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ORIGINAL PAPER

Structural studies of shikimate dehydrogenase from *Bacillus anthracis* complexed with cofactor NADP

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Abstract *Bacillus anthracis* has been employed as an agent of bioterrorism, with high mortality, despite anti-microbial treatment, which strongly indicates the need of new drugs to treat anthrax. Shikimate pathway is a seven step biosynthetic route which generates chorismic acid from phosphoenol pyruvate and erythrose-4-phosphate. Chorismic acid is the major branch point in the synthesis of aromatic amino acids, ubiquinone, and secondary metabolites. The shikimate pathway is essential for many pathological organisms, whereas it is absent in mammals. Therefore, these enzymes are potential targets for the development of nontoxic antimicrobial agents and herbicides and have been submitted to intensive structural studies. The forth enzyme of this pathway is responsible for the conversion of dehydroshikimate to shikimate in the presence of NADP. In order to pave the way for structural and functional efforts toward development of new antimicrobials we describe the molecular modeling of shikimate dehydrogenase from *Bacillus anthracis* complexed with the cofactor NADP. This study was able to identify the main residues of the NADP binding site responsible for ligand affinities. This structural study can be used in the design of more specific drugs against infectious diseases.

Keywords Bacillus anthracis · Bioterrorism · Drug-design · Molecular modeling · Shikimate dehydroganase

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Introduction

Anthrax has been employed in bioterrorism attacks and it is related with high mortality, despite antimicrobial treatment and therapy [1]. Therefore there is an urgent need of new therapies to treat anthrax. Among potential targets, to be employed on the development of drugs against bacterial diseases we could mention shikimate pathway enzymes and 2-*trans*-enoyl-ACP (CoA) reductase [2–13]. The focus of this work is on Shikimate Dehydrogenase. The shikimate pathway links the metabolism of carbohydrates to biosynthesis of aromatic compounds. This seven-step metabolic pathway leads from phosphoenolpyruvate and eritrose 4-phosphate to chorismate, the common precursor for many aromatic products [14]. Such as folic acid, vitamins E and K, ubiquinone and three aromatic amino acids: tryptophan, phenylalanine and tyrosine.

In bacteria, fungi, plants and apicomplexan parasites, chorismate, the final product of the shikimate pathway, is the branch point in the biosynthesis for all these products that are essential for these species. Because of numerous enzymatic reactions, there is great potential for the design and synthesis of enzyme inhibitors which may selectively block specific enzyme-catalyzed transformations along the seven steps of this pathway [15, 16].

Generally, bacteria have at least two orthologues for every other enzyme in the shikimate pathway. Although the redundancy of these enzymes in many pathogenic organisms suggests that their function is important under physiological conditions [17]. Crystal structures for all of the pathway enzymes have been determined [18–24]. The absence of shikimate pathway in mammals makes these enzymes attractive targets for the development of new antibacterial agents [25, 26] especially broad antibiotic drugs [27]. Shikimate dehydrogenase (SD) catalyzes the fourth step of

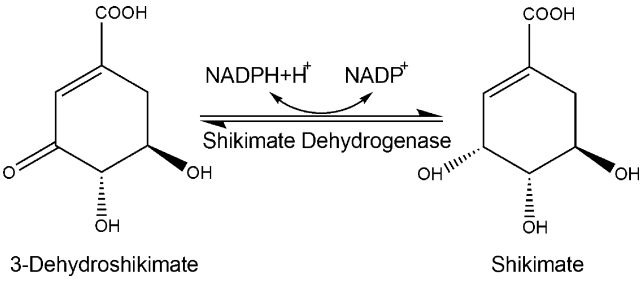


Fig. 1 The reversible reaction catalyzed by *Ba*SD

the biosynthetic pathway and is responsible for the reduction of 3-dehydroshikimate to shikimate in the presence of NADP, shown in Fig. 1 [28].

This enzyme belongs to the super family of NADP-dependent oxireductases [29]. Due to its occurrence in an early step of the pathway and the availability of its synthetic substrate analogs, SD is considered as a good target for inhibition [30, 31]. Further structural studies of enzyme-ligand complexes will enhance understanding of the pathway and may reveal avenues for new drug and herbicide design [32].

In this work we present the structural model of shikimate dehydrogenase from *Bacillus anthracis* (*Ba*SD) in order to describe its interactions with the cofactor NADP. The NADP binding site present in the structure was analyzed. The analysis was carried out with NADP in order to identify the structural basis for the specificity of different inhibitors against SDs. The knowledge of this may help in the design of specific inhibitors.

Materials and methods

Molecular modeling

Homology modeling is usually the method of choice when there is a clear relationship of homology between the sequence of a target protein and at least one known structure. This computational technique is based on the assumption that tertiary structures of two proteins will be similar if their sequences are related, and this approach is the most likely to give accurate results [33].

The starting point of homology modeling is the identification of proteins in the protein data bank (PDB) [34] that are related to the target sequence and then select the templates [35]. To search the sequence from *Ba*SD we used SWISS-PROT [36–38]. Model building of *Ba*SD was carried out using the program PARMODEL [39]. PARMODEL is a parallelized version of the MODELLER9v1 [40].

The modeling procedure begins with an alignment of the sequence to be modeled (target) with related known three-dimensional structures (templates). This alignment is usually the input to program. The output is a three-dimensional model for the target sequence containing all main-chain and side chain non-hydrogen atoms (PDB file) [41–43].

An important step before modeling is the multiple sequence alignment, to improve the sensitivity of the search and to find regions with high similarity [35]. Possible templates and target sequence alignments were performed with CLUSTALW [44, 45]. The atomic coordinates of all waters were removed from the templates before sending the modeling to PARMODEL [39]. A total of 1000 models were generated for each binary complex (*Ba*SD: NADP) and the final model was selected based on the MODELLER [40] objective function. The optimization process was performed on a Beowulf cluster with 16 nodes.

Evaluation of binding affinity

Analysis of the interaction between a ligand and a protein target is still a scientific endeavor. The analysis of the interaction between a ligand and a protein target is an important scientific tool to predict new potential [45]. The affinity and specificity between a ligand and its protein target depends on several structural features such as directional hydrogen bonds and ionic interactions, as well as on shape complementarity of the contact surfaces of both partners [46].

We used the program XSCORE [47] to evaluate the binding affinity of the cofactor NADP against *Ba*SD. According to this method, the binding affinity of the ligand can be decomposed to the contribution of individual atoms. Each ligand atom obtains a score, called the atomic binding score, indicating its role in the binding process.

The program reads the structure, assigns atom types and parameters, performs the calculation, and gives the dissociation constant of the given protein-ligand complex. The computational results are outputted into a text file in which

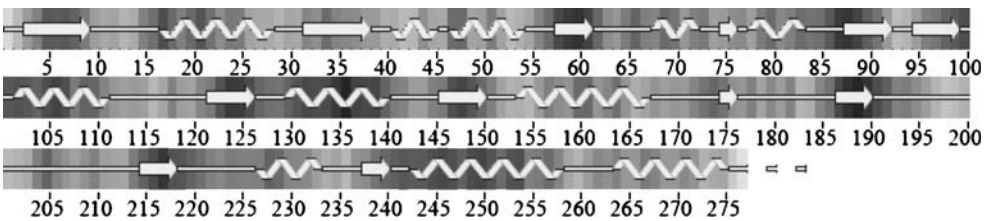


Fig. 2 The secondary structure assessment performed by the program PROCHECK [49]

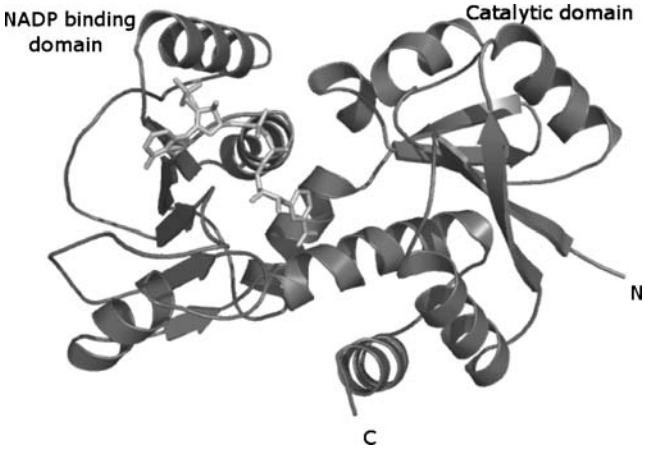


Fig. 3 Structural model for *Ba*SD monomer in complex NADP, the NADP is shown in stick representation. The protein is essentially composed of two distinct domains, the NADP binding domain and the substrate binding domain (catalytic domain) which are linked by two helices. Figure generated with the program PYMOL [53]

the detailed information of each ligand atom, including the atomic binding score, is tabulated [48].

Analysis of the models

The overall stereo chemical quality of the final models for *Ba*SD were assessed by the program PROCHECK [49]. Protein-ligand interactions were analyzed with LIGPLOT [50]. Protein parameters, such as pI and molecular weight were determinated with EXPASY [51]. The models were analyzed and with VMD [52] and all protein figures were prepared with PYMOL [53]. The cutoff for hydrogen bonds was 3.3 Å. The contact area for the complexes was calculated using AREAIMOL [62].

Results and discussion

Overall description

The structure of *Ba*SD is a compact α/β sandwich with two distinct domains, responsible for binding substrate and NADP cofactor, respectively. The first three β-strands follow a regular α/β succession, with the helices α1 and α2 parallel to β-strands, flanking opposite sides of the sheet (Figs. 2 and 3). The domain is completed by a C-terminal α-helical hairpin (α9 and α10), which packs against the β-sheet on the same side as α-1 [21].

A systematic survey of NADP protein complexes showed that NADP interacts with a variety of proteins more variably than does NAD [54]. Analysis of the present structural model reveals an enzyme with a deep cleft, which contains the active site formed on the junction of two domains. The C-terminal domain is easily recognized as a Rossmann-fold dinucleotide binding domain, responsible for binding the NADP cofactor.

The N-terminal domain is responsible for substrate binding [55, 56]. Among available templates we chose the

Table 1 Shikimate dehydrogenases analyzed in this work

Enzyme	PDB access code	Resolution (Å)	References	Sequence identity (%)
*Ba*SD*	2HK9	2.2	[58]	40%
*Mt*SD*	2CY0	1.9	[32]	28%
*Hp*SD*	2CY0	1.9	[32]	32%
*Af*SD*	2CY0	1.9	[32]	39%
AaSD**	2HK9	2.2	[58]	-
*Ec*SD**	1NYT	2.3	[21]	-

*Molecular modeling **Structure solved by X-ray diffraction

Table 2 Intermolecular interactions and protein-ligand affinities

Enzyme	pKd (Experimental Data)	pKd (Xscore)	Contact surface(Å^2)	Intermolecular H-Bonds
*Aa*SD	4.37	6.82	556	12
*Af*SD	3.72	6.22	523	10
*Ec*SD	4.23	6.64	505	13
*Mt*SD	4.20	5.79	491	11
*Hp*SD	3.73	5.80	558	16
*Ba*SD	ND	6.06	524	7

ND. Not determined.

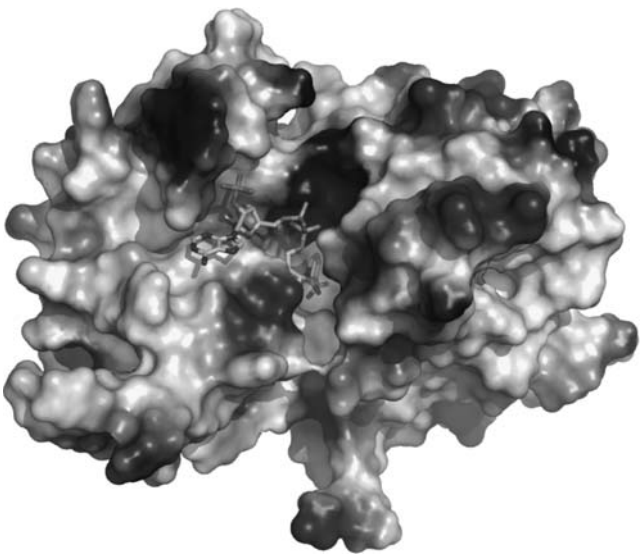


Fig. 4 Electrostatic potential surface for the *Ba*SD:NADP complex. The partial positive charge presents the N-terminal of alpha helices interacting with phosphates from NADP calculated with PYMOL [53]

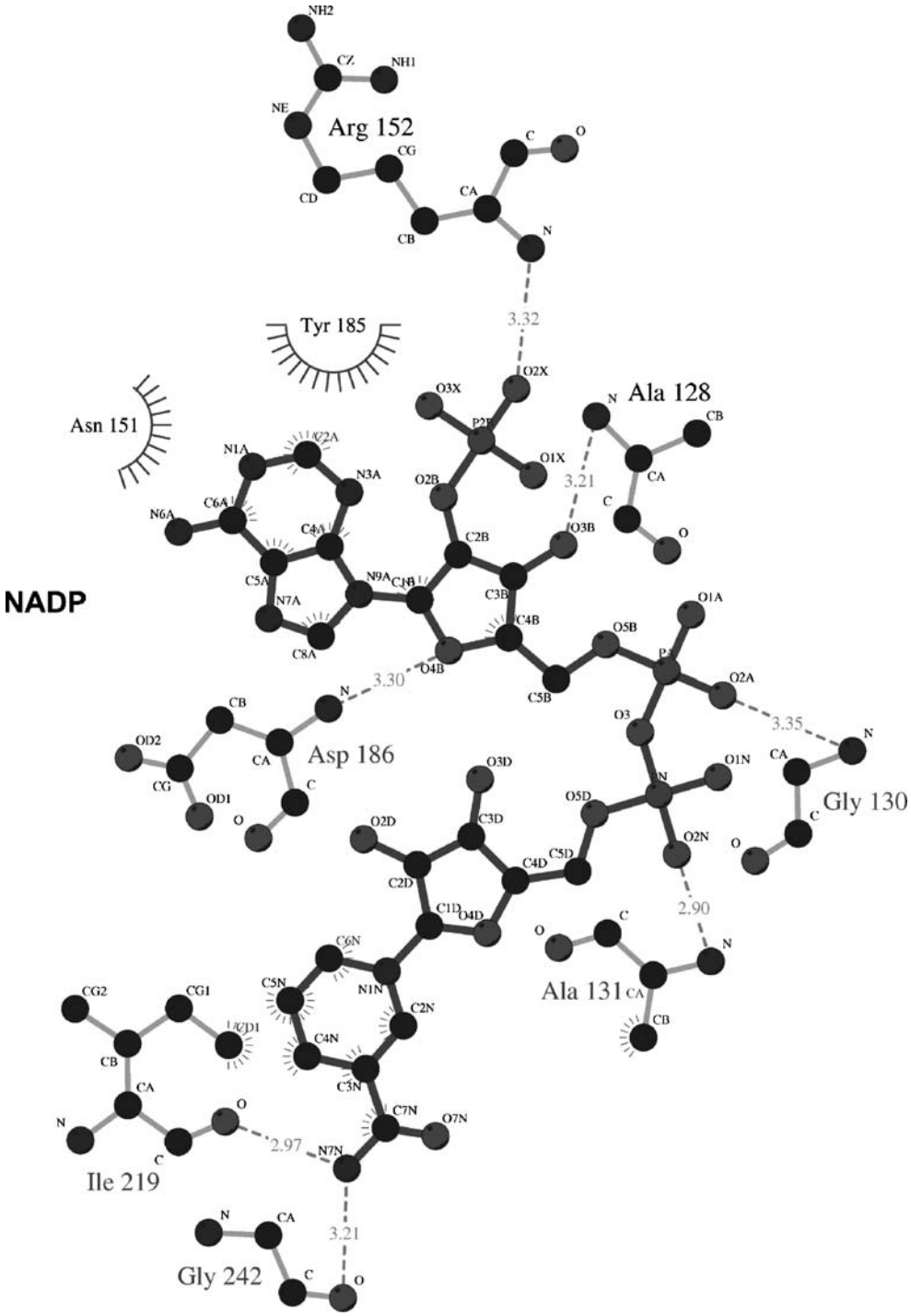


Fig. 5 LIGPLOT [50] representation of NADP binding site to *Ba*SD. Dotted lines represent the hydrogen bonds and the half circles the hydrophobic contacts

one with highest identity and similarity: the shikimate dehydrogenase from *Aquifex aelicus* (solved by 2.2 Å resolution), PDB access code: 2HK9 [57]. The substrate binding site in the SD has been identified by the position of the nicotinamide ring of the cofactor and was delineated almost entirely by residues from the N-terminal domain [21]. The *Ba*SD consists of 277 amino acids with a molecular weight 30163.9 Da and theorical pI of 6.55.

Quality of the model

There is no crystallographic structure available for *Ba*SD, however the similarity between *Ba*SD and the template *Aa*SD sequence makes this crystallographic structure a reasonable template for modeling of *Ba*SD. In order to evaluate the possible differences in the target and template structure we superposed the structures of *Aa*SD and *Ba*SD.

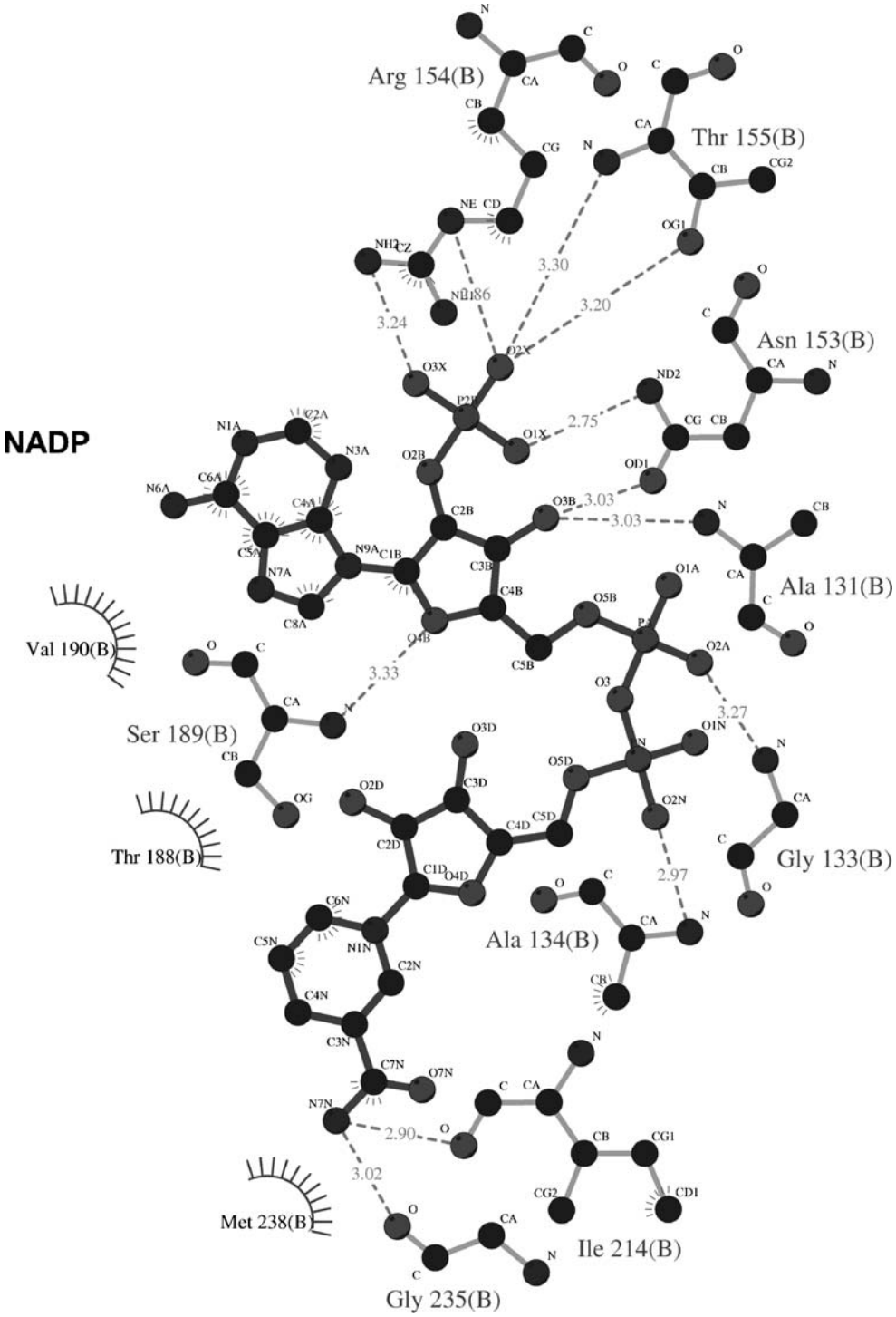


Fig. 6 LIGPLOT [50] representation of NADP binding site to *Aa*SD. Dotted lines represent the hydrogen bonds and the half circles the hydrophobic contacts

The atomic coordinates of crystallography structures of templates were used as basic models for modeling *Ba*SD. The analysis of the Ramachandran diagram Φ–Ψ plots for the templates was used to compare the overall stereo chemical quality of the *Ba*SD structure against those of templates solved by X-ray crystallography.

The model presented over 93.4% of the residues in the most favorable regions, higher than the template *Aa*SD: 89.8%. From analysis of the overall stereo chemical quality of the molecular model we conclude that this model is good enough for structural studies.

Attempts to confirm the pKd results

In order to confirm our results obtained for the affinities (pKd values) of NADP against *Ba*SD, we used data from

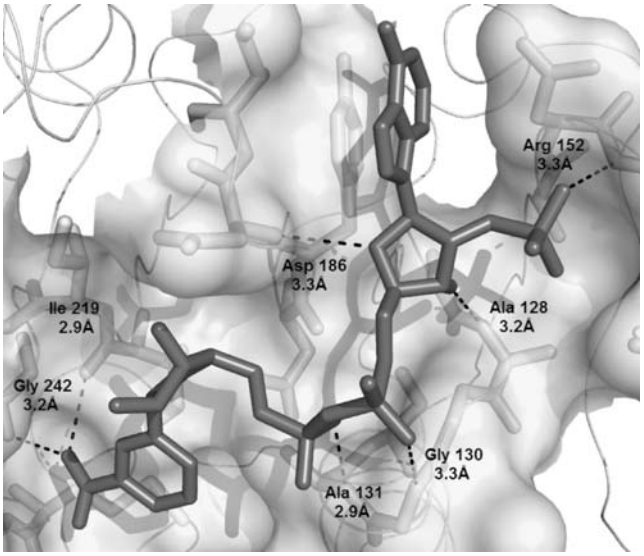


Fig. 7 The binding site of the *Ba*SD in complex with NADP. Figure generated with the program PYMOL [53]

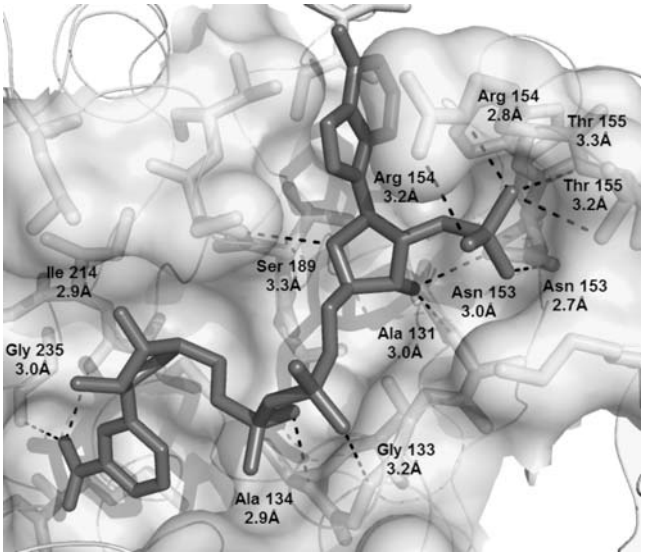


Fig. 8 The binding site of the *Aa*SD in complex with NADP. Figure generated with the program PYMOL [53]

Gan et al. 2007 [57]; Lim et al. 2004 [58]; Michel et al. 2003 [21]; Zhang et al. 2005 [59] and Han et al. 2006 [60]. Those are K_M from the organisms *Aquifex aeolicus* (*As*SD), *Archaeoglobus fulgidus* (*Af*SD), *Escherichia coli* (*Ec*SD), *Mycobacterium tuberculosis* (*Mt*SD) and *Helicobacter pylori* (*Hp*SD).

To calculate pKd values we modeled the structures of SDs from these organisms with the NADP using molecular modeling approach (Table 1). As templates we used structures deposited in the Protein Data Bank, with PDB access codes 2HK9, 1NYT and 2CY0 in agreement with molecular modeling procedure described in materials and methods. The program XSCORE was employed to evaluate the affinity constant of the NADP cofactor against SD structures. The calculated pKd values are shown in Table 2.

Comparing the results obtained with XSCORE against experimental determinated affinities, we can observed that the pKd values obtained are fairly close to the experimental results however they can be employed for a qualitative analysis only. Using XSCORE results we may suggest that NADP presents a high affinity for *Ba*SD. The lower affinity of *Ba*SD for NADP when compared with to *Aa*SD is probably due to the lower number of hydrogen bonds.

In order to estimate the solvent accessible area (ASA) and cofactor surface of the proteins cited above, we had performed analysis using AREAIMOL which is a program from CCP4 package (Collaborative Computational Project, Number 4, 1994) [61] using default settings. The results showed a correlation between the contact surface and the lower pKd value for *Ba*SD therefore contact surfaces contribute to increase protein-ligand affinity, as can be observed in the empirical scoring functions employed in the program XSCORE. However for *Hp*SD and *Mt*SD this relation was not observed.

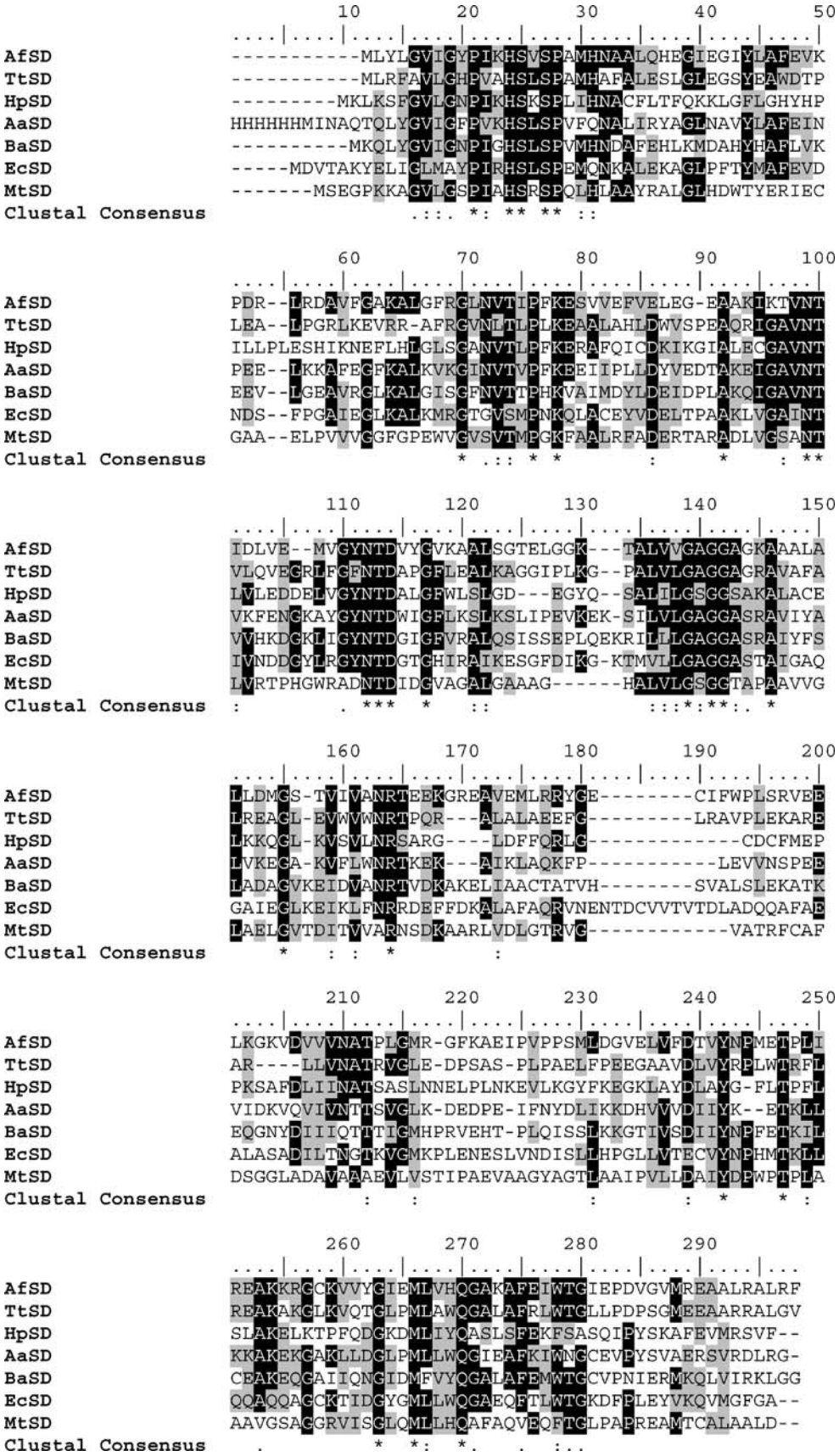
NADP interactions analysis

Figure 4 shows a deep grove is formed between the two domains in which the cofactor NADP is located. This feature of the fold creates a natural cavity contributing to the binding of the adenine ring of the NADP [62]. The hydrophobic contacts and all hydrogen bonds between *Ba*SD and *Aa*SD with the cofactor NADP are shown in Figs. 5 and 6.

The domain structure is very similar to those of NADP binding domains in other dehydrogenases [63]. Figures 7 and 8 show hydrogen bonding between the N-H groups in the last turn of the helix and the phosphate oxygens. Analysis of the electrostatics interactions in the structure of *Ba*SD indicates a cluster of positive charges in N-terminal of helix (Fig. 4). This analysis was carried out with the program PYMOL [53].

These interactions involve residues Gly130 and Ala131; those are present in *Ba*SD and *Aa*SD and highly conserved in all orthologues proteins shown in the multiple alignment

Fig. 9 Sequence alignment for shikimate dehydrogenase from *Archaeoglobus fulgidus, Thermus thermophilus, Helicobacter pylorii, Aquifex aeolicus, Bacillus anthracis, Escherichia coli, Mycobacterium tuberculosis*. The multiple alignment was performed using ClustalW [43] (*) indicates positions which have a single, fully conserved, (:) indicates that one of the following 'strong' groups is fully conserved, and (.) indicates that one of the following 'weaker' groups is fully conserved ▶


AfSD
TtSD
HpSD
AaSD
BaSD
EcSD
MtSD
Clustal Consensus

(Fig. 9). The multiple alignment shows to that Arg154 and Gly235 are 100% conserved in *Af*SD, *Tt*SD, *Hp*SD, *Mt*SD, *Ec*SD [17] and *Aa*SD [57]. Due to this strong conservation of residues in NADP binding site the design of broad spectrum inhibitors may be a possibility. Analysis of the intermolecular interactions in the template A*a*SD indicates that Asn153 and Thr155 also participate in electrostatic interactions with phosphate.

Unexpectedly these interactions were missing in *Ba*SD. Despite conservation of the NADP binding site the residue Ser189 was mutated in the model to Asp186 nevertheless in this novel interaction this residue may act as the general acid/base catalyst during the hydride transfer reaction.

Comparatively *Aa*SD has more electrostatic interactions (12) than the *Ba*SD (seven). The hydrophobic contacts observed in *Aa*SD were not conserved in *Ba*SD: Val190 was mutated to Tyr185, Thr188 and Met 238 were missing. Although the model presented an extra contact, Asn151. These contacts all together may influence the affinity between the *Ba*SD and NADP.

Conclusions

We have obtained a molecular model of *Ba*SD based on the crystal structure of SD from *A. aeolicus*. The residues Gly130 and Ala131 are present in the N-terminal of the helix and those make positive electrostatic interactions with the phosphates of NADP cofactor therefore these residues are present in *Ba*SD and the template *Aa*SD highly conserved in all orthologues proteins. All models show good shape complementarity and a high number of intermolecular hydrogen bonds.

The model strongly indicates that the NADP binding site is conserved in SD structures. Furthermore, the alignment of seven SD sequences indicates that the main residues involved in intermolecular hydrogen bonds are conserved in all sequences. This observation suggests that competitive inhibitors with NADP could be able to inhibit SDs from other organisms, since specificity and affinity between enzyme and its inhibitor depend on directional hydrogen bonds and ionic interactions, as well as on shape complementarity of the contact surfaces of both partners [64–69].

Further inhibition experiments may confirm this prediction hence the knowledge of interactions between proteins and ligands will undoubtedly aid in the design and identification of useful inhibitors that may be used as bactericide against *Bacillus anthracis*.

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