THERMOCHEMISTRY OF FUMARASE-INHIBITOR BINDING

Jasna KNIEWALD and Pavao MILDNER
Laboratory of Biochemistry, Technological Faculty, University of Zagreb, 41000 Zagreb, Yugoslavia

Received 27 February 1975

1. Introduction

The steady state kinetics of the reaction catalysed by fumarase (fumarate hydratase, EC 4.2.1.2) has been studied in the past [1–3]. The activity of fumarase was found to be affected by the addition of inorganic salts, by variation of temperature and pH, as well as by addition of inhibitors [4–8].

In the present study, binding enthalpies of two competitive inhibitors of pig heart fumarase, succinate and citrate, to enzyme protein were determined at different temperatures between 25 and 37°C, but under a fixed set of conditions, such as medium composition (10 mM phosphate buffer pH 7.4) and ionic strength ($\Gamma/2 = 0.03$).

The binding of structural analogues of the substrate, like succinate and citrate, can serve as a model for the first step in the enzymatic conversion. The experiments performed at various temperatures allowed a check to be made of van 't Hoff enthalpy value for inhibitor binding reported in a paper by Massey [8], wherein binding enthalpies of malonate and succinate were found to be negative at low temperature and change sign above 27°C. By measuring the enthalpy change of succinate and citrate binding to fumarase under the experimental conditions specified above, we found that these interactions were endothermic. The experimental design was such as to ensure that different degrees of binding occurred, which permitted calculation of equilibrium constants in addition to binding enthalpies. Knowledge of equilibrium constants, in turn, enabled the calculation of free energies (ΔG_B) and entropies (ΔS_B) of binding. In addition, heat capacity changes (ΔC_p) were derived from binding enthalpies in order to discriminate between conformational alterations and hydrophobic-binding effects possibly occurring in enzyme–inhibitor complex formation.

2. Materials and methods

Pig heart fumarase (fumarate hydratase, EC 4.2.1.2) was purchased from Boehringer Mannheim GmBH, as a 1% crystalline suspension in ammonium sulphate solution, and was used without further purification. All chemicals were analytical grade commercial preparations. The inhibitors, sodium succinate and sodium citrate, were salts manufactured by N.B.Company, Cleveland and by Kemika, Zagreb, respectively. Water used for preparing the buffer solutions was first demineralized and glass-distilled.

Fumarase activity was determined with freshly prepared enzyme solutions, at ambient temperature (25°C), by means of a Varian Techtron Model 635 spectrophotometer at 240 nm. Incubation mixtures were 100 mM in phosphate buffer pH 7.4, with L-malate as the substrate ($K_m = 4.1 \times 10^{-3}$ M). In calculations, the mol. wt of fumarase was assumed to be 194 000 [9].

Calorimetry of inhibitor binding at different temperatures was carried out in 10 mM phosphate buffer pH 7.4, (as checked with a Beckman Expandomatic pH-meter, standardized at pH 7.41). The inhibitor solutions were prepared on the day of the experiment, and had varying concentrations within a range 2.64–29.82 mmol · kg^{-1} for succinate and 4.70–27.36 mmol · kg^{-1} for citrate. The concentration of fumarase in the calorimetric liquids was 171.8 μmol · kg^{-1} in all experiments. Molar ratios of inhibitor to enzyme were

determined on the basis of total enzyme and total inhibitor concentrations present in the calorimetric system. The actually employed ratios were 15–174 : 1 and 27–159 : 1 for succinate : fumarase and citrate : fumarase, respectively. The temperatures at which the measurements were carried out were 25, 28, 30, 32, 35 and 37°C.

The microcalorimetric measurements were performed with an LKB Model 10700-2 Batch Micro Calorimeter, using 18-carat gold reaction vessels. The differential voltage signal obtained from the calorimeter was amplified by a Keithly 150 B Microvolt Ammeter and the ammeter output was recorded by a Goerz Elektro Servogor recorder fitted with a ball-and-disc integrator. Calorimetric vessels were filled by means of glass syringes and the amounts of samples were determined by differential weighing. The two compartments in the reaction vessel received 1.00 ml of fumarase solution and 2.00 ml of the inhibitor solution, respectively, and corresponding compartment fillings in the reference vessel were 1.00 ml of buffer solution and 2.00 ml of the inhibitor solution. Thus, the enthalpy of dilution of inhibitor during experiments was automatically corrected for. Separate experiments were made to determine the enthalpy of dilution of fumarase, using the same buffer and the same temperature as under reaction conditions.

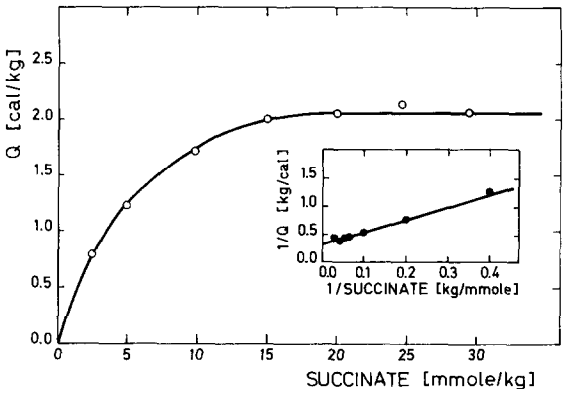

Fig.1. Calorimetrically measured heats of succinate binding to pig heart fumarase, as a function of final inhibitor concentration. Temp. 25°C, 10 mM phosphate buffer, pH 7.4, Γ/2=0.03. The concentration of fumarase was 171.8 μmol/kg in all experiments. Inset: Double-reciprocal plot of $1/Q$ vs. 1/(succinate). Each point in fig.1 and in the inset represents the average of at least triplicate determinations.

3. Results and discussion

Spreading the microcalorimetric measurements over a wide range of temperatures, without changing other conditions as described in the preceding Section, was a modus operandi adopted in view of results previously reported by Massey [8]. This author found that the inhibition of fumarase by competitive inhibitors was dependent on temperature, and our experiments were intended, among other things, to check whether inhibitor binding is likewise dependent on temperature.

A typical diagram showing the dependence of succinate binding heats Q (corrected for dilution heats) on inhibitor concentration is presented in fig.1, with a corresponding double reciprocal plot of $1/Q$ vs. $1/I$ inserted, according to Wadsö et al. [10,11]. This plot is linear, and its intercept, $1/Q_m$, permits a graphical determination of ΔH. The equilibrium constant, K_{ass}, is obtained from the slope of the line, $1/Q_m \cdot K_{ass}$), where Q_m is the reciprocal of the intercept and represents the extrapolated binding heat for complete saturation of enzyme by the inhibitor. Line fitting was carried out by computer treatment of experimental data using a least-square program.

Further use of binding data, such as shown on fig.1, requires certain assumptions, the most important one concerning the number and behavior of binding sites. Our arguments are based on the assumption that there are four independent binding sites per molecule, as suggested by Kanarek and others [9,12–14]. We further assume that these sites are saturated at inhibitor concentrations corresponding to the plateau regions of the experimentally obtained binding-heat curves, and that the heat of binding change per ligand is independent of the extent of binding.

In designing calorimetric measurements of ligand binding, the fact that foreign ligands, usually buffer components, may competitively bind to the macromolecule and thereby significantly disturb the thermodynamic parameters of the process under investigation is often ignored. By measuring the magnitude of this side effect and using the largest heat value obtained by Lovrien and Sturtevant [15], we calculated that the binding enthalpies for inhibitor–enzyme complex as

Table 1
Thermodynamic parameters per site per mol of protein for succinate binding to pig heart fumarase at $\Gamma/2$=0.03 and pH 7.4

Temperature (°C)	ΔH_B* (kcal · mol^{-1})	ΔS_B (cal · mol^{-1} · deg^{-1})	ΔG_B (kcal · mol^{-1})	K_{ass} (M^{-1})
25	2.847	−7.731	5.152	1.670×10^{-4}
28	3.356	−6.176	5.216	1.634×10^{-4}
30	3.396	−5.733	5.134	1.984×10^{-4}
32	3.719	−4.883	5.209	1.853×10^{-4}
35	4.001	−3.761	5.160	2.186×10^{-4}
37	4.051	−3.173	5.035	2.824×10^{-4}

* Calculated from the experimental data assuming 4 binding sites/mol of fumarase.

determined in our system, may be too low by 1 kcal per mol of ligand.

After correction for the binding effect of ionic ligands and calculation of binding enthalpy per site (assuming four independent binding sites per molecule of fumarase), we were also able to calculate free energy (ΔG_B) and entropy (ΔS_B) per site. The calculations of ΔG_B and ΔS_B are based on Eqns (1) and (2).

$$\Delta G_B = -RT \ln K_{ass} \quad (1)$$

$$\Delta G_B = -\Delta H_B - T\Delta S_B \quad (2)$$

Thermodynamic magnitudes and binding constants are summarized in tables 1 and 2 for succinate and citrate, respectively.

The inhibitors studied, succinate and citrate, are closely structurally related to the natural substrate of fumarase, with citrate differing from succinate by one hydroxyl and one carboxymethyl group. Calorimetric measurements of binding heats, when structures such as these interact with fumarase, may demonstrate, generally, the changing roles of enthalpy and entropy in these processes as the temperature is varied. However, despite differences between ΔH_B values for succinate and citrate binding, these differences cannot be specifically attributed to any definite structural features.

Earlier studies, in which van 't Hoff enthalpies for succinate binding were obtained [8] indicated that ΔH_B was negative below 27°C and positive above that temperature. Our data on succinate binding presented in table 1 are in good agreement with the values cited above [8]. Moreover, tables 1 and 2, summarize not only the temperature dependences of ΔH_B, but also those of ΔG_B and ΔS_B, for succinate and citrate binding to fumarase at pH 7.4. At all temperatures there is a strong enthalpy–entropy compensation,

Table 2
Thermodynamic parameters per site per mol of protein for citrate binding to pig heart fumarase at $\Gamma/2$=0.03 and pH 7.4

Temperature (°C)	ΔH_B* (kcal · mol^{-1})	ΔS_B (cal · mol^{-1} · deg^{-1})	ΔG_B (kcal · mol^{-1})	K_{ass} (M^{-1})
25	2.599	−8.680	5.187	1.574×10^{-4}
28	2.896	−7.647	5.199	1.683×10^{-4}
30	3.034	−7.079	5.180	1.838×10^{-4}
32	3.387	−5.987	5.214	1.839×10^{-4}
35	3.870	−4.537	5.268	1.831×10^{-4}
37	4.155	−3.343	5.192	2.188×10^{-4}

* Calculated from the experimental data assuming 4 binding sites/mol of fumarase.

resulting in free energy values which are less dependent on temperature.

Macromolecule–ligand interactions may be accompanied by either conformational alternations or hydrophobic effects or both, and such processes have been used to interpret heat capacity changes observed in another enzyme–inhibitor interaction [16]. Only few ΔC_p values have been reported for the binding of low molecular weight compounds to proteins. All values found so far have been surprisingly large and negative (in the range of –5 to –10 kcal · mol^{-1} · deg^{-1}), suggesting either conformational changes of the protein molecule, or hydrophobic binding effects of some groups of the low molecular weight compounds [16]. Hydrophobic effects are always expected to cause a marked decrease of the apparent heat capacity of the reaction system. For the present purposes ΔC_p values have been derived from enthalpy values in tables 1 and 2 at temperatures of 25° and 37°C, respectively. In the instance of succinate binding we obtained a ΔC_p value of +0.100 kcal · mol^{-1} · deg^{-1} and in citrate binding a ΔC_p of +0.129 kcal · mol^{-1} · deg^{-1}. These values are close to zero, indicating that the binding reactions result in no decreased exposure of hydrophobic groups to the bulk of water, and that only minor conformational changes take place when succinate or citrate are bound to fumarase.

In summary, complete sets of thermodynamic parameters are presented for binding of two competitive inhibitors to fumarase. The data indicate that the enthalpies of binding of succinate and citrate, to pig heart fumarase are positive in the range of 25–37°C and pH 7.4. The differences in the contribution of ΔH_B and ΔS_B to the free energy of inhibitor binding, as calculated from actually measured data, cannot be related easily to differences in structures. The results of heat capacity changes obtained show that the binding of inhibitors to fumarase is accompanied by minor conformational changes of the enzyme.

Acknowledgement

This study was supported by the Grant No. VII/2-72 from the Croatian Republican Research Fund.

References

[1] Frieden, C. and Alberty, R. A. (1955) J. Biol. Chem. 212, 859–868.
[2] Frieden, C., Wolfe, R. G., Jr. and Alberty, R. A. (1957) J. Am. Chem. Soc. 79, 1523–1525.
[3] Brant, D. A., Barnett, L. B. and Alberty, R. A. (1963) J. Am. Chem. Soc. 85, 2204–2210.
[4] Mann, P. J. G. and Woolf, B. (1930) Biochem. J. 24, 427–434.
[5] Massey, V. (1951) Nature, 167, 769–770.
[6] Massey, V. (1953) Biochem. J. 53, 67–71.
[7] Massey, V. (1953) Biochem. J. 53, 72–79.
[8] Massey, V. (1953) Biochem. J. 55, 172–177.
[9] Kanarek, L., Marler, E., Bradshaw, R. A., Fellows, R. E. and Hill, R. L. (1964) J. Biol. Chem. 239, 4207–4211.
[10] Bjurulf, C., Laynez, J. and Wadsö, I. (1970) Eur. J. Biochem. 14, 47–52.
[11] Bjurulf, C. and Wadsö, I. (1972) Eur. J. Biochem. 31, 95–102.
[12] Kanarek, L. and Hill, R. L. (1964) J. Biol. Chem. 239, 4202–4206.
[13] Frieden, C., Bock, R. M. and Alberty, R. A. (1954) J. Am. Chem. Soc. 76, 2482–2484.
[14] Robinson, G. W., Bradshaw, R. A., Kanarek, L. and Hill, R. L. (1967) J. Biol. Chem. 242, 2709–2718.
[15] Lovrien, R. and Sturtevant, J. M. (1971) Biochemistry 10, 3811–3815.
[16] Wadsö, I. (1972) in MTP International Review of Science, Physical Chemistry, Vol. 10 (H. A. Skinner, ed.) Butterworth, London.