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On the structural affinity of macromolecules with different biological properties: Molecular dynamics simulations of a series of TEM-1 mutants

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ABSTRACT

Molecular Dynamics simulations have been carried out in order to provide a molecular rationalization of the biological and thermodynamic differences observed for a class of TEM β-lactamases. In particular we have considered the TEM-1(wt), the single point mutants TEM-40 and TEM-19 representative of IRT and ESBL classes respectively, and TEM-1 mutant M182T, TEM-32 and TEM-20 which differ from the first three for the additional of M182T mutation. Results indicate that most of the thermodynamic, and probably biological behaviour of these systems arise from subtle effects which, starting from the alterations of the local interactions, produce drastic modifications of the conformational space spanned by the enzymes. The present study suggests that systems showing essentially the same secondary and tertiary structure may differentiate their chemical–biological activity essentially (and probably exclusively) on the basis of the thermal fluctuations occurring in their physiological environment.

1. Introduction

One of the major intellectual advances in the comprehension of the molecular basis of biological processes has been achieved, in the last century, with the possibility of determining the three-dimensional structure of macromolecules. However the complexity of nature is always a little ahead of the efforts of the human mind. In fact, several observations have repeatedly demonstrated that a simple relationship between tridimensional structure and thermodynamic and kinetic feature of a macromolecule is not always possible. In this study we have used the standard tools of Molecular Dynamics simulations to address a case study in which a family of enzymes, structurally very similar, do show significant thermodynamic and biological differences. Among the various possibilities we have chosen the class A TEM β-lactamases and related mutations based on the M182T mutation. Class A TEM β-lactamases, according to Ambler classification [1] are the predominant source of bacterial resistance. They can hydrolyze the four-membered β-lactam ring of penicillins, utilizing a catalytic serine, and render the antibiotic ineffective [2,3]. Several mechanism-based inhibitors of these enzymes were designed to reverse the resistance and a large number of inhibitor-resistant TEM (IRT) mutants have evolved. The TEM-1 wild type enzyme, hereafter termed as TEM-1(wt) (Fig. 1), is a globular protein behaving as excellent penicillinase but showing little activity toward third generation cephalosporines that possess oxyimino side chains with an enhanced steric hindrance for the TEM-1 active site (Fig. 1).

However, since these drugs were introduced to fight bacterial resistance, microrganisms evolved clever mechanisms of resistance, such as extended spectrum β-lactamase (ESBL) mutants, conferring resistance also to these drugs. All ESBLs have reduced penicillinase activities, but increased oxyimino-cephalosporinase activities and show lower thermodynamic stabilities with respect to TEM-1(wt). In fact, ESBLs should enlarge the active site in order to accomodate the bulky oxyimino side chain of cephalosporines.

In this scenario, it has been proposed the crucial role of the M182T substitution in restoring stability to the ESBL mutants. The M182T substitution, far from the enzyme active site (Fig. 1), has always been found in combination with other substitutions not only in ESBLs, but also in IRT mutants, and it has been found alone in TEM-135 enzyme isolated in clinical strains [4]. Moreover, it has been shown [5] that this substitution stabilizes thermody-

Abbreviations: MD, molecular dynamics; ED, essential dynamics; IRT, inhibitor-resistant TEM; ESBL, extended-spectrum beta-lactamases.

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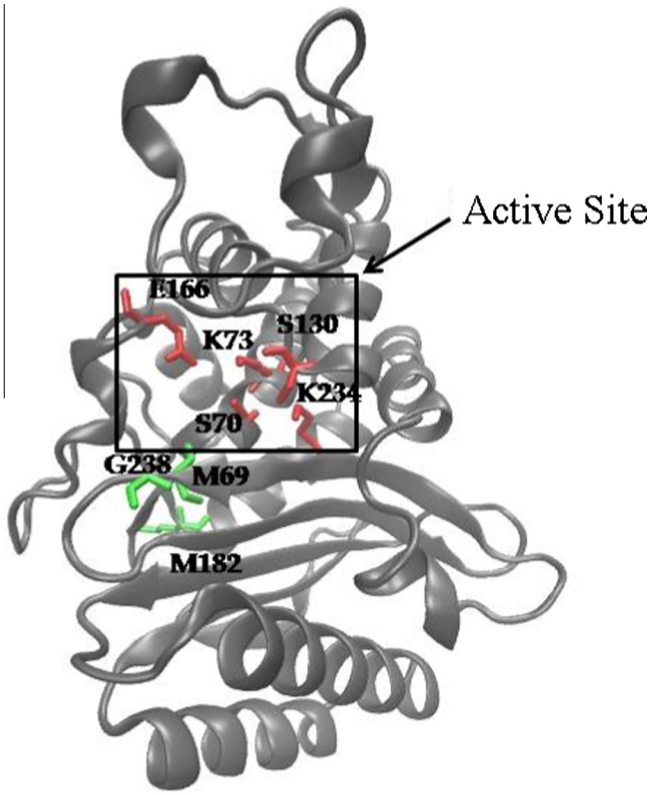

Fig. 1. Cartoon-like representation of TEW1(wt) including the schematic localization of the active site (green residues) and mutations (red) considered in the present study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

namically TEM enzymes and generally occurs only in mutants that would be destabilized with respect to the TEM-1.

In this study we have considered the following systems: (i) the TEM-1(wt); (ii) the single point mutants M182T, TEM-40(M69I) and TEM-19(G238S) with M69I and G238S representative of IRT and ESBL classes respectively; (iii) the doubly substituted TEM-32(M69I, M182T) and TEM-20(M182T, G238S).

The main goal of the present computational investigation is to provide convincing evidences able to suggest plausible interpretations of the observed biological differences shown by the above enzymes in spite of their structural similarity. It is important to note that, in principle, significant structural and biological differences may also be induced by the inclusion of a ligand. However, in this specific study we have tried to follow a more general approach focussing our attention on the *intrinsic* features of TEM-1(wt) and the related mutants.

2. Methods

The Molecular Dynamics (MD) simulations were performed utilizing the Gromacs package [6]. The initial coordinates for TEM-1(wt), TEM-1 mutant M182T and TEM-32(M69I, M182T) were taken from the crystal structures available on PDB with code 1BTL, 1JWP and 1LI0, respectively. The initial coordinates of the mono-substituted TEM-40(M69I) were obtained by modifying those of TEM-32(M69I, M182T). The initial coordinates of TEM-19(G238S) and TEM-20(M182T, G238S) were derived from those of TEM-1(wt). Each enzyme was put in a dodecahedral box whose dimension prevents self-interaction, and then solvated with 7020 molecules of water, at the typical density of water at 298 K and 1.0 atm, utilizing the single point charge (SPC) model. [7] A proper number of counterions (sodium ions) were added to ensure the electrical neutrality of the whole system. All the simulations were performed adopting the following protocol:

(i) after an energy minimization, the whole system was slowly heated up to 300 K using short (100.0 ps) MD runs. (ii) The simulation was extended up to 140 ns for TEM1(wt) and 80 ns for all other simulated enzymes, at 300 K in an isothermal/isochoric ensemble, using the Berendsen thermostat [8]. It is important to note that on the basis of the backbone Root Mean Square Deviation (RMSD) with respect the TEM-1(wt) crystal structure, the first 15 ns were disregarded from the analyses for all the investigated systems. (iii) The simulated enzymes were described using the OPLS force field [9], the LINCS algorithm was adopted to constrain all bond lengths [10], and the long range electrostatics were computed by Particle Mesh Ewald method [11] with 34 wave vectors in each dimension and a 4th order cubic interpolation. The trajectories were analyzed either using standard Gromacs tools or using home-made routines. To analyze protein collective motions we adopted Essential Dynamics (ED) analysis [12]. On the purpose the covariance matrix of the atomic C^{α} positional fluctuations was built from the MD trajectory and then diagonalized producing an orthonormal set of eigenvectors defining a new set of generalized coordinates along which the enzyme fluctuations occur. The M eigenvectors with the largest eigenvalues, i.e. with the largest associated fluctuation (large amplitude motions) allow to define the essential M-dimensional subspace onto which the trajectory is projected. From the obtained projection it is possible to identify the conformations sampled by the whole system, e.g. the protein in the present case and, hence, it might be relatively straightforward to concisely represent and compare the conformational space spanned by the investigated proteins. In this case, since we are interested in *differences* between the fluctuation pattern characterizing TEM-1(wt) and all the mutants, we have linked all the equilibrated trajectories. Hence ED analysis was performed on the whole linked trajectory. It follows that the resulting essential eigenvectors do not precisely coincide with the eigenvectors of the covariance matrix of each enzyme but allow to directly identify the mechanical-dynamical analogies and differences between the investigated systems.

3. Results and discussion

3.1. Morphological analyses of TEM(wt) and selected mutants

Preliminary analysis show high morphological affinities between the investigated enzymes. As reported in Fig. 2, and in line with the available experimental data, the simulated enzymes, all characterized by relatively low structural deviations from the TEM-1(wt) crystal structure (RMSD between 0.1 and 0.25 nm), are expected to show e relatively high structural affinity. Significant deviations are observed only in some solvent exposed loops. In this respect, it is important to remark that in all the investigated cases the secondary structure is maintained basically unaffected if compared to that observed in the TEM-1(wt) crystal structure. Consistently, all the enzymes also show a globular and similar shape as witnessed by the eigenvalues of the inertia tensors reported in the Table 1. Analysis of the C^{α} Root Mean Square Fluctuations (RMSFs) reported in Fig. 3 also indicates that all the enzymes are characterized by a relatively low fluctuation pattern.

Similarly to the RMSD, higher RMSF values have been found only in solvent exposed loops. If we focus our attention on the RMSF of the catalytic residues, reported in Table 2, we note that the mechanical stability is even more pronounced.

These results clearly indicate that the highly similar structural affinities, shown by these systems in the crystal state (when available), are confirmed in solution at least according to the presently utilized simulation features and that, despite their biological

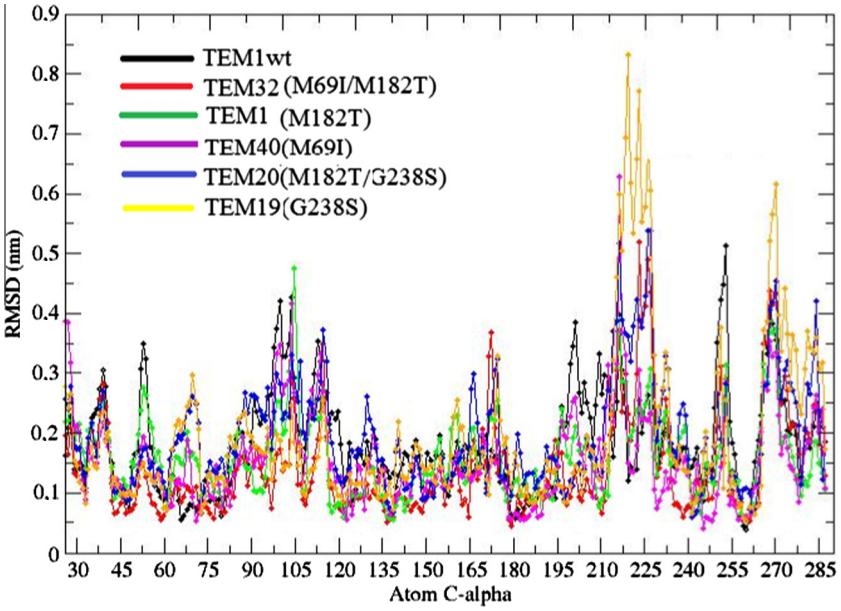

Fig. 2. C-alpha Root Mean Square Deviation (nm) calculated along the equilibrated portion of the trajectories.

Table 1
Average moments of inertia (amu $*$ nm^2) evaluated along the simulation. The standard deviation is reported in parenthesis.

Enzyme	I_{TOT}	I_1	I_2	I_3
TEM-1wt	38388 (300)	15879 (199)	23524 (265)	25846 (264)
TEM32(M69I/M182T)	39012 (360)	15793 (182)	24016 (292)	26429 (327)
TEM-1(M182T)	38629 (346)	15300 (295)	23983 (300)	26167 (372)
TEM-40(M69I)	38694 (347)	15780 (205)	23954 (272)	25967 (272)
TEM20(M182T/G238S)	39593 (470)	15467 (244)	24527 (397)	26954 (415)
TEM-19(G238S)	38728 (480)	16237 (269)	23765 (385)	25909 (430)

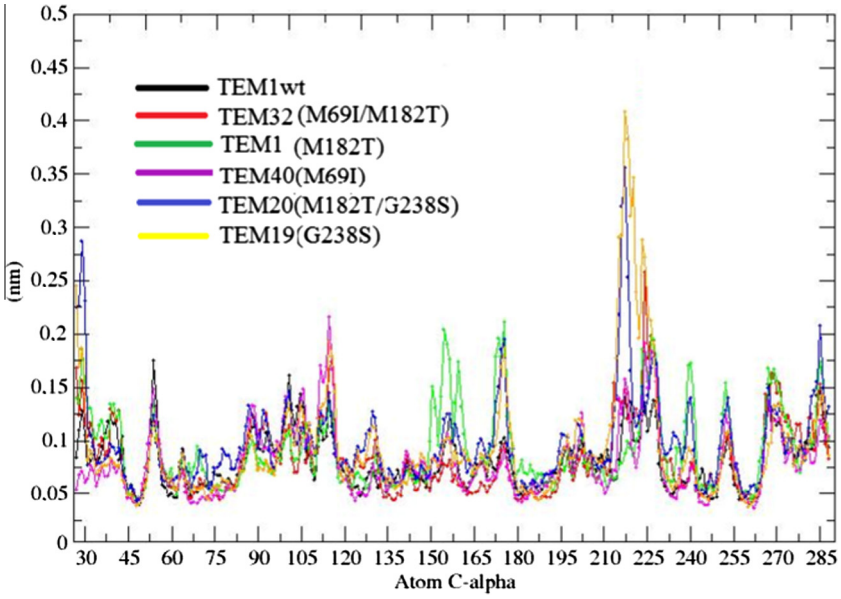

Fig. 3. C-alpha RMSF on the equilibrated portion of the trajectory.

differences, all the systems are characterized by active site with almost identical structural and mechanical features. It follows that further aspects should be considered as plausible determinants of the different enzymes behavior. Hence we have concentrated our attention on the conformational space spanned by the enzymes.

3.2. Essential dynamics

In order to better identify the conformational space spanned by the different enzymes, we carried out ED analysis as reported in the Experimental Section.

Table 2
C^{α} Root Mean Square Fluctuation (nm) for the active-site residues.

Enzyme	S70	K73	S130	E166	K234
TEM-1wt	0.06	0.06	0.06	0.08	0.05
TEM-32(M69I/M182T)	0.06	0.05	0.10	0.05	0.07
TEM-1(M182T)	0.07	0.05	0.08	0.07	0.06
TEM-40(M69I)	0.05	0.04	0.07	0.08	0.05
TEM-20(M182T,G238S)	0.08	0.06	0.12	0.10	0.11
TEM-19(G238S)	0.05	0.05	0.11	0.07	0.05

First of all it is important to remark that from ED analysis on the concatenated trajectories more than 50% of the overall fluctuation turns out to be described by the first two eigenvectors. This finding, not unexpected for systems as the ones investigated in this study [12], allows to qualitatively identify the conformational space spanned by the systems by projecting the trajectory onto the plane defined by the first two eigenvectors (hereafter termed as essential plane). Consequently, the conformational space sampled by each enzyme is represented by a number of spots. In order to help the not-expert reader, it should be noted that enzymes characterized by restricted conformational motions will produce spots of limited areas. On the other hand, spots of larger areas are produced by enzymes undergoing larger conformational transitions. Moreover, superposition of spots on the same plane occurs only when the enzymes span very similar or identical conformational repertoire. As a final remark it is important to note that the fluctuations along the first two eigenvectors do not cover the whole conformational repertoire of the enzymes. However this limitation would not be severe in the presence of scarcely overlapping spots. On the other hand, if the use of the first two eigenvectors produce a very high affinity, it cannot be excluded that differences in the projections might arise when additional eigenvectors beyond the second are used. The projections of the simulated and concatenated trajectories of the six enzymes under investigation, reported in Fig. 4, are characterized by relatively small areas: this is in accordance with their low RMSFs pattern already reported in Fig. 3.

Moreover, and most importantly, all the spots are characterized by scarce mutual overlap clearly suggesting *differences* in the fluctuation *pattern* of the enzymes. Moreover, if we compare the spots related to the single-mutants systems with the TEM-1(wt) we can conclude that all the mutations heavily affect the conformational repertoire. Particularly severe is the effect of M182T and G238S. It is also important to remark the presence of some overlap between the doubly-mutants. In this respect it might be important to remind the all these enzymes, although presenting the same secondary structure and similar shape in solution, show rather different thermodynamic properties [13]. Hence our finding suggests that for the investigated enzymes a single mutation, although scarcely affecting the geometrical parameters, is able to produce significant changes in the conformational repertoire and, probably, in the thermodynamic (equilibrium) property. This implies that a 'dynamical' view of the enzyme might be of fundamental importance for rationalizing the properties in physiological conditions.

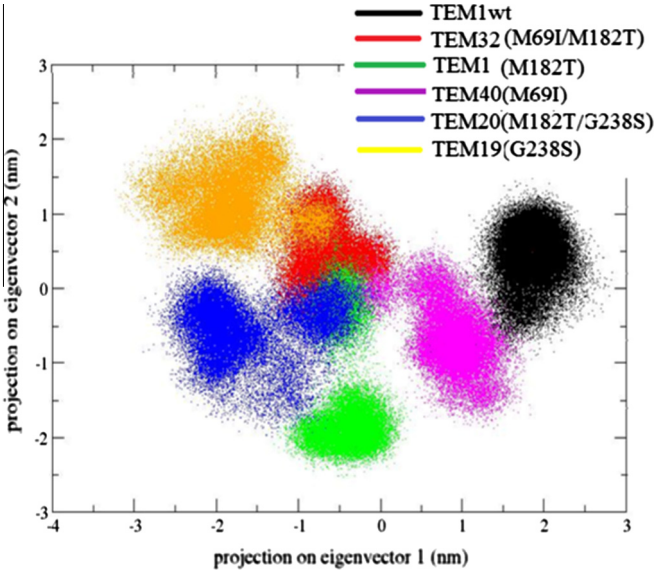

Fig. 4. Projection of the different trajectories onto the essential plane (see text).

3.3. Analysis of the active-site volume and flexibility

Further analysis has been carried out for evaluating geometrical and mechanical features of the active site of the different enzymes. On the purpose, we have estimated the average value of the catalytic-site volume ($\langle V \rangle$) using the standard criteria as the thermally averaged volume of a sphere that can be included in the active site without energy expenditure, considering van der Waals interactions. In order to take into account flexibility effects we adopted the standard formula $\langle V \rangle = V_i * X_i$ where V_i is the volume of the active-site ith configuration and X_i is the active-site ith configuration probability (Boltzmann weight). Each of the active-site ith configuration was evaluated by carrying out an ED analysis (similarly to that reported in Fig. 4) limitedly to residues forming the active-site. The flexibility of the active site was evaluated by the standard deviation of the previously defined $\langle V \rangle$. The above computed values have been reported in the Table 3 with the whole enzyme flexibility expressed as the trace of the backbone covariance matrix (see previous subparagraph) and compared to the thermodynamic data available from literature.

Among the six examined enzymes the TEM-1(wt) has the lowest average volume of the active site, the lowest flexibility of the active site, the lowest global flexibility and an intermediate thermodynamical stability.

With respect to the TEM-1(wt), the introduction of a single point mutation in the natural mutant TEM-19 gives rise to a significant increase of the active site volume, the active site flexibility, the whole enzyme flexibility and also to a sharp decrease in the thermodynamic stability. The same destabilization is also observed for the natural mutant TEM-40 in which, however we do observe a significant increase of active site volume and flexibility. The introduction of the M182T mutation in the three mentioned enzymes has the same qualitative effect, consisting in an increase of volume and flexibility of the active site and also an increase of the flexibility of the whole enzyme with a regain of the thermodynamical stability. The last effect emerged from the present study can be correlated to the character of the mutants in the following way. The TEM-1(wt), possessing the lowest volume, is an excellent penicillinase but has little effect on third generation cephalosporines like ceftazidime (CAZ) and cefotoxime (CTX) that have bulky oxyimino side-chains too large to be accomodated in the wt active site. On the other hand, ESBLs show reduced penicillinase activity relative to wt, since their enlarged active sites could be less appropriate to the smaller side chain of penicillins, but they show cephalosporinase activities 100-fold increased, since their enlarged active sites are capable to accept the larger oxyimino side chains of cephalosporines to be processed.

3.4. Analysis of H-bonds

Following the previous analyses, focused on the whole structural and conformational features, we have undertaken a deeper investigation at atomistic level on the active sites. In particular we have monitored the H-bonds involving the most important

Table 3
Correlation among geometrical and mechanical data from MD simulations and experimental thermodynamical data (unfolding temperature,Tm, and enthalpy) of the enzymes. Estimated error in the trace of the covariance matrix is equal to the standard error evaluated by dividing each trajectory in three sub-portions.

Enzyme	⟨V⟩(nm^3)	Covariance Matrix Trace (nm^2)	Tm (°C)	ΔH (kcal/mol)
TEM-1(wt)	3.08 ± 0.03	1.86 ± 0.05	51.5[a]	139.5[a]
TEM-1(M182T)	4.5 ± 0.1	2.10 ± 0.08	57.7[a]	142.2[a]
TEM-32(M69I/M182T)	4.3 ± 0.1	2.50 ± 0.08	55.2[b]	141.1[b]
TEM-40(M69I)	3.4 ± 0.1	1.90 ± 0.05	48.6[b]	138.2[b]
TEM-20(M182T/G238S)	4.6 ± 0.9	2.97 ± 0.08	54.0[a]	126.5[a]
TEM-19(G238S)	4.5 ± 0.4	3.05 ± 0.08	47.0[a]	103.7[a]

[a] Ref. [13].
[b] Ref. [14].

catalytic residues, namely S70, K73, S130, E166 and K234. On the purpose, we have utilized the Gromacs criteria for the definition of H-bonds and have evaluated the stability of each H-bond by calculating its Helmholtz Free energy of formation (ΔA_f) from the ratio between the frames in which the H-bond is found and the frames in which the H-bond is not formed. Moreover, we have considered as 'stable' (basically permanent hydrogen bond) the interactions showing ΔA_f lower than the thermal energy at 298 K (approximately 2.5 kJoule/mol). The results are reported in the Table 4. Some couples of residues are permanently H-bonded along the entire length of simulation performed for the six different enzymes. In fact, the carbonyl group of S70 is always H-bonded to K73 with the lowest ΔA_f values, indicating higher stability of H-bond for every simulated enzyme. Moreover, these two residues form the additional less stable H-bond CO(S70)–N^{ζ}(K73) in three mutants, as indicated in Table 4. Other direct H-bonds found in all enzymes, and reported in the same Tble Table, are those occurring between the hydroxyl group of S130 and the side-chain NH of K234, and those between the side-chain NH of K73 and the two carboxylic oxygens of E166. In any case it is important to remark that none of these H-bonds appear as particularly stable (formation free energy larger than thermal energy) in the simulated conditions. Worth to note is the case of TEM-1(wt) simulation where the hydroxyl group of S70 gives rise only to a scarcely stable H-bond with the carboxyl group of E166, while direct H-bond between these two residues is never formed in the other five variants. As a consequence, the glutamate carboxylate could not directly accept the proton from S70. The lack of direct H-bond between these two catalytic important residues has been already demonstrated, in the TEM-1 wt, to be essentially due to the presence of water molecules bridging them. [15] As a matter of fact, water molecules H-bonded to catalytic residues are supposed to lower the energy barrier in the proton transfer reactions involved in the acylation step of substrates. Hence their presence in the catalytic site is highly favoured. [15].

While direct H-bond interaction is observed between S70 and S130 for three enzymes, direct H-bond formation between K73 and K234 does not appear in any enzyme. Finally, the carboxylate group of E166, in addition to the above discussed interaction with S70, gives rise to a permanent salt bridge with the ammonium group of K73 in all cases. As a final step, we have analyzed the H-bond a weak-interactions experienced by the residue in the position 182 on the basis of the already remarked crucial role. The T residue, replacing the M in position 182, is capable to form a network of multiple H bonds with the surrounding hydrophilic groups of the two-domain protein. At this regard, Table 5 summarizes the ΔA_f for the H bonds that the OH of T182 forms during the simulations performed for the three enzymes containing this substitution. However, as emerged from the same Table, the T residue only forms stable H-bonds with residues A185, in TEM-1(M182T) and TEM-32, and with E64 in TEM-20.

In summary, we have performed MD simulations in aqueous solution for six TEM variants. The enzymes considered in this study are the TEM-1(wt), the single point mutants TEM-40 and TEM-19 representative of IRT and ESBL classes respectively, and TEM-1(M182T), TEM-32 and TEM-20 which differ from the first three for the additional of M182T mutation. All these enzymes, in spite of their high structural affinity are characterized by thermodynamic and biological differences.

From a comparison of their simulated trajectories, we have observed that these enzymes, although maintaining basically unaltered their secondary structure and not showing drastic differences in the H-bond network in the key residues, span a markedly different conformational space suggesting that, for a better comprehension of the thermodynamical and biological features of bio-macromolecules, analysis of the mechanical and dynamical properties represent a crucial starting point for better understanding the molecular basis of the biological activity.

The most evident effect produced by the M182T substitution, emerging from the present study, is associated with the increase of volume of the active site. The last effect suggests a way to understand the β-lactamase evolution under the pressure of different types of adopted inhibitors. Evolution has taken place from primary substitution of residues of the active site to increase the

Table 4
298 K Free energy of formation (kJoule/mole) of H-bonds between catalytic important residues. Stable H-bonds are indicated in bold. Entries not included refer to H-bonds never formed during the siimulations.

	TEM1(wt)	TEM1 (M182T)	TEM32 (M69I/M182T)	TEM40 (M69I)	TEM20 (M182T/G238S)	TEM19 (G238S)
NH(K73)-CO(S70)	**2,1**	**2,6**	3,9	**1,4**	**1,8**	**1,5**
N^{ζ}(K73)-CO(S70)			14,5	4,7		13,2
OH(S70)-OH (S130)				17,3	15,5	6,8
OH(S70)-γCOO(E166)	17,3					
N^{ζ}(K234)-CO (S70)					13,2	
N^{ζ}(K234)-OH (S70)	15,5			8,8	8,9	4,3
N^{ζ}(K73)-CO (S130)		15,5	6,9			
N^{ζ}(K73)-OH (S130)	13,8			15,5		
N^{ζ}(K73)-γCOO-(E166)	4,8	5,2	3,6	4,5	5,1	5,4
N^{ζ}(K73)-γCOO-(E166)	4,5	5,8	7,2	4	3,6	3,1
N^{ζ}(K234)-OH (S130)	6,1	3,7	**2,1**	6,6	15,5	5,1

Table 5
298 K Free energy of formation (kJoule/mole) of H-bonds between OH(T182) and other residues found in the three enzymes containing the substitution M182T. Stable H-bonds are indicated in bold. Entries not included refer to direct H-bonds never formed during the simulations.

H-bond partners	TEM-1 (M182T)	TEM-32 (M69I/M182T)	TEM-20 (M182T/G238S)
OH(T182)-CO(P62)	9,8	12,4	10.2
OH(T182)-COOH(E63)	12,1	14,5	
OH(T182)-CO(E64)		17,3	**0,2**
OH(T182)-NH(A184)	14,5	15,5	
OH(T182)-NH(A185)	**0,6**	**1,3**	15.5

hydrolytic activity, to secondary substitutions involving residues far away from the active site, but capable to cause the enlargement of the active site for accommodating larger β-lactams stable to the hydrolysis to be processed with the same catalytic machinery.

Recently, the following substitutions A184V, T265M, R275Q and N276D found in ESBLs have been reported [16] as having similar effect as that of M182T, that is they can compensate for primary destabilizing substitution. Nowadays, this kind of substitutions are present in over one third of IRT and ESBL mutants.

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