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Analytical Techniques for Cell Fractions

XXIII. A Stable Thermal Gradient Device for Heat Denaturation Studies on Proteins[1,2]

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The ISO–DALT two-dimensional electrophoretic system (1,2), based on the method of O'Farrell (3), is capable of performing large numbers of analyses on complex mixtures of proteins. However, both separations employed are carried out under dissociating or denaturing conditions and no enzyme activities are readily observable in the analyzed proteins. In order to identify the spots corresponding to particular enzymes, it is therefore necessary to employ some nondestructive resolving technique first and as a second step to perform both enzyme and two-dimensional electrophoretic analyses on the fractions generated. By correlating enzyme activity with intensity of various spots on the two-dimensional gels throughout the series of initial fractions, identifications can be made. This approach, unlike the more direct immunoprecipitation methods (4), requires the running of large numbers of enzyme analyses and two-dimensional gels and some convenient initial resolving procedure. Convenient and rapid techniques for the analyses (5,6) and gels (1,2) have been described previously in this series and elsewhere. This paper deals with the use of selective denaturation in a temperature gradient as an initial resolving procedure and describes a simple thermal gradient device for generating such a gradient.

STABLE THERMAL GRADIENT DEVICE

The simplest arrangement by which a temperature gradient may be established is one in which a long thermal conductor is heated at one end

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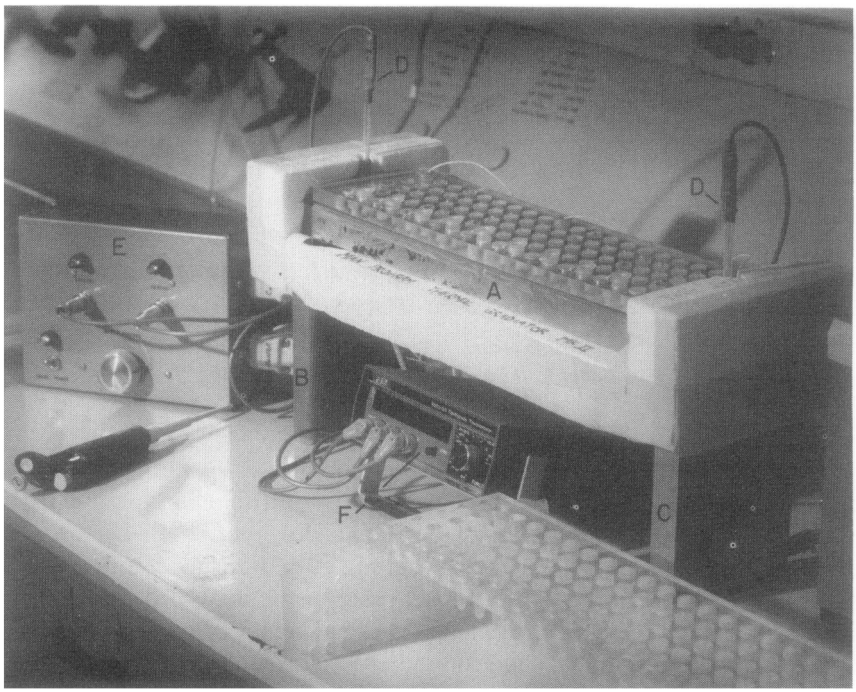

FIG. 1. Stable thermal gradient device and controller. The aluminum conductor strip (A), along which the temperature gradient is formed, has 5 rows of 20 wells and is supported in a flat horizontal position by two piers. The left pier (B) is wrapped with an electric heating element and the right pier (C) has an internal serpentine cooling chamber provided, at the back, with inlet and outlet hose connections. Mercury thermoregulators (D; set for 100 and 40°C) are mounted in vertical holes in the left and right piers. These are connected to the controller (E) which switches the heater on and off to maintain the left pier center at 100°C and the flow of tap water on and off (by solenoid valve) to maintain the right pier at 40°C. An additional variable resistance is provided to adjust the "on-cycle" heat output of the electric heater. The conductor strip is insulated on its top surface with 1/16-in. sheet silicon rubber and along its bottom surface with polyurethane. A digital thermistor thermometer (F) is connected to three probes in water-filled Microfuge tubes situated at positions 1, 10, and 20 in the back row. The temperature gradient is thereby continuously monitored. One Lucite acrylic resin tube-holding rack is shown in position on the thermal gradient device and another (bottom right) is being loaded.

and cooled at the other. Provided that the end temperatures and the geometry are constant, a stable temperature gradient will be produced. The device shown in Fig. 1 follows this plan and consists of a 16 × 4.5 in. strip of aluminum 1 in. thick. This thermal conductor is bolted at either end to supporting piers (also 4.5 in. wide and 1 in. thick) which are kept at two constant temperatures by thermoregulators controlling, in one case, an electric heater, and in the other, the flow rate of cold tap water through a cooling chamber in the pier. Temperature is monitored at various points along the

gradient by a switchable digital thermometer. Some insulation is used to reduce external heat leakage.

Five parallel rows of 20 wells (7/16 in. diameter, 7/8 in. deep) in the conducting strip constitute the experimental temperature points. In operation, 200-μl aliquots of sample protein solution are placed in Beckman Microfuge B tubes in rows of 20 (one row per experiment) in a special Lucite acrylic resin rack [a sheet of 14 × 4.5 × 1/4-in. Lucite drilled with the same pattern of 20 × 5 holes (7/16 in. diameter) as the conductor strip and capable of snugly holding the plastic Microfuge tubes by their lips]. At time 0, this rack containing up to five rows of tubes is placed on top of the conducting strip so that the bottom 7/8 in. of each Microfuge tube extends into a well (half filled with water to speed temperature equilibration). After some set interval (typically 5 min), the rack is removed and placed on crushed ice to cool all the samples. Each sample is then spun in the Microfuge for 2 min to sediment denatured protein and supernatants are collected for enzyme and ISO–DALT analysis. When the piers are maintained at 40 and 100°C, a uniform temperature gradient of about 2.1°C/well is established along the conductor strip 1 h from start-up (Fig. 2).

It must be emphasized that this device and method do not yield precise physico-chemical data on heat denaturation kinetics, since thermal equilibration of samples with the strip is not instantaneous (taking 3–5 min) and indeed proceeds at different rates according to position in the gradient. Nevertheless, as an empirical technique it is reproducible and hence valuable.

RESULTS

As shown in Fig. 3, various red cell enzymes lose their activities smoothly over different, rather narrow temperature intervals. Although this technique is only capable of resolving denaturation temperatures separated by 1 or 2° using the gradient shown, it is evident that enzymes from a single source display widely varying thermolabilities and can therefore be easily distinguished. Most enzymes disappear according to smooth, nearly symmetrical curves, but the temperature interval involved varies from enzyme to enzyme. A few enzymes, like red cell malate dehydrogenase, consistently show a biphasic curve indicating the presence of two forms having widely different thermostabilities. It is obvious that the temperature range employed can be adjusted at will, since maximum and minimum can be separately set to give any mean and any range. Thus enzymes barely resolved in Fig. 3 could be further separated by narrowing the temperature range across the device.

DISCUSSION

When ISO–DALT two-dimensional gels are run on each sample as well as enzyme analyses, temperature gradient thermal denaturation studies

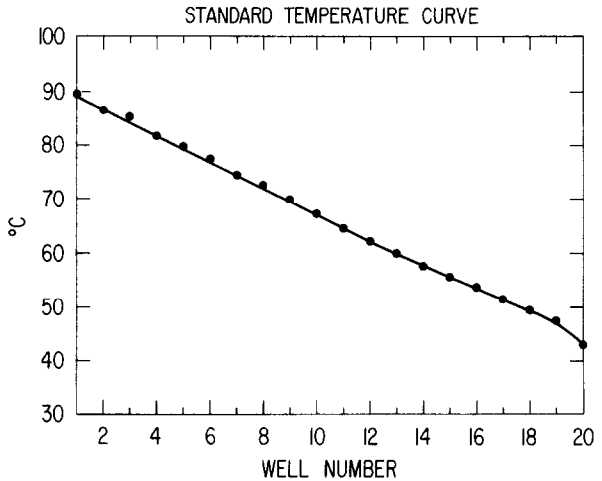

FIG. 2. Plot of temperature vs well number for the arrangement shown in Fig. 1 (100 and 40°C pier temperatures). Measurements were made using a small thermistor probe and a digital thermometer.

can be of great value in identifying enzymes in the ISO-DALT pattern. To make full use of this possibility requires quantitative densitometry of the gel patterns and computerized reduction of the data; these aspects will be dealt with in a later paper. When an accurate list of spot abundances is

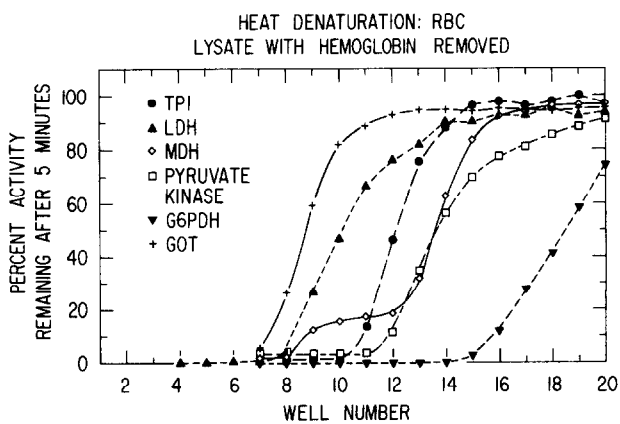

FIG. 3. Enzyme activity vs well (temperature) for six red cell enzymes. The sample was a human red cell lysate from which hemoglobin had been removed by treatment with DEAE-cellulose. Temperature gradient was as shown in Fig. 2, with hot end to the left. Enzyme analyses were performed using a centrifugal fast analyzer (5,6) and commercial reagents. GOT, Aspartate aminotransferase, EC 2.6.1.1; LDH, lactate dehydrogenase, EC 1.1.1.27; MHD, malate dehydrogenase, EC 1.1.1.37; pyruvate kinase, EC 2.7.1.40; G-6-PDH, glucose-6-phosphate dehydrogenase, EC 1.1.1.49; TPI, triosephosphate isomerase, EC 5.3.1.1.

obtainable for each sample, then these abundances can be matched to enzyme activities over the temperature range using sophisticated mathematical techniques.

It is possible to achieve similar results by heating samples at a constant temperature for varying lengths of time. Such a technique is, however, more time consuming and more difficult to use on a wide variety of enzymes simultaneously (i.e., denaturing both the very thermolabile and the very thermostable at a single temperature). In the latter case, time points from milliseconds to years would be needed to span some varied groups of proteins at a single temperature. Hence the temperature gradient is more useful.

An intriguing aspect of thermal denaturation studies is the ability of substrates, cofactors, and allosteric effectors to substantially alter the denaturation temperatures of some enzymes. Thus, when denaturation studies are performed as described here on a mixture of enzymes, only those enzymes which bind an added small (or large) molecule may be shifted in denaturation temperature. In practice, addition of cofactors can produce large stabilizations: NADP shifts the denaturation curve of glucose-6-phosphate dehydrogenase (G6PD) upward by about 17°C (data not shown). Such effects can be used, in conjunction with ISO-DALT analysis, to determine which spots in the two-dimensional pattern correspond to proteins which bind a given compound, irrespective of whether the enzyme activities of any of the spots are known.

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