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A "Slow" Temperature Jump Apparatus Built from a Stopped-Flow Machine

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A simple modification to a standard thermostated stopped-flow machine is described which allows it to be used as a temperature jump machine. Temperature jumps larger than 10°C can be achieved in <150 ms which makes it useful for the range of times where conventional rapid temperature jumps are not applicable. The apparatus has a sample size of 300 μl and can produce temperature jumps both above and below the initial temperature.

Temperature relaxation is a well-established method for following the fast kinetics of chemical and biochemical systems at equilibrium (1–3). The standard methods use electrical discharge (4) or laser flash (5) to increase the temperature in a few microseconds, and the subsequent chemical relaxation of the system to the new equilibrium can be followed between 10^{-6} and 10^{-1} s. The loss of heat to the surrounding thermostated cell holder, which is at the starting temperature, prevents observations of longer relaxation times unless some form of temperature clamping is employed. Slower relaxations have been studied by switching between two circulating thermostated baths. In 1968 Pohl published details of a device which could equilibrate a spectrophotometer cell within a few seconds and many subsequent designs have been based on this principle (6–9). More recently, Nakatani (10) designed a machine which pushed a solution at an initial temperature (T_1) through a heat exchanger before entering an observation chamber thermostated at a second temperature (T_2).

We report here a simple modification of a standard stopped flow machine for use as a temperature jump apparatus capable of producing changes of 15°C in <150 ms using a sample volume of 300 μl.

MATERIALS AND METHODS

The Apparatus

A SF4 stopped-flow machine (Hi-Tech Scientific Ltd., Salisbury, UK) was adapted as a temperature jump apparatus and required very little modification. In principle, most thermostated stopped flow machines could be adapted easily. A diagrammatic representation of the equipment is shown in Fig. 1. A single pneumatically driven syringe containing the reaction solution is held at temperature (T_1). The outlet from the syringe is connected to the observation cell via a delay line. The observation cell is a standard quartz cell (10 × 2 × 2 mm (i.d.)) with windows to allow absorbance or fluorescence observations along either a 2- or a 10-mm light path. The outlet of the observation cell is connected to the stopping syringe which does not need to be thermostated. The delay line is designed to allow the solution to equilibrate to the new temperature (T_2) by the time it arrives at the observation cell. Consideration must be given to the length, internal diameter, and material of the delay line and to the speed at which the sample travels through the tube in order to achieve maximum speed of heat exchange with minimum sample volume. Teflon (i.d. 1.5 mm; o.d. 3 mm) and stainless steel (i.d. 0.73 or 0.18 mm; o.d. 1.5 mm) tubing was used to connect the syringes with the observation chambers. Low dead volume polypropelene or stainless steel connectors were used throughout supplied by Anachem (Luton, UK).

The observation cell and most of the tubing is immersed in a thermostated tank containing 50% ethylene glycol. A fixed temperature is maintained (±0.1°C) by an internal heater and controlled by a thermostat. Cooling, if required, is supplied by liquid nitrogen. In the apparatus described here the sample syringe is held at room temperature; the observation cell is held either above or below ambient temperature.

Optics and Data Acquisition

For absorbance measurements light was supplied from a 100-W tungsten lamp driven by a power supply (Constant Electronics Ltd., Reading, UK). The light was passed through a Schoeffel Instruments monochromator and the light was collected by a quartz light guide with a slit-shaped entrance giving an effective band-

0003-2697/91 $3.00

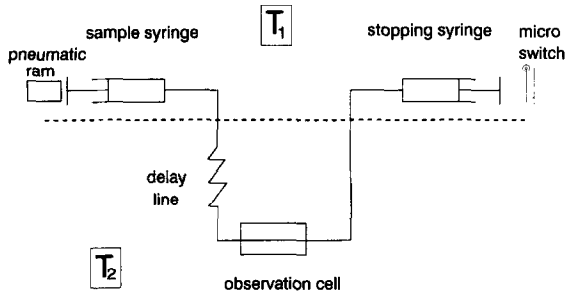

FIG. 1. Diagrammatic representation of the temperature jump apparatus. Components below the dotted line are thermostated at temperature (T_2) and those above are at ambient temperature (T_1).

width of 2 nm. The output from the photomultiplier (EMI 9526B) was electronically filtered using an unity gain amplifier.

The voltage output from the photomultiplier was then digitized by a 1401, 1-MHz analog digital converter (Cambridge Electronic Devices, Cambridge, UK). The data were collected as 400 12-bit data points and then transferred to a Hewlett–Packard 300 computer. The data were analyzed by a nonlinear least-squares fitting routine (11). Data collection was initiated by a microswitch on the stopping syringe. The speed and duration of the push was recorded by a linear potentiometer attached to the sample syringe.

For fluorescence measurements light was supplied from a 100-W high-pressure mercury lamp (Wotan HBO). Light was monochromated through a Bausch and Lomb monochromator and then filtered (Schott UG 11). Emission was detected at an angle of 90° to the exciting light through a Schott KV 393 filter.

Proteins

Myosin subfragment 1 (S1) was prepared by a chymotryptic digest of rabbit muscle myosin (12). F-actin was purified by the method described by Lehrer and Kerwar (13) and was covalently labeled at Cys-374 with *N*-(1-pyrenyl)iodoacetamide (14). The fluorescence of the pyrene group was excited at 365 nm and emission was measured at 407 nm. The fluorescence was quenched by 70% when S1 bound to actin (15).

RESULTS

The proton equilibrium of an indicator in a temperature-dependent buffer provides a convenient test of a temperature jump apparatus as protons equilibrate in less than 5 μs (16). The indicator used was Phenol red in 0.1 M Tris/HCl, pH 8.2, at 570 nm.

The temperature dependence of the absorbance of 25 μM phenol red in the observation chamber is shown in Fig. 2. This plot has a slope of −0.011 absorbance unit/°C over the range 5–35°C. The same result was obtained in a standard Pye-Unicam SP8-200 uv/vis spectrophotometer. Pushing a solution at ambient temperature from the syringe into the thermostated observation chamber at a temperature that is 15°C higher or lower will, therefore, induce a 0.165 absorbance change (=32% change in transmission). If temperature equilibration has occurred in the time taken to travel from the syringe to the observation chamber then no transient absorbance change will be observed. Any transient changes in absorbance will, therefore, indicate incomplete temperature equilibration. The results of a series of tests are shown in Fig. 3. Using a combination of Teflon tubing (i.d. 1.5 mm) and stainless steel tubing (i.d. 0.73 mm) a transient absorbance change was observed which was complete in 20 s. Replacing Teflon with stainless steel tubing (i.d. 0.18 mm) gave relaxation amplitudes of 2% (±0.5%) transmission change which corresponds to 0.8°C (±0.2°C) temperature change or 95% of the expected relaxation. Using the two stainless steel tubes, the total volume from the syringe to the optical path is approximately 200 μl. Routinely, 300-μl volumes were used to ensure complete wash out of the old solution. The pressure of the pneumatic ram was varied between 1.5 and 3.5 bar and the relaxation amplitude was independent of the pressure over this range. Analysis of linear potentiometer traces gave the total pushing times as 230–150 ms for 1.5–3.5 bar and terminal flow rates of 2.3–1.3 cm^3/s, respectively. For a dead volume of 200 μl this would give a minimum dead time of 87 ms at 3.5 bar.

As a demonstration of the apparatus, we examined the association of actin with myosin subfragment 1 (S1). This reaction is known to have a strong temperature dependence (17,18). The change in fluorescence of a pyrene group covalently attached to actin provides a convenient monitor of the association reaction (15).

The apparatus was loaded with 5 μM actin and 5 μM S1 in 0.3 M KCl, 20 mM cacodylate, 5 mM $MgCl_2$ at pH 7. Three pushes of 300 μl were carried out so as to fill both the tubing and cell and, therefore, eliminate dilution.

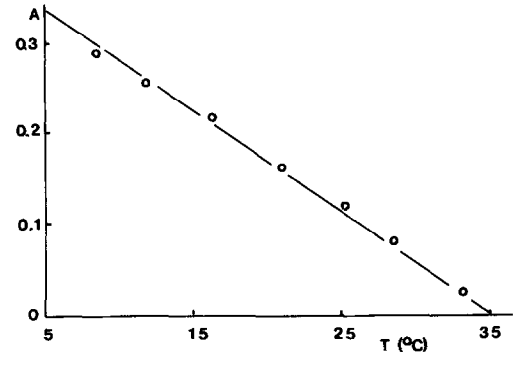

FIG. 2. Temperature dependence of the absorbance of 25 μM phenol red. Conditions: 0.1 M Tris/HCl, pH 8.2, at 570 nm. Slope of the computer fitted line = −0.011 (absorbance units/°C).

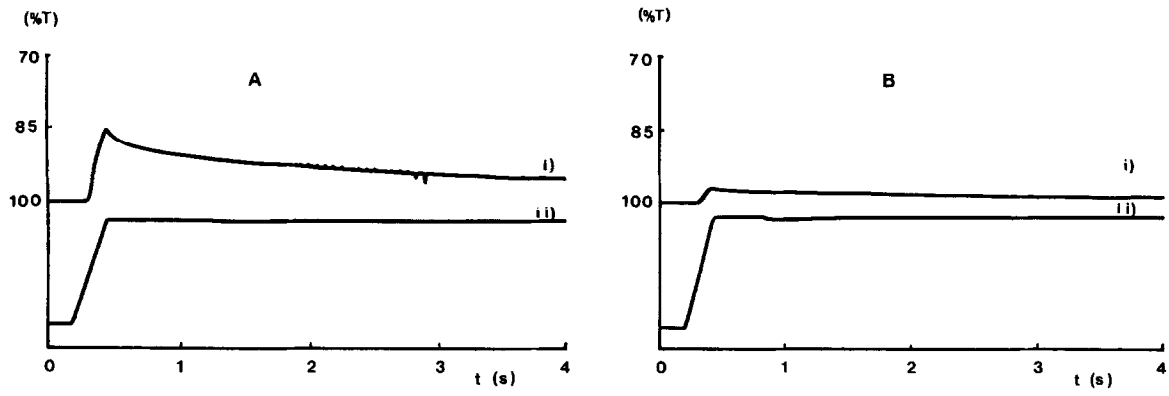

FIG. 3. Change in transmission following a 15°C temperature jump. 25 μM phenol red in 0.1 M Tris/HCl, pH 8.2, 570 nm was jumped from 20 to 5°C. (i) Represents an average of three relaxations. (ii) Recorded voltage output from a linear movement transducer attached to the driving syringe. (A) Delay tube 10 cm of 1.5 mm (i.d.) Teflon and 13 cm of 0.73 mm (i.d.) stainless steel tubing. Driving pressure, 1.5 bar; pushing time 280 ms; observed transmission change, 15%. (B) Delay tube Teflon replaced with 10 cm of 0.18 mm (i.d.) stainless steel tubing. Driving pressure, 1.5 bar; pushing time, 230 ms; observed transmission change, 2%.

The solution was left for 10 min to equilibrate to the preset temperature. A volume of 300 μl of acto.S1 was then pushed from 20 to 5°C. The resulting fluorescence change is shown in Fig. 4a. The solution arrived within 150 ms in the cell and the increase in fluorescence followed the dissociation of acto.S1 as the binding is weakened at lower temperature. The observed reciprocal relaxation time was 0.64 s^{-1} and a plot of $1/\tau$ as a function of ($[\bar{A}] + [\bar{S}1]$) is shown in Fig. 4b. The equilibrium concentration of free proteins was calculated from the dissociation constant (K_d = 1.66 μM) which was obtained using the same proteins in a separate fluorescence titration experiment (as described in Ref. (15)). The data in Fig. 4b are shown with the best fit straight line superimposed and with a slope of 1×10^5 M^{-1} s^{-1} and an intercept of 0.208 s^{-1}. This data is consistent with a single-step binding model,

$$A + M \underset{k-a}{\overset{k+a}{\rightleftharpoons}} A \cdot M$$

where A is actin and M is myosin subfragment 1 (S1). In this model the observed reciprocal relaxation time ($1/\tau$) is given by

$$1/\tau = k_{+a}([\bar{A}] + [\bar{M}]) + k_{-a},$$

where the slope in Fig. 4b defines k_{+a} and the intercept k_{-a}. However, it has been shown, using pressure relaxation methods, that this reaction is a three step reaction (19);

$$A + M \overset{K_0}{\rightleftharpoons} AM \overset{K_1}{\rightleftharpoons} A\text{–}M \overset{K_2}{\rightleftharpoons} A \cdot M$$

where steps 0 and 2 are rapid equilibrium steps. Provided that $([\bar{A}] + [\bar{M}]) \times K_0 \ll 1$, then on the time scale of this experiment $k_{+a} = K_0 k_{+1}$ and $k_{-a} = k_{-1}/(1 + K_2)$.

The ratio of $k_{-1}/(1 + K_2)$ and $K_0 k_{+1}$ = 2.08 μM which is in good agreement with the independently measured K_d of 1.66 μM. $K_0 k_{+1}$ and $k_{-1}/(1 + K_2)$ were measured in a separate stopped-flow experiment (as described in Ref. (15)) under the same experimental conditions and gave 1.2×10^5 M^{-1} s^{-1} and 0.27 s^{-1}, respectively, again in good agreement with the values obtained here.

DISCUSSION

Data presented here demonstrate that a temperature jump apparatus can be built by simple modification of the stopped-flow apparatus, using readily available components. The wide availability of stopped-flow equipment will make it an effective tool for the study of reversible reactions. The apparatus described has a sample volume of 300 μl and can produce a 15°C temperature jump in <150 ms. However, the apparatus is not fully optimized and reductions in both the sample volume and the time resolution may be possible. Using the current equipment relatively large volumes exist in the connectors between the sample syringe and the stainless steel tubing and between the tubing and the observation cell. Customized connectors could eliminate these volumes. Small-bore stainless steel tubing is used for the heat exchanger as this material is reasonably inert and readily available for use with high-performance liquid chromatography systems. However, stainless steel is not the first choice of material for a heat exchanger and a better material may be available depending on the sensitivity of the sample to metals. The addition of independent thermostating of the sample syringe will allow a wider range of temperature jumps to be studied.

Advantages of the apparatus are that large temperature jumps (>10°C) can be made to both higher and lower temperatures. There is no limit on the repeat rate

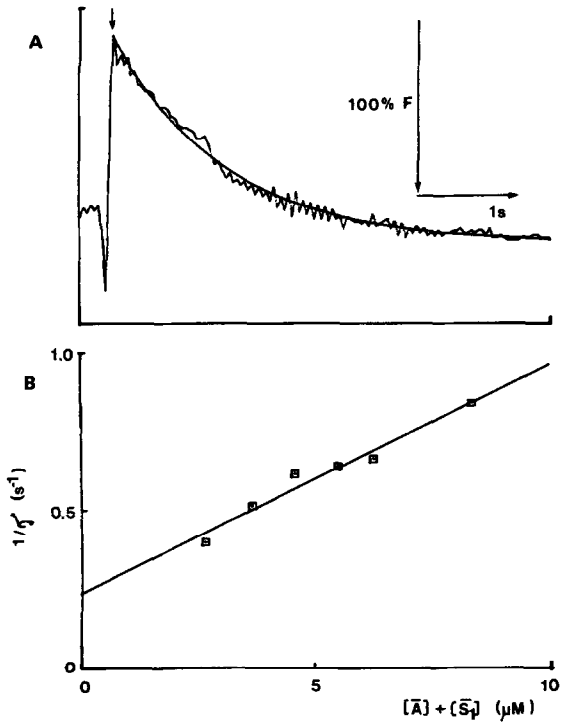

FIG. 4. Temperature-induced relaxation of a solution of pyrene-labeled actin and S1. (A) Temperature-induced changes in the fluorescence of a 0.3 ml solution of 5 μM pyrene-labeled acto.S1 at 3 bar. The arrow indicates the end of the push of the solution. The trace is an average of three traces and the computer-drawn best-fit exponential is superimposed, $1/\tau = 0.64\ s^{-1}$. Fluorescence is referred to the value before the start of the temperature jump. (B) Plot of the observed reciprocal relaxation times ($1/\tau$) against free pyrene-actin and S1 concentrations. The line represents the best-fit-line to the data and gives a gradient of $1 \times 10^5\ M^{-1}\ s^{-1}$ and an intercept of $0.208\ s^{-1}$. Conditions, T-jump from 20 to 5°C; buffer, 0.3 M KCl, 20 mM cacodylate, 5 mM $MgCl_2$, pH 7.

for the temperature jumps as a fresh sample can be examined as soon as observation of the preceding sample is complete. There is no delay while the sample chamber reequilibrates to the initial temperature as in conventional temperature jump equipment. This allows rapid repeated data collection under conditions where signal averaging is necessary because of low signal-to-noise ratio. The repeat rate is also an advantage when labile systems are being studied.

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