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ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES ON SULPHYDRYL-CATALYSED STRUCTURAL ALTERATIONS OF BOVINE MERCAPTALBUMIN

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SUMMARY

The N–A isomerization (the intramolecular SH/S–S exchange reaction) of bovine mercaptalbumin (BMA) in alkaline medium was studied by using ion-exchange high-performance liquid chromatography (HPLC) and moving-boundary electrophoresis. Results obtained by ion-exchange HPLC on the N–A isomerization of BMA were consistent with those by moving-boundary electrophoresis and showed at least two kinds of the A-form, A_1 and A_2, indicating that the N–A isomerization is a multi-step reaction. The rate of the N–A isomerization was strongly suppressed in [^{2}H]water solution. The suppresion by [^{2}H]water might support the current view that intra- and intermolecular hydrophobic and/or hydrogen bonds are strengthened in [^{2}H]water.

INTRODUCTION

At alkaline pH, bovine mercaptalbumin (BMA) isomerizes to the aged form (A-form) by the intramolecular SH/S–S exchange reaction shown in the following equation

$$N \rightleftharpoons A_1 + mH^+ \rightleftharpoons A_2 + nH^+ \rightleftharpoons \cdots \quad (1)$$

where N and A_i are the N- and A-forms, respectively, as reported by Sogami *et al.*[1], Nikkel and Foster[2], Stroupe and Foster[3], Wallevik[4,5] and Inouye *et al.*[6] The N–A isomerization has been studied by using the pH–solubility profile[1,2,7,8], reversible-boundary spreading[9,10], moving-boundary electrophoresis[2,6,10], gel electrophoresis[3], isoelectric focusing[4,5] and ion-exchange chromatography[2]. However, these methods

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are complicated and time consuming compared with ion-exchange high-performance liquid chromatography (HPLC). Ion-exchange HPLC is a convenient technique for assaying the A-form in order to study the kinetics and equilibria of the N–A isomerization in more detail. We have compared data obtained by ion-exchange HPLC with those obtained by other methods[2–6] and studied the suppressing effect of [^{2}H]water on the N–A isomerization.

EXPERIMENTAL

Materials

Crystallized bovine plasma albumin (BPA, Lot N 72905) and Fraction V of BPA (Lot V 75208) were purchased from Armour Pharmaceutical (Chicago, IL, U.S.A.) and were defatted by the modified Chen method[11,12]. Defatted BPA was filtered through a Triton X-100-free Millipore membrane (0.45 μm) (Millipore, Bedford, MA, U.S.A.). Prior to use, defatted BPA was dialysed against appropriate salt solutions or deionized, distilled water, using pre-treated Cellophane tubing[12]. Defatted bovine mercaptalbumin (BMA) was prepared from defatted Fraction V of BPA, using SE-Sephadex C-50 according to the Hagenmaier and Foster method[10]. The SH content of BMA before freeze-drying was 1.00 ± 0.02 mol/mol BMA by the modified Ellman method[6,13,14] at pH 8.02 (0.10 I/2 Tris buffer). In the circular dichroism-resolved secondary structure of BMA, the fractions of α-helix, β-structure and unordered form determined by Chen's method[15,16], were 0.68, 0.13 and 0.19, respectively. BPA dimer was prepared as the S–S dimer in 0.10 *M* potassium chloride at pH 3.3, *i.e.*, the mid-point of the acid-induced expansion, in the presence of a catalytic concentration of Cu^{2+}[17,18], as previously described[19]. The degrees of dimerization and polymerization were determined by HPLC on TSK G 3000SW (Toyo Soda, Tokyo, Japan) with 0.10 *M* sodium phosphate buffer–0.30 *M* sodium chloride (pH 6.86) as eluent. SH-blocked BMA or BPA was prepared as previously described by using iodoacetamide (Nakarai Chemicals, Kyoto, Japan, Lot V2H 9676)[6,12,19]. All of the protein solutions were filtered through a Millex HA (0.45 μm) filter (Millipore) before HPLC analysis[19].

In the preparations of aged BPA, a solution of charcoal-defatted BPA was exhaustively dialysed against several changes of deionized, distilled water in a cold-room. Dialyse BPA was deionized by passing it through a Dintzis column[1,6,12]. The pH of the defatted, deionized BPA solution was then raised to the desired value below pH 8.7 by adding 0.30 *M* tris(hydroxymethyl)aminomethane (Tris) and above pH 8.7 by adding 0.30 *M* Tris and 0.10 *M* potassium hydroxide[6]. The solution was then filtered through a Millex HA (0.45 μm) filter into sterilized glassware with a silicone-rubber stopper. Ageing pH values given in the text are the average of pH value before and after ageing[6]. After ageing for the appropriate time at the appropriate pH and temperature, the pH and sodium acetate concentration were adjusted to 4.82 and 0.03 *M* respectively, by adding 1/50 volumes of concentrated sodium acetate buffer. In the preparations of aged BMA, BMA was dissolved in deionized, distilled water. Aged BMA was prepared as described in the aged BPA preparation. In the ageing experiments with BMA in water–[^{2}H]water, the pL was adjusted to the appropriate value by adding 0.30 *M* Tris (water or [^{2}H]water) according to the following equation:

$$pL = (\text{meter reading}) + 0.3314\,n + 0.0766\,n^2$$

where n is the deuterium atom fraction and pL is the generalized equivalent of pH (ref. 20). After adjusting the pH to 4.82 and the sodium acetate concentration to 0.03 *M*, BPA and BMA, aged in water or water–[^{2}H]water, were dialysed against 0.03 *M* sodium acetate buffer at 3°C for at least 1 day and were analysed by moving-boundary electrophoresis and/or ion-exchange HPLC. Some HPLC experiments was carried out without dialysis. Dialysed, aged BMA and BPA were filtered through a Millex HA filter before HPLC analysis.

Methods

Moving-boundary electrophoresis was carried out on a Hitachi HTD-1 Tiselius type instrument (Hitachi, Tokyo, Japan) with a microcell (1.00 ml, 0.180 cm^2 cross-section). The electrophoresis of a dialysed 0.5% solution was conducted at 2.5°C for 1.5–2 h. The buffer was 0.03 *M* sodium acetate buffer (pH 4.82). The results obtained were analysed as previously reported[6]. The conductivities of a dialysed protein solution and buffer were measured with a Yanagimoto Model MY-7 conductometer (Yanagimoto, Osaka, Japan) at 2.5°C.

The two kinds of HPLC system used in these experiments were as follows: (A) an Erma Degasser ERC-3110 (Erma Optical Works, Tokyo, Japan); Toyo Soda HLC-803A HPLC system with a 500 μl sample loop; TSK G 3000SW column (60 × 0.75 cm I.S.); Toyo Soda LS-8 low-angle laser light scattering photometer (632.8 nm); and Jasco Uvidec 100-IV UV monitor (Japan Spectroscopic, Tokyo, Japan); (B) Toyo Soda Gradientor (HLC-803D with a 100-μl sample loop and GE-4); TSK SP-5PW column (7 × 0.75 cm I.D.); and Jasco Uvidec 100-IV UV monitor. System A with the TSK G 3000SW column was used for the determination of monomer, dimer and oligomer contents with 0.10 *M* sodium phosphate buffer–0.30 *M* sodium chloride (pH 6.86) at a flow-rate of 0.50 ml/min and 23 ± 1°C. The weight-average molecular weight was obtained using UV and low-angle laser light scattering profiles, as described previously[19]. System B with the TSK SP-5PW column was used for the determination of the fraction of the A-form. Almost all of the ion-exchange HPLC experiments were carried out on BMA. The N- and A-forms of aged BMA were separated by a 30-min linear gradient elution with increasing sodium chloride concentration from 0.085 to 1.50 *M* in 0.02 *M* sodium acetate buffer (pH 5.00) at a flow-rate of 1.00 ml/min at 23°C. The TSK SP-5PW column was equilibrated with 0.02 *M* sodium acetate buffer (pH 5.00), containing 0.085 *M* sodium chloride, at 23°C. The pH of the 0.02 *M* sodium acetate buffer, containing 1.5 *M* sodium chloride, was adjusted to exactly 5.00 because the pH value of the eluent is important for the separation of the N- and A-forms on the TSK SP-5PW column. Deaeration of buffer solutions was carried out by bubbling helium (initial flow-rate, 1 l/min for 10–20 min; flow-rate during operation, 10–30 ml/min).

The concentrations of BPA and BMA were determined with a Hitachi 320 spectrophotometer, assuming $A_1^{1\%}{}_{cm}$ at 279 nm to be 6.67. The pH measurements were made with a Hitachi–Horiba F-7SS instrument, equipped with an expanded scale, using a Radiometer GK-2401C combined electrode. Deionized, distilled water was prepared by passing glass-distilled water (reflux column height, 1.4 m; specific resistance, $0.9 \cdot 10^6$ Ω cm) through a mixed-bed column and had a specific resistance

far greater than 5 10^6 cm. For HPLC experiments, glass-distilled water was used as a solvent, because it was practically free of dust and was suitable for the low-angle laser light scattering photometer. However, in order to remove dust from buffer salts, buffer solutions were filtered through a Triton X-100-free Millipore membrane (0.45 μm) or a Millipore Sterivex-GS filter (0.22 μm). Cellophane tubing (Visking) was pre-treated by boiling in 50% saturated $NaHCO_3$ (half-saturation at 25°C) and by exhaustive washing with deionized, distilled water[12]. Pre-treated cellophane tubing was stored in a cold-room at 3°C in deionized, distilled water.

All chemicals employed were of analytical-reagent grade. [^{2}H]water (degree of deuteration 99.75%) and [^{2}H]hydrochloric acid and [^{2}H]hydroxide solutions were purchased from Merck (Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Ion-exchange HPLC of dimerized BPA

As previously described[18,19], Cu^{2+}-catalysed dimerization was found to arise from the intermolecular disulphide formation of BMA. Thus, the monomer in dimerized BPA might be mainly BPA, devoid of SH groups, *i.e.*, bovine nonmercaptalbumin (BNA). The HPLC profile of dimerized BPA, eluted from TSK G 3000SW with 0.10 *M* sodium phosphate buffer–0.30 *M* sodium chloride (pH 6.86) at 23°C, showed at least four components corresponding to monomer, dimer, oligomer, etc. The weight-average molecular weight (M_w) of each component was obtained according to the following equation, assuming the M_w of BMA monomer to be 66000:

$$h(\mathrm{LS})/h(\mathrm{UV}) = L_0 M_w \qquad (2)$$

where h(LS) and h(UV) are the peak heights of the light-scattering intensity at 632.8 nm and the absorbance at 279 nm, respectively, and L_0 is a constant, as described previously[19,21–23]. The values of M_w, corresponding to monomer, dimer and trimer were estimated to be 68100, 132000 and 183000, respectively. Fractions of monomer, dimer and oligomers were 0.37, 0.54 and 0.09, respectively. The ion-exchange HPLC profile of Cu^{2+}-catalysed dimerized BPA, eluted from TSK SP-5PW, showed several peaks, corresponding to monomer, dimer and oligomers. The elution volume of a major peak (the secondary peak), corresponding to dimer, was almost equal to that of the A-form, indicated as A_1 in Fig. 1. Hence small peaks in the HPLC profile of non-aged BMA, eluted from TSK SP-5PW, might be dimer and oligomers, as indicated in Fig. 1. Sogami *et al.*[1] and Inouye *et al.*[6] reported that no formation of intermolecular disulphide bonds with resultant formation of dimeric and oligomeric species during the ageing reaction was observed in ultracentrifugal and HPLC (TSK G 3000SW) analyses. Therefore, assuming the small peaks in the HPLC profile of non-aged BMA, eluted from TSK SP-5PW (left upper HPLC profile in Fig. 1), to be dimer and oligomers, the fraction of the A-form was calculated as shown in Figs. 2–4.

Ion-exchange HPLC on TSK SP-5PW

HPLC profiles of BMA, aged at pH 8.6 without added salt at 25°C for 0 min, 80 min and 26 h, are shown on the left side of Fig. 1. The schlieren patterns of BMA,

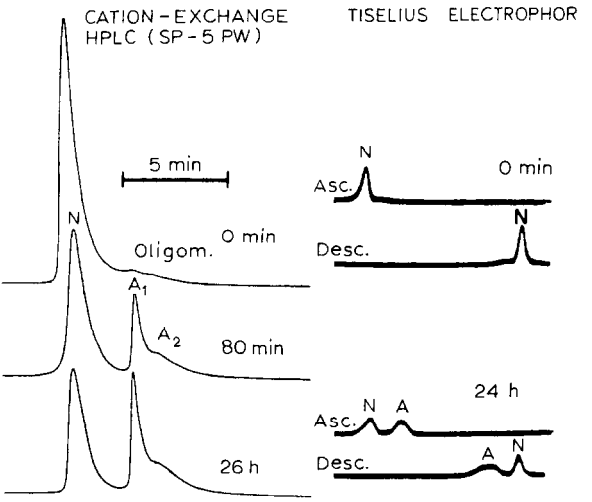

Fig. 1. Moving-boundary electrophoresis (right side, 0.03 *M* sodium acetate buffer, pH 4.82, 2.5°C) and ion-exchange HPLC (left side) of BMA, aged at pH 8.6 with no added salt at 25°C for 0 min, 80 min, 24 h and 26 h. Ion-exchange HPLC on TSK SP-5PW (7 × 0.75 cm I.D.) was carried out by 30-min linear gradient elution with increasing sodium chloride concentrations from 0.085 to 1.50 *M* in 0.02 *M* sodium acetate buffer (pH 5.00) at a flow-rate of 1.00 ml/min at 23°C. N and A (A_1, A_2) indicate the N- and A-forms, respectively. The secondary and tertiary small peaks in the ion-exchange HPLC profile of non-aged BMA might be the dimer, and oligomer, respectively. TISELIUS ELECTROPHOR indicates moving-boundary electrophoresis by a Tiselius type instrument. Asc. and Desc. indicate ascending and descending schlieren patterns, respectively.

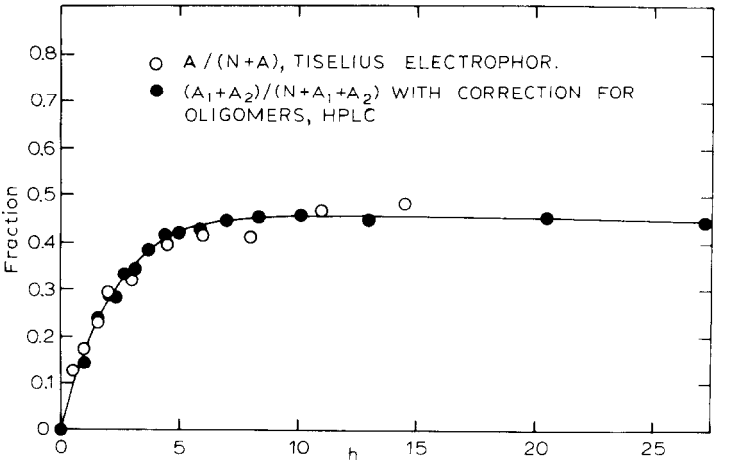

Fig. 2. Fraction of the A-form of aged BMA, $(A_1 + A_2)/(N + A_1 + A_2)$ (●), obtained by ion-exchange HPLC as a function of the time of ageing at pH 8.6 with no added salt at 25°C. Data obtained by moving-boundary electrophoresis [A/(N + A), O] were taken from ref. 6. Electrophoresis: $K_{app} = 0.95$; $k_{+1} = 4.1 \cdot 10^{-5}$ sec^{-1}; $k_{-1} = 4.3 \cdot 10^{-5}$ sec^{-1}. HPLC: $K_{app} = 0.89$; $k_{+1} = 5.7 \cdot 10^{-5}$ sec^{-1}; $k_{-1} = 6.4 \cdot 10^{-5}$ sec^{-1}. See the text for definitions of K_{app}, k_{+1} and k_{-1}.

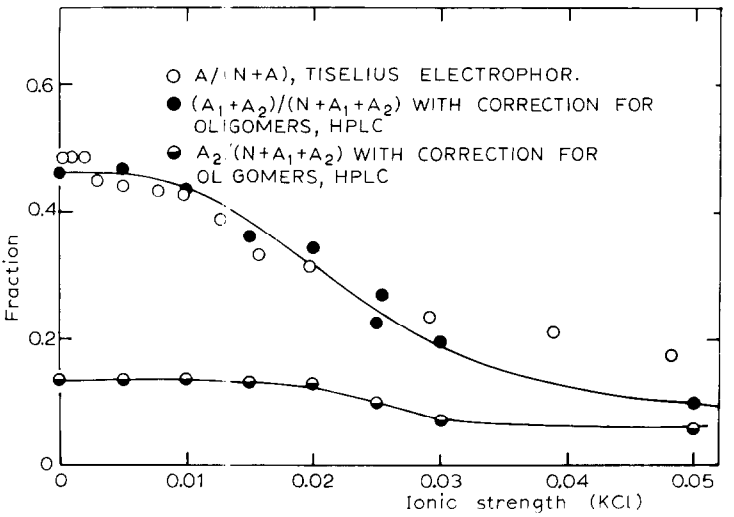

Fig. 3. Fractions of the A-form [$(A_1 + A_2)/(N + A_1 + A_2)$, ●] and the A_2-form [$A_2/(N + A_1 + A_2)$, ◒] of aged BMA, obtained by ion-exchange HPLC, as a function of the ionic strength (potassium chloride for aging at pH 8.6 at 25°C for 15 h. Data obtained by moving-boundary electrophoresis [$A/(N + A)$, O] were taken from ref. 6. A_1 and A_2 indicate two kinds of the A-form (see A_1 and A_2 in Fig. 1).

aged at pH 8.6 for 0 min and 24 h under the same ageing conditions as for HPLC experiments, are shown on the right side of Fig. 1. The schlieren patterns of the aged BMA were similar to those given by Nikkel and Foster[2] and Inouye *et al.*[6]. As reported by Nikkel and Foster[2], the A-form is more positive than the N-form below pH 5.3 and more negative above pH 5.3. Therefore, HPLC on TSK SP-5PW at pH 5.00 and moving-boundary electrophoresis at pH 4.82 could resolve the N- and A-forms, as shown in Fig. 1. The principal, secondary and tertiary (broad) peaks in the

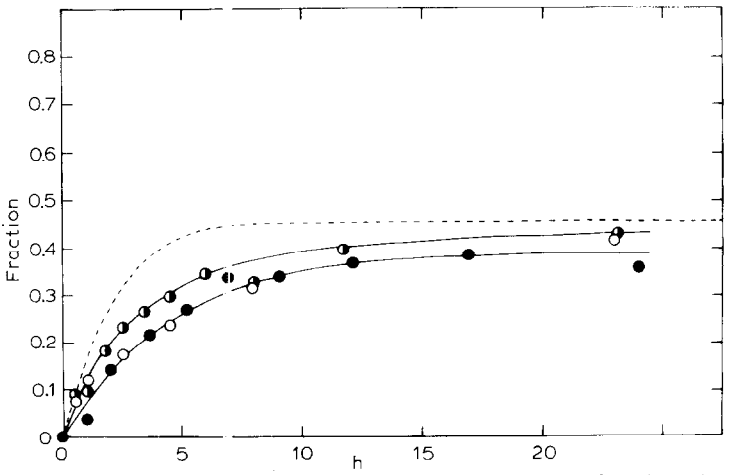

Fig. 4. Fractions of the A-form, $(A_1 + A_2)/(N + A_1 + A_2)$, of aged BMA, obtained by ion-exchange HPLC as function of [^{2}H]water volume fraction and the time of ageing at pL 8.6 with no added salt at 25°C. ----, Deuterium a om fraction, $n = 0$; ◑, $n = 0.494$; O, $n = 0.897$; ●, $n = 0.996$. pL is the generalized equivalent o ` pH in water–[^{2}H]water[20].

HPLC profiles on TSK SP-5PW might correspond to the N-, A_1- and A_2-forms, respectively, as indicated in eqn. 1.

The HPLC of aged BMA, eluted from TSK SP-5PW, was similar to that obtained by SE-Sephadex C-50 chromatography with 0.02 *M* sodium acetate buffer (pH 4.70) at 4°C and a linear salt gradient of 0.1–0.7 *M* sodium chloride[2]. In our HPLC experiments, we found it difficult to elute the A-form completely from TSK SP-5PW under the conditions given by Nikkel and Foster[2], *i.e.*, a linear salt gradient of 0.10–1.0 *M* sodium chloride in 0.02 *M* sodium acetate buffer (pH 4.85). Under these conditions, although the HPLC profile of the A-form, eluted from TSK SP-5PW, showed at least three components, the HPLC profile of the N-form was less symmetrical. We therefore used 0.02 *M* sodium acetate buffer (pH 5.00) and a 30-min linear gradient from 0.085 to 1.50 *M* at a flow-rate of 1.00 ml/min. Under these conditions, the N- and A-forms were almost completely separated with a nearly symmetrical principal peak, corresponding to the N-form.

Comparison of HPLC data with those obtained by moving-boundary electrophoresis

The kinetics of the N–A isomerization reaction of BMA at pH 8.6 without added salt at 25°C was studied by ion-exchange HPLC. Assuming the fraction of the A-form of the aged BMA obtained by ion-exchange HPLC to be $(A_1 + A_2)/(N + A_1 + A_2)$, the time course of the ageing reaction of BMA, obtained by ion-exchange HPLC, was in good agreement with that by the moving-boundary electrophoresis of Inouye *et al.*[6]. The solid curve in Fig. 2 was calculated from the equation for a reversible first-order reaction of the type $N \rightleftharpoons A$, as reported by Stroupe and Foster[3], using ion-exchange HPLC data:

$$[K_{app}/(1 + K_{app})] \ln(A_\infty - A_t)/A = -k_{+1}t \quad (3)$$

where $K_{app} = k_{+1}/k_{-1}$ is the apparent equilibrium constant and A_t and A_∞ are the amounts of A, *i.e.*, $(A_1 + A_2)$ at time t and infinite time, respectively. The solid curve in Fig. 2 corresponds to $k_{+1} = 5.7 \cdot 10^{-5}$ sec^{-1}, $k_{-1} = 6.4 \cdot 10^{-5}$ sec^{-1} and $K_{app} = 0.89$. These values were comparable to $k_{+1} = 4.1 \cdot 10^{-5}$ sec^{-1}, $k_{-1} = 4.3 \cdot 10^{-5}$ sec^{-1} and $K_{app} = 0.95$ obtained by Inouye *et al.*[6] by moving-boundary electrophoresis.

It has been reported by several workers[1–3,6,10] that the ageing reaction is suppressed by increasing the ionic strength. As shown in Fig. 3, the $(A_1 + A_2)/(N + A_1 + A_2)$ profile of the aged BMA, obtained by ion-exchange HPLC, was in agreement with that by the moving-boundary electrophoresis [A/(N + A)] of Inouye *et al.*[6]. Fig. 3 also shows a fraction of the A_2-form, $A_2/(N + A_1 + A_2)$. The pH profile of the ageing reaction of BMA, obtained by ion-exchange HPLC, was also in agreement with that obtained by the moving-boundary electrophoresis by Inouye *et al.*[6] (data not shown).

From the experimental evidence in Figs. 1, 2 and 3, it is possible to say that chromatography on TSK SP-5PW can resolve the aged BMA into the N- and A-forms, as in the case of moving-boundary electrophoresis. Ion-exchange HPLC appears to be a convenient method of assaying for the A-form and for studying the kinetics and equilibria of the N–A isomerization in more detail, because the amount of sample is small (less than 1 mg) and the method is less time consuming.

Effect of [²H]water on the ageing reaction

It was reported by several workers[24–26] that [²H]water might strengthen the intra- and intermolecular hydrophobic and/or hydrogen bonds. We therefore studied the effect of water–[²H]water solvents on the ageing reaction of BMA and used ion-exchange HPLC on TSK SP-5PW for analysis. The ageing reaction of BMA was carried out at pL 8.6 without added salt at 25°C. The H–²H exchange reaction (exchange-in) of BMA (*ca.* 12%) was carried out at pD 4.02 for 10 days at 3°C. The deuterated BMA was diluted 50-fold, using water–[²H]water solvents, and the pL was adjusted to 8.6 by adding 0.30 *M* Tris in [²H]water. In these experiments, the final salt concentrations were approximately 3 m*M*, and the deuterium atom fractions of the ageing solutions were 0.996 (● in Fig. 4), 0.897 (○) and 0.494 (◑). Although 3 m*M* sodium chloride or potassium chloride had no stabilizing effect on the ageing reaction of BMA in water[6], as shown in Fig. 3, rates of the ageing reaction were strongly suppressed in 50% (deuterium atom fraction 0.494), 90% (0.897) and 100% (0.996) [²H]water solutions, as shown in Fig. 4. The suppression of the ageing reaction might be due to the strengthening of intramolecular hydrophobic and/or hydrogen bonds of BMA, resulting in the suppression of the intramolecular sulphydryl–disulphide exchange reaction.

There are several reports on H–²H exchange (exchange-in) and ²H–H exchange (exchange-out) of BPA and human serum albumin[27–30]. For the H–²H exchange reaction of proteins, Hvid and Nielsen[27] proposed the following exchange scheme:

$$\mathrm{M} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{I} \xrightarrow{k_3} \text{exchange} \qquad (4)$$

in which the hydrogen in the shielded conformation, M, is not accessible to exchange with bulk solvent, and in the open conformation, I, is accessible. Benson and Hallaway[29] suggested that BPA is highly "motile", *i.e.*, it fluctuates rapidly at pH 7 and above between the M and I states. Benson and Hallaway[29] also studied the effect of ionic strength on the ²H–H exchange reaction of BPA at pH 7.7 at 0°C and found a suppression of exchange rates on increasing the ionic strength from 0 to 0.20 *M* potassium chloride, indicating that the equilibrium between M and I is shifted to the M state. However, even in 0.20 *M* potassium chloride at pH 7.7 and 0°C, significant amounts of hydrogen were exchanged[29,30]. On the other hand, the N–A isomerization, *i.e.*, the ageing reaction of BMA, was almost completely suppressed on increasing the ionic strength to 0.20 *M* potassium chloride (Fig. 3 and ref. 6). In the H–²H exchange reaction, the restricted *local* motilities might be sufficient. However, the *global* structural fluctuations of helical segments or subdomains[31] might be necessary for the ageing reaction of BMA (the intramolecular sulphydryl–disulphide exchange reaction). In the motile state above pH 7 without added salt, the restricted *local* motions might be superimposed on the *global* structural motions of helical segments or subdomains of BMA, like a librational motion[32], which is important for the intramolecular sulphydryl–disulphide exchange reaction.

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