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A chiquimato desidrogenase de *Mycobacterium tuberculosis*: mutagênese sítio-direcionada, expressão, purificação e caracterização da enzima mutante K69A

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ÍNDICE

LISTA DE ABREVIATURAS

DHQ-SD – desidroquinato desidratase-chiquimato desidrogenase;

DHS – 3-desidrochiquimato;

DNA – ácido desoxirribunucléico;

DOTS - Directly Observed Treatment Short-course*;*

HIV – vírus da imunodeficiência humana;

IPTG – isopropil-beta-D-tiogalactopiranosídeo;

Kcat – constante catalítica;

Kcat/Km – eficiência catalítica;

Kd – constante de dissociação;

kDa – quilodaltons;

Km – constante de Michaelis-Menten;

LB – Luria-Bertani;

MDR-TB – cepa de *Mycobacterium tuberculosis* resistente a múltiplas drogas;

*Mtb*SD – chiquimato desidrogenase de *Mycobacterium tuberculosis*;

$NADP^+$ - nicotinamida adenina dinucleotídeo fosfato, forma oxidada;

NADPH – nicotinamida adenina dinucleotídeo fosfato, forma reduzida;

OMS – Organização Mundial da Saúde;

PCR – reação em cadeia da DNA polimerase;

SD – chiquimato desidrogenase;

SDS-PAGE – eletroforese em gel de poliacrilamida sob condições desnaturantes com dodecil sulfato de sódio;

SHK – chiquimato ou ácido chiquímico;

TB – tuberculose;

Vmáx – velocidade máxima de reação;

XDR-TB – tuberculose extensivamente resistente;

RESUMO

As enzimas da via do chiquimato são alvos atraentes para o desenvolvimento de agentes para tratar a tuberculose já que esta via é essencial para *Mycobacterium tuberculosis* e está ausente em humanos. A enzima chiquimato desidrogenase de *M. tuberculosis* (*Mtb*SD), codificada pelo gene *aro*E, catalisa a quarta reação na via do chiquimato. Estudos de estrutura tridimensional, de perfis de pH e de mutagênese sítio-direcionada envolvendo muitas chiquimato desidrogenases sugerem a participação da Lisina-69 e do Aspartato-105 (numeração de acordo com a seqüência da *Mtb*SD) na catálise e/ou na ligação ao substrato. A determinação das propriedades cinéticas de enzimas mutantes pode permitir importantes proposições acerca do papel destes resíduos para a enzima *Mtb*SD. A mutagênese foi realizada usando uma técnica de amplificação por PCR para as seguintes proteínas mutantes: K69A, K69H, K69I, K69Q, D105A, D105N. Testes de diversas condições experimentais foram feitos para obter a expressão das proteínas *Mtb*SD mutantes na fração solúvel. Além disso, empregamos um protocolo de purificação otimizado para obter as enzimas *Mtb*SD não mutante e K69A aparentemente homogêneas. Realizamos ensaios cinéticos em estado estacionário e medidas espectrofluorimétricas destas duas enzimas, e os resultados indicam que o resíduo conservado Lisina-69 tem função catalítica e não está envolvido na ligação ao substrato para a *Mtb*SD. Estudos estruturais, de mutagênese sítio-direcionada e de cinética enzimática podem trazer importantes contribuições para o desenho racional de novos e efetivos fármacos para tratar a tuberculose.

ABSTRACT

The shikimate pathway is an attractive target for the development of antitubercular agents because it is essential in *Mycobacterium tuberculosis* but absent in humans. *Mycobacterium tuberculosis aro*E-encoded shikimate dehydrogenase (*Mtb*SD) catalyzes the forth reaction in the shikimate pathway. Three-dimensional structure studies, pH-rate profiles and site-directed mutagenesis studies involving many shikimate dehydrogenases have suggested participation of Lysine-69 and Aspartate-105 (*M. tuberculosis* numbering) in catalysis and/or substrate binding. Importantly, investigation of the kinetic properties of mutant enzymes can bring important insights about the role of these residues for the *Mtb*SD enzyme. Mutagenesis was performed using a PCR-amplification technique for the following mutant proteins: K69A, K69H, K69I, K69Q, D105A, D105N. Screening of experimental conditions has been performed to obtain the expression of the *Mtb*SD mutant proteins in the soluble fraction. In addition, an improved purification protocol was used to obtain homogeneous wild-type *Mtb*SD and K69A mutant enzymes. We have carried out steady-state kinetic assays and spectrofluorimetric measurements for the wild type and the K69A enzymes. Results indicate that the conserved Lysine-69 residue in *Mtb*SD plays a catalytic role and is not involved in substrate binding. Enzyme kinetics, site-directed mutagenesis and structural studies provide a framework on which to base the rational design of new and effective agents to treat tuberculosis.

1 INTRODUÇÃO

1.1 Tuberculose

A tuberculose (TB) humana é uma doença infecto-contagiosa causada principalmente pelo bacilo *Mycobacterium tuberculosis*, uma bactéria aeróbica que causa infecção usualmente nos pulmões (COLE *et al.*, 1998). A TB foi responsável por milhões de mortes no passado, quando não existia tratamento adequado para a doença e se desconhecia seu agente causal. No século XIX, a TB foi uma doença avassaladora, com altas taxas de transmissão e que levava a um número de mortes muito elevado. Esta época corresponde ao início da revolução industrial, onde houve o surgimento dos aglomerados urbanos, muitas vezes sem estruturas de higiene e habitação, o que colaborava para a disseminação e o estabelecimento de inúmeras doenças, dentre elas a TB (DORMANDY, 2002). Na Inglaterra vitoriana, a TB foi romantizada e suas vítimas se tornaram padrão de beleza dada a esqualidez e a brancura pálida e também porque já não era uma doença exclusiva das camadas pobres, atingindo, inclusive, muitos intelectuais da época (DUCATI *et al.*, 2006).

Somente em 1882, o médico e bacteriologista alemão Robert Koch tornou pública a identificação do *M. tuberculosis* como agente etiológico da TB, durante o IV Congresso Mundial de TB (KAUFMANN *et al.*, 2003). O *M. tuberculosis* é geralmente transmitido entre os indivíduos através do ar por aerossóis que contêm o bacilo. Essas pequenas gotículas podem permanecer por longos períodos de tempo em ambientes fechados. Quando inalada, a micobactéria é fagocitada pelos macrófagos alveolares nos bronquíolos

respiratórios e nos alvéolos. O bacilo inalado poderá ou não estabelecer a infecção e isso dependerá da virulência bacteriana e da capacidade bactericida dos macrófagos do hospedeiro (DUNLAP *et al.*, 2000).

A descoberta da estreptomicina, em 1944, marcou a era de ouro no desenvolvimento de drogas para tratar a TB; a seguir, outros fármacos usados atualmente no tratamento da doença foram introduzidos, são eles: ácido p-aminosalicílico (1946), isoniazida (1952), ciclosserina (1955), canamicina (1957), rifampicina (1965), etionamida (1966), etambutol (1968) e pirazinamida (1970) (DUNCAN, 2003). O desenvolvimento destes fármacos e o emprego de medidas profiláticas proporcionaram uma diminuição no número de mortes, que se manteve por algumas décadas (BLOOM e MURRAY, 1992; DANIEL, 1997)

Contudo, a TB ressurgiu com força e é, atualmente, considerada a principal causa de morte devido a um único agente infeccioso (ENARSON e MURRAY, 1996). A epidemia do vírus da imunodeficiência humana (HIV), a deterioração dos programas de saúde pública visando o controle da TB, a multiplicação de aglomerados urbanos sem estrutura sanitária e o número elevado de pessoas sem moradia são alguns dos fatores apontados como responsáveis pelo aumento dos números de TB em muitos países, inclusive desenvolvidos (BLOOM e MURRAY, 1998). Em 1993, a Organização Mundial da Saúde (OMS) declarou a TB como emergência de saúde global, estimando-se que um terço da população humana esteja infectada pelo bacilo e que na última década 30 milhões de pessoas morreram desta doença (CORBETT *et al.*, 2003; DYE *et al.*, 1999). Outros números mostram que 9 milhões de pessoas são infectadas com o bacilo anualmente, o que leva a 2 milhões de mortes, principalmente na África e na Ásia (WHO, 2006).

Assim, a TB permanece como uma das principais causas de morte em todo o mundo devido, também, à grande capacidade de adaptação do bacilo, que consegue sobreviver a variadas condições dentro e fora do hospedeiro humano. O *M. tuberculosis* tem sido apontado como o patógeno de maior sucesso no planeta, de todos os tempos, conseguindo muitas vezes permanecer silencioso e latente dentro do hospedeiro, desviando das defesas do sistema imune (ENSERINK, 2001; WICKELGREN, 2000). O bacilo pode permanecer dormente até que as defesas do hospedeiro sejam diminuídas como no caso da infecção pelo HIV e por imunossupressão por fármacos. Com o aumento no número de infectados com o HIV, torna-se preocupante o numero de hospedeiros ativamente contaminados e capazes de transmitir a doença (MANABE e BISHAI, 2000).

TB e HIV são tão proximamente relacionados que o termo co-epidemia tem sido usado para descrever sua relação. A taxa de reativação da TB latente em pessoas imunocompetentes é de 0,2 % por ano. Já em pacientes com HIV, este risco de reativação da TB latente pode chegar até 13,3 % ao ano, dependendo do nível de imunossupressão (KNIGGE *et al.*, 2000). Pacientes HIV-poitivos que são infectados com TB desenvolvem a TB ativa em uma tava de 37 % nos primeiros 6 meses; já nos pacientes imunocompetentes, essa taxa é de até 5 % nos dois primeiros anos. Além disso, pacientes HIV-positivos apresentam mal-absorção de algumas drogas, talvez devido à enteropatia relacionada ao HIV, o que pode comprometer os tratamentos da TB e também levar ao surgimento de cepas resistentes a fármacos usados para tratar a TB (SEPKOWITZ, 1995).

O tratamento da TB teve melhoria significativa com a expansão do programa *Directly Observed Treatment Short-course* (DOTS), que combina compromisso político, serviços de microscopia, suprimento de medicamentos, sistemas de monitoramento e observação direta ao tratamento (PASQUALOTO e FERREIRA, 2001). Atualmente, o tratamento recomendado pela OMS consiste na administração combinada dos fármacos isoniazida, rifampicina, pirazinamida e etambutol durante dois meses. O tratamento deve prosseguir por mais quatro meses, quando se administra isoniazida e rifampicina. Infelizmente, alguns países não conseguem implantar o programa DOTS para a totalidade dos seus pacientes, reforçando a hipótese de que tratamentos mais curtos e com menores efeitos colaterais melhorariam a adesão do paciente e isso levaria a uma maior eficácia no tratamento da TB (DUNCAN, 2003).

De fato, tratamentos inapropriados e a não adesão do paciente ao tratamento são comumente associados com a emergência de cepas de TB resistentes a múltiplas drogas (MDR-TB), cujos isolados são resistentes a pelo menos isoniazida e rifampicina, dois dos principais fármacos usados no tratamento padrão da TB (DUNCAN, 2003; BASSO e BLANCHARD, 1998).

Pacientes com MDR-TB devem ser tratados com uma combinação de drogas de segunda linha que, além de serem significativamente mais caras, possuem mais efeitos tóxicos e são menos efetivas que as drogas de primeira linha (O'BRIEN e NUNN, 2001). Em países industrializados, o tratamento normal custa em torno de 2.000 dólares por paciente, mas alcança 250.000 dólares para pacientes com MDR-TB (PASQUALOTO e FERREIRA, 2001).

Casos de coinfecção de HIV e MDR-TB alcançam taxas de mortalidade próximas a 100 %, e esta é definida como a infecção oportunista mais maligna associada à AIDS (FÄTKENHEUER *et al.*, 1999). Cerca de 300.000 novos casos de MDR-TB são diagnosticados a cada ano, sendo que de 4 a 20 % destes são classificados como TB extensivamente resistente (XDR-TB), definida como casos de TB cujos isolados são resistentes à isoniazida, rifampicina e a pelo menos três das seis principais classes de drogas de segunda linha (aminoglicosídeos, polipetídeos, fluoroquinolonas, tiamidas, ciclosserina e ácido p-aminosalicílico) (DORMAN e CHAISSON, 2007; CDC, 2006). XDR-TB está sendo relatada em todo o mundo, inclusive nos Estados Unidos, onde a TB estava sendo considerada sob controle. A ocorrência já difundida de XDR-TB traz discussões sobre a drástica situação de casos de TB virtualmente incuráveis e aponta para a urgente necessidade de introduzir novos e eficazes fármacos anti-TB (DORMAN e CHAISSON, 2007).

Já se passaram quase 40 anos desde a introdução da última droga para tratar a TB. Estima-se que o desenvolvimento completo de uma nova droga anti-TB custe de 100 a 800 milhões de dólares (DUNCAN, 2003). Considerando-se que 95 % dos casos de TB ocorram em países subdesenvolvidos e em desenvolvimento, a indústria farmacêutica parece não estar suficientemente atraída, em termos financeiros, para o desenvolvimento de novos fármacos para tratar a TB (O'BRIEN e NUNN, 2001).

Em 1998, Stewart Cole e colaboradores publicaram a seqüência completa do genoma de *M. tuberculosis* (COLE et al., 1998) proporcionando novas abordagens de pesquisas sobre este microrganismo e, até mesmo,

abrindo perspectivas para o futuro desenvolvimento de fármacos para tratar a TB.

1.2 Via do ácido chiquímico

A via do ácido chiquímico (**Figura 1**) liga o metabolismo dos carboidratos com a biossíntese de compostos aromáticos, convertendo eritrose-4-fosfato em corismato, que é o precursor para a síntese de metabólitos importantes como aminoácidos aromáticos, ubiquinona e folato (PARISH e STOKER, 2002). Esta via é essencial no metabolismo de algas, plantas, bactérias, parasitos do filo Apicomplexa e fungos, mas é ausente em mamíferos (BENTLEY, 1990; ROBERTS *et al.*, 1998). Assim, as enzimas desta via recebem muita atenção como potenciais alvos para o desenvolvimento de fármacos antimicrobianos não tóxicos, herbicidas e drogas antiparasitárias (COGGINS *et al.*, 2003). Um grande exemplo do emprego exitoso de inibidores específicos desta via é o glifosato, inibidor da 5-enolpiruvilchiquimato-3-fosfato sintase e um dos herbicidas de amplo espectro mais usados em todo o mundo. Este composto também demonstrou atividade inibitória do crescimento de parasitos do filo apicomplexo *in vitro* (AMRHEIN *et al.*, 1980; ROBERTS *et al.*, 1998). É comprovado que a via do chiquimato é crucial para a viabilidade de *M. tuberculosis* (PARISH e STOKER, 2002). Já que esta via está ausente no hospedeiro humano e é essencial para a micobactéria, suas sete enzimas são alvos atrativos para o desenvolvimento de agentes que possam tratar a TB (PARISH e STOKER, 2002; BENTLEY, 1990). Com o objetivo de desenhar racionalmente novas e efetivas drogas antimicobacterianas, muitos estudos de caracterização molecular e bioquímica têm envolvido as enzimas da via do chiquimato de *M. tuberculosis* como, por exemplo, 3-desóxi-D-arabino-heptulosonato-7-fosfato sintase (RIZZI *et al.*, 2005), desidroquinato sintase (MENDONÇA *et al.*, 2007), chiquimato quinase (PEREIRA *et al.*, 2004), 5-

enolpiruvilchiquimato-3-fosfato sintase (OLIVEIRA *et al.*, 2003) e corismato sintase (DIAS *et al.*, 2004; FERNANDES *et al.*, 2007).

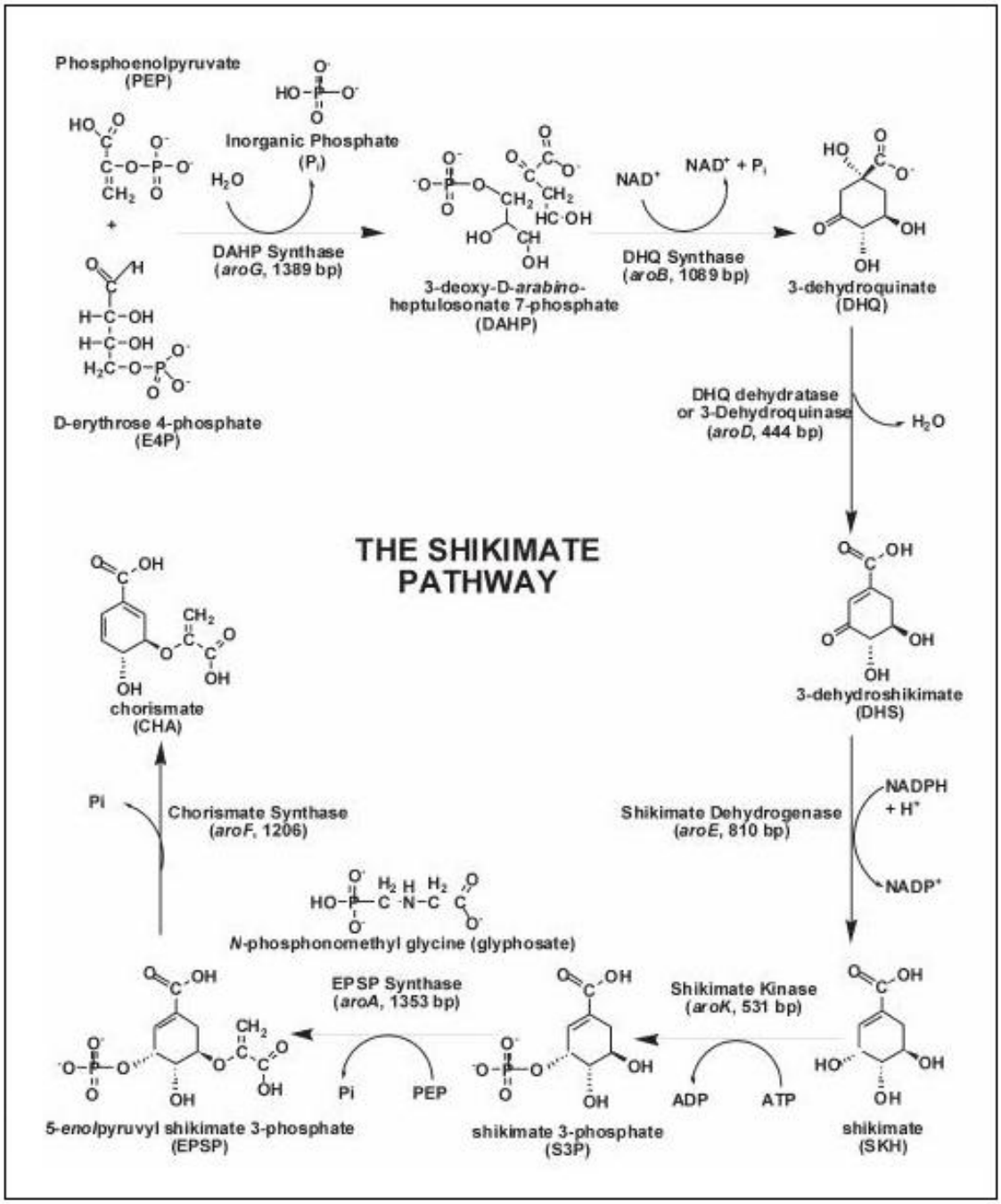

Figura 1: A via do ácido chiquímico, mostrando seus sete passos enzimáticos (BASSO *et al*., 2005).

1.3 Chiquimato desidrogenase

A quarta reação enzimática da via do ácido chiquímico é catalisada pela chiquimato desidrogenase (SD, EC 1.1.1.25), que é codificada pelo gene *aro*E de *M. tuberculosis* e catalisa a redução reversível do 3-desidrochiquimato (DHS) em chiquimato (SHK), na presença do NADPH como o doador de hidreto (**Figura 2**; BENTLEY, 1990).

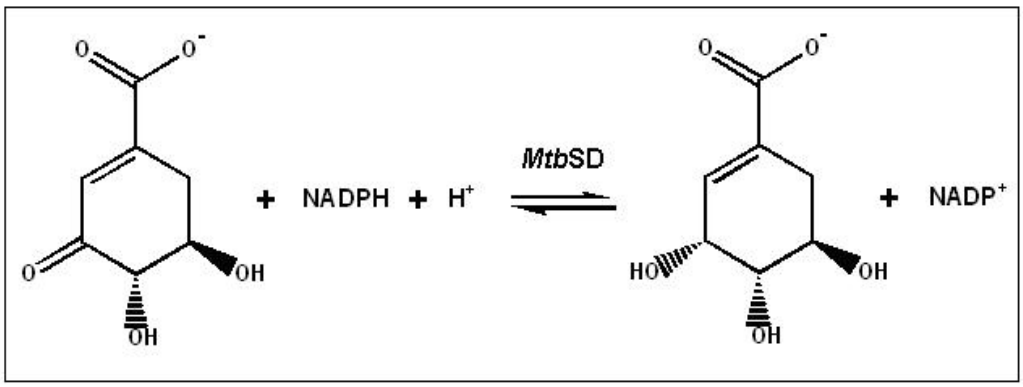

Figura 2: Reação catalisada pela enzima chiquimato desidrogenase (*Mtb*SD).

Em plantas, a SD é parte de uma enzima bifuncional, a desidroquinato desidrogenase-chiquimato desidrogenase (DHQ-SD) (DEKA *et al.*, 1994; SINGH e CHRISTENDAT, 2006). Em fungos e leveduras, a SD compõe um complexo enzimático pentafuncional (CHARLES *et al.*, 1986; DUNCAN *et al.*, 1987). Em bactérias, estudos bioquímicos e filogenéticos identificaram a existência de três subclasses de SD, até então: AroE, YdiB e SD-like. AroE é a SD de ocorrência mais ampla e é uma enzima monofuncional. YdiB e SD-like foram identificadas em um número reduzido de microorganismos; YdiB é uma SD bifuncional que também catalisa a redução reversível do desidroquinato a quinato. Até então, a SD-like é uma enzima não muito bem caracterizada, e foi sugerida como monofuncional em *H. influenzae*, catalisando a oxidação do

SHK a DHS com uma constante catalítica bastante reduzida se comparada com a da AroE (MICHEL *et al.*, 2003; SINGH *et al.*, 2005). Recentemente, um estudo relatou inibidores específicos da SD de *Helicobacter pylori*, o que reforça a importância do desenvolvimento de novos agentes antimicrobianos e da diversificação de alvos para novas drogas (HAN *et al.*, 2006).

A enzima SD de *M. tuberculosis* (*Mtb*SD), codificada pelo gene *aro*E (Rv2552c), possui 269 aminoácidos e pertence à superfamília das oxirredutases dependentes de NAD(P)H, que atuam em rotas anabólicas e catabólicas (BENACH *et al.*, 2003); além disso, encontra-se na forma de dímero em solução (FONSECA *et al.*, 2006).

Em 2002, foi publicada a clonagem do gene *aro*E da cepa *M. tuberculosis* H37Rv em vetor de expressão pET23a(+) (Novagen®) e a expressão da enzima recombinante na fração solúvel, em células de *Escherichia coli* BL21(DE3) (MAGALHÃES *et al.*, 2002). Em seguida, nosso grupo publicou a purificação da enzima recombinante, sua caracterização cinética e estudos estruturais (FONSECA *et al.*, 2006; ARCURI *et al.*, 2008). Além disso, foram propostos os mecanismos cinético e químico da reação catalisada pela *Mtb*SD (FONSECA *et al.*, 2007). Entretanto, as bases moleculares da ligação ao substrato DHS e da redução catalítica ainda não foram completamente elucidadas.

Estudos de alinhamento de várias seqüências de SDs demonstram que os aminoácidos lisina (Lis, K) da posição 69 e aspartato (Asp, D) da posição 105 (Lis69 e Asp105, numeração de acordo com a seqüência da AroE de *M. tuberculosis*) são, entre outros resíduos, completamente conservados nas cadeias polipeptídicas das seguintes enzimas: AroE de *M. tuberculosis, E. coli*,

H. influenzae, *Methanococcus jannaschii, Thermus thermophilus, Neisseria meningitides*, *H. pylori*, *Archaeoglobus fulgidus, Aquiflex aeolicus, Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa,* YdiB de *E. coli, Corynebacterium glutamicum,* SD-like de *Haemophilus influenzae*, DHQ-SD de *Arabidopsis thaliana* e *Lycopersicon esculentum* (MICHEL *et al.*, 2003; HAN *et al.*, 2006; FONSECA *et al.*, 2007; YE *et al.*, 2003; BAGAUTDINOV e KUNISHIMA, 2007, **Anexo 6.1**). A seguir, referir-se-á aos números dos resíduos sempre de acordo com a numeração da seqüência polipeptídica da *Mtb*SD.

Estudos das estruturas cristalinas das enzimas AroE e YdiB de *E. coli* sugerem que Lis69 e Asp105 estejam localizados no sítio de ligação ao substrato destas enzimas e que estes resíduos provavelmente tenham um papel fundamental na catálise acido-básica da reação da SD neste organismo (MICHEL *et al.*, 2003). Ainda em 2003, outro estudo de cristalografia reforçou esta nova proposição para a YdiB de *E. coli*, onde o resíduo conservado Asp105 possivelmente atue como catalisador ácido-básico durante a transferência de hidreto (BENACH *et al.*, 2003).

As estruturas tridimensionais da AroE de *H. influenzae* e de *M. jannaschii* sugerem Lis69 e Asp105, dentre outros resíduos, como parte do sítio ativo destas enzimas e que estes aminoácidos possam contribuir para a catálise (YE *et al.*, 2003; PADYANA e BURLEY, 2003). Muito importante foi a determinação dos complexos ternários da AroE de *T. termophilus* e *A. aeolicus* com o cofator $NADP^+$ e o substrato SHK; estas estruturas permitiram uma boa compreensão sobre o mecanismo enzimático (**Figura 3**; BAGAUTDINOV e KUNISHIMA, 2007; GAN *et al.*, 2007).

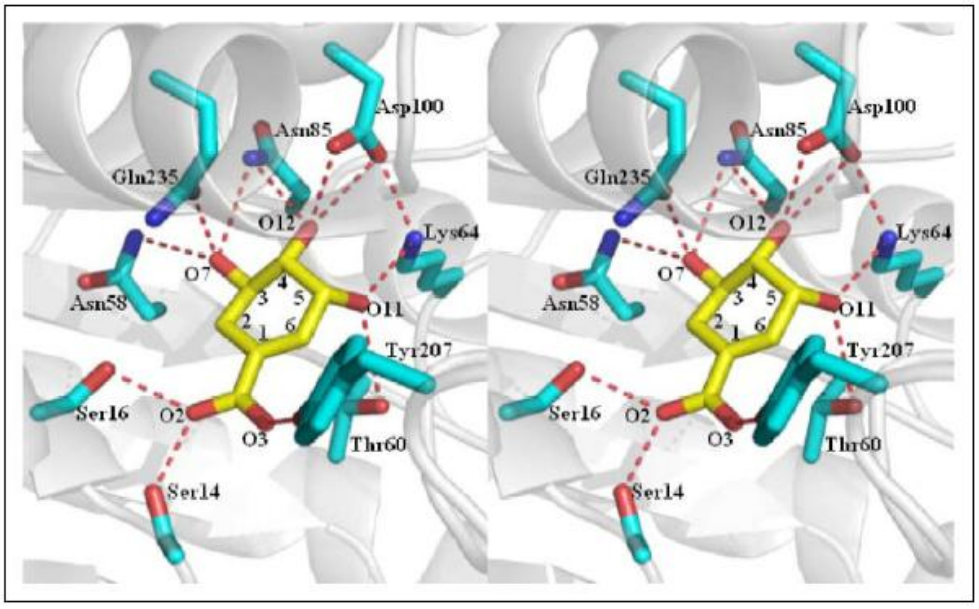

Figura 3: Visão do SHK ligado ao complexo ternário AroE de *Thermus termophilus*:NADP(H):SHK (BAGAUTDINOV e KUNISHIMA, 2007).

Todas essas estruturas revelaram uma arquitetura geral bem conservada para estas enzimas, composta de dois domínios: o domínio catalítico N-terminal, onde Lis69 e Asp105 estão localizados, e o domínio C-terminal, de ligação ao NAD(P); estes dois domínios estão separados por um grande sulco central, onde está localizado o sítio catalítico destas enzimas (**Figura 4**).

A determinação da estrutura cristalina, estudos de mutagênese e de efeito de pH indicam que Lis69 e Asp105 estão criticamente envolvidos na catálise na enzima SD-like de *H. influenzae* (SINGH *et al.*, 2005). Entretanto, um trabalho de mutagênese sítio-direcionada envolvendo a enzyma YdiB de *E. coli* sugere que estes dois aminoácidos são muito importantes para a ligação ao substrato e que a catálise não ocorre por um mecanismo geral ácido-básico, como fora inicialmente sugerido (LINDNER *et al.*, 2005). Recentemente, outra

análise de enzimas mutantes mostrou que o resíduo que corresponde à Lis69 e ao Asp105 da enzima DHQ-SD de *A. thaliana* tem grande importância na atividade catalítica desta enzima (SINGH e CHRISTENDAT, 2006). Estudos do efeito do pH com a SD de *A. aeolicus* também indica que um resíduo, provavelmente a Lis69, funciona como uma base durante a catálise (GAN *et al.*, 2007). Nosso grupo publicou a determinação dos mecanismos cinético e químico da *Mtb*SD, incluindo os efeitos do pH, e os dados são consistentes com a provável participação de um resíduo lisina tanto na catálise quanto na ligação ao substrato (FONSECA *et al.*, 2007).

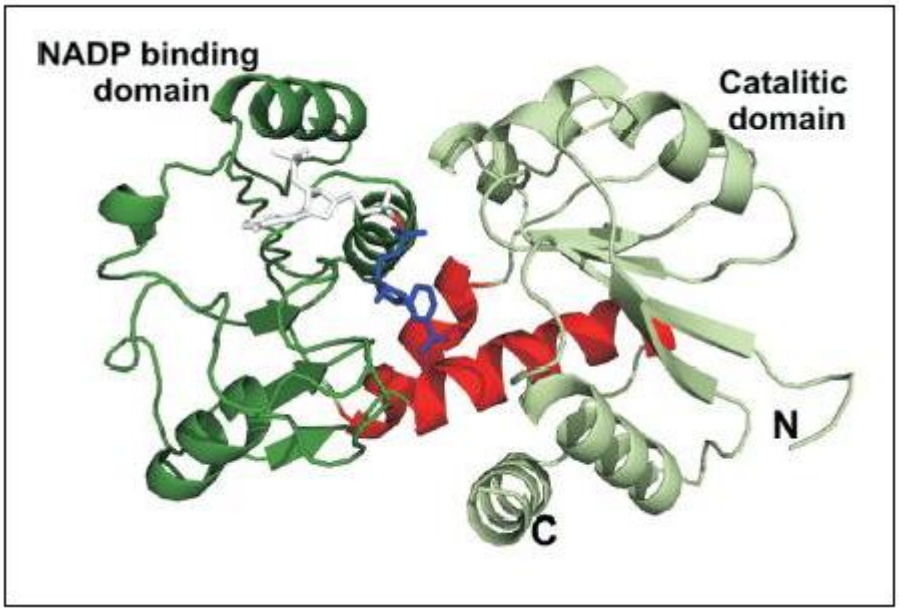

Figura 4: Modelo estrutural do monômero da enzima *Mtb*SD em complexo com o $NADP^+$, composta pelos domínios catalítico e de ligação ao $NADP^+$ (ARCURI *et al.*, 2008).

Com base nestes estudos, os resíduos Lis da posição 69 e Asp da posição 105 da enzima SD de *M. tuberculosis* foram escolhidos para investigação da sua

importância, se alguma, na ligação do substrato e/ou na catálise enzimática, por meio da técnica de mutagênese sítio-direcionada.

2 OBJETIVOS

Este trabalho foi desenvolvido com vistas a determinar o papel da cadeia lateral de aminoácidos do sítio ativo da enzima SD de *M. tuberculosis*, buscando o melhor entendimento do seu mecanismo cinético e, assim, a caracterização refinada deste alvo molecular. Os nossos resultados poderão servir como base para o desenho racional de fármacos para o tratamento da tuberculose.

Para tanto, alguns objetivos específicos foram traçados:

I. Planejamento das mutações e projeção de oligonucleotídeos iniciadores específicos para as mutações desejadas;
II. Mutagênese sítio-direcionada do gene *aro*E de *M. tuberculosis* H37Rv;
III. Seqüenciamento do gene *aro*E após a técnica de mutagênese;
IV. Super-expressão e purificação das proteínas mutantes;
V. Caracterização cinética da proteína mutante K69A e comparação com o perfil da enzima não mutante.

3 MÉTODOS E RESULTADOS

Incialmente, idealizamos as mutações a serem realizadas. Este planejamento inicial do trabalho está apresentado na próxima seção, intitulada “Planejamento das mutações”.

As seções de métodos e de resultados serão apresentadas na forma de manuscritos, intitulados **“Screening of experimental conditions to express soluble shikimate dehydrogenase mutants from *Mycobacterium tuberculosis* H37Rv”** e **“The conserved Lysine 69 residue plays a catalytic role in *Mycobacterium tuberculosis* shikimate dehydrogenase”**, já submetidos (**Anexos 6.5 e 6.6**).

No primeiro manuscrito constam os métodos e resultados referentes à mutagênese sítio-direcionada, à super-expressão das enzimas *Mtb*SD mutantes na fração solúvel e à purificação da proteína mutante K69A. No segundo manuscrito está descrita a caracterização cinética da *Mtb*SD mutante K69A.

3.1 Planejamento das mutações

Quatro resíduos foram escolhidos para substituir a Lis, totalizando quatro enzimas mutantes distintas (**Figura 5**):

- Alanina (Ala, A): com esta mutação se consegue retirar a cadeia carbônica e também o grupamento amino da Lis;

- Histidina (His, H): assim como a Lis, é um aminoácido básico; pode-se investigar, portanto, se o grupamento básico da Lis tem algum papel importante para esta enzima;

- Glutamina (Gln, Q): possui uma cadeia lateral com três carbonos e um grupamento amida que permite que este resíduo mantenha a propriedade de realizar interações por meio de pontes de hidrogênio, assim como a Lis pode realizar pelo seu grupamento amino;

- Isoleucina (Ile, I): assim como a Lis, possui em sua cadeia lateral quatro carbonos unidos por ligações simples; logo, possui a propriedade de fazer interações do tipo van der Waals.

Duas enzimas mutantes foram idealizadas para estudar o papel do aminoácido Asp, são os seguintes resíduos substituintes (**Figura 6**):

- Ala: retira-se o grupamento carboxílico do Asp;

- Asparagina (Asn, N): possui uma cadeia lateral com dois carbonos e um grupamento amida que permite que este resíduo mantenha a propriedade de realizar interações através de pontes de hidrogênio, assim como o Asp pode realizar pelo seu grupamento carboxílico.

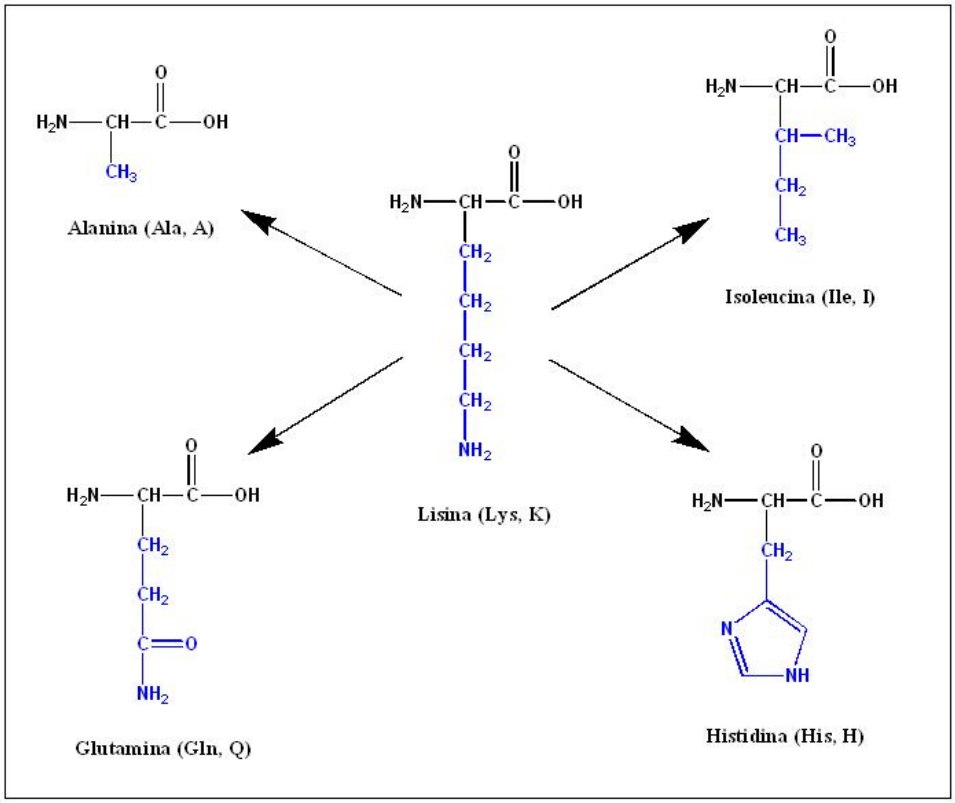

Figura 5: Resíduos idealizados para substituir a Lisina 69 da *Mtb*SD.

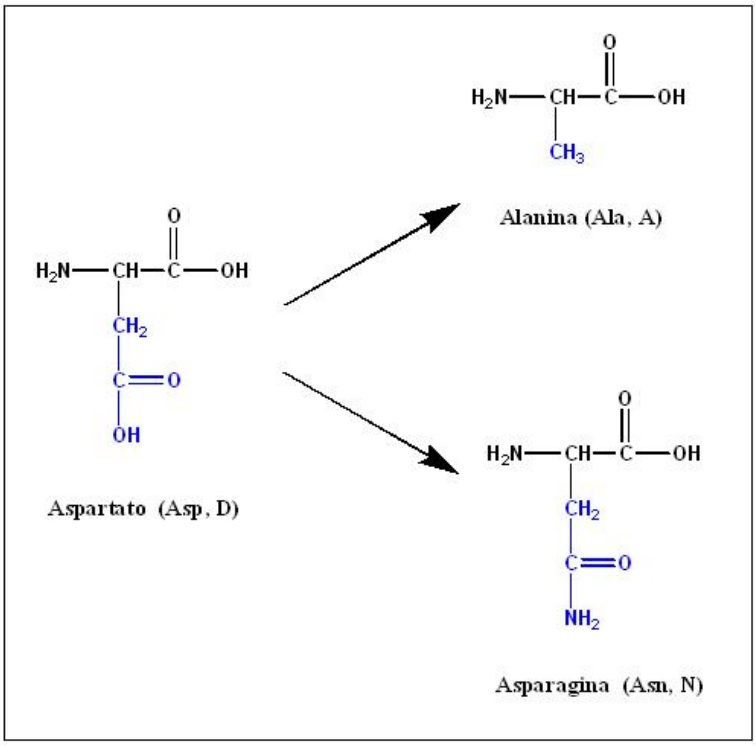

Figura 6: Resíduos idealizados para substituir o Aspartato 105 da *Mtb*SD.

3.2 Manuscrito 1

Screening of experimental conditions to express soluble shikimate dehydrogenase mutants from *Mycobacterium tuberculosis* H37Rv

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Keywords: *Mycobacterium tuberculosis*, shikimate pathway, shikimate dehydrogenase, site-directed mutagenesis, protein expression, protein purification

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Short title: *Mycobacterium tuberculosis* shikimate dehydrogenase

ABSTRACT

The shikimate pathway is an attractive target for the development of antitubercular agents because it is essential in *Mycobacterium tuberculosis*, the causative agent of tuberculosis, but absent in humans. *Mycobacterium tuberculosis aro*E-encoded shikimate dehydrogenase (*Mtb*SD) catalyzes the forth reaction in the shikimate pathway. Three-dimensional structure studies, pH-rate profiles and site-directed mutagenesis studies involving many shikimate dehydrogenases have suggested participation of Lysine and Aspartate amino acid side chains in catalysis and/or substrate binding. Importantly, investigation of the kinetic properties of mutant enzymes can bring important insights about the role of these residues for the *Mtb*SD enzyme. Mutagenesis was performed using a PCR-amplification technique for the following mutant proteins: K69A, K69H, K69I, K69Q, D105A, D105N. Screening of *E. coli* strains, growth temperature, hours of growth, and the need for isopropyl β-D-thiogalactopyranoside (IPTG) induction, cell disruption methods, and additives were employed to optimize *Mtb*SD production yield. In addition, an improved purification protocol to obtain homogeneous *Mtb*SD is presented. The kinetic parameters for the wild type enzyme were also determined. Enzyme kinetics, site-directed mutagenesis and structural studies provide a framework on which to base the rational design of new agents with antitubercular activity. However, the availability of sufficient amounts of proteins of *M. tuberculosis* still remains an essential and laborious step. The results presented here show that optimization of expression, disruption and purification protocols resulted in a

higher yield of functional *Mtb*SDs enzyme, which is an essential step towards target-based development of chemotherapeutic agents to treat tuberculosis.

ABREVIATIONS USED

TB, tuberculosis; MDR-TB, multi-drug resistant strains of *Mycobacterium tuberculosis*; XDR-TB, extensively-drug resistant tuberculosis; SD, shikimate dehydrogenase; *Mtb*SD, *Mycobacterium tuberculosis* shikimate dehydrogenase; DHS, 3-dehydroshikimate; SHK, shikimate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; DHQ-SD, dehydroquinate dehydratase-shikimate dehydrogenase; PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; LB, Luria-Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; OD_{600}, optical density at 600 nm; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, continues to be a threat to human health worldwide. It has been estimated that one-third of the world population is infected with the tubercle bacilli and that in the last decade 30 million people died from this disease [1]. Among the possible factors involved in the resurgence of TB are the epidemic of the human immunodeficiency virus, the increase in the homeless population, and the decline in health care structures and national surveillance [2]. Inappropriate treatment regimens and patient noncompliance in completing the therapies are commonly associated with the emergence of multi-drug resistant TB (MDR-TB), whose isolates are resistant to at least isoniazid and rifampicin, two pivotal drugs used in the standard treatment of TB [3,4]. More recently, the emergence of extensively drug resistant TB (XDR-TB) strains were also described [5], which are also a contributing factor to the alarming emergence of TB cases worldwide. XDR-TB is defined as resistant to isoniazid and rifampicin and at least three of the six main classes of second-line drugs (aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and para-aminosalicylic acid) and its high occurrence raises the prospect of virtually incurable TB [5,6]. Hence, the alarming rise of strains of *M. tuberculosis* resistant to current drugs stimulates an urgent need to discover novel antitubercular agents acting on new drug targets.

The shikimate pathway links the metabolism of carbohydrates to the biosynthesis of aromatic compounds, converting erythrose 4-phosphate in chorismate, which is the precursor for the synthesis of important metabolites,

such as aromatic amino acids, ubiquinone and folate [7]. This pathway is essential for algae, higher plants, bacteria, and fungi, but it is absent in mammals [8]. Importantly, the shikimate pathway has been shown to be essential for the viability of *M. tuberculosis* [7]. Since this pathway is absent in human host and is essential for mycobacteria, its seven enzymes are attractive targets for the development of antitubercular agents [7,8]. With a view to the design of effective antimycobacterial drugs, efforts have been made to characterize at the molecular and biochemical levels many enzymes from this pathway in *Mycobacterium tuberculosis*, such as 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase [9], dehydroquinate synthase [10], shikimate kinase [11], 5-enolpyruvylshikimate-3-phosphate synthase [12] and chorismate synthase [13,14].

The fourth reaction of the shikimate pathway is the *aro*E-encoded shikimate dehydrogenase (SD, EC 1.1.1.25) in *M. tuberculosis* that catalyzes the reversible reduction of 3-dehydroshikimate (DHS) to shikimate (SHK) in the presence of NADPH as the hydride donor [8] (Figure 1).

Figure 1

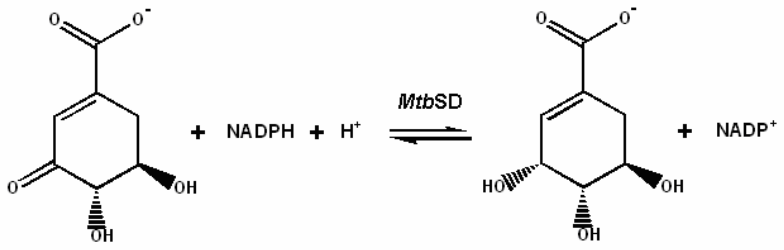

Figure 1: The shikimate dehydrogenase-catalyzed reaction.

In plants, SD is part of a bifunctional dehydroquinate dehydratase-shikimate dehydrogenase (DHQ-SD) enzyme [15,16]. In fungi and yeast, SD participates of a pentafunctional enzyme complex [17,18]. In bacteria, biochemical and phylogenetic studies have identified the existence of three subclasses of SD to date: AroE, YdiB, and SD-like. AroE is a widely distributed monofunctional enzyme. YdiB and SD-like enzymes have been identified in a lower number of organisms. The *ydi*B-encoded protein is a bifunctional SD enzyme that also catalyses the reversible reduction of dehydroquinate to quinate. SD-like is not well characterized, and was suggested to be monofunctional in *Haemophilus influenzae*, catalyzing the oxidation of SHK with a lower rate when compared with that of AroE [19,20]. Recent report of specific inhibitors against the shikimate dehydrogenase from *Helicobacter pylori* has reinforced the importance of the target diversity and the introduction of novel effective antimicrobial agents [21]. Our group has previously reported cloning, expression, purification, kinetic properties and structural studies of the *aro*E-encoded shikimate dehydrogenase from *M. tuberculosis* (*Mtb*SD) [22-25]. However, the molecular basis of DHS recognition and the catalytic mechanism for *Mtb*SD are not yet known.

Multiple sequence alignment studies showed that the amino acids lysine in the position 69 and aspartate in the position 105 (Lys69 and Asp105, *M. tuberculosis* numbering) are, amongst other residues, fully conserved in the polypeptide chain of the following aligned enzymes: AroE from *M. tuberculosis, Escherichia coli*, *Haemophilus influenzae*, *Methanococcus jannaschii, Thermus thermophilus, Neisseria meningitides*, *Helicobacter pylori*, *Archaeoglobus fulgidus, Aquiflex aeolicus, Pseudomonas aeruginosa,* YdiB from *Escherichia*

coli, Corynebacterium glutamicum, SD-like from *Haemophilus influenzae*, SD parts of *Arabidopsis thaliana* and *Lycopersicon esculentum* DHQ-SDs [19,21,24,26,27]. In the following, we will refer to residues according to the *M. tuberculosis* AroE numbering. Crystal structure determination of AroE and YdiB proteins from *E. coli* suggest that a lysine and an aspartate (Lys69 and Asp105 in *Mtb*SD) residues are located in the substrate binding site and probably play critical roles in the acid-base catalysis of the SD reaction in this organism [19]. In the same year, other crystallographic work reinforced this novel proposal for YdiB from *E. coli*, in which the conserved aspartate residue (Asp105 in *Mtb*SD) is supposed to act as the acid-base catalyst during the hydride transfer [28]. Three-dimensional structures of both *H. influenzae* and *M. jannaschii* AroE suggest conserved lysine and aspartate, amongst other residues, as part of the active site of these enzymes and that these amino acids may contribute to catalysis [26,29]. Importantly, ternary complexes for AroE from both *Thermus termophilus* and *Aquiflex aeolicus* in complex with $NADP^+$-SHK were determined and provided important insights into the enzymatic mechanism [27,30]. These structures have revealed a common overall architecture of these enzymes, comprising two domains: the N-terminal catalytic domain, where the conserved lysine and aspartate residues (Lys69 and Asp105 in *Mtb*SD) are located, and the C-terminal NAD(P)-binding domain. Mutagenesis studies, determination of the crystal structure and pH-rate profiles indicate important roles in catalysis for the lysine and aspartate residues in the SD-like enzyme from *H. influenzae* [20]. However, a site-directed mutagenesis work on the YdiB enzyme from *E. coli* suggests important roles for these amino acids in substrate binding and that catalysis does not occur through a general acid-base

mechanism, as initially thought [31]. More recently, another mutational analysis has shown that the corresponding lysine and aspartate residues play critical roles in the catalytic activity of *Arabidopsis thaliana* DHQ-SD enzyme [16]. The pH-rate profile of the *A. aeolicus* SD also indicates that a residue, probably a lysine, functions as a general base during catalysis [30]. We have recently reported determination of kinetic and chemical mechanisms for *Mtb*SD, including pH-rate profiles, and the data are consistent with the probable participation of a lysine residue in catalysis and/or substrate binding [24].

In an attempt to probe the role, if any, of Lys69 and Asp105 residues in *Mtb*SD, we replaced Lys69 residue by alanine, isoleucine, histidine and glutamine, and Asp105 by alanine and asparagine. Here, we describe site-directed mutagenesis and an expression protocol to obtain both wild type and mutant enzymes in soluble form. In addition, we developed a new and improved purification protocol to obtain homogeneous wild type *Mtb*SD. We have also used this protocol to purify the K69A mutant.

Enzyme kinetics and structural studies provide a framework on which to base the target-based rational design of new agents with antitubercular activity. However, the availability of sufficient amounts of proteins of *M. tuberculosis* still remains an essential and laborious step. Unfortunately, even when a genome can be sequenced, only up to 20 % of the protein targets can produce soluble proteins under very basic experimental conditions [32]. Thus, expression of proteins in soluble form has been identified as an important bottleneck in efforts to determine biological activity and crystal structure of *M. tuberculosis* proteins [33]. It should be pointed out that expression of *Mtb*SD in soluble and active form proved to be laborious to achieve [22]. Accordingly, the results here

described represent an important step towards providing recombinant *Mtb*SD proteins to allow functional and structural efforts on which to base the rational design of antitubercular agents.

MATERIALS AND METHODS

Site-directed mutagenesis of the *aro*E gene

Mutagenesis experiments were performed using the QuickChange site-directed mutagenesis kit from Stratagene, according to the manufacturer's instructions. This technique consists of a PCR-amplification procedure that uses the *PfuTurbo*® DNA polymerase and a pair of specific antiparallel primers, carrying codons for the required substitutions. The oligonucleotide primers were individually designed, and both primers must contain the desired mutation as given in Table 1. The recombinant vector, pET23a(+)::*aro*E, was used as template since the method allows specific mutations in virtually any double-stranded plasmid. The PCR amplification program was as follows: one step of 95º C for 30 s, 16 cycles at 95º C for 30 s, 55º C for 1 min, and 68º C for 10 min, followed by a final extension step at 68º C for 10 min. The PCR products were treated with *Dpn*I endonuclease to select for the mutation-containing synthesized DNA. The mutant *aro*E genes were entirely sequenced to both confirm the insertion of the desired mutation and to ensure that no unwanted mutations were introduced by the PCR step.

Table 1

Designed primers for the desired mutations

Mutant	Mutagenic primer*
K69A	forward 5’ ggtgtttcgg tgaccatgcc gggc**gcg**ttc gccgccctgc ggttcg 3’
K69A	reverse 5’ cgaaccgcag ggcggcgaa**c gc**gcccggcat ggtcaccga aacacc 3’
K69I	forward 5’ ggtgtttcgg tgaccatgcc gggc**atc**ttc gccgccctgc ggttcg 3’
K69I	reverse 5’ cgaaccgcag ggcggcgaa**g at**gcccggcat ggtcaccga aacacc 3’
K69H	forward 5’ ggtgtttcgg tgaccatgcc gggc**cac**ttc gccgccctgc ggttcg 3’
K69H	reverse 5’ cgaaccgcag ggcggcgaa**g tg**gcccggcat ggtcaccga aacacc 3’
K69Q	forward 5’ ggtgtttcgg tgaccatgcc gggc**cag**ttc gccgccctgc ggttcg 3’
K69Q	reverse 5’ cgaaccgcag ggcggcgaa**c tg**gcccggcat ggtcaccga aacacc 3’
D105A	forward 5’ ggctggcggg ccgacaacac c**gcg**atcgac ggggtggccg gggcg 3’
D105A	reverse 5’ cgccccggcc accccgtcga t**cgc**ggtgtt gtcggcccgc cagcc 3’
D105N	forward 5’ ggctggcggg ccgacaacac c**aac**atcgac ggggtggccg gggcg 3’
D105N	reverse 5’ cgccccggcc accccgtcga t**gtt**ggtgtt gtcggcccgc cagcc 3’

3.2 In bold are the nucleotides corresponding to the substituting amino acid

Overexpression and release of the mutant *Mtb*SD

Recombinant protein expression tests were carried out at various experimental conditions including screening of *E. coli* strains, growth temperature, hours of growth, and the need for isopropyl β-D-thiogalactopyranoside (IPTG) induction. In addition, we tried to solubilize the insoluble aggregates with 50 mM Tris-HCl, pH 7.8 buffer containing the agents sarcosyl, triton X-100, zwittergent 3-14 or urea. The concentrations employed were as follows: 0.2, 1 or 2 % for sarcosyl; 0.1, 1 or 2 % for triton X-100; 0.1, 1, 3 or 6 M for urea; 0.1, 0.5 or 5 % for zwittergent 3-14. The solubilization test consisted of treating the insoluble fraction with each agent for 30 minutes of stirring at 4 ºC. Finally, the disruption methods of French press and freeze-thaw

were tested. Satisfactory results were obtained with the *E. coli* C41 (DE3) strain, grown at 37 ºC for 24 h with IPTG induction. The optimized protein expression protocol and release of the soluble recombinant proteins are given hereafter. The recombinant plasmid pET23a(+)::*aro*E (either wild-type or mutants) was transformed into *E. coli* C41 (DE3) electrocompetent cells by electroporation, and selected on LB agar plates containing 50 μg mL^{-1} ampicillin. Single colonies were used to inoculate 2 L of LB medium containing 50 μg mL^{-1} ampicillin, and 1 mM IPTG was added to cultures reaching an OD_{600} of 0.4 – 0.6. The cells were grown for additional 24 h at 37º C at 180 rpm, after induction. Cells (5 g) were harvested by centrifugation at 14,900*g* for 30 min at 4º C, and stored at -20 ºC. The freeze-thaw method was used to release the proteins in the soluble fraction [34]. Cells were placed into metal containers, allowing fast temperature equilibrium to be reached, and placed into a dry-ice/ethanol bath for 2 min and transferred to an ice-water bath for 8 min, and this cycle was repeated 5 times. These cells were dissolved in 25 mL of 50 mM Tris-HCl, pH 7.8 and placed on ice for additional 30 min. The sample was centrifuged at 4 ºC for 1h at 48,000*g* and the soluble fraction containing the protein of interest was collected and employed in the optimization of recombinant protein purification protocols.

Purification steps

The sample containing the soluble enzyme was incubated with 1 % (w/v) of streptomycin sulfate for 30 min and centrifuged at 48,000*g* for 30 min. The supernatant was dialyzed against 50 mM Tris-HCl, pH 7.8 (buffer A), using a

dialysis tubing with molecular weight exclusion limit of 12-14 kDa. Ammonium sulphate was added to a final concentration of 1 M. The sample was clarified by centrifugation and the supernatant was loaded on a Phenyl-Sepharose Fast Flow hydrophobic interaction column pre-equilibrated with 50 mM Tris-HCl, pH 7.8, 1 M $(NH_4)_2SO_4$ (buffer B). The column was washed with 10 column volumes of buffer B and the bound proteins were fractionated with a 20-column volume linear gradient from 1 to 0 M $(NH_4)_2SO_4$. The fractions containing *Mtb*SD were pooled, concentrated to 10 mL and loaded on a Sephacryl S-200 HR column pre-equilibrated with buffer A. Fractions containing the recombinant protein were pooled and loaded on a MonoQ anion exchange column previously equilibrated with buffer A. The column was washed with 5 column volumes of buffer A and the adsorbed proteins were eluted using a 20-column volume linear gradient (0- 100%) of 50 mM Tris-HCl, 0.5 M NaCl, pH 7.8 buffer. The fractions containing the purified protein were pooled, concentrated and dialyzed against the enzymatic assay buffer. All the purification steps were performed at 4 ºC. Samples of the purification steps were analyzed by SDS-PAGE [35] and the protein content was determined by the Bradford method [36], using the Bio-Rad protein assay kit (Bio-Rad) and bovine serum albumin as standard.

RESULTS AND DISCUSSION

Sequence alignment analysis of many shikimate dehydrogenase polypeptide chains showed that, amongst other amino acids, lysine and aspartate residues (Lys69 and Asp105 in *Mtb*SD) are fully conserved, and it has been suggested that these residues are of fundamental importance in catalysis and/or substrate binding [19,21,26,27]. Three-dimensional structure determination of shikimate dehydrogenase enzymes (AroE, YdiB and SD-like) from several organisms have also suggested the involvement of these amino acids in catalysis and/or substrate binding and have revealed the conserved overall architecture of the shikimate dehydrogenase family, composed by a catalytic domain and a NAD(P)-binding domain [19,20,26,27,29-31]. In addition, site-directed mutagenesis results have confirmed the importance of lysine and aspartate for catalysis (in SD-like from *H. influenzae* and *A. thaliana* DHQ-SD) and for substrate binding (in YdiB from *E. coli*) [16,20,31]. Based on structural and functional studies, it is likely that the conserved lysine in the position 69 and aspartate in the position 105 of the *Mtb*SD enzyme may play an important role either in catalysis or binding or both. To investigate the functional role of these residues, we have replaced Lys69 with alanine, isoleucine, histidine or glutamine, and Asp105 with alanine or asparagine by site-directed mutagenesis. The PCR-amplification mutagenesis technique proved to be an effective procedure, allowing mutation in the double-stranded recombinant plasmid, pET23a(+)::*aro*E. It should be pointed out the importance of treating the PCR product with the *Dpn*I endonuclease that specifically digests the

methylated DNA template, and selects for the mutation-containing synthesized DNA.

After sequencing of the recombinant genes in their entirety, the mutant plasmids were transformed in *E. coli* for protein expression tests. Recombinant protein expression of *Mtb*SD has proved to be a difficult task to achieve [22]. Thus, a range of variable experimental conditions were tried to improve recombinant protein expression. Tests in LB medium using various *E. coli* strains, at different cultivation temperatures, either with or without IPTG induction (Table 2) as well as cell growth for additional 3, 6, 12, 18, 24, or 36 hours of cultures after reaching an OD_{600} of 0.4 – 0.6. Disappointingly, these attempts to express the wild-type and mutant *Mtb*SDs in soluble form were unsuccessful. The best recombinant protein expression protocol of *Mtb*SD K69A mutant in insoluble form was using *E. coli* C41(DE3) strain, grown at 37 ºC for 24 hours after induction with 1 mM IPTG, and cell disruption by sonication (Figure 2). The need for the presence of IPTG to allow expression is consistent with the use of the vector pET23a(+). The pET expression system uses the powerful T7 RNA polymerase, under control of IPTG-inducible lacUV5 promoter, to transcribe genes of interest [37].

Table 2

Expression tests for the *Mtb*SD mutant enzymes

E. coli strain	Strain characteristics	Temperature tested (º C)	1 mM IPTG induction
BL21(DE3)	Deficiency of the protease *lon* e *omp*T, reducing the degradation of the expressed recombinant proteins	37	Yes
BL21(DE3)		37	No
BL21(DE3)		30	Yes
BL21(DE3)		30	No
BL21(DE3)		20	Yes
BL21(DE3)		20	No
C41(DE3)	Derived from BL21(DE3); prevents cell death associated with expression of many recombinant toxic proteins	37	Yes
C41(DE3)		37	No
C41(DE3)		30	Yes
C41(DE3)		20	Yes
C43(DE3)	Derived from C41(DE3); express a different set of toxic proteins as compared with C41(DE3)	37	Yes
C43(DE3)		37	No
C43(DE3)		30	Yes
C43(DE3)		20	Yes
C41(DE3)pLysS	Express T7 lysozyme, natural inhibitor of T7 RNA polymerase; stabilize recombinant encoding mainly toxic proteins	37	Yes
C41(DE3)pLysS		37	No
C43(DE3)pLysS		37	Yes
C43(DE3)pLysS		37	No

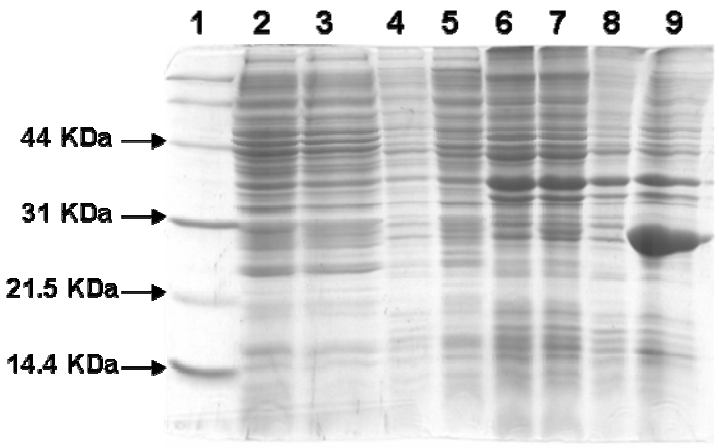

Figure 2: SDS-PAGE analysis of protein extracts from the mutant SD K69A in *E. coli* C41(DE3) host cells grown at 37 ºC for 24 h. Lane 1, molecular weight marker Low Range (Bio-Rad); Lanes 2-5, soluble fractions: 2, control without IPTG; 3, K69A without IPTG; 4, control with 1 mM IPTG; 5, K69A with 1 mM IPTG; Lanes 6-9, insoluble fractions: 6, control without IPTG; 7, K69A without IPTG; 8, control with 1 mM IPTG; 9, K69A with 1 mM IPTG.

After cellular growth under the conditions determined above, we tried to solubilize the insoluble bodies using the detergents sarcosyl, triton X-100, zwittergent 3-14, or with the chaotropic agent urea. Solubilization of recombinant wild-type and K69A *Mtb*SD enzymes in inclusion bodies was achieved by either treating the pellet with the denaturant agent urea (3 or 6M) or by treating with the non ionic detergent zwittergent 3-14 (5 %). These soluble fractions were dialyzed against Tris HCl 50 mM pH 7.8 buffer and the protein of interest remained in the soluble fraction after *in vitro* refolding (Figure 3).

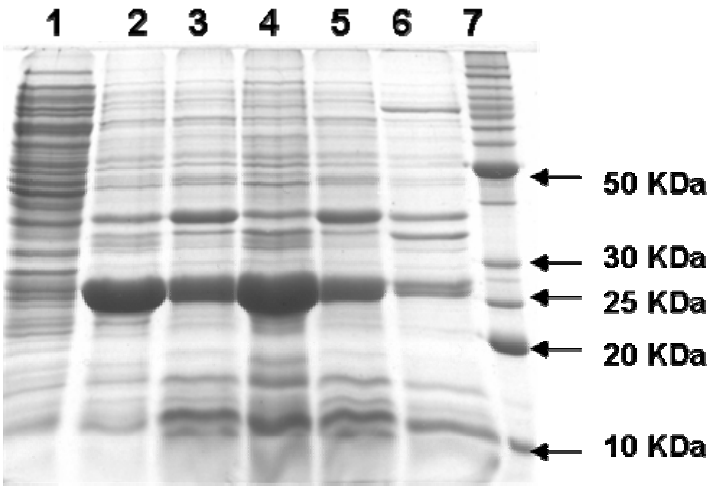

Figure 3: SDS-PAGE analysis of total protein extracts from the K69A SD mutant after solubilization using 5 % zwittergent 3-14. Lane 1, soluble protein fraction after disruption by sonication; 2, insoluble protein fraction after sonication; 3, soluble protein fraction after zwittergent 3-14 treatment; 4, insoluble protein fraction after zwittergent; 5, soluble protein fraction after *in vitro* refolding; 6, insoluble protein fraction after refolding; 7, Bench Mark Protein Ladder (Invitrogen).

However, the control experiment in which wild-type *Mtb*SD was solubilized by urea or zwittergent 3-14 showed that no measurable enzyme activity could be detected. Although inclusion body formation can greatly simplify protein purification, there is no guarantee that the *in vitro* refolding will yield large amounts of biologically active product. Moreover, inclusion body purification schemes present a number of problems such as: use of denaturants that are expensive and can cause irreversible modifications of protein structure that will elude all of the most sophisticated analytical tests, refolding usually must be done in very dilute solution and the protein reconcentrated, and refolding encourages protein isomerization leading to precipitation during

storage [38]. Since we aim at comparing steady-state kinetic parameters of the mutant enzymes with those of the wild-type to determine the catalytic mechanism of *Mtb*SD, the use of these solubilizing agents should be avoided. Accordingly, efforts were made to express recombinant *Mtb*SD proteins in its soluble form. The use of French press (Cell Disrupter System, Constant Systems Ltd, UK) as the cell disruption method, unfortunately, led to recombinant proteins in the insoluble fraction at all pressures tested: 20, 24, 27, 32 and 40 kpsi (40 kpsi = 2700 bar for 18 mm cylinder diameter). On the other hand, the freeze-thaw method was able to yield K69I, K69Q, K69H and D105N mutant proteins in soluble form (Figure 4). This method had previously been described to be an efficient procedure in releasing the soluble and active wild-type *Mtb*SD [22]. The freeze-thaw is not a lysis method and release of soluble proteins is through transient pores in the cellular envelope [34]. No differences were observed when 5, 10 or 15 cycles of the freeze-thaw procedure were tested (data not shown), and 5 cycles was then chosen. SDS-PAGE with Coomassie blue staining analysis showed a protein band with an apparent molecular mass of approximately 27 kDa, which is in agreement with the expected molecular mass value [23].

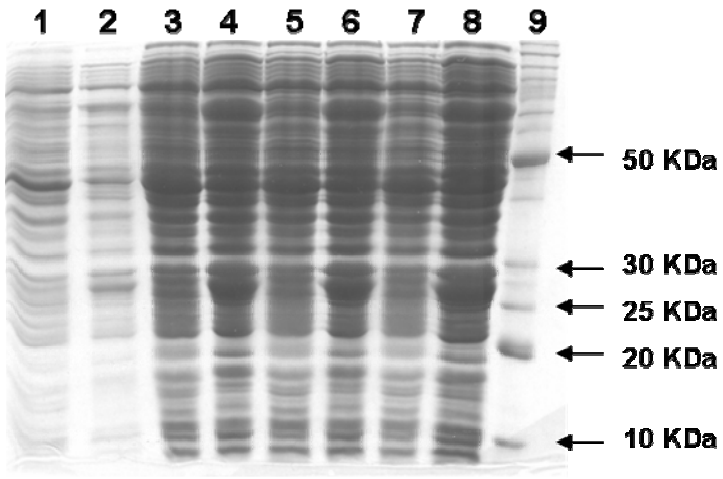

Figure 4: SDS-PAGE analysis of soluble protein extracts after 5 cycles of the freezing-and-thawing disruption method. Lanes 1, 3, 5, 7, control; 2, SD K69I mutant; 4, K69Q; 6, K69H; 8, D105N; 9, Bench Mark Protein Ladder (Invitrogen).

A purification protocol using four chromatographic steps was previously described [23]. Here, we describe optimization of recombinant *Mtb*SD protein purification protocol using three chromatographic steps (Table 3). This optimized protocol resulted in increased homogeneous recombinant protein yield (13.5 %) as compared to a previous protocol (8.9 %). Moreover, the protocol here described resulted in an 11.8-fold purification as compared to 8.5-fold purification as previously described. The latter needed 49 g of *E. coli* BL21(DE3) host cells harboring the recombinant pET23a(+)::*aro*E gene to yield 10.8 mg of homogeneous *Mtb*SD (0.2 mg *Mtb*SD/g cells). The protocol here described yields 1.5 mg of homogeneous *Mtb*SD protein from 5 g of *E. coli* C41(DE3) host cells (0.3 mg *Mtb*SD/g cells) that represents an improvement of approximately 50 % in the recombinant protein yield. The optimized protein purification protocol here described removed the first anion-exchange Q-

Sepharose Fast Flow column step and thus the supernatant of streptomycin precipitation step was loaded on a hydrophobic interaction Phenyl Sepharose Fast Flow column.

Table 3

Improved purification protocol for *Mtb*SD

Purification step	Total protein (mg)	Total activity (U)	Specific activity[a] (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	130.3	156.5	1.2	1	100
Phenyl-Sepharose FF	25.1	113.0	4.5	3.8	72.2
Sephacryl S-200	8.9	81.9	9.2	7.7	52.3
Mono-Q HR	1.5	21.2	14.1	11.8	13.5

The results presented are for a purification protocol from 5 g of *E. coli* host cells
[a] U mL^{-1} mg^{-1}.

It should be pointed out that the purification protocol described here does not depend on a histidine tag for the purification purpose of recombinant proteins. The strategies to obtain homogeneous shikimate dehydrogenases from *H. pylori* [21] and *H. influenzae* [26], YdiB protein from *E. coli* [28], SD-like enzyme from *H. influenzae* [20] employed recombinant proteins with histidine tags. Recombinant *Mtb*SD has also been tagged to a histidine tract to facilitate protein purification [39]. Although many protocols use histidine tags to facilitate protein purification by the nickel-affinity chromatography strategy, adding histidine tags may alter the protein structure and the biological activity [40,41]. Although addition of histidine tags to recombinant proteins facilitate purification protocols, we have made efforts to produce recombinant *Mtb*SD without any

fusion partner to avoid any possible effect that the latter may have on the enzyme. Thus improvement of protocols previously described was the strategy of choice, which proved fruitful because homogeneous recombinant K69A (Figure 5) and wild-type (data not shown) *Mtb*SD proteins were obtained by a three-step purification protocol, as assessed by SDS-PAGE, with increased protein yield (Table 3).

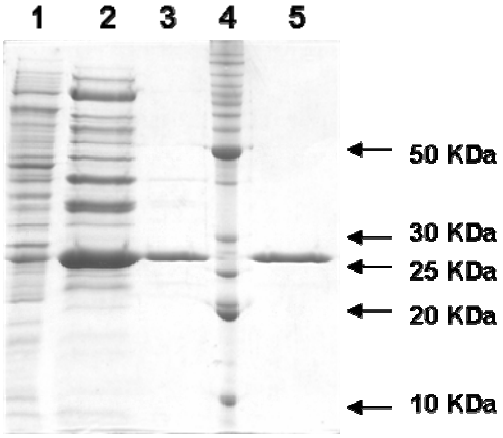

Figure 5: SDS-PAGE analysis of the protein fractions from purification steps of the mutant K69A *Mtb*SD. Lane 1, crude protein extract, injected on Phenyl Sepharose FF; 2, pooled protein fractions injected on Sephacryl S-200; 3, pooled protein fractions injected on Mono-Q HR; 4, Bench Mark Protein Ladder (Invitrogen); 5, purified K69A *Mtb*SD enzyme.

To ascertain that the apparent steady-state kinetics parameters of recombinant *Mtb*SD proteins were comparable with the ones using a previously reported protocol [23], determination of Km and Vmax were carried out for wild-type *Mtb*SD. All kinetic experiments were performed in potassium phosphate 100 mM buffer since in phosphate [24] the catalytic rate value for this enzyme seems to be larger than when the assay is carried out in Tris-HCl buffer [23].

The values obtained for the apparent steady-state kinetic parameters were as follows: Km = 29 (±2 μM) for DHS, Km = 11 (±1 μM) for DHS, k_{cat} = 50 (±1) s^{-1}. These values are in agreement with previously reported parameters [23,24]. It should be noted that here we determined apparent steady-state kinetic parameters and not true parameters as described elsewhere [24]. These data provide a solid foundation on which to base analysis of recombinant *Mtb*SD mutant enzymes to determine the amino acid residues that are essential for catalysis and/or binding of the substrates.

Here we report, to the best of our knowledge, the first site-directed mutagenesis work on *Mtb*SD. We also describe an efficient expression protocol and an improved recombinant protein purification protocol that should be employed to obtain homogeneous *Mtb*SD mutant proteins. It should be pointed out that expression and purification of functional wild-type and mutants of *Mtb*SD have proved a laborious goal to achieve. This is probably the reason for only a scarce number of papers having appeared on studies of *Mtb*SD mechanism of action. A thorough characterization of wild-type and mutants of *Mtb*SD should provide a better understanding of the catalytic and chemical mechanisms of this enzyme. However, these studies hinge on the availability of *Mtb*SD proteins in soluble and functional forms. We thus hope that the results here presented will pave the way for the target-based rational design of novel effective antimicrobial agents.

ACKNOWLEDGMENTS

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3.3 Manuscrito 2

The conserved Lysine 69 residue plays a catalytic role in *Mycobacterium tuberculosis* shikimate dehydrogenase

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Keywords: *Mycobacterium tuberculosis*, shikimate pathway, shikimate dehydrogenase, site-directed mutagenesis

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Short title: Role of Lysine 69 in *Mtb*SD

ABSTRACT

The *Mycobacterium tuberculosis* shikimate dehydrogenase (*Mtb*SD) is a target for the development of antitubercular agents. Structural and functional studies indicate that Lysine69 may be involved in catalysis and/or substrate binding in *Mtb*SD. Here we show that this residue plays a catalytic role and is not involved in substrate binding.

Tuberculosis (TB) remains a major global health concern. It has been estimated that one-third of the world population is infected with *Mycobacterium tuberculosis*, the causative agent of TB, and that 30 million people died from this disease in the last decade (1). Among the possible factors involved in the resurgence of TB are the epidemic of the human immunodeficiency virus, the increase in the homeless population, and the decline in health care structures and national surveillance (2). Inappropriate treatment regimens and patient noncompliance in completing the therapies are commonly associated with the emergence of multi-drug resistant TB (MDR-TB), defined as strains of *M. tuberculosis* resistant to at least isoniazid and rifampicin, two pivotal drugs used in the standard treatment of TB (3, 4). More recently, it has been reported the emergence of extensively drug-resistant (XDR) TB cases, defined as cases in persons with TB whose isolates are resistant to isoniazid and rifampicin as well as resistant to any one of the fluoroquinolone drugs and to at least one of the three injectable second-line drugs (5). XDR-TB is widespread raising the prospect of virtually incurable TB worldwide (6). There is thus an urgent need for new drugs to improve the treatment of MDR- and XDR-TB, and to provide more effective drugs to shorten the duration of TB treatment.

The shikimate pathway is an attractive target for the development of herbicides and antimicrobial agents because it is essential in algae, higher plants, bacteria, and fungi, but absent from mammals (7). The mycobacterial shikimate pathway leads to the biosynthesis of precursors of aromatic amino acids, naphthoquinones, menaquinones, and mycobactins (8). This pathway has been shown to be essential for the viability of *M. tuberculosis* (9). Accordingly, the enzymes of the shikimate pathway represent promising targets

for the development of non-toxic antimycobacterial agents. Shikimate dehydrogenase (SD; EC 1.1.1.25), the fourth enzyme of this pathway, catalyzes the NADPH-dependent reduction of 3-dehydroshikimate (DHS) to shikimate (SHK). We have previously reported the cloning and expression of functional *aroE*-encoded SD from *M. tuberculosis* H37Rv strain (*Mtb*SD) (10). We have also reported purification to homogeneity of recombinant functional *Mtb*SD, *N*-terminal amino acid sequencing, electrospray ionization mass spectrometry analysis, size exclusion chromatography, determination of the apparent kinetic parameters for all substrates, thermal stability, and activation energy for the enzyme-catalyzed chemical reaction (11). More recently, we have described the kinetic and chemical mechanisms of recombinant *Mtb*SD (12). Multiple sequence alignment, homology modeling, and pH-rate profiles suggest that the side chain of Lys-69 in the DHS/SHK binding site of *Mtb*SD may play a role in substrate binding and/or catalysis (12, 13). Here we describe site-directed mutagenesis, steady-state kinetics and fluorimetric measurements to probe the role of Lys-69 in *Mtb*SD and provide insight into the molecular basis of DHS/SHK recognition and/or catalysis.

A previously constructed pET-23a(+)::*aro*E recombinant vector (10) was used as a template for site-directed PCR-based mutagenesis using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis primers were designed to change AAG (encoding a lysine residue) to GCG (encoding an alanine residue). The synthetic oligonucleotides employed as primers were as follows: 5' ggtgtttcggtgaccatgccgggc**gcg**ttcgccgccctgcggttcg 3' (forward) and 5' cgaaccgcagggcggcgaa**cgc**gcccggcatggtcaccgaaacacc 3' (reverse) (in bold is the codon for alanine). The PCR amplification program

using *PfuTurbo*® DNA polymerase was as follows: one step of 95º C for 30 s, 16 cycles at 95º C for 30 s, 55º C for 1 min, and 68º C for 10 min, followed by a final extension step at 68º C for 10 min. The PCR product was treated with *Dpn*I endonuclease that specifically digests the methylated DNA template, and selects for the mutation-containing synthesized DNA. Sequencing of mutagenic *aroE* gene in pET-23a(+) vector from a single colony confirmed that the mutation was introduced into the expected site and that no unwanted mutations were introduced by the PCR amplification step (data not shown). This recombinant plasmid was introduced into *E. coli* C41 (DE3) host cells (Novagen, Madison, WI) by electroporation. Single colonies were used to inoculate 2 L of LB medium containing 50 μg mL^{-1} ampicillin, and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to cultures reaching an OD_{600} of 0.4 - 0.6, and grown for 24 h at 37º C at 180 rpm. Cells (5 g) were harvested by centrifugation at 14,900*g* for 30 min at 4º C, and stored at -20º C. The freeze-thaw method was used to release the proteins in the soluble fraction as previously described (10). The purification protocol was essentially as previously described (11) except for removal of the first anionic exchange chromatographic step (a thorough description of this improved protocol will be described elsewhere – manuscript in preparation). Samples of the purification steps were analyzed by SDS-PAGE (14) and the protein content was determined by the Bradford method (15), using the Bio-Rad protein assay kit (Bio-Rad) and bovine serum albumin as standard.

Steady-state kinetics measurements of homogeneous *Mtb*SD K69A mutant activity were carried out for the forward direction at 25º C in 100 mM potassium phosphate buffer, pH 7.3, by monitoring the decrease in absorbance at 340 nm

(ε=6220 $M^{-1}cm^{-1}$ for NADPH) accompanying the conversion of NADPH and DHS to $NADP^+$ and SHK. The K69A activity was measured at various final concentrations of DHS (20, 50, 70, 100, 150, 200, 250, 300 μM) while NADPH was maintained at a saturating level (200 μM), and various concentrations of NADPH (20, 30, 50, 70, 100, 150, 200, 250 μM) while DHS was maintained at a saturating level (250 μM). The wild-type (WT) SD activity was measured at various concentrations of DHS (10, 20, 30, 40, 50, 80, 100 μM) while NADPH was maintained at a saturating level (200 μM), and various concentrations of NADPH (5, 10, 15, 20, 40, 50 μM) while DHS was maintained at a saturating level (250 μM). All measurements were in duplicate. Kinetic constants were obtained by non-linear regression analysis of the kinetic data using the SigmaPlot software (SPSS, Inc) and the Michaelis-Menten equation ($v = V_{max} \times [S]/K_m + [S]$). The apparent steady-state kinetic parameters (Table 1) show that the catalytic constant (k_{cat}) value for wild-type *Mtb*SD (50 s^{-1}) is 68-fold larger than K69A (0.73 s^{-1}). There was a modest increase in the K_m values for DHS (K69A=76 μM; WT *Mtb*SD=29 μM) and NADPH (K69A=30 μM; WT *Mtb*SD=11 μM). The apparent second-order rate constant (k_{cat}/K_m) values for DHS (K69A=9.6 x 10^3 $M^{-1}s^{-1}$; WT *Mtb*SD=1.7 x 10^6 $M^{-1}s^{-1}$) and NADPH (K69A=24 x 10^3 $M^{-1}s^{-1}$; WT *Mtb*SD=4.5 x 10^6 $M^{-1}s^{-1}$) may apparently indicate that the mutant has a lower specificity constant for both substrates. However, it should be noted that the dominant feature is a decrease in the catalytic constant and only a modest decrease in the Michaelis-Menten constants for both substrates. These results suggest that the Lysine69 residue in *Mtb*SD is involved in catalysis and plays a minor role in substrate binding. To confirm this proposal, spectrofluorimetric assays were carried out to determine the dissociation

constant for enzyme-substrate binary complex formation. Fluorescence titration of substrate was carried out at 25 ºC making microliter additions of concentrated substrate solutions to 2 mL of 1 µM of *Mtb*SD monitoring the intrinsic protein fluorescence (λexc=300 nm; 310 ≤ λem ≤ 450nm; maximum λem at 340 nm). The fluorescence intensity at varying DHS concentration (2-450 µM) yielded dissociation constant (Kd) values of 32 (± 4) µM for WT *Mtb*SD and 134 (± 21) µM for K69A mutant (Table 1).

TABLE 1

Apparent steady-state kinetic parameters and equilibrium binding constants for wild type and K69A mutant *Mtb*SD

Parameter	Wild type	K69A
V_{max} (U mg^{-1})[a]	110 ± 2	1.61 ± 0.03
K_m DHS (µM)[a]	29 ± 2	76 ± 4
K_m NADPH (µM)[a]	11.0 ± 0.6	30 ± 2
k_{cat} (s^{-1})[a]	50 ± 1	0.73 ± 0.01
k_{cat}/K_m DHS (M^{-1} s^{-1})[a]	1.7 (± 0.1) x 10^6	9.6 (± 0.5) x 10^3
k_{cat}/K_m NADPH (M^{-1} s^{-1})[a]	4.5 (± 0.2) x 10^6	24 (± 2) x 10^3
Kd DHS (µM)[b]	32 ± 4	134 ± 21

[a] steady-state kinetic parameters

[b] spectroscopic measurements of intrinsic protein fluorescence

Interestingly, measurements of changes in nucleotide fluorescence upon NADPH binding to WT *Mtb*SD (λexc=370 nm; 380 ≤ λem ≤ 600nm; maximum λem at 445 nm) did not show any saturation, which indicates a very large Kd value. This is consistent with a steady-state ordered bi-bi mechanism with DHS binding first followed by NADPH binding to *Mtb*SD active site (12). The steady-

state kinetics and spectrofluorimetric measurements indicate that the conserved Lys69 residue in *Mtb*SD plays a critical role in catalysis, but plays no role in substrate binding.

Analysis of the three-dimensional structures for the ternary complexes of SDs from *Thermus termophilus* (16) and *Aquifex aeolicus* (17) in complex with $NADP^+$ and SHK suggest that the protonated lysine residue (Lys64 in *T. termophilus* and Lys70 in *A. aeolicus*) interacts with carbon-3 of DHS, thereby acting as an acid-base catalytic group that donates a proton to the carbonyl of DHS during reduction and that removes a proton during oxidation of SHK. The Lys67 in *Haemophilus influenzae* SD (18) and Lys385 in *Arabidopsis thaliana* dehydroquinate dehydratase-SD (19) play a critical role in catalysis. Interestingly, substitution of the conserved lysine residue (Lys71) in *E. coli* quinate/shikimate dehydrogenase YdiB with alanine caused only a modest decrease in k_{cat} and a large increase in K_m for SHK (20). These results demonstrate that enzymes that catalyze the same chemical reaction may do so by employing different catalytic mechanisms. Based on double isotope effects and pH-rate profiles, we have previously proposed that the chemical mechanism for *Mtb*SD involves hydride transfer and solvent proton transfer in a concerted mechanism, and an amino acid residue with pK_a value of 8.9 is involved in catalysis. Here we demonstrate that the *Mtb*SD Lys69 is important for catalysis and is likely involved in stabilization of the developing negative charge at the hydride-accepting C-3 carbonyl oxygen of DHS for the forward reaction. This knowledge should assist in the rational design of antitubercular agents.

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4 DISCUSSÃO

4.1 Mutagênese sítio-direcionada do gene *aro*E

Estudos de alinhamentos de seqüências de muitas SD mostram que, dentre outros aminoácidos, os resíduos Lis e Asp (Lis69 e Asp105 da *Mtb*SD) são completamente conservados e sugerem a provável importância destes aminoácidos para a catálise e/ou ligação ao substrato (MICHEL *et al.*, 2003; HAN *et al.*, 2006; FONSECA *et al.*, 2007; YE *et al.*, 2003; BAGAUTDINOV e KUNISHIMA, 2007). A determinação da estrutura tridimensional de enzimas SD (AroE, YdiB e SD-like) de muitos organismos também sugere o envolvimento destes aminoácidos na catálise e/ou ligação ao substrato e revela a conservação do padrão estrutural dentre as enzimas da família da SD (MICHEL *et al.*, 2003; SINGH *et al.*, 2005; YE *et al.*, 2003; BAGAUTDINOV e KUNISHIMA, 2007; PADYANA e BURLEY, 2003; GAN *et al.*, 2007; LINDNER *et al.*, 2005). Além disso, estudos de mutagênese sítio-direcionada confirmaram a importância destes dois resíduos para a catálise (na SD-like de *H. influenzae e na* DHQ-SD de *A. thaliana*) e para a ligação ao substrato (na YdiB de *E. coli*; LINDNER *et al.*, 2005; SINGH e CHRISTENDAT, 2006; SINGH *et al.*, 2005). Baseados em estudos estruturais e funcionais, é provável que os resíduos conservados Lis69 e Asp105 tenham importância na catálise, na ligação ao substrato, ou em ambos, na enzima *Mtb*SD. Para investigar a importância, se existir alguma, destes resíduos nós substituímos a Lis69 por Ala, Ile, His ou Gln, e o Asp105 por Ala ou Asn através da técnica de mutagênese sítio-direcionada. Primeiramente, projetamos os pares de oligonucleotídeos iniciadores para as mutações desejadas, seguindo as instruções do fabricante:

ambos devem conter a mutação e anelar à seqüência oposta de cada fita do plasmídeo; devem possuir de 25 a 45 bases; a trinca correspondente ao aminoácido substituinte deve estar aproximadamente no meio da seqüência iniciadora; cada oligonucleotídeo iniciador deve ter um conteúdo mínimo de 40 % de citosina e guanina (**Tabela 1** do Manuscrito 1). A técnica de mutagênese através de amplificação por PCR foi um procedimento eficaz, que permitiu mutação no plasmídeo recombinante dupla fita, pET23a(+)::*aro*E. Cabe ressaltar a importância de tratar o produto da amplificação por PCR com a endonuclease *Dpn*I. Esta enzima cliva especificamente o DNA metilado que fora usado como molde, selecionando, assim, o DNA sintetizado possivelmente contendo a mutação e excluindo o DNA não mutado. Após os procedimentos de mutagênese, clones de cada mutação tiveram seu DNA plasmidial clivado com as enzimas de restrição *Nde*I e *Bam*HI, flanqueando o gene de interesse, para verificar a presença do plasmídeo recombinante possivelmente mutado (**Anexo 6.4**).

Na seqüência, alguns dos clones cujos plasmídeos liberaram o inserto adequadamente foram enviados para o seqüenciamento de DNA. As seqüências dos genes *aro*E mutantes foram verificadas para confirmar a alteração dos nucleotídeos desejados e também para certificar que nenhuma outra mutação indesejada tenha sido introduzida aleatoriamente durante o passo de PCR (dados não mostrados).

4.2 Super-expressão das proteínas mutantes

Após os resultados do seqüenciamento, os plasmídeos mutantes foram eletroporados em células de *E. coli* para testes de expressão protéica. Um protocolo de expressão da enzima *Mtb*SD recombinante havia sido descrito usando a cepa de *E. coli* BL21 (DE3), sem indução com IPTG (MAGALHÃES *et al.*, 2002). Infelizmente, não foi observada a expressão das proteínas mutantes na fração solúvel, nem na insolúvel, seguindo este protocolo. Então, testamos outras condições de crescimento como temperaturas reduzidas de cultura, outras cepas de *E. coli*, com e sem indução por IPTG (**Tabela 2** do Manuscrito 1), além de diferentes tempos de crescimento. Conseguimos a expressão destas proteínas na cepa *E. coli* C41 (DE3), com crescimento por 24 h a 37º C e a 180 rpm, depois da indução com 1 mM de IPTG e rompimento celular por sonicação (**Figura 2** do Manuscrito 1). A necessidade da adição de IPTG para favorecer a expressão é consistente com o uso do vetor pET23a(+). O sistema de expressão pET utiliza a T7 RNA polimerase, sob controle do promotor lacUV5 induzido por IPTG, para transcrever genes de interesse (KELLEY *et al.*, 1995).

Depois da multiplicação celular nas condições determinadas acima, testamos a solubilização das proteínas de interesse, insolúveis, usando os detergentes sarcosil, triton X-100, zwittergent 3-14 ou o agente caotrópico uréia. Assim, conseguimos obter uma fração da proteína mutante K69A na fração solúvel após tratar o agregado insolúvel com o agente desnaturante uréia (3 e 6 M) e com o detergente não-iônico zwittergent 3-14 na concentração de 5 %; a proteína de interesse permaneceu na fração solúvel após *refolding in vitro* (**Figura 3** do Manuscrito 1). Entretanto, os procedimentos de *unfolding* e

refolding podem causar modificações irreversíveis na estrutura protéica, inclusive podem afetar a sua atividade biológica (SCHEIN, 1989).

De fato, quando testamos a atividade da enzima *Mtb*SD sem mutação após utilizar os protocolos de solubilização que utilizam uréia e zwittergent 3-14, esta enzima não apresentou atividade mensurável (dados não mostrados). Já que o nosso objetivo é comparar os perfis cinéticos das proteínas mutantes com o da não mutante, o uso destes agentes que podem modificar a atividade enzimática deve ser evitado. Então, os nossos esforços foram focalizados no sentido de obter a expressão destas proteínas na fração solúvel.

O método de rompimento celular que utiliza a prensa de French levou as proteínas recombinantes de interesse à fração insolúvel após testes com variadas pressões (dados não mostrados). Felizmente, o método de congelamento e descongelamento foi testado e, com sucesso, conseguiu liberar uma fração das proteínas de interesse do citoplasma da célula (**Figura 4** do Manuscrito 1).

Este método havia sido previamente usado para obter a enzima *Mtb*SD não mutada na fração solúvel e em sua forma ativa (MAGALHÃES *et al.*, 2002). Cabe ressaltar que este não é um método de lise como a sonicação e a prensa de French; as proteínas solúveis são liberadas através de poros transientes que se formam na membrana celular durante os ciclos de congelamento e descongelamento (JOHNSON e HECHT, 1994).

De uma maneira geral, dificuldades na expressão de proteínas na fração solúvel são consideradas importantes empecilhos aos estudos bioquímicos e cristalográficos das proteínas de *M. tuberculosis* (VICENTELLI *et al.*, 2003). Anteriormente, a expressão da *Mtb*SD na fração solúvel havia sido uma tarefa

difícil de atingir (MAGALHÃES *et al.*, 2002). O protocolo de expressão estabelecido aqui representa um importante passo para conseguir as proteínas mutantes na fração solúvel e que permitirá os estudos funcionais desejados.

4.3 Purificação da enzima mutante K69A

Conseguimos purificar a enzima mutante K69A até a aparente homogeneidade utilizando um protocolo otimizado em relação ao descrito anteriormente (FONSECA *et al.*, 2006). Este novo protocolo envolve três etapas cromatográficas, enquanto o anterior possui quatro. Com o protocolo otimizado obteve-se aumentos de 8,9 para 13,5 % no rendimento da purificação e de 8,5 para 11,8 na taxa de purificação (**Tabela 3** do Manuscrito 1).

O protocolo anterior produzia 10,8 mg de *Mtb*SD homogênea a partir de 49 g de células *E. coli* BL21(DE3) (0,2 mg *Mtb*SD/g célula). O protocolo utilizado neste trabalho produz 1,5 mg da proteína *Mtb*SD a partir de 5 g de células *E. coli* C41(DE3) (0,3 mg *Mtb*SD/g célula), o que representa um aumento próximo a 50 % na produção desta proteína recombinante. Além disso, deve-se ressaltar que este protocolo de purificação não utiliza a adição de cauda de histidina a fim de facilitar a purificação de proteínas recombinantes. As estratégias para purificar as enzimas AroE de *H. pylori* (HAN *et al.*, 2006), AroE de *H. influenzae* (YE *et al.*, 2003), YdiB de *E. coli* (BENACH *et al.*, 2003), SD-like de *H. influenzae* (SINGH *et al.*, 2005) resultam em proteínas recombinantes com caudas extras de histidina. Um protocolo de purificação que adiciona cauda de histidina à SD recombinante de *M. tuberculosis* já foi publicado (ZHANG *et al.*, 2005).

Embora muitos protocolos que utilizam esta estratégia de cromatografia de afinidade por níquel compreendam uma única coluna de purificação, a adição de cauda de histidina pode alterar significativamente a estrutura protéica e sua atividade biológica (CHANT *et al.*, 2005; FONDA *et al.*, 2002). Tendo isto

em mente, evitou-se utilizar técnicas que pudessem alterar o perfil protéico e impedir que nossos objetivos de caracterização enzimática fossem alcançados.

Assim, as enzimas *Mtb*SD sem mutação e a mutante K69A (**Figura 5** do Manuscrito 1) foram purificadas até a aparente homogeneidade usando o protocolo de apenas três passos cromatográficos. Determinamos os parâmetros cinéticos aparentes da enzima *Mtb*SD não mutante após purificação utilizando o protocolo otimizado (**Tabela 1** do Manuscrito 2). Os valores de Km e Vmáx determinados neste trabalho são muito semelhantes às constantes aparentes já publicadas para esta enzima (FONSECA *et al.*, 2006), provando que o protocolo de purificação otimizado resulta na enzima *Mtb*SD ativa e permitindo futuras análises funcionais das proteínas mutantes após purificação por este protocolo.

4.4 Caracterização da enzima mutante K69A

Depois da purificação da enzima *Mtb*SD K69A, realizamos imediatamente os experimentos de caracterização enzimática, determinando as constantes cinéticas aparentes, Km e Vmáx desta enzima mutante, no sentido direto da reação.

Os parâmetros cinéticos aparentes (**Tabela 1** do Manuscrito 2) mostram que o valor da constante catalítica (Kcat) da enzima *Mtb*SD sem mutação é 68 vezes maior do que o valor da K69A. Foram observados aumentos modestos nos valores de Km para o DHS e para o NADPH. A enzima mutante apresentou diminuição de 182 vezes na eficiência catalítica (Kcat/Km) para o substrato DHS e de 189 vezes para o cofator NADPH. A diminuição na eficiência catalítica foi devida predominantemente ao valor de Kcat diminuído, com pouco efeito dos ligeiros aumentos nas constantes de Michaelis-Menten para ambos os substratos. De fato, o valor de Kcat para a enzima mutante permaneceu apenas 1,46 % daquele da enzima não mutante.

Estes resultados sugerem que a o resíduo Lis69 da *Mtb*SD está envolvido na catálise e que tem pouca ou nenhuma importância na ligação ao substrato. Para confirmar essa proposição, ensaios de espectrofluorimetria foram realizados para determinar a constante de dissociação (Kd) para a formação do complexo binário enzima-substrato. Os resultados destes experimentos apontam diferenças modestas nos valores de Kd para as enzimas *Mtb*SD não mutante e K69A (**Tabela 1** do Manuscrito 2). Assim, ensaios cinéticos em estado estacionário e medidas espectrofluorimétricas indicam que o resíduo conservado Lis69 tem importante função na catálise e que não está envolvido na ligação ao substrato na enzima *Mtb*SD.

Análise de estruturas tridimensionais dos complexos ternários de SDs de *Thermus termophilus* (BAGAUTDINOV e KUNISHIMA, 2007) e de *Aquifex aeolicus* (GAN *et al.*, 2007) complexados com $NADP^+$ and SHK sugerem que o resíduo protonado Lis (Lis64 de *T. termophilus* and Lis70 in *A. aeolicus*) interage com o carbono-3 do DHS, atuando, então, como um grupo catalítico ácido-básico que doa um próton para o grupamento carbonila do DHS durante a redução e que remove um próton durante a oxidação do SHK.

A Lis67 da SD-like de *Haemophilus influenzae* (SINGH *et al.*, 2005) e a Lis385 da DHQ-SD de *Arabidopsis thaliana* (SINGH e CHRISTENDAT, 2006) têm função crítica na catálise. Interessantemente, a substituição do resíduo Lis conservado (Lis71) na quinato/SD YdiB de *E. coli* por Ala causou apenas uma pequena diminuição no Kcat e um grande aumento no Km do SHK (LINDNER *et al.*, 2005). Estes resultados demonstram que enzimas que catalisam as mesmas reações químicas podem usar mecanismos catalíticos diferentes. Aqui, demonstramos que a Lis69 da *Mtb*SD é importante para a catálise e que provavelmente esteja envolvida na estabilização da carga negativa gerada no átomo oxigênio da carbonila do DHS, na reação direta.

De certa forma, parecia intrigante que os resultados de mutagênese sítio-direcionada para as enzimas SDs, até então, sugeriam papéis diferentes para este resíduo nestas enzimas: a Lis69 (numeração de acordo com a sequência da *Mtb*SD) é crucial para a catálise na enzima DHQ-SD de *A. thaliana* (SINGH e CHRISTENDAT, 2006) e na SD-like de *H. influenzae* (SINGH *et al.*, 2005), mas é importante somente para a ligação ao substrato na YdiB de *E. coli* (LINDNER et al., 2005).

As enzimas AroE, YdiB e SD-like têm estruturas tridimensionais muito similares mas suas propriedades bioquímicas como especificidade por substratos e parâmetros cinéticos são muito distintos (SINGH *et al.*, 2005). Embora AroE, YdiB e SD-like consigam catalisar a reação de chiquimato desidrogenase, esta pode ser somente uma atividade secundária para YdiB e SD-like; YdiB possui outros substratos específicos, catalisando a redução reversível do desidroquinato a quinato; os valores de Kcat para YdiB de *E. coli* e para SD-like de *H. influenza* são muito baixos comparados aos Kcat das enzimas AroE. (LINDNER et al., 2005; SINGH *et al.*, 2005). Estas diferenças funcionais e bioquímicas podem estar relacionadas com os prováveis distintos papéis que o resíduo Lis69 possa ter dentre as enzimas da família chiquimato desidrogenase.

Os nossos resultados estão de acordo com os estudos de mutagênese envolvendo a enzima bifuncional DHQ-SD de *A. thaliana* e a SD-like de *H. influenzae*, já que os valores de Kcat estão bastante diminuídos e mudanças insignificantes são observadas nos valores de Km e nos resultados de fluorimentria, comparando a enzima mutante K69A com a não mutante. (SINGH *et al.*, 2005; SINGH e CHRISTENDAT, 2006).

Este trabalho apresentou a primeira identificação de um aminoácido cataliticamente importante para a enzima *Mtb*SD, através da técnica de mutagênese sítio-direcionada. A purificação e os ensaios cinéticos das outras enzimas mutantes K69I, K69H, K69Q, D105A, D105N poderão ajudar a melhor caracterizar o mecanismo catalítico desta enzima de *M. tuberculosis*. Um entendimento mais detalhado, a nível molecular, das enzimas da via do

chiquimato pode servir como base para o desenho racional de novos e efetivos agentes antimicrobianos.

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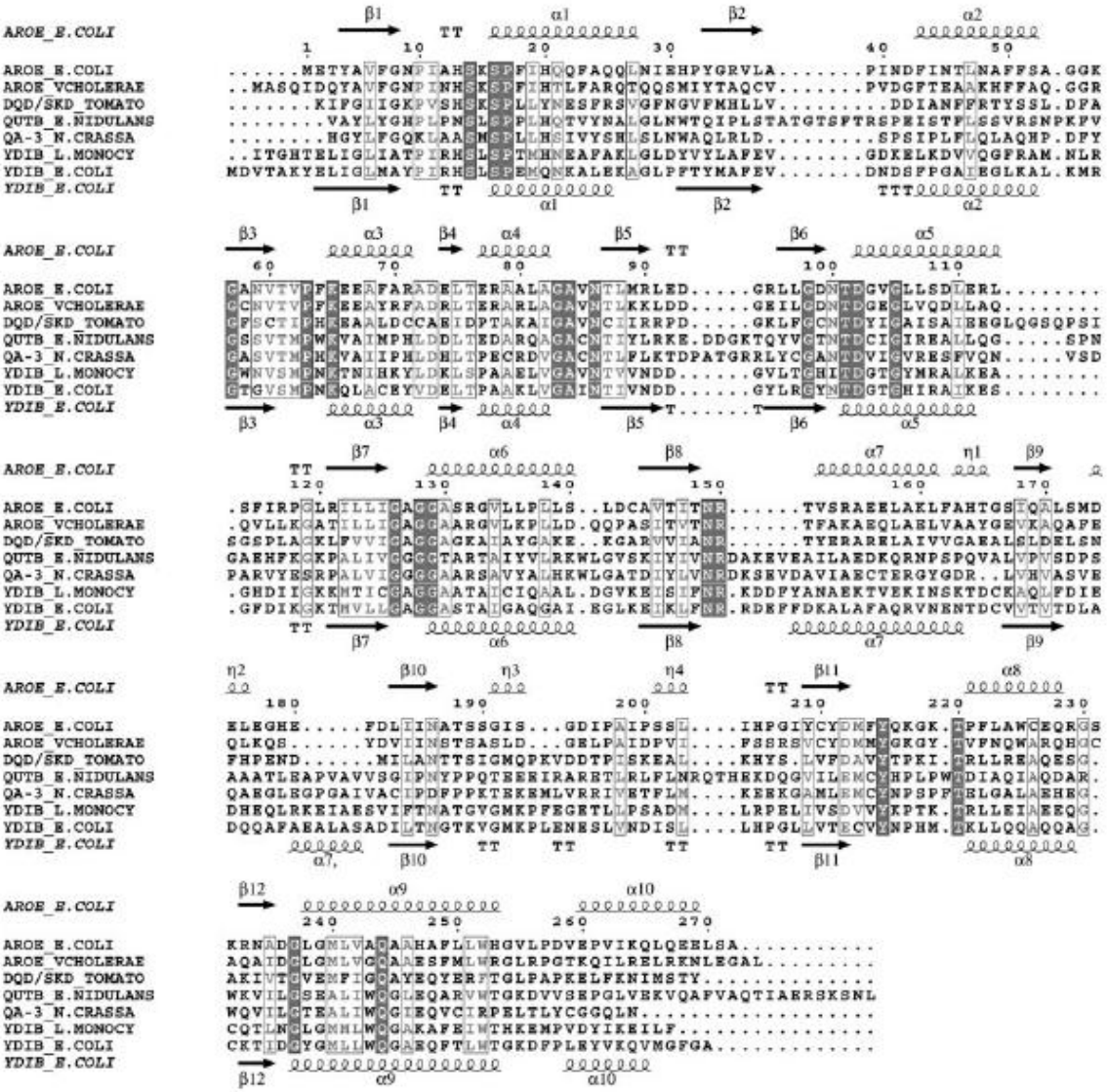
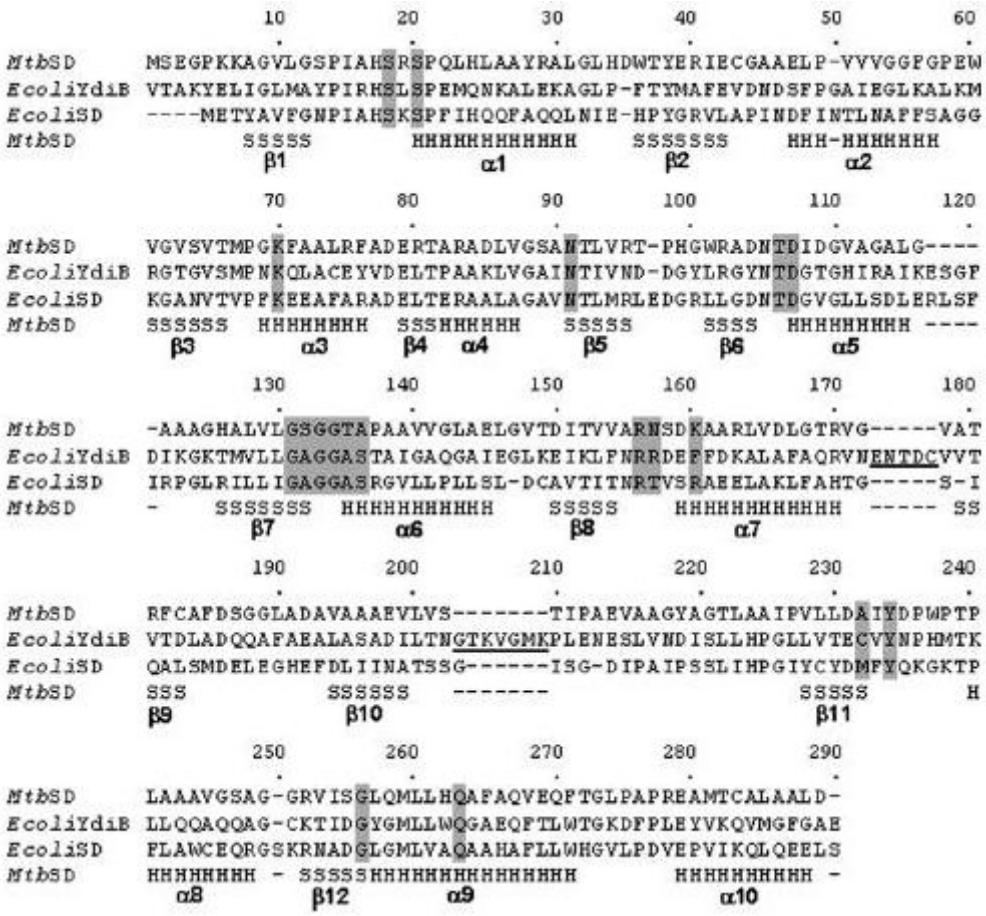
6 ANEXOS

6.1 Alinhamentos de sequências de chiquimato desidrogenases

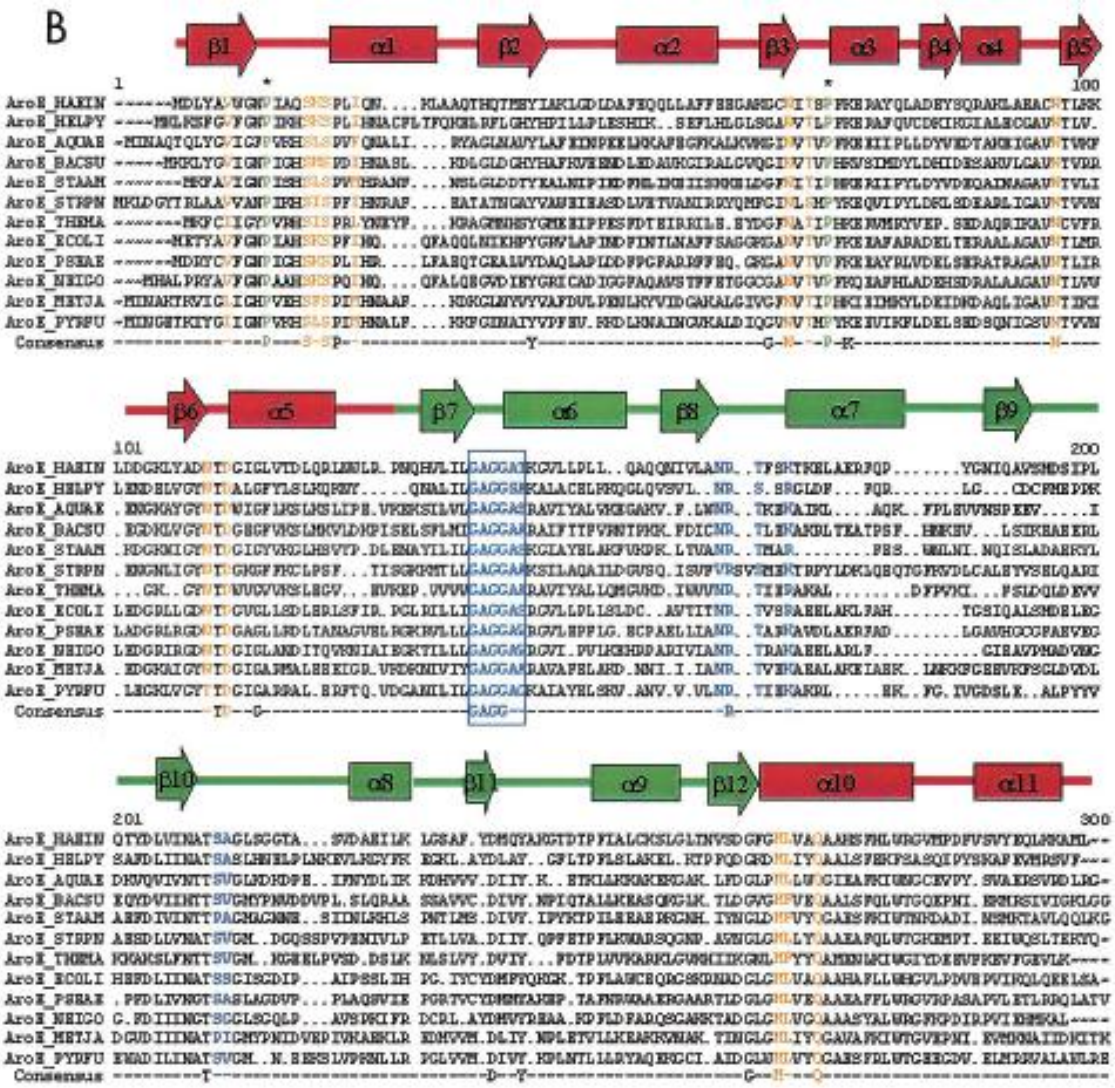


Alinhamento múltiplo de seqüências das enzimas AroE de *E. coli*, *Vibrio cholerae*, QutB de *E. nidulans*, Qa-3 de *N. crassa*, YdiB de *E. coli* e *L. monocytogenes*, DHQ-SD de *Lycopersicon esculentum* (MICHEL et al., 2003).

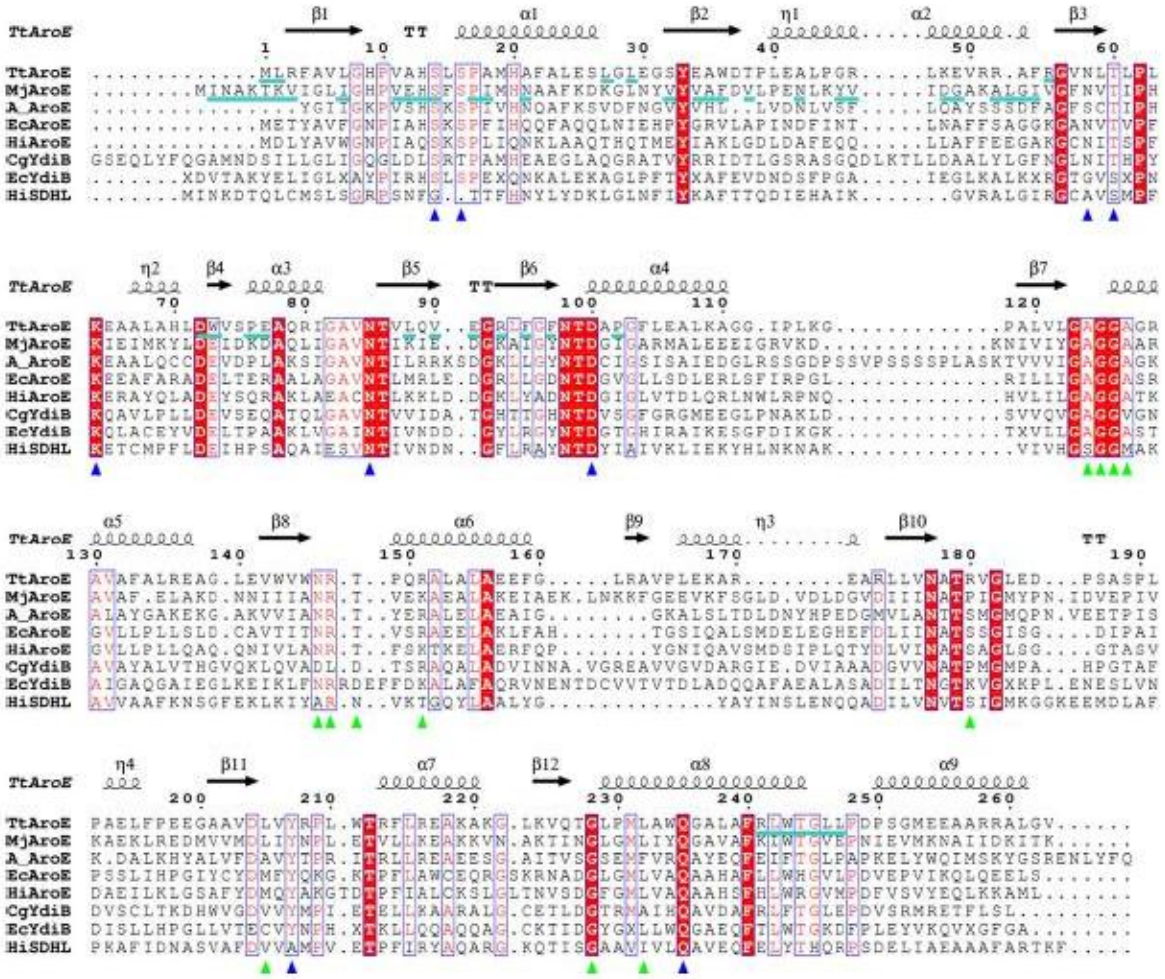
Alinhamento de seqüências de SD de várias bactérias: *E. coli*, *H. influenzae*, *N. meningitidis*, *M. jannaschii*, *A. fulgidus*, *M. tuberculosis* e *H. pylori* (HAN et al., 2006).



Alinhamento múltiplo de seqüências das enzimas AroE de *Mycobacterium tuberculosis*, AroE de *E. coli* e YdiB de *E. coli* (FONSECA et al., 2007).



Alinhamento múltiplo de seqüências de AroE dos seguintes organismos: *H. influenzae*, *Helicobacter pylori*, *Aquifex aeolicus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Thermotoga marítima*, *E. coli*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Methanococcus jannaschii* e *Pyrococcus furiosus* (YE et al., 2003).



Alinhamento múltiplo de seqüências das enzimas AroE de *Thermus thermophilus*, *E. coli*, *H. influenzae* e *M. jannaschii*, YdiB de *E. coli* e *Corynebacterium glutamicum*, SD-like de *H. influenzae*, DHQ-SD de *A. thaliana* (BAGAUTDINOV e KUNISHIMA, 2007).

6.2 Seqüência dos resíduos de aminoácidos da *Mtb*SD [#]

MSEGPKKAGV LGSPIAHSRS PQLHLAAYRA LGLHDWTYER IECGAAELPV

VVGGFGPEWV GVSVTMPG**K**F AALRFADERT ARADLVGSAN TLVRTPHGWR

ADNT**D**IDGVA GALGAAAGHA LVLGSGGTAP AAVVGLAELG VTDITVVARN

SDKAARLVDL GTRVGVATRF CAFDSGGLAD AVAAAEVLVS TIPAEVAAGY

AGTLAAIPVL LDAIYDPWPT PLAAAVGSAG GRVISGLQML LHQAFAQVEQ

FTGLPAPREA MTCALAALD

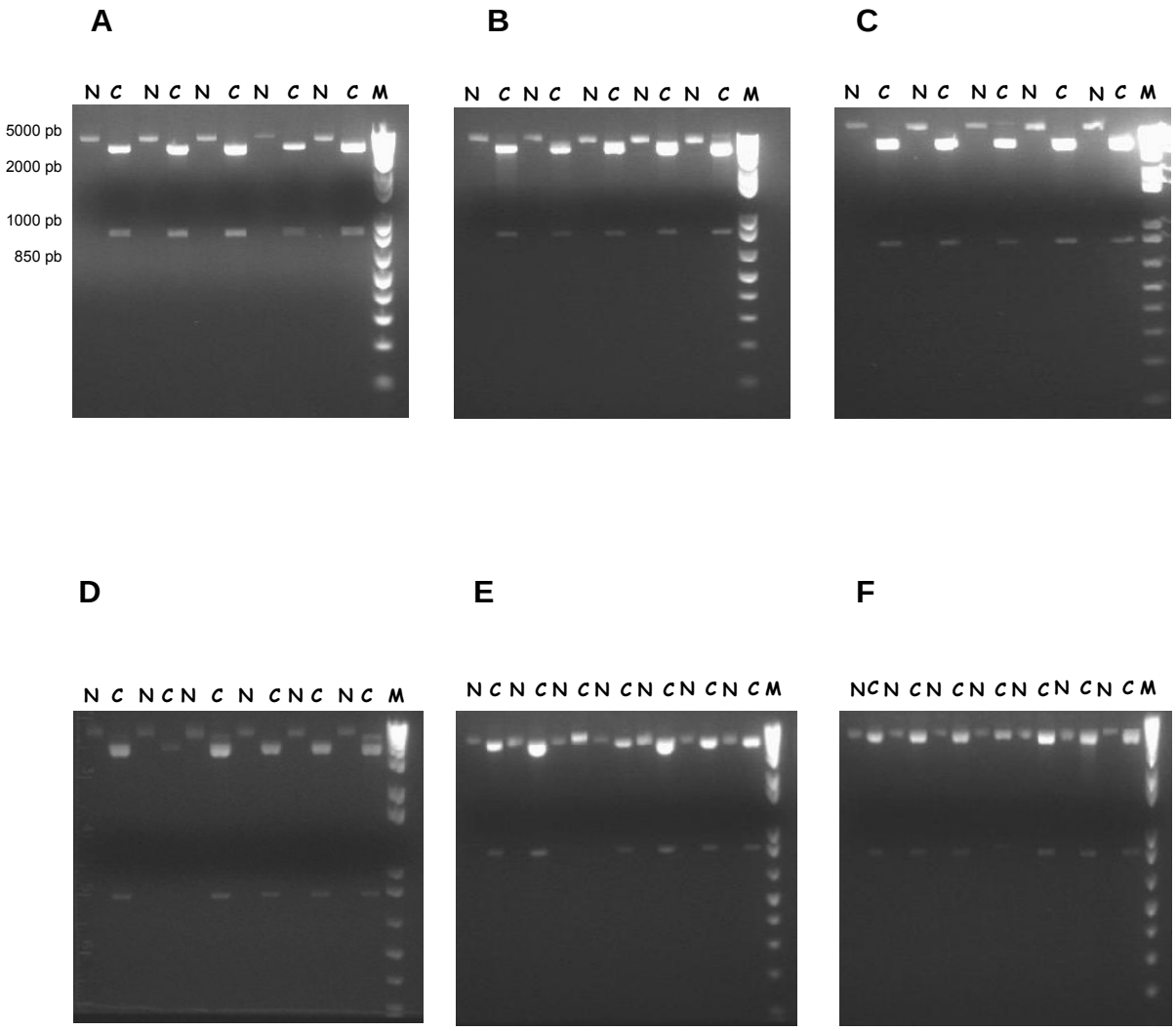
[#] Os resíduos em vermelho foram mutados

6.3 Seqüência de nucleotídeos do gene *aro*E de *M. tuberculosis* *

```
   1 atgagcgaag gtcccaaaaa agccggcgtg cttggttcgc cgatcgcgca ttcccgctcc
  61 ccgcagctgc acctggccgc ctaccgggcg ttggggctgc acgactggac ctatgagcgc
 121 atcgaatgcg gtgcggccga gttgcccgtc gtggtcggtg gtttcggacc ggagtgggtc
 181 ggtgtttcgg tgaccatgcc gggcaagttc gccgccctgc ggttcgccga cgagcgcacc
 241 gcacgcgcgg accttgtcgg ttcggccaac accctggttc ggacgccgca tggctggcgg
 301 gccgacaaca ccgacatcga cggggtggcc ggggcgttgg gggcggctgc tggacacgcg
 361 ctggtgctgg ggtccggggg gaccgcaccg gcggccgtgg tggggctggc cgaactcggg
 421 gtcaccgaca tcaccgtggt ggcgcgcaac tcggacaagg cggcccggct ggtggacctg
 481 ggcacacggg tcggcgtggc gacccggttc tgcgcgttcg acagcggtgg gttggccgat
 541 gcggtggccg ccgcggaagt gctggtcagc accattccag cggaggtggc cgcggggtat
 601 gccggcacct tggccgcgat cccggtgctg ttggacgcca tctacgatcc gtggcccaca
 661 ccgctggccg ccgcggtcgg atcggcgggc gggcgggtga tcagcgggct gcagatgttg
 721 ctgcatcagg cgttcgcgca ggtggagcag ttcaccgggc tacccgcccc ccgcgaagcg
 781 atgacttgcg cgctggccgc gttggactag
```

* Em negrito estão as seqüências definidas como base para o desenho do par de oligonucleotídeos iniciadores para cada mutação: de 181 a 226 para as mutações da lisina 69 e de 292 a 336 para as mutações do aspartato 105; em vermelho estão as duas trincas de nucleotídeos mutadas.

6.4 Géis de agarose a 2 %



Eletroforese em gel de agarose a 2 % do vetor pET23a(+) contendo ou não o inserto de interesse, *aro*E, após a mutagênese sítio-direcionada e após clivagem com as enzimas de restrição *Nde*I e *Bam*HI. Possíveis mutações: A, K69A; B, K69I; C, K69H; D, K69Q; E, D105A; F, D105N. N = não clivado; C = clivado; M = marcador 1 Kb plus DNA Ladder.

6.5 Carta de submissão do artigo "Screening of experimental conditions to express soluble shikimate dehydrogenase mutants from *Mycobacterium tuberculosis* H37Rv" à revista Protein Expression and Purification

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Authors: Valnês S Rodrigues-Junior, MSc; Diogenes S Santos, PhD

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6.6 Carta de submissão do artigo "The conserved Lysine 69 residue plays a catalytic role in *Mycobacterium tuberculosis* shikimate dehydrogenase" à revista Journal of Bacteriology

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