pyridoxal 5'-phosphate; SEC, size-exclusion chromatography.

* *Present address:* University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, UK.

Correspondence address: B. Leistler, Hewlett-Packard GmbH, Waldbronn Analytical Division, PO Box 1280, W-7517 Waldbronn 2, Germany. Fax: (49) (7243) 602 212.

Published by Elsevier Science Publishers B.V.

separate experiments at low protein concentration could be largely avoided. Our results suggest that PLP dissociates during the M ⇌ M* transition, supporting the previous view that M is in fact the correctly folded monomer of cAAT and still able to bind the coenzyme.

2. EXPERIMENTAL

All reagents of purest available grade were purchased from Fluka (Buchs, Switzerland). Water was purified by filtration through an ion exchange system. Holo- and apo-cAAT were isolated and prepared as described previously [6].

All experiments were carried out on a Hewlett-Packard (HP) 1050 Ti Series quarternary pump. A gradient pump was used to change the GuCl concentration automatically by mixing two different solvents. The samples were injected with an HP 1050 Ti Series autosampler, and the signals were recorded with a HP 1050 Series multiple wavelength detector and a HP 1046A fluorescence detector. An HP HPLC Chem-Station (DOS Series) operating under Windows 3.0 was used for data acquisition and calculation. The column used was a TSK 3000 SWLX (300 × 7.5 mm) packed with 5 μm particles (Tosoh Haas, Yamaguchi, Japan).

Fluorescence emission spectra were measured with a Perkin Elmer 650-10S fluorimeter equipped with a thermostated cuvette holder. Measurements were made at 25°C in 1-cm path-length cuvettes. Fluorescence was excited at 280 nm, and the slit widths were set to 2 nm on both sides. Protein concentration was 0.9 μM in buffer A (see below) without NaCl.

cAAT was stored in the following buffer: 10 mM HEPES at pH 7.4, 5 mM DTE, 1 mM EDTA. Buffer A used for the SEC was 10 mM HEPES at pH 7.0, 1 mM DTE, 1 mM EDTA, 300 mM NaCl. NaCl was added to suppress unwanted electrostatic interaction between the enzyme and the column matrix [10]. Buffer B was the same as buffer A, but contained additionally 6 M GuCl. All chromatographic experiments were performed at 23 ± 2°C at a flow rate of 0.5 ml/min. The experimental procedure was automated by increasing the GuCl concentration in steps every 50 min by appropriate mixing of buffer A and B with automated injection of apo-cAAT 20 min after changing the percentage of B. Each chromatogram was measured at least twice. The average deviation of the elution volumes was below 1%. The absorbance of the eluate was monitored at 230 and 280 nm, band width 8 nm, and a reference wavelength of 550 nm, band width 100 nm. The fluorescence was detected at 335 nm (excitation 280 nm, cut-off filter 295 nm, PMT gain 10). The normalized fluorescence was calculated from the ratio of fluorescence-to-UV signal at each GuCl concentration. The fluorescence of native apo-cAAT (0 M GuCl) was arbitrarily set to 1.0. The elution time profiles were normalized by extrapolating the linear change of the elution volume above 4.8 M GuCl to zero GuCl concentration. The normalized elution volume is the apparent deviation from this base line.

3. RESULTS AND DISCUSSION

3.1. *Unfolding of apo-cAAT*

We investigated the unfolding of apo-cAAT as a reference for the PLP binding studies. Dissociation and unfolding of apo-cAAT at equilibrium with GuCl have been investigated previously in separate fluorescence [6] and SEC-HPLC [7] studies.

In each analysis 2 μg (10 μl) were injected onto the column which was equilibrated with different concentrations of GuCl. The chromatograms of apo-cAAT at increasing concentrations of GuCl are shown in Fig. 1. The change of the hydrodynamic volume or Stokes' radius with GuCl concentration is very similar to that reported from the previous experiments [7] under the same conditions. Moreover, the transition profile obtained by following the change of protein fluorescence (Fig. 2) coincides well with that obtained from separate test tube experiments [6]. The apparently biphasic transition of the hydrodynamic volume together with the non-coincidence of the fluorescence transition between

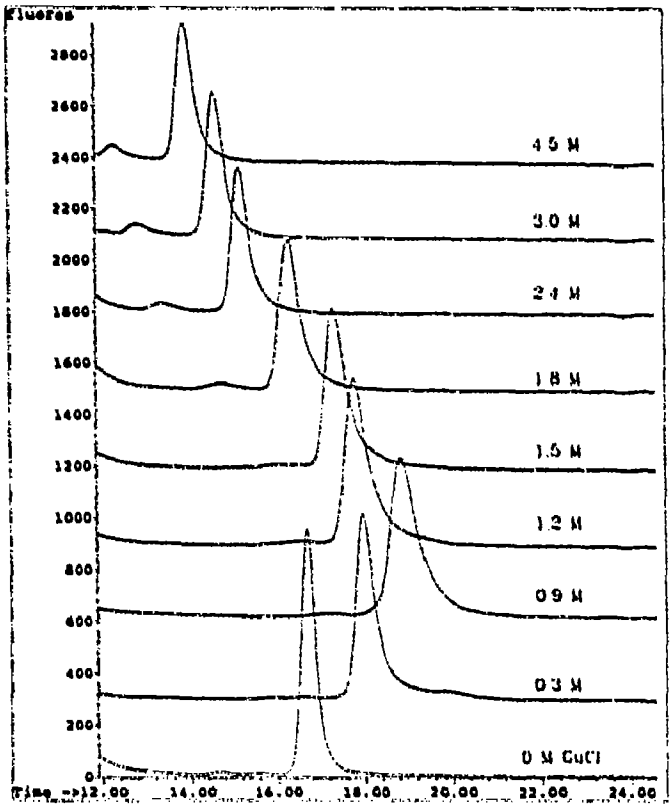

Fig. 1. Elution profile of 10 μl (2 μg) apo-cAAT at the indicated GuCl concentrations. Flow rate 0.5 ml/min, fluorescence detection at 335 nm (excitation 280 nm).

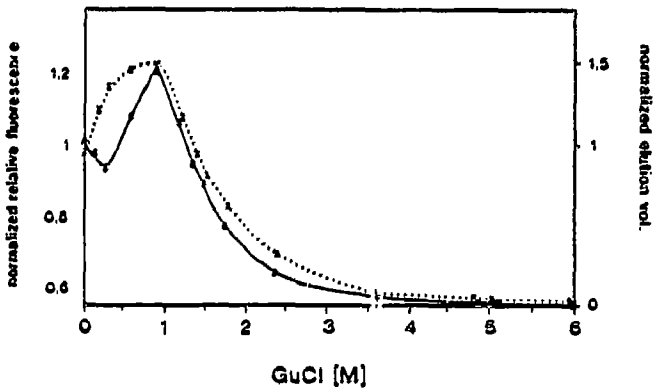

Fig. 2. Normalized elution volume (×) and normalized relative fluorescence (●) of apo-cAAT in Fig. 1 as a function of GuCl concentration.

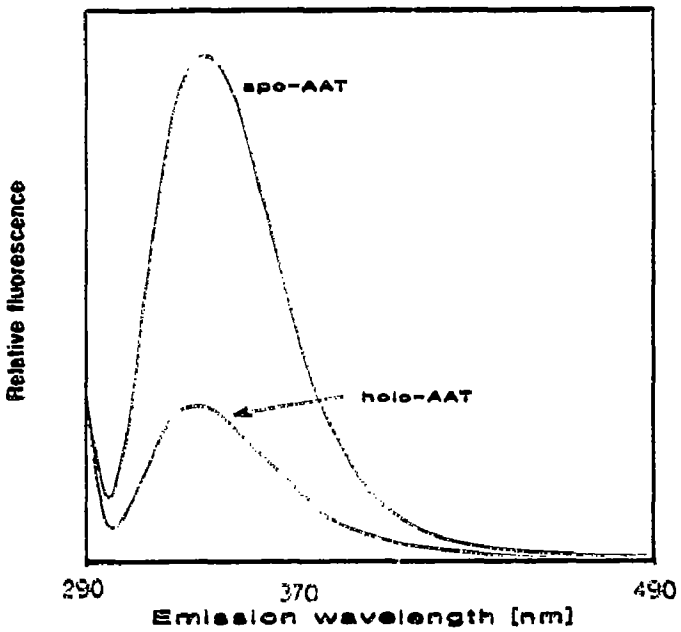

Fig. 3. Binding of PLP quenches the fluorescence of cAAT. Protein concentration 0.9 μM. Excitation wavelength 280 nm.

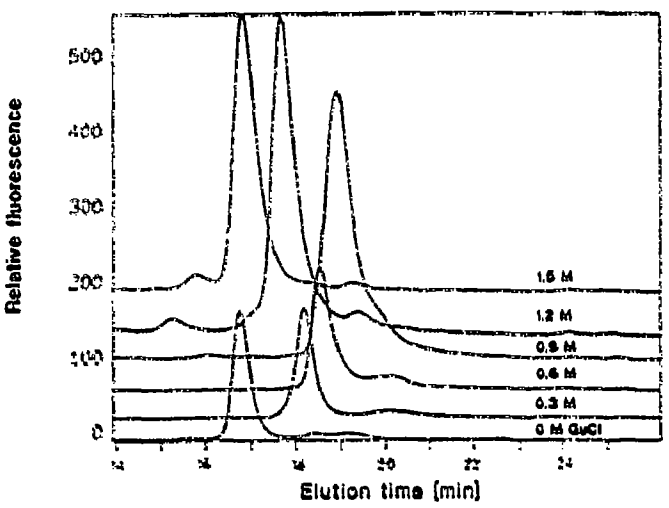

Fig. 4. Elution profiles of 10 μl (0.7 μg) pre-equilibrated holo-cAAT at the indicated GuCl concentrations. Flow rate 0.5 ml/min, fluorescence detection at 335 nm (excitation 280 nm).

0 and 1 M GuCl (Fig. 2) demonstrates the existence of the two compact monomeric intermediates, M and M*.

3.2. *PLP binding to monomeric cAAT*

Fig. 3 shows the fluorescence spectra of holo- and apo-cAAT in buffer A. As the fluorescence of cAAT is strongly quenched by bound PLP, this signal difference can be used to monitor the loss of PLP during unfolding of holo-cAAT. The fluorescence of PLP is only marginal under these conditions.

PLP is bound covalently to apo-cAAT by a hydrolyzable Schiff base. To ensure that the equilibrium of unfolding and of binding is attained prior to chromatography, 0.07 mg/ml holo-cAAT was incubated in buffer A containing different GuCl concentrations for 16 h at 4°C. Under these conditions, the folding intermediates are stable at equilibrium [6]. No precipitation of protein was observed after incubation. 10 μl (0.7 μg) of each pre-incubated sample was injected onto the column equilibrated with the same GuCl concentration.

The elution time profiles (Fig. 4) were very similar to those of apo-cAAT under identical conditions, indicating the presence of compact monomeric species between 0.3 and 1.0 M GuCl. The relative fluorescence, however, was constant between 0 and 0.6 M GuCl, and makes up one third of native apo-cAAT (Fig. 4). It increased with a transition around 0.75 M GuCl to the same value observed for apo-cAAT (Fig. 2). These data suggest that the protein fluorescence is quenched by bound PLP between 0 and 0.6 M GuCl. Taken together, these results show that between 0.3 and 0.6 M GuCl a largely monomeric species is present whose fluorescence is quenched as that of the native dimer. We therefore claim that PLP is lost during the M ⇌ M* transition (i.e. between 0.6 and 0.9 M GuCl), and that the intermediate, M, is able to bind PLP. M* does not bind the coenzyme, which has been demonstrated earlier [9].

The unaltered fluorescence of M furthermore suggests that a substantial part of the monomer, at least the coenzyme-binding domain, which makes up the largest part of the protein monomer and contains most of the chromophores, is in the native conformation. This interpretation is consistent with the previous finding that the coenzyme binding domain is stable as a monomer and still capable of a strong and specific binding of PLP [8]. Similar to the findings in the present study, the P-domain loses PLP during a transition of the folded monomer into a compact intermediate conformation. One may speculate whether cAAT has been derived from a monomeric ancestor, or probably from a monomeric

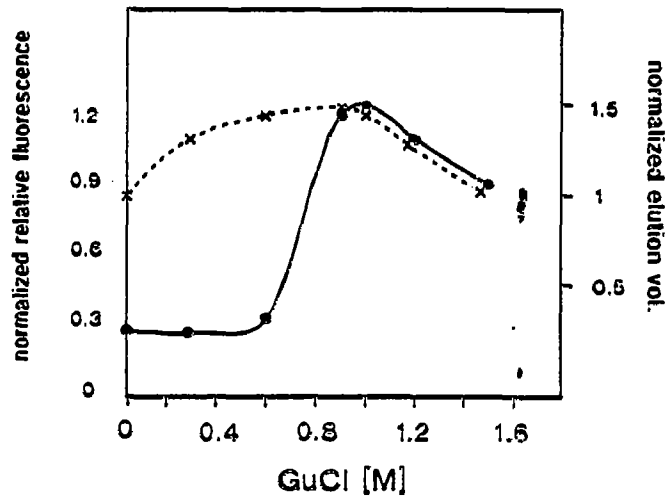

Fig. 5. Normalized elution volume (×) and normalized relative fluorescence (●) of holo-cAAT in Fig. 4 as a function of GuCl concentration.

PLP-binding domain, from which also other aminotransferases have been derived.

4. CONCLUSION

By means of SEC-HPLC with coupled fluorescence and absorbance detection we were able to investigate the binding of the coenzyme PLP to the monomeric folding intermediates, M and M*, of eAAT. As M only accumulates at very low protein concentrations [6], it was important to have a sensitive method, and a separate check of the monomeric state of the enzyme. The sensitive fluorescence detection demonstrated clearly specific binding of PLP to M, whereas the eluted M* was completely free of PLP.

Acknowledgements: We thank Cornelia Stoppkotte for experimental support, and Prof. Kasper Kirschner for critical comments. The work of B.L. was supported by the Swiss National Science Foundation, Grant 31.25711.88.

REFERENCES

[1] Jaenicke, R. (1991) Biochemistry 30, 3147–3160.
[2] Rudolph, R., Siebendritt, R., Nesslauer, G., Sharma, A.K. and Jaenicke, R. (1990) Proc. Natl. Acad. Sci. USA 87, 4625–4629.
[3] Ptitsyn, O.B. (1987) J. Prot. Chem. 6, 273–293.
[4] Kuwajima, K. (1989) Proteins 6, 87–103.
[5] Kim, P.S. and Baldwin, R.L. (1990) Annu. Rev. Biochem. 59, 631–660.
[6] Herold, M. and Kirschner, K. (1990) Biochemistry 29, 1907–1913.
[7] Herold, M. and Leistler, B. (1991) J. Chromatogr. 539, 383–391.
[8] Herold, M., Leistler, B., Huge, A., Luger, K. and Kirschner, K. (1991) Biochemistry 30, 3612–3620.
[9] Leistler, B., Herold, M. and Kirschner, K. (1992) Eur. J. Biochem. 205, 603–611.
[10] Kopaciewic, M. and Regnier, F.E. (1982) Anal. Biochem. 126, 8–16.