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# Revealing electronic features governing hydrolysis of cephalosporins in the active site of the L1 metallo- $\beta$ -lactamase†

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The QM/MM simulations followed by electron density feature analysis are carried out to deepen the understanding of the reaction mechanism of cephalosporin hydrolysis in the active site of the L1 metallo- $\beta$ -lactamase. The differences in reactivity of ten similar cephalosporin compounds are explained by using an extended set of bonding descriptors. The limiting step of the reaction is characterized by the proton transfer to the nitrogen atom of the cephalosporin thiazine ring accompanied with formation of the  $C_4=C_3$  double bond in its  $N-C_4-C_3$  fragment. The temporary  $N\cdots H-O_w$  hydrogen bond, which is formed in the transition state of the limiting step of the reaction was recognized as a key atomic interaction governing the reactivity of various cephalosporins. Non-local real-space bonding descriptors show that different extent of localization of electron lone pair at N atom in the transition state affect the reactivity of compounds: smaller electron localization is typical for the less reactive species. In particular, the Fermi hole analysis shows how exchange electron correlation in the  $N\cdots H-O_w$  fragment control electron lone pair localization. Delocalization tensor, linear response kernel and source function indicate that features of electron delocalization in the  $N-C_4-C_3$  fragment of cephalosporins in the transition state complexes determine the differences in  $C_4-C_3$  bond for substrates with high and low rate constants. The  $C_4-C_3$  bond of the  $N-C_4-C_3$  fragment at the transition state is similar to that of the preceding intermediate for the less reactive species and resembles the features of the enzyme-product complex for more reactive compounds. The power and limitations of the descriptors applied for solving the problem are discussed and the generality of approach is stressed.

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## 1. Introduction

Enzymes are complex finely tuned machines that efficiently perform certain chemical reactions.<sup>1</sup> The rate constants may vary dramatically depending on the particular substrate involved in enzymatic reaction. It is still an open question, what particular features of the substrates govern their reactivity in the active site of an enzyme.<sup>1-4</sup> Existing experimental techniques are able to register the difference in reactivity of a series of similar compounds although they cannot explain its origin. The molecular modeling and quantum chemistry are the proper tools that shed light on this point. Analysis of electron density<sup>5</sup> is able to locate specific inter- and intramolecular interactions in the

chemical systems while various bonding descriptors allow one to analyze and quantify the features of particular chemical interactions.<sup>6-23</sup> Recently we have demonstrated<sup>24-26</sup> that there are certain key interactions among a great number of all interatomic interactions in the active site of enzyme that mainly determine the observed macroscopic property, the steady-state rate constant. We pointed out that these key interactions can be defined at the stationary points of the reaction path on the potential energy surface (PES) from quantum mechanics/molecular mechanic (QM/MM) approach<sup>27,28</sup> combined with subsequent electron-density analysis.

Molecular modeling in combination with electron-density analysis is already used for organic reaction studies. Equilibrium geometry configurations and corresponding calculated electron densities of reagents, intermediates, transition states and products of chemical reactions are analyzed by various approaches as QTAIM (Quantum Theory of Atoms in Molecules)<sup>29-41</sup> or conceptual DFT (Density Functional Theory)<sup>23,36,42-44</sup> as well as diverse bonding descriptors.<sup>33-37,45,46</sup> Current studies of enzymatic reactions<sup>47-49</sup> also demonstrate an enhanced interest in the application of such combined methods in biochemistry. For example, the Fukui function

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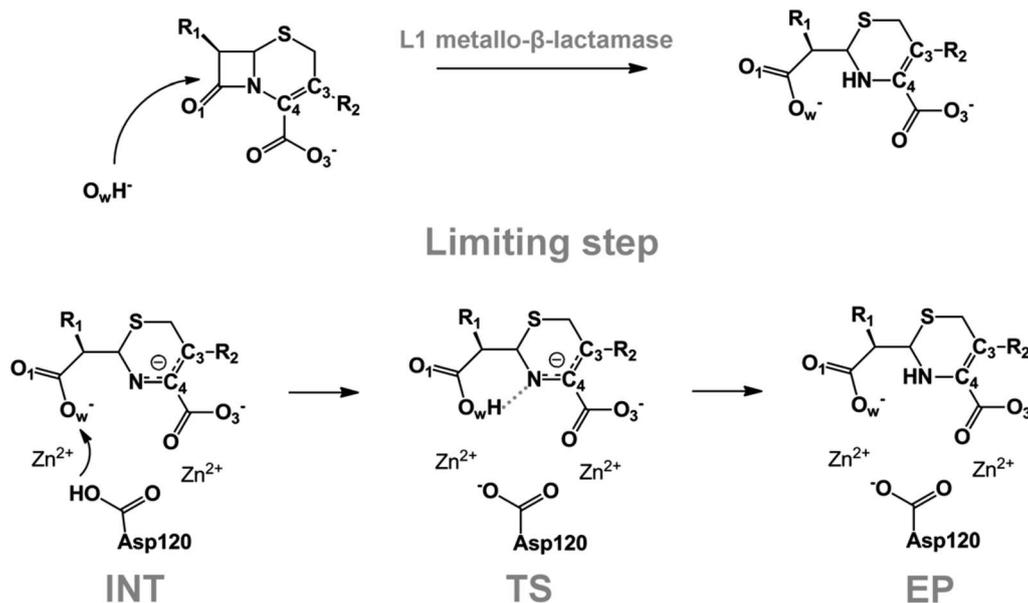


Fig. 1 Mechanism of cephalosporin hydrolysis in the active site of L1 MβL. The intermediate (INT), transition state (TS) and product (EP) of the limiting step. The intramolecular  $N \cdots H-O_w$  hydrogen bond is indicated by grey dotted line.

interpreted the residue–residue interactions in biomolecular systems simulated by QM/MM method,<sup>47</sup> electrostatic potential allowed to identify regions of electrophilic/nucleophilic attack in the QM/MM structural models,<sup>48,49</sup> *etc.* Thus, here we aim to systematically examine the applicability of various available bonding descriptors in the QM/MM models of enzymatic reactions.

We focus on the following issues: (i) what electronic features of key interactions are responsible for different reactivity of similar substrates in the active site of a particular enzyme? (ii) What electronic features and related bonding descriptors allow to quantitatively characterize the progress of such reactions?

We examine below cephalosporin hydrolysis induced by L1 metallo-β-lactamase (L1 MβL). This bacterial enzyme is responsible for destruction of β-lactam antibiotics<sup>50,51</sup> that is one of the known reasons of bacterial resistance.<sup>52,53</sup> This chemical reaction exhibits outstanding mechanism (Fig. 1).<sup>54–56</sup> Typically, proton transfer happens with low energy barrier along the hydrogen bond in enzymatic reactions.<sup>57,58</sup> Here, the proton is transferred from the carboxylate group of catalytic aspartate to the nitrogen atom of the substrate forming temporary O–H covalent bond at the transition state of the limiting step (Fig. 1).<sup>54</sup>

We utilize molecular models obtained *via* QM/MM approach together with the detailed electron density study to follow the progress of this reaction in a set of 10 similar systems. Those are composed of the L1 MβL and various cephalosporin compounds differing in their reactivity in the active site of the selected enzyme.<sup>59,60</sup> We focus on the limiting stage of the reaction and discriminate the electronic features determining the reactivity of compounds. We analyze the key interaction (the temporary hydrogen bond formed in the transition state suggested in ref. 24) and electronic features in the N–C<sub>4</sub>–C<sub>3</sub> fragment of cephalosporins to establish the influence of conjugated

system (Fig. 1) on this interaction. Finally, we formulate how these features are related to each other and affect the observed reaction rate constant.

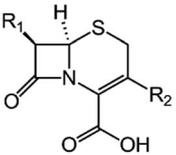
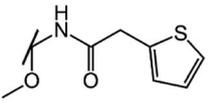
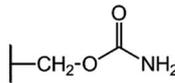
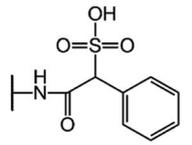
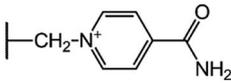
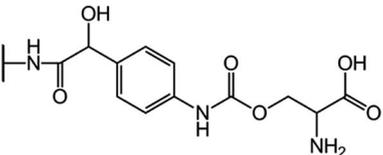
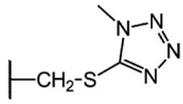
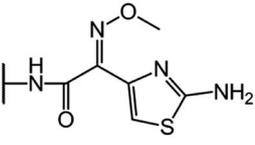
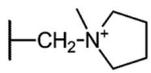
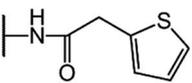
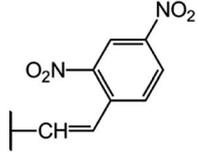
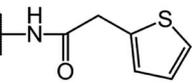
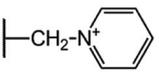
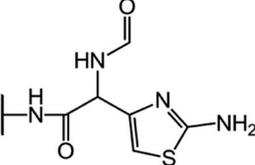
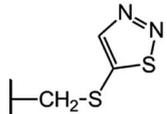
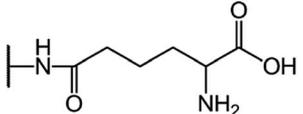
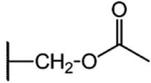
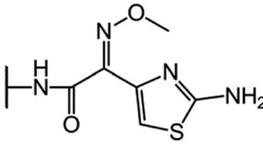
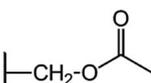
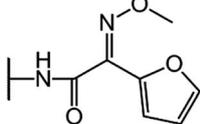
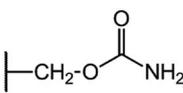
## 2. Methodology

We utilize the two-step methodology to study electronic features governing the reactivity of 10 cephalosporins in the active site of L1 MβL. First, the QM/MM approach is utilized to obtain equilibrium geometry configurations on the potential energy surface corresponding to the reaction intermediates (minima) and transition states (saddle points). Second, we explicitly reveal the electronic features in the active sites of the complexes. We apply the QTAIM<sup>5,61</sup> and other advanced bonding descriptors: source function,<sup>11,62</sup> Fermi hole,<sup>15,63</sup> electron delocalization indices,<sup>16</sup> delocalization tensor,<sup>64</sup> condensed linear response kernel<sup>23</sup> to analyze the QM part of each system, *i.e.* its active site. The basic information about these bonding descriptors is summarized in Table 2; more information is given in the ESI.†

We consider cephalosporin antibiotics with rate constants from 1.1 to 80 s<sup>−1</sup> (Table 1).<sup>59,60</sup> The electronic structure analysis is carried out for stationary points on PES obtained in the QM/MM simulations. The structures of intermediates, transition states and products at the limiting step were taken from our previous study.<sup>24</sup> Each QM subsystem included cephalosporin, two Zn<sup>2+</sup> ions and amino acid residues (Tyr32, His116, His118, Asp120, His121, His196, Ser221, His263) in the active site of L1 MβL, atoms of catalytic O<sub>w</sub>H<sup>−</sup> ion, performing the nucleophilic attack, and two water molecules (see Fig. S1†). The QM subsystems were described at the PBE0-D3/6-31G\*\* level of DFT using the NWChem program package,<sup>65–67</sup> while the MM parts (included protein and solvation water shell) were modeled with AMBER force field parameters.<sup>68</sup> The electronic embedding



Table 1 Atomic structures of considered cephalosporins and rate constants,  $k_{\text{cat}}$ ,<sup>59,60</sup> of their hydrolysis by the L1 MβL

		$R_1$	$R_2$	$k_{\text{cat}}, \text{s}^{-1}$
Cefoxitin (CFX)			1.1	
Cefsulodin (CFS)			7.5	
CGP-17520A (CF0)			8.5	
Cefepime (CFP)			15	
Nitrocefin (NCF)			20	
Cefaloridine (CFR)			28	
GCP-31523A (CF3)			38	
Cephalosporin C (CFL)			62	
Cefotaxime (CFT)			66	
Cefuroxime (CFU)			80	



scheme was applied assuming contributions of the partial charges from all MM atoms to one-electron part of QM Hamiltonian. Other details of QM/MM computational protocol are presented in ref. 54.

The electron densities in the QM subsystems were calculated at stationary points on the PES obtained in the QM/MM calculations. The environmental effects of the MM subsystem were included explicitly by adding terms to the one-electron part of the QM Hamiltonian corresponding to the interactions of the partial atomic charge of the MM atoms with the QM subsystem. The QTAIM analysis<sup>5</sup> and the bonding descriptor calculations were performed using Multiwfn program.<sup>69</sup> More computational details can be found in the ESI.†

### 3. Results and discussion

The role of electronic features in the active sites of 10 cephalosporin – L1 MβL complexes and influence of the immediate and distant surrounding on the bonding picture along the limiting step of reaction (INT → TS → EP) (Fig. 1) is examined below in detail. The non-covalent atomic interactions in the INT, TS and EP systems were located using the QTAIM criteria, namely the existence of the bond path, *i.e.* bond critical point (bcp) and two corresponding gradient lines, between atoms.<sup>6</sup> Characteristics of main atomic interactions in the enzyme–substrate complexes are collected and discussed in the ESI.† We focus further on the two pivotal processes happening at the limiting step: proton transfer, which occur with formation of the key atomic interaction, temporary N··H–O<sub>w</sub> hydrogen bond in the TS, and bond

**Table 2** Chemical bond descriptors used for investigation of electronic features of QM subsystems obtained in the QM/MM simulations of hydrolysis of cephalosporins in the active site of the L1 MβL. For more details see the ESI

Descriptor	Expression	Meaning
Ellipticity of electron density ( $\epsilon$ )	$\epsilon = \frac{\lambda_1}{\lambda_2} - 1$ , where $\lambda_1$ and $\lambda_2$ are negative eigenvalues of the Hesse matrix of electron density ( $ \lambda_2  <  \lambda_1 $ )	It is a measure of the axial symmetry deviation of electron density along the bond path; indicates existence of the $\pi$ component of chemical bonds <sup>61</sup>
Source/influence function (SF)	$G(\mathbf{r}, \mathbf{r}') = -\frac{1}{4\pi} \frac{\nabla^2 \rho(\mathbf{r}')}{ \mathbf{r} - \mathbf{r}' }$ , where $\rho(\mathbf{r})$ is electron density at point $\mathbf{r}$ and $\nabla^2 \rho(\mathbf{r})$ is its Laplacian	SF represents the influence of electron density curvature in point $\mathbf{r}'$ on electron density at point $\mathbf{r}$ <sup>11,62</sup>
Atomic source function	$SF(\mathbf{r}, \Omega_i) = -\frac{1}{4\pi} \int_{\Omega_i} \frac{\nabla^2 \rho(\mathbf{r}')}{ \mathbf{r} - \mathbf{r}' } d\mathbf{r}'$ , where $\Omega_i$ is $i$ -th QTAIM zero-flux atomic basin	Atomic SF measures total influence of $i$ atom on the electron density at point $\mathbf{r}$ . <sup>11</sup> The percentage form of SF <sup>70</sup> is $SF\% = \frac{SF(\mathbf{r}, \Omega_i)}{\rho(\mathbf{r})} \times 100\%$
Fermi hole	$h_x(\mathbf{r}, \mathbf{r}') = \frac{\rho_2(\mathbf{r}, \mathbf{r}')}{\rho(\mathbf{r})} - \rho(\mathbf{r}')$ , where $\rho_2(\mathbf{r}, \mathbf{r}')$ is the electron pair density	Fermi hole <sup>71,72</sup> expresses the decrease in probability of finding electron with spin $\sigma$ at a point $\mathbf{r}'$ while the reference electron of the same spin is at a position $\mathbf{r}$ . It measures spatial localization of the electron of given spin $\sigma$
Electron delocalization index	$\delta_{AB} = -\frac{1}{2} \int_{\Omega_A} d\mathbf{r} \int_{\Omega_B} d\mathbf{r}' \rho(\mathbf{r}) h_x(\mathbf{r}, \mathbf{r}')$	Electron delocalization index shows the number of electron pairs spreading out between basins of A and B atoms <sup>16,17</sup>
Delocalization tensor density (DTD)	$\mathbf{D}(\mathbf{r}) = \frac{1}{2} \langle \Psi_0   \{ \Delta \hat{\mathbf{Q}}(\mathbf{r}) \otimes \Delta \hat{\mathbf{R}} \}   \Psi_0 \rangle$ , where $\Psi_0$ is the ground-state many-electron wave function of a system; $\Delta \hat{\mathbf{R}} = \hat{\mathbf{R}} - \langle \mathbf{R} \rangle$ is the fluctuations of the total electron position operator, $\hat{\mathbf{R}} = \sum_i^N \hat{\mathbf{r}}_i$ , relative to its mean value $\langle \mathbf{R} \rangle$ ; $\hat{\mathbf{Q}}(\mathbf{r})$ is a local analog of $\hat{\mathbf{R}}$ (local electron position operator) <sup>64</sup>	DTD evaluates the contribution of spatial fluctuations of $\mathbf{Q}(\mathbf{r})$ at the point $\mathbf{r}$ to the total fluctuations of the center of masses $\mathbf{R}$ of electron subsystem. DTD expresses the contribution of small space volume near $\mathbf{r}$ to the total electron delocalization resulting from various physical effects (position quantum uncertainty of each particle; mutual correlations in electron motion, <i>etc</i> )
Condensed linear response kernel (CLRK)	$\chi_{A,B} = \int_{\Omega_A} d\mathbf{r} \int_{\Omega_B} d\mathbf{r}' \chi(\mathbf{r}, \mathbf{r}')$ , $\chi(\mathbf{r}, \mathbf{r}') = \frac{\delta \rho(\mathbf{r})}{\delta v(\mathbf{r}' )}$ is the linear response of electron density at point $\mathbf{r}$ to variation of external potential at point $\mathbf{r}'$ ; $\Omega_A$ and $\Omega_B$ are atomic basins of A and B atoms, respectively <sup>23</sup>	It measures propensity of electron density in the atomic basin, $\Omega_A$ , to flow towards atomic basin, $\Omega_B$ , induced by the difference in the electric potential between these basins. <sup>73</sup> CLRK reflects influence of the changes of the nuclei configuration in molecular systems to changes in electron density



transformations in the N-C<sub>4</sub>-C<sub>3</sub> fragment of the substrate (Fig. 1). The complexity of these processes assumes usage of diverse bonding descriptors to reveal the impact of electronic structure features and effects of surrounding on the chemical bonding and the rate constants of cephalosporin hydrolysis by the L1 MβL.

### 3.1. The proton transfer in terms of bonding characteristics

The significance of TS intramolecular N···H-O<sub>w</sub> hydrogen bond (see Fig. 1) in the cephalosporin hydrolysis is established in our recent studies.<sup>24–26</sup> We found that electron density, kinetic and potential energy densities, atomic source function and other electron-density based descriptors calculated at bcp of this H-bond correlate with the experimental hydrolysis rate constants,  $k_{\text{cat}}$ .<sup>24–26</sup> It allows us to consider this H-bond as a key atomic interaction responsible for reactivity of the substrates. At that, atomic source function, SF, and its relative form, SF%,<sup>11</sup> calculated at the N···H-O<sub>w</sub> bcp in TS complexes show the best correlation with  $k_{\text{cat}}$  values among other studied descriptors.<sup>24</sup> Therefore, we study more deeply the influence of atomic surrounding on the N···H-O<sub>w</sub> hydrogen bond by the analysis of atomic source function contributions to the  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$  from the entire active site in the TS complexes, see Table 3. The SF% contribution from all atoms of the substrate is being larger than 70%. The core of cephalosporin (all atoms in a cephalosporin molecule excluding R<sub>1</sub> and R<sub>2</sub> substituting groups) contributes to  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$  55–62% depending on the particular compound (Table 3) and the other part comes from the substituents R<sub>1</sub> and R<sub>2</sub> (Fig. 1). The impacts of the neighboring amino acids, water molecules and zinc cations are much lower and do not exceed 15% for each of these species. The atomic source contributions to  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$  for the same substituents (R<sub>1</sub> or R<sub>2</sub>) are similar for the compounds with different properties (Table S1†). This is in line with the idea of chemical transferability of the atomic groups: we may expect that similar groups will give similar electron density contributions to the  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$ .<sup>62,70</sup>

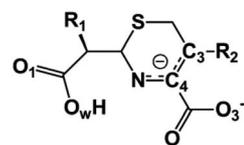
However, the N and O<sub>w</sub>H atomic fragments, which form the N···H-O<sub>w</sub> bond in TS complexes, are not chemically transferable, as is usually observed for hydrogen bonds of close length.<sup>11</sup> The electron density contributions to the  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$  from the N and O<sub>w</sub>H groups vary significantly for the substrates with different reactivity in spite of close N···H-O<sub>w</sub> bond lengths ( $\Delta R_{\text{N}\cdots\text{H}} = 0.2 \text{ \AA}$ ,  $\Delta R_{\text{N-O}_w} = 0.2 \text{ \AA}$ ). The difference between the highest and the lowest SF(O<sub>w</sub>H)% values amounted to 9.4%, the range for the SF(N)% is even larger and reaches 16.8%, see Table 3. This difference in SF values highlights the dissimilarities in the N···H-O<sub>w</sub> bond nature in the TS complexes of cephalosporins despite close N···H bond lengths.

To reveal the influence of delocalization of the lone pair of N atom (see Fig. 1) on the features of N···H-O<sub>w</sub> bonds in TS complexes and reactivity of the cephalosporins, we analyzed relative delocalization indices,  $\delta_{\text{N,H}}^{\text{rel}} = \frac{\delta_{\text{N,H}}}{\sum \delta_{\text{N},i} + \sum \delta_{\text{H},j}}$  (see ESI† for definition) (Fig. 2). The  $\delta_{\text{N,H}}^{\text{rel}}$  expresses the ratio of number of electrons delocalized between N and H atoms and all other atomic basins in the TS complexes. The higher  $\delta_{\text{N,H}}^{\text{rel}}$  value the more electrons are delocalized between N and H atoms. It was found, that  $\delta_{\text{N,H}}^{\text{rel}}$  indices in TS complexes correlate with the  $k_{\text{cat}}$  values (Fig. 2): cephalosporins with high  $k_{\text{cat}}$  values have less delocalized electrons along the N···H-O<sub>w</sub> bond than those with low  $k_{\text{cat}}$ . This indicates the role of N electron lone pair delocalization in the N···H fragment in the transition state on the rate of hydrolysis.

The interrelation of SF(N)% and  $\delta_{\text{N,H}}^{\text{rel}}$  with  $k_{\text{cat}}$  values originates from the internal link between these descriptors:<sup>11</sup> the atomic source function and delocalization indices values are complied with each other for similar systems. Other essential fact is that delocalization indices for some molecules correlate with the Lewis basicity constants.<sup>74</sup> From chemical viewpoint, correlation of  $\delta_{\text{N,H}}^{\text{rel}}$  with  $k_{\text{cat}}$  means that in the transition state of the limiting step substrates with low  $k_{\text{cat}}$  values (and high  $\delta_{\text{N,H}}^{\text{rel}}$  values) shows higher Lewis basicity than substrates with

**Table 3** The atomic source function contributions to the  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$  in the TS complexes. SF(core)% is total atomic source contribution of all atoms in a cephalosporin molecule excluding R<sub>1</sub> and R<sub>2</sub> substituting groups; SF(R<sub>1</sub>)% and SF(R<sub>2</sub>)% are atomic source contributions from R<sub>1</sub> and R<sub>2</sub> groups; SF(A<sub>i</sub>)% are main atomic source contributions at the  $\rho(r_{\text{b}})_{\text{N}\cdots\text{H}}$  of the TS complexes

				SF(A <sub>i</sub> )%							
	SF(core)%	SF(R <sub>1</sub> )%	SF(R <sub>2</sub> )%	S	C <sub>3</sub>	C <sub>4</sub>	N	H	O <sub>w</sub>	O <sub>1</sub>	O <sub>3</sub>
CFX	61.8	11.6	3.7	5.8	3.7	5.1	0.2	-11.4	31.4	15.2	6.9
CFS	67.5	6.9	2.8	6.0	2.6	5.0	0.8	-10.6	34.3	13.6	5.6
CF0	67.1	6.8	3.2	6.2	3.2	4.9	-0.8	-10.2	32.7	14.5	6.1
CFP	65.4	8.0	2.2	6.2	3.4	4.9	-0.1	-13.1	34.9	15.5	6.2
NCF	67.5	8.5	3.5	6.0	2.8	7.9	-2.1	-12.2	32.5	16.6	8.3
CFR	62.6	8.9	3.3	6.7	4.7	5.6	-6.0	-17.0	36.6	16.4	8.8
CF3	60.3	9.4	3.7	6.7	4.0	4.9	-6.8	-12.8	31.0	17.5	7.2
CFL	60.4	8.6	3.8	6.7	4.9	5.0	-8.6	-16.2	34.6	17.2	8.7
CFT	56.5	10.6	4.3	7.6	5.3	5.4	-14.8	-15.4	30.7	20.3	9.5
CFU	55.9	10.8	4.5	7.9	4.9	6.1	-16.0	-12.2	26.5	21.2	9.8



high  $k_{\text{cat}}$  values. Recalling the definition of basicity as capability of electron pair donation,<sup>75</sup> we conclude that interpretation in terms of Lewis basicity supports the observed features of N $\cdots$ H bonding.

Fermi hole (see Table 2) originates in Pauli exclusion principle and measures spatial localization of the electron with given spin relatively to a reference same-spin electron. Electron delocalization indices are expressed through the electron Fermi hole (see eqn (6) in ESI†).<sup>76</sup> Therefore, it is fruitful to compare the spatial details of the Fermi hole distribution in the region of the N $\cdots$ H–O<sub>w</sub> bond for the reference electron placed at the N $\cdots$ H bcp. Fig. 3 compares two substrates with high  $k_{\text{cat}}$  values (CFT and CFU), two substrates with low  $k_{\text{cat}}$  values (CFP and CFX) and two substrates with high and low  $k_{\text{cat}}$  values (CFU and CFX). The almost identical Fermi hole distribution near N atoms for substrates with close catalytic rate constants is remarkable. Isolines around N atom are close to each other both in CFU–CFT and CFP–CFX pairs (Fig. 3a and b). Fig. 3c demonstrates that the Fermi hole is more compact and locates around N atom for substrate with high  $k_{\text{cat}}$  value and is more extended along hydrogen bond for substrate with low  $k_{\text{cat}}$  values (Fig. 3c). Moreover, this behavior is also observed for all other considered complexes.

Fermi hole also provides information about electron pair localization. Expanded Fermi hole distribution in the N $\cdots$ H–O<sub>w</sub> bond for TS complexes of substrates with low  $k_{\text{cat}}$  values shows that electron lone pair of N atom stronger delocalize along N $\cdots$ H bond comparing with cephalosporins with high rate constants. One can say that proton transfer process is slow insofar as electron lone pair of N atom spreads towards H–O<sub>w</sub> fragment.

Thus, the reactivity of cephalosporins is controlled by the N electron lone pair delocalization in the N $\cdots$ H–O<sub>w</sub> fragment in the transition state of the limiting reaction step. The high delocalization of N electron lone pair towards H–O<sub>w</sub> group in the N $\cdots$ H–O<sub>w</sub> fragment makes hydrolysis of cephalosporins by L1 MβL slow. The Fermi hole analysis showed how electronic exchange in the N $\cdots$ H–O<sub>w</sub> fragment is responsible for different reactivity of cephalosporins when hydrolyzing by the L1 MβL.

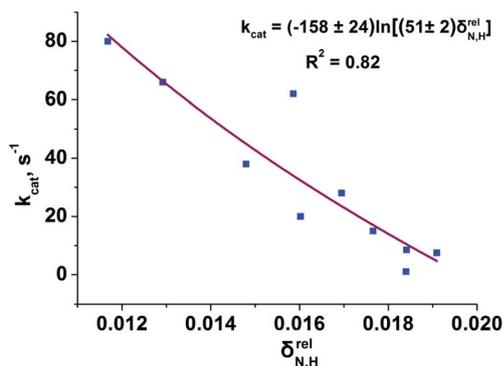


Fig. 2 Dependency of the experimental  $k_{\text{cat}}$  values on the relative delocalization indices,  $\delta_{\text{N,H}}^{\text{rel}}$ , for N $\cdots$ H–O<sub>w</sub> fragment in the set of TS complexes. The logarithmic function approximating the correlation prevents unphysical negative values of  $\delta_{\text{N,H}}^{\text{rel}}$ .

### 3.2. Analysis of the C<sub>3</sub>=C<sub>4</sub> double bond formation

The double bond formation between the C<sub>4</sub> and C<sub>3</sub> atoms is another significant phenomenon that occurs in the cephalosporins at the limiting stage (Fig. 1). To study the  $\pi$  character of C<sub>4</sub>–C<sub>3</sub> bond in the INT, TS and EP complexes, the QTAIM bond ellipticity indices,  $\varepsilon$ , along the C<sub>4</sub>–C<sub>3</sub> bond path were computed (see Fig. 4, 5 and S4†). Their remarkable asymmetry in case of INT complexes reflects the existence of carbanion,<sup>77</sup> caused by the delocalized electron lone pair between the N and C<sub>3</sub> atoms. This feature is preserved for TS structures of cephalosporins with low  $k_{\text{cat}}$  values. For substrates with high rate constants nearly bell-shaped  $\varepsilon$  profiles are observed (Fig. 4). In the EP complexes the bond ellipticity maxima ( $\varepsilon_{\text{max}}$ ) are close to the C<sub>4</sub> atoms. This shift in  $\varepsilon_{\text{max}}$  position signals about completion of double bond formation. The asymmetry of  $\varepsilon$  profiles for EP complexes are caused by chemically non-equivalent

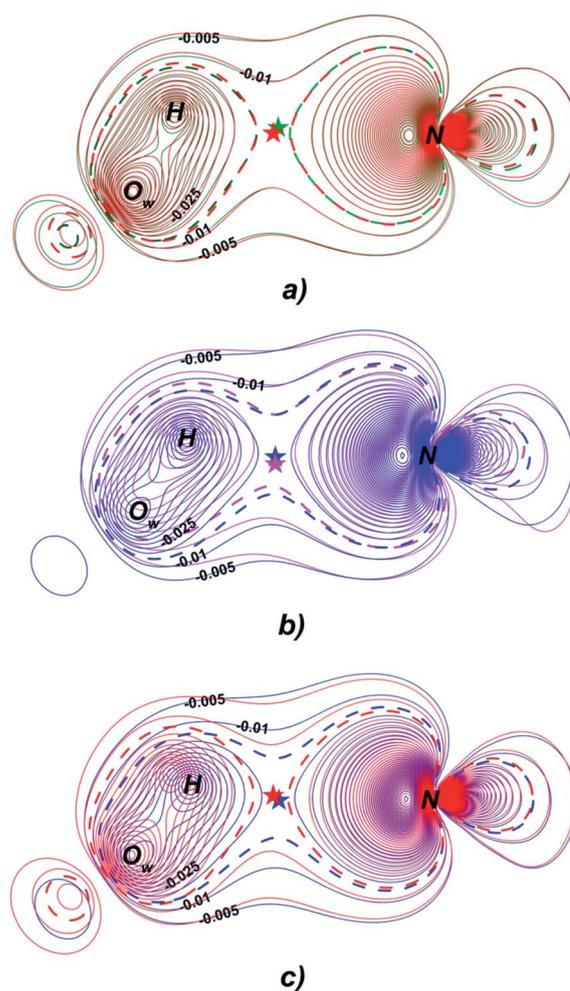


Fig. 3 Distribution of the Fermi holes pairwise laid one on another in the plane of N $\cdots$ H–O<sub>w</sub> bond for substrates with (a) high  $k_{\text{cat}}$  – CFT (green) and CFU (red); (b) low  $k_{\text{cat}}$  – CFP (pink) and CFX (blue); (c) high and low  $k_{\text{cat}}$  – CFU (red) and CFX (blue). In each case the reference electron is placed at N $\cdots$ H bcp (bcp positions are marked by stars). Isolines are in interval  $-0.4 \div 0.0$  a.u. with a 0.005 a.u. step. Bold dashed isolines correspond to the  $-0.015$  a.u. Distribution of the Fermi holes for the other cephalosporins is given in the ESI (Fig. S3†).



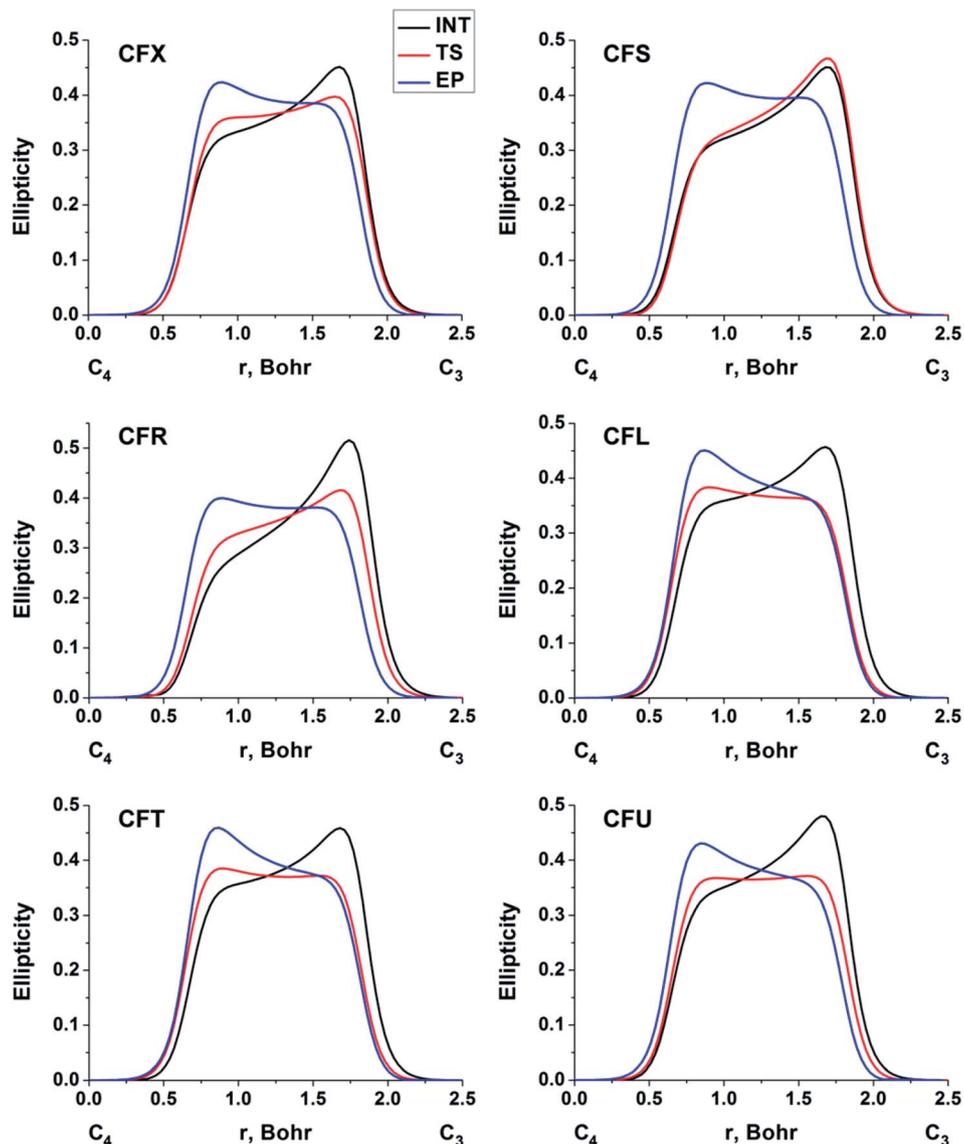


Fig. 4 The bond ellipticity index profiles along the  $C_4$ – $C_3$  bond path for the INT, TS and EP structures in case of substrates with low (CFX, CFS), medium (CFR) and high (CFL, CFT, CFU) rate constants. Profiles for the other cephalosporins are given in ESI (Fig. S4†).

substituting groups at the  $C_3$  and  $C_4$  atoms. The electron-withdrawing carboxylate group at the  $C_4$  atom provides the charge transfer to the  $C_4$  atomic basin.

We conclude that the significant feature of  $C_4=C_3$  bond formation for cephalosporins with high  $k_{cat}$  values is similarity of the bonds between  $C_3$  and  $C_4$  atoms in the TS and EP complexes. The opposite is true for substrates with low  $k_{cat}$  values:  $C_4$ – $C_3$  bond in the TS structures is similar to that in the INT complexes.

At the same time, the  $\varepsilon$  profiles in NCF considerably differ from the  $\varepsilon$  profiles of other considered compounds (Fig. 5a). NCF has long conjugated  $\pi$ -system in the  $R_2$  substituting group, see Table 1, Fig. 5b. This suggests dissimilar type of electron delocalization in the  $N$ – $C_4$ – $C_3$  fragment for NCF and other cephalosporins. Nevertheless, due to the semi-local nature of the ellipticity index, its profiles fail to manifest effects of

extensive electron delocalization in chemical bond picture. Therefore, to study electron delocalization in the  $N$ – $C_4$ – $C_3$  fragment in more detail and to reveal the influence of the conjugated bonds in  $R_2$  on the  $C_4$ – $C_3$  bond in the NCF complexes, we applied the electron delocalization tensor  $D$  (see the ESI,† eqn (7)–(9)),<sup>64</sup> which is a quantitative measure of uncertainty in electrons' positions and reflects various physical effects: from quantum uncertainty in position of each particle to mutual correlations in electron motion. The major eigenvalue of delocalization tensor density  $D(r)$ ,  $\lambda_1(r)$ , expresses delocalization magnitude along the direction of maximum electron delocalization in a system. A continuous domain in the distribution of  $\lambda_1(r)$  in the  $N$ – $C_4$ – $C_3$  area is the typical feature of INT and TS structures (at least at one side of 6-membered ring), see Fig. 6. EP complexes of substrates without conjugated bonds in  $R_2$  exhibit isolated domains in the  $C_4$ – $C_3$  and  $N$ – $C_4$  bond regions



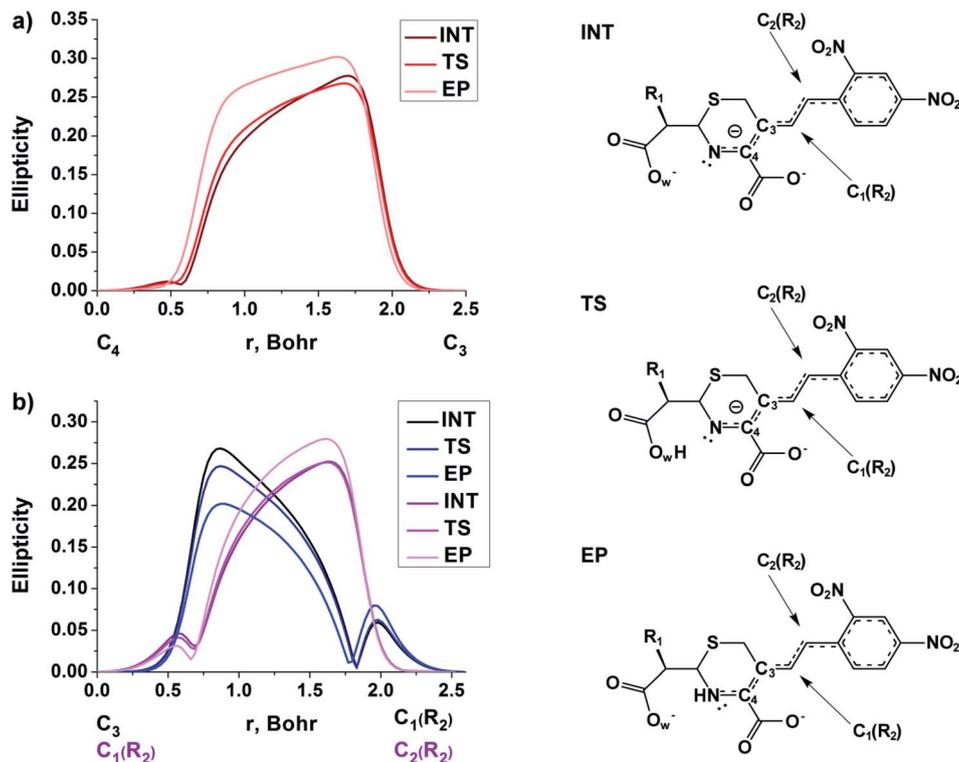


Fig. 5 The bond ellipticity profiles along the (a)  $C_4-C_3$ ,  $C_3-C_1(R_2)$  and  $C_1(R_2)-C_2(R_2)$ , (b) bond paths for the INT, TS and EP structures in case of NCF.

(Fig. 6, left panel). It signals about completion of double bond formation. In the EP structure of NCF the isolated domains in the distribution of  $\lambda_1(r)$  along conjugated system are absent. Delocalization of electrons gradually increases in the transition from INT to EP complexes (Fig. 6b). This feature may be compared to the changes in the bond ellipticity along the  $C_4-C_3$  bond paths for INT, TS and EP structures of NCF (Fig. 5a).

The influence of conjugated bonds in  $R_2$  in the NCF complexes on the  $C_4-C_3$  bond features was studied by the source function,  $G(r, r')$  (see ESI† for definition).<sup>11</sup> The  $C_4-C_3$  bcp was treated as a reference point. The source function local maxima at the  $C_1(R_2)-C_2(R_2)$  bond become higher for the EP structure (Fig. 7) in comparison with the TS complex. It illustrates increased influence of  $C_1(R_2)-C_2(R_2)$  chemical bond on the  $\rho(r_b)_{C_4-C_3}$  value. Therefore, the SF analysis in combination with delocalization tensor study of NCF complexes implies, that changes in electron delocalization in the  $C_1(R_2)-C_2(R_2)$  fragment predominantly affect features of  $C_4=C_3$  bond formation during the INT  $\rightarrow$  EP reaction step.

We conclude, that features of electron delocalization in the  $N-C_4-C_3$  fragment in cephalosporins plays pivotal role in the double  $C_4=C_3$  bond formation at the limiting step of reaction. They lead to differences in  $C_4-C_3$  bonds in the TS complexes for substrates with high and low  $k_{cat}$  values. First of all, these differences are reflected in the bond ellipticity profiles along  $C_4-C_3$  bond path. However, correct interpretation of  $\epsilon$  profiles demands the non-local bonding description. Delocalization tensor and source function revealed how electron delocalization

in the conjugated bond system of cephalosporins in the TS structures influences the reactivity of these compounds in the active site of the L1 M $\beta$ L.

### 3.3. Relationship between the proton transfer and the double bond formation

The linear response kernel (CLRK)  $\chi_{N,B}$  (where  $B = C_3, C_4$ , see eqn (11) in ESI†) for the  $N-C_4-C_3$  fragment in the TS complexes of cephalosporins allows to have a close look of the cumulative impact of the close and distant effects in the proton transfer and  $C_4=C_3$  double bond formation on the rate constants of cephalosporin hydrolysis by L1 M $\beta$ L. The CLRK matrix elements  $\chi_{N,B}$  (Fig. 8) reflect the response of electron density of N atom upon a perturbation in the external potential  $\delta\nu$  (*i.e.* infinitesimal nucleus shift) at one of the atoms of  $C_4-C_3-R_2$  fragment (*i.e.*  $B = C_4, C_3$  and atoms of the  $R_2$  substituting group) of cephalosporins in TS structures. The  $\chi_{N,C_3}$  and  $\chi_{N,C_4}$  values are higher for cephalosporins with low catalytic rate constants in comparison with the substrates with high  $k_{cat}$  values (Fig. 8a). Since linear response function is associated with electron delocalization in the molecular system,<sup>73</sup> one can conclude that higher  $\chi_{N,C_3}$  and  $\chi_{N,C_4}$  values indicate higher delocalization in the  $N-C_4-C_3$  fragment. In other words, high electron delocalization in the  $N-C_4-C_3$  fragment in the TS complexes leads to lower reactivity of cephalosporins. Recall that the N electron lone pair delocalization in the  $H\cdots N$  fragment also influences the rate constants of cephalosporin hydrolysis (see Section 3.2): the faster the



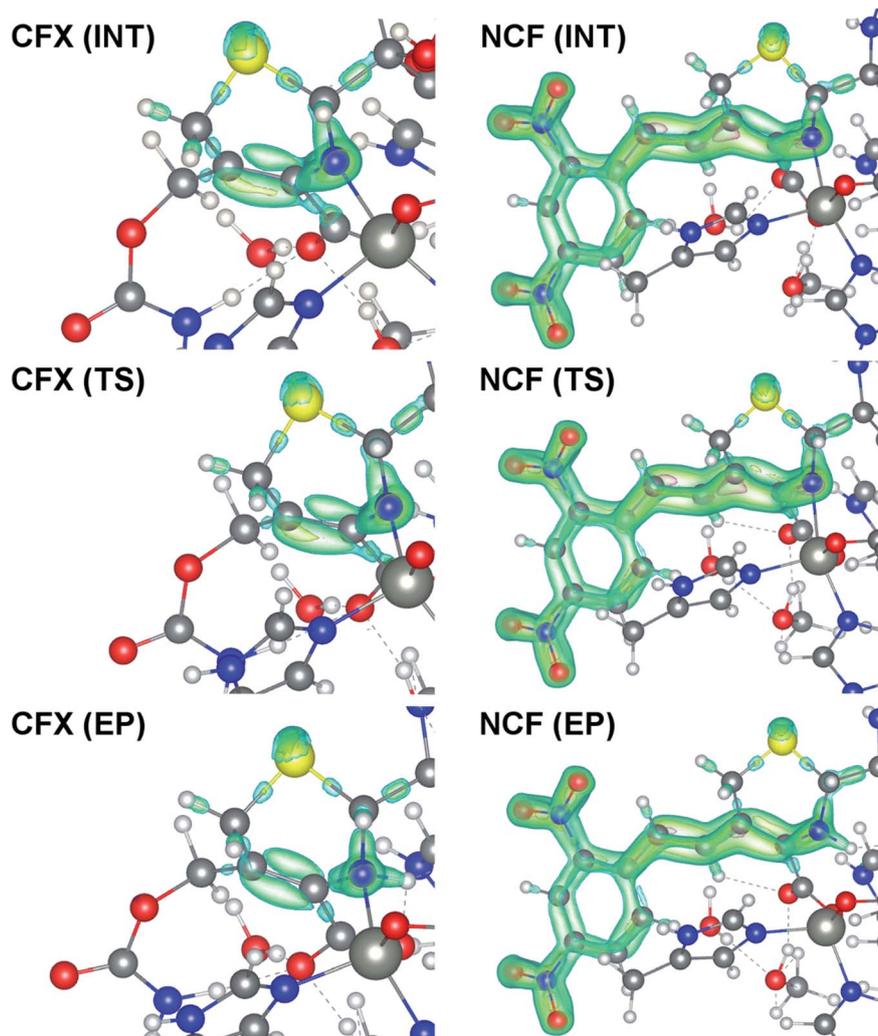


Fig. 6 Spatial distribution of major eigenvalue of delocalization tensor density  $D(r)$ ,  $\lambda_1(r)$ , for the INT, TS and EP complexes of CFX and NCF. Only domains in the 6-membered ring and  $R_2$  substituting group (for NCF) are plotted. The 0.28, 0.3, 0.4 and 0.6 isosurfaces are marked by blue, green, yellow and pink, respectively. The yellow, blue, red, gray and white spheres represent the S, N, O, C and H atoms, respectively. The  $Zn^{2+}$  ions are displayed as large grey circles.

proton transfer and double bond formation are, the smaller delocalization in the  $H\cdots N-C_4-C_3$  fragment in TS complexes of cephalosporins is.

Additionally, the CLRK analysis of the TS structure of NCF reveals influence of long conjugated bond system in the  $R_2$  substituting group on the delocalization of lone pair electrons of N atom. The maxima and minima positions at  $R_2$  correspond to the mesomeric active and passive atoms, respectively (Fig. 8b). This result supports conclusions obtained through delocalization tensor and source function analysis (Section 3.2).

To summarize, the delocalization of N atom lone pair along  $H\cdots N-C_4-C_3$  fragment governs the process of proton transfer in cephalosporin hydrolysis at the limiting step of reaction. The more lone pair localizes at N atom in TS complexes, the faster hydrolysis reaction is. On the contrary, high delocalization of lone electron pair of N atom in the  $H\cdots N-C_4-C_3$  fragment of transition state structures leads to the low rate constants of cephalosporin hydrolysis by L1 M $\beta$ L.

## 4. Conclusions

We demonstrated that the reaction mechanism in the active site of enzyme and the origin of different reactivity of a set of similar compounds can be deeply studied and interpreted using the QM/MM simulations followed by modern electron-density based bonding analysis. We considered the practically important reaction mechanism of hydrolysis of cephalosporin compounds by L1 metallo- $\beta$ -lactamase. The set of considered compounds has the same core and different substituents that affect their reactivity. We have already showed<sup>24</sup> that the temporary  $N\cdots H-O_w$  hydrogen bond formed in the transition state of limiting step of reaction in these enzymatic complexes acts as a key atomic interaction governing the reactivity of cephalosporins. Here we deepen the understanding of the reaction mechanism by analysis of the non-local real-space distributed bonding descriptors.



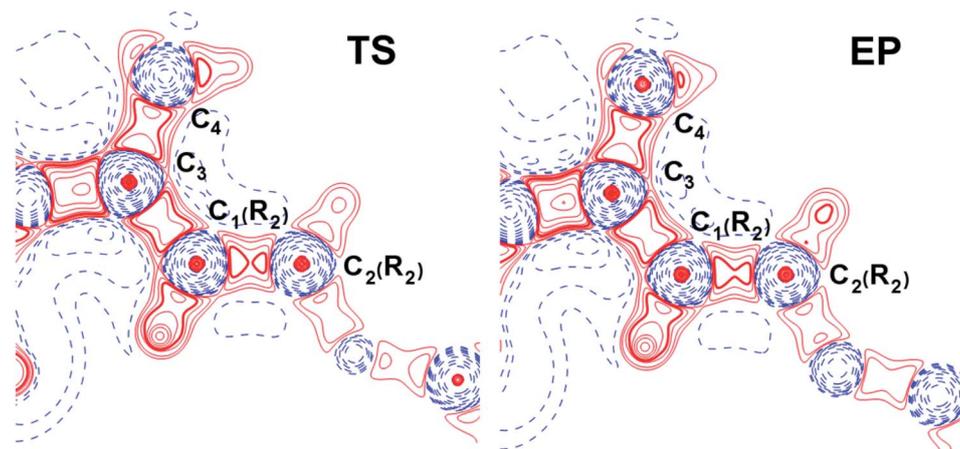


Fig. 7 The source function  $G(r,r')$  (see ESI† for definition) for TS and EP complexes of NCF plotted in the plane of  $C_3-C_1(R_2)-C_2(R_2)$  atoms. The  $C_4-C_3$  bcp was regarded as a reference point. The solid red and broken blue lines represent positive and negative contours, correspondingly. The contour lines range from  $-500$  to  $1200$  a.u. All contour lines are drawn at intervals of  $\pm 100$  a.u., except the additional 24 contours in range from  $-10$  to  $10$  a.u. (each new value is equal to the half of the previous one). The changes in the  $C_1(R_2)-C_2(R_2)$  bond region are marked by a bold line.

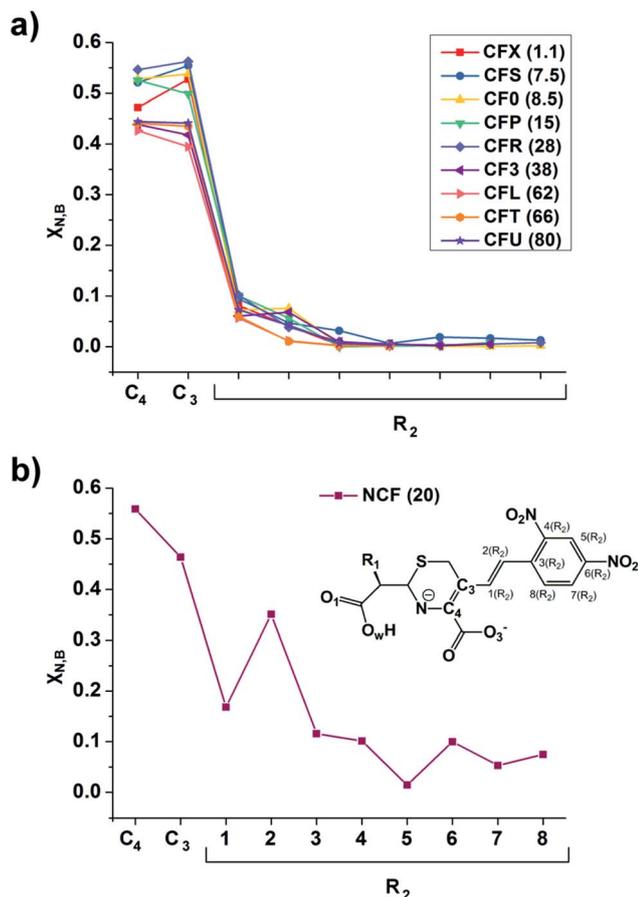


Fig. 8 The linear response elements  $\chi_{N,B}$  (see the ESI† eqn (11)) between the proton and various atoms of  $C_4-C_3-R_2$  fragment (see Fig. 1 and Table 1) of cephalosporins except NCF (a) and NCF (b) in the TS structures. The  $k_{cat}$  values for each of substrates are given in parenthesis.

Fermi hole and delocalization indices analysis demonstrate that exchange electron correlation in the  $N\cdots H-O_w$  fragment influence greatly the different reactivity of cephalosporins in the reaction with L1 M $\beta$ L. It is determined by different localization of electron lone pair at N atom for cephalosporins with high and low reaction rate constants. The less lone pair localizes at the N atom in transition state complexes, the slower hydrolysis reaction is. This is in line with the previously found dependency between the reactivity of compounds and the atomic influence to the electron density at the bond critical point of the  $N\cdots H-O_w$  hydrogen bond<sup>24</sup> as the source/influence function and delocalization indices are related descriptors.<sup>11</sup>

Analysis of the alternation of the  $N-C_4-C_3$  fragment along the limiting stage demonstrate the following features. The  $C_3-C_4$  bond at the transition state is similar to that of the preceding intermediate for the less reactive species and resembles the features of the enzyme-product complex for more reactive compounds. Bond critical point characteristics for thiazine ring of cephalosporins do not show correlation with  $k_{cat}$  values. At the same time, the electron delocalization in the  $N-C_4-C_3$  fragment of thiazine ring strongly affects hydrolytic activity of cephalosporins. It was established by delocalization tensor analysis combined with condensed linear response kernel study. We suppose that the applied protocol of bonding analysis has the general character and it is applicable to other enzymatic reactions.

## Conflicts of interest

There are no conflicts to declare.

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