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Unfolding of truncated and wild type aspartate aminotransferase studied by size-exclusion chromatography

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ABSTRACT

The reversible unfolding of globular proteins with increasing concentration of guanidinium chloride (GuCl) can be analysed by size-exclusion chromatography, because the hydrodynamic volume of the proteins increases during unfolding. The dimeric enzyme aspartate aminotransferase (AAT) shows an uncoupled dissociation of the identical subunits followed by the unfolding of the monomers. During the monomer unfolding formation of an intermediate is observed. A monomeric mutant of AAT unfolds with a similar shape of the unfolding transition phase, but is less stable, as shown by a shift of the transition mid-point from 1.7 *M* GuCl for the wild type to 1.3 *M* GuCl for the mutant.

INTRODUCTION

It has been demonstrated for many globular proteins that reversible unfolding can be studied by size-exclusion chromatography (SEC). Fundamental work in this field [1,2] showed that the guanidinium chloride (GuCl)-dependent unfolding transition monitored by SEC is the same as the unfolding transition observed by column-independent spectroscopic methods. The hydrodynamic radius, *i.e.*, the Stoke's radius, of proteins clearly increases during denaturation by chaotropic salts such as urea or GuCl. In this study, GuCl was used because it is more chaotropic and comparison with previous GuCl-dependent unfolding studies is possible.

High-performance liquid chromatography (HPLC) is a rapid and powerful method for comparing the conformational stability of site-directed mutants with that of the wild type proteins. Isoenzymes from different organisms can also be easily compared with this method. Moreover, the refolding conditions of proteins which precipitate in "inclusion bodies" during overexpression in the cell can be analysed. Recently, many refolding studies have shown (for a review, see ref. 3) that most globular proteins can be refolded *in vitro*, which is a prerequisite for reproducible stability studies.

The enzyme AAT (aspartate aminotransferase, E.C. 2.6.1.1) from *Escherichia*

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coli has a relative molecular mass of 87 146 (sequence data). It is a dimer consisting of two identical subunits. The crystal structure of the enzyme is known [4,5]. AAT catalyses the reversible transamination of L-amino acids into the corresponding α-keto acids by its covalently bound cofactor pyridoxal 5'-phosphate (PLP). AAT can be reversibly unfolded with GuCl up to protein concentrations of 1 mg/ml [6]. The uncoupled dissociation and unfolding of the dimeric AAT can be analysed by SEC, which is difficult to do by conventional methods. Each subunit of AAT consists of two domains containing no S–S bridges. It has a p*I* value of 4.6. The large domain of AAT, the PLP binding domain (P-domain) [7], was constructed and isolated by site-directed mutagenesis [8]. The P-domain consists of 283 amino acids and has a molecular mass of 32,367 dalton (sequence data). In this paper we compare the stability and GuCl-dependent unfolding of the isolated P-domain with wild type AAT.

EXPERIMENTAL

Materials

All reagents of the purest available grade were purchased from Fluka (Buchs, Switzerland). Water was purified by filtration through an ion-exchange system.

AAT and the mutated protein called "P-domain" were isolated as described previously [6]. The proteins used for column calibration were purchased from Sigma (St. Louis, MO, U.S.A.).

Instrumentation and columns

All experiments were carried out on a Hewlett-Packard (HP) 1050 Ti Series quaternary HPLC pump. To avoid crystallization of salt on the pump pistons, the seal wash option was used with filtered water. A gradient pump was necessary so that the GuCl concentration could be changed automatically by mixing two different solvents. The samples were injected with an HP 1050 Ti Series autosampler and the signals were measured with an HP 1050 Series multiple wavelength detector. The data were transferred from the detector via an HP Series 35900 analog–digital converter to an HP HPLC ChemStation Series 9000. The column used was a TSK 3000 SWXL (300 × 7.5 mm I.D.) packed with 5-μm particles (Tosoh, Yamaguchi, Japan).

Buffers and chromatographic conditions

AAT and P-domain were stored at concentrations of 1.3 and 0.7 mg/ml, respectively, in the following buffer: 10 m*M* HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid] (pH 7.4)–5 m*M* DTE (dithioerythritol)–1 m*M* EDTA (ethylenediaminetetraacetate)–10 μ*M* PLP (100 μ*M* PLP for the P-domain)–1 m*M* 2-oxoglutarate. PLP was added to ensure that the enzymes were always saturated with the coenzyme. The size-exclusion column was calibrated with the following proteins of known molecular mass: thyroglobulin (660 000), aldolase (160 000), AAT (87 000), bovine serum albumin (67 000), ovalbumin (45 000) and RNase A (13 700). The flow-rate was 0.5 ml/min.

Solvent A used for the calibration was 10 *M* HEPES (pH 7.0)–1 m*M* DTE–1 m*M* EDTA–300 m*M* NaCl. For the unfolding studies solvent B contained the same components plus 6 *M* GuCl. All experiments were carried out at room temperature (23 ± 2°C). 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.

Fluorescence unfolding studies

Fluorescence measurements were carried out with an SLM 8000 single-photon counting recording fluorimeter. For equilibrium studies the protein was preincubated in solvent A containing the different concentrations of GuCl for at least 3 h. The measurements were performed at 20°C. All solutions were sterilized by filtration directly before use. The measured protein fluorescence spectra were corrected for the small apparent fluorescence of the solutions of GuCl in solvent A. The shift of the protein fluorescence emission maximum (excitation at 280 nm) was monitored depending on the GuCl concentration.

RESULTS AND DISCUSSION

Unfolding of AAT

In each analysis, 26 μg (20 μl) of native AAT were injected onto a TSK 3000 SWXL column which was equilibrated with different GuCl concentrations by mixing solvents A and B. The procedure was automated with the following step gradient from 0 to 6 *M* GuCl: *X*% increase of solvent B every 50 min with automatic injection of protein 20 min after changing %B. Each chromatogram was measured at least three times. The relative standard deviation of the elution volume was 1%.

At 0 *M* GuCl AAT had a retention volume of 8.45 ml, which is consistent with an apparent molecular mass of 86 000. This demonstrates that AAT is in the dimer conformation. With increasing GuCl concentration the retention volume of the protein increased owing to the dissociation of AAT into monomers. The retention volume at 0.7 *M* GuCl was 9.3 ml (see Figs. 1 and 2) which gives an apparent molecular

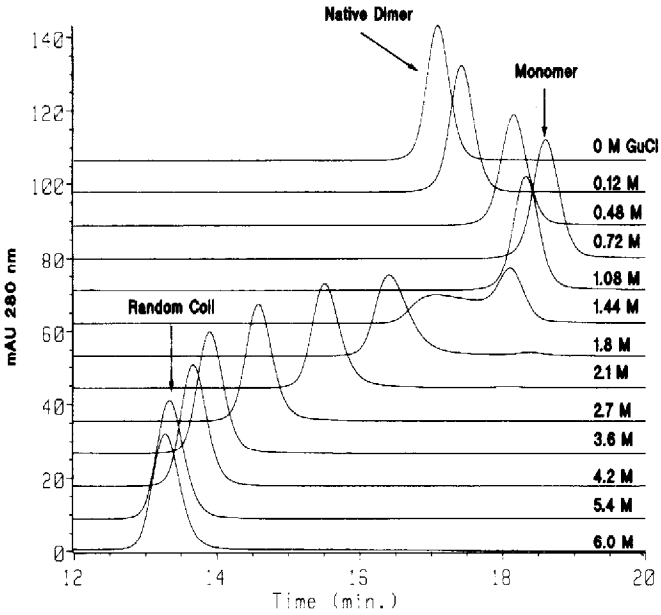

Fig. 1. Elution profiles of holo-AAT at the indicated concentrations of GuCl. Flow-rate, 0.5 ml/min; detection, 280 nm; injection, 20 μl (26 μg) automatically every 20 min after changing the concentration of B.

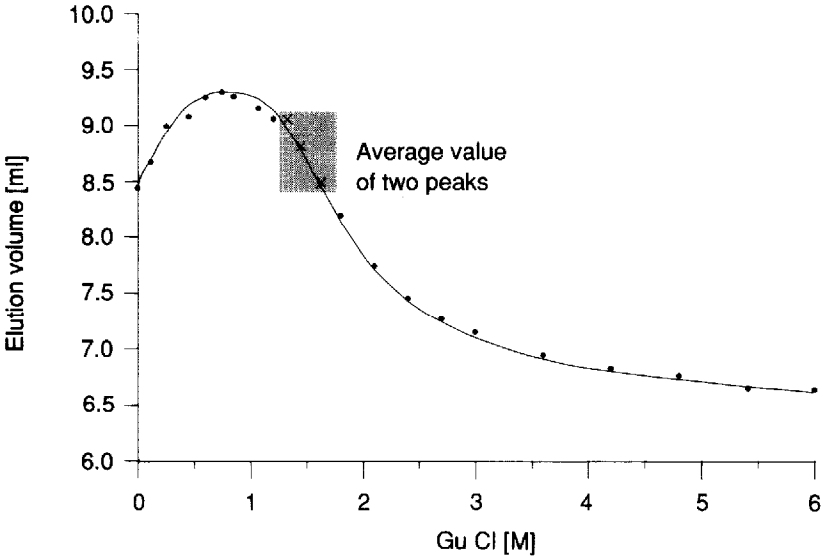

Fig. 2. Change in elution volume of holo-AAT as a function of GuCl concentration. ×, Average value of the retention volume and peak area if two peaks were observed.

mass of 48 000. The true molecular mass of the subunit is 43 573. The apparent molecular mass indicates that the subunits are in a globular conformation, although they are not as compact as in the dimer conformation. The dissociation of the enzyme at 0.7 *M* GuCl into monomers of slightly relaxed globular conformation has been confirmed independently by analytical ultracentrifugation [6].

Further increase in the GuCl concentration beyond 1.2 *M* led to a gradual decrease in elution volume, which is due to the increase in the hydrodynamic volume during the unfolding of the monomers. The transition phase between 1.2 and 3.5 *M* GuCl does not fit to a two-state process and has an overall transition mid-point at 1.7 *M* GuCl (Fig. 2). At concentrations higher than 3.5 *M* GuCl, the change in elution volume is almost linear, indicating that the protein is in the random coil state. The reason for the further decrease in the elution volume is not completely clear. Because it has been observed with all proteins studied so far [2,9,10], this effect seems to be due only to the high GuCl concentration. Even proteins being folded or unfolded over the whole concentration range of GuCl and the small molecule vitamin B_{12} displayed a continuous small decrease in the elution volume with increasing GuCl concentration [9]. Therefore, it is evident that the linear part of the elution volume decrease is not due to a conformational change of the protein. The increase in the GuCl concentration results in an increase in viscosity and might inhibit the diffusion of the molecules into the pores. Shalongo *et al.* [9] assumed a short-time binding of the protein to the column matrix which is diminished with increasing denaturant concentrations. A Superose 12 column (Pharmacia) also showed this effect [6].

In the first part of the monomer unfolding transition, two peaks at 1.44 *M* GuCl were observed simultaneously (see Fig. 1), which indicates the transiently accumulated "structured monomers". The GuCl concentration range with two peaks observed at

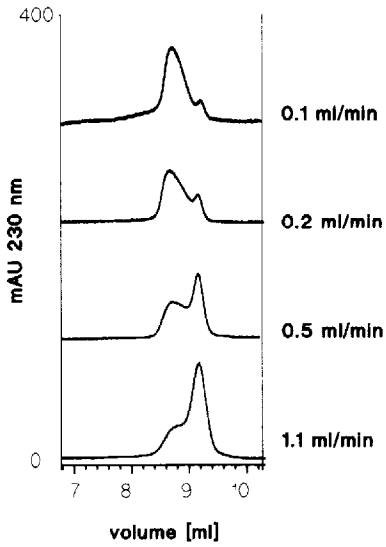

Fig. 3. Elution profiles of AAT in 1.44 *M* GuCl at four flow-rates.

a flow-rate of 0.5 ml/min is shadowed in the unfolding curve in Fig. 2. The formation of the intermediate is relatively slow at room temperature; during the retention time of 18 min only about 50% intermediate is formed. In order to obtain more information about the rate of the intermediate formation, the runs at 1.44 *M* GuCl were repeated with different flow-rates. At the fastest flow-rate of 1.1 ml/min, a small amount of intermediate is formed (*ca.* 15%). In contrast, at the very slow flow-rate of 0.1 ml/min the intermediate is formed to the extent of 90% during 87 min (see Fig. 3). The data were used for a rough estimation of the rate constant of intermediate formation, which gave a value of about $1.1 \cdot 10^{-3}$ s^{-1}.

This strategy can always be used to determine the rate constants of a change of protein conformation provided that the species with different conformations can be separated on the column and that the process is in the time range of the retention time of the column. Generally this requires HPLC size-exclusion columns with a high pressure resistance to allow short retention times and a high separation performance, which is the case with the TSK 3000 SWXL column. A further possibility of detecting folding intermediates is to slow the rate of intermediate formation by decreasing the column temperature.

Unfolding of the P-domain

The conformational stability of mutant proteins compared with the wild type is always of great interest. The AAT subunit consists of two domains. The larger one, the coenzyme binding domain, is compactly folded [7] and binds the coenzyme PLP even in the absence of the truncated small domain [8]. Two questions are of interest concerning the isolated mutated protein: first, is the isolated P-domain still a dimer, and second, how stable is the P-domain compared with the wild type?

The retention volume of 9.6 ml (Fig. 4) clearly indicates that the P-domain is

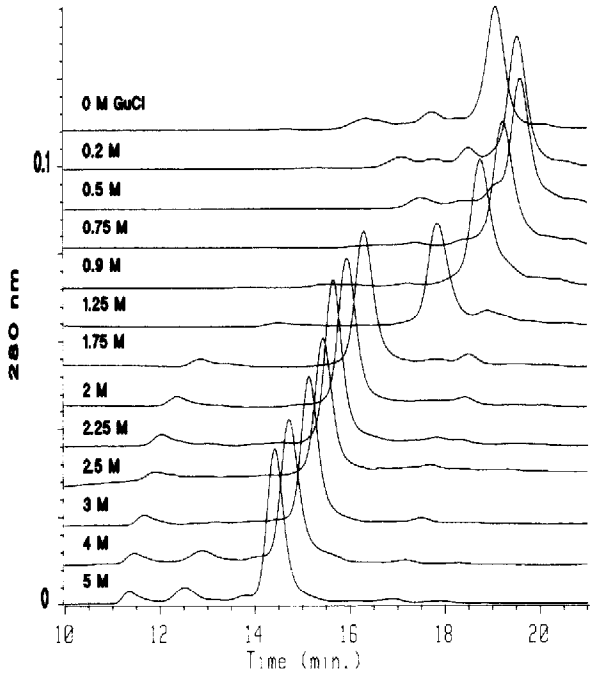

Fig. 4. Elution profiles of P-domain at the indicated concentrations of GuCl. Flow-rate, 0.5 ml/min; detection, 280 nm; injection, 20 μl (14 μg) automatically every 20 min after changing the concentration of GuCl in solvent B.

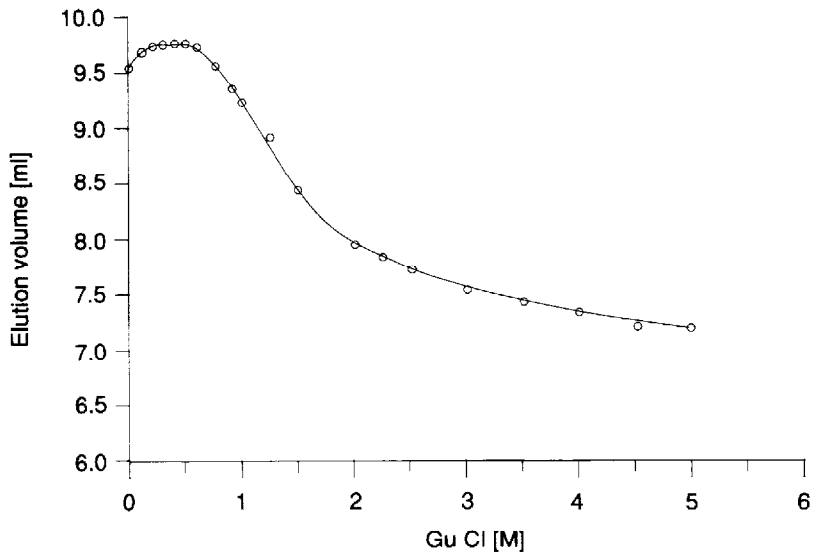

Fig. 5. Change in elution volume of P-domain as a function of GuCl concentration.

monomeric. The apparent molecular mass is 33 500 dalton, which is close to the true value of 32 367 dalton (sequence data). The retention volume of the P-domain increases slightly on adding 0.3 *M* GuCl (see Figs. 4 and 5), suggesting increasing compactness. It is known that the salt concentration in the elution buffer can influence the elution volume of proteins owing to the change in their "viscosity radius" [11]. The explanation given [11] was that the surrounding counter-ion layer of proteins in buffer becomes negligibly small at high ionic strength. Therefore, the elution volume of many proteins increases slightly with increasing salt concentration.

To evaluate the influence of the salt concentration in solvent A, the unfolding experiment shown in Fig. 5 was repeated in the presence of 0.1 *M* instead of 0.3 *M* NaCl (data not shown). Also under these conditions the elution volume increases up to 0.3 *M* GuCl and then decreases with the same unfolding transition phase starting at concentrations higher than 0.5 *M* GuCl. Unfolding was completed at about 3.5 *M* GuCl. The transition mid-point was at 1.3 *M* GuCl, as shown by the normalized curve in Fig. 6. The curve shows the relative change in elution volume; the conformation-independent decrease in the elution volume due to the increase in the salt was subtracted graphically (see als legend of Fig. 6). The measurements repeated at a flow-rate of 0.9 ml/min gave the same transition curve, demonstrating that the experiments were carried out at the thermodynamic equilibrium.

To rule out a column dependent influence on the unfolding curve, the unfolding was additionally monitored by the GuCl-dependent change of the protein fluorescence emission maximum (Fig. 6). The red shift of the fluorescence emission maximum monitors the exposure of the buried tryptophan residues to the solvent during the unfolding of the proteins [12]. Both methods displayed an identical unfolding transition mid-point, whereas the fluorescence method was not able to detect the first conformational change of the protein. This is a further example of the accurate determination of the unfolding transition of a protein by SEC.

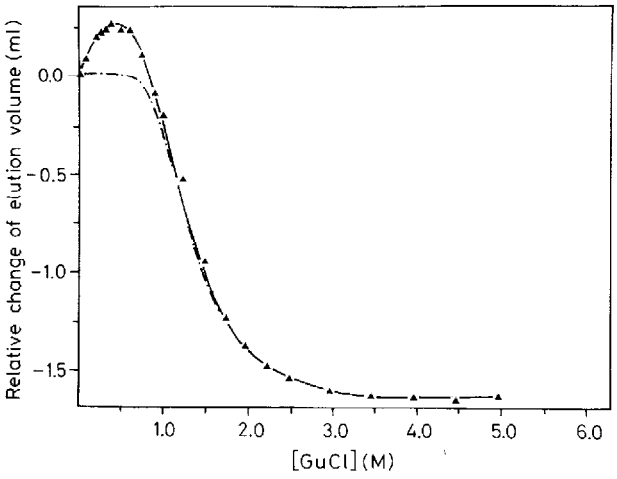

Fig. 6. Normalized unfolding curve of the P-domain. The curve was normalized by extrapolating the linear change in elution volume above 3.5 *M* GuCl to zero GuCl concentration. The relative change in elution volume is the apparent deviation from this baseline. Normalized change in elution volume (▲) compared with the change of the fluorescence emission maximum (– · –, excitation at 280 nm).

Comparison of the stability of AAT and P-domain

The unfolding transition curve measured in the presence of increasing concentrations of a strong denaturant aspect is a measure of the conformational stability of the protein [13]. The unfolding curves revealed by GuCl-dependent SEC can therefore be used to compare the stabilities of related proteins. The P-domain is monomeric under all conditions tested. The small increase in the elution volume of the P-domain at concentrations up to 0.3 *M* GuCl may be due to a conformational change of the protein. This effect has to be confirmed by an independent method. The main unfolding phase is characterized by a strong increase in the hydrodynamic radius between 0.5 and 3.5 *M* GuCl (see Figs. 5 and 6). The transition phase does not fit to a two-phase process, indicating that unfolding of the P-domain is not a simple two-state mechanism. The shape of the unfolding curve looks very similar to the second phase of the unfolding of wild type AAT (the unfolding of the monomers; compare Figs. 2 and 5), indicating that the unfolding of AAT monomers is mainly due to the unfolding of the large PLP-binding domain. The main difference is the shift of the overall transition mid-point from 1.7 *M* (AAT) to 1.3 *M* GuCl (P-domain), demonstrating the lower stability of the isolated P-domain.

Both unfolding curves were confirmed with column-independent spectroscopic methods (ref. 6 and Fig. 6), demonstrating that the SEC unfolding curves show the equilibrium state.

CONCLUSIONS

Protein folding studies with SEC are fast and easy if automated inject and mixing of two solvents are available. Thermodynamic studies do not depend on the type of SEC column. However, for kinetic studies, a column with a high pressure resistance and low elution volume is recommended to facilitate the resolution of time-dependent processes. The following properties of the HPLC system are prerequisites for reproducible results: (i) the pump should work reliably at low pressure and with viscous solutions; (ii) the system should be resistant to corrosion for maximum reliability with very high salt concentrations; a seal washing feature is recommended; and (iii) a gradient system is necessary to allow precise automatic changes of the salt concentration.

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