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Monitoring of hapten–protein coupling reactions by capillary zone electrophoresis: improvement of abscisic acid–bovine serum albumin coupling and determination of molar coupling ratios

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Abstract

The utility of capillary zone electrophoresis (CZE) was investigated for the analysis of the coupling reaction of abscisic acid (ABA) – a plant hormone – on bovine serum albumin (BSA), a model hapten–protein coupling reaction. Through two different coupling reactions using the C1 carboxyl group or the C4′ carbonyl group of ABA, we demonstrate a correlation between the ABA/BSA molar coupling ratio and the modification of the electrophoretic mobility of BSA in CZE, in order to develop a rapid and efficient tool for coupling ratio determination. In addition, preliminary ABA activation steps were analysed by CZE to specify the stoechiometric and kinetic conditions of these reactions. These results show that CZE is an effective and fast technique for the monitoring of hapten–protein coupling reactions.

Keywords: Capillary electrophoresis; Coupling reactions; Hapten; Proteins; Abscisic acid; Bovine serum albumin; Albumin

1. Introduction

The use of protein conjugates is a very general process, used frequently in various fields of scientific work. For example, in our field of study concerning plant hormones, hapten–protein conjugates are used to produce antibodies [1,2], for immobilisation of plant hormones to proteins in immunochemistry [3], for quantitative ELISA [4,5], to isolate hormone-binding proteins by affinity-based procedures and to test hormone/receptor interactions [6].

Many protein coupling protocols are described [1,2,7], but close examination of these protocols shows that some reaction parameters are not often optimised (stoechiometric and kinetic conditions), because of a lack of tools for monitoring reactions. Furthermore, estimation of the hapten–protein molar coupling ratio is generally obtained by the use of radioactively labelled molecules or spectrophotometric absorption modification. These methods are not always easy

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to use, and in some cases are even totally unusable if labelled substances are not commercially available or if the UV absorption is too weak (e.g. gibberellin, a plant hormone). For these reasons, we were interested in the development of a new coupling reaction monitoring technique. With the development of capillary zone electrophoresis (CZE)[1] for protein study and analysis [8–12], we decided to investigate the use of this new technology to monitor hapten–protein coupling reactions.

Here we describe the use of CZE to analyse two coupling reactions of a small molecule as a plant hormone: for example abscisic acid (ABA) with a widely used carrier protein, bovine serum albumin (BSA). This work allowed us to monitor the first ABA activation steps and to specify reaction parameters. We also demonstrate a correlation between the ABA–BSA molar coupling ratio and the electrophoretic mobility (μ) of BSA in CZE in order to determine the hapten–protein coupling ratio. The features of CZE analysis — rapidity, reproducibility of results and very little material consumption — allowed us a proximate monitoring of coupling reactions.

2. Experimental

2.1. Chemicals and reagents

Reagents were obtained as follows: (±)*cis*, *trans*-ABA (Fluka, New York, NY, USA); *cis*,*trans*-[G-^{3}H]ABA (Amersham, Little Chalfont, UK); *p*-aminobenzoyl hydrazide (Aldrich, Milwaukee, WI, USA). NHSC and all other reagents were from Sigma (St. Louis, MO, USA).

[1] Abbreviations used: ABA, *cis*, *trans*-abscisic acid; ABH, *p*-aminobenzoyl hydrazide; BSA, bovine serum albumin; CZE, capillary zone electrophoresis; DMF, N,N-dimethylformamide; EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EOF, electroosmotic flow; μ, electrophoretic mobility; NHSC, N-hydroxysuccinimide; TH, tyrosine hydrazide.

2.2. Coupling of ABA on BSA through the carbonyl group (C4') of ABA: ABA–C4'-BSA

Activation of ABA using ABH: ABA–ABH

ABH (30.2 mg, 200 μmol) was dissolved in 2 ml of methanol containing acetic acid (20% v/v). This solution was added to ABA (52.9 mg, 200 μmol) and [^{3}H]ABA (60 pmol, 4 μCi). The mixture was stirred at room temperature in the dark for 24 h. Aliquots were taken every hour for CZE analysis of the kinetics of the activation reaction. After activation, solvents were removed under nitrogen and the brown residue was taken up in 0.2 ml of methanol. $NaNO_2$ solution at 25 mg/ml (0.6 ml, 220 μmol) and 1 ml of 0.5 *M* hydrochloric acid were added to the ABA–ABH solution for diazotization. This step was performed at 0°C in ice for 20 min.

ABA–C4'-BSA coupling

After diazotization, the pH of the solution was neutralised with 2 *M* NaOH and added slowly to BSA (6.6 mg, 0.1 μmol) dissolved in 0.5 ml of ultrapure water (Milli-Q Water System, Millipore, Bedford, MA, USA). The reactions were performed in the dark and stirred at 4°C. For the analysis of the kinetics of the reaction, the ABA–ABH/BSA molar ratio was fixed to 100:1 and aliquots were taken every hour, for 5 h. For the analysis of the correlation between CZE displacement and the molar coupling ratio, increasing ABA–ABH/BSA molar ratios were chosen (5:1, 10:1, 25:1, 50:1, 100:1 and 250:1). The reaction time was 20 h.

2.3. Coupling of ABA on BSA through the carboxyl group (C1) of ABA: ABA–C1-BSA

Activation of ABA using EDAC and NHSC: ABA–NHSC ester

ABA (26.4 mg, 100 μmol), [^{3}H]ABA (30 pmol, 2 μCi) and NHSC (23.0 mg, 200 μmol) were dissolved in 1.8 ml of DMF and added to EDAC (38.3 mg, 200 μmol) dissolved in 0.2 ml of ultrapure water. The reaction was performed with stirring at room temperature for 2 h.

Aliquots were taken for CZE analysis of the kinetics of the reaction.

ABA–C1-BSA coupling

The pH of the ABA–NHSC solution was adjusted to 8.7 with 2 *M* NaOH and then the solution was added slowly to BSA (6.6 mg, 0.1 μmol) dissolved in 0.5 ml of ultrapure water. The reactions were performed with stirring at 4°C. Experimental conditions for the analysis of the coupling reaction kinetics were similar to those used in ABA–C4′-BSA synthesis: the ABA–NHSC/BSA molar ratio was fixed to 100:1 and aliquots were taken every hour, for 5 h. For the analysis of the correlation between CZE displacement and the molar coupling ratio, five increasing ABA–ABH/BSA molar ratios were chosen (5:1, 10:1, 25:1 and 100:1). The reaction time was 20 h.

2.4. Determination of the molar coupling ratio of ABA–BSA conjugates

Coupling ratios were calculated from the [^{3}H]ABA incorporated in ABA–BSA conjugates: an aliquot of the conjugate was taken and dialysed against water by ultrafiltration in order to eliminate non-incorporated [^{3}H]ABA (UltraFree-MC 10 NMWL, Millipore) prior to counting with a liquid scintillation system (LS 6000IC, Beckman Instruments, Palo Alto, CA, USA).

2.5. CZE analysis

All CZE experiments were performed using a P/ACE System 2100 HPCE instrument (Beckman) controlled by a computer fitted with P/ACE software (Beckman). The capillary used was a neutral hydrophilic phase capillary (CElect-P175, Supelco, Bellefonte, PA, USA) of length 67 cm; distance between injection and detection, 60 cm; I.D., 75 μm. The wavelength of the UV detector was 214 nm.

In order to reduce protein adsorption to the capillary wall that interferes with the reproducibility of the CZE measurements, the capillary was systematically flushed for 1 min with 100 m*M* NaOH and equilibrated for 3 min with electrophoresis buffer (10 m*M* phosphate, pH 6.0) prior to each sample injection. Samples were pressure injected and an EOF standard was co-injected: benzyl alcohol, 1 s pressure injected (ca. 7 nl). In all experiments a constant voltage of 30 kV was applied for 8 min and the temperature of the capillary was controlled at 25 ± 0.1°C.

In order to allow precise measurement of mobility, results were expressed as electrophoretic mobility (μ) calculated according to the equation [13]:

$$\mu = \left(\frac{L^2}{V}\right)\left(\frac{1}{T} - \frac{1}{T_{eof}}\right)$$

where μ is electrophoretic mobility, L is length of the capillary (cm), V is voltage (kV), T is migration time of sample peak (min), and T_{eof} is migration time of standard peak (benzyl alcohol) (min).

2.6. Sample preparation and separation

Aliquots of ABA activation mixtures were diluted in ultrapure water (dilution 1:100) and 1 s pressure injected (ca. 7 nl). Required quantities for CZE analysis are ca. 10 nmoles for ABA, ABH, NHSC or EDAC. Owing to the presence of excess activated ABA, the ABA–BSA CZE analysis required a fast purification step before injection. The ABA–BSA solutions were passed through a size-exclusion column (PD10, Pharmacia Biotech, Uppsala, Sweden) eluted with ultrapure water to separate ABA–BSA from ABA–ABH or ABA–NHSC. ABA–BSA fractions were 4 s pressure injected (ca. 28 nl).

For the study of the kinetic and stoechiometric conditions of ABA–BSA coupling, CZE analysis of each sample was repeated 4 times. To give an idea of the precision of the CZE procedure, coefficients of variation (expressed in percent) of migration time of neutral marker or protein samples are about 1.5%, and coefficients of variation of calculated electrophoretic mobility are about 3.0%.

3. Results and discussion

3.1. ABA activation reactions

The use of CZE for ABA activation monitoring was very simple since mixtures can be injected directly in water. Electrophoretic separations (presented in Fig. 1) were fast (6 min) and only minute quantities of reaction mixture were necessary, which allowed the monitoring of activation reactions in real time. The disappearance of the initial compounds (ABA and ABH) and appearance of the product (ABA–ABH) was obvious for ABA–ABH activation. The ABA–NHSC activation was monitored by the removal of the initial compounds (ABA, EDAC and NHSC) since no separation of the product of the reaction (ABA–NHSC) could be achieved. The persistence of the EDAC peak was due to the excess of EDAC (EDAC/ABA = 2:1).

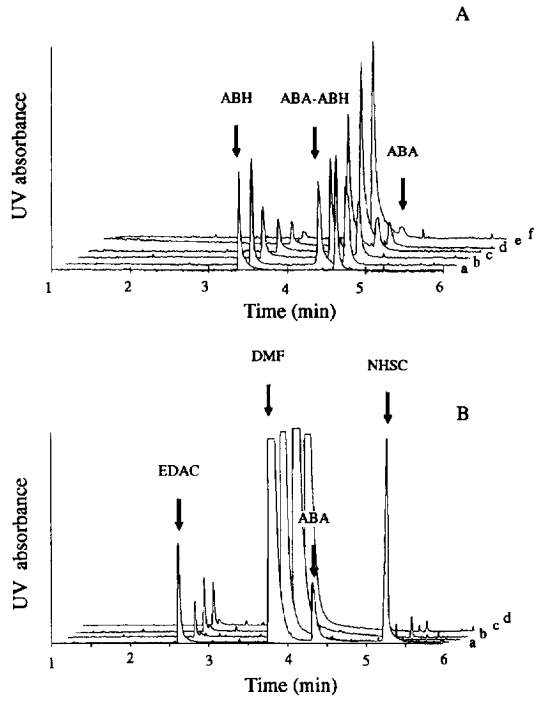

Fig. 1. CZE analysis of ABA activation. (A) CZE separation of ABA–ABH mixture at different activation times: 0 h (a), 1 h (b), 2 h (c), 5 h (d), 10 h (e) and 25 h (f). (B) CZE separation of ABA–EDAC–NHSC mixture at different activation times: 0 min (a), 10 min (b), 20 min (c) and 30 min (d).

In addition to this qualitative monitoring of the activation reaction, a semi-quantitative analysis can be performed by integration of the peak areas of the different compounds. Results of this analysis are presented in Fig. 2. According to the protocols used, the yield of ABA activation for both reactions is high (more than 90%). The kinetics of the reactions are quite different.

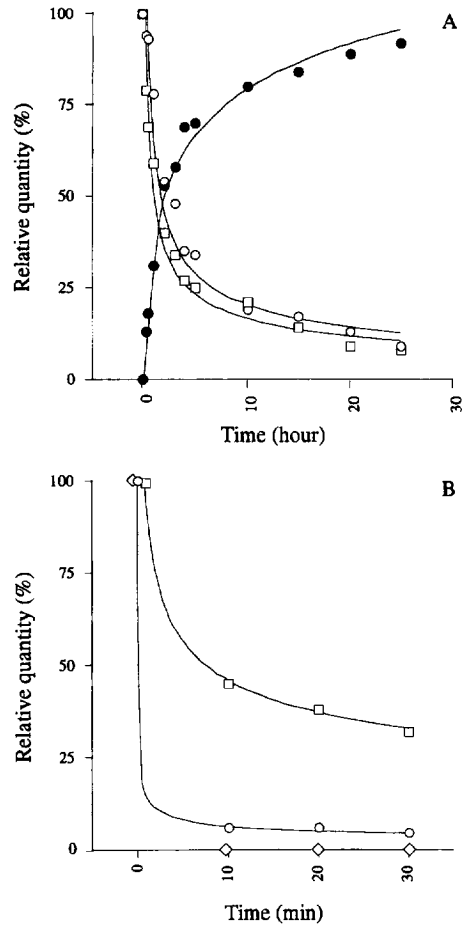

Fig. 2. Kinetics of ABA activation. Data (relative quantities) were calculated from the relative area of integrated CZE peaks. (A) ABA–ABH activation [ABA(○), ABH (□), ABA–ABH (●)]. The regression used for ABA–ABH is a logarithmic regression ($r = 0.989$). The regression used for ABA and ABH is a power type regression ($r = 0.969$ and 0.973, respectively). (B) ABA–NHSC activation [ABA (○), EDAC (□), NHSC (◇)]. The regression used for ABA and EDAC is a power type regression ($r = 0.988$ and 0.998, respectively). No regression of NHSC experimental points was possible owing to its rapid disappearance.

ABA–ABH activation needs several hours: 80% of the initial compounds disappear after 10 h of incubation and 92–96% of ABA–ABH was produced in 25 h. Further extension of the reaction time (up to 72 h) could not be monitored precisely by CZE, because of the very low concentration of the remaining reactants. ABA–NHSC activation is almost immediate and, after 10 min reaction, the quantities of ABA or NHSC remaining were not significant.

The use of CZE monitoring allowed us to define the optimal steochiometric conditions precisely and to reduce significantly the time for activation: ABA–C4′-BSA coupling protocol was based on two publications that reported a reaction time of 3 days for ABA–ABH activation [7] and 15 days for ABA–TH activation — a similar reaction [14].

3.2. ABA–BSA coupling

Correlation between the molar coupling ratio and electrophoretic mobility (μ) ABA–BSA conjugates

The first step was to establish the correlation between the molar coupling ratio and the modification of the electrophoretic mobility of ABA–BSA. Electrophoregrams presented in Fig. 3 show a clear shifting of the BSA peak as the quantity of activated ABA is increased, for both types of coupling reactions. Correlation between ABA–BSA peak shifting and the molar coupling ratio is illustrated in Fig. 4.

In both reactions, the ABA–BSA molar coupling ratio (moles of conjugated ABA/moles of protein) calculated from incorporated [^{3}H]ABA, was ca. 17:1 (16.7:1 for ABA–C1-BSA and 16.8:1 for ABA–C4′-BSA). According to Dayhoff [15], 19 phenol groups (Tyr) are theoretically available on BSA for ABA–C4′-BSA coupling. For ABA–C1-BSA, 59 ε-NH_2 groups (Lys) are theoretically available on BSA, but the number of real accessible groups is not well defined (30–35 according to Dayhoff [15]). The molar coupling ratio that we obtained was much lower than this value. But, on the other hand, we obtained the same molar coupling ratio and the same electrophoretic mobility for 50:1 and 100:1 activated

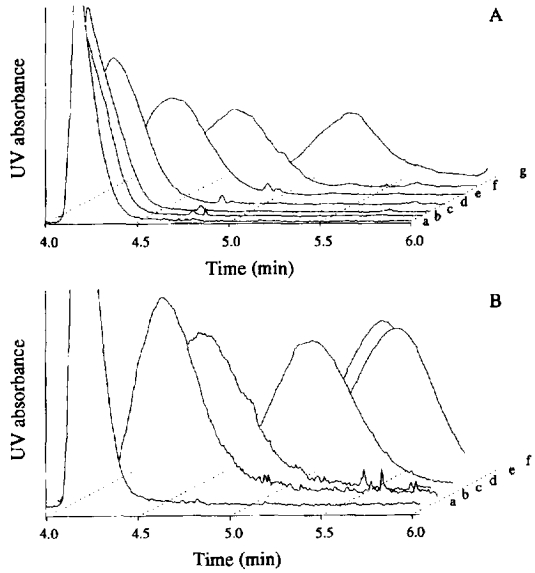

Fig. 3. Shifting of ABA–BSA peak in CZE as a function of steochiometric coupling reaction conditions. (A) ABA–C4′-BSA coupling. Activated ABA–ABH:BSA coupling conditions were 0:1 (a), 5:1 (b), 10:1 (c), 25:1 (d), 50:1 (e), 100:1 (f) and 250:1 (g). (B) ABA–C1-BSA coupling. Activated ABA–NHSC–BSA coupling conditions were 0:1 (a), 5:1 (b), 10:1 (c), 25:1 (d), 50:1 (e) and 100:1 (f).

ABA–BSA stoechiometric conditions. Nonetheless, these values for the molar coupling ratio did not express the real maximal coupling capacity, but rather the mean of a gaussian distribution of different ABA–BSA conjugates according to their coupling ratio. This point will be discussed later.

The displacement of the BSA peak was more important in the ABA–C1-BSA coupling: maximal electrophoretic mobilities of ABA–C1-BSA and ABA–C4′-BSA were −14.95 and −12.60 respectively, which represent a difference of about 35% according to the electrophoretic mobility of native BSA. This result is consistent with the modification of the electrostatically charged C1 carboxyl group.

3.2.2. Kinetics of ABA–BSA coupling

Kinetics of coupling reactions have not been widely studied previously. The use of CZE allowed us to analyse the kinetics of the two coupling reactions (Fig. 5). Numeric data pre-

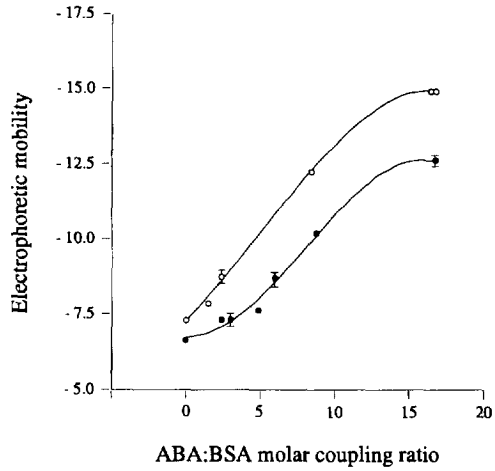

Fig. 4. Correlation between CZE electrophoretic mobility (μ) of ABA–BSA conjugates and ABA–BSA molar coupling ratio: ABA–C1-BSA (○), ABA–C4′-BSA (●). Each value was calculated on the average of four experimental CZE measurements. Standard deviations are not visible when they are less than 0.004. The regression coefficient is 0.999 for polynomial regression (magnitude 3) of ABA–C1-BSA and 0.996 for polynomial regression (magnitude 3) of ABA–C4′-BSA.

sented in Fig. 6 show that these reactions are surprisingly quite fast, since maximal electrophoretic mobility was achieved in 3 h for both coupling reactions.

3.2.3. Evaluation of molar coupling ratio distribution of ABA–BSA conjugates

The shifting of the ABA–BSA peak according to the coupling ratio was simultaneously accompanied by a modification of the shape of the BSA peak. Graphically, peak widening and flattening is quite obvious. We could explain this by the fact that the result of coupling reactions is not a single ABA–BSA conjugate, but a statistical heterogeneous mixture of different molar coupling ratio ABA–BSA classes. The gaussian shape of the ABA–BSA peak allowed us to evaluate the distribution of these ABA–BSA conjugates around an average coupling ratio — measured by [^{3}H]ABA incorporation. Results are presented in Fig. 7 as a projection of calculated molar coupling ratios on CZE peaks of ABA–BSA. These projections were calculated

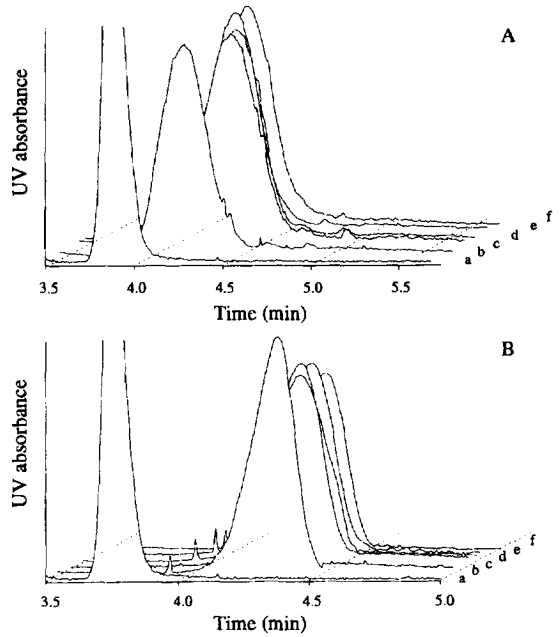

Fig. 5. Shifting of ABA–BSA peak in CZE as a function of coupling reaction time. (A) ABA–C4′-BSA coupling. Coupling reaction times were 0 h (a), 1 h (b), 2 h (c), 3 h (d), 4 h (e) and 5 h (f). (B) ABA–C1-BSA coupling. Coupling reaction times were 0 h (a), 1 h (b), 2 h (c), 3 h (d), 4 h (e) and 5 h (f).

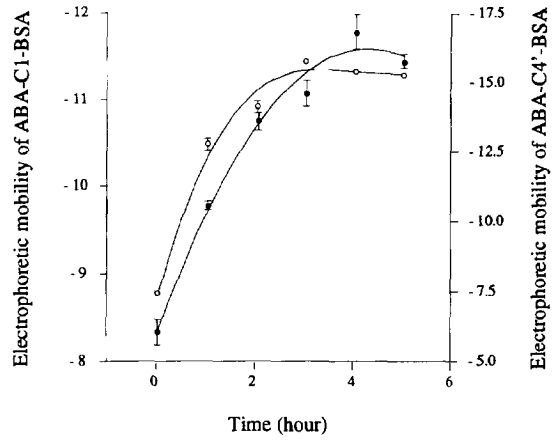

Fig. 6. Correlation between CZE electrophoretic mobility (μ) of ABA–BSA conjugates and coupling reaction time: ABA–C1-BSA (○), ABA–C4′-BSA (●). Each value was calculated on the average of four experimental CZE measurements. Standard deviations are not visible when they are less than 0.004. The regression coefficient is 0.994 for polynomial regression (magnitude 3) of ABA–C1-BSA and 0.993 for polynomial regression (magnitude 3) of ABA–C4′-BSA.

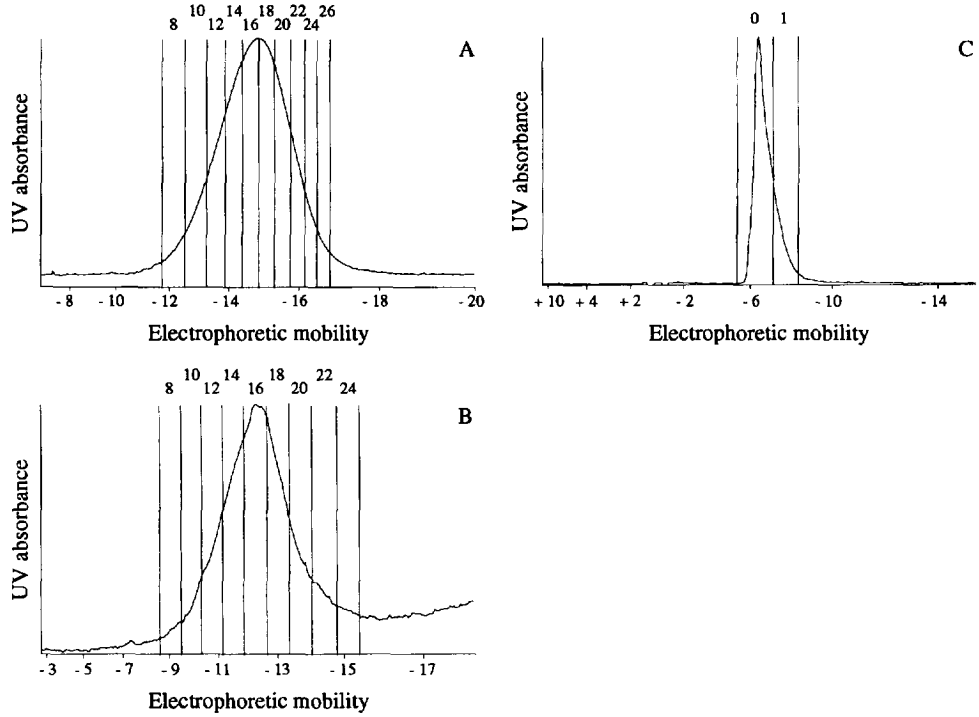

Fig. 7. Projection of molar coupling ratios on CZE peaks of ABA–C1-BSA (A), ABA–C4′-BSA (B) and native BSA (C). Molar coupling ratios (indicated on the top of electropherograms) were calculated from polynomial regressions (magnitude 3) presented in Fig. 4. The polynomial regression of ABA–C1-BSA coupling was used for native BSA projection.

from the equation of polynomial correlation curves (magnitude 3) presented in Fig. 4.

Projections show that the ABA–BSA molar coupling ratio distribution is ca. 17:1 ± 8 for ABA–C1-BSA (93% of the total peak area) and 17:1 ± 6 for ABA–C4′-BSA (89% of the total peak area). In order to verify the accuracy of this evaluation, a projection was calculated from ABA–C1-BSA coupling polynomial regression on native BSA (Fig. 7C). The native BSA peak was well centred on 0 coupling ratio, even if the shape of this peak was not symmetrical.

The maximal molar coupling ratio of ABA–C1-BSA coupling calculated by this means (25:1) is compatible with the number of accessible lysine residues (30–35 residues). On the other hand, the maximal ratio of ABA–C4′-BSA coupling (23:1) exceeds the number of tyrosine residues (19 residues). This contradiction can be explained by the fact that these values are calculated from an extended polynomial interpolation (to a non-experimental area of interpolation) with some imprecision. In other respects, ABA–BSA conjugates have different velocities according to their coupling ratios — this is the principle of CZE separation. Conjugates which reach the maximal coupling ratio shift slowly in the UV detector, therefore representation of these conjugates, calculated from area integration, is artificially more important.

In conclusion, projections give some qualitative elements concerning the distribution of ABA–BSA conjugates, but numerical values must be analysed cautiously.

4. Conclusions

Electrophoretic mobility in CZE was used for monitoring the hapten–protein coupling reaction of ABA on BSA, using two different C groups of ABA. A correlation between the ABA–BSA

molar coupling ratio and the electrophoretic mobility of the ABA–BSA conjugate was established. Under the conditions here described, we reached an optimal molar coupling ratio of 16.7:1 for the ABA–C4′-BSA coupling reaction, and of 16.8:1 for ABA–C1-BSA. CZE also allowed the monitoring of ABA activation reactions (synthesis of ABA–ABH and ABA–NHSC intermediates) and in that way specified yield, kinetic and steochiometric conditions of these reactions.

In comparison to classical tools for hapten–protein coupling ratio determination by evaluation of labelled hapten incorporation or spectrophotometric absorption modification, the use of CZE is an attractive alternative. The analysis of one sample takes less than 20 min (10 min for sample pre-purification by size exclusion, 8 min for CZE separation) and requires a limited quantity of ABA–BSA conjugate (about 1 nmol of BSA). The efficiency of this method is obvious in comparison with the time required for the process of dialysis of some milligrams of conjugate required for incorporated [^{3}H]ABA evaluation. Without CZE analysis characteristics, we were not able to study the kinetic and steochiometric conditions of these coupling reactions. Furthermore, when labelled standards are not available, CZE provides a new way to estimate the coupling ratio. The molar coupling ratio can be estimated as a relative coupling ratio from the maximal electrophoretic mobility. Furthermore, CZE analysis of conjugates provides some new information: the gaussian shape of the ABA–BSA peak gave us a qualitative evaluation of the distribution of ABA–BSA conjugates according to their coupling ratios.

These results show the benefit of CZE monitoring of coupling reactions. In that direction, the use of a photodiode array detector should provide very interesting information since the shifting of the hapten–protein conjugate according to the coupling ratio could be correlated to modifications of the protein absorption spectrum.

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