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# Calorimetric Study of Ligand Binding to Fumarase

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A microcalorimetric method has been used to study the binding of inhibitors, succinate and citrate, to pig heart fumarase at single conditions, i. e. 37 °C, pH 7.4 and Γ/2 0.03, but by using three buffer systems with different heats of ionization. Binding processes with succinate and citrate were endothermic and the same general pattern was obtained for the inhibitors used ($\Delta G^0 \sim 37$ or 38 kJ. mol$^{-1}$). It has been shown that 0.1 or 0.2 moles of $H^+$ per mol of enzyme was absorbed when the enzyme was saturated with succinate or citrate, respectively. Binding processes in buffer systems with different heats of ionization made possible the calculations of enthalpies of binding ($\Delta H^{0\prime} \sim 16.4$ or 16.8 kJ mol$^{-1}$) in a hypothetical buffer system with zero heat of ionization.

## INTRODUCTION

Enzyme action has been the subject of innumerable kinetic studies directed toward understanding the details of catalysis. None of these studies has progressed to the point where the complete process could be described in quantitative terms. A set of thermodynamic parameters for all process steps would provide a framework within which kinetic parameters should fit. The binding of structural analogs of the substrate to the enzyme can serve as a model for the first step in catalysis. By measuring the enthalpies of succinate and citrate binding to fumarase in phosphate buffer[1] and in Krebs mammalian Ringer solution[2], we found that those interactions were endothermic. In the present study the enthalpies of binding of the same inhibitors to pig heart fumarase were determined at single conditions, i. e. 37 °C, pH 7.4 and Γ/2 0.03, but by using three buffer systems with different heats of ionization. This enabled us to calculate the enthalpies of binding in a hypothetical buffer with zero heat of ionization, which were also found to be endothermic. It was found that binding of ligands was accompanied by proton uptake ranging from 0.1 to 0.2 mol of $H^+$ per mol of enzyme.

## EXPERIMENTAL

Pig heart fumarase (fumarate hydratase, EC 4.2.1.2) was purchased from Boehringer Mannheim GmbH, as a 1% crystalline suspension in ammonium sulphate and was used without further purification. The inhibitors were analytical grade; sodium succinate was supplied by N. B. Company and sodium citrate was from Kemika, Zagreb. Buffers, Pipes (Piperazine-1,4-diethane-sulphonic acid) and Hepes (2-/4-(2--Hydroxyethyl)-1-piperazinyl/-ethanesulphonic acid) were purchased from E. Merck, Darmstadt. The water used for preparing the buffer solutions was demineralized and distilled in a glass still.

Measurements of fumarase activity were performed at ambient temperature (25 °C) in a Varian Techtron Model 635 spectrophotometer at 240 nm in 100 mM phosphate buffer pH 7.4 with L-malate as substrate ($K_m = 4.1 \times 10^{-3}$ M). The molecular weight of fumarase was assumed to be 194,000.[3]

Calorimetric measurements were performed in 42 mM Pipes pH 7.4 and 42 mM Hepes pH 7.4, both with ionic strenght Γ/2 0.03. The pH was checked by Beckman Expandomatic pH-meter, standardized at pH 7.41. Enzyme solutions were prepared immediately prior to measurements; the fumarase concentration was 171.8 μmol $kg^{-1}$ in the calorimetric liquids and was kept constant in all experiments. Fresh inhibitor solutions were prepared daily with varying concentrations within a range of 2.47 to 29.65 mmol $kg^{-1}$ for succinate and 4.53 to 27.19 mmol $kg^{-1}$ for citrate.

Microcalorimetric measurements were performed with LKB Batch Micro Calorimeter Model 10700-2, using 18-carat gold reaction vessels; details were presented in our previous paper[1]. The calorimeter performance was repeatedly checked by determining the enthalpy of dilution of sucrose solutions. The calorimetric vessels were filled by means of glass syringes and the sample quantities were determined by differential weighing. The two compartements in the reaction vessel were filled with 1.00 ml of fumarase solution and 2.00 ml of inhibitor solution. The two compartements in the reference vessel were filled with 1.00 ml of buffer solution (Pipes or Hepes) and 2.00 ml of inhibitor solution. Thus, the enthalpy of dilution of inhibitor was corrected for. Separate experiments were made to determine the heat of dilution of fumarase, using the same buffer and the same temperature as under reaction conditions.

## RESULTS AND DISCUSSION

The heat absorbed ($Q$) when succinate is bound to the enzyme, plotted as a function of inhibitor concentration, at 37 °C in Pipes buffer, is presented in Figure 1. At a constant temperature, a typical saturation curve is obtained. From the experimental heat values ($Q$) corrected for dilution heats, $\Delta H_{SC}$,

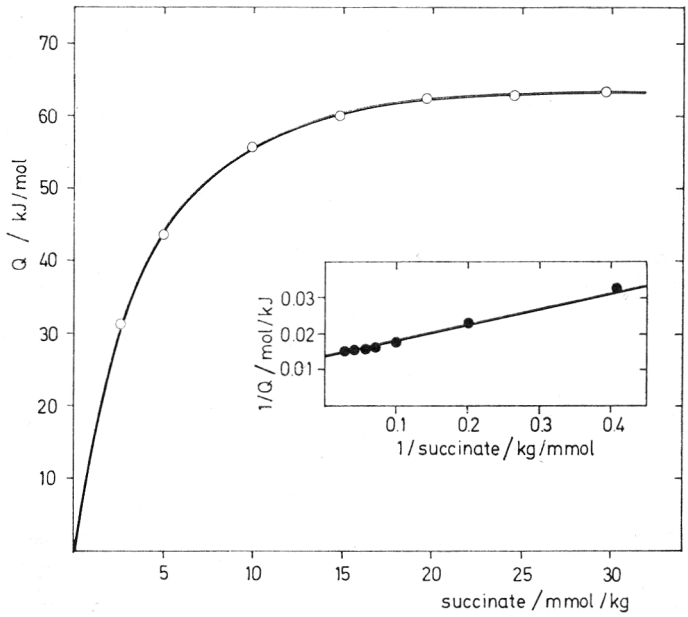


Figure 1. Calorimetrically measured heats of succinate binding to fumarase, as a function of final inhibitor concentration. Temp. 37 °C, 42 m*M* Pipes buffer, pH 7.4, Γ/2 0.03. The concentration of fumarase was 171.8 μmol/kg in all experiments. Inset: Double reciprocal plot of 1/$Q$ vs. 1/(succinate). Each point in Figure 1 and in the inset represents the average of at least triplicate determinations.

the enthalpy under saturated conditions was obtained according to Wadsö et al.[4,5], by plotting $1/Q$ vs. $1/I$ as shown on the inset of Figure 1. Line fitting was carried out by computer treatment through minimizing the square error sum for experimental points forming a straight line.

Typical binding results, as shown in Figure 1, can be utilized for calculations based on certain assumptions. The most important one is that in fumarase there are four independent binding sites per molecule, as suggested by Kanarek and others[3,6–8]. We further assumed that those sites are saturated at inhibitor concentrations corresponding to the plateau regions of the experimentally obtained heat-binding curves, and that the heat of binding per molecule of ligand is independent of the extent of binding of previously bound ligand.

In making calorimetric measurements of the binding of ionic ligands to the macromolecule, competition by buffer ions for binding sites should be considered. Two of the buffers used in this study, Pipes and Hepes have monovalent anions, while the phosphate buffer has a bivalent anion. The fumarase binding sites can bind anions and the measured heats ($\Delta H_{SC}$) include the heat of binding of the anion. Therefore, we performed our experiments in three buffers with the same ionic strenght, but with different heat of ionization ($\Delta H_i$) (phosphate buffer 4.73[9], Pipes 11.46[10] and Hepes 20.96 kJ/mol of proton[10]). Values for the limiting enthalpy change $\Delta H_{SC}$ were plotted as functions of the buffer heats of ionization $\Delta H_i$ in Figure 2. The linearity between the

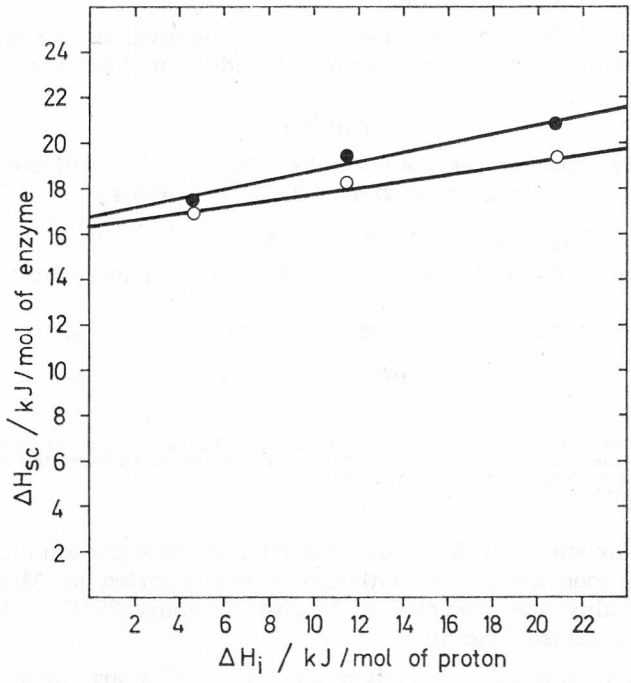


Figure 2. The variation of $\Delta H_{SC}$, the heat of association of ligand, with $\Delta H_i$, the heat of ionization of the buffer, for saturating levels of succinate (○—○) and citrate (●—●) with pig heart fumarase, at 37 °C, pH 7.4 and $\Gamma/2$ 0.03. The heat of ionization ($\Delta H_i$ kJ/mol) for phosphate buffer 4.73, Pipes 11.46 and for Hepes 20.96.

variation of $\Delta H_{SC}$ and $\Delta H_i$ (shown in Figure 2) indicates that undoubtedly there is a change in protonation of the protein when the inhibitor is bound. The values in phosphate buffer are taken from our previous paper[1].

The interaction between inhibitor and enzyme at saturation in an adequately buffered solution can be separated into two processes (eqs. 1 and 2):

$$E + mI + nH^+ \rightleftharpoons E\,I_m\,H_n^{n+}; \quad \Delta H_B \tag{1}$$

$$n\,BH^+ \rightleftharpoons nB + nH^+; \quad n\,\Delta H_i \tag{2}$$

E refers to enzyme, I to inhibitor and B to unprotonated buffer. Two straight lines in Figure 2, for succinate and citrate binding, correspond to the following equation:

$$\Delta H_{SC} = n\Delta H_i + \Delta H_B \tag{3}$$

where $\Delta H_B$ is the enthalpy of binding in a hypothetical buffer with zero heat of ionization, calculated from the ordinate intercept (on Figure 2) by computer treatment of experimental data, using a least-square program. Corrections were not made for buffer anion binding in calculations of $\Delta H_B$ and $n_B$ from the data in Figure 2. Numbers of moles of proton binding per mol of protein, $n_B$, calculated from the slope, leads to the conclusion that 0.15 moles of $H^+$ per mol of protein were absorbed when succinate is bound and in the case of citrate 0.20 moles of $H^+$ per mol of protein were absorbed, at 37 °C and $\Gamma/2$ 0.03. Values are presented in Table I with other derived thermodynamic parameters and binding constants.

The structural features that cause the thermodynamic parameters, cited in Table I, to be different for two various ligands cannot be specified precisely.

TABLE I

*Thermodynamic Parameters per Mol of Binding Sites for Ligand Binding to Pig Heart Fumarase at 37 °C, $\Gamma/2$ 0.03 and pH 7.4*

| Ligand | $n_B$ mol of $H^+$ $mol^{-1}$ | $\Delta H_B^{0\prime a}$ kJ $mol^{-1}$ | $\Delta G_B^{0\prime b}$ kJ $mol^{-1}$ | $\Delta S_B^{0\prime c}$ J $mol^{-1}$ $deg^{-1}$ | $K_B$ $M^{-1}$ |
|---|---|---|---|---|---|
| Succinate | + 0.15 | 16.35 | 37.6 | — 68.7 | $0.5 \times 10^{-6}$ |
| Citrate | + 0.20 | 16.83 | 38.8 | — 70.7 | $0.3 \times 10^{-6}$ |

[a] Calculated from experimental data assuming 4 binding sites per mol of fumarase.
[b] Calculated from the value of $\Delta G_B^{0\prime} = -RT \ln K_B$ obtained at 37 °C, using the Gibbs-Helmholtz equation and the enthalpies given in column 3.
[c] $\Delta S_B^{0\prime} = (\Delta H_B^{0\prime} - \Delta G_B^{0\prime})/T$.

Our data for succinate-fumarase and citrate-fumarase binding at 37 °C in Table I are in good agreement with the values reported by Massey[11], where van't Hoff enthalpy was indicated to be positive above 27 °C, and was 17.6 kJ for fumarate-fumarase binding.

In summary, sets of thermodynamic parameters are presented for the binding of two competitive inhibitors to fumarase. The data indicate that in a hypothetical buffer system with zero heat of ionization the binding reactions of succinate and citrate are endothermic. In addition to the data of binding of

the same inhibitors in phosphate buffer at different temperatures[1] and in quasi physiological conditions[2], our results might be representative of possible binding effects of such inhibitors to fumarase, an enzyme from the Krebs cycle.

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## SAŽETAK

### Kalorimetrijski studij vezivanja liganada na fumarazu

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Mikrokalorimetrijskom metodom praćeno je vezivanje inhibitora na fumarazu izoliranu iz svinjskog srca pod istim uvjetima, tj. 37 °C, pH = 7,4 i $\Gamma/2 = 0{,}03$ mol dm$^{-3}$. Upotrijebljena su tri puferska sistema s različitim toplinama ionizacije. Studirani su inhibitori sukcinat i citrat, te je nađeno da su procesi vezivanja endotermi, i dobivene su uglavnom jednake veličine ($\Delta G^{0\prime} \sim 37$ ili 38 kJ mol$^{-1}$) za oba inhibitora. Nađeno je da se 0,1 ili 0,2 mola $H^+$ po molu enzima veže kada je enzim zasićen sukcinatom ili citratom. Procesi vezivanja u puferskim sistemima s različitim toplinama ionizacije omogućuju izračunavanje entalpije vezivanja ($\Delta H^{0\prime} \sim 16{,}4$ ili 16,8 kJ mol$^{-1}$) u hipotetskom puferu s toplinom ionizacije nula.

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