Denaturation of proteins and nucleic acids by thermal-gradient electrophoresis

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A polyacrylamide-gel-electrophoresis method has been developed that permits the analysis of conformational changes that occur during the thermal denaturation of macromolecules. A stable transverse temperature gradient was produced in an aluminium heating jacket clamped around a vertical polyacrylamide slab gel. After temperature equilibration, gels were loaded with either a layer of protein solution (20–200 µg/gel) or a solution of double-stranded DNA (20 µg/gel) and electrophoresis begun. At the end of the run the gels were stained and the effect of temperature on mobility observed. The technique proved informative both for the irreversible unfolding of proteins (*Drosophila* alcohol dehydrogenase and lactic acid dehydrogenase) and for a protein that was reversibly denatured by heat (β -lactamase). In the latter case a clear transition between the native enzyme and a slower-migrating denatured state was observed. The patterns obtained were analogous to the type produced by the transverse-urea-gradient-electrophoretic method of Creighton [(1979) *J. Mol. Biol.* **129**, 253–264]. The method also resolved a complex mixture of double-stranded-DNA restriction-digest fragments.

Gel electrophoresis is an analytical method that is capable of resolving gross changes in the overall conformation of macromolecules, as well as distinguishing differences in net electrostatic charge. The present paper describes the application of this method to the study of temperature-induced transitions in the structure of proteins and nucleic acids.

Temperature effects on the structure of proteins and nucleic acids have been studied by a wide variety of other biophysical techniques. At a critical specific temperature a rapid transition usually occurs from the native state to a state or states of relatively little organized tertiary structure. The effective volume of such denatured states is consequently much larger than the native state and theoretically they should migrate at significantly slower rates during electrophoresis in a concentrated gel matrix. The analysis of such changes in electrophoretic mobility can be conveniently determined in a single experiment by slab-gel electrophoresis into a stable transverse temperature gradient. This method was initially devised to study the relative thermostability of the alcohol dehydrogenase alloenzymes of *Drosophila* [see the following paper (Thatcher & Sheikh, 1981)] and is conceptually analogous to the transverse-urea-gradient-electrophoresis methods of Creighton (1979) and Fischer & Lerman (1979).

Experimental

Materials

Acrylamide monomer was obtained from Eastman-Kodak, Kirby, Liverpool, U.K., and was recrystallized from chloroform before use. *NN'*-Methylenebisacrylamide, ribonuclease A (EC 3.1.27.5) and pig heart lactate dehydrogenase (EC 1.1.1.27) were purchased from Sigma, Poole, Dorset, U.K. All other biochemicals and laboratory reagents were obtained from BDH, Poole, Dorset, U.K. A sample of β -lactamase (penicillinase, EC 3.5.2.6) from *Staphylococcus aureus* was kindly given by Dr. J. Moulton, and Dr. R. Everett supplied purified DNA from the plasmid PED822 (this is a pBR322 clone containing a 3.1-kilobase insert of DNA).

Apparatus and methodology

An ordinary vertical slab-gel apparatus was used in these experiments. The top buffer reservoir was adjustable in height and could accommodate the gel and heating jacket assembly. The jacket was made from two pieces of aluminium plate (12.5 cm $\times$ 21.5 cm $\times$ 1.3 cm) and the ends of each were engineered so that hot or cold liquid could be circulated through them. Thermal conduction led to

the development of identical temperature gradients along each plate of the jacket and along the gel mould clamped between them (Fig. 1a). Each plate end had two vertical holes, 2 cm apart, drilled through (holes labelled A in Fig. 1b). Two more holes were then drilled in horizontally (holes labelled B in Fig. 1b) so that the upper hole fused with the inner vertical hole and the lower horizontal hole

Fig. 1. Construction of the heating jacket

(a) Assembly of the heating jacket and the operating principle. Water at two different temperatures is circulated through the ends of two aluminium plates by using two thermostatically controlled circulating water baths (A and B). The plates are connected in series so that an identical thermal gradient develops across each plate by conduction. An ordinary vertical polyacrylamide slab gel is then clamped between the plates and electrophoresis performed as usual. (b) Construction of one end of the aluminium plates. The letters refer to various details of construction referred to in the text.

joined the outer vertical hole. A brass plug was then inserted in the outer vertical hole (plug labelled C in Fig. 1b) and a channel milled at the bottom of the aluminium plates to provide a connection between the vertical holes. The top and bottom of the plates were then capped with brass (plates labelled D in Fig. 1b). Nipples were then screwed into the entry and exit ports so that each end could be connected to Haake circulating water baths. Holes 1 cm apart and 1 cm deep were drilled into the top of the outer plate of the jacket (holes labelled E in Fig. 1b). These holes were half-filled with liquid paraffin and were used to monitor the temperature gradient in conjunction with a thermistor probe and chart recorder. The outer faces of the jacket were lagged with 6 mm-thick asbestos sheeting in order to minimize heat loss.

Protein gels were prepared by using two polyacrylamide-gradient recipes (20–12%, w/v and 10–7%, w/v) at either pH 4 or pH 8.6. No attempt was made to choose electrophoresis buffers whose pH would not be expected to vary with temperature. However, the buffers that were employed actually varied less than 0.3 pH unit over the temperature ranges used, and the pH chosen for a particular experiment was always at least 2 pH units away from the pI of the proteins under study. Therefore the effect of temperature on the ionization of the components of the electrophoresis buffers had a minimal effect on the net charge of the proteins being electrophoresed.

The inside faces of the aluminium plates were smeared with silicone grease and the glass plates enclosing the polymerized gel inserted between them and clamped, the top of the heating jacket being 5 mm below the gel meniscus. The circulation baths were switched on and after at least 1 h of equilibration the sample was loaded. Between 20 and 200 μ g of protein or 20 μ g of DNA were dissolved in 400 μ l of electrophoresis tank buffer. After mixing with 200 μ l of 50% (w/v) sucrose the sample was loaded quickly on to the top of the gel with a 100 μ l Eppendorf micropipette. During electrophoresis runs the temperature at the central thermistor position in the heating jacket was measured and routinely varied less than 1°C. At the end of each electrophoresis run the positions of the thermistor probes were marked on the glass gel plates and the temperature at each position recorded. The apparatus was then dismantled and the inner glass plate, facing the reservoir, was carefully separated from the gel. The position of the thermistor probes could then be marked on the gel with a hypodermic syringe loaded with Indian ink. The gels were either stained with 0.1% (w/v) Coomassie Blue or with ethidium bromide (1 μ g/ml).

Electrophoresis details. The gels were poured in moulds consisting of two glass plates (220 mm $\times$

200 mm × 3 mm) separated by 1.5 mm Perspex (Lucite) spacers. Protein gels were transverse polyacrylamide gradient gels, gradients running either from 20 to 12% (w/v) polyacrylamide or from 10 to 7% (w/v) polyacrylamide.

10–7% gels. Two solutions, A and B, were prepared. Solution A contained 5.63 ml of Stock A [40% (w/v) acrylamide monomer], 0.9 ml of Stock B [3% (w/v) *NN'*-methylenebisacrylamide], 20 μ l of *NNN'*-tetramethylethylenediamine, 2 ml of riboflavin solution (8 mg/100 ml of water) and either 1.25 ml of the Tris/EDTA/boric acid buffer, pH 8.6, described by Ursprung & Carlin (1968) or 2.5 ml of Tris/acetate buffer, pH 4.0 (0.3 M-Tris adjusted to pH 4.0 with acetic acid). The final solution A volume was made up to 25 ml with distilled water. Solution B contained 4.0 ml of Stock A, 0.6 ml of Stock B and the same volumes of the other solutions added to solution A to a final volume of 25 ml. A transverse polyacrylamide gradient gel was then poured in such an orientation that in the final assembly the concentrated-acrylamide end of the gel would be at the warmer end of the temperature gradient.

20–12% gels. The gel solutions were prepared as described above, except that solution A in this case contained 11.5 ml of Stock A and 2 ml of Stock B and solution B contained 6.75 ml of Stock A and 1.22 ml of Stock B.

The gels were polymerized by exposure to fluorescent light for 30 min. The reservoir buffers were diluted from the gel buffer concentrates as follows: Tris/EDTA/Boric acid, pH 8.6, was diluted 1 + 19; Tris/acetate, pH 4.0, was diluted 1 + 9. Electrophoresis was performed at 2 mA/cm of gel for 2–4 h.

The gel used for DNA separation was a non-gradient slab gel prepared by mixing 4.5 ml of Stock A with 0.8 ml of Stock B, 5 ml of the pH 8.6 buffer, 12 g of urea, 50 μ l of *NNN'*-tetramethylethylenediamine and 500 μ l of 1% (w/v) ammonium persulphate to a final volume of 50 ml with distilled water.

Results and discussion

Temperature obviously influences many other components of the gel-electrophoretic system apart from affecting the shape of the macromolecules being sieved through the gel. The *pK* of the ions in the electrophoresis buffer, the ionization of charged groups on the amphoteric sample molecule and the structure of the polyacrylamide gel itself will all be affected by temperature. Most importantly, the velocity of migration of all ions in the system will be increased at higher temperatures. The rationale adopted in these experiments has been to compensate for the latter effect empirically, and to minimize the former effects by a judicious choice of tempera-

ture gradient and pH for each application. Preliminary experiments established that ribonuclease A, exposed to a non-denaturing temperature gradient of 20–60°C (2°C/cm gel) at pH 4.0, migrated as a horizontal band through either a 20–12% (w/v) transverse polyacrylamide gradient or a 10–7% (w/v) transverse polyacrylamide gradient. By using these gradient gels and similar temperature gradients, the denaturation of three proteins has been studied.

The denaturation of *Drosophila* alcohol dehydrogenase has been investigated in detail by thermal gradient electrophoresis [see the following paper (Thatcher & Sheikh, 1981)]. In these experiments the alcohol dehydrogenase migrated as a distinct horizontal band at both pH 8.6 and pH 4.0. At the 'melting' temperature this native enzyme band ceased abruptly and no transition zone between this state and the heat-denatured aggregate, smeared at the top of the gel, could be discerned. Pig heart lactate dehydrogenase showed an identical denaturation pattern. On electrophoresis at pH 8.9 the enzyme 'melted' at 58°C (120 min electrophoresis). Most proteins that undergo irreversible thermal denaturation probably show similar patterns. Denaturation presumably involves two inseparable processes: unfolding, followed by aggregation and a rapid decrease in solubility. Experiments with two electrophoretic variants of *Drosophila* alcohol dehydrogenase showed that the method is capable of revealing the effect of temperature on subunit exchange in multimeric proteins [see the following paper (Thatcher & Sheikh, 1981)].

At high temperature, many of the proteins commonly used in denaturation studies unfold reversibly. Sharp transitions are observed at the 'melting' temperature, and the denatured states are quite soluble. Unfortunately the 'melting points' of most of these proteins are beyond the practical limits of the thermal-gradient-gel method (i.e. 10–60°C). However, Brandts *et al.* (1965) have shown that the 'melting' temperature of one of these proteins, ribonuclease A, decreases rapidly as the pH is decreased, and at pH 3.0 undergoes a transition with a mid-point of approx. 40°C. Thermal-gradient gel electrophoresis at this pH revealed no obvious unfolding transition of native ribonuclease A to a state having decreased electrophoretic mobility (Fig. 2). At approx. 50°C a second slower-migrating species did appear above the native band. It is not known whether this band represents a slower refolding form of the enzyme or an irreversibly unfolded state generated by thiol oxidation at high temperature.

The β -lactamase of *Staphylococcus aureus* is relatively sensitive to solvent denaturation (Robson & Pain, 1976*a,b*; Carrey & Pain, 1978; Creighton & Pain, 1980) and the process appears to be fully

Fig. 2. Thermal-gradient electrophoresis of ribonuclease A at pH 3.0

A 20–12% (w/v) polyacrylamide gel was poured transversely, incorporating a 0.1 M (final concn.) -glycine/HCl buffer at pH 3.0. This gel was loaded with 200 μ g of ribonuclease A and run for 120 min at a current of 1.5 mA/cm of gel. The temperature gradient increased linearly from 20°C (right) to 60°C (left) and was quantified by measuring the local temperature at various points along the adjacent heating jacket, as described in the text.

reversible. Thermal-gradient electrophoresis at pH 4.0 shows that the enzyme is also relatively thermolabile, and irreversible aggregation of the denatured state was also not apparent (Figs. 3 and 4). β -Lactamase migrated as a sharp band, showing a well-defined transition to a slower-migrating discrete electrophoretic species at higher temperature (above 50°C). The decrease in electrophoretic mobility of the heat-denatured form was unlikely to have been due to a decrease in net charge of the enzyme as Bull & Breeze (1973) have presented evidence that only small changes in isoelectric point accompany thermal denaturation. The gel patterns obtained in these β -lactamase experiments are remarkably similar to those obtained by Creighton (1979) with the urea-gradient-electrophoresis method. By analogy with this technique, the slower migrating β -lactamase species formed at high temperature consists of a preponderance of protein molecules in open unfolded conformations. These denatured states are retarded more effectively by the polyacrylamide gel during electrophoresis and form a compact band of decreased mobility. The distinct transition zone forms because of the rapid inter-conversion of molecules between the native and denatured states. At lower temperatures most of the β -lactamase molecular population is in the native conformation, whereas at higher temperatures the equilibrium begins to favour the open conformational states. The actual position of the protein band in the transition zone is a function of the average mobility of the molecular population. On urea-gradient electrophoresis, β -lactamase forms a diffuse transition zone, which is due to the slow kinetic equilibrium between the native enzyme and a partially folded intermediate state, H (Creighton &

Fig. 3. Thermal-gradient electrophoresis of β -lactamase: unfolding gel

Native *Staphylococcus aureus* β -lactamase (200 μ g) was loaded on to a 20–12% (w/v) transverse polyacrylamide gradient gel (pH 4.0). The gel was run for 200 min and stained for protein as described in the text. T_m is the 'melting' temperature.

Fig. 4. Thermal-gradient electrophoresis of β -lactamase: refolding gel

β -Lactamase (200 μ g) was dissolved in the sucrose sample buffer (pH 4.0) and heated in a water bath at 70°C for 5 min. The gel was then loaded and run under conditions identical with those used for the unfolding gel (Fig. 3).

Pain, 1980). By contrast, the thermal-gradient gels for β -lactamase always showed a distinct transition zone. This pattern suggests that the thermal denaturation of *S. aureus* β -lactamase can be described in terms of a rapid, reversible and co-operative unfolding process, with no detectable intermediates forming between the native conformation and the unfolded states.

Two small spurs were always present on the shoulder of the temperature transition suggesting (again by analogy with the urea-gradient-gel method) that two or more minor conformational varieties of the denatured state are unable to refold rapidly. When β -lactamase was preheated to 70°C for 5 min just before electrophoresis, these spurs were intensified and stretched well into the low-temperature zone of the gel (Fig. 4). A second, completely separate, protein band of lower electrophoretic mobility was also formed at high temperature and resembled the band formed in the pH 3.0 gels for ribonuclease A. Both of these slow-refolding

Fig. 5. *Separation of DNA fragments by thermal-gradient electrophoresis*

Purified DNA (20 μ g) from the plasmid PED822 (a pBR322 derivative clone containing a 3.1-kilobase DNA insert) was digested with the restriction endonuclease *Hae* III in 6 mM-NaCl/6 mM-Tris/HCl (pH 7.4)/6.6 mM-MgCl₂/10 mM-2-mercaptoethanol. The digest was then diluted into Tris/EDTA/borate sample buffer, pH 8.6, and applied to a 3.6% (w/v)-polyacrylamide slab gel, pH 8.6, containing 4 M-urea. The DNA was electrophoresed into a 30 (right)–75°C (left) temperature gradient for 150 min. After soaking the gel in ethidium bromide (1 μ g/ml) for 30 min, the gel was photographed under u.v. light.

species could conceivably have been produced by some physical barrier to rapid refolding, like the isomerization of proline residues (Brandts *et al.*, 1975), but the phenomenon is more likely due to the temperature-induced chemical modification of one or more labile amino acid residues. The deamidation of glutamine and asparagine are the only likely sources of covalent modification of the unfolded β -lactamase as cysteine and tryptophan are not present in the amino acid sequence. At least five such residues have been shown to be prone to deamidation (Ambler, 1975).

The reproducibility of the thermal-gradient-electrophoresis method was estimated by using the β -lactamase enzyme pattern. In six experiments the 'melting point' of the enzyme, i.e. the mid-point of the transition, was $49.5 \pm 1^\circ\text{C}$ (S.D.).

DNA denaturation

Gel electrophoresis under non-denaturing conditions separates double-stranded DNA fragments on a molecular-weight basis. As the conditions required to denature double-stranded DNA are dependent on base sequence and not on the number of bases in that sequence, gel electrophoresis in the presence of denaturants can add another dimension to the analytical power of the technique. Fischer & Lerman (1979) have shown that a complex restriction-enzyme digest of bacteriophage DNA is resolved by transverse urea/formamide-gradient

electrophoresis at 60°C. Fig. 5 shows that the thermal-gradient-gel-electrophoresis method resolves a mixture of double-stranded DNA fragments with comparable sensitivity.

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