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Orientation and structure-building role of the water molecules bound at the contact surface of the dihydrofolate reductase–methotrexate complex

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SUMMARY

Orientation of ten water molecules bound strongly at the contact surface of the dihydrofolate reductase–methotrexate enzyme–inhibitor complex was determined theoretically. To optimize the orientation of the water molecules, a recent method based on a simple electrostatic model was applied. The electrostatic complementarity in the binary complex was investigated using the lock-and-key model, considering the effect of the water molecules as well. The strongly bound water molecules improve the electrostatic fit in the pteridine region of methotrexate. Their role in the benzoic amide and γ-glutamate region is to decrease the internal energy by creating water bridges among remote polar sites making it possible to form H-bonds. Some modifications in the inhibitor structure were proposed for achieving greater inhibitor potency. The presumably enhanced effect is ascribed to the free energy gain in repelling the water molecules from the contact surface to the bulk of the solvent, and, in other cases, to internal energy decreases due to better electrostatic fit in the enzyme–inhibitor complex.

INTRODUCTION

The enzyme dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate, necessary for the biosynthesis of DNA. Since the inhibition of DHFR leads to stopping DNA replication, DHFR inhibitors may play a very important role in cancer therapy by controlling the cell-growth process.

There has been wide experimental and theoretical interest in investigating the DHFR–inhibitor interaction in the past decades. A general review up to 1984 is given by Blaney et al. [1]. Theoretical studies on this subject have been continuing also since that time using molecular graphics [2–8], calculating quantum-chemical structural parameters [9–17] or carrying out QSAR predictions on the inhibiting potency of non-tested compounds [6, 18, 19].

Theoretical investigations of the DHFR–inhibitor interactions are generally based on the experimentally determined or hypothetical geometric structure of the enzyme–inhibitor complex. The X-ray structure determined by Bolin et al. [20] and Filman et al. [21] was used for the novel calculations of the most extensively studied DHFR–MTX (methotrexate) complex (with or without the coenzyme). None of the models, however, took into consideration the presence of the strongly-bound water molecules at the contact surface between DHFR and MTX.

The water molecules near the interacting sites of an enzyme and a substrate, as well as the more loosely bound elements of the hydrate shell around the complex, play a fundamental role in defining the protein–substrate structure [22]. Due to the solvent environment, interaction energy, energy of protonation or deprotonation of the protein side chains, etc. differ considerably from the corresponding gas phase values [23,24]. The significance of the strongly bound water molecules in the enzyme–inhibitor models was also demonstrated in our earlier work [25,26]. The role of water molecules in structure building, and a method to theoretically determine their optimal orientation at the contact surface are the subjects of the present paper.

MODEL AND CALCULATIONS

In our previous calculations of the electrostatic fit between DHFR and MTX [27,28], the electrostatic lock-and-key model was used without taking into consideration the effect of the bound water molecules. In this paper, the molecular electrostatic potential [29–31] values are calculated in the reference points of MTX considering the effect of ten water molecules nearest to the pteridine, benzoic amide and γ-glutamate regions. The definition and numbering of the reference points are given in Ref. 28; for the sake of easy comparison, the same points are used here as well (Fig. 1). The Descartes coordinates for the DHFR–MTX... 10 H_2O system were taken from the refined X-ray structure by Filman et al. [32] published in the Protein Data Bank [33]. The structural values differ somewhat from the ones used by us in Refs. 27 and 28, so the potential values

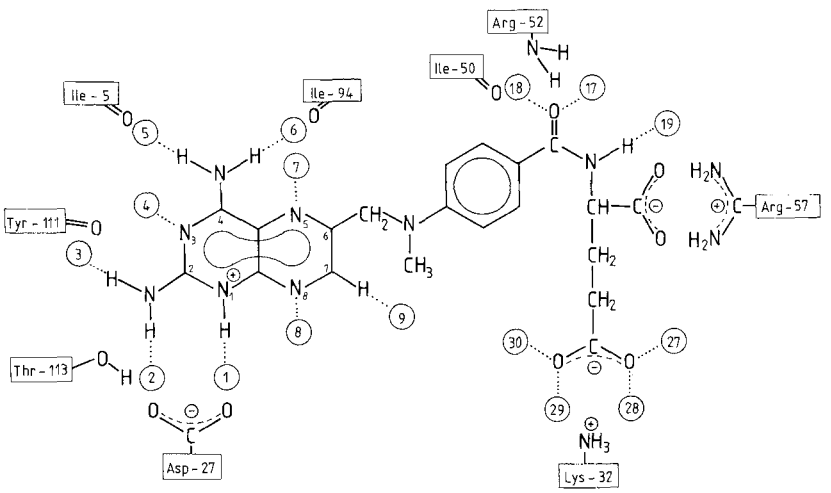

Fig. 1. The reference points (as defined in Ref. 28) in the investigated regions of MTX (numbers in circles) and the nearest amino-acid fragments of DHFR.

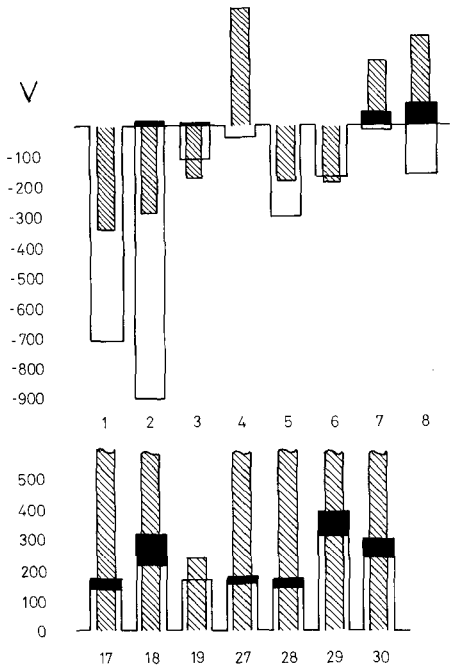

Fig. 2. The electrostatic potential (kJ/mol) pattern of DHFR (empty bars), MTX (hatched bars) and the potential contribution of the W603, W702, W639, W614, W685, W703 and W760 water molecules (full bars) in the reference points of MTX. The values are indicated with their correct sign for DHFR and water molecules, with opposite sign for MTX. Bars pointing into the same and opposite directions correspond to attractive and repulsive interaction, respectively, in the given point.

are recalculated here. We used the PROTPOT programming package [34–36] for generating hydrogen atoms and ionized side chains in the enzyme. Further details of the calculations and model are given in Ref. 28.

Applying the so-called potential key, the electrostatic fit is demonstrated by the potential patterns of DHFR and MTX (Fig. 2). The molecular electrostatic potential contributions of some water molecules are also indicated in this figure. However, to calculate these values, the orientations of the water molecules relative to their environment must be known. Since the hydrogen positions were not obtained from the X-ray data, their values had to be determined. Recently, a method was proposed by the author [37] to optimize the orientation of a water molecule with fixed oxygen position in polar environment. The method is based on a simple electrostatic approximation (justified by ab initio quantum chemical calculations) and involves the molecular electrostatic potentials of both the water molecule and its environment in special reference points. These points are defined in the directions of the O-H bonds and the oxygen lone pairs in the water molecule, 100 pm apart from the H and O atoms, respectively. By obtaining the minimum of F(Eq.1) as a function of the polar angles defining the orientation of the water molecule relative to the enzyme–inhibitor complex, the optimal position of the water molecule can be located.

$$F = \sum_{i=1}^{4} V_W(i) * V_{env}(i) \qquad (1)$$

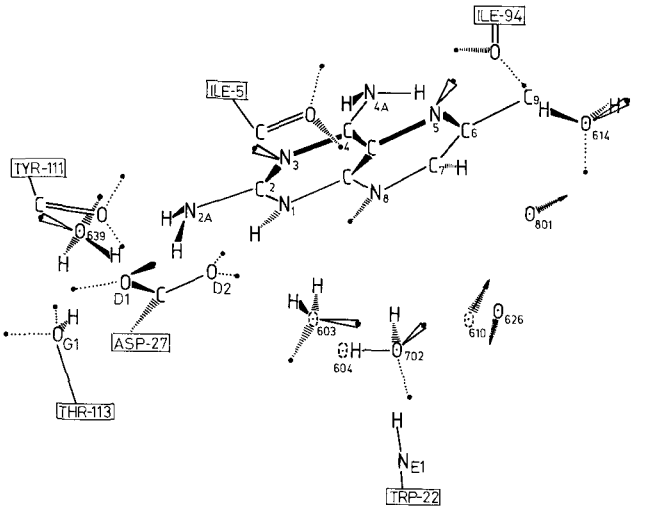

Fig. 3. The geometric arrangement of the DHFR–MTX complex in the pteridine region. Near amino-acid fragments and centres of eight water molecules (603–801) are also indicated. Circles with full or dashed lines at the water centres indicate the location of the O atom above and below the drawing plane, respectively. Arrows at 610, 626 and 801 show the orientations of the dipole vectors.

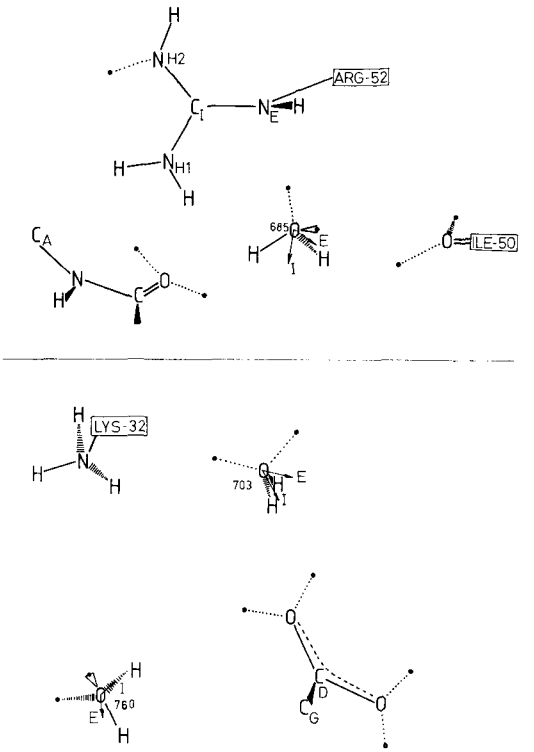

Fig. 4. Nearest environment of W685 (upper figure) and of W703 and W760 (lower figure) in the complex. Arrows E and I indicate the electric field generated by the enzyme and inhibitor itself, respectively, at the O sites of the water molecules.

where $V_W(i)$ and $V_{env}(i)$ are the molecular electrostatic potential values of the water molecule and the environment, respectively, in reference point i. The potential values were calculated in the framework of the bond-increment method [38–40] using localized molecular orbitals. The $V_W(i)$ values are -325 and 186 kJ/mol for the lone pair and the hydrogen regions, respectively, in the reference points of the water molecule. Their ratio is very similar to that found by Kollman and coworkers [41–43] who calculated the interaction energy of H-bonded complexes succesfully with a simple product of the molecular electrostatic potentials of the component molecules of dimers.

In investigating the effect of water on the electrostatic fit, the W603, W702, W610, W626, W639, W614 and W801 water molecules were considered in the pteridine region, W685 in the benzoic amide, and W703 and W760 in the γ-glutamate regions. The molecules were divided into two groups. Orientations of the six molecules nearest to MTX and the surrounding enzyme chains (see Table 1 and Figs. 3 and 4) were fully optimized, and the remaining ones were optimized at different levels.

At full optimization level (W603, W639, W614, W685, W703 and W760), the water molecules were rotated about three perpendicular axes. The F values were calculated using the water and the environment (DHFR + MTX) potentials. In the starting orientations of the water molecules, the directions of the dipole-moment vectors coincided with those of the electric field vectors generated by the environment at the oxygen sites. The molecular electric field calculated with the bond-increment method supplies good dipole orientation for the water molecule in a polar environment [40]. The contributions of DHFR and MTX to the total electric field, as well as the angles between the components and the resulting field vector are shown in Table 2. The minimum value of the F function and its separate contributions from DHFR and MTX are also collected here. Further-

TABLE 1
THE DISTANCES (R/pm) OF THE WATER (W) MOLECULES FROM SOME NEAR-LYING ATOMS OF THE ENZYME AND MTX AS WELL AS FROM THE O ATOMS OF THE NEIGHBOURING WATER MOLECULES

	MTX	R	Enzyme	R	Water	R
W603	$H_{(N1)}$	357	O_{D2}(Asp 27)	282		
	N_8	352	N_{E1}(Trp 22)	345	W702	271
W702	N_8	321	N_{E1}(Trp 22)	303	W610	438
					W626	264
W626	N_8	378			W610	564
					W603	515
W610	N_8	530			W603	377
W639	$N_{(2A)}$	290	O_{D1}(Asp 27)	421		
			O_{G1}(Thr 113)	286		
			O(Tyr 111)	303		
W614	N_5	398	O(Ile 94)	269	W801	230
W801	N_5	434				
W685	O	292	N_{H1}(Arg 52)	303		
			N_E(Arg 52)	286		
			O(Ile 50)	322		
W703	O_{E2}	345	N_Z(Lys 32)	359		
W760	O_{E2}	429	N_Z(Lys 32)	487		

TABLE 2
THE ABSOLUTE VALUE OF THE ELECTRIC FIELD (E, [V/nm]) AT THE O SITES OF THE WATER MOLECULES AND THE MINIMAL F VALUES OBTAINED AT THE ORIENTATION-OPTIMIZATION PROCESS

	$\|E_i\|$	$\|E_e\|$	$\|\Sigma E\|$	$\alpha(E_i E_e)$	$\alpha(E_e \Sigma E)$	F_i	F_e	ΣF	$\Delta\alpha$
W703	14.89	12.63	21.88	75°	41°	−8.3	−16.5	−24.8	30°
W603	4.49	17.74	15.08	132°	13°	+1.5	−15.2	−13.7	30°
W639	6.10	13.17	11.39	120°	28°	−3.8	−11.7	−15.5	0°
W685	12.86	9.84	8.76	137°	87°	−1.4	−3.5	− 4.9	21°
W614	2.73	9.38	7.14	150°	11°	+0.2	−14.1	−13.9	21°
W760	6.73	6.44	5.92	127°	66°	+5.6	−13.1	− 7.5	15°

Indices e and i refer to the enzyme and inhibitor components, respectively, symbol Σ refers to the sum of components. α is the angle between the field vectors given in brackets, $\Delta\alpha$ is the change of the dipole direction during the optimization

more, Table 2 contains the change in the dipole vector orientation ($\Delta\ \alpha$) obtained in the F-function process as compared to the simple electric field method.

For W702, the dipole direction was made to coincide with that of the electric field vector generated by DHFR + MTX plus the six water molecules whose orientations were previously determined at full optimization level. The optimal arrangement of W702 was obtained by minimizing the F function at the rotation about the dipole axis.

The binding energy of W603 (i.e., proportional with the electric field in the point-dipole approximation) is greater than that of W702 (Tables 2 and 3). This suggests that the binding of W603 precedes that of W702. The influence of the nearest neighbours on the resulting field at the oxygen sites of some water molecules studied is shown in Table 3. The considerable effect calculated explains why the orientations of the six most strongly bound water molecules had to be determined in a previous step.

In the case of W610, W626 and W801 that are water molecules farther from the contact surface, only the dipole orientations were determined considering also the effect of the neighbouring water molecules. The values of the resulting field are given in Table 3, and the dipole orientations are shown in Fig. 3.

The contribution of the W603, W702, W639, W614, W685, W703 and W760 molecules to the resulting potential at the reference points of MTX are indicated in Fig. 2. The contribution due to W610, W626 and W801 are discussed in the next section.

RESULTS AND DISCUSSION

The electrostatic pattern of DHFR and MTX in reference points 1–8 show some difference from our earlier results due to the somewhat different geometric structure of the complex.

The interaction is repulsive at points 4 and 7 and is attractive at point 3, contrary to previous results. Nevertheless, the repulsion is small at points 4 and 7 (see Fig. 2), and the same is valid at point 3 in the earlier work*. Thus, the results may be considered to be independent of the uncer-

*Due to an unfortunate error, half of the correct potential values are shown in Fig. 7 of Ref. 28.

TABLE 3
THE ELECTRIC FIELD OF THE ENVIRONMENT AT THE O SITES OF WATER MOLECULES ORIENTATED AT NON-FULL OPTIMIZATION LEVEL (SEE TEXT)

	$\lvert E_i \rvert$	$\lvert E_e \rvert$	$\lvert EE \rvert$	$\lvert + E_{W603} \rvert$	$\lvert + E_{W702} \rvert$	$\lvert + E_{W614} \rvert$
W702	5.09	10.40	6.64	8.02		
W626	6.12	3.32	5.15	5.34	8.10	
W610	3.06	1.49	4.31	3.14	3.33	
W801	3.20	8.22	5.59			8.28

tainties in the geometry if the calculated potential values are at least 60–80 kJ/mol. This is true for the other points (1, 2, 5, 6, 8, 17–19, and 27–30) where the qualitative features of the patterns are maintained as compared to the previous results.

The potential contributions of the bound water molecules should be added to that of DHFR if the recognition and binding of MTX by the solvated enzyme is investigated. The components of the total electric field and αs show that W603, W702, W639 and W614 are much more orientated by the enzyme than the inhibitor (Tables 2 and 3). (The changes in the dipole directions, $\triangle\alpha$, amounted to only 0–30° in the optimization process.)

The electric field of the enzyme is sufficiently high to locate these water molecules. (For the minimal $|\boldsymbol{E}|$ value necessary to locate a water molecule observable by X-ray crystallography, $\sim$ 4V/nm was found in the β-trypsin–BPTI complex [36].) On this basis, these water molecules may be thought to be bound to the enzyme alone, so they should be taken as elements of the environment.

The components of F reflect the relative weight of the enzyme and the inhibitor in orienting the W603, W639 and W614 molecules. The F_e components have strongly negative values indicating the favourable orientations to the enzyme in every case. The F_is are slightly negative or positive depending on the relative significance of the inhibitor in determining the water orientation. For W639, where the inhibitor electric field is sufficiently high to influence the final orientation, the F_i value is negative, indicating an orientation somewhat preferable also to the inhibitor. (The sign of the potential product corresponds to that of the interaction energy [42].) This is, however, at the expense of F_e, which is considerably less negative than in the case of W603 or W614 with a weak inhibitor field.

The presence of the water molecules improves the electrostatic fit in all points except 2 and 3. Here, the potentials of the water molecules slightly oppose the local potential of DHFR. This may be explained by the strong orientating effect of the Asp27 ionic carboxylic group (Fig. 3) resulting in an unfavourable interaction in the amine region. The hydration of the ionic side chain, however, probably compensates this slight energy loss. (The overall interaction is very attractive at both points.)

At point 7, the resulting potential of the environment (DHFR + water) is +31 kJ/mol, and having an opposite sign as compared to that of the single DHFR. Thus taking into account the potential contribution of the strongly bound water molecules, the interaction becomes attractive in the lone pair region of N_5 of MTX. If the effect of W610, W626 and W801 is also considered, the environmental electrostatic potential decreases to 17–29 kJ/mol. Due to the uncertainties in the model geometry and the approximations made, these values can hardly be considered of significance.

Nevertheless, the model suggests that the role of the strongly bound water molecules at and near the contact surface is to decrease the total internal energy of the system by improving the electrostatic fit between the inhibitor and its immediate environment.

This conclusion is in accordance with the results obtained at point 8 (N_8 lone pair). The potential contribution of the strongly bound water molecules is +69 kJ/mol, which decreases to 49–62 kJ/mol when W610, W626 and W801 are also taken into consideration. This range of values is still sufficient to considerably improve the fit at point 8, though the overall interaction remains repulsive.

In other regions (reference points 17–19 and 27–30), the role of the water molecules is less meaningful from the point of view discussed, since here even the enzyme–inhibitor interactions themselves are favourable. The binding of the water molecules at these sites (Fig. 4) should be explained with local factors.

In fact, despite the potential complementarity, the fitting of the electric fields is not satisfactory here [28]. The large electric field of MTX encounters relatively small values from the enzyme in the reference points. This is valid even for reference points 27–39, where oppositely charged groups are facing each other. These sites are, however, far from each other, explaining the calculated low field values of the enzyme at the reference points of MTX. It does not mean, of course, that the enzyme electric field would be small near to the (Lys32)-NH_3^+ group. A possible way of forming strong H-bonds is the mutual hydration of the side chains by W703 and W760. Similar arguments may also be used to explain the binding of W685. There is a crevice created by Arg52, Ile50 and the amide region of MTX at the formation of the complex. The H-bonding sites are generally far from each other, three H-bonds can be formed only by locating a water molecule.

The water molecules W685, W703 and W760 experience electric fields of similar magnitude from the enzyme and the inhibitor (Table 2). Where the angle of the two electric field vectors is relatively small (75°, W703), the optimal orientation is favourable for both the enzyme and the inhibitor, and the F components are highly negative. In the case of W685, the resulting orientation is rather unfavourable for both the elements of the complex, and the F components are of much less negative values. At W760, the positive F_i value indicates a very unfavourable orientation of the water molecule with respect to the inhibitor. This is mainly due to the repulsive interactions among the lone pairs in the γ-carboxylate of MTX and those of W760. The negative ΣF value reflects, however, an overall preference for this arrangement when considering the whole system.

It is probably not rare for a crevice created by polar groups to be filled by a water molecule. Two such cases were found in the present investigation, and a similar situation was encountered in Ref. 25. Common to all these examples is the distance of the polar groups and/or the unfavourable orientation of the bonds and lone pairs to form a H-bridge (Fig. 5). The water molecules are mobile elements of the complicated substructures having polar groups of much more restricted motion. The easier rotation and translation of the water molecules make possible their optimal arrangement relative to the environment in order to form favourable H-bonds.

The gain in the free energy of the system via the decrease of the internal energy is, however, partly balanced by entropy loss upon demobilizing a water molecule. Its return to the bulk of the solvent makes the solvation free energy at least 13 kJ/mol more negative [44]. It means that suitable modifications of the inhibitor structure to repel the strongly bound water molecules from the contact surface would result in inhibitors that could presumably bind to the enzyme more strongly than MTX.

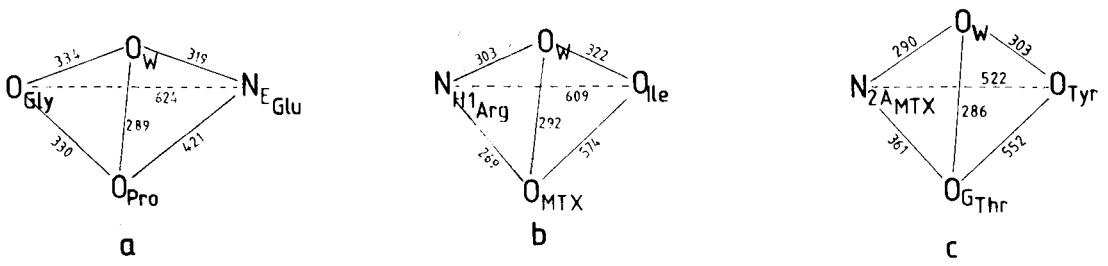

Fig. 5. Tetrahedrals of strongly bound water O atoms and the nearest heteroatoms capable of forming H-bonds. W403 (a, Ref. 25); W685 (b, this work); W639 (c, this work).

Incorporating a water molecule, like W685 in the present environment, into the inhibitor was outlined in Ref. 25. By replacing the carbonyl group in the benzoic amide region with a $\succ$CH-$(CH_2)_{1\text{-}2}$-OH group, the new side chain could repel W685, resulting in an overall 6 kJ/mol free energy gain estimated according to Andrews et al. [45]. Lengthening the γ-glutamate side chain with one or two CH_2 groups [28] would not only strengthen the (Lys32)NH_3^+...COO^- (MTX) bond, but also partly release the W703 and W760 molecules resulting in a gain in entropy. A similar effect may be expected by replacing the γ-glutamate carboxylic group with HO-$(CH_2)_{1\text{-}2}$-CH-$(CH_2)_{1\text{-}2}$-OH.

Further proposals for the modification of the inhibitor structure are based on the analysis of the role of W603-W801 molecules in the pteridine region. Replacing the N_3, N_5 and N_8 ring atoms by groups with a better electrostatic fit (e.g., CH) would make the binding stronger, as was also stressed by Cuthbertson and Richards [11].

The predictive power of the electrostatic lock-and-key model in modifying the inhibitor structure may be assessed by comparing the present conclusions with other theoretical and experimental results. Table 4 (taken from Blaney et al. [1] and references therein) shows the effects of the N to CH changes on the inhibitory potency of some 2,4-diaminopteridines. While the prediction concerning the effect of the N_3 change is only partly supported, the N_5 and N_8 replacements are generally preferred. An indirect support of the electrostatic lock-and-key concept comes from the N_8-NO replacement [47]. It results in much weaker binding (see below), in accordance with the considerably worse electrostatic fit near to reference point 8 (the N^+-O^- group is more repulsive to the negative enzyme environment, see Fig. 2).

By leaving off the glutamic acid in the inhibitor, the measured value of concentration for 50% inhibition [47] increased by two orders of magnitude in accordance with the loss of the ion-pair interactions among the carboxylate groups of MTX and the protonated side chains of DHFR (Fig. 1). Forming ethyl ester at the acidic sites and replacing N_8 of the pteridine ring by NO, the above concentration increased by a factor of 10^4 as compared to MTX. Here the loss of the ion-pair interactions was followed by an even worse electrostatic fit.

The methyl substitution at C_7 (reference point 9, Fig. 1) diminishes the binding energy according to both theoretical [13] and experimental [48] results. The measured ID_{50} increases by a factor of 10^4. While the positive DHFR and negative MTX potentials fit in this reference point (Ref. 28, recalculated in the present work), the methyl substituent makes the local potential of MTX less

TABLE 4
EFFECT OF N-CH SUBSTITUTION ON THE INHIBITORY POWER OF 2,4-DIAMINOPTERIDINE DERIVATIVES (FROM REF. 1 AND REFERENCES THEREIN)

K_i in mol, X = $CH_2N(CH_3)C_6H_4$, Y = X-4-CO-Glu

Position in pteridine			Subst.	pK_i	Source of DHFR
3	5	8	6		
N	N	N	CH_3	3.7	pigeon liver
CH	N	N	CH_3	4.0	"
N	N	N	XH	5.4	"
CH	N	N	XH	4.2	"
N	N	N	Y (MTX)	7.6–7.9	"
CH	N	N	Y	7.2	"
N	CH	CH	Y	7.8[a]	"
N	N	N	H	3.6	rat liver
N	CH	N	H	3.7	"
N	CH	CH	H	4.7	"
N	N	N	CH_3	4.1	"
N	CH	CH	CH_3	5.7	"
N	N	N	CH_3	5.3	M species 607
N	N	CH	CH_3	5.8	"
N	N	N	n-C_4H_9	9.0	"
N	N	CH	n-C_4H_9	7.1	"

[a]The measured pK_i value for MTX is 7.7, as published in Ref. 46.

negative, thereby weakening the binding. On the contrary, the fluoro substituent at C_7 was found favourable theoretically [12]. Similar predictions follow from the present method by considering the better fit of the more negative potential of the fluoro-substituted key to the positive potential of the enzyme lock.

CONCLUSIONS

A simple electrostatic model is useful in optimizing the orientation of structural water molecules in a polar environment, namely at the contact surface of the DHFR–MTX complex. Analysis of the role of the strongly bound water molecules revealed that there are at least two types of functions to be fulfilled by these molecules. They are orientated by their environment to improve the electrostatic fit between the elements of the enzyme–inhibitor complex. Filling in crevices created by the enzyme and/or the inhibitor, the water molecules form bridges among remote polar functions and the through-space interactions are supplemented with H-bonds, decreasing the total internal energy. Explaining the role of the water molecules in structure building provides ideas for modification of the inhibitor structure to attain stronger enzyme–inhibitor binding. Some applications of these hypotheses are shown; the predictions made are generally in agreement with the available experimental results.

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