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Changes in the Electron Density of the Cofactor NADPH on Binding to *E. coli* Dihydrofolate Reductase

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ABSTRACT **Quantum-mechanical electron density calculations reveal that a significant polarization is induced in the cofactor NADPH (reduced nicotinamide adenine dinucleotide phosphate) on binding to the enzyme dihydrofolate reductase. The calculations indicate that electron density corresponding to ~0.7 electron charges is shifted within the molecule, extending over more than 20 Å. Further calculations on proposed enzyme mutants show that the polarization of NADPH on binding to DHFR is, in large part, induced by a motif of three positively charged residues. This motif was also identified to be directly responsible for the positive electrostatic potential surrounding the cofactor binding site in the enzyme. The possibility of this long-range polarization of NADPH was originally proposed based on a previous study of ligand binding to DHFR where a conserved structural motif of three positively charged residues was found to play a major role in polarizing the substrate folate over its entire length of 18 Å.**

Key words: protein–ligand interactions, electrostatics, density functional theory, protein structure–function relationship

INTRODUCTION

Dihydrofolate reductase (DHFR) has been an extensively studied enzyme system in efforts to improve the understanding of protein-substrate and protein-drug interactions as well as enzyme catalysis.[1–10] DHFR catalyzes the NADPH-dependent reduction of folate to 7,8-dihydrofolate and of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate.[11] We are studying *Escherichia coli* DHFR (ecDHFR) in its ternary complex with the cofactor NADPH and folate using ab initio quantum mechanical techniques. Recently, it was shown that folate is significantly polarized when it binds to the enzyme.[12] A conserved structural motif of three positively charged amino acids, Arg-32, Lys-52, and Arg-57, was identified to play a major role in the observed substrate polarization. Difference electron density (between the bound and unbound substrates) calculations revealed an increase in σ and a decrease in π density upon binding in the 7–8 bond of the pteridine ring (the bond that is reduced when folate is converted to dihydrofolate). These changes in the electronic structure of the substrate may be involved in the reduction of the double bond that occurs during the enzymatic reaction. Equivalent effects were observed in dihydrofolate on binding to DHFR,[13] suggesting an explanation for the specific reaction sequence catalyzed by the enzyme. Electrostatic potential calculations using the Poisson–Boltzmann technique[14,15] have shown that the structural motifs formed by (1) Lys-32, Arg-52, Arg-57 and (2) Arg-44, Lys-76, Arg-98 are directly responsible for pockets of positive potential around the ligand binding sites for (a) folate and (b) NADPH. These pockets are formed within the overall negative electrostatic environment of the enzyme (ecDHFR carries a net charge of −10 at neutral pH).[16] The first motif has also been, as mentioned above, identified as a major cause of the long-range polarization of folate (this polarization extends over its entire length of 18 Å). Since the similar structural motif (2) (and the positive electrostatic potential induced by this motif) is found at the entrance to the NADPH binding site, the question arises as to whether NADPH undergoes an equivalent polarization upon binding to the enzyme. This is not at all obvious, considering the features of the NADPH molecule (schematic in Fig. 1a). This relatively large molecule, containing 74 atoms, has a length of over 25 Å in its binding conformation and has no extensive π electron system (which would favor electronic polarization effects). This study of NADPH also addresses the question of whether a direct correlation exists between local electrostatic enzyme potentials and ligand polarization effects in the DHFR system. We present the results of quan-

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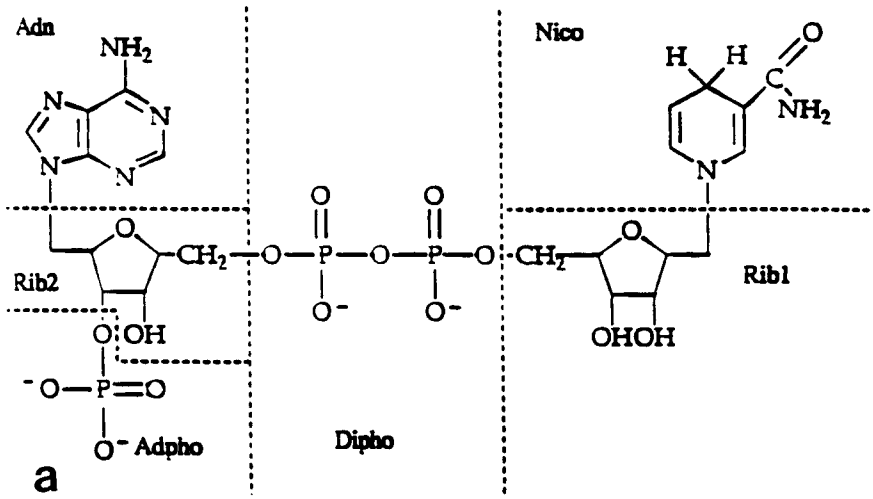
This paper is dedicated to the memory of Cyrus Levinthal.



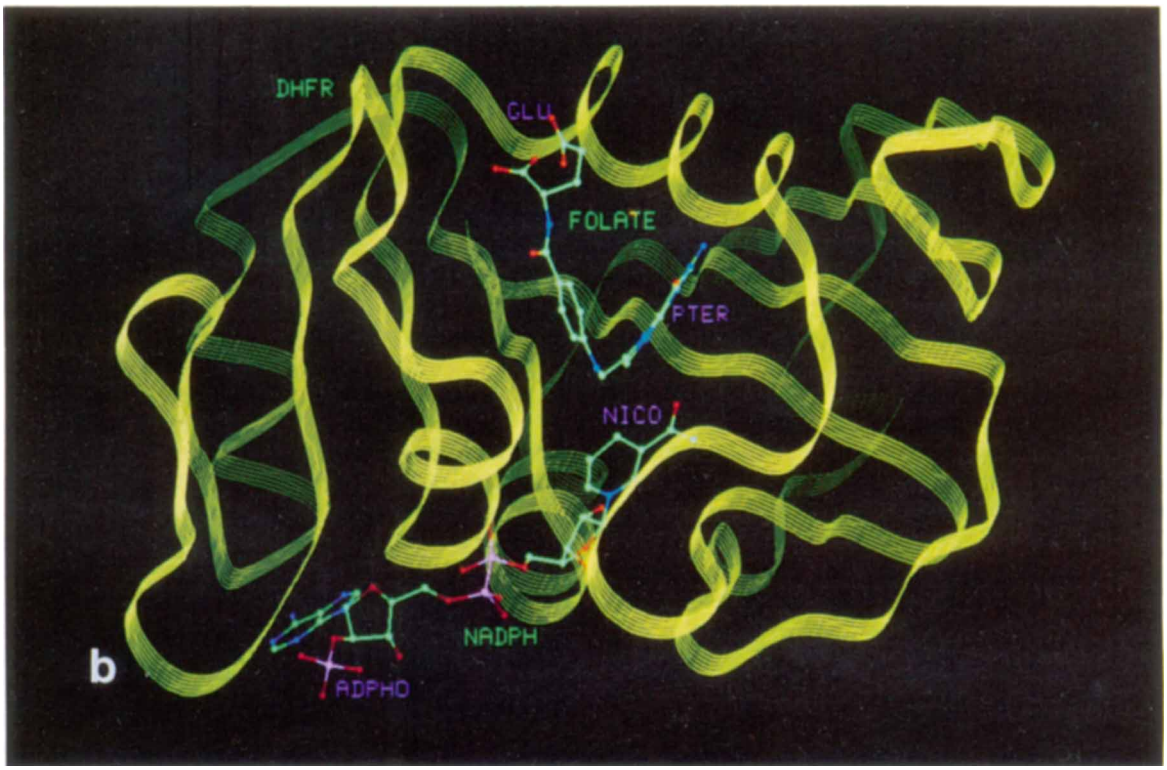


Fig. 1. **(a)** Schematic diagram of NADPH. The NADPH fragments are partitioned by dashed lines. Nico, nicotinamide; Rib1, ribose; Dipho, diphosphate; Rib2, ribose; Adpho, adenosine phosphate; Adn, adenine. This fragment scheme is used in the analysis of the charge migration in NADPH on binding to ecDHFR. **(b)** The ternary complex of ecDHFR (shown as a yellow ribbon), the substrate folate, and the cofactor NADPH. The pteridine (PTER) and glutamate (GLU) moieties in folate and the adenosine phosphate (ADPHO) and nicotinamide (NICO) moieties in NADPH are labeled.

tum mechanical calculations on the changes that take place in the electron density of NADPH on binding to wild-type ecDHFR and to enzyme mutants. Quantum mechanical treatment of molecules of the size of NADPH using conventional Hartree–Fock ab initio calculations,[17] is a very difficult, if even tractable problem, in terms of the computer time and disk space needed. As described below, we, therefore, need to employ alternative quantum mechanical techniques that are still accurate, but that are more efficient.

METHODS

Local density functional (LDF) theory is an ab initio electronic structure method,[18–20] originally developed in solid-state physics to study metallic

systems and, more recently, introduced in the field of chemistry.[21–24] Unlike Hartree–Fock calculations,[17] which express the energy of a molecular system as a function of the wavefunction, the LDF method expresses the energy as a function of the electron density and this results in significant computational advantages.[25] The total electron density of molecules with as many as 100 atoms can be obtained by minimizing the total electronic energy of the system until a self-consistent solution of the quantum mechanical calculation is obtained. We are employing the LDF method to explore changes in the electron density of ligands (substrates, cofactor, and inhibitors) on binding to DHFR. Electron density calculations on small molecules using the LDF method have been shown to be in good agreement with experimental data.[26–28] The calculations described here on the protein–ligand systems are carried out in three steps. First, the electron density of the isolated ligand in its binding conformation is calculated. Then, the electron density calculation of the ligand is repeated in the electrostatic field of the hydrated holoenzyme. Finally, the difference electron density (bound-isolated) is calculated and examined using computer graphics.[29] In these calculations, all atoms of the ligand are treated quantum mechanically, employing a basis set with polarization functions, equivalent in size to a Gaussian 6-31G** basis set. To mimic the electrostatic environment formed by the hydrated enzyme complex in the calculation of the bound ligand (NADPH in this case), the protein, the substrate folate, and the water molecules of the hydration shell are represented as point charges at the position of each of the atoms, including all hydrogens. The Coulombic potential arising from all point charges is then included in the Hamiltonian of the LDF calculation. The self-consistent LDF solution including this potential gives the electron density of the ligand when bound to the enzyme. Atomic charges for the ligand are calculated using a Mulliken population analysis.[30] To evaluate the influence of individual amino acids on the electron distribution of the ligands, enzyme mutants are constructed by replacing amino acids using computer graphics model building or by selectively setting the partial charges on all atoms of these amino acids to zero. In the LDF calculation, these mutations lead to a modified Coulombic potential in the effective Hamiltonian. To analyze the effects of these mutations, difference densities [bound(wild-type)−bound(mutant)] are calculated. For each of the quantum mechanical calculations on NADPH, ~10 hrs of computer time, depending on the convergence behavior, were needed on a CRAY Y-MP supercomputer. An extensive description of the computational details, including the vectorization procedure for the DMol program,[31] the LDF code used here, will be presented elsewhere.[32] The model system used for the calculations, the ecDHFR–folate–NADPH ternary complex (Fig. 1b), was derived from the crystal structure of ecDHFR–folate–$NADP^+$[33] as described previously.[12] The entire enzyme complex is surrounded by a 5 Å hydration shell, equilibrated by molecular dynamics,[34] in order to provide a solution-like environment. NADPH was modeled based on its crystal structure in the ecDHFR complex[33] by adding hydrogen atoms, the positions of which are not experimentally available, to the molecule and refining these positions using energy minimization, during which the nonhydrogen atoms of NADPH were held fixed.[35,36]

RESULTS AND DISCUSSION

Long-Range Polarization of NADPH on Binding to the Enzyme

The NADPH difference electron density (bound vs. unbound) calculation reveals, as previously observed for folate,[12] a clear polarization of the cofactor NADPH, induced by the enzyme (Fig. 2). Small electron density changes occur on several fragments of the NADPH molecule (Fig. 2a). Very significant changes, however, take place at the adenosine phosphate group (which becomes more negative, i.e. accepts charge density) and the nicotinamide moiety (which becomes more positive, i.e., loses charge density), in which the amide group is most affected. The distance between these two fragments is approximately 21 Å. This illustrates the long-range character of the observed polarization. Table I gives the corresponding Mulliken charge population analysis for bound and unbound NADPH. The calculation of the partial charges, summed over the different NADPH fragments, confirms the significance of the calculated effect. The adenosine phosphate group gains ~0.4 and the diphosphate group gains ~0.2 electron charges on binding, the nicotinamide ring, ~21 Å away, loses ~0.5 and the adjacent ribose loses ~0.2 electron charges. Ribose 2 and the adenine ring are largely unaffected. This polarization has a comparable magnitude to that observed for folate[12] (and dihydrofolate[13]) where a migration of ~0.6 (~0.5) electron charges over 18 Å was found. The magnitude of the polarization would be expected to be reduced once repolarization effects on protein atoms are included. Repolarization of protein atoms will not, however, alter the qualitative effects observed here.

Short-Range Electrostatic Interactions

Short-range electrostatic contacts play an important role in enhancing the protein-ligand interactions in order to compensate for the energy needed to polarize NADPH upon binding. The dominant short-range interaction between ecDHFR and NADPH is the strong salt bridge between the adenosine phosphate group in NADPH and Arg-44 (which has a length of ~2.7 Å). The adenosine phosphate group was the most significantly affected in terms of a gain

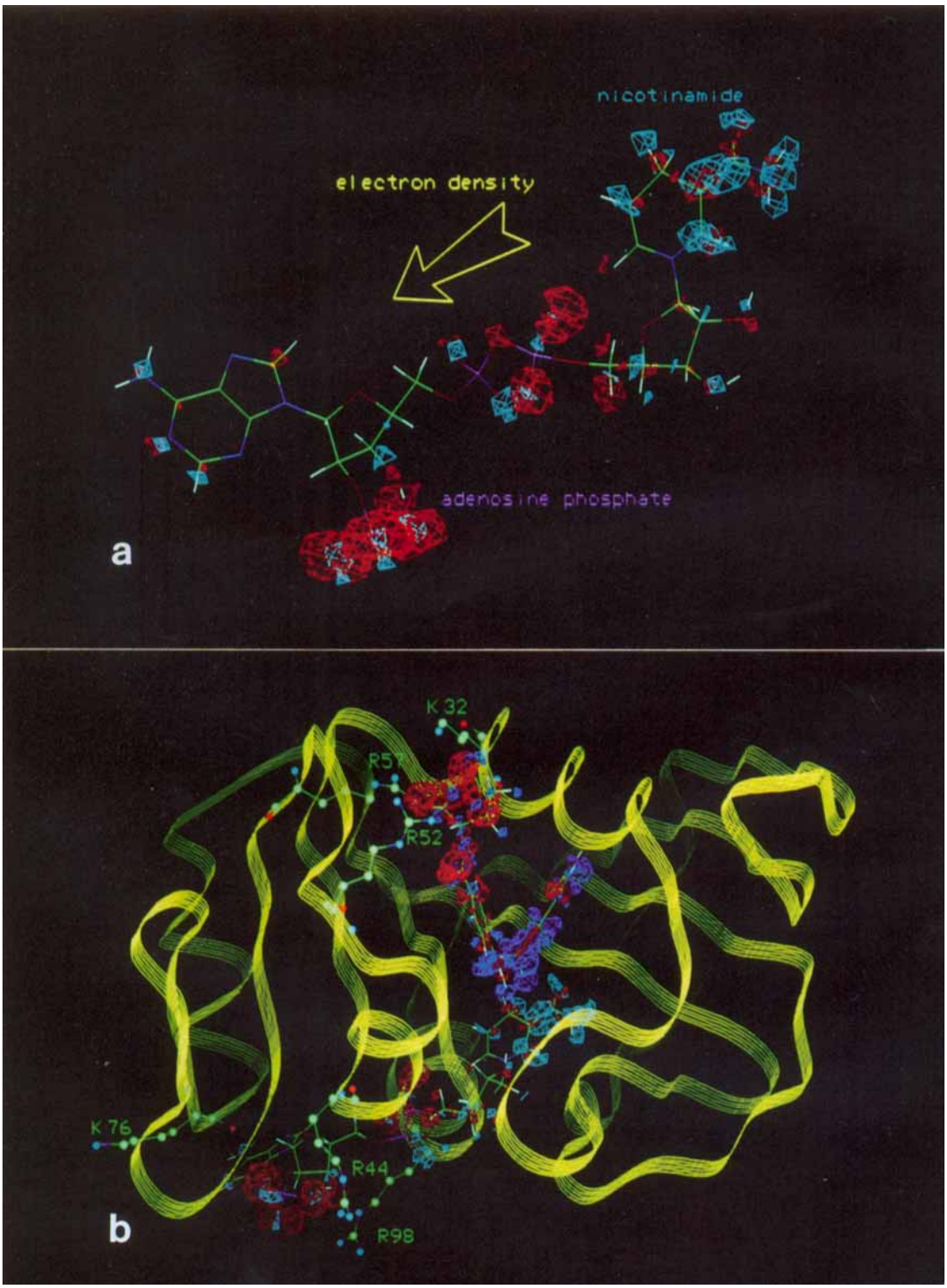


Fig. 2. **(a)** Migration of electrons in NADPH, induced by binding to ecDHFR. The change in charge is shown by difference electron density contours (bound−unbound) at two levels: 0.014 electrons/Å^3 (e/Å^3) (red) (i.e., these regions gain electrons and become more negative upon binding); and −0.014 e/Å^3 (blue) (these regions become more positive upon binding). The difference electron density contours are superimposed on NADPH in its binding conformation. This figure illustrates the long-range polarization effect that is induced in NADPH on binding to the enzyme. As can be clearly seen, the nicotinamide moiety and the adenosine phosphate group are the most significantly affected regions of the NADPH molecule. The nicotinamide moiety loses electron density corresponding to ~0.5 electron charges whereas the adenosine phosphate group, which forms a salt bridge to Arg-44, gains 0.4 electron charges. The polarization effect takes place over a distance of ~21 Å. **(b)** The difference electronic densities (bound-unbound) of NADPH and folate[12] are shown for the ligands bound to ecDHFR. The contour levels are as described in **a.** It should be noted that the polarization of the ligands is in the direction of the two clusters of positively charged residues that are located at the entrances to the ligand binding sites (residues 32, 52, and 57 at the folate site and residues 44, 76, and 98 at the NADPH site). These motifs are responsible for the positive electrostatic potential "pockets" around the ligand binding sites in ecDHFR[16] and play a major role in polarizing the ligands.

TABLE I. Influence of the Enzyme Environment on the Charge Distribution Within NADPH*

Fragment†	Unbound charge‡	Wild type		R^{44}, K^{76}, R^{98}		$R^{44} \rightarrow L$		Long-Range	
		Charge§	ΔCharge**	Charge††	ΔCharge**	Charge‡‡	ΔCharge**	Charge§§	ΔCharge**
Nico	−0.73	−0.22	0.51	−0.40	0.33	−0.24	0.49	−0.59	0.14
Rib1	0.12	0.37	0.25	0.29	0.17	0.38	0.26	0.28	0.16
Dipho	−1.86	−2.10	−0.24	−2.07	−0.21	−2.10	−0.24	−1.84	0.02
Rib2	0.42	0.38	−0.04	0.47	0.05	0.43	0.01	0.34	−0.07
Adpho	−1.52	−1.96	−0.44	−1.73	−0.21	−1.79	−0.27	−1.56	−0.11
Aden	−0.43	−0.47	−0.04	−0.56	−0.13	−0.68	−0.25	−0.63	−0.20

*The charges of the fragments were determined by summing the charges on the constituent atoms (determined by a Mulliken population analysis[30]).

†Nico, nicotinamide; Rib1, ribose; Dipho, diphosphate; Rib2, ribose; Adpho, adenosine phosphate; Aden, adenosine (see Fig. 1a).

‡These are the charges for unbound NADPH.

§The charges for NADPH bound to the wild-type enzyme.

**The difference in charge (bound−unbound) caused by binding.

††The charges for NADPH bound to ecDHFR in which the charges on the atoms of R^{44}, K^{76}, and R^{98} have been set to zero.

‡‡The charges for NADPH bound to the $R^{44} \rightarrow L$ mutant.

§§The charges for NADPH bound to ecDHFR in which the charges have been set to zero on all atoms of residues that have at least one atom within 3.5 Å of the cofactor. The charges on the water and substrate atoms have also been omitted from this calculation.

of electron density upon binding (Fig. 2b). Calculations of the electrostatic interaction energy[35] between this group, consisting of one phosphorus and four oxygen atoms, and all atoms of residue Arg-44, assuming a dielectric constant of 3 for the protein environment,[14,37] show that the polarization of the adenosine phosphate group results in enhanced electrostatic interactions with Arg-44 of ~14 kcal/mol.

The Role of the Structural Motif Arg-44, Lys-70, Arg-98

In order to evaluate whether, as hypothesized, the three positively charged residues at positions 44, 70, and 98 in ecDHFR (which are directly responsible for the positive electrostatic potential around the NADPH binding site) are a major factor in polarizing the cofactor, calculations on the "electrostatic triple mutant" (with the charges switched off on these three residues) were carried out and compared to the results obtained for the wildtype enzyme. Table I gives the results of this comparison. It is seen that the polarization of NADPH is significantly reduced when the charges on these three residues are not included in the calculation of the density of enzyme-bound NADPH. The polarization (loss of electron density) of the nicotinamide moiety, more than 20 Å away from the structural motif, is reduced by ~36%, demonstrating the significant influence of these three charged residues on the long-range polarization of NADPH. A difference electron density map [bound(wild-type)−bound(mutant)], illustrating these effects, is shown in Figure 3a.

Correlation of Electrostatic Enzyme Potentials and Ligand Polarization

The role of the structural motif Arg-44, Lys-76, Arg-98 in the polarization of the cofactor NADPH is very similar to the role of the structural motif Lys-32, Arg-52, Arg-57 in the polarization of folate,[12] as described previously. Both structural motifs have been identified, through analysis of the electrostatic potential of ecDHFR using the Poisson–Boltzmann method, as being responsible for the positive potentials around the binding sites in ecDHFR that enable the negatively charged cofactor (charge −4) and substrate (charge −2) to bind to the net negatively charged enzyme (charge −10).[16] We have now, therefore, obtained a consistent picture of enzyme electrostatics and long-range polarization effects in the ligands upon binding. The results suggest a direct correlation of the electrostatic potential and polarization effects.

Sequence Comparison of DHFRs and the Role of Arg-44

A sequence alignment of DHFRs from various species[38] shows that Lys-76 and Arg-98 are not conserved in bacterial and vertebrate DHFRs. We, therefore, cannot conclude that the structural motif 44, 76, 98 is equivalent to the more conserved motif 32, 52, 57 at the entrance of the substrate binding site in DHFR (which is postulated to be important for the catalytic function of DHFR[16]). Arg-44, however, is strictly conserved among all bacterial DHFRs and corresponds to Lys-46 in the vertebrate enzymes. This amino acid also forms a strong salt-bridge to the adenosine phosphate group of NADPH—the strongest enzyme-cofactor contact. These observations imply a key role for Arg^{44} within the structural motif 44, 76, 98 and suggest the study of DHFR mutants at position 44.

ecDHFR Mutants at Position 44

Experimental data for mutants at position 44 are available. For example, the mutant Arg-44 → Leu shows reduced catalytic activity,[39] implying a desta-

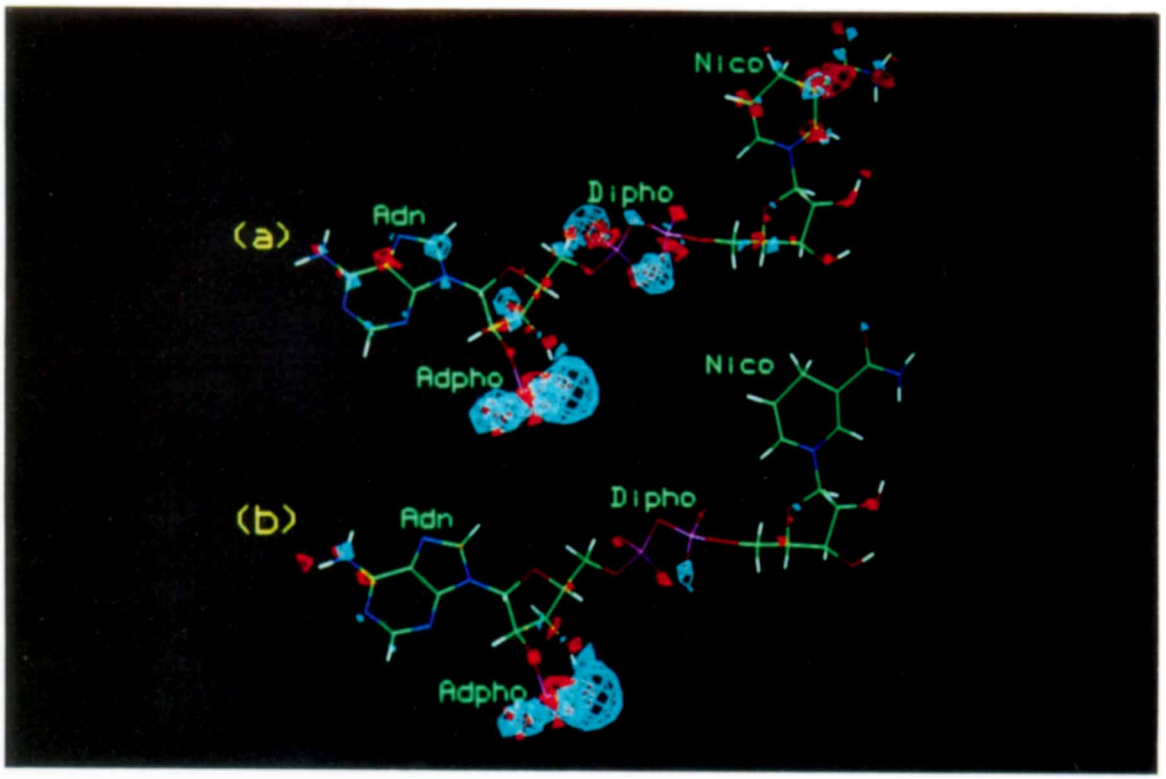


Fig. 3. Difference electron densities of NADPH bound to wild-type DHFR and "electrostatic" enzyme mutants. In **a,** the difference electron density of NADPH bound to ecDHFR with Arg-44, Lys-76, and Arg-98 uncharged relative to the density of NADPH bound to fully charged (wild-type) ecDHFR [i.e., bound (wildtype) − bound (mutant)] is shown superimposed on NADPH in its binding conformation. Contours are shown at levels of 0.007 e/Å^3 (red) and −0.007 e/Å^3 (blue) (i.e., at 50% of the contour levels shown in Fig. 2). These electron densities illustrate the repolarization within the NADPH molecule that is a consequence of the absence of electrostatic interactions with the structural motif of residues 44, 76, and 98 and reveals the major role of this motif in the polarization of NADPH by ecDHFR. The influence of these charges extends to the nicotinamide moiety in NADPH, which regains charge density in the mutant. The adenosine phosphate group becomes significantly more positive, relative to NADPH bound to the wild-type enzyme, in the absence of electrostatic interactions with the positively charged residues at positions 44, 76, and 98. In **b,** difference density, equivalent to that shown in **a,** but now comparing the electron redistribution in NADPH on binding to the wild-type enzyme and the ecDHFR mutant Arg-44 → Leu. The conserved residue Arg-44 forms a salt bridge to NADPH and is the "key" residue of the structural motif 44, 76, 98. The difference electron density shows, however, that changes in the electronic structure of bound NADPH, induced by the absence of Arg-44, do not significantly change the electronic structure of the nicotinamide moiety ~20 Å away. We can, therefore, conclude that Arg-44 does not contribute to the function of the enzyme via electronic structure changes in the cofactor. The presence or absence of the electrostatic influence of Arg-44 alone induces a significant, but local, electron density effect in the substrate that affects mainly the adenosine phosphate group and the adjacent adenine ring in NADPH. The overall effect is, as would be expected, reduced relative to the effects induced by three positive residues, illustrated in **a.**

bilization of the transition state of the enzymatic reaction. This indicates that position 44 is a functionally important site in DHFR. In order to evaluate if electronic structure effects are responsible for this decrease in catalytic activity, we constructed a model of the mutant Arg-44 → Leu. This was accomplished with only slight changes in neighboring side chain orientations and the repositioning of two water molecules. We therefore assumed that the binding conformation of NADPH is not affected by this mutation and that no significant structural changes are induced in the enzyme and we calculated the difference electron density for NADPH bound to this mutant relative to unbound and wild-type bound NADPH. These calculations, therefore, evaluate the electrostatic contribution of Arg-44 since, by this mutation, this charged residue is replaced by a nonpolar one. The results, given in Figure 3b and Table I, show that, as expected, strong local electron density changes are induced due to this mutation, predominantly at the adenosine phosphate group and the adjacent adenine moiety. The nicotinamide moiety, locus of the hydride transfer reaction, however, remains virtually unaffected. We would postulate, based on this result, that electronic structure effects in NADPH would not be responsible for the observed[39] changes in the enzymatic reaction caused by mutations at the (conserved) position 44. It is hypothesized, therefore, that Arg-44 mutants may induce structural changes into the enzyme or NADPH which could lead to altered activity, for example, by changing the NADPH binding conformation rela-

tive to the substrates folate or dihydrofolate. Conformational changes as a consequence of this mutation have been suggested based on kinetic data.[39]

Long-Range Electrostatic Interactions

In order to evaluate the influence of longer range electrostatic interactions[40,41] between the enzyme and the cofactor on the electron distribution of NADPH, additional LDF calculations of bound NADPH were carried out. In these calculations, all charges of protein residues with at least one atom within 3.5 Å of the cofactor were set to zero. In order to include only protein–cofactor interactions, waters and the substrate were omitted. The results, given in Table I, show that these long-range electrostatic interactions contribute to ~20–30% of the polarization of NADPH. A similar influence of longer range electrostatic interactions, with comparable magnitude, was found for folate on binding to DHFR.[12] These results illustrate the significant influence of the overall electrostatic field of the enzyme on the electronic structure of the ligands.

CONCLUSIONS

The cofactor NADPH undergoes a significant, and long-range, polarization on binding to DHFR. Long-range electrostatic interactions contribute to ~20–30% of the overall polarization of the cofactor. The structural motif Arg-44, Lys-76, Arg-98, shown to be directly responsible for the positive electrostatic potential around the NADPH binding site in DHFR, contributes to ~35% of the polarization of NADPH and, therefore, plays a comparable role in enzyme–cofactor interactions as does the conserved structural motif Lys-32, Arg-52, Arg-57 in the enzyme–substrate interactions in DHFR. The studies confirm a direct correlation of electrostatic enzyme potentials and ligand polarization in the *E. coli* DHFR system, revealing a novel aspect of the structure–function relationship. Arg-44 is the only conserved amino acid within the structural motif 44, 76, 98 and, as our calculations on enzyme mutants suggest, is not likely to play a role in DHFR function via induced changes in the electronic structure of the cofactor. This conserved amino acid, however, is a key factor in NADPH binding, forming a strong salt bridge to the cofactor. The polarization of NADPH leads to significantly enhanced short-range electrostatic interactions between Arg-44 and the adenosine phosphate group of the cofactor. These enhanced electrostatic interactions, may, in consequence, significantly stabilize the particular binding conformation of NADPH in the fully active enzyme.

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