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Separation and Biospecific Identification of Subviral Particles of Human Rhinovirus Serotype 2 by Capillary Zone Electrophoresis

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During infection, human rhinoviruses undergo structural rearrangements of their capsid proteins from D-antigenic native virus (sedimenting at 150S upon sucrose density gradient centrifugation) to C-antigenic A-particles (sedimenting at 135S) and B-particles (sedimenting at 80S); the latter remain after release of the viral genomic RNA into the cytosol. Subviral particles with very similar properties can also be produced in vitro upon exposure to elevated temperatures or to low-pH buffers. This paper reports on the successful separation of native virus and 80S B-particles by capillary zone electrophoresis. Separation was carried out in an untreated fused-silica capillary (50 μm i.d., total length 50.0 cm, effective length 41.5 cm) at 20 °C and monitored with UV detection. The separation buffer was 100 mmol/L boric acid/borate (pH 8.3) and contained 0.5% sodium deoxycholate, 0.05% SDS, and 0.5% Triton X100R; the detergents were required to prevent viral aggregation and adsorption to the capillary wall. The analytes were identified from their characteristic spectra as determined by fast spectral scanning. Final confirmation was obtained by comparison of electropherograms from samples prior and after immunodeplition with antibodies specifically precipitating D- or C-antigen. The present method enables one to easily monitor and quantify these structural changes and thus to determine the most favorable conditions for complete conversion of native virus to 80S B-particles.

Human rhinoviruses (HRVs), the cause of ∼50% of all common cold infections, are particles with a diameter of ∼35 nm and a molecular mass of 8×10^6 Da. They are composed of 60 copies each, of 4 viral capsid proteins (VP1−VP4) forming an icosahedral shell that encloses an RNA genome with positive (messenger sense) polarity (for a recent review see, e.g., ref 1). These highly ordered structures easily undergo conformational modifications in vivo during the infection pathway or in vitro upon exposure to low pH or to elevated temperature.[2−4] Even prolonged storage above −80 °C results in viral damage.

Native virions exhibit a sedimentation coefficient of 150S upon rate zonal centrifugation in sucrose density gradients; structural modification results in subviral 135S A-particles and 80S B-particles.[2,5] With respect to the native structure, A-particles, which are considered intermediates in the uncoating process, have lost the innermost capsid protein VP4 but still contain RNA. Additional release of the RNA results in subviral B-particles, the end product of uncoating.

As native virus and subviral particles exhibit distinct surface charges and zeta potentials, their separation in an electric field is principally possible and has been demonstrated previously for poliovirus, which is related to rhinovirus, using small glass tubes.[6] Relying on the separation power of capillary zone electrophoresis (CZE) techniques, we have recently developed a very efficient method for the rapid assessment of the purity of rhinoviral preparations with the potential for quantification of native virus.[7] This method is now routinely used in the laboratory for the quality control of viral batches. However, whereas native virus could be unequivocally identified with this method, subviral particles could not be positively correlated with any of the peaks observed in the electropherograms. Apparently, they were not stable under the conditions used and the peaks represented products of further disintegration. Aiming at the determination of the three-dimensional structure of a complex between subviral particles and the monoclonal antibody 2G2[8] by electron cryomicroscopy,[9,10] a method for the rapid and easy identification and quantification of B-particles was required in order to adjust production conditions. It was thus a necessity to develop a buffer system, which preserves the integrity of virus as well as of 80S particles during CZE analysis. Off-line biospecific identification of the analytes was used to establish a correlation between the peaks seen in CZE and the respective viral particles.

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The choice of the separation buffer in capillary electrophoresis is not as trivial for viruses as it is for small ions. The problem here is similar to that often encountered in protein analysis. In many cases, one is confronted with a severe adsorptive interaction with the capillary wall leading to peak distortion or even to loss of the analyte due to irreversible adsorption (cf., e.g., ref 11). Another complication is the particular lability of rhinoviruses. The separation medium has to be chosen to minimize structural modifications of the virus during electrophoresis which might lead to peak broadening, loss of material, appearance of degradation products (which were not present in the original sample), or a combination of these. For these reasons, use of detergents, which are often added to the separation buffer to reduce protein adsorption, must be carefully evaluated. We here describe the result of the assessment of various buffer components and CZE conditions for their applicability in virus analysis. Biospecific techniques were combined off-column with CZE in order to aid in identification of the peaks. As a result, a method is presented that is able to efficiently separate native virus from 80S subviral particles.

EXPERIMENTAL SECTION

Apparatus. A ^{3D}Capillary Electrophoresis System (Hewlett-Packard, Waldbronn, Germany), with a diode-array detector was used to carry out all CZE experiments. Fused-silica capillaries, packed in a standard HP cassette, were purchased from Polymicro Technologies (Phoenix, AZ). Total capillary length was 50.0 cm, effective length 41.5 cm, i.d. 50 μm. The capillary was thermostated at 20 °C during all experiments.

Injection was accomplished by application of 50 mbar pressure to the inlet vial for 9 s. Positive polarity mode with 25 kV was used in all experiments (detector placed at the cathodic side of the capillary). Current generated was typically ~18 μA.

The detector signals were recorded at 205 and 254 nm. In some cases, the fast spectral scanning mode was used to aid in peak identification. Data were collected using the Hewlett-Packard Chemstation Software.

Reagents. Human rhinovirus serotype 2 (HRV2) was produced and purified from infected cell pellets as described previously.[7] The background electrolyte (BGE) was 100 mmol/L boric acid adjusted to pH 8.3 with 1 mol/L NaOH and containing 0.5% sodium deoxycholate, 0.05% SDS, and 0.5% Triton X100R as detergents. All samples were prepared in sample buffer, 100 mmol/L borate buffer (pH 8.3) without or with detergents added. In the latter case, the concentration of detergents corresponded to a 1/5 dilution of BGE.

Monoclonal antibodies (mAb) 3B10 directed against native virus[10] and mAb 2G2 directed against an epitope only present on subviral particles[8] were purified from hybridoma tissue culture supernatants by standard procedures. All other chemicals were purchased from E. Merck (Darmstadt, Germany) and were used without further purification. Reagent solutions were prepared in deionized water. Buffers were filtered through 0.45 μm cellulose nitrate membranes. All solutions were centrifuged for 2 min in a tabletop centrifuge at 5000*g* prior to CZE analysis.

Procedures. A new capillary was conditioned by flushing with 100 mmol/L hydrochloric acid, followed by water, 1 mol/L NaOH, and water for 10 min, each. Between the runs, capillary was flushed with 100 mmol/L NaOH, water, and BGE, for 2 min each, by applying ~950 mbar pressure. The sample buffer was supplemented with *o*-phthalic acid (20 μg/mL) as an internal standard (IS).

Immunodeplition. Immunodeplition with mAbs of different specificity was performed as described previously.[7] Briefly, to 40 μL of virus solution (~0.1 mg/mL) in sample buffer (with or without detergents added) between 10 and 250 μg/mL of the respective mAb in the same buffer (without IS) was added sequentially. Every addition was followed by sample incubation at room temperature for 1 h and centrifugation at 8000 rpm for 10 min whereupon the supernatant was analyzed by CZE.

Sucrose Density Gradient Centrifugation. A 15 μL aliquot of virus stock solution (~15 μg) was mixed with 5 μL (40 000 cpm) of [^{35}S]methionine-labeled HRV2, prepared essentially as described,[8] and clarified in an Eppendorf centrifuge at 13 000 rpm for 10 min. The supernatant was layered onto a continuous 10−40% sucrose density gradient in 50 mmol/L Tris-HCl (pH 8.0) containing 2 mmol/L $CaCl_2$ and spun at 40 000 rpm for 1 h in a Beckman TLS 55 rotor. The gradient was fractionated into 6-drop fractions (~70 μL) from the bottom. Where indicated, fractions were pooled, mixed with an equal volume of the sample buffer, and concentrated in Centricon tubes (10-kDa cutoff) to 1/8 of the original volume.

RESULTS AND DISCUSSION

Composition of Background Electrolyte. Subviral 80S B-particles are produced in vitro upon heating rhinoviruses to 56 °C for 10 min[3] or to 50 °C for 15 min.[4] When a viral sample was subjected to the former conditions (incubation at 56 °C for 10 min in sample buffer without SDS) and analyzed by CZE (using SDS-containing BGE as described in our previous paper[7]), however, no defined peaks were seen (data not shown). This might be due either to aggregation or to instability of the subviral particles in the SDS-containing BGE. Therefore, we investigated whether the presence of SDS (at 10 mmol/L) in the sample buffer during heating would result in dissociation of putative aggregates; this was indeed the case as five highly reproducible peaks were seen, indicating that the 80S B-particles are unstable in SDS at elevated temperatures, even at a concentration as low as 10 mmol/L. Apparently, they were completely disintegrated into capsid proteins and RNA. However, only one of these peaks could be identified as viral RNA whereas the others remained elusive, as they could not be correlated unambiguously with any of the capsid proteins (data not shown and ref 7). Upon elimination of SDS from the heated sample by centrifugation dialysis using Centricon tubes prior to CZE, again no defined peaks were apparent (data not shown). This suggests that the material reaggregated when SDS was removed. It thus became clear that another buffer system was necessary for the identification of subviral particles; nevertheless, detergent was required in both the sample buffer and BGE to prevent aggregation and capillary wall adsorption.

Radioimmunoprecipitation Assay (RIPA) Buffer as a Basis for BGE. The combination of detergents in RIPA buffer[12] is very effective in solubilizing protein aggregates but does not

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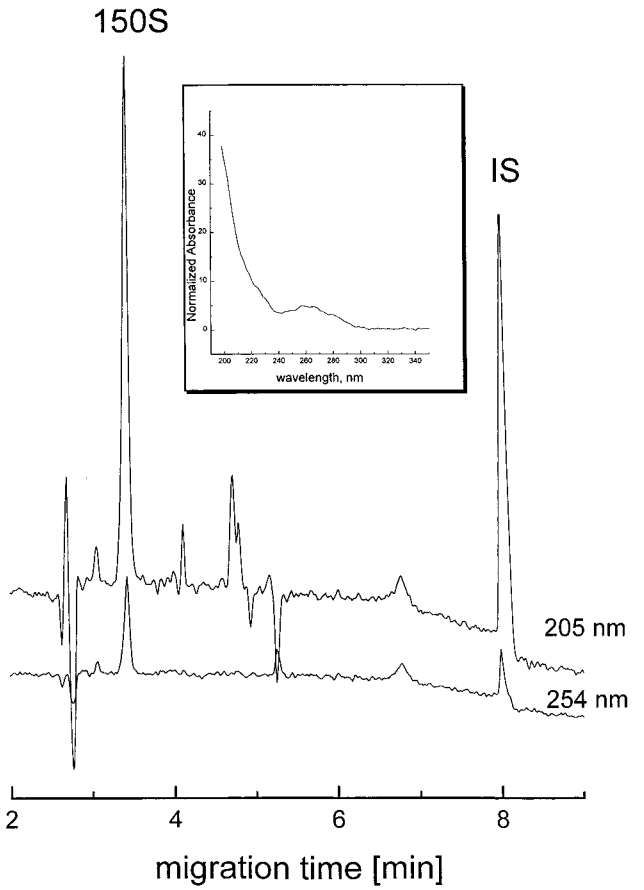

Figure 1. Electropherogram of purified HRV2 obtained with standard BGE (100 mmol/L sodium borate/boric acid (pH 8.3) containing 0.5% sodium deoxycholate, 0.05% SDS, and 0.5% Triton X100R as detergents). Uncoated capillary, 50 μm i.d. × 50.0 cm, effective length 41.5 cm. Virus (~200 μg/mL) was injected in 100 mmol/L sodium borate/boric acid (pH 8.3) without detergents. Voltage, +25 kV. IS, internal standard (*o*-phthalic acid, 20 μg/mL). The inset represents the normalized UV spectrum derived from the apex of the peak at 3.4 min.

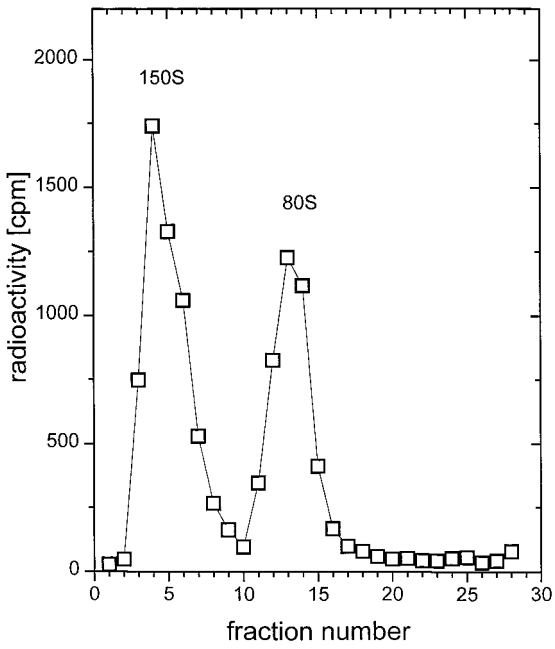

Figure 2. Separation of 150S native HRV2 and subviral particles by sucrose density gradient ultracentrifugation. About 15 μg of purified native HRV2 was mixed with ~40 000 cpm of [^{35}S]methionine-labeled HRV2 to be used as a marker. Centrifugation was for 1 h at 40 000 rpm in a TLS 55 Beckman rotor. Radioactivity in the fractions was determined from aliquots by liquid scintillation counting.

inhibit the interaction between various antigens and their cognate antibodies. Furthermore, this buffer has been used previously for *Staphylococcus aureus*-aided immunoprecipitation of subviral HRV2 particles.[8] RIPA buffer contains 1% sodium deoxycholate, 0.1% SDS, and 1% Triton X100. The latter compound was replaced by its reduced homologue Triton X100R, which has lower UV absorption. Furthermore, the concentration of the detergents was reduced; 100 mmol/L sodium borate/boric acid (pH 8.3), 0.5% sodium deoxycholate, 0.05% SDS, and 0.5% Trition X100R proved to be the best choice as BGE. This was used in all further experiments and is termed "standard BGE".

CZE Analysis of Native Virus in Standard BGE. A typical electropherogram of native HRV2 using standard BGE is shown in Figure 1. As indicated from the spectrum (inset in Figure 1), the main peak recorded at 205 and 254 nm with a migration time of 3.4 min contains RNA and thus most probably represents native virus. As observed previously, a peak with a migration time of 3.0 min corresponds to an impurity usually found in virus prepared from the infected cell pellet by poly(ethylene glycol) precipitation followed by sucrose gradient centrifugation.[7] The peak at 4.2 min and the negative peaks at 4.9 and 5.1 min are all "system" or "vacancy" peaks; they result from the difference in composition between BGE and sample buffer as seen from control runs (not shown). Generally, the electropherogram appears very similar to that reported in our previous paper and shows one major peak of native HRV2 with a negative effective mobility (10.9×10^{-9} m^2 V^{-1} s^{-1}) and some minor impurities.[7]

Confirmation of Peak Identity with Native HRV2. Rate zonal ultracentrifugation in sucrose density gradients is commonly used for virus purification and analysis, since it reliably separates native virus, subviral particles, and low-molecular-weight impurities. Thus, we first investigated whether it was possible to identify by CZE those fractions of the gradient that contained native virus. Purified HRV2 was mixed with [^{35}S]methionine-labeled HRV2 (used as a marker) and placed on top of a preformed 10−40% (w/v) sucrose density gradient. Twenty-eight fractions were collected after centrifugation from the bottom of the gradient by puncturing the tube with a needle, and radioactivity was determined from aliquots by liquid scintillation counting. As expected from the calculation of the centrifugation time, the peak of native HRV2 was recovered in fraction 4 whereas a substantial amount of 80S B-particles was present in fractions 13 and 14 (Figure 2). The small shoulder at fraction 6 most probably represents 135S A-particles. Subviral particles were only present in the radiolabeled virus because it had undergone substantial denaturation upon storage. Initially, the fractions were directly analyzed by CZE, but it turned out that the concentration of virus in the fractions was below the detection limit. Therefore, fractions 1−3, 4−6, and 7−9 (see Figure 2) were combined and concentrated 8-fold by centrifugation dialysis in Centricon tubes. As depicted in Figure 3, the results of CZE analysis of the pooled fractions closely reflected the distribution of the virus in the gradient fractions. The peak in the electropherogram originating from the 150S particles collected from fractions 4−6 corresponds to maximum

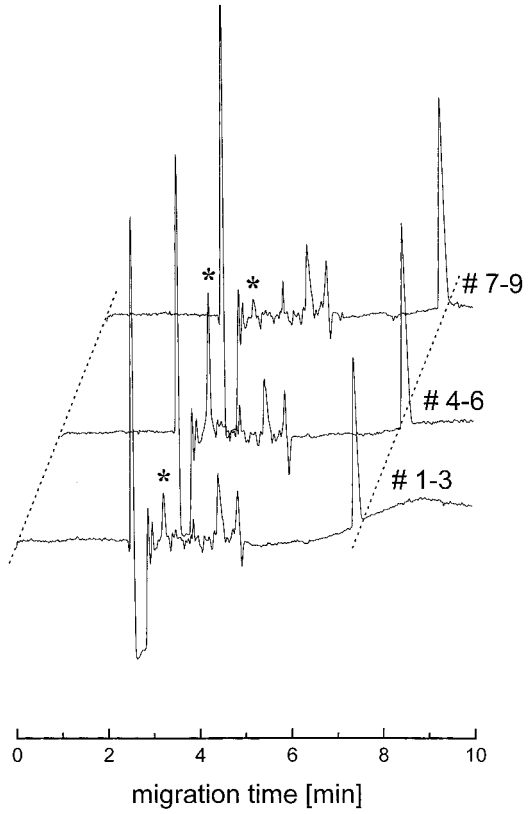

Figure 3. CZE analysis of three pools of fractions collected from sucrose density gradient centrifugation. Fractions 1−3, 4−6, and 7−9 (see Figure 2) were combined and concentrated 8-fold by centrifugation dialysis. The peak marked with an asterisk represents 150S viral particles. All other conditions were as in Figure 1 except that detergents (corresponding to 1/5 diluted BGE) were present in the sample buffer.

radioactivity in Figure 2. As expected, smaller amounts of virus were found in the pools of fractions 1−3 and 7−9.

Fractions corresponding to subviral particles were not analyzed in this experiment because only tiny amounts of unlabeled 80S and 135S particles were expected to be present in this recently prepared virus batch. Attempts at isolating 80S subviral B-particles (prepared by incubation of virus at 56 °C) from sucrose density gradients in concentrations sufficient for subsequent CZE analysis failed due to extensive aggregation.

CZE Analysis of Heated Virus. Native HRV2 was converted into 80S subviral particles by heating ∼200 μg of HRV2 to 56 °C for 10 min[3] in sample buffer containing detergents and analyzed by CZE. As seen in Figure 4A, the peak corresponding to native HRV2 at a migration time of 3.4 min virtually disappeared whereas a new major peak at 3.9 min emerged. Fast-scanning spectral analysis revealed an extinction maximum at 280 nm indicating the presence of protein but the absence of RNA. These findings allowed tentative assignment of this peak to 80S subviral B-particles. With the exception of a minor peak at 4.4 min, no additional distinct peaks were seen. This result thus differs significantly from that obtained previously where both heating of the virus and subsequent analysis were carried out in the presence of 10 mmol/L SDS; under these latter conditions, five new peaks had emerged.[7]

No peak attributable to intact RNA was seen in the electropherogram. However, when compared to a control sample that had not been heated (compare to Figure 1), it was noted that the baseline exhibited more chemical noise with some tiny peaks exhibiting absorption at 254 nm. These might originate from partially degraded RNA caused by RNases probably present as contaminants in the viral sample.

Immunological Identification of the Major Peak as 80S Subviral B-Particles. One of the most accurate means of identification of a given component is by a change of the corresponding electropherogram upon an appropriate biospecific reaction, the principle of affinity capillary electrophoresis. Immunodeplition was thus used for further identification. Monoclonal antibody 3B10 recognizes a conformational epitope on the viral capsid which is not lost upon heating; however, as the amino acid residues making up the binding site are contributed from different proteins, dissociation of the capsid into single polypeptides by more drastic measures leads to loss of affinity.[10] On the other hand, mAb 2G2 does not bind to native virions but rather recognizes a structural epitope only present on subviral particles.[8] Reaction of these antibodies with their cognate antigens leads to aggregation, facilitating the removal of the complexes by centrifugation.

The heated virus sample shown in Figure 4A was therefore incubated with increasing amounts of mAb 3B10, and aggregates formed between antibody and any viral particles were removed by centrifugation under conditions (10 min at 8000 rpm) described in a previous paper.[7] Subsequent analysis of the supernatant by CZE showed that the peak putatively identified as 80S particles successively diminished (not shown) and finally disappeared completely (compare Figure 4A and B). These results exclude that the peak at 3.9 min corresponds to any isolated viral protein and thus provides additional evidence for its representing 80S subviral particles.

In a second set of experiments, mAb 2G2 was used. To allow for discrimination between native HRV2 and 80S subviral B-particles during the same analysis, the sample was incubated for 2.5 min at 56 °C in order to allow for the conversion of only ∼50% of native virus to subviral particles. Immunodeplition was carried out as for 3B10. Comparison of parts A and B of Figure 5 demonstrates that the peak assigned to 80S subviral particles strongly decreased upon addition of 2G2 and centrifugation. However, it failed to disappear completely but rather became broader. This might indicate the formation of heterogeneous complexes containing different ratios of 2G2 and HRV2. Apparently, such complexes could not be quantitatively removed by centrifugation. As expected, the peak corresponding to native virus remained unaffected.

Monitoring Conformational Changes of HRV2 Induced by Heating. To determine favorable conditions for the preparation of 80S subviral particles, the degree of structural modification of native virus as a function of incubation time at 50 [4] or at 56 °C[3] was monitored by CZE. HRV2 (8 μg/40 μL) was incubated at 50 °C in sample buffer with detergents for the time periods indicated in Figure 6 and subjected to CZE analysis. The heights of peaks corresponding to the 150S and 80S particles, respectively, were determined and plotted against the time of incubation. Notably, the conversion from native virus to 80S particles at 50 °C occurred much more slowly than anticipated from the paper by Lonberg Holm and Noble Harvey, who reported on extensive modification

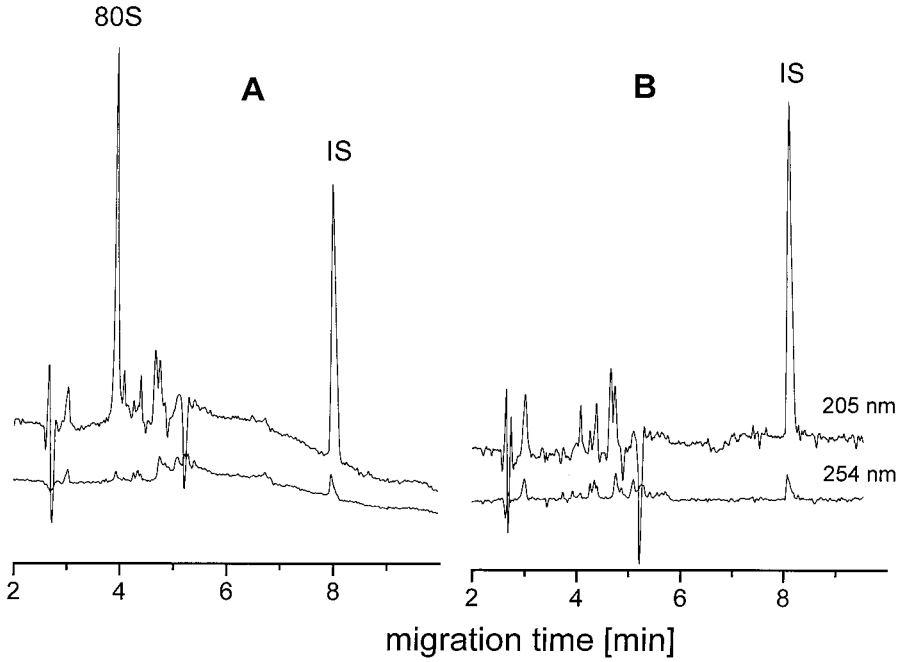

Figure 4. Electropherogram of heated HRV2. A viral sample as in Figure 1 was incubated at 56 °C for 10 min in sample buffer containing detergents prior to analysis (A) to the heated viral sample, monoclonal antibody 3B10 was added in excess and precipitates were removed by centrifugation for 10 min at 8000 rpm prior to CZE analysis (B). All other conditions were as specified in Figure 3.

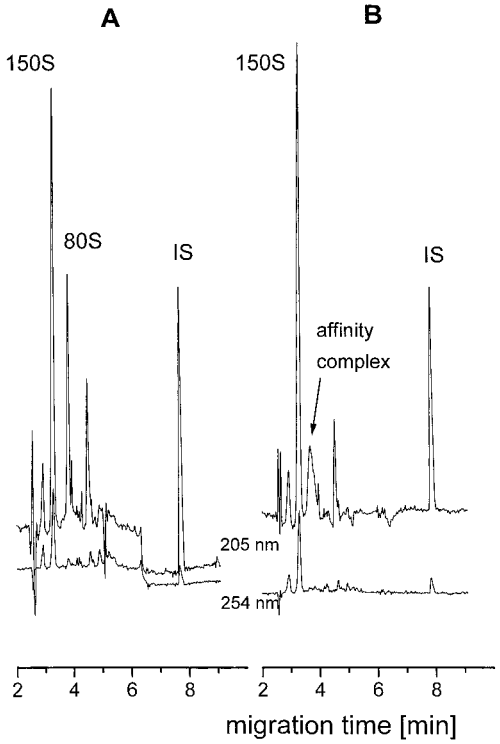

Figure 5. CZE separation of subviral 80S particles and native virus 150S. Viral sample as used in Figure 1 was heated at 56 °C for 2.5 min in a sample buffer containing detergents (corresponding to 1/5 BGE) prior to analysis (A); to the heat-treated viral sample, an excess amount of monoclonal antibody 2G2 was added and precipitates were removed by centrifugation (B). For other conditions, see Figure 3.

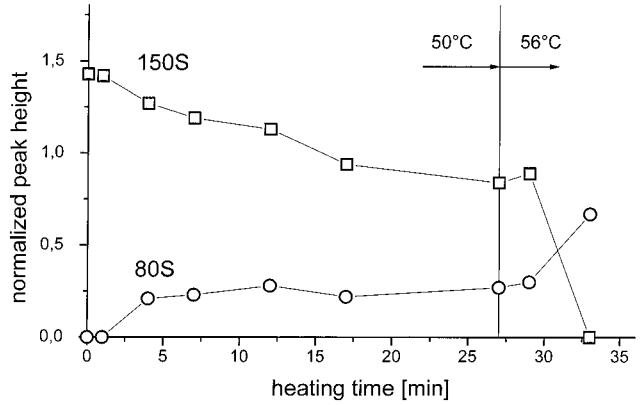

Figure 6. Time course of conformational change of HRV2 induced by incubation at elevated temperature. A viral sample as used in Figure 1 was incubated at 50 °C in sample buffer (containing detergents) for up to 27 min, then the temperature was raised to 56 °C, and incubation was continued for additional 6 min. At the time points indicated, a sample was analyzed by CZE. The normalized peak height is given as the ratio of the peak height corresponding to native virus or to 80S subviral B-particles, respectively, to that of the internal standard. Other conditions as in Figure 3.

upon incubation for 15 min.[3] Even after 27 min, native virus was only converted to an extent of ~40%. However, when the temperature was raised to 56 °C, the rate of conversion was found to be drastically increased and the reaction was essentially complete within 5 min. The reason for the enhanced stability of HRV2 at 50 °C with respect to that reported in the earlier work by Lonberg Holm and Noble Harvey might be the presence of SDS in the sample buffer; this detergent has been demonstrated to substantially protect HRV2 against denaturation.[4] In addition to the peaks from native virus and from 80S particles, no other peaks were observed and the total amount of native virus plus 80S particles remained constant over time (data not shown). This indicates that no further modification of the subviral particles had taken place during the time scale observed and incubation at 56 °C for 10 min appears to be optimal for the large-scale production of homogeneous 80S B-particles.

CZE Analysis of Acidified HRV2. The buffer conditions described above were best suited for the separation of 150S and 80S viral particles. However, no peak, which might have corresponded to 135S particles, could be assigned. These particles can be produced in vitro upon treatment at pH values below 5.6.[3,4,13] Virus (∼100 μg/mL) was thus incubated in 50 mmol/L acetic acid/acetate buffer (pH 5.0) for 30 min at room temperature and neutralized, and CZE analysis was carried out. This treatment resulted in a large number of small peaks most probably resulting from extensive aggregation due to the increased hydrophobicity of the acidified virus.[3] Increasing the detergent concentration in the sample was not beneficial to the resolution (data not shown).

CONCLUSIONS

In the present paper, it is demonstrated that native virus and 80S subviral particles from human rhinovirus serotype 2 can be separated by CZE using a ternary mixture of detergents as background electrolyte. The detergents appeared to be important for the prevention of aggregation of subviral particles and adsorption to the capillary wall. Immunodeplition with monoclonal antibodies with distinct specificities toward the different viral conformations was used to confirm the identity of the peaks. This CZE method provided the power and speed of analysis that allowed adjusting the particular conditions of complete conversion of native HRV2 to 80S B-particles. These latter particles can now be prepared under favorable conditions in quantities sufficient for the determination of the binding topology of monoclonal antibody 2G2; this antibody recognizes a particular epitope which is absent from native virus but appears on subviral particles upon conversion. Knowledge of the processes involved in these conversions will be gained from structural analyses to be carried out in the future; these will help to understand the mechanism of release of the viral RNA into the cytoplasm.

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