Allosteric Properties of Inosine Monophosphate Dehydrogenase Revealed through the Thermodynamics of Binding of Inosine 5′-Monophosphate and Mycophenolic Acid. Temperature Dependent Heat Capacity of Binding as a Signature of Ligand-Coupled Conformational Equilibria†

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ABSTRACT: The thermodynamic properties of binding of the substrate, inosine monophosphate (IMP), and the uncompetitive inhibitor, mycophenolic acid, to inosine monophosphate dehydrogenase (IMPDH) were measured. Specifically, the free energy, enthalpy, entropy, and heat capacity changes were determined for each ligation state of the tetrameric enzyme, over a temperature range from 2.5 to 37 °C by high-precision titration microcalorimetry. It was discovered that IMP binds to IMPDH in a negatively cooperative fashion and that mycophenolic acid binding is critically dependent on the presence of IMP. Moreover, the binding of IMP is entropically driven at low temperatures and enthalpically driven at high temperatures, with an unusually large, temperature dependent heat capacity change. A thermodynamic argument, based on the general nature of the heat capacity function for a binding reaction and its temperature dependence, is used to infer the existence of an equilibrium mixture of at least two structural forms of apo-IMPDH. The equilibrium is perturbed in the presence of IMP and mycophenolic acid, suggesting a mechanism for the ligand-linked conformational changes. An allosteric model, incorporating subunit−subunit interactions nested within a concerted conformational change involving the entire tetrameric macromolecule, is proposed to account for the observed binding behavior. The implications of these findings for the design of novel "allosteric-effector" inhibitors of IMPDH, to be used for the purpose of immunosuppression, are discussed.

Inosine monophosphate dehydrogenase (IMPDH),[1] the first enzyme in the committed pathway of the *de novo* biosynthesis of guanine nucleotides, catalyzes the NAD-dependent conversion of inosine 5′-monophosphate to xanthosine 5′-monophosphate. Mycophenolic acid is a tight binding, uncompetitive inhibitor of IMPDH that acts as a potent immunosuppressive agent by depleting intracellular GTP pools of lymphocytes, leading to inhibition of lymphocyte proliferation. Lacking metabolic salvage pathways for the production of guanine nucleotides used by most cells, lymphocytes must rely solely on *de novo* biosynthesis, rendering mycophenolic acid a T-cell and B-cell specific antiproliferant (Allison *et al.*, 1977). As clinical proof-of-principle for IMPDH as a viable molecular target, the U.S. Federal Drug Administration has recently approved mycophenolate mofetil, an oral prodrug of mycophenolic acid, for use in combination therapy with other immunosuppressive agents to prevent acute rejection of kidney transplants (Shaw *et al.*, 1995; Sollinger, 1995).

Despite its efficacy, mycophenolic mofetil produces a number of undesirable side effects in patients. Toxicities of other immunosupressive drugs currently used for combating rejection following tissue or organ transplant surgery are well-known, and the mechanism of toxicity of mycopheolic mofetil has been the focus of ongoing pharmacological investigations (Shaw *et al.*, 1995). The identification of substructural elements in mycophenolic acid that are responsible for its recognition and inhibition of IMPDH, and those substructural elements that are responsible for the toxicity, allows one to assess and develop drug design strategies aimed at achieving more efficacious, less toxic immunosuppressive agents. Additionally, the identification of new properties of the enzyme can open up novel strategies for the design of inhibitors that may lead to the development of more effective immunosuppressive agents. Therefore, as part of a research program in the molecular biochemistry and biophysics of immunosuppression and autoimmune diseases, we have investigated the energetics of binding of substrates and inhibitors to IMPDH.

Analysis of the effects that IMP and XMP have on the binding of MPA, in view of the proposed mechanisms of catalysis by IMPDH (Antonino & Wu, 1995), has led to the proposal of a model for the enzymatic mechanism of action of mycophenolic acid [Fleming *et al.*, 1996; Link & Straub, 1996; see also Hedstrom and Wang (1990)]. Additionally, the crystal structure of the tetrameric form of IMPDH bound with mycophenolic acid and a partially turned over substrate has been solved, allowing a detailed structural interpretation of the mechanism of mycophenolic acid inhibition (Sintchak

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[1] Abbreviations: IMP, = inosine 5′-monophosphate; IMPDH, inosine monophosphate dehydrogenase; BME, β-mercaptoethanol; Gdn·HCl, guanidinium hydrochloride.

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et al., 1996). In this report, we present results on the thermodynamics of ligand binding in a fashion that draws attention to some important principles of molecular recognition in the IMPDH system that have not been realized from previous biochemical or structural analyses. In particular, our findings indicate that IMPDH functions as an allosteric protein.

Specifically, stepwise changes in the enthalpy, entropy, free energy, and heat capacity of IMP to the tetrameric form of IMPDH, in the temperature range from 5 to 40 °C, have been determined by high-precision titration calorimetry. The thermodynamic functions for the binding of MPA to IMPDH in the presence of IMP have likewise been measured. A thermodynamic argument is presented that identifies a temperature dependent heat capacity change of binding as a hallmark of a conformational equilibrium of distinct structural states in an unbound protein. In addition to revealing the allosteric properties of IMPDH, the results illustrate the general utility of investigating thermodynamic linkage properties for guiding the design of small molecule inhibitors for multi-ligand, multisubunit protein targets.

EXPERIMENTAL PROCEDURES

Materials. Hamster IMPDH type II was purified by the methods of Gilbert *et al.* (1976) and Ikegami *et al.* (1987) with modifications. *Escherichia coli* cell paste containing overexpressed hamster IMPDH (type II) was suspended in 50 mM Tris·HCl, pH 8.0, with 300 mM KCl, 2 mM EDTA, 10 mM BME, 1.5 mM urea, and then lysed with a microfluidizer. The following inhibitors were added prior to cell lysis to avoid proteolysis: 0.2 mM PMSF, 1 μg/L each of Pepstatin/Leupeptin/E-64. Cellular debris was removed by centrifugation at 19 000 rpm for 80 min at 4 °C. Ammonium sulfate was added slowly to bring the sample to a final concentration of 25% w/v, before centrifuging at 19 000 rpm for an additional 80 min. The pellet was resuspended in 50 mM Tris·HCl, 300 mM KCl, 2 mM EDTA, 10 mM BME, 10% glycerol at pH 8.0, and loaded onto an IMP affinity column (Gilbert *et al.*, 1976). IMPDH activity in the fractions was determined by monitoring the absorbance at 340 nm indicating the formation of NADH. The enzyme-containing fractions were then pooled and concentrated prior to being applied to a Sephacryl S-300 gel filtration column to remove IMP and any aggregates of IMPDH. In order to completely remove IMP, purified IMPDH was extensively dialyzed into the desired buffer conditions. Absense of protein aggregation was confirmed by light-scattering measurements. All buffers were degassed and contained fresh BME as a continuous purge of N_2 gas was bubbled through the dialyzate. To ensure that IMP was absent after dialysis, protein samples were checked for residual IMP by capillary electrophoresis. No traces of IMP were detected. In order to ensure that the association state of the protein remained constant under all conditions employed in these studies, analytical size exclusion chromatography and light-scattering investigations were performed, revealing that the protein remained in the tetrameric state (Fleming *et al*., 1996; Sintchak *et al*., 1996). Additionally, binding constants of IMP were determined at various protein concentrations. No change in binding constants were observed, again confirming the absence of ligand linked change in the association state of the protein. Finally, to ensure that the protein remained in a native, folded state under conditions employed here, the thermal unfolding of the protein was monitored by fluorescence spectroscopic, circular dichroism, and differential scanning calorimetry (F. J. Bruzzese, unpublished results). The protein had a midpoint unfolding temperature of 71 °C and was fully folded at the highest temperature (37 °C) employed in this study.

Titration Calorimetry. The binding of IMP or mycophenolic acid was measured by titrating (typically) 4−6 μL injections of 2 mM ligand into 100−250 μM protein in a MicroCal OMEGA titration microcalorimeter (Northampton, MA). The concentration of apo-IMPDH was determined optically with an HP-8452A diode array spectrophotometer [ϵ(IMPDH) = 0.441 cm^2/mg in 8.0 M Gdn·HCl at 276 nm (Fleming *et al*., 1996)]. The concentrations of IMP was determined by weight and then checked spectrophotometrically [ϵ(IMP) = 12.7 cm/mM in 0.1 M potassium phosphate, pH 7.0, at 249 nm (Sigma technical support, personal communication)]. The concentration of mycophenolic acid was determined by weight. Experiments were carried out under the following solution conditions: 150 mM bicine, 300 mM KCl, 2 mM EDTA, 10 mM BME, with 10% glycerol.

Since IMPDH is composed of four identical subunits and remains as a tetramer under the conditions employed here, the general phenomenological description of binding may be cast in terms of the Adair formalism (Adair, 1925). The heat absorbed or released, Δq_i, after each injection of ligand solution into a solution of IMPDH, as detected by the calorimeter, is given by

$$\Delta q_i = q_i + \frac{v_i}{V_o}\left(\frac{q_i + q_{i-1}}{2}\right) - q_{i-1} \qquad (1)$$

where q_i, the heat content of the cell after the ith injection is given by

$$q_i = M_t V_o \sum_{j=1}^{4} n_j \qquad (2)$$

where V_o is volume of the calorimetric cell (1.38 mL), v_i is the volume of the ith injection, and M_t is the concentration of protein in the cell prior to the titration (Wiseman *et al*., 1989). The η_j's are the fractions (α_j) of protein in a given state of ligation (α_j, $j = 1-4$), weighted by the sum of the average intrinsic enthalpy changes for binding the first j ligands, ΔH_j. We term these quantities the enthalpically weighted species fractions:

$$n_j = \alpha_j \sum_{j=1}^{j} \Delta H_j \qquad (3)$$

We can also relate the fractional amount of macromolecule with 0, 1, 2, 3 or 4 ligand molecules bound (α_j) to the partition function (binding polynomial) for a four-site, nondissociating macromolecule as expressed with free ligand activity x, and (1) the overall Adair parameters (β_j − eq 4a) reflecting the reaction from the unliganded macromolecule to the jth state of ligation; (2) the stepwise binding constants (K_j − eq 4b) reflecting the equilibrium constants for the four successive binding reactions; or (3) the intrinsic stepwise

Table 1: Intrinsic Binding Constants and Enthalpy Changes for IMP Binding to IMPDH at Various Temperatures Derived from the Nonlinear Least-Square Analyses of Calorimetric Titrations Illustrated in Figure 1

T (°C)	$\kappa_1 = \kappa_2 = \kappa_3$ (M^{-1})	κ_4 (M^{-1})	Δh_1 kcal/mol	Δh_2 kcal/mol	Δh_3 kcal/mol	Δh_4 kcal/mol
2.5	$5.6\ (\pm1.5) \times 10^5$	$4.1\ (1.1) \times 10^5$	11.1 ± 0.2	31 ± 1	41 ± 1	14.1 ± 0.7
8.2	$6.7\ (\pm1.6) \times 10^6$	$5.3\ (\pm0.6) \times 10^5$	8.9 ± 0.2	12.0 ± 0.3	11.0 ± 0.3	11.5 ± 0.2
17.4	$4.5\ (\pm1.1) \times 10^6$	$3.5\ (\pm0.5) \times 10^5$	0.9 ± 0.2	5.0 ± 0.3	0.6 ± 0.3	-1.8 ± 0.2
25.0	$2.4\ (\pm0.6) \times 10^6$	$2.3\ (\pm0.2) \times 10^5$	-6.5 ± 0.2	-9.4 ± 0.5	-12.1 ± 0.4	-18.1 ± 0.4
37.0	$1.1\ (\pm0.1) \times 10^6$	$1.3\ (\pm0.4) \times 10^5$	-30.2 ± 0.3	-41.0 ± 0.7	-29.0 ± 0.6	-30.4 ± 0.6

Table 2: Overall Thermodynamic Parameters for the Binding of IMP to IMPDH

T (°C)	ligation state, j	overall binding parameter, β_j (M^{-j})	free energy change, ΔG_j (kcal mol^{-1})	enthalpy change, ΔH_j (kcal mol^{-1})	entropy change, $T\Delta S_j$ (kcal mol^{-1})	heat capacity change, ΔC_j (kcal K^{-1} mol^{-1})
2.5	1	2.3×10^6	-8.0 ± 0.2	11.1 ± 0.2	19.1 ± 0.4	-0.054 ± 0.003
2.5	2	1.9×10^{12}	-15.4 ± 0.3	42.2 ± 1.2	57.6 ± 1.5	-1.5 ± 0.2
2.5	3	7.2×10^{17}	-22.5 ± 0.5	53.8 ± 2.2	76.3 ± 2.7	-2.1 ± 0.2
2.5	4	7.4×10^{22}	-28.8 ± 0.7	67.9 ± 1.9	96.7 ± 2.6	-2.6 ± 0.1
8.2	1	2.7×10^7	-9.6 ± 0.1	8.9 ± 0.2	18.5 ± 0.3	-0.4 ± 0.2
8.2	2	2.7×10^{14}	-18.5 ± 0.2	20.9 ± 0.5	39.4 ± 0.7	-2.0 ± 0.2
8.2	3	1.2×10^{21}	-27.2 ± 0.5	31.9 ± 0.8	59.1 ± 1.3	-2.9 ± 0.3
8.2	4	1.6×10^{26}	-33.8 ± 0.6	43.4 ± 1.0	77.2 ± 1.6	-3.8 ± 0.2
17.4	1	1.3×10^7	-9.5 ± 0.1	0.9 ± 0.2	10.4 ± 0.3	-1.01 ± 0.05
17.4	2	1.2×10^{14}	-18.7 ± 0.3	5.9 ± 0.5	24.7 ± 0.8	-2.9 ± 0.3
17.4	3	3.7×10^{20}	-27.3 ± 0.4	6.5 ± 0.8	33.8 ± 1.2	-4.1 ± 0.4
17.4	4	3.2×10^{25}	-33.9 ± 0.6	4.7 ± 1.0	38.6 ± 1.6	-5.8 ± 0.2
25.0	1	9.6×10^6	-9.5 ± 0.1	-6.5 ± 0.2	3.0 ± 0.3	-1.49 ± 0.04
25.0	2	3.4×10^{13}	-18.5 ± 0.3	-15.8 ± 0.7	2.6 ± 1.0	-3.6 ± 0.2
25.0	3	5.5×10^{19}	-26.9 ± 0.5	-27.9 ± 1.1	-1.0 ± 1.6	-5.1 ± 0.2
25.0	4	3.3×10^{24}	-33.4 ± 0.5	-46.0 ± 1.5	-12.6 ± 2.0	-7.4 ± 0.1
37.0	1	4.4×10^6	-9.4 ± 0.1	-30.2 ± 0.3	-20.8 ± 0.4	-2.3 ± 0.1
37.0	2	7.3×10^{12}	-18.3 ± 0.2	-71.2 ± 1.0	-53.0 ± 1.2	-4.7 ± 0.5
37.0	3	5.3×10^{18}	-26.6 ± 0.2	$-100. \pm 1.6$	-73.6 ± 1.8	-6.7 ± 0.7
37.0	4	3.6×10^{23}	-33.4 ± 0.4	$-151. \pm 2.2$	$-117. \pm 2.8$	-9.9 ± 0.4

binding constants (κ_j − eq c), reflecting the equilibrium constants for the four successive binding reactions weighted by the appropriate statistical factors (Wyman & Gill, 1990):

$$P = 1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 \quad (4a)$$

$$P = 1 + K_1 x + K_1 K_2 x^2 + K_1 K_2 K_3 x^3 + K_1 K_2 K_3 K_4 x^4 \quad (4b)$$

$$P = 1 + 4\kappa_1 x + 6\kappa_1 \kappa_2 x^2 + 4\kappa_1 \kappa_2 \kappa_3 x^3 + \kappa_1 \kappa_2 \kappa_3 \kappa_4 x^4 \quad (4c)$$

Each term in the binding polynomial reflects the relative concentration of IMPDH with a specific number of ligand molecules bound. The fractional concentrations of the species are then given by the appropriate term in the binding polynomial divided by P:

$$\alpha_j = \frac{\beta_j x^j}{P} \quad (5)$$

where β_0 is defined as 1, so that the unliganded species fraction is simply P^{-1}.

RESULTS

IMP Binds to Apo-IMPDH with Negative Cooperativity and a Large Heat Capacity Change That Is Temperature Dependent. The binding of IMP to IMPDH produces a titration profile that departs significantly from that described by a model representing a macromolecule with independent and non-interacting binding sites (Figure 1). These data were analyzed according to eq 1, with the binding polynomial expressed with intrinsic constants as in eq 4c. Upon analyzing the calorimetric data at each temperature, we noticed that the parameters were converging on similar values for the first three intrinsic binding constants. The data were then fit to a model which incorporated the constraint, $\kappa_1 = \kappa_2 = \kappa_3$, with no significant change in the standard error of a point. Such a constraint is justified mathematically, since no significant difference in the fit is obtained by including the imposed constraint (Gill *et al*., 1988). It is motivated by the interest in obtaining higher resolution of the other adjustable phenomenological parameters. Physically, this model indicates that the first three IMP molecules bind with the same intrinsic affinity, albeit with different enthalpies and intrinsic entropies of binding, and that the fourth IMP molecule binds with significantly different affinity than the first three. The values of the intrinsic binding constants and associated enthalpies are given in Table 1. Table 2 provides values of the overall equilibrium constants for each of the four sites, and the associated enthalpy, entropy, and heat capacity changes, as a function of temperature. For each temperature in which IMP binding was studied, the first three intrinsic binding constants were the same, and the fourth constant indicated significantly weaker binding.

Often, ligand binding can have a significant pH dependence indicating that protons are absorbed or released upon the binding of ligand. Calorimetrically, the number of protons absorbed or released in the reaction can be measured by performing titrations in two different buffers having different heats of ionization. We therefore performed additional titrations of IMP in bis-Tris buffer at pH 8.0 and 25 °C. However, we observed no difference in the derived

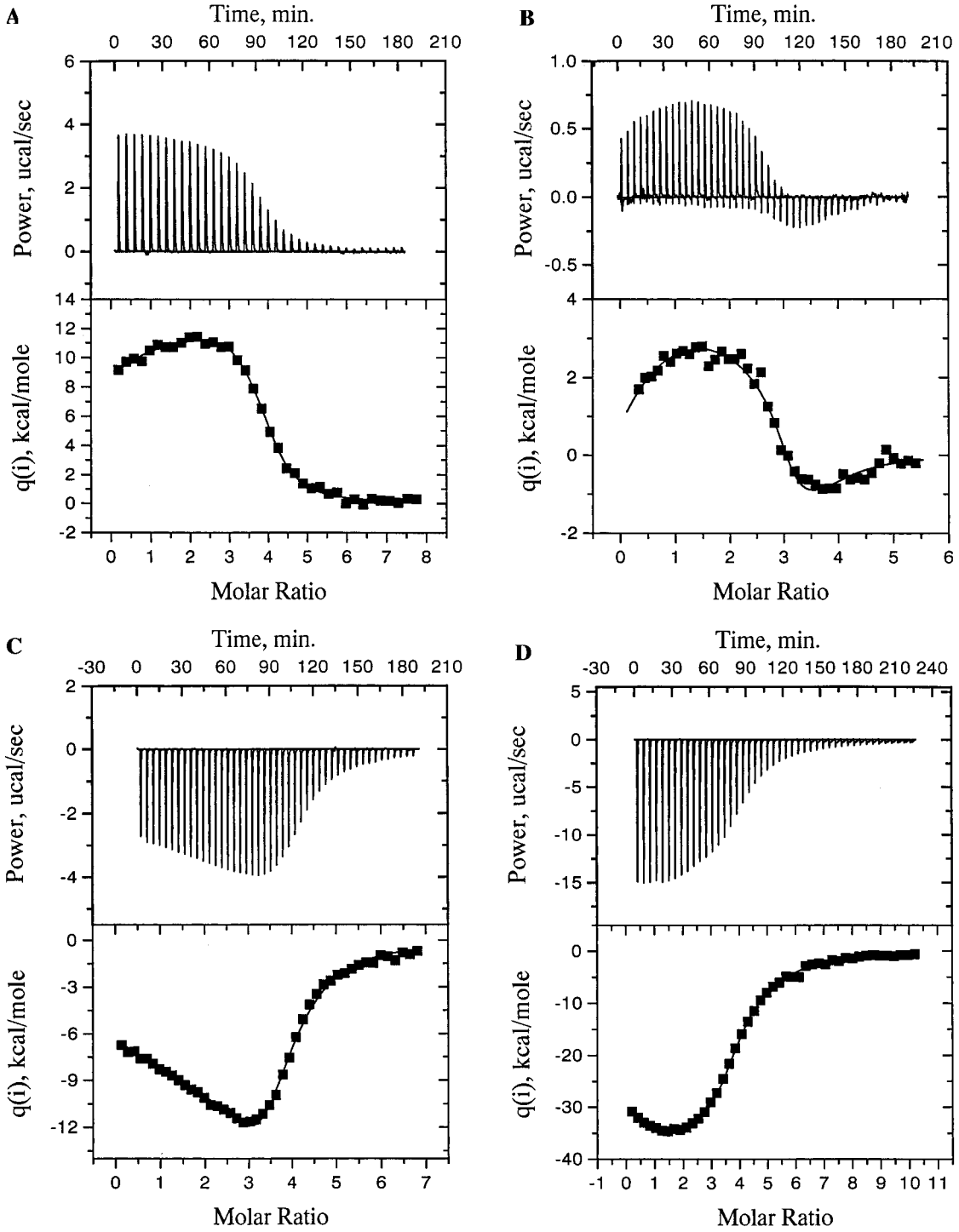


FIGURE 1: Titration calorimetric isotherms showing the heat observed upon injecting aliquots of IMP into a solution of IMPDH at (A) 8.2 °C, (B) 17.1° C, (C) 25 °C, and (D) 37 °C. Lines through the points represent best fits to the data as analyzed by eq 1.

enthalpy changes and binding constants in the two buffers, indicating that there is no detectable change in proton uptake upon binding of IMP at pH 8.0.

From the data in Table 1 and Figure 1, it is apparent that the enthalpy change is strongly temperature dependent. This is illustrated in a striking fashion by the series of titrations of IMP into IMPDH at different temperatures shown in Figure 1. At high temperature, the binding of IMP is strongly exothermic with an overall enthalpy change of −151 kcal/mol-of-tetramer; at 25 °C the overall enthalpy change is less exothermic, −46 kcal/mol-of-tetramer. At 17.4 °C, the overall enthalpy change for IMP binding is nearly zero, +5 kcal/mol-of-tetramer. In fact, at 17 °C, the first several injections are endothermic while the last few are exothermic (Figure 1)! At lower temperatures, the binding is completely endothermic (43 kcal/mol-of-tetramer at +8.2 °C and +68 kcal/mol-of-tetramer at 2.5 °C).

Since the enthalpy change of a reaction is related to the variation of the binding constant with temperature, and we have determined both the temperature dependencies of each of the binding constants and the associated calorimetric enthalpies, there is a redundancy of thermodynamic data. To ensure the overall consistency of the data set, we performed a global analysis of the enthalpy and free energy data as function of temperature (Varadarajan *et al.*, 1992). This was accomplished by simultaneously fitting the enthalpy

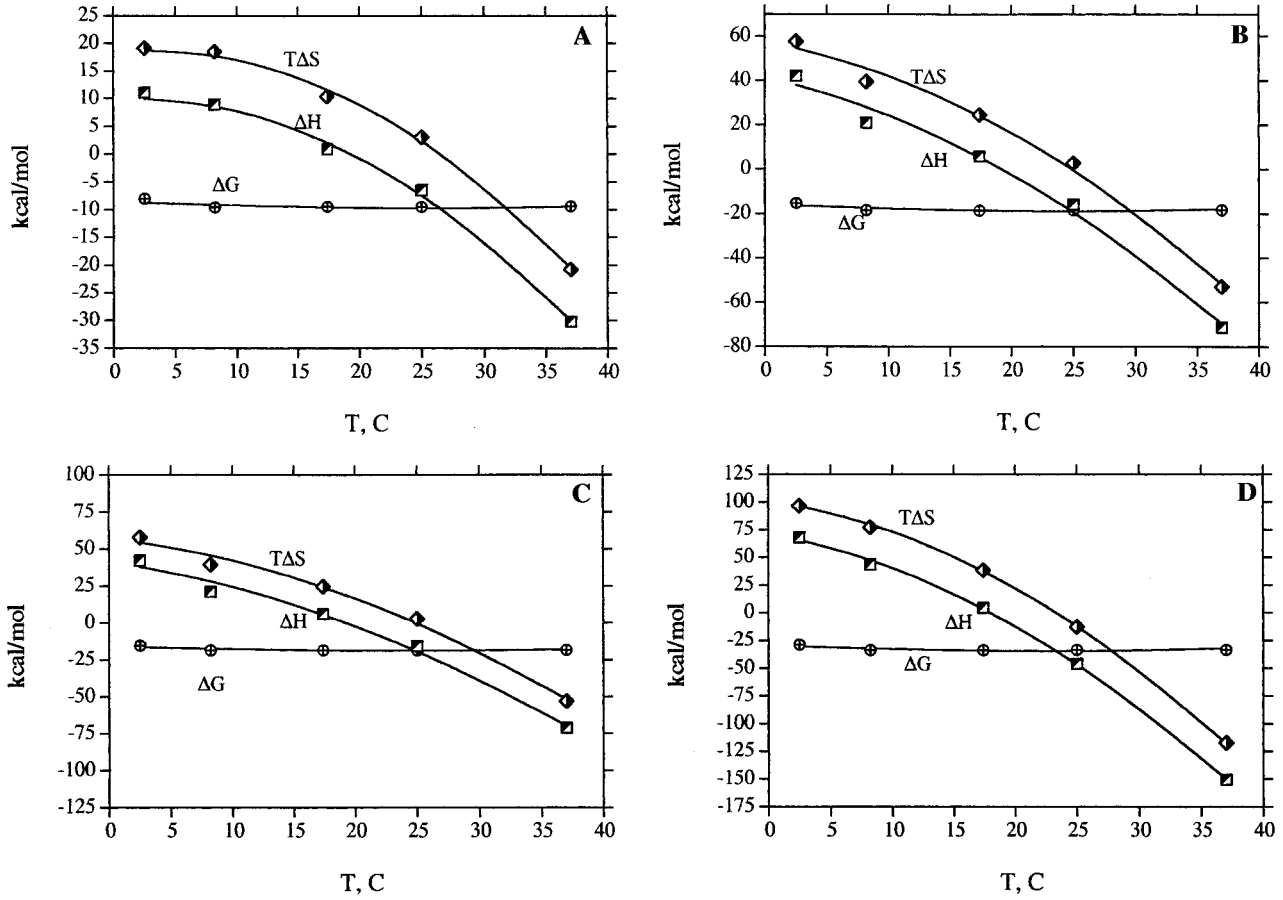


FIGURE 2: Global analysis of enthalpy and free energy changes for IMP binding to IMPDH as a function of temperature according to eqs 6 and 7. The binding over the entire temperature range could be described the following best fit overall parameters at $T^* = 25$ °C, for each ligation state (j), which describe the solid curves in the figures: (A) $j = 1$, $\Delta H^* = -7.5 \pm 0.6$, $\Delta G^* = -9.8 \pm 0.4$, $\Delta C^* = -1.49 \pm 0.04$, $\Delta\varphi_p = -0.064 \pm 0.008$; (B) $j = 2$, $\Delta H^* = -19.4 \pm 2.6$, $\Delta G^* = -18.9 \pm 1.7$, $\Delta C^* = -3.6 \pm 0.2$, $\Delta\varphi_p = -0.095 \pm 0.027$; (C) $j = 3$, $\Delta H^* = -29.4 \pm 1.8$, $\Delta G^* = -27.5 \pm 1.2$, $\Delta C^* = -5.1 \pm 0.2$, $\Delta\varphi_p = -0.13 \pm 0.02$; (D) $j = 4$, $\Delta H^* = -46.7 \pm 1.5$, $\Delta G^* = -34.5 \pm 1.0$, $\Delta C^* = -7.4 \pm 0.1$, $\Delta\varphi_p = -0.210 \pm 0.016$. Units on parameters are the same as that given in Table 2; units on $\Delta\varphi_p$ are kcal mol^{-1} K^{-2}.

and free energy data as a function of temperature, derived from the data in Figure 1, to the following set of equations which have three of the four parameters in common:

$$\Delta G = \Delta G^* + T\int_{\tau^*}^{\tau}\Delta H\, d\tau \quad (6)$$

$$\Delta H = \Delta H^* + \int_{T^*}^{T}\Delta C\, dT \quad (7)$$

where $\tau = 1/T$. Four parameters suffice to describe the temperature dependence of the thermodynamic functions: the free energy (ΔG^*), enthalpy (ΔH^*), and heat capacity changes (ΔC_p^*) at $T^* = 25$ °C, and a term reflecting a linear temperature dependence of the heat capacity change ($\Delta\varphi_p$),

$$\Delta C_p = \Delta C_p^* + \Delta\varphi_p(T - T^*) \quad (8)$$

As shown in Figure 2, the data sets for each site fit well to this consistent pair of thermodynamic expressions which embody the van't Hoff equation that links the variation of the free energy with temperature to the enthalpy function. The converged estimates are given in the legend to Figure 2. The fact that good global fitting was obtained for each of the overall free energy and enthalpy data sets indicates that the van't Hoff enthalpy and calorimetric enthalpy are consistent for this system.

Mycophenolic Acid Associates Non-Cooperatively with the IMP−IMPDH Complex. When mycophenolic acid was titrated into a solution of apo-IMPDH, no heats were observed, indicating that binding does not occur or that it is extremely weak. Although possible, it is unlikely that mycophenolic acid binds appreciably, with an enthalpy change close to zero. However, the fact that mycophenolic acid does not bind appreciably to apo-IMPDH is consistent with the uncompetitive mechanism of inhibition (Hedstrom & Wang, 1990). In the presence of IMP, mycophenolic acid binds and produces a simple non-interacting binding site titration isotherm. Analysis of the data yielded the binding constants and enthalpy changes of the reaction. Global analysis of the free energy and enthalpy changes over a range of temperature according to eqs 6 and 7 produced a good fit (Figure 3). The converged estimates of the free energy, enthalpy, and heat capacity changes at 25 °C, and a parameter reflecting a linear temperature dependence of the heat capacity change ($\Delta\varphi_p$) in the temperature range from 2 to 37 °C, are indicated in the legend to Figure 3.

DISCUSSION

Ligand Linkage and Conformational Change. The importance of the observation that the binding of one ligand to a macromolecule could have an effect on the binding of another ligand to that same macromolecule was first emphasized in the work of Bohr *et al*. (1904) on the binding of oxygen and carbon dioxide to hemoglobin. In the case of oxygen binding, it is now well-known that the binding of

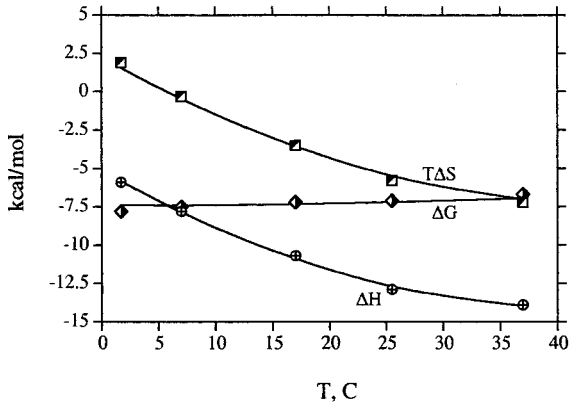


FIGURE 3: Global analysis of enthalpy and free energy changes for mycophenolic acid binding to IMPDH in the presence of IMP, as a function of temperature according to eqs 6 and 7. The binding over the entire temperature range could be described by the following best fit overall parameters at $T^* = 25$ °C: $\Delta H^* = -12.6 \pm 0.2$, $\Delta G^* = -7.2 \pm 0.1$, $\Delta C^* = -0.174 \pm 0.013$, $\Delta\varphi_p = +.010 \pm 0.002$.

the first oxygen molecule makes it subsequently easier for the second oxygen molecule to bind, which in turn facilitates the binding of the next oxygen molecule, and so on, until the protein is saturated with oxygen. Also, the binding of carbon dioxide causes the release of oxygen by decreasing its effective affinity and vice versa—that is, the binding of oxygen causes the release of carbon dioxide in a reciprocally linked fashion (Wyman & Gill, 1990). With the macromolecule acting as a transducer of free energy, such phenomena illustrate the coupling of discrete binding events at the molecular level in a biochemical system.

The binding of oxygen to hemoglobin is an example of *positive homotropic* cooperativity: *homotropic*, because the interactions are between sites that bind the same ligand (oxygen), and positive because it is energetically more favorable for ligand molecules to bind in succession. The binding of IMP to IMPDH is an example of *negative* homotropic cooperativity, since the binding of the first 3 equiv of IMP results in the last equivalent of IMP binding more weakly. The effects that oxygen and carbon monoxide exert on one another's binding to hemoglobin is an example of *negative heterotropic* interaction: heterotropic, because the ligands are different, and negative, because they antagonize each other's binding. The binding of IMP and mycophenolic acid to IMPDH reported here is an example of *positive* heterotropic interaction. In fact, it is a very extreme case of positive heterotropic interaction since the binding of mycophenolic acid does not take place at all (or at best binds very weakly) in the absence of IMP.

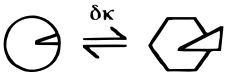
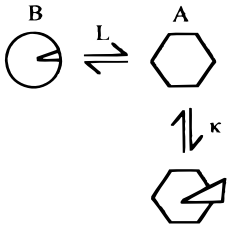
What is the molecular basis for positive and negative cooperativity, and for the existence of heterotropic interactions, in macromolecular binding processes? One needs to consider each macromolecular system separately to fully answer this question, but the 1960s gave rise to the development and popularization of the concept of allosterism (or allostery) as a general means to describe these phenomena that were observed in a number of enzyme systems, in addition to the earlier binding studies of hemoglobin [for historical details on the development of the concept of allostery see Wyman and Gill (1990), Edsall (1980), and Debru, (1983)]. The key concept of allostery is that macromolecules can exist in more than one structural form. The structure of the protein determines the ligand binding properties, and the concentrations or chemical potentials of the ligands determine the populations of the different macromolecular structural forms.

Initial Evidence for Conformational Change upon IMP Binding. A number of dehydrogenases exhibit positive and negative cooperative ligand binding behavior that has been described in terms of various allosteric models (Perutz, 1993). The structural basis for allosteric interaction in bacterial L-lactate dehydrogenase was recently described by Iwata and Ohta (1993). In addition to the observation of positive or negative homotropic cooperativity as evidence that proteins exhibit allosteric behavior, a large and temperature dependent heat capacity change of binding has been taken as a signature of ligand-induced conformational change in a protein (Fischer, 1988), particularly with regard to dehydrogenases. The results in Table 1 and Figure 2 show the large heat capacity change and its strong temperature dependence for the binding of IMP to IMPDH. If the binding of IMP took place without any change in conformation, one would predict the heat capacity change to be greater than or equal to -0.29 kcal K^{-1} mol-of-sites^{-1} (Murphy & Friere, 1992), given that the total amount of solvent accessible surface area buried upon IMP binding as calculated by the method of Lee and Richards (1971), is 650 Å^2. Upon comparison of this value, based on a rigid body binding model, with the experimental value of -1.8 kcal K^{-1} mol-of-sites^{-1} at 25 °C, it is clear that a much larger change in the solvent accessible surface area is taking place, suggesting that a conformational change accompanies binding. Taken with the observations of negative cooperativity of IMP binding, positive heterotropic interaction of IMP and mycophenolic acid binding, and the fact that other NAD-dependent dehydrogenases exhibit conformational changes, the large heat capacity change for IMP binding provided a first clue for a conformational change. Following up on our observations, Nimmersegen *et al.* (1996) investigated the susceptibilities of IMPDH and IMP-IMPDH to *in vitro* proteolysis. Their subsequent findings were consistent with our initial interpretation of a conformational change on the binding of IMP, first indicated through our analysis of the thermodynamics of binding.

A Simple Pre-Existing ("Implicit") Conformational Equilibrium Model. Two general types of ligand promoted conformational changes can be envisioned. One type of conformational change occurs when more than one structural form of the protein pre-exists in a conformational equilibrium prior to ligand binding. The addition of ligand which binds preferentially to one form can cause a *coupling* of binding and conformational change, as the "implicit" or "hidden" equilibrium is shifted. The fact that there is a large heat capacity change alone does not necessarily indicate that more than one structural form of the protein is present *prior* to the binding of a ligand. Certainly it is conceivable that a ligand can *induce* a structural change in a protein to populate a form that did not pre-exist. This type of conformational change is referred to as an induced fit. We argue below that the existence of a temperature dependent heat capacity change is consistent with a coupled conformational equilibrium and not an induced fit. To introduce the proof more clearly, we consider at first, a simple model of a coupled conformational equilibrium.

If IMDPH were composed of two macromolecular forms (A and B), then the observed temperature dependence of the enthalpy and the heat capacity changes of IMP binding to

Table 3: Outline of the Argument Stating That a Large Temperature Dependent Heat Capacity Change of Binding Is Indicative of a Pre-Existing Conformational Equilibrium Model

model	induced fit model (one-site KNF model)	pre-existing conformational equilibrium model (one-site MWC model)
description		
partition function	$P = 1 + \delta\kappa\, x$	$P = 1 + [\kappa(1 + L)^{-1}]x$
free energy change	$\Delta G^\circ = \Delta G_{bind} + \Delta G_{conf}$ $\Delta G^\circ = -RT \ln \kappa - RT \ln \delta$	$\Delta G = \Delta G_{bind} + \Delta G_{conf}$ $\Delta G^\circ = -RT \ln \kappa + RT \ln(1 + L)$
enthalpy change	$\Delta H = \Delta H_{bind} + \Delta H_{conf}$	$\Delta H = \Delta H_{bind} + \alpha^\circ \Delta H_{conf}$ where $\alpha^\circ = (1 + L)^{-1}$
heat capacity change	$\Delta C = \Delta C_{bind} + \Delta C_{conf}$	$\Delta C = \Delta C_{bind} + \alpha^\circ\, \Delta C_{conf} + \Delta H_{conf}(d\alpha^\circ/dT)$
structural basis for heat capacity change	the amount of polar and nonpolar surface area buried due to ligand binding and the accompanying induced conformational change: $[a\Delta A_{np,bind} + b_\Delta A_{p,bind}] + [a\Delta A_{np,conf} + b\Delta A_{p,conf}]$	the amount of polar and nonpolar surface area buried due to binding, and the amount of polar and nonpolar surface area buried due to the conformational change weighted by the fraction of macromolecule existing in form B prior to binding: $[a\Delta A_{np,bind} + b\Delta A_{p,bind}] + \alpha^\circ\, [a\Delta A_{np,conf} + b\Delta A_{p,conf}]$
temperature dependence of heat capacity change	$\Delta\varphi \sim 0$, since the heat capacity change is independent of temperature, provided that the amount of solvent accessible surface area of the liganded and unliganded macromolecules doesn't change appreciably from 0 to 35 °C.	$\Delta\varphi = \Delta H_{conf}\, (d^2\alpha^\circ/dT^2) + (d\alpha^\circ/dT)\, \Delta C_{conf}$ The temperature dependence of the heat capacity change arises chiefly from the difference in population of the A and B forms as a function of temperature in the absence of ligand (i.e., the pre-existing conformational equilibrium is temperature dependent in the 0−35 °C range).

IMPDH could be described as follows. Consider the association of IMP and IMPDH to be composed of a rigid-body "pure" intermolecuclar binding interaction event and an intramolecular conformational event. Suppose that the enthalpy due to binding interactions is exothermic ($\Delta H_{bind} < 0$) and that the heat capacity change is negative ($\Delta C_{bind} < 0$), as they are for most biomolecular reactions that do not involve conformational changes. Further suppose that the heat capacity for the conformational transition is positive ($\Delta C_{conf} > 0$) and that the heat of conformational change is large and endothermic ($\Delta H_{conf} > 0$), as if the conformational change caused a hydrophobic binding pocket to be opened up for binding. Then one can account for the variation of enthalpy with temperature in the binding of IMP to IMPDH by recognizing that at low temperature, IMPDH could exist mostly in form B prior to the addition of IMP. When IMP is added at low temperature, the exothermic enthalpy of binding is overwhelmed by the large endothermic enthalpy of the conformational transition that takes place upon binding, with the result of an overall observed endothermic heat of binding (such as the case at 2.5 °C, overall $\Delta H = +67.9$ kJ/mol, Table 2). At slightly higher temperatures, there is a greater fraction of IMPDH existing in the A form prior to binding so that the binding of IMP is largely canceled by the heat of the conformational transition (as in the case of near zero enthalpy change for IMP binding at 17 °C). At higher temperatures, the overall observed enthalpy changes will be negative since most of the IMPDH is in the A form prior to the addition of IMP (not much of a transition need take place), as in the cases of IMP binding at 25 and 37 °C. Notice that this model also accounts for the observation that the negative heat capacity change will be smaller in magnitude at low temperature, becoming increasingly larger in magnitude at higher temperature, as is observed experimentally. It also accounts for the observation that the binding of IMP is entropically driven at low tempertature and enthalpically driven at high temperature. One might now ask if the observed thermodynamic behavior could be accounted for by an induced fit model as well. The answer is "no", as we now show.

Temperature Dependence of the Heat Capacity Change as a Signature for a Ligand Coupled Pre-Existing Conformational Equilibrium Mechanism. We wish to argue quite generally that the temperature dependence of the heat capacity change that we observe for IMP binding points to the existence of a pre-existing population of protein forms (i.e., a ligand-coupled conformational equilibrium). To see this, consider again the simple case in which two forms of a macromolecule, A and B, coexist at equilibrium with equilibrium constant $L = [A]/[B]$ (see Table 3 for an outline of the argument that is considered presently). Upon binding of a ligand X that favors the A form, the enthalpy change for the reaction could then be divided into two components: a component reflecting only the binding interaction between the macromolecule and the ligand, ΔH_{bind}, and a component reflecting the heat of conformational change (B → A) of the protein, ΔH_{conf}. If the fraction of protein present as form B prior to the introduction of ligand to the system is α°, the enthalpy change for this reaction can be written as

$$\Delta H = \Delta H_{bind} + \alpha^\circ \Delta H_{conf} \quad (9)$$

(In the absence of a pre-existing equilibrium between forms of the protein, the same equation would apply with $\alpha^\circ = 1$). The heat capacity change is then given by the temperature derivative of ΔH:

$$\Delta C = \Delta C_{\text{bind}} + \alpha^{\circ}\Delta C_{\text{conf}} + \Delta H_{\text{conf}}\left(\frac{d\alpha^{\circ}}{dT}\right) \quad (10)$$

Notice that it contains three terms. The first heat capacity term reflects only the heat capacity change due to direct protein ligand interaction, and the second component reflects the contribution to the heat capacity change due to a change in population of macromolecular conformation that is brought about by the addition of ligand. These two terms would be present even if the two conformations did not coexist prior to binding (with $\alpha^{\circ} = 1$, see Table 3). It is the third term which expresses an explicit temperature dependence of the observed heat capacity that applies specifically to the pre-existing conformational equilibrium model and is of particular interest. It attributes the temperature dependence of the heat capacity to a shift in the pre-existing conformational equilibrium between structural forms due to a temperature change, as has been pointed out previously for macromolecular systems by Sturtevant (1977), Etfink *et al.* (1983), Fischer (1988), and Ferrari and Lohman (1994). This third term in eq 10 would not be present if there were no pre-existing equilibrium of forms, as prescribed by the induced fit model.

Let us now examine and compare the temperature dependencies of the heat capacity changes for the induced fit model and the pre-existing conformational equilibrium model. It has been shown for a variety of biomolecular interactions, including the folding and binding reactions of proteins, that the heat capacity change can be accounted for, in large measure, by the change in solvent accessible surface areas of polar and nonpolar groups on the molecules involved in the reaction (Spolar *et al*., 1989; Murphy & Friere, 1992; Connelly & Thomson, 1992; Connelly *et al*., 1993; Connelly, 1997). The heat capacity changes for binding (ΔC_{bind}) and conformational change (ΔC_{conf}) are therefore temperature independent, provided the average accessible surface areas of the unliganded and liganded forms do not change appreciably over the narrow temperature range from 0 to 40 °C (as they could be in the case of a protein with temperature-sensitive conformational forms). Thus, for the induced fit model, there is no temperature dependence of the heat capacity change. However, for the pre-existing conformational equilibrium model, there is a significant temperature dependence to the heat capacity change given by

$$\Delta\varphi = \Delta H_{\text{conf}}\left(\frac{d^2\alpha^{\circ}}{dT^2}\right) + \Delta C_{\text{conf}}\left(\frac{d\alpha^{\circ}}{dT}\right) \quad (11)$$

The temperature dependence of the heat capacity change, $\Delta\varphi$, immediately follows from eq 11, so that the simple pre-existing conformational equilibrium model would appear to account for the thermochemcial data of IMPDH and IMP. This model does indeed account for the temperature dependence of the heat capacity change. However, from a consideration of this multisite binding system with successive binding constants presented in Table 2, and an understanding of the behavior of allosteric models, this simple model is not entirely appropriate as we show in the next section. The simple model presented above considers only an identical and independent site macromolecule. We must account for the full binding behavior of IMPDH and, in particular, the negative cooperativity of IMP binding.

It is useful at this juncture to refer to the recent work of Ferrari and Lohman (1994). They present a particularly elegant description of an observed negative heat capacity change that can be accounted for by a pre-existing, temperature dependent conformational equilibrium in the binding of oligodeoxyadenylate to single-stranded binding protein with $\Delta C_{\text{conf}} = 0$ and $\Delta C_{\text{bind}} = 0$ (in other words, they attribute the observed heat capacity change in their system to the third term on the right-hand side of eq 10) . However, in their careful analysis, Ferrari and Lohman (1994) point out that the variation of the binding constant with temperature cannot rule out a second model, that the origin of the heat capacity change is derived from the release of water molecules from nonpolar surface (i.e., ($\Delta C_{\text{bind}} < 0$, with no conformational change occurring). Our analysis suggests that one could actually distinguish between these two models for the molecular origin of the heat capacity change by calorimetrically measuring the temperature dependence of the enthalpy and looking for evidence of a temperature dependent heat capacity change.

We would also like to draw attention to the work of Fischer (1988). In a very thorough and searching analysis of the origin of the temperature dependence of the enthalpy changes in the binding of ligands to dehydrogenases, Fischer analyzes calorimetric data in terms of the equivalent of our eqs 10 and 11 but with the assumptions that $\Delta C_{\text{conf}} = 0$ and $\Delta C_{\text{bind}} = 0$. By drawing on the recent results of Spolar *et al.* (1992) and Murphy and Friere (1992) on the relationship between heat capacity changes and the changes in the solvent accessible polar and nonpolar surfaces in macromolecular reaction processes, we were able to extend the previous arguments in the literature concerning implicit equilibria and heat capacity changes to demonstrate that it is *the temperature-dependence of the heat capacity (not the enthlapy) that is the distinguishing feature of the pre-existing conformational equilibrium model.* Our description of the heat capacity change developed above, and in Chart 1, includes terms due to coupled equilibria *and* the changes in exposure of groups to water upon binding.

Standard Allosteric Models and Their Inadequacies for Describing IMP Binding. Two specific models were put forth in the 1960s to account for the properties of proteins that exhibited allosteric behavior: the MWC model (Monod *et al*., 1965) and the KNF model (Koshland *et al*., 1966). The MWC model was based on the equilibria between two overall macromolecule forms (R and T), each form binding a ligand non-cooperatively but with different affinities. This model can be used to quantitatively analyze binding data through the following binding polynomial representation:

$$P_{\text{MWC}} = (1 + \kappa_{\text{R}}x)^4 + L(1 + \kappa_{\text{T}}x)^4 \quad (12)$$

where κ_{R} is the binding constant for the R form, κ_{T} is the binding constant for the T form, and L is the equilibrium constant between the two unliganded forms, $L = [\text{T}_o]/[\text{R}_o]$. The MWC model explicitly states that the two forms of the protein exist prior to the introduction of binding a ligand (pre-existing conformational equilibrium). It is therefore an attractive choice of model for IMP binding based on the temperature dependence of the heat capacity. However, one limiting feature of the MWC model is that it can account only for positive homotropic cooperativity, not negative

cooperativity. Therefore, it would not adequately account for the binding of IMP to IMPDH.

In contrast, the KNF induced fit model proposes that each subunit of a multisubunit macromolecule exists in two structural forms based solely on their ligation states, and that interactions among the binding sites are the result of interactions among the subunits that change, depending on the degree of occupancy of the macromolecule (Koshland *et al*., 1966). This class of models, which can account for both positive and negative cooperativity, can be represented by the following binding polynomial:

$$P_{KNF} = 1 + 4\kappa\delta^2 x + 4\kappa^2\delta^3 x^2 + 2\kappa^2\delta^4 x^2 + 4\kappa^3\delta^4 x^3 + \kappa^4\delta^4 x^4 \quad (13)$$

with intrinsic site binding constant κ and a site−site interaction factor δ (Wyman & Gill, 1989). For homotetrameric proteins, the KNF model, which predicts that the binding curve will be symmetric about the median ligand, is therefore inconsistent with an asymmetric binding curve (Wyman & Gill, 1989). To see this analytically, one need transform the binding polynomial to a normalized binding polynomial with the transformation $x = x_m x'$, where x_m is the median ligand activity equal to $(\beta_4)^{-1}$, so that the leading and constant coefficients are both 1. If the coefficients of the jth and $(4 - j)$th terms of the normalized polynomial are identical, then the binding polynomial will produce a symmetric binding curve. To be symmetric, the coefficients of a binding polynomial must therefore obey the following relation: $\beta_j(x_m)^j = \beta_{4-j}(x_m)^{4-j}$. Stated another way, the ratios of the jth and $(4-j)$th normalized binding coefficients must be equal to unity. For IMP binding to IMPDH, the ratios are 0.88, 0.25, 0.30, 0.27, and 0.43 at temperatures 2.5, 8.2, 17.4, 25.0, and 37.0 °C, respectively. In all cases, except for the very lowest temperature, the binding curves are highly asymmetric.

It is useful to summarize the important points of the discussion at this stage. The thermochemical data presented here, especially the large temperature dependence of the heat capacity change, in addition to the known allosteric behavior of a number of other dehydrogenases, argue for some change in structural forms of the protein: an allosteric model. What type of allosteric model would be consistent with the data presented here? The MWC model, invoking two forms which are conformationally equilibrated prior to ligand binding, is ruled out since it is inconsistent with negative cooperativity. The KNF model is ruled out by the asymmetry of the binding curve and corresponding binding polynomial. The large temperature dependence of the heat capacity function further suggests that the KNF model is inadequate.

A Nested Allosteric Model Can Account for the Observed Binding Behavior of IMPDH. Among the possible models to account for the observations are those representing the class of proteins exhibiting nested allosterism (Robert *et al.*, 1987; Connelly *et al*. 1989a,b). For IMPDH, there may be just two overall quarternary conformational forms as in the MWC model, with each form behaving according to KNF-like tetramers *nested* within the two "overall" conformational forms (R and T). Each overall conformational form is composed of tetramers that exhibit negative cooperativity, rather than the usual assumption of non-cooperativity dictated by the MWC model. Upon IMP binding, there would be a concerted conformational change involving the entire tetramer, as well as more localized negative subunit−subunit interactions. Such nested models have been proposed to explain the behavior of the large multimeric hemocyanins, the chaperonin assemblies, and human hemoglobin (Perutz, 1992). A model of this class can account for the asymmetry and negative cooperativity of IMP binding to IMPDH *and* for the thermochemical data. The binding polynomial for a nested model applied to IMP binding to IMPDH can be written as

$$P_{NEST} = P_{KNF,R} + (L')P_{KNF,T} \quad (14)$$

from which it can be surmised that there are two overall pre-existing conformational forms of the macromolecule, R and T. The R and T forms behave according to KNF models with different binding parameters. Unfortunately, in order to quantitatively determine these binding constants, one would be forced to analyze a single calorimetric binding titration by fitting for ten parameters, five equilibrium constants each with their associated enthalpy changes, a procedure that has proved intractable upon initial analysis. We are currently developing methods that, when combined with other experimental data, may allow us to estimate these parameters to quantitatively test the proposed model.

Implications for Drug Design. Having provided evidence that IMPDH is an allosteric protein and proposed a possible model for its binding behavior, we find it worthwhile to point out the implications for the design of novel inhibitors for use as drug candidates. The structure that has been solved recently and reported by Sincheck *et al.* (1996) contains mycophenolic acid with a partially turned over IMP intermediate bound in the active site. This represents one possible structure out of the allosteric forms indicated in our model, and our results suggest it would closely approximate one of the R-state conformations to which mycophenolic acid binds. If one could inhibit IMPDH by designing an allosteric effector inhibitor that binds to one of the T conformations, it is likely to be structurally distinct from mycophenolic acid. This is highly desirable since the prodrug of mycophenolic acid, although efficacious, leads to severe toxicity. Such an "allosteric-effector" strategy for ligand design was successfully employed in the design of antilipidemic drugs which bind to the T state of human hemoglobin, as part of an effort to prevent the aggregation of deoxyhemoglobin S in patients with sickle cell anemia (Lalezari *et al.*, 1988). It is desirable to obtain a measure of the relative amounts of each of the conformations of IMPDH present under physiological conditions in order to better assess the feasibility of the "allosteric-effector" approach to inhibition. One step in the implementation of this strategy is to solve the structure of the unliganded form of IMPDH, a feat that has thus far not been reported. Our finding that more than one conformational form of IMPDH exists in solution suggests a reason why it has proved so difficult to crystallize apo-IMPDH.

In this connection, it is curious that mycophenolic acid binding to IMPDH in the presense of IMP takes place with a negative heat capacity change but with a positive temperature dependence to the negative heat capacity change. So at low temperature, the heat capacity change for mycophenolic acid binding to the IMPDH−IMP complex is more negative than it is at higher temperature (Figure 3). According to general arguments on the temperature dependence

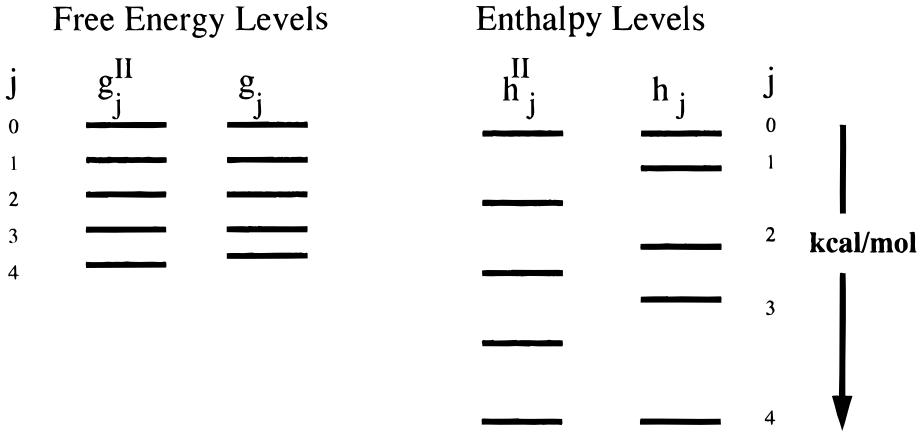


FIGURE 4: Free energy (g_j) and enthalpy levels (h_j) of IMP binding to tetrameric IMPDH. Notice that the free energy levels are very evenly spaced (similar to the free energy levels of a non-cooperative system, g^{II}), masking any difference in the properties of each ligation state. In contrast, the uneven spacing of enthalpy levels (as compared to the enthalpy levels of a non-cooperative binding system, h^{II}) show that the transition to each ligation state from the previous is not equivalent energetically or, therefore, structurally. The negatively cooperative IMP binding properties of IMPDH are easily measured by calorimetric methods, whereas they go undetected by spectroscopic methods.

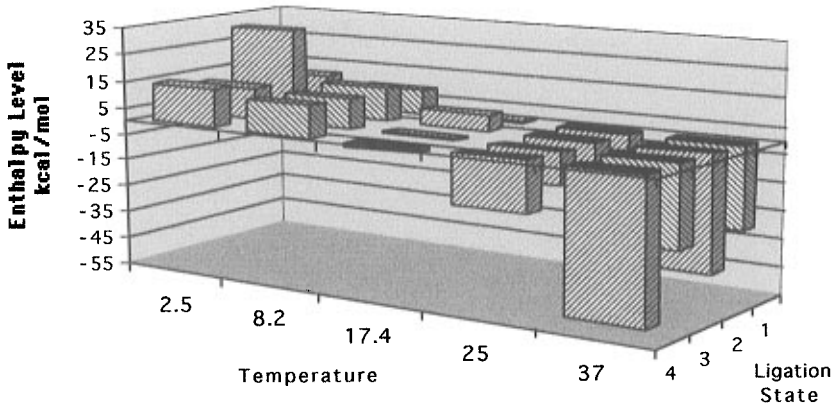


FIGURE 5: Differential enthalpy levels as a function of temperature and ligation state for the binding of IMP to IMPDH. The staggered heights reflect the uneven spacing of the successive energies of binding IMP at a given temperature. The heights of the columns in the figure are given by $\Delta H_j - \Delta H_{j-1}$ for $j = 1-4$. In contrast, an identical and independent site binding system would given heights (differential enthalpy levels) of equal magnitude.

of the heat capacity made above, we interpret this result as an indication that, when IMPDH is saturated with IMP, it still contains an equilibrium mixture of conformational forms. The relative amount of these forms is shifted with temperature.

Detection of Negative Cooperativity and the Nature of Conformational Change. A large body of literature on the enzymology of IMPDH exists (Hupe *et al.*, 1986; Antonino & Wu, 1994; Hedstrom & Wang, 1990; Antonino *et al.*, 1994; Hager *et al.*, 1995). Why has the negative cooperativity of IMP binding to IMPDH never been observed? Certainly negative cooperativity in the binding of substrates to other dehydrogenases is well documented (Henis & Levitzski, 1980). The answer to this question lies in the way one typically measures the association of IMP with the tetrameric IMPDH. In the case of an enzyme assay carried out at saturating levels of the co-substrate NAD, while varying the concentration of IMP, a spectroscopic signal is used for detecting turnover (the increase in absorbance at 340 nm due to the formation of NADH), allowing a determination of K_m. In principle, two, three or four K_m's could be determined this way if their values differed significantly or if there was a way to label the activity of each subunit. We have found that it is only the last site to bind IMP that differs significantly in its affinity, and not by an enormous margin (a factor of 10 at most temperatures, Table 1). Since the heat of binding IMP at each ligation stage is markedly different, and since we employed a direct calorimetric method, we were afforded a unique "detection label" for the binding activity of each site, the heat of binding. Another way of indicating the advantage of employing titration calorimetry to investigate the binding of IMP, is to say that the free energy levels of binding IMP are spaced rather evenly, whereas the enthalpy levels or IMP binding are very unevenly spaced (Figure 4). Since one is restricted to measuring *differences* in energy levels in practice, one has a better chance of detecting differences in the binding properties of the various ligation states by measuring heat in the case of IMP binding to IMPDH (Figure 5).

A related point concerns the inference that a conformational change in the protein occurs upon binding IMP. We had first suggested that a conformational change occurs when IMP binds, on the basis of the large heat capacity change that we observed. Our colleagues strengthened our hypothesis by demonstrating the differential susceptibility of IMPDH and IMP−IMPDH to *in vitro* proteolysis (Nimmersegen *et al.*, 1996). It was only after careful consideration of the nature of the temperature dependence of the heat capacity change that we were able to argue for a pre-existing conformational equilibrium of apo-IMPDH. The only method capable of detecting a temperature dependent heat capacity

change with adequate precision, in the temperature range in which we conducted our experiments, is microcalorimetry. The recent commercialization of high-precision microcalorimeters will undoubtedly assist in the exploration of the nature and magnitudes of a variety of conformational changes linked to binding reactions (Connelly, 1994).

Conclusion. We have employed a temperature dependent thermodynamic investigation of the binding of the substrate IMP and the small molecular weight inhibitor mycophenolic acid to IMPDH. A thermodynamic argument based on the nature of the heat capacity function and its temperature dependence was used to demonstrate the existence of multiple forms of apo-IMPDH. The negatively cooperative properties of IMP binding and allosteric properties of IMPDH have not been reported previously, having eluded detection by the prior enzymatic analyses employed for investigating this enzyme. Our findings support the use of high-precision microcalorimetric binding studies for investigating the functional properties of multisubuit protein targets. The discovery of the allosteric properties of IMPDH opens a new strategic window for the design of drugs which target this enzyme which plays a key metabolic role in proliferating cells of the human immune system.

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