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High-performance gel-permeation chromatographic analysis of protein aggregation

Application to bovine carbonic anhydrase

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ABSTRACT

Protein association in bovine carbonic anhydrase (bCA) was investigated by high-performance gel-permeation chromatography (HPGPC). It was shown that bCA undergoes a time-dependent aggregation after freezing and storing at −20°C. The addition of 50% ethylene glycol or glycerol prevented the aggregation. The HPGPC data were confirmed by the use of cross-linking with subsequent sodium dodecyl sulphate–polyacrylamide gel electrophoretic analysis. The esterase activity of the aggregated bCA was comparable to that of the monomer, suggesting that the observed protein association does not affect the esterase catalytic centre of bCA. This study illustrates how HPGPC can be a convenient and reliable tool for monitoring storage-induced aggregation of proteins.

INTRODUCTION

Many proteins show aggregation or changes in solubility under certain solvent conditions [1,2]. As aggregation can directly affect the chromatographic behaviour of proteins in high-performance liquid chromatography (HPLC), and possibly limit analysis and purification, a systematic analysis of protein–protein interactions is important to produce optimized separations in both analytical- and preparative-scale operation [3]. To date, chromatographic studies of protein aggregation have chiefly been performed by size exclusion [4,5], although hydrophobic interaction chromatography has recently been applied to the aggregation of β-lactoglobulin A [3].

A high-performance gel-permeation chromatographic (HPGPC) method with non-ionic detergent in the eluent has previously been re-

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ported to be a convenient, sensitive and high-yield technique for protein separation according to molecular mass [6]. In this study, an application of this HPGPC method to the analysis of protein aggregation by use of bovine carbonic anhydrase (bCA) was investigated.

EXPERIMENTAL

Chemicals and reagents

Carbonic anhydrase (carbonate dehydratase; carbonate hydro-lyase; EC 4.2.1.1) from bovine erythrocytes was obtained from two sources: Sigma carbonic anhydrase (Lot No. 115F94102) was from Sigma (S. Louis, MO, USA), and Boehringer Mannheim carboanhydrase (Lot No. 13026121-31) was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Dimethyl suberimidate dihydrochloride (DMS) was purchased from Sigma, veronal buffer from Whittaker Bioproducts (Walkersville, MD, USA), triacetin (glycerol triacetate), sodium acetate, anhydrous and bromothymol blue from Wako (Osaka, Japan) and Nonidet P-40 from Nacalai Tesque (Kyoto, Japan).

Determination of bCA by HPGPC

A Model 2150 HPLC pump with a Model 2152 LC controller (Pharmacia LKB, Uppsala, Sweden) was utilized. Sample injection was carried out with a U6K universal liquid chromatographic injector (Millipore, Milford, MA, USA) with a 2-ml loop. A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the column. The flow-rate was 0.4 ml/min with a column inlet pressure of *ca.* 40–50 kg/cm^2 when two JASCO Bio-Fine GFCSI150-K columns (300 × 7.9 mm I.D.) and a glass column (GlasPac Column TSK G 3000SW, 300 × 8 mm I.D.; Pharmacia LKB Biotechnology, Bromma, Sweden) were used. When using a column consisting of the usual type of stainless-steel tube (Shimpack Diol-300, 250 × 7.9 mm I.D.; Shimadzu, Kyoto, Japan), the flow-rate was 1.2 ml/min with a column inlet pressure of *ca.* 100–150 kg/cm^2. A sodium phosphate buffer (0.1 *M* NaH_2PO_4, pH 6.0) solution containing sodium chloride (0.3 *M*), glycerol (30%, v/v) and the non-ionic detergent Nonidet P-40 (NP-40) (0.15%, v/v) was utilized as the eluent. Detection of bCA was carried out with a UV detector (Model 655A-21; Hitachi, Tokyo, Japan) at 280–290 nm with a range of 0.64 absorption units full-scale (a.u.f.s.). A synthetic polymer gel column (TSK 4000 PW, 300 × 7.5 mm I.D.; Tosoh, Tokyo, Japan) was also tested.

Aggregation regime of bCA

A solution of 0.1 g/ml of bCA in 0.6 *M* sodium phosphate buffer (pH 6.0) containing 0.6 *M* sodium chloride, 2.5% (v/v) glycerol and 0.15% (v/v) NP-40 was stored in aliquots of 0.1 ml at −20°C for 12 h. Under the described conditions, a two-peak chromatogram was obtained by HPGPC.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the procedure of Laemmli [7] using 7.5% and 12% acrylamide concentrations. Proteins were detected by Coomassie Brilliant Blue staining.

Cross-linking analysis

To confirm aggregation, DMS was used as a cross-linking agent at a final concentration of 1 mg/ml according to the procedure of Hitchcock [8]. After reaction for 30 min, samples were analysed by SDS-PAGE. Acrylamide concentrations of 7.5% and 12% were used.

Assay of carbonic anhydrase and esterase activity

The carbon dioxide hydration activity was assayed according to the procedure of Armstrong *et al.* [9]. The esterase activity of bCA was characterized by the hydrolysis of the ester triacetin. The reaction was monitored by the liberation of acetic acid. This product was measured by salting-out reversed-phase HPLC, as described previously [10], except that acetate was detected by UV absorption at 210 nm. Esterase activities of both aggregated and monomeric bCA were assayed. Specific activity was expressed as picomoles of acetate liberated per minute per milligram of protein.

Protein content

Protein concentrations were assayed by using a BCA protein assay kit from Pierce (Rockford, IL, USA). BSA was used as a standard protein.

Statistical analysis

The statistical evaluation of the difference in esterase activity between aggregated and monomeric bCA was carried out by Wilcoxon's test. p values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

A high-recovery protein separation system containing a non-ionic detergent (NP-40) and 2.5% (v/v) glycerol has been developed previously [6]. However, the bCA peak was asymmetric under the conditions originally described. Sufficient peak symmetry could be obtained only by using high glycerol concentrations (30%, v/v) (Fig. 1), and no further improvement in resolution was achieved by changing the phosphate (0.05, 0.15 *M*), chloride (0.2, 0.4 *M*) or NP-40 (0.1, 0.2%, v/v) concentrations or by adding 0.1 *M* sodium sulphate.

A two-peak chromatogram was observed after freezing and storage of bCA (0.1 g/ml) in a glycerol–phosphate–chloride solution at −20°C for 12 h (Fig. 2B). A two-peak chromatogram was also observed using a Tosoh TSK 4000 PW column (data not shown). As under the same conditions similar chromatographic patterns with both silica and synthetic polymer gel columns were observed, it seems unlikely that mechanisms other than gel permeation play a role in this chromatographic separation.

Reproducible bCA aggregation was obtained under the following incubation conditions: solution composition 0.1 g/ml bCA, 0.6 *M* NaH_2PO_4, 0.6 *M* NaCl, 0.15% (v/v) NP-40 and 2.5% (v/v) glycerol, storage temperature −20°C and storage time 12 h. A time course analysis was also carried out: the percentage of bCA multimers, as assessed by the aggregated/monomer peak-height ratio, increased during the first 3 h, before reaching a peak-height plateau rate

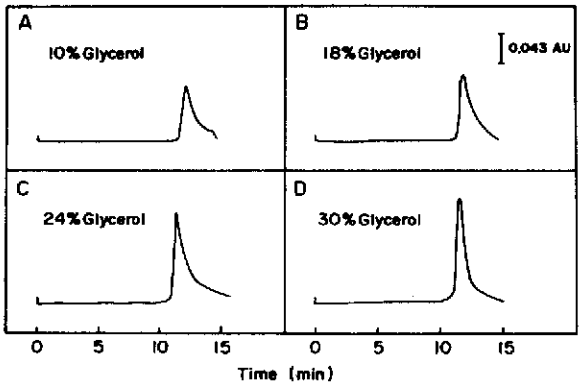

Fig. 1. Effect of different glycerol concentrations in the eluent on the peak shape of bCA chromatogram. A TSK G 3000 SW column (300 × 8 mm I.D.) was used. The eluent conditions except glycerol concentration were as described under Experimental. bCA (0.1 mg) was injected into the HPGPC system. Glycerol concentration: (A) 10; (B) 18; (C) 24; (D) 30% (v/v).

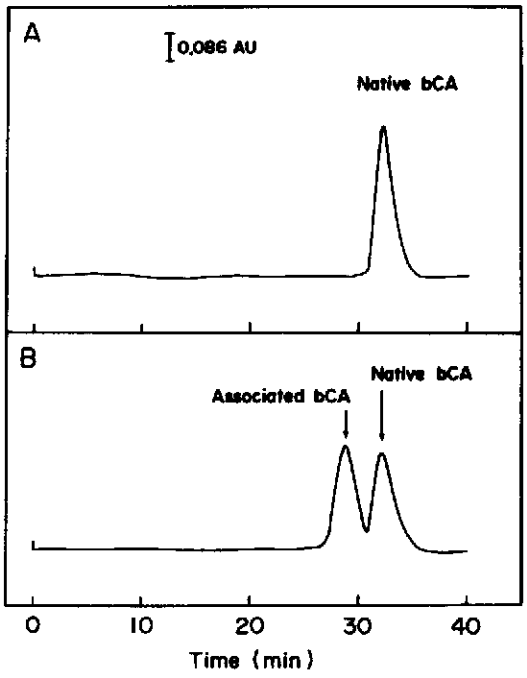

Fig. 2. Separation of aggregated bCA and bCA monomer. A three-column system (as described previously [6]) was used: a TSK G 3000 SW column (300 × 8 mm I.D.) and two JASCO Bio-Fine GFC SI 150-K columns (300 × 7.9 mm I.D.) were joined in series. Other conditions were as described under Experimental. Frozen-stored and freshly dissolved samples of bCA (0.5 mg) were injected into the HPGPC system.

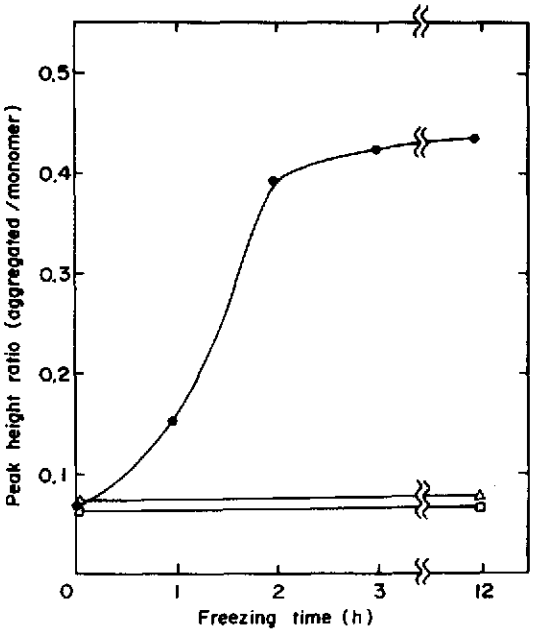

Fig. 3. Time course analysis of aggregation in bCA and the preventive effect of glycerol and ethylene glycol on the formation of bCA multimer species. Protein aggregation was analysed with the Shimpack Diol-300 column as described under Experimental. Peak heights were measured manually. ● = bCA, aggregation regimen as described under Experimental; □ = bCA, addition of 50% (v/v) glycerol; △ = bCA, addition of 50% (v/v) ethylene glycol.

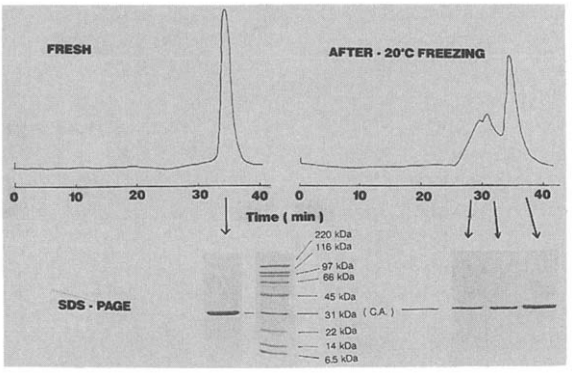

Fig. 4. SDS-PAGE analysis of associated bCA and bCA monomer. Fractionation was carried out by separating bCA (1 mg) with a three-column system as described in Fig. 2. Samples of 0.02 ml of protein peak fractions were analysed by SDS-PAGE at an acrylamide concentration of 12%. Protein bands were detected by Coomassie Brilliant Blue staining. Molecular mass standards for SDS-PAGE (Bio-Rad Labs.) were used. kDa = kilodalton.

of about 0.45 (Fig. 3). On the other hand, in the presence of 50% (v/v) glycerol or 50% (v/v) ethylene glycol, multimer species were not detectable (Fig. 3). Moreover, a two-peak chromatogram was not observed when the samples were frozen and stored at −80°C for as long as 3 weeks. The results of a standard SDS-PAGE analysis of the proteins in the HPGPC peak fractions are shown in Fig. 4. After boiling with 1% (w/v) SDS in Laemmli sample buffer the apparently higher-molecular-mass fraction, *i.e.*, the faster eluting peak, contained only M_r 31 000 bCA monomer (Fig. 4, bottom right). On the other hand, the inability of NP-40, a milder detergent than SDS, to separate the aggregated bCA into monomer suggests the presence of a hydrophobic interaction mechanism under the observed aggregation. In order to confirm aggre-

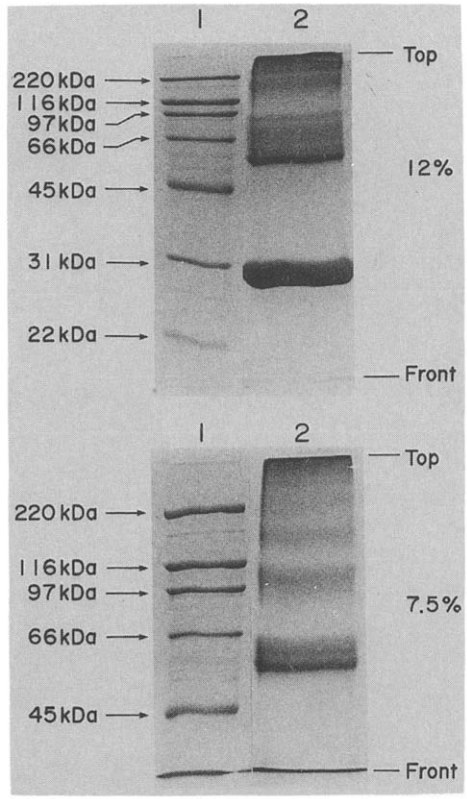

Fig. 5. Demonstration of bCA multimer species formation by cross-linking method. Details of the procedure are described under Experimental. Lanes: 1 = standard proteins (Bio-Rad Labs.); 2 = two-peak bCA treated with DMS. Top, 12% acrylamide; bottom, 7.5% acrylamide. Aliquot samples of 0.01 mg of bCA were analyzed.

gation, a cross-link analysis was carried out as described under Experimental. SDS-PAGE results of cross-linking confirmed the presence of multimer species, mainly dimers in two-peak bCA (Fig. 5). In contrast, no corresponding multimer bands were detectable in single-peak bCA (data not shown). Cross-linked dimer species migrated in a broad band at an apparent molecular mass of 54 000–66 000, while trimers and tetramers were detectable in fainter bands (Fig. 5, bottom).

In order to identify functional changes possibly related to aggregation, the hydrolytic activities of the bCA multimers on triacetin were determined and compared with the bCA monomer. As shown in Table I, the esterase activity of the multimeric species did not differ significantly from that of the bCA monomer. The preserved esterase activity of the bCA multimers indicates that the aggregation occurs without affecting the esterase catalytic centre of the enzyme. Therefore, the presence of intact bCA enzymatic activity despite aggregation suggests that the observed phenomenon consistently differs from that occurring under denaturing conditions, such as in the presence of guanidine hydrochloride, in which the catalytic activity is significantly lost [2]. Moreover, the observation of the formation of bCA multimer species after freezing and storage at −20°C but not at −80°C may provide additional insight into the development of methods to avoid aggregation in enzyme solutions.

Thus, given the combined characteristics of high sensitivity, analytical speed and wide commercial availability of its components, this HPGPC method has been shown to be a simple, applicable and useful tool for the systematic analysis of protein–protein interactions.

TABLE I

COMPARISON OF ESTERASE ACTIVITIES BETWEEN MONOMERIC AND ASSOCIATED bCA

Esterase activity using triacetin as substrate was measured as described under Experimental. Specific activity was expressed as picomoles of acetate liberated per minute per milligram of protein. No statistically significant difference was observed between the esterase activities of aggregated bCA and bCA monomer (Wilcoxon's test, $p > 0.05$).

Sample No.	Monomeric bCA	Associated bCA
1	24.7	31.0
2	19.8	22.6
3	43.5	38.0
4	42.7	28.6
5	36.5	28.9
6	38.1	27.3
7	31.1	35.3
8	35.1	33.2
9	31.0	25.3
Mean ± S.D.	30.0 ± 4.9	33.6 ± 7.9

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