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The Action of Inhibitors (Histidine and AMP) on the ATP Phosphoribosyltransferase of *E. coli*

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The inhibitors histidine and AMP cause the enzyme ATP phosphoribosyltransferase of *E. coli* to associate into a hexamer from its initial dimeric form. The behaviour of these inhibitors has been studied by three different methods. I) Equilibrium dialysis studies have shown that one mole of dimeric enzyme (67,000 g) binds one mole of histidine. II) By kinetic inhibition of the reaction studied at 21, 25 and 38° C the enthalpy changes in the process of histidine and of AMP inhibition have been deduced. The inhibition has also been studied in function of enzyme concentration and temperature. The inhibition appears to be slightly negatively cooperative for histidine and positively cooperative for AMP. In neither case is it possible to obtain 100 % maximal inhibition. III) By microcalorimetric analysis the values obtained for the enthalpies of histidine and of AMP interaction with the enzyme are similar.

The enzyme ATP phosphoribosyltransferase catalyses the first step of the pathway for histidine biosynthesis (1), namely the reversible reaction between 5-phosphoribosyl α-l-pyrophosphate (PRibPP) and ATP, and it is feedback inhibited by the end product, histidine (14, 18). In *Salmonella typhimurium* the enzyme is known to be a hexamer (210,000 mol wt.) of identical subunits (26, 28) and only drastic and non-physiological conditions can dissociate it (20). Several reports have appeared concerning the action of histidine studied by kinetic inhibition (2, 28) and equilibrium dialysis (3). MARTIN (14), studying the conformational changes associated with feedback inhibition by histidine, found that histidine binding does not in itself cause feedback inhibition. Later, BLASI *et al.* (5) studied the conformational changes associated with histidine inhibition, by fluorescence, circular dichroism and difference spectroscopy. On the other hand, the *Escherichia coli* enzyme is normally a dimer (67,000 daltons) that can be

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associated by histidine into a hexamer (11, 24). The effect of histidine has also been studied by fluorescence titration (25).

The fact that AMP has an inhibitory effect on the reaction catalysed by ATP phosphoribosyltransferase of *E. coli* was first reported by ATKINSON and his collaborators (9, 10), who showed that AMP is able to bind to the enzyme and is synergictic with histidine in the inhibition of the enzyme. This behaviour was also studied by KRYVI and KLUNGSÖYR (12). Moreover, as demonstrated by KLUNGSÖYR, and KRYVI (11), AMP associates the enzyme from its initial dimeric form stabilizing the hexameric species. DALL-LARSEN and KLUNGSÖYR have recently reported (6) that the enzyme binds approximately one molecule of AMP per dimer.

In the study of the *E. coli* enzyme in our laboratory we have been interested in the binding of histidine and the subsequent association that it causes. We now present data on the association process effected by histidine and AMP, studied by kinetic inhibition, equilibrium dialysis and microcalorimetry.

Materials and Methods

The source of the enzyme was the OA111 mutant derived from *E. coli* K12 and described by MARTÍN DEL RÍO *et al.* (16). ATP phosphoribosyltransferase was purified following the method of WHITFIELD (28) but omitting the DEAE step and including acid precipitation (pH 4.7) (19); the enzyme was finally purified by an $(NH_4)_2SO_4$ precipitation between 15 and 28 g/100 ml. The purity of the enzyme was estimated by gel filtration and polyacrylamide gel electrophoresis. The specific activity of the pure enzyme was 150 nkat, based on the data of VOLL *et al.* (26) that a change of 0.02 A_{290} per min corresponds to the formation of 1.67 nmoles of phosphoribosyl-ATP at 27° C. Enzyme concentration was determined by LOWRY's method (13). 0.1 M Tris-HCl, pH 8.0, was the only buffer employed in all the studies reported in this paper.

Initial velocities for the ATP phosphoribosyltransferase were measured according to the method of MARTIN *et al.* (15). To determine the percentage of inhibition caused by histidine or AMP, either the ligand was included in the reaction mixture before starting the reaction, or the reaction was started in the absence of inhibitor and after a few minutes the inhibitor was added; both experimental designs grave identical results.

Equilibrium dialysis experiments were done at 25° C. Each cell contained two compartments separated by a dialysis membrane; 300 μl of enzyme (33 μM, dimer) were equilibrated with 300 μl of L-[G-^{3}H] histidine (from Amersham, England) solution. The cells were mounted in a rotating wheel and left for 14 h; after this time, in control experiments without enzyme, both compartments were completely equilibrated. The histidine bound to the enzyme was evaluated in a Nuclear-Chicago Mark II scintillation spectrometer, using a 0.5 % solution of 2-(4-t-butyl-phenyl)-5(4-biphenyl)-1,2,4-oxidiazole (from Ciba) in toluene.

Calorimetric measurements of reaction heat in enzyme and ligand mixing were made at 25° C in a LKB Model 10700 bath type microcalorimeter (4). Periodically the calorimeter performance was checked by determining the enthalpy of dilution of sucrose solutions. Two ml of solution containing effector were combined with 3 ml of enzyme solution. In the reference calorimeter vessel, the design was the same but omitting the enzyme. The dilution heat of the enzyme was so negligible that it could be ignored in the calculations.

Results

In the equilibrium dialysis experiments, figure 1, shows the amount of histidine bound as a function of the concentration

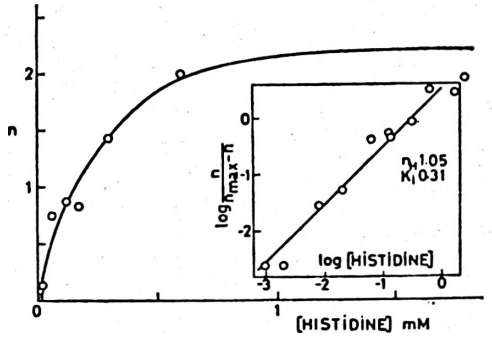

Fig. 1. *Equilibrium dialysis experiments.* Moles (n) of histidine bound per mole (200,000 daltons) of enzyme as a function of total histidine concentration. The buffer used was 0.1 M Tris-HCl, pH 8.0. The enzyme concentration was 2.2 mg/ml. (Inset) Hill plot of the data. (All the Hill graphs reported in this paper have been analysed by the least square method.)

of the ligand. A double reciprocal plot gives a straight line, from which a value of 2.6 moles of histidine bound/200,000 *g* of enzyme is obtained. The modified Hill plot (17) presented as an inset has a slope of 1.05 indicating that the binding process of histidine to ATP phosphoribosyltransferase seems not to be cooperative. The half saturation by histidine ($K_{0.5}$) is 0.31 mM. Another plot (Scatchard, not shown) of the data of figure 1 yields a straight line, indicating again an absence of cooperativity; extrapolation of the line for infinite histidine concentration indicates that there are 3 binding sites per 200,000 daltons of enzyme. This value agrees with previous results (6, 9) and is approximately half of that obtained in the *S. typhimurium* enzyme (3).

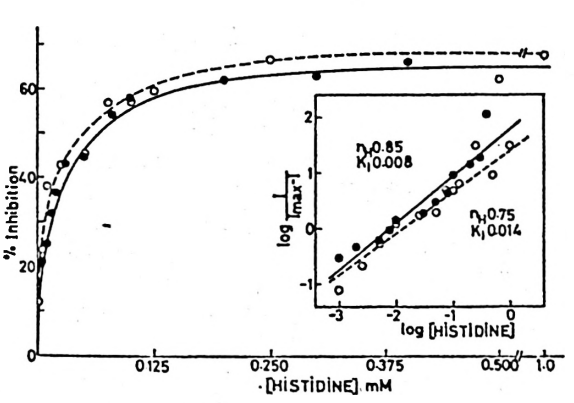

Fig. 2. *Percentage of inhibition caused by histidine.*
The enzyme concentration was 8 μg/ml. O, at 38° C; ●, at 21° C. (Inset) Hill plot.

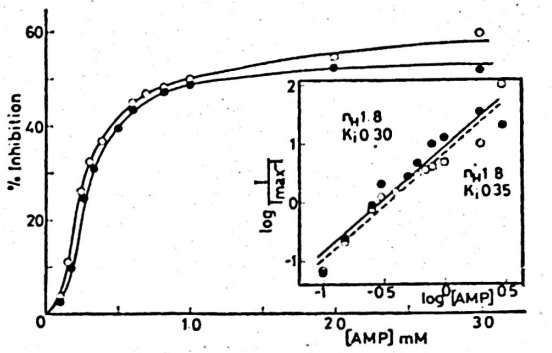

Fig. 3. *Percentage of inhibition caused by AMP.*
The enzyme concentration was 8 μg/ml. O, at 38° C; ●, at 21° C. (Inset) Hill plot.

In figure 2 the inhibition of the enzyme activity caused by histidine at 21 and 38° C, is presented. Double reciprocal plots of the data (not shown) are concave downward, indicating negative cooperativity; from them it can be deduced that the maximum inhibition by histidine at

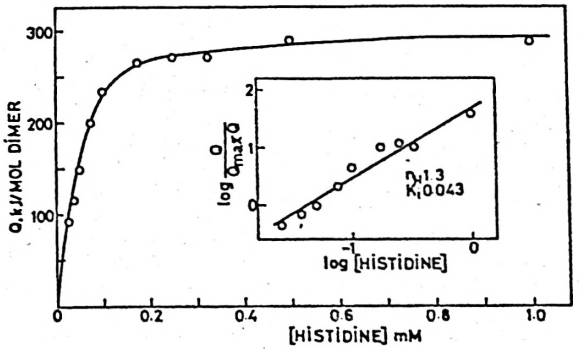

Fig. 4. *Calorimetric titration of enzyme and histidine interaction.*
The heat evolved is plotted *vs* histidine concentration. The experiments were done in 0.1 M Tris-HCl, pH 8.0. The enzyme concentration was 3 μM (dimer). (Inset) Hill plot.

21° C is 66.5 % of the initial activity, and at 38° C, 69 %. From the Hill representation, values of n_H (Hill coefficient) of 0.85 (at 21° C) and 0.75 (at 38° C) are obtained. The midpoints of inhibition by histidine (K_i) are 8 μM and 14 μM, respectively. Taking these values as the apparent dissociation constants of the binding and using the equation:

$$\ln \frac{K_{s1}}{K_{s2}} = \frac{\Delta H^\circ (T_2 - T_1)}{T_2 T_1 R} \qquad [1]$$

where K_{s1} and K_{s2} are the apparent dissociation constant at T_1 (294° K) and T_2 (311° K), respectively, the enthalpy change (ΔH°) for the histidine inhibition has been calculated assuming that ΔH° does not change appreciably in this temperature range. A value of —25 kJ/mol of histidine binding site is obtained.

In figure 3 the inhibition of the enzyme activity caused by AMP at 21 and 38° C is presented. Double reciprocal plots of the data (not shown) are concave upward indicating positive cooperativity; from them it can be deduced that the maximum inhibition by AMP at 21° C is 54 % of the initial activity, and at 38° C, 60.5 %. Following the same procedure as in the case of histidine, from the inset to the figure the same parameters are calculated: Hill coeficient, 1.8: K_i, 0.30 mM (21° C) and 0.35 mM (38° C); ΔH°, —7 kJ/mol of AMP binding site.

In figure 4 the heat liberated by a fixed concentration of enzyme as a function of histidine concentration is shown. A double reciprocal plot indicates slightly positive cooperativity and yields a Q_{max} of 295 kJ/mol of dimer. From the Hill representation (inset), values of 1.2 and 0.043 mM for n_H and $K_{0.5}$, respectively, are derived.

Similarly, figure 5 presents a calorimetric titration of the enzyme with AMP. The Q_{max} is now 315 kJ/mol of dimer, similar to the enthalpy of titration with histidine. The values for n_H and $K_{0.5}$ are now 1.3 and 0.11 mM, respectively.

In the experiments described above, two kinds of heat were measured: the heat evolved or absorbed by the ligand binding and the heat evolved or absorbed by the enzyme association process induced by histidine or AMP (11, 12, 24). In order to distinguish both processes the following experiment was carried out: the concentration of the enzyme was varied keeping constant the inhibitor concentration and the liberated heat was monitored (fig. 6). In both cases the dependency is clearly

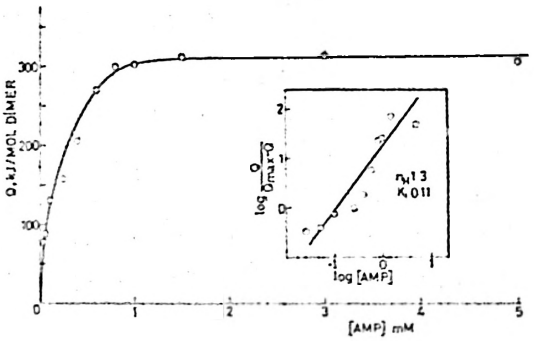

Fig. 5. *Heat evolved in the calorimetric titration of enzyme with AMP.*
The buffer employed was 0.1 M Tris-HCl, pH 8.0. The enzyme concentration was 6 μM (dimer). (Inset) Hill plot.

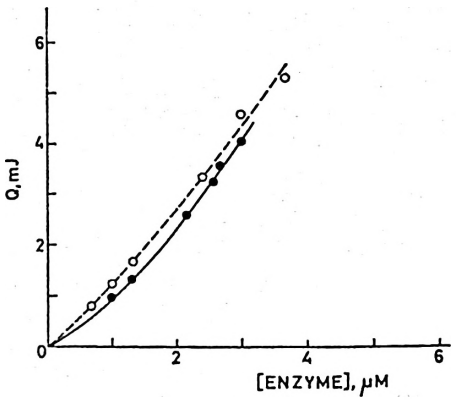

Fig. 6. *Heat evolved by a fixed ligand concentration as a function of enzyme (dimer) concentration.*
The buffer was 0.1 M Tris-HCl, pH 8.0. ●, histidine (0.25 mM); ○, AMP (1 mM).

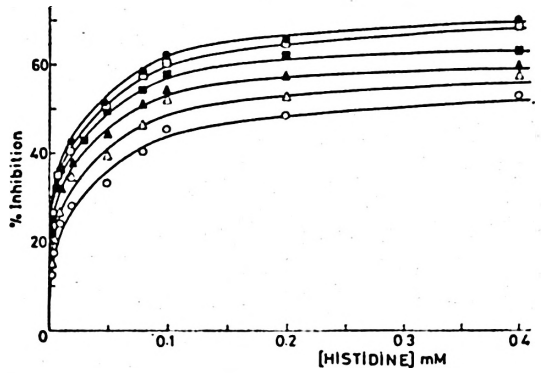

Fig. 7. *Inhibition caused by histidine at 25° C at different enzyme concentration.*
O, 0.074 μM (dimer); △, 0.127; ▲, 0.200; ■, 0.272; □, 0.363; and ●, 0.441 μM.

not linear but concave upward, indicating that the heat produced is dependent on the enzyme concentration, i.e. association of the enzyme by histidine or AMP is important in the production of heat. The same type of non-linear dependency was reported by Ho and WANG (8) in another case of ligand binding and subsequent enzyme association.

KRYVI and KLUNGSÖYR (12) found that the specific activity of the enzyme decreases with increasing concentration of the enzyme, indicating aggregation to less active forms. In other words, in addition

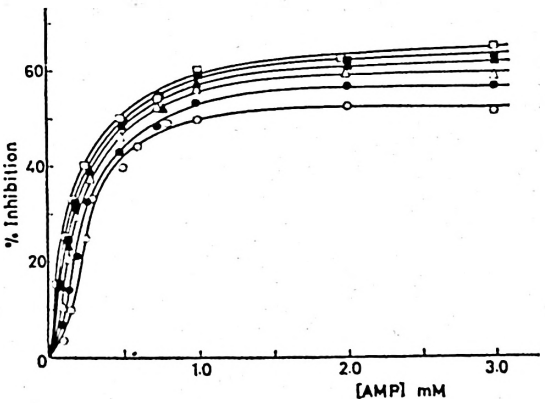

Fig. 8. *Inhibition caused by AMP at 25° C at different enzyme concentrations.*
O, 0.074 μM (dimer); ●, 0.111; △, 0.181; ▲, 0.272; ■, 0.328; and □, 0.441 μM.

Table I. *Maximal inhibition and Hill parameters as a function of enzyme concentration.*

Enzyme concentration (μM)	I_{max} %	n_H	K_i (μM)
	Histidine inhibition		
0.074	56.5	0.75	18
0.127	61	0.65	13
0.200	63.5	0.70	10
0.272	66.2	0.65	8
0.363	74.6	0.65	11.5
0.441	76.5	0.62	12.5
	AMP inhibition		
0.074	53.8	1.90	285
0.111	58.8	1.70	275
0.181	60.6	1.55	238
0.272	65	1.25	238
0.328	64.5	1.35	228
0.441	66.7	1.25	231

to the effect of specific ligands, the enzyme association process also depends on the concentration of the enzyme. It seems reasonable to assume that because of the very low enzyme concentration (about 0.1 μM, dimer) in kinetic inhibition studies, the enzyme is unable to become completely associated even at saturant histidine concentrations (figs. 7 and 8). When inhibition studies at 25° C are done at different enzyme concentrations, the extent of maximal inhibition mediated by histidine or AMP increases with the concentration of the enzyme (table I). In each case the maximum inhibition has been calculated from double reciprocal plots of the data in figures 7 and 8. Again, double reciprocal plots of the values in the first two columns of table I, show that the extrapolated maximum inhibition caused by histidine is 77 % and that caused by AMP is 69 %. The data of figs. 7 and 8 have also been analyzed by the Hill treatment and in table I the values of the Hill coefficient (n_H) and K_i are presented. The kinetic inhibition of the enzyme has also been tested at different temperatures. Figure 9 shows clearly

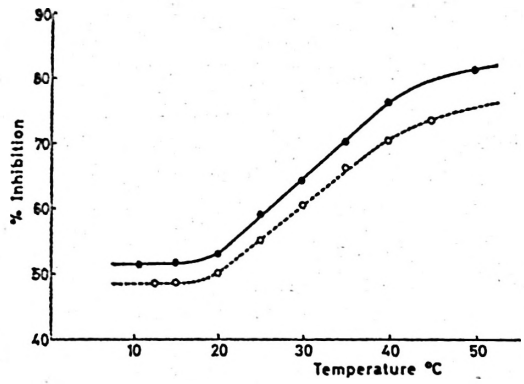

Fig. 9. *Percentage of Inhibition of the activity of the enzyme as a function of temperature.*
The enzyme concentration was 0.02 μM. ●, histidine (2 mM); ○, AMP (4 mM).

that increasing the temperature enhances the extent of maximal inhibition obtained. All these data show that the process of enzyme aggregation is very complicated: in addition to the effect of specific inhibitors such as histidine and AMP, association is also obtained by increasing temperature or/and concentration of the enzyme.

Discussion

It is known that the apparent Hill coefficient and binding constant depend upon the enzyme concentration (27, 29). Thus the values obtained in the present study cannot be compared since the concentration of the enzyme is very different in the three experimental approaches used. In the kinetic inhibition by histidine (enzyme concentration, 0.12 μM [dimer]), K_i is (at 25° C) 0.009 mM (calculated using equation [1]). In the microcalorimetric studies (enzyme concentration, 3 μM), K_i is 0.043 mM. In equilibrium dialysis (enzyme concentration, 33 μM), K_i has a value of 0.290 mM. Similarly, in the case AMP, K_i takes a value of 0.31 mM (at 25° C) (calculated using equation [1]) in the kinetic inhibition (enzyme concentration, 0.066 μM, dimer) and 0.11 mM in the calorimetric measurements (enzyme concentration, 6 μM). On the other hand, in the kinetic experiments the substrates and products of the reaction were present and this circumstance could alter the affinity of the enzyme for the inhibitors histidine and AMP. In fact, the dissociating effect of the substrate PRibPP on the enzyme has been proved (24).

The calculated reaction heats for histidine and AMP inhibition (+25 and +7 kJ/mol, respectively) are very different from those obtained in the microcalorimetric experiments (295 and 315 kJ/mol). In the kinetic inhibition we consider that there is only binding of the inhibitor: the low concentration of the enzyme and the presence of the dissociating substrate PRibPP (24) would hinder the process of enzyme association. On the contrary, in the calorimetric studies we are measuring both processes of binding of the inhibitor and aggregation of the enzyme.

Histidine has a synergistic inhibitory effect with one of the products released in the enzymatic reaction, phosphoribosyl-ATP (9). This behaviour could explain why low concentrations of histidine (ca. 10 μM) cause half of the maximal inhibition. For the same reason the enzyme substrates and products could cause the histidine inhibition to be slightly negatively cooperative (n_H, 0.8), white the histidine binding measured by equilibrium dialysis is of Michaelian type, and when measured by microcalorimetry is slightly positively cooperative. The practical absence of cooperativity in the binding of histidine found in our laboratory by fluorimetric measurements at 22° C ($n = 1.03$) (25) agrees with the value ($n_H = 1.05$) obtained here in the equilibrium dialysis at 25° C. However, DALL-LARSEN and KLUNGSÖYR (6) working at low temperature (2-4° C) have recently reported that 200,000 daltons of enzyme bind indeed 3 molecules of histidine but with slight positive cooperativity ($n_H = 1.23$).

In previous studies (11, 24) it was de-

monstrated that histidine (0.05 to 1 mM) associated the enzyme from dimer to hexamer. Later using several spin labels covalently bound to this enzyme we were able to detect the association effected by histidine with as low as 10 μM histidine (23). In the present report, in the microcalorimetric experiments we are measuring the heat of binding plus subsequent association, and from the concavity of figure 6 it can be deduced that association of the enzyme by AMP or histidine is already apparent at 1 μM (from this concentration up, the curves display nonlinearity).

We have seen that histidine is unable to inhibit the enzyme activity completely; this has been already found in previous work in our laboratory (22). However, in *in vivo* experiments the histidine analogue, thiazolealanine, a false feedback inhibitor, can shut down the biosynthesis of histidine (7 and references therein); this would mean that *in vivo* histidine does completely inhibit enzyme activity. This discrepancy can be explained assuming that there is an interrelationship between the extent of association and the extent of inhibition, i. e., that enzyme association is necessary for enzyme inhibition. In fact, KLUNGSÖYR and KRYVI (11) and TÉBAR and BALLESTEROS (22) have suggested that the hexameric form of the enzyme is inactive or less active. TÉBAR (21) in gel filtration experiments, reported that a semipurified feedback resistant enzyme was unable to become associated into a hexamer even at a very high concentration [0.1 M] of histidine. Moreover in the conditions of the experiments, owing to the high dilution of the enzyme, some of it could be denatured and unable to aggregate under histidine action. The same is valid for the AMP; the enzyme cannot be completely associated by the inhibitor under the conditions of the enzymatic assay.

We have tried to follow the reasoning of HO and WANG in the case of AMP binding to phosphorylase *b* (8), which is accompanied by association of the enzyme (dimer to tetramer). Using a mathematical iterative procedure, they calculated the equilibrium constant for the enzyme association and the enthalpies of AMP binding and tetramer formation and with these data, they evaluated the thermodynamic parameters of the interaction. In this case the system is much more complicated. First of all, the association is dependent on the enzyme concentration (figs. 6-8 and [12]). Second, the association is also dependent on the temperature (fig. 9). Third, the aggregation process does not stop at the hexamer, but aggregates of higher order are formed (11, 24). And fourth, the equilibrium dimer-hexamer is not clean, since with 0.4 mM histidine the tetrameric species was also present (24). Furthermore, new gel filtration experiments made in the same starting conditions as in the microcalorimetric experiments (25° C and equal enzyme concentration) (J. C. LEYVA, unpublished data) show that while in the absence of ligands the enzyme elutes as a dimer, in the presence of histidine (2 mM) or AMP (4 mM) the elution pattern is similar in both cases: the ratio of hexamer:tetramer:dimer is 3:1:1. These facts complicate the picture and do not allow further elaboration of the data.

Acknowledgements

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Resumen

Los inhibidores histidina y AMP asocian al enzima ATP fosforibosiltransferasa de *E. coli* de dímero a hexámero. La acción de ambos inhibidores se ha estudiado por tres métodos diferentes. 1) Por diálisis de equilibrio se demuestra que un mol de dímero (67,000 g) liga un mol de histidina. 2) Estudiando la cinética

de inhibición de la reacción a 21, 25 y 38° C, se han podido deducir los cambios de entalpía que ocurren en la inhibición del enzima por histidina o AMP. La inhibición, estudiada también en función de la concentración de enzima y de la temperatura, parece seguir cooperatividad positiva para el AMP y ligeramente negativa para la histidina, y en ninguno de los dos casos es posible alcanzar el 100 % de inhibición máxima. 3) Por microcalorimetría se han obtenido valores similares para las entalpías de la interacción del enzima con AMP e histidina. Este resultado podría indicar que ambos inhibidores llevan al enzima hasta un mismo estado final de asociación.

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