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Calorimetric Studies of Binding of Substrates and Inhibitors to Triosephosphate Isomerase

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CALORIMETRIC and kinetic studies of the binding of substrates and inhibitors to triosephosphate isomerase (TIM, E.C. 5.3.1.1) from chicken-breast muscle are intended to complement the crystallographic studies of this enzyme which are now well advanced (Banner *et al.*, 1971). TIM is the enzyme of the glycolytic pathway responsible for the rapid interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP), and the system offers an exciting opportunity for studying both the structural and thermodynamic features of the interaction of an enzyme with its true substrate.

Chicken TIM, molecular weight about 54,000 (Furth, Milman, Offord and Priddle, unpublished), is a dimer consisting of two apparently identical subunits. The overall equilibrium constant of the reaction, including hydration effects (Trentham *et al.*, 1969; Reynolds *et al.*, 1971) [DHAP]/[GAP] = 22, is known to be independent of temperature (Meyerhof and Junowicz-Kocholaty, 1943; Lowry and Passonneau, 1964; Burton and Waley, 1968; Veech *et al.*, 1969), implying that the enthalpy of isomerization is close to zero. We have confirmed this by microcalorimetry (ΔH = 0 ± 0.5 kJ/mole). Thus, it is possible to measure directly the binding of true substrates to the enzyme in the microcalorimeter, unimpeded by the large enthalpies of subsequent catalytic processes which usually mask thermochemical studies of enzyme–substrate interaction. There is the technical difficulty that traces of aldolase frequently present in preparations of TIM will catalyse the strongly exothermic reaction GAP + DHAP → fructose 1,6-diphosphate and for this reason much of our preliminary work has concentrated on the binding to TIM of the powerful competitive inhibitor 2-phosphoglycollate (PGA), a possible analogue of the transition state of the isomerization process proposed by Wolfenden (Wolfenden, 1969, 1970; Johnson and Wolfenden, 1970).

Enzyme–ligand titration curves are obtained from the heats of reaction in the standard manner (Bjurulf *et al.*, 1970) using an LKB batch microcalorimeter at 25°C in 0.02 M triethanolamine–HCl buffer, pH 7.7 throughout. Ligand titration curves are typically hyperbolic (Fig. 1A) and double-reciprocal plots are linear (Fig. 1B).

Table 1 gives values of the TIM–PGA dissociation constant, K_I, and enthalpy, $n\Delta H$, at a series of enzyme concentrations, obtained by iterative least-squares fitting of the data to the equation of the hyperbolic binding curve:

$$-Q = \tfrac{1}{2}\Delta H(nc_E + C_I + K_I)\left\{1 - \sqrt{\left(1 - \frac{4nc_E C_I}{(nc_E + C_I + K_I)^2}\right)}\right\} \qquad (1)$$

where Q = heat of reaction/unit volume, n = number of ligand binding sites/enzyme molecule (assumed identical and independent), and C_E and C_I are the total enzyme and ligand concentrations, respectively.

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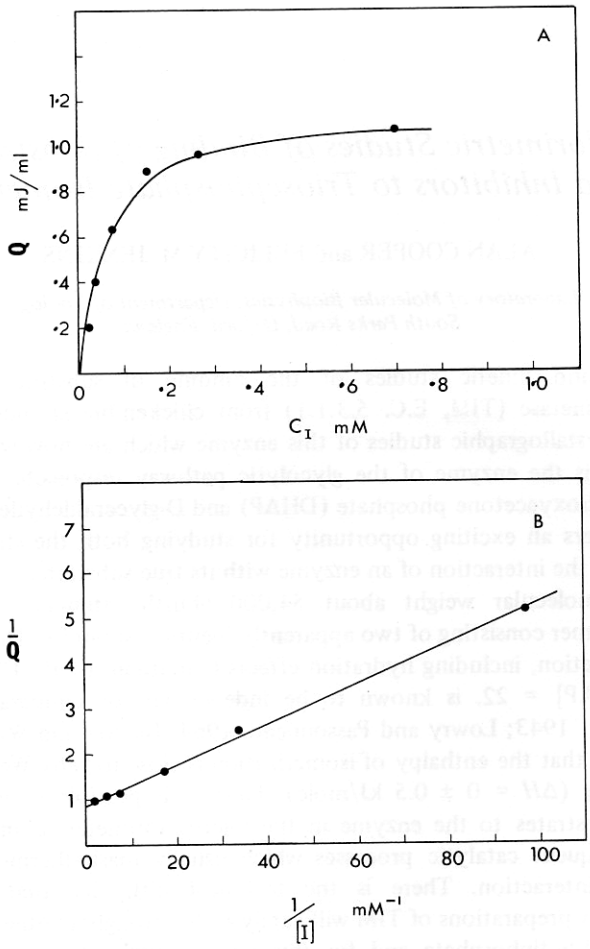

FIG. 1. Binding of PGA to chicken TIM (1.5 mg/ml) in 0.02 M triethanolamine-HCl buffer, pH 7.7, at 25°C. (A) Heat of reaction as a function of total inhibitor concentration. (B) Double-reciprocal plot: inverse of the heat of reaction versus the inverse free inhibitor concentration.

TABLE 1. CALORIMETRIC DATA FOR TIM–PGA BINDING

TIM concentration (mg/ml)	$-n\Delta H$ (kJ/mole)	K_I (μM)
5.4	41.2 ± 1.0	360 ± 17
1.5	41.7 ± 0.8	48 ± 3
1.18	41 ± 3	18 ± 5
0.71	45.6 ± 1.3	15 ± 4

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Although the enthalpy of binding remains relatively constant over the concentration range studied, there is a marked variation in the apparent dissociation constant. K_I determined at very low enzyme concentrations (about 2×10^{-6} mg/ml) from the effect of PGA on the kinetics of isomerization [see eqn. (2)] has the value 7.5 (±1) μM.

Modified Hill plots of the calorimetric data (Fig. 2) are linear, with a least-squares slope (Hill coefficient, n_H) of 1.005 ± 0.037. This justifies our assumption that the inhibitor sites are identical and independent, and also seems to rule out aggregation of TIM as the source of variation of K_I since this would give non-linear Hill plots with slopes not equal to unity (Wyman, 1964). Furthermore, we have observed no measurable heat of dilution of the protein, and ultracentrifuge studies show no sign of TIM aggregation (M.P. Esnouf, private communication). Heterogeneity of the protein preparation is also ruled out by the Hill plot, and all preparations when diluted for kinetic studies gave the same value for K_I (i.e. about 7.5 μM). The source of this concentration dependence continues to elude us.

The stoichiometry (n) of ligand binding can in principle be determined by fitting the data to eqn. (1), and for PGA a value of $n = 1$ gave consistently better results than higher n values. However, this is not a very sensitive test, especially in cases where the enzyme concentration is very much lower than the apparent dissociation constant. Calorimetric binding curves give $n\Delta H$, and an independent measure of ΔH is needed. The method of Bolen *et al.* (1971) is inapplicable

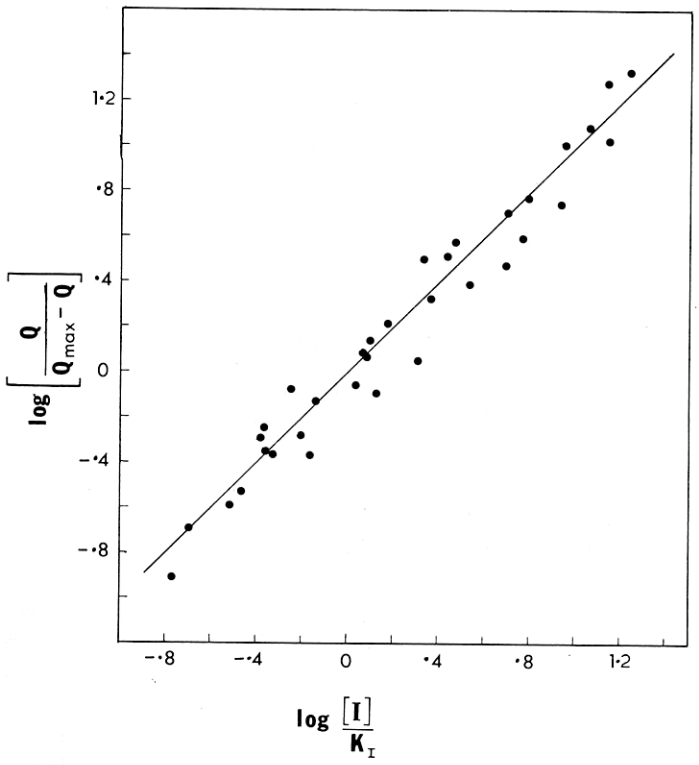

FIG. 2. Modified Hill plot of TIM–PGA binding data. Points include data for enzyme concentrations in the range 0.54 to 5.4 mg/ml, plotted with log ($[I]/K_I$) as abscissa to reduce them all to the same line.

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here because of the variation of K_I with enzyme concentration, but we can use kinetic data. The velocity (V) of isomerization in the presence of a competitive inhibitor obeys the simple Lineweaver–Burk type relation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}\,[S]}\left(1 + \frac{[I]}{K_I}\right) \qquad (2)$$

where $[S]$ is the substrate concentration and K_m the Michaelis constant for the enzyme–substrate complex. Thus, K_I may be determined independent of any assumption of stoichiometry, and for experiments done over a range of temperatures the enthalpy of inhibitor binding, ΔH, will be given by the Van't Hoff plot, log K_I versus $1/T$. $-\Delta H$ obtained in this manner for the TIM–PGA reaction is 41.5 (±3) kJ/mole. Comparison with the $n\Delta H$ values in Table 1 confirms that the value $n = 1$ is most likely for the binding of PGA to the TIM dimer.

A similar analysis for the binding of substrate (GAP) is possible using the Van't Hoff plot of K_m to determine ΔH. This is not really so rigorous, since K_m is not necessarily the same as the true substrate binding constant, K_S, found from the calorimetric binding curve. They are, however, indistinguishable in this case (Table 2), and the procedure should be valid.

TABLE 2. CALORIMETRIC AND KINETIC DATA FOR TIM–SUBSTRATE BINDING

Calorimetry:	TIM concentration = 0.75 mg/ml
	K_S = 1.4 (±0.1) x 10^{-4} M
	$-n\,\Delta H$ = 62.8 (±2.4) kJ/mole
Kinetics:	TIM concentration = 2.4 x 10^{-6} mg/ml
	K_m = 1.5 (±0.3) x 10^{-4} M
	$-\Delta H$ = 32 (±5) kJ/mole

It follows that $n = 2$ is the likely value for binding of substrate to TIM. Hill plots of the kinetic data are linear with $n_H = 1$, indicating that the substrate binding sites are identical and that there is no interaction between them.

This presents us with an intriguing problem: if the TIM dimer has two independent binding sites for the substrate, but only one for the inhibitor PGA, how is it that PGA seems to act as such a good competitive inhibitor? A first assumption might be that the PGA molecule binds at some unique site, perhaps at the junction of the protein subunits, and somehow blocks catalysis at both active sites simultaneously. However, none of the possible variations of this model gives the perfectly linear Lineweaver–Burk type plots that are observed (Wolfenden, 1969, 1970; and our own work). Furthermore, there is direct crystallographic evidence that PGA binds at the same site as the substrate molecules (G.A. Petsko, unpublished). We are forced to conclude that the binding of PGA at one of the substrate sites somehow blocks catalysis at the other,

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possibly by inducing a conformational change at the other site. There are two ways this might occur: (a) by preventing the binding of substrate at the second site, or (b) by allowing binding of the substrate at the other site, but preventing isomerization. Only alternative (b) gives kinetic equations consistent with the observations. In support of this we have shown with calorimetric experiments that substrate molecules will bind to TIM even in the presence of saturating concentrations of PGA, when of course no isomerization occurs.

If PGA really is a transition-state analogue (Wolfenden, 1969, 1970) then it would appear that only one transition state configuration is allowed on the enzyme at any one time. Binding of PGA might be thought to "freeze" the enzyme in this configuration whereupon binding of a substrate molecule can occur, but isomerization is blocked because this would require the presence, albeit transient, of a second transition state configuration on the enzyme.

Further consideration shows that the determination of stoichiometry from a comparison of calorimetric and Van't Hoff heats of binding is not entirely unambiguous. If the binding of ligand to enzyme involves a simultaneous binding (or release) of a proton then the calorimetric enthalpy will include a heat of protonation (or deprotonation) of the buffer which will not appear in the Van't Hoff heat (if, as here, the measurements are corrected for the variation of buffer pH with temperature). Direct binding studies are in progress to resolve this ambiguity.

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