The Temperature-Dependence of Adenylate Cyclase from Baker's Yeast

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The Michaelis constant of membrane-bound adenylate cyclase increased from 1.1 to 1.8 mM between 7 and 38°C ($\Delta H = 13$ kJ/mol). Over this temperature range, the maximum velocity increased 10-fold, and the Arrhenius plot was nearly linear, with an average ΔH^* of 51 kJ/mol. The temperature-dependence of the reaction rate at 2 mM-ATP was examined in more detail: for Lubrol-dispersed enzyme, Arrhenius plots were nearly linear with average ΔH^* values of 45 and 68 kJ/mol, respectively, for untreated and gel-filtered enzymes; for membrane-bound enzyme, ΔH^* changed from 40 kJ/mol above about 21°C to 62 kJ/mol below 21°C, but this behaviour does not necessarily indicate an abrupt, lipid-induced, transition in the reaction mechanism.

Studies of the temperature-dependence of membrane-bound enzymes have frequently revealed apparent transition temperatures at which the slopes of their Arrhenius plots appear to change abruptly. These effects have often been attributed to changes in the physical state of the membrane lipids. Some of the most dramatic effects have been observed with adenylate cyclases. Thus rate measurements at a single substrate concentration have shown that the basal activities of adenylate cyclase in the plasma membranes of rat liver cells (Houslay & Palmer, 1978) and fat-cells (Counis & Jutisz, 1977) become nearly independent of temperature (with enthalpies of activation, ΔH^* , of about 7 kJ/mol) above 27°C, although they have large enthalpies of activation below this temperature. Such behaviour may contain information about the interaction between lipids and enzyme (reviewed by Sandermann, 1978). It would also cause the intracellular cyclic AMP concentration of an organism such as *Saccharomyces cerevisiae* to decrease sharply when the temperature rose above the transition temperature for its adenylate cyclase, unless the activity of its cyclic AMP phosphodiesterase exhibited similar temperature discontinuities. We have therefore examined the temperature-dependence of adenylate cyclase from baker's yeast.

The presence of very high ATPase activities in most preparations of membrane-bound adenylate cyclase means that an ATP-regenerating system must be included in the assay mixtures, and complicates studies of the temperature-dependence. The effective ATP concentration is the steady-state concentration determined by the relative activities of the ATPase and the ATP-regenerating system, and a finite amount of ADP is always present. Since the enthalpies of activation of ATPases are often very large

near 0°C, but decrease sharply at higher temperatures (e.g. Silvius *et al.*, 1978), the steady-state concentrations of ATP and ADP may change markedly with temperature. This will not necessarily be revealed by non-linear progress curves, and must be specifically excluded by varying the ratio of ATPase-containing adenylate cyclase to ATP-regenerating system at each temperature. In addition, many adenylate cyclases show pronounced substrate inhibition at ATP concentrations close to the K_m , so that measurements at a single substrate concentration may be very misleading, and V_{max} and K_m are both obtained only by long extrapolation. Both these problems are acute with adenylate cyclase from baker's yeast.

Experimental

Enzyme assay

Adenylate cyclase was measured as described by Varimo & Londeborough (1976). Reaction mixtures contained 100 mM-1,4-piperazinediethanesulphonic acid/KOH, pH 6.2, 5 mM-MnCl₂, 1.6 mM-MgCl₂, 10 mM-phosphoenolpyruvate (potassium or tricyclohexylammonium salt), 0.25 mg of pyruvate kinase/ml, 0.4 mM-cyclic AMP and 0.25–4.0 mM-[8-¹⁴C]ATP. A milliunit of enzyme produces 1 nmol of cyclic AMP/min at 2 mM-ATP and 30°C in this assay system. The [8-¹⁴C]ATP was purified at a specific radioactivity of 3000 c.p.m./nmol by ion-exchange chromatography, as described by Moffatt (1964), and stored as the dried sodium salt at –20°C. Reactions were started by addition of enzyme that had been incubated at the desired temperature for 5–8 min, and stopped after intervals between 0.5 and up to 40 min. Initial rates were estimated from three or four time points in the

linear part of the progress curves. The amounts of radioactivity found in cyclic AMP at extrapolated zero time were between 0 and 15 c.p.m. above background (15–20 c.p.m.) per 15×10^4 c.p.m. of ATP.

Adenylate cyclase preparations

Commercial baker's yeast from our Rajamäki factories was used. Cell envelopes were made by the method of Nurminen *et al.* (1970), except that the buffer was 25 mM-triethanolamine hydrochloride/NaOH/0.3 mM-EDTA/0.5 mM-MnCl₂, pH 7.2. The 100 000g sediment was prepared by disintegrating yeast in this buffer, but with a Mini Mill (Gifford Wood Co., Hudson, NY, U.S.A.) as earlier described (Varimo & Londesborough, 1976): after removal of unbroken cells and cell walls by centrifugation for 10 min at 2000g, the material sedimenting in 90 min at 100 000g was collected. Crude Lubrol-dispersed enzyme was made by the same method, but by using buffer containing also 1% (w/v) Lubrol PX and 0.3 M-KCl: after centrifugation for 90 min at 100 000g, the clear supernatant beneath the fatty layer was collected. Gel-filtered enzyme was prepared as follows: 28 ml of crude Lubrol-dispersed enzyme was concentrated to 9.8 ml by keeping it for 2 h in a dialysis sac surrounded by powdered poly(ethylene glycol) at 5°C, and 9.0 ml of the concentrated enzyme was applied to a column (3.3 cm $\times$ 67 cm) of Sepharose 6B equilibrated with 25 mM-triethanolamine hydrochloride/NaOH/0.3 mM-EDTA/0.5 mM-MnCl₂, pH 7.2, containing 1% Lubrol PX. Adenylate cyclase appeared about 50 ml after the void volume, but

before the main protein peak, as described earlier (Varimo & Londesborough, 1976).

The membrane-bound preparations were usually frozen and thawed before use. This increased their adenylate cyclase activity by about 20% and extended the linearity of assays, probably by partially inactivating ATPase, but did not cause significant solubilization of the adenylate cyclase.

Materials

Chemicals were from the sources described earlier (Varimo & Londesborough, 1976).

Results

Double-reciprocal plots obtained at four temperatures with adenylate cyclase in twice-frozen-and-thawed cell envelopes are shown in Fig. 1. Initial rates of reaction at the ATP concentrations between 2.0 and 0.38 mM were proportional to enzyme concentration (and therefore independent of the ratio of ATPase to pyruvate kinase) over a 2-fold range (0.17–0.38 munit/ml at 30 and 7°C, and 0.1–0.23 munit/ml at 38°C; the pyruvate kinase concentration was kept at 0.25 mg/ml). Median estimates of the maximum velocity ($\bar{V}_{max}$) and Michaelis constant ($\bar{K}_m$) were obtained from direct linear plots (Eisenthal & Cornish-Bowden, 1974). Substrate inhibition appeared at smaller ATP concentrations at 7°C than at higher temperatures, and the point at 4 mM-ATP was excluded from the analysis at 7°C. The values of $\bar{V}_{max}$ and $\bar{K}_m$ at the first and third quartiles of the

Fig. 1. Double-reciprocal plots at four temperatures

Initial rates, v_0 (nmol of cyclic AMP/min), were determined in standard assay mixtures containing 0.25–4.00 mM-ATP and e munits of adenylate cyclase. Lines are drawn for values of $\bar{K}_m$ and $\bar{V}_{max}$ determined by direct linear plots (Eisenthal & Cornish-Bowden, 1974).

Fig. 2. Temperature-dependence of K_m and V_{max} for membrane-bound enzyme

Logarithms of K_m (mM) and V_{max} ($\mu\text{mol}/\text{min}$ per unit) are plotted against the reciprocal temperature. The meaning of the error bars is described in the text.

sets of estimates of V_{max} and K_m provided by the direct linear plots were used to indicate the errors in V_{max} and K_m . Plots of the logarithms of these values against the reciprocal temperature were linear within this experimental error (Fig. 2). K_m increased slowly with temperature, from 1.1 mM at 7°C to 1.8 mM at 38°C, corresponding to an enthalpy of 13 kJ/mol. V_{max} increased nearly 10-fold over the same temperature range, with an apparent enthalpy of activation of 51 kJ/mol (solid line in Fig. 2).

The shape of the Arrhenius plots was examined in more detail with rate measurements at 2 mM-ATP, because this was the largest ATP concentration that could be used without danger of substrate inhibition at lower temperatures. For the adenylate cyclase activity of frozen-and-thawed cell envelopes, the apparent enthalpy of activation changed from 40 kJ/mol above an apparent discontinuity at 21°C to 62 kJ/mol below this temperature (Fig. 3). The shape of the Arrhenius plot was independent of enzyme concentration, and a similar plot, with ΔH^*

changing from 38 to 62 kJ/mol, was obtained (results not shown) with frozen-and-thawed 100000g sediment, assayed in the presence of 20 μg of oligomycin/ml (which did not alter the activity of adenylate

Fig. 3. Temperature-dependence of the activity at 2 mM-ATP of membrane-bound and Lubrol-dispersed enzyme. Rates (v_t) at 2 mM-ATP are expressed relative to that at 30°C (v_{30}). For clarity, the upper three plots have been lifted by 0.7, 0.5 and 0.2 log unit respectively. Final enzyme concentrations were, for cell envelopes, 0.1 munit/ml ($\bullet$) or 0.4 munit/ml ($\circ$); for crude Lubrol-dispersed enzyme, 0.16 and 0.32 munit/ml ($\blacktriangle$) or 0.5 munit/ml ($\triangle$); and for gel-filtered enzyme, 0.23 munit/ml ($\square$). In two experiments ($\bullet$ and $\blacktriangle$) the stock enzyme preparation was first incubated at 35°C for 8 min before measurement of rates at other temperatures in a random order. In the other experiments, rates below 30°C were measured with enzyme that had not been exposed to temperatures above 30°C. The data for membrane-bound enzyme have been superimposed on either the theoretical curve for $V_{max} = k_a k_b / (k_a + k_b)$ with $k_a = k_b$ at 22°C and ΔH_a^* and ΔH_b^* respectively 20 and 78 kJ/mol (topmost plot), or two straight lines intersecting at 21°C.

cyclase at 30°C, but decreased ATPase activity). For freshly prepared cell envelopes, the average enthalpy of activation between 7 and 38°C (about 43 kJ/mol) was slightly less than that of frozen-and-thawed material, but the shape of the Arrhenius plot was not investigated in detail.

In these experiments with membrane-bound enzyme we found no evidence that the rate of reaction at any temperature was altered by previous exposure of the enzyme to temperatures up to 38°C for times of up to 10 min. However, for crude Lubrol-dispersed enzyme, the ratio of activities at 23 and 30°C (v_{23}/v_{30}) decreased by about 15% when the enzyme was preincubated at 35°C. Thus, when enzyme prepared and maintained at less than 5°C was assayed first at 23°C and then at 30°C, v_{23}/v_{30} was about 0.74 (0.69–0.78), but after exposure to 35°C for 8 min this ratio decreased to about 0.66 (0.63–0.69), mainly because of an increase in the activity at 30°C. The effect of preincubation at 35°C persisted through storage at 0°C for 4.5 h. For enzyme preincubated at 35°C, the Arrhenius plot was linear within experimental error, with a ΔH^* of 45 kJ/mol (Δ , solid line in Fig. 3), but between 13 and 25°C data obtained with enzyme not preincubated at 35°C lay above this linear plot (Δ , broken line in Fig. 3). The Arrhenius plot with Lubrol-dispersed enzyme after gel filtration was a smooth curve, with an average ΔH^* of 68 kJ/mol between 9 and 38°C.

Discussion

The results of Figs. 2 and 3 suggest that the deviations of the temperature-dependence of adenylate cyclase of baker's yeast from 'normal' (i.e. an approximate doubling of reaction rate for every 10°C rise in temperature) are too small to cause a marked temperature-dependence of the intracellular concentration of cyclic AMP. The effect of temperature on the activities of the cyclic AMP phosphodiesterases of yeast has not yet been examined.

Silvius *et al.* (1978) have shown that the apparent discontinuities sometimes observed in the slopes of Arrhenius plots can be artefacts, especially when rates are measured at a single substrate concentration. The ratio observed reaction rate/ $V_{\max}$ may then change with temperature, because both K_m and substrate inhibition may be temperature-dependent. With the membrane-bound adenylate cyclase, we found that 2 mM-ATP did not cause substrate inhibition between 7 and 38°C, and that the effect of temperature on the K_m was small, as is also the case for the adenylate cyclase from ascites-tumour cells (Bär, 1974) and fat-cells (Counis & Jutisz, 1977). If the K_m of the membrane-bound enzyme increases steadily from 1.1 mM at 7°C to 1.8 mM at 38°C (Fig. 2), then for this membrane-bound enzyme the rates of

reaction at 2 mM-ATP decrease from 65% of $V_{\max}$ at 7°C to 53% of $V_{\max}$ at 38°C. The average ΔH^* is therefore underestimated by 4.8 kJ/mol, but the shape of the Arrhenius plot in Fig. 3 is not distorted by the temperature-dependencies of K_m or substrate inhibition. Although the Arrhenius plot of $V_{\max}$ was linear within experimental error (Fig. 2, solid line), straight lines drawn through the actual median estimates, $\bar{V}_{\max}$, intercept at 21°C, and yield ΔH^* values of 45 and 60 kJ/mol above and below this temperature (Fig. 2, broken lines). This is in good agreement with the more detailed data at 2 mM-ATP, which cannot be fitted by a single straight line. Finally all rate determinations were made under conditions where the rates of reaction were proportional to the enzyme concentration, so that they were not affected by the temperature-dependencies of the activities of ATPase or pyruvate kinase. The change in the apparent enthalpy of activation of membrane-bound adenylate cyclase from about 60 kJ/mol at around 10°C to about 45 kJ/mol at around 30°C therefore appears to be a real phenomenon.

Temperature-dependent changes in the ΔH^* of membrane-bound enzymes are often explained by assuming a sudden change in the reaction mechanism of the enzyme, induced by a temperature-dependent change in the physical state or composition of the lipid adjacent to the enzyme. In the present case, a simpler explanation will also suffice. Since the dissociation steps of both pyrophosphate and cyclic AMP must appear in the kinetic expression for $V_{\max}$, the simplest form this can take is $k_a k_b / (k_a + k_b)$, where k_a and k_b are the rate constants for these dissociation steps (in this paper, enzyme concentration is expressed in activity units, so that $V_{\max}$, as defined in Fig. 2, is a dimensionless ratio of rates, and similarly k_a and k_b are ratios of the sizes of the rate constants at $t^\circ\text{C}$ to those at a standard temperature). If k_a and k_b are both kinetically significant, but have different enthalpies of activation, then the Arrhenius plot will bend where k_a and k_b become equal, and this bend can be very sharp (J. Londeborough, unpublished work). In the topmost plot of Fig. 3 the data for membrane-bound enzyme have been superimposed on the theoretical curve for $V_{\max} = k_a k_b / (k_a + k_b)$, with $k_a = k_b$ at 22°C and $\Delta H_a^* = 20 \text{ kJ/mol}$ and $\Delta H_b^* = 78 \text{ kJ/mol}$. Within experimental error, this theoretical curve fits the data as well as any pair of straight lines. The correct kinetic expression for $V_{\max}$ may be more complicated, but any expression containing both multiples and sums of individual rate constants will lead to a curved Arrhenius plot. In the present case, this kind of explanation has the advantage that it predicts that the Arrhenius plot will be continuous, whereas explanations based on a sudden change in reaction mechanism predict an abrupt change in $V_{\max}$ at the

same temperature as the abrupt change in ΔH^* (Kumamoto *et al.*, 1971; J. Londesborough, unpublished work).

The K_m for ATP at 30°C is not changed by 'solubilization' of yeast adenylylase with Lubrol PX and gel-filtration (Varimo & Londesborough, 1976). For crude Lubrol-dispersed enzyme that had been preincubated at 35°C, the Arrhenius plot at 2mM-ATP was linear within experimental error. Elimination of apparent discontinuities in the slopes of Arrhenius plots of membrane-bound enzymes by treatment with detergent has sometimes been taken as evidence that these discontinuities are caused by changes in the physical state of lipid in the membrane, and is at least consistent with this hypothesis. However, even if the reaction mechanism of membrane-bound enzyme were not affected by changes in the physical state of adjacent lipid, it very probably would be affected by removal of the enzyme from the membrane, and formation of a detergent-enzyme complex. Merely altering the relative sizes of k_a and k_b , so that they are never equal within the observable temperature range of 0–40°C, would eliminate the bend in the Arrhenius plot. There is some evidence that treatment with Lubrol increases the size of the smallest rate constant contributing to V_{max} . This is the roughly 2-fold activation that occurs, at constant K_m , during 'solubilization' with Lubrol PX (Varimo & Londesborough, 1976; the suggestion then made was that this might be caused by increased accessibility).

Below about 25°C the enthalpy of activation of crude Lubrol-dispersed enzyme that had not been exposed to 35°C appeared to be greater than that of exposed enzyme (Fig. 3, broken line), but exposed and unexposed enzyme behaved similarly above 30°C. The unexposed enzyme appeared to have a discontinuity of about 25% in V_{max} between 25 and

30°C. Incubation of Lubrol-dispersed enzyme at 35°C may permit a rearrangement of the mixed Lubrol/lipid/protein micelle, which presumably contains the adenylylase. Gel filtration increased the average enthalpy of activation. During gel filtration, the mixed micelles containing adenylylase are partially equilibrated with a large excess of 'pure' Lubrol micelles, so that their content of yeast lipid probably decreases (Tanford & Reynolds, 1976). These changes in the behaviour of Lubrol-dispersed enzyme do indicate that the details of the reaction mechanism are probably affected by the structure of the Lubrol-adenylylase complex, but this does not imply that the change in the slope of the Arrhenius plot of membrane-bound enzyme is necessarily due to a temperature-dependent change in the state of the lipid adjacent to the enzyme.

References

- Bär, H.-P. (1974) *Mol. Pharmacol.* **10**, 597–604
Counis, R. & Jutisz, M. (1977) *Mol. Cell. Endocrinol.* **7**, 313–324
Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
Houslay, M. D. & Palmer, R. W. (1978) *Biochem. J.* **174**, 909–919
Kumamoto, J., Raison, J. K. & Lyons, J. M. (1971) *J. Theor. Biol.* **31**, 47–51
Moffatt, J. G. (1964) *Can. J. Chem.* **42**, 599–604
Nurminen, T., Oura, E. & Suomalainen, H. (1970) *Biochem. J.* **116**, 61–69
Sandermann, H., Jr. (1978) *Biochim. Biophys. Acta* **515**, 209–237
Silvius, J. R., Read, B. R. & McElhaney, R. N. (1978) *Science* **199**, 902–904
Tanford, C. & Reynolds, J. A. (1976) *Biochim. Biophys. Acta* **457**, 133–170
Varimo, K. & Londesborough, J. (1976) *Biochem. J.* **159**, 363–370