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Enthalpy analysis of horseradish peroxidase in the presence of Ni^{2+}: a stabilization study

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Abstract

The interaction of horseradish peroxidase (HRP) with Ni^{2+} has been investigated by isothermal titration calorimetry (ITC), potentiometry, equilibrium dialysis, and spectrophotometry. Total enthalpy of cited interaction was measured by ITC at 27 °C, pH = 7.0.The enthalpy of ionization (ΔH_{ion}) is obtained by acid–base titration at 25 and 50 °C in 100 mM NaCl. Enthalpy of binding is obtained by equilibrium dialysis at 27 and 37 °C based on the Wyman binding potential to evaluate equilibrium constants at two temperatures. The van't Hoff relation is used for calculation of enthalpy of binding (ΔH_{bin}) and also a modified differential relation is applied for estimation of ionization enthalpy (ΔH_{ion}). Denaturation profiles on HRP with and without the presence of Ni^{2+} using *n*-dodecyl trimethylammonium bromide (DTAB) as a denaturant, has been studied. Using the Pace theory to evaluate free energy in the absence of denaturant ($\Delta G^{\circ}_{H_2O}$) is the best parameter for determination of protein stability. The results show 4.9 kJ mol^{-1} higher free energy for stabilization of HRP in the presence of Ni^{2+}. Enthalpy of conformational change of HRP by Ni^{2+} (3.5 mM) is determined by resolving the contributions of calorimetric enthalpy, binding enthalpy and ionization enthalpy. The enthalpy of stabilization for HRP in the presence of Ni^{2+} (3.5 mM) is equal to -118.62 kJ mol^{-1} which was obtained by differential relation between enthalpies of unfolding and conformation.

Keywords: HRP stabilization; Ni^{2+}; Enthalpy of stabilization; Free energy; Enthalpy analysis

1. Introduction

Peroxidases are ubiquitous heme-containing enzymes that catalyze the oxidation of a wide variety of organic and inorganic substances by hydrogen peroxide. Horseradish peroxidase (HRP) is the most thoroughly studied member of this enzyme superfamily [1].The enzyme in its resting state reacts with H_2O_2 in a two-electron oxidation process to result in an oxy-ferryl moiety and a porphyrin π cation radical (compound I). Compound I is reduced by a hydrogen donor (AH) in two steps to yield compound II and resting enzyme, respectively.

$$\mathrm{HRP\,(Fe^{3+})} + \mathrm{H_2O_2} \rightarrow \mathrm{Compound\,I\,\{(Fe^{4+}{=}O)P^{\bullet+}\}} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{Compound\,I\,\{(Fe^{4+}{=}O)P^{\bullet+}\}} + \mathrm{AH} \rightarrow \mathrm{Compound\,II\,\{(Fe^{4+}{=}O)P\}} + \mathrm{A^{\bullet}} \quad (2)$$

$$\mathrm{Compound\,II\,\{(Fe^{4+}{=}O)P\}} + \mathrm{AH} \rightarrow \mathrm{HRP\,(Fe^{3+})} + \mathrm{H_2O} + \mathrm{A^{\bullet}} \quad (3)$$

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The enzyme has found various applications in analytical biochemistry (e.g. enzyme immunoassay; cytochemistry; DNA probes and biosensors) and biotechnology [2–5].

Therefore, due to its wide spread utilization, stability studies have received increasing interest [6,7]. Various strategies to increase the stability of enzyme include: chemical modification [8–10], using osmolytes [11] and special organic solvents (e.g. polyethylene glycol) [12].

The interaction of protein with metal ion is of interest because of various reasons: this includes a biochemical standpoint and a pathological perspective. An understanding of physiological and indeed pathological interactions of metal ions with proteins are important especially with respect to their thermodynamic and kinetic stabilities [13,14]. While all proteins in general can bind with metals, the number and the strength of binding vary with the metal and the nature of the protein [15,16]. For a protein that is folded into its native state, however, binding is likely to take place only where the metal ion has access to the potential binding site. In fact, almost any protein will bind with metal ions weakly, the most likely sites for weak binding are the exterior hydrophobic amino acid side chains [14].

Survey of literature shows that some work has been carried out to assess the structural stability of the enzyme which emphasizes the importance of hydrogen-bonding network on the stability of the structure of the enzyme [6,7,17–21]. Although some work on the metal ion-peroxidase has been reported [22–25], the use of metal ion to enhance the life span of enzyme is a subject of valuable practical interest [26].

In this paper we report our results on the interaction of Ni^{2+} with peroxidase from a thermodynamic point of view, the effect of Ni^{2+} on the stabilization of the enzyme, and a reliable enthalpy analysis.

2. Experimental

2.1. Materials

HRP type II with a purity index $RZ \approx 2.5$; $NiCl_2{\cdot}6H_2O$ were purchased from Merck and *n*-dodecyl trimethylammonium bromide (DTAB) was obtained from Sigma. Solutions were prepared using CO_2-free deionized water (Barnstead NANOpure D4742 EC = 18 MΩ). A 100 mM NaCl solution was used for potentiometric titrations and the other solutions were prepared in phosphate buffer 2.5 mM, pH = 7.0.

2.2. Methods

2.2.1. Spectrophotometry

HRP–Ni^{2+} interaction profiles were carried out using a Shimadzu 2101 PC spectrophotometer in order to obtain stability parameters. A HRP solution (5 mM) in a 1.5 ml thermostated cell was titrated with aliquots of a Ni^{2+} solution (0.3 M). Chemical denaturation of HRP by DTAB was performed using a pre-incubated enzyme solution with Ni^{2+} (3.5 mM). Concentration of protein was checked by $\varepsilon_{403} = 1.02 \times 10^5\ cm^{-1}\ M^{-1}$ [27]. Details of experimental conditions are indicated in figures.

2.2.2. Isothermal titration calorimetry

The calorimetric experiments were carried out with a four channel microcalorimeter (Thermal Activity Monitor 2277, Thermometric, Sweden). Ligand solution (200 mM) was injected with the help of a Hamilton syringe into the calorimetric stirred vessel, which contained 2 ml solution of protein (0.6 mg/ml) in phosphate buffer (2.5 mM, pH = 7.0). Thin (0.15 mm i.d.) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimeter vessel. The injection of the ligand into the perfusion vessel was repeated 20 times, and each injection included 35 μl of ligand. The rate of heat output from the sample cell with respect to the reference cell (including 2 ml of phosphate buffer) was recorded with an accuracy of 0.1 μW by a computerized recording system. The enthalpy change for each injection was calculated by Digitam 3 software. Also in a same manner, enthalpies of dilution of ligand solutions were measured in the absence of the protein and used for correction of the enthalpy of protein–Ni^{2+} interaction. The enthalpy of dilution of HRP solution is negligible.

2.2.3. Potentiometric titration

Acid–base titration is performed in nitrogen atmosphere by means of an automatic and thermostated microtitrator, model Titroline alpha (Schott Co.). For determination of ionization enthalpy (ΔH_{ion}), to

4 ml of a solution of 0.1% (w/v) HRP in NaCl (100 mM) in the presence and absence of Ni^{2+} at isoionic pH, known amounts of acid or alkali were added followed by potentiometric measurement of pH (2–12.0) at 25 and 50 °C. Theory and details of experiments and derivation of equations for estimation of ionization enthalpy were described elsewhere [27–29].

2.2.4. Equilibrium dialysis

Binding enthalpy (ΔH_{bin}) was determined using the common equilibrium dialysis technique as used frequently [7,30–32,15,16]. Concentration of protein was 0.03% (w/v), and raw data were collected at two temperatures of 27 and 37 °C. The concentration of unbound Ni^{2+} was determined by atomic absorption spectrophotometry (type AA-200 Varian).

3. Results and discussion

Fig. 1 represents the denaturation profile of HRP by Ni^{2+} monitored at wavelength of 275 nm as the probe of conformational change of the protein. The process is based on the two-state mechanism and the Pace theory was used to find the transition concentration $[Ni^{2+}]_{1/2}$ and the free energy of conformational stability ($\Delta G^{\circ}_{H_2O}$) [33–35,6,19]. At lower concentration of Ni^{2+} (≈5 mM), no considerable conformational change can be detected (pre-transition region). This concentration range of Ni^{2+} (up to 5 mM) is corresponded to the activatory concentration range of Ni^{2+}, and upper concentrations >5 mM denotes to the inhibitory behavior of Ni^{2+} on HRP [36]. The subsequent experiments satisfy the long-term stability and increased activity of HRP by 3.5 mM Ni^{2+}. So this concentration of Ni^{2+} is selected to evaluate the conformational stability of the protein. In this respect, a reliable method of choice to evaluate effect of Ni^{2+} on conformational stability of HRP is using a suitable denaturant, e.g. DTAB. So in order to characterize the effect of Ni^{2+} on the stability of HRP, sigmoidal denaturation curves were plotted by measuring absorbance at 403 nm as a function of DTAB concentration in the absence and presence of Ni^{2+}(3.5 mM), as shown in insets of Fig. 2. Assuming the two-state transition model as follows [6]:

$$\Delta G^{\circ}_{D} = -RT \ln K_D = -RT \ln\left(\frac{A_N - A_{obs}}{A_{obs} - A_D}\right) \quad (4)$$

where R is the universal gas constant, ΔG°_{D} the free energy of denaturation, K_D the equilibrium denaturation constant, A_{obs} the observed absorbance, and A_N and A_D are the absorbances of the native and denatured species, respectively. According to the Pace or linear extrapolation method (LEM) we can write [6]

$$\Delta G^{\circ}_{D} = \Delta G^{\circ}_{H_2O} - m\,[\text{denaturant}] \quad (5)$$

where $\Delta G^{\circ}_{H_2O}$ is the free energy of conformational stability in the absence of denaturant and m is a measure of the dependence of ΔG°_{D} on the denaturant concentration. Fig. 2 shows linear plots of Eq. (5) for denaturation of HRP by DTAB in the presence and absence of 3.5 mM Ni^{2+}. $\Delta G^{\circ}_{H_2O}$ in absence and presence of Ni^{2+} (3.5 mM), is obtained at the intercept of plot at Fig. 2b. These are compatible with previously reported values of $\Delta G^{\circ}_{H_2O}$ for HRP/surfactant interactions [7,19]. So that, Ni^{2+} at the activatory concentration range (e.g. 3.5 mM) in addition to the functional stability, stabilizes the HRP structure to an extent of 4.9 kJ mol^{-1} in terms of Gibbs free energy. The figure also shows that $[DTAB]_{1/2}$ shifts from 5.65 mM (in the absence of Ni^{2+}) to 10 mM (in the presence of 3.5 mM Ni^{2+}) which denotes the structural stabilization behavior of the Ni^{2+}.

We have also performed an enthalpy analysis in order to resolve the above stabilization effect in

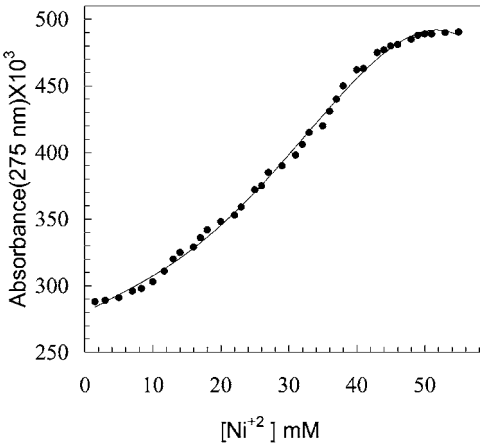


Fig. 1. Variation of Absorption (at 275 nm) as a function of Ni^{2+} concentrations. Experimental conditions: $T = 27$ °C; buffer = 2.5 mM phosphate; pH = 7.0; [HRP] = 1×10^{-5} M. Absorbance change due to addition of Ni^{2+} has been corrected.

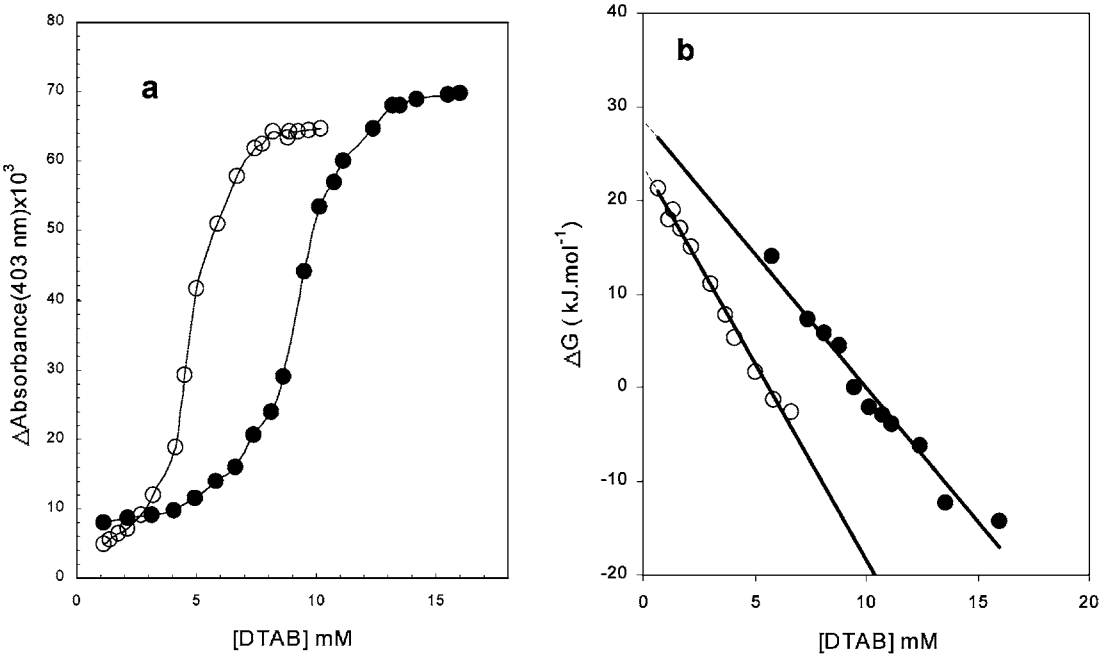


Fig. 2. (a) Denaturation profile for HRP solution by DTAB monitored at soret band (403 nm) in the absence and presence of Ni^{2+} (3.5 mM). (b) The Gibbs free energy vs. DTAB concentrations in the absence and presence of Ni^{2+} (3.5 mM); [HRP] = 5×10^{-6} M ((○) in the absence of Ni^{2+}; (●) in the presence of Ni^{2+} (3.5 mM)).

respect of enthalpy of interaction between Ni^{2+} and HRP. The total enthalpy change associated with interaction of HRP and Ni^{2+} could be estimated by isothermal titration calorimetry. The interaction includes: (a) binding of Ni^{2+} to the enzyme molecule, (b) conformational change of the enzyme molecule, and (c) deprotonation of acidic groups impart in the interaction. Thus for the interaction of HRP with Ni^{2+} the calorimetric enthalpy (ΔH_{cal}) consists of contributions from enthalpy of binding (ΔH_{bin}), enthalpy of conformational change (ΔH_{con}) and enthalpy of ionization (ΔH_{ion}). Hence we can write [27,37]

$$\Delta H_{cal} = \Delta H_{bin} + \Delta H_{con} + \Delta H_{ion} \quad (6)$$

Fig. 3 shows the enthalpy of binding versus Ni^{2+} concentrations. The enthalpies of binding (ΔH_{bin}) were obtained from the temperature dependence of the equilibrium binding constant using the van't Hoff relation [38].

$$\Delta H_{bin} = -R\frac{d(\ln K_{app})}{d(1/T)} \quad (7)$$

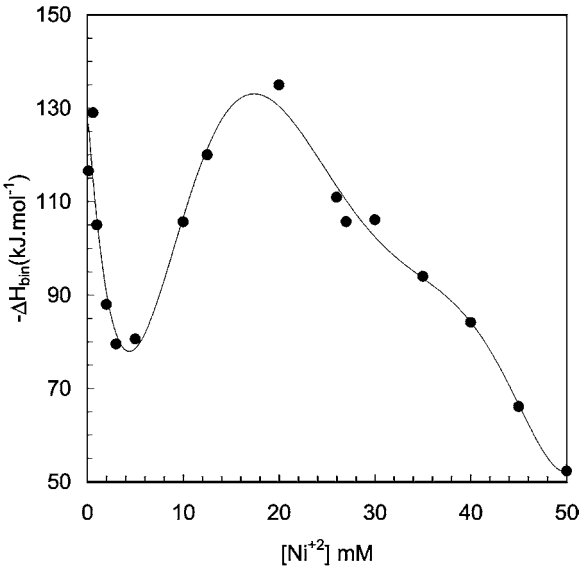


Fig. 3. Enthalpy of binding interaction of HRP and Ni^{2+} as a function of Ni^{2+} concentration at pH = 7.0, and 27 °C, 2.5 mM phosphate buffer and [HRP] = 7×10^{-6} M.

The Fig. 3 shows decreasing of the exothermicity of the Ni^{2+}/HRP interaction and continue up to borderline of activatory region (<5 mM) and increasing of the exothermicity corresponding to inhibitory region (activity data are not shown [36]), the curve falls towards the $[Ni^{2+}] \approx 20$ mM and after this point the curve corresponds to simultaneous binding and denaturation processes.

Due to strong electrostatic effect regarding the binding of such ligands to proteins, the pK_a shifts were occurred. Therefore we can write a modified van't Hoff relation as follows [27]:

$$\Delta H_{\text{ion}} = 2.303R\left[\frac{\partial \text{pH}}{\partial(1/T)}\right] \quad (8)$$

Fig. 4 shows the apparent enthalpy of various titratable groups of HRP in presence of $[Ni^{2+}] =$ 3.5 mM. These enthalpies are obtained from temperature dependence of pH for each solution at a fixed amount of added acid or alkali using Eq. (8). Fig. 4 depicts the ΔH_{ion} for HRP in the absence and presence of Ni^{2+} that is showing the exothermic pattern for 3.5 mM Ni^{2+} and mostly endothermic process in the absence of Ni^{2+}. Binding of Ni^{2+} to protein facilitates deprotonation of titratable groups, and increasing of pH (at 3.5 mM of Ni^{2+}) induced more negative values for ΔH_{ion}. Of the possible candidates for interaction of Ni^{2+} with HRP, especially those present at active site [39], binding to carboxylic residues (Asp. and Glu.) do not cause high amount of heat, the fact which is consistent with higher bond length cited in literature [40], while binding to N-donor residues (imidazole and amine groups) results in higher heat evolution, leading to much more bond strength (lower bond length) as shown in Fig. 4.

Fig. 5 shows the variation of calorimetric enthalpy as a function of $[Ni^{2+}]$. ΔH_{cal} is a measure of overall interaction enthalpy between Ni^{2+} and HRP (at pH = 7.0, $T = 27\ ^\circ$C and phosphate buffer 2.5 mM) and confirming the mid-point transition concentration of Ni^{2+}, $[Ni^{2+}]_{1/2}$, obtained by experiments of Fig. 1. Now by substituting the respected values of enthalpies in Eq. (6), ΔH_{con} can be obtained at given conditions. The value of enthalpies (ΔH_{bin}, ΔH_{ion}, ΔH_{cal}) at $[Ni^{2+}] = 3.5$ mM could be as follows: $\Delta H_{\text{bin}} = -78.77$ kJ mol^{-1} (see Fig. 3); $\Delta H_{\text{ion}} = -63.5$ kJ mol^{-1} (see Fig. 4); $\Delta H_{\text{cal}} = 0.55$ kJ mol^{-1} (see Fig. 5) and by inserting these into Eq. (6), ΔH_{con} equals to 142.82 kJ mol^{-1}.

Scheme 1 was constructed to show the enthalpy relationship between various enzyme conformations. Considering the native state as the reference point of

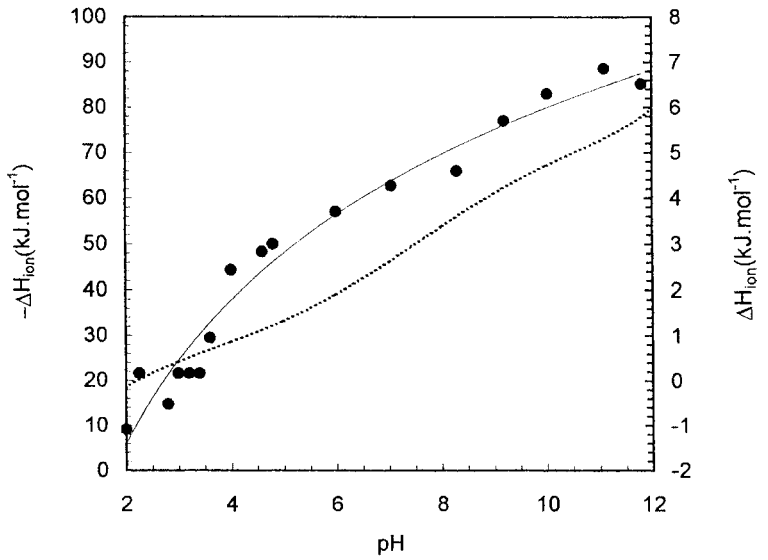


Fig. 4. Ionization enthalpy (ΔH_{ion}) for titratable groups of HRP as a function of pH in 100 mM NaCl solution. Enthalpy values are calculated from titration curves at 25 and 50 °C using Eq. (8). ((. . .) for native HRP in the absence of Ni^{2+} that is directly taken from [24]; (●) in the presence of Ni^{2+} (3.5 mM)).

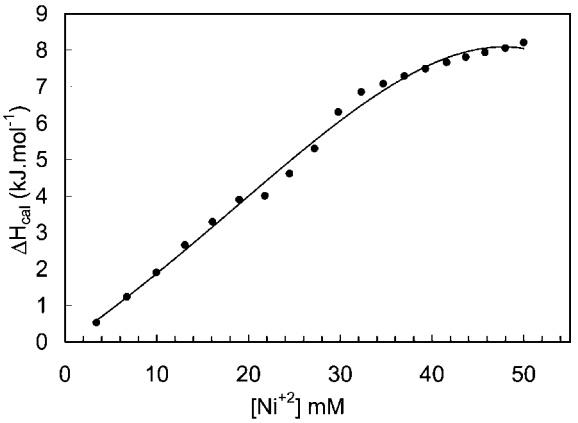
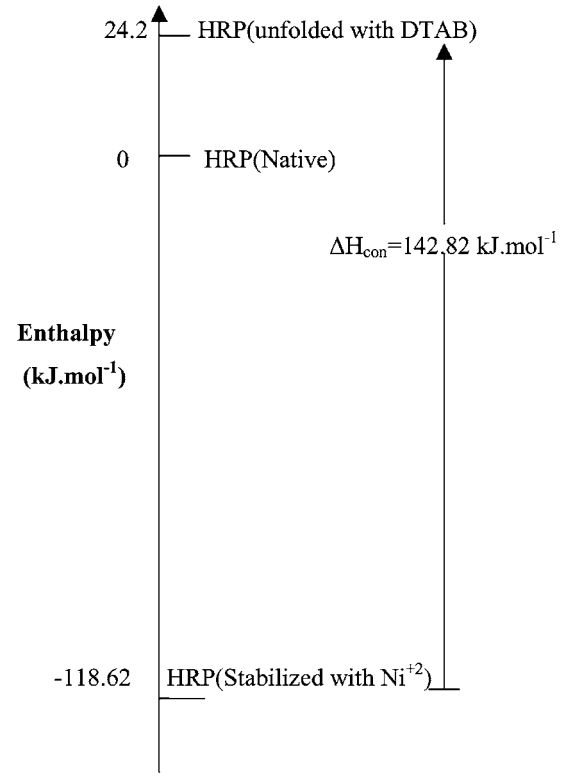


Fig. 5. Calorimetric enthalpy change on the interaction of HRP with Ni^{2+} at pH = 7.0, 27 °C using 2.5 mM phosphate buffer.

enthalpy change equal to zero (an arbitrary point), the other states (unfolded and stabilized forms) could be distinguished truely. Converting the native state to denatured state through denaturation by denaturant, like DTAB, involves an enthalpy change of 24.2 kJ mol^{-1} (ΔH_{unf}), as reported earlier [30] and ΔH_{unf} for HRP in the absence of 3.5 mM Ni^{2+} (stabilized form) is equal to 142.82 kJ mol^{-1}. The difference between these two quantities ($\Delta H_{con} - \Delta H_{unf} = \Delta H_{stab}$) is a measure of enthalpy of stabilization that is equal to −118.62 kJ mol^{-1}. This value for enthalpy of stabilization is corresponding to 4.9 kJ mol^{-1}as Gibbs free energy of stabilization (see Fig. 2).

We can conclude that the small amount of Ni^{2+} (<5 mM) induced the structural stabilization effect for HRP. This is associated with long term functional activity for this enzyme. Knowing that the enthalpy is an extensive parameter as a function of state, it is possible to resolve the contribution of the stabilization enthalpy (ΔH_{stab}) from enthalpy of conformational change (ΔH_{con}) using equation: $\Delta H_{con} = \Delta H_{unf} + \Delta H_{stab}$, which is depicted in Scheme 1.



Scheme 1. Illustrative representation of enthalpy of stabilization, enthalpy of conformational change, and enthalpy of unfolding.

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