Functional shikimate dehydrogenase from *Mycobacterium tuberculosis* H37Rv: Purification and characterization

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Abstract

Tuberculosis (TB) poses a major worldwide public health problem. The increasing prevalence of TB, the emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis*, the causative agent of TB, and the devastating effect of co-infection with HIV have highlighted the urgent need for the development of new antimycobacterial agents. Analysis of the complete genome sequence of *M. tuberculosis* shows the presence of genes involved in the aromatic amino acid biosynthetic pathway. Experimental evidence that this pathway is essential for *M. tuberculosis* has been reported. The genes and pathways that are essential for the growth of the microorganisms make them attractive drug targets since inhibiting their function may kill the bacilli. We have previously cloned and expressed in the soluble form the fourth shikimate pathway enzyme of the *M. tuberculosis*, the *aroE*-encoded shikimate dehydrogenase (mtSD). Here, we present the purification of active recombinant *aroE*-encoded *M. tuberculosis* shikimate dehydrogenase (mtSD) to homogeneity, N-terminal sequencing, mass spectrometry, assessment of the oligomeric state by gel filtration chromatography, determination of apparent steady-state kinetic parameters for both the forward and reverse directions, apparent equilibrium constant, thermal stability, and energy of activation for the enzyme-catalyzed chemical reaction. These results pave the way for structural and kinetic studies, which should aid in the rational design of mtSD inhibitors to be tested as antimycobacterial agents.

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Keywords: *Mycobacterium tuberculosis*; Shikimate pathway; Shikimate dehydrogenase; *aroE*; Enzyme kinetics; Protein purification; Drug target

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The causative agent of tuberculosis (TB), 1 *Mycobacterium tuberculosis*, infects approximately 32% of the world's human population. TB remains the leading cause of mortality due to a bacterial pathogen. Currently, there are 8 million new cases and 2 million deaths annually from tuberculosis, and it is predicted that a total of 225 million new cases and 79 million deaths will occur between 1998 and 2030 [1]. Approximately 2 billion individuals are believed to harbor latent TB based on tuberculin skin test surveys [2], which represents a considerable reservoir of bacilli. Possible factors underlying the resurgence of TB worldwide include the HIV epidemic, increase in the homeless population, and decline in health care structures and national surveillance [3]. The pandemic of AIDS has had
a major impact on the TB problem, owing not only to increased reactivation of latent TB but also to acceleration of transmission in HIV/TB co-infected patients following the increase in the number of smear-positive infectious pulmonary TB cases [4]. Another contributing factor is the evolution of multi-drug TB (MDR-TB), defined as resistant to isoniazid and rifampicin, which are the most effective first-line drugs [5]. MDR-TB is more difficult and more expensive to treat, and more likely to be fatal [6].

According to the 2004 Global TB Control Report of the World Health Organization, there are 300,000 new cases per year of MDR-TB worldwide, and 79% of MDR-TB cases are now “super-strains,” resistant to at least three of the four main drugs used to treat TB [7]. The factors that most influence the emergence of MDR-TB strains include inappropriate treatment regimens, and patient non-compliance in completing the prescribed courses of therapy due to the lengthy standard “short-course” treatment or when the side effects become unbearable [8]. Thus, there is a need for the development of new antimycobacterial agents to both treat M. tuberculosis strains resistant to existing drugs and shorten the duration of short-course treatment to improve patient compliance [9].

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 [10]. The mycobacterial shikimate pathway leads to the biosynthesis of chorismic acid, which is a precursor of aromatic amino acids, naphthoquinones, menaquinones, and mycobactins [11]. The salicylate-derived mycobactin siderophores have been shown to be essential for M. tuberculosis growth in macrophages [12]. In addition, the shikimate pathway has more recently been shown to be essential for the viability of M. tuberculosis [13]. Accordingly, the essentiality of mycobacterial shikimate pathway and its absence from human host indicate that any enzyme of this pathway represents a promising target for the development of non-toxic antimycobacterial agents.

Analysis of the complete genome sequence of M. tuberculosis shows the presence of seven aroE genes predicted to be involved in the shikimate pathway [14]. Amongst them, the aroE-encoded shikimate dehydrogenase (SD) has been predicted by DNA sequence homology to be present in M. tuberculosis H37Rv strain. Shikimate dehydrogenase (EC 1.1.1.25) catalyzes the fourth reaction in the shikimate pathway. We have previously reported the cloning and expression of M. tuberculosis SD (mtSD) [15]. In addition, measurements of the NADPH-dependent reduction of 3-dehydroshikimate to shikimate catalyzed by mtSD confirmed the correct assignment to the structural gene encoding SD in M. tuberculosis [15]. Here, we report the purification to homogeneity of recombinant and functional mtSD. The purification protocol yielded approximately 11 mg of homogeneous recombinant mtSD from 14 L of Escherichia coli cell culture. We also present N-terminal amino acid sequencing and electrospray ionization mass spectrometry (ESI-MS) data that unambiguously demonstrate the identity and purity of homogenous recombinant mtSD protein. The estimated molecular mass of native homogeneous recombinant protein determined by gel filtration indicates that mtSD enzyme is a dimer in solution with a subunit molecular mass value of 27,207 Da determined by ESI-MS. The apparent kinetic parameters for mtSD were determined for all substrates in both forward and reverse reactions. The mtSD thermal stability was evaluated, and an estimate for the activation energy ($E_a$) was obtained from a linear plot of $\log k$ versus $1/T$ ($K^{-1}$). A comparison of polypeptide sequences of SDs from M. tuberculosis, E. coli, Haemophilus influenzae, and Methanococcus jannaschii allowed identification of amino acid residues that are likely to be involved in 3-dehydroshikimate/shikimate binding.

The results presented here will pave the way for structural and functional efforts currently underway in our laboratory. It is hoped that these studies will provide a framework on which to base the design of new agents with antitubercular activity and low toxicity to humans.

**Materials and methods**

**Overexpression and release of mtSD**

The recombinant plasmid pET23a(+)::aroE was transformed into E. coli BL21 (DE3) host cells by electroporation, and selected on LB agar plates containing 50 μg mL$^{-1}$ carbenicillin. Single colonies were used to inoculate 14 L of LB medium containing 50 μg mL$^{-1}$ carbenicillin with no addition of isopropyl β-D-thiogalactopyranoside (IPTG), and grown for 24 h at 37 °C at 180 rpm as described elsewhere [15]. Cells (49 g) were harvested by centrifugation at 14,900g, for 30 min at 4 °C, and stored at −20 °C. Cells expressing recombinant mtSD were placed into metal containers to allow fast temperature equilibrium to be reached, which is necessary for increased efficiency of cell disruption by the freeze–thaw method [15]. Cell paste was placed into a dry-ice/ethanol bath for 2 min and immediately transferred to an ice-water bath for no longer than 8 min; this cycle was repeated 10 times. The cells were dissolved in 196 mL of 50 mM Tris–HCl, pH 7.8 (buffer A). After incubating the mixture for 30 min on ice, cell debris was removed by centrifugation (48,000g for 1 h) and the supernatant containing soluble mtSD was collected.

**Purification of recombinant mtSD**

All steps of the purification protocol of recombinant mtSD were performed on ice or at 4 °C. The supernatant containing soluble mtSD was incubated with 1% (w/v) of streptomycin sulfate for 30 min and centrifuged at 48,000g for 30 min. The supernatant was dialyzed twice against buffer A, using a dialysis tubing with molecular weight exclusion limit of 6000–8000 Da. This sample was
clarified by centrifugation (48,000g for 30 min) and loaded on a Q-Sepharose fast flow (26 cm × 9.5 cm) column (Amersham Biosciences) pre-equilibrated with the same buffer. The column was washed with 5 column volumes of buffer A and the absorbed material was eluted with a linear gradient (0–100%) of 20 column volumes of 50 mM Tris–HCl, pH 7.8, 0.5 M NaCl (buffer B) at 1 mL min⁻¹. The fractions containing mtSD were pooled (55 mL) and ammonium sulfate was added to a final concentration of 1 M, and clarified by centrifugation (48,000g for 30 min). The supernatant was loaded on a Phenyl-Sepharose High Performance (Amersham Biosciences) column pre-equilibrated with 50 mM Tris–HCl, pH 7.8, 1 M (NH₄)₂SO₄ (buffer C). The column was washed with 5 column volumes of buffer C and the bound proteins were eluted with a 20-column volume linear gradient (0–100%) of buffer A at 1 mL min⁻¹. The mtSD-containing fractions were pooled (43 mL), concentrated to less than 4 mL using an Amicon ultrafiltration cell (MWCO 10,000 Da), and loaded on a Sephacryl S-200 (26 cm × 60 cm) (Amersham Biosciences) column pre-equilibrated with buffer A. The recombinant mtSD protein was eluted in a total volume of 29 mL at a flow rate of 0.5 mL min⁻¹, and loaded on an anion-exchange Mono-Q column (Amersham Biosciences) pre-equilibrated with buffer A. The column was washed with 5 column volumes of buffer A and the absorbed material was eluted with a 20-column volume linear gradient (0–100%) of buffer B at 1 mL min⁻¹. Elution profiles were followed at 280 and 215 nm. Homogeneous mtSD was eluted in a total volume of 12.5 mL and stored at −20 °C. Protein purification was monitored by SDS–PAGE [16], and the protein concentration was determined by the method of Bradford et al. [17] using the Bio-Rad protein assay kit (Bio-Rad) and bovine serum albumin as standard.

**Enzyme activity assay of mtSD**

The screening of fractions of the purification protocol containing shikimate dehydrogenase was performed by assaying enzyme activity in the reverse direction in 100 mM Tris–HCl, pH 9.0, at 25 °C. The 500 μL assay mixture contained 2 mM NADP⁺ and 4 mM D-shikimate (SHK) [18], and the reaction was initiated with addition of 1 μL of the chromatographic fractions. Measurement of the NADP⁺-dependent oxidation of SHK to form NADPH and 3-dehydroshikimate (DHS) catalyzed by mtSD was continuously monitored by the increase in absorbance at 340 nm (εNADPH = 6.18 × 10³ M⁻¹ cm⁻¹). One unit of enzyme activity (U) is defined as the amount of enzyme catalyzing the conversion of 1 μmol of NADP⁺ per minute at 25 °C.

**Determination of apparent kinetic parameters**

Determination of the apparent steady-state kinetics parameters, \( V_{\text{max}} \) and \( K_m \), for DHS and NADPH in the forward reaction, was carried out at varying concentration of one substrate (5, 10, 20, 30, 50, 100, and 200 μM) while the other was maintained at constant saturation level. The reverse reaction was performed in the same conditions of pH (100 mM Tris–HCl buffer, pH 7.0), temperature (25 °C) and range of concentrations of substrates. The reaction was initiated with addition of 6 pmol of homogenous \( M. tuberculosis \) SD enzyme and was monitored for 1 min. The kinetic data were analyzed by double reciprocal plots.

Shikimate dehydrogenase activity measurements were based on decreasing concentration of NADPH upon DHS reduction or on increasing concentration of NADPH upon SHK oxidation. The reaction catalyzed by mtSD was continuously monitored by measuring change in absorbance at 340 nm (εNADPH = 6.18 × 10³ M⁻¹ cm⁻¹).

**Determination of the energy of activation and thermal stability of recombinant mtSD**

The energy of activation \( (E_a) \) was estimated for recombinant mtDS by the following Arrhenius equation:

\[
    k = A e^{-E_a/RT},
\]

where \( k \) is the rate constant of the reaction at temperature \( T \) (in Kelvin), \( A \) is a pre-exponential factor (related to collision frequency and a steric factor); \( E_a \) is the activation energy; \( R \) is universal gas constant (8.3145 J K⁻¹ mol⁻¹) and \( e^{-E_a/RT} \) is the fraction of molecular collisions that have energy equal to or greater than the measured energy of activation \( (E_a) \) of the system at a particular temperature \( T \). The \( E_a \) value was calculated from the slope of the linear plot log \( k \) versus \( 1/T \) (K⁻¹) fitting the data to the following equation: log \( k = (E_a/2.3RT) + \log A \). Measurements of mtSD enzyme activity were in triplicates and performed in 100 mM Tris–HCl buffer, pH 7.0, in the presence of saturating concentrations of SHK (200 μM) and NADP⁺ (100 μM) at the following temperatures: 15, 20, 25, 30, and 37 °C.

For thermal stability determination, mtSD was incubated at 15, 25, 37, and 55 °C and the remaining enzyme activity was measured at different times of incubation up to 1 h, monitoring the mtSD reverse reaction in an assay mixture containing saturating concentrations of substrates (200 μM SHK and 100 μM NADP⁺) in 100 mM Tris–HCl, pH 7.0, at 25 °C.

**Mass spectrometry analysis**

The homogeneity of recombinant protein preparation was assessed by mass spectrometry (MS), employing some adaptations made to the system described by Chassaigne and Lobinski [19]. Samples were analyzed on a triple quadrupole mass spectrometer, model QUATTRO II, equipped with a standard electrospray (ESI) probe (Micromass, Altrincham), adjusted to ca. 250 μL min⁻¹. The source of temperature (80 °C) and needle voltage (3.6 kV) were maintained constant throughout the experimental data collection, applying a drying gas flow (nitrogen) of 200 L h⁻¹ and a nebulizer gas flow of 20 L h⁻¹. The mass
spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments. The subunit molecular mass of \textit{M. tuberculosis} SD was determined by ESI-MS, adjusting the mass spectrometer to give a peak width at half-height of 1 mass unit, and the cone sample to skimmer lens voltage controlling the ion transfer to mass analyzer was set to 38 V. About 50 pmol sample was injected into electrospray transport solvent. The ESI spectrum was obtained in the multi-channel acquisition mode, scanning from \textit{m/z} 500 to 2000 at scan time of 7 s. The mass spectrometer is equipped with MassLynx and Transform softwares for data acquisition and spectra handling.

\textbf{N-terminal amino acid sequencing}

The N-terminal amino acid residues of purified recombinant mtSD were identified by automated Edman degradation sequencing using a PPSQ 21A gas-phase sequencer (Shimadzu).

\textbf{Determination of native mtSD molecular mass}

The molecular mass of native mtSD homogenous protein was estimated by gel-permeation chromatography on a Superdex \textit{200} HR column (1.0 cm \times 30 cm) (Amersham Biosciences). The column was eluted with 50 mM Tris–HCl containing 0.2 M NaCl, pH 7.8, at a flow rate of 0.4 mL min\(^{-1}\). The eluate was monitored at 215 and 280 nm and the column was calibrated with the following protein standards (Amersham Biosciences): ribonuclease A (13,700 Da) from bovine pancreas, chymotrypsinogen (25,000 Da) from bovine pancreas, ovalbumin (43,000 Da) from hen egg, and albumin (67,000 Da) from bovine serum. Blue Dextran 2000 was used to determine the void volume (\(V_0\)). The \(K_v\) value was calculated for each protein using the equation \((V_e - V_0)/(V_t - V_0)\), where \(V_e\) is the elution volume for the protein and \(V_t\) is the total bed volume, and \(K_v\) was plotted against the logarithm of standard molecular weights.

\textbf{Results and discussion}

Expression in \textit{E. coli} BL21 (DE3) of recombinant \textit{M. tuberculosis} shikimate dehydrogenase (mtSD) and the method of disruption of transformed host cells were as described elsewhere \cite{15}. Recombinant mtSD was purified as described under Materials and methods and the samples of each chromatographic step were analyzed by SDS-PAGE with Coomassie blue staining and assayed for enzyme activity in the reverse direction, following the increase in absorbance at 340 nm due to the NADP\(^{+}\)-dependent oxidation of \(\delta\)-shikimate to form NADPH and 3-dehydroshikimate. The recombinant protein was purified 8.5-fold (Table 1) to electrophoretic homogeneity (Fig. 1). The relative mobility of the polypeptide chain in SDS-PAGE indicates a homogeneous protein with a subunit molecular mass value of approximately 27 kDa (Fig. 1). Even though a large amount of cells were needed for recovery of homogeneous target protein in quantities necessary for kinetic and structural studies, the freeze–thaw method \cite{20} was previously shown to be, amongst a number of experimental protocols tested to reduce insoluble protein production, the method of choice to obtain soluble mtSD in its active form \cite{15}. Approximately 11 mg of homogeneous recombinant mtSD could be obtained from 49 g of \textit{E. coli} BL21 (DE3) host cells following the purification protocol presented in Table 1, which required four chromatographic steps to obtain homogeneous mtSD. The recombinant protein eluted with approximately 50\% of 50 mM Tris–HCl, pH 7.8, containing 0.5 M NaCl (buffer B), consistent with the theoretical value of 5.11 for the mtSD isoelectric point. Since the theoretical isoelectric point values for mtSD and \textit{E. coli} SD (5.12) are quite similar, the anion-exchange chromatographic step using Q-Sepharose Fast Flow resin is unlikely to have separated these

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|l|}
\hline
\textbf{Purification step} & \textbf{Total protein (mg)} & \textbf{Total activity (U)} & \textbf{Specific activity \(^a\) (U mg}^{-1}\text{)} & \textbf{Purification (fold)} & \textbf{Yield (\%)} \\
\hline
Crude extract & 1040.4 & 475.3 & 0.46 & 1.0 & 100.0 \\
Q-Sepharose & 82.5 & 110.0 & 1.33 & 2.9 & 23.1 \\
Phenyln-Sepharose & 24.1 & 69.2 & 2.87 & 6.2 & 14.6 \\
Sephacryl S-200 & 17.4 & 65.3 & 3.75 & 8.2 & 13.7 \\
Mono-Q & 10.8 & 42.1 & 3.90 & 8.5 & 8.9 \\
\hline
\end{tabular}
\caption{Purification of \textit{M. tuberculosis} shikimate dehydrogenase from \textit{E. coli} BL21 (DE3) [pET23a(+):\textit{aroE}] cells}
\end{table}

\textsuperscript{a} U mL\(^{-1}\) mg\(^{-1}\)

The results presented are for a typical purification protocol from 49 g of \textit{E. coli} host cells.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{SDS-PAGE (12.5\%) analysis of pooled fractions from the various steps of the purification protocol of mtSD. Lane 1, crude extract (50 \mu g); lane 2, Q-Sepharose fast flow ion exchange (30 \mu g); lane 3, Phenyl-Sepharose hydrophobic interaction (10 \mu g); lane 4, Sephacryl S-200 gel permeation (10 \mu g); lane 5, Mono-Q ion exchange (17 \mu g); lane 6, MW marker High Range (Gibco).}
\end{figure}
enzymes. Nevertheless, this chromatographic step resulted in 2.9-fold protein purification (Table 1) and removal of some noticeable contaminants with subunit molecular weight values larger than 29 kDa (Fig. 1). The recombinant mtSD protein in 50 mM Tris–HCl, pH 7.8, containing 1 M (NH₄)₂SO₄ adsorbed to a Phenyl-Sepharose High Performance column and was eluted with approximately 57% of 50 mM Tris–HCl, pH 7.8, buffer resulting in a 6.2-fold purification with removal of substantial amount of contaminants (Fig. 1). Gel filtration on Sephacryl S-200 resin was performed to desalt and further purify the recombinant protein. Elution of fractions containing mtSD from an anion-exchange Mono-Q column with approximately 50% of 50 mM Tris–HCl, pH 7.8, buffer containing 0.5 M NaCl resulted in homogeneous mtSD, with a protein yield of approximately 9% (Table 1). It is noteworthy that there was no need for elution with coenzyme from an affinity column as described for E. coli shikimate dehydrogenase [21]. Accordingly, the purification protocol here described represents an efficient and low-cost method to obtain homogeneous mtSD. The purification of M. jannaschii shikimate dehydrogenase by glutathione and Sepharose Q chromatography yielded, after proteolytic removal of the affinity tag, a protein with five N-terminal residues resulting from cloning artifact [22]. The strategy followed to obtain homogeneous H. influenzae shikimate dehydrogenase provided a protein with a C-terminal hexahistidine tag [23]. It has been shown that N- and C-terminal hexahistidine tags have a noticeable negative effect on protein solubility of recombinant proteins expressed in E. coli [24]. In addition, His-tagged proteins may have different structure [25] or biological activity [26] as compared to their native form. The mtSD cloning strategy we described elsewhere [15] and protein purification we presented here yielded homogeneous polypeptide chain with no extra amino acid residues.

The subunit molecular mass of active mtSD was determined to be 27,076 Da by electrospray ionization mass spectrometry (ESI-MS), indicating removal of the N-terminal methionine residue (predicted molecular mass: 27,207 Da). The ESI-MS result also revealed a peak at 54,150 Da, indicating that the enzyme could have a dimeric form. No peak could be detected at the expected molecular mass for E. coli SD (29,413 Da) and a degree of purity of 98% could be estimated by ESI-MS, thus providing evidence for the identity and purity of the recombinant protein. The first 11 N-terminal amino acid residues of mtSD were identified as SEGPKKAGVLG by the Edman degradation method. This result unambiguously identifies the homogeneous recombinant protein as mtSD and confirms removal of the N-terminal methionine. Modification at the N-termini is a common type of co-/post-translational alteration of proteins synthesized in prokaryotic cells. Methionine aminopeptidase-catalyzed cleavage of initiator methionine is usually directed by the penultimate amino acid residues with the smallest side chain radii of gyration (glycine, alanine, serine, threonine, proline, valine, and cysteine) [27]. Removal of N-terminal methionine from mtSD polypeptide chain conforms to this rule since serine is the penultimate amino acid residue.

The enzymatic activity of homogeneous recombinant mtSD purified was assayed in the reverse direction by continuously monitoring the increase in absorbance at 340 nm upon NADP⁺-dependent oxidation of D-shikimate to form NADPH and 3-dehydroshikimate. The activity of mtSD was linearly dependent on sample volume added to the reaction mixture (Fig. 2), thereby showing that the initial velocity is proportional to total enzyme concentration and that true initial velocities are being measured. The M. tuberculosis SD was stable at −20 °C for at least 1 year.

A value of 58,367 Da for the molecular mass of homogeneous mtSD protein was estimated by gel filtration (data not shown). This result suggests that mtSD is a dimer in solution, in agreement with the ESI-MS results. Shikimate dehydrogenase from M. jannaschii has recently been shown to be a dimer in solution [22]. Whereas dehydrogenases usually form oligomers, shikimate dehydrogenase is present as a monomer in both E. coli [18] and H. influenzae [23].

The apparent kinetics parameters obtained are presented in Table 2. The plots fitted to a hyperbolic equation, indicating that the recombinant enzyme-catalyzed chemical

![Fig. 2. Linear dependence of mtSD activity on homogeneous protein volume. The rates of mtSD enzyme activity were followed in the reverse reaction by continuously monitoring the increase of NADPH concentration at 340 nm. Reactions were started by addition of varying volumes of homogeneous mtSD protein solution.](Image 319 to 547)

Table 2

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$V_{max}$ (U mg⁻¹)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHS</td>
<td>108 ± 5</td>
<td>31 ± 2</td>
<td>49 ± 2</td>
<td>1.6 (±0.1) × 10⁶</td>
</tr>
<tr>
<td>NADPH</td>
<td>100 ± 5</td>
<td>10 ± 1</td>
<td>45 ± 2</td>
<td>4.5 (±0.5) × 10⁶</td>
</tr>
<tr>
<td>SHK</td>
<td>18 ± 1</td>
<td>50.18 ± 0.01</td>
<td>8.2 ± 0.5</td>
<td>1.63 (±0.01) × 10⁷</td>
</tr>
<tr>
<td>NADPH⁺</td>
<td>12.9 ± 0.7</td>
<td>22 ± 2</td>
<td>5.9 ± 0.3</td>
<td>2.68 (±0.03) × 10⁷</td>
</tr>
</tbody>
</table>

*All constants were measured in Tris–HCl 100 mM (pH 7.0) at 25 °C.*
reaction obeys Michaelis–Menten kinetics for all substrates (Fig. 3). The $K_m$ and $V_{max}$ values for DHS were found to be, respectively, 31 $\mu$M and 108 U mg$^{-1}$; and for NADPH they were 10 $\mu$M and 100 U mg$^{-1}$. The $k_{cat}$ for DHS at saturating NADPH was 49 s$^{-1}$ yielding a $k_{cat}/K_m$ of $1.6 \times 10^6$ M$^{-1}$ s$^{-1}$. The $K_m$ value for DHS is lower than the value for SD purified from Pisum sativum (340 $\mu$M); but the $K_m$ value for NADPH is in agreement with the value of 4.3 $\mu$M determined at pH 7.4 [28]. For the reverse reaction, the $K_m$ and $V_{max}$ values for SHK were found to be, respectively, 50.18 $\mu$M and 18 U mg$^{-1}$; and for NADP$^+$ they were 22 $\mu$M and 12.9 U mg$^{-1}$. The $k_{cat}$ for SHK at saturating NADP$^+$ was 8.2 s$^{-1}$ yielding a $k_{cat}/K_m$ of $1.6 \times 10^5$ M$^{-1}$ s$^{-1}$. For the reverse reaction catalyzed by P. sativum SD, the $K_m$ values were 10.3 $\mu$M for NADP$^+$ and 600 $\mu$M for SHK [28]. The $K_m$ value for SHK is thus 10 times larger for P. sativum SD than for mtSD. A comparison of the SHK and NADP$^+$ $K_m$ values for E. coli SD [29] with those obtained for mtSD shows that these two enzymes catalyze the reverse reaction with similar kinetic constants. A value of 19.6 for the apparent equilibrium constant ($K_{eq}$) under the experimental conditions given in the Materials and methods section was estimated by the Haldane equation using the apparent steady-state kinetic parameters for mtSD. An apparent equilibrium constant value of 10.3 for the reaction at pH 7.4 has been determined by finding a mixture of substrate concentrations that showed no measurable change in optical density at 340 nm [28]. However, it should be pointed out that the Haldane equation we used here is the kinetic relationship for rapid-equilibrium random Bi Bi system, and it was implicitly assumed that the dissociation constant value for a substrate binding second is not changed by the substrate binding first [30].

The temperature effects on recombinant homogeneous mtSD stability are presented in Fig. 5. Aliquots were removed for assay of residual enzyme activity after heating for 1, 5, 10, 15, 30, 45, and 60 min at temperature values of 15, 25, 37, and 55 $^\circ$C. The homogeneous mtSD is very stable, maintaining the specific activity, measured at 25 $^\circ$C, unchanged in the reverse reaction up to 37 $^\circ$C for at least 1 h of incubation (Fig. 4). However, at 55 $^\circ$C, there is a gradual loss of recombinant mtSD biological activity, with only 20% of the initial enzyme activity remaining after 1 h of incubation.

Fig. 3. Reciprocal plots with 3-dehydroshikimate (DHS), NADP$^+$, d-shikimate (SHK) or NADPH as variable substrate (5, 10, 20, 30, 50, 100, and 200 $\mu$M) while the concentration of the other substrate was maintained at constant saturation level in the forward and reverse reaction. The enzyme activity was assayed at 25 $^\circ$C in 100 mM Tris–HCl buffer, pH 7.0. The reaction catalyzed by mtSD was continuously monitored by the absorbance at 340 nm ($\varepsilon_{\text{NADPH}} = 6.18 \times 10^3$ M$^{-1}$ cm$^{-1}$). The kinetics data were analyzed by linear regression fit Michaelis–Menten kinetics. (A) 3-dehydroshikimate as variable substrate (5, 10, 20, 30, 50, 100, and 200 $\mu$M) while the NADPH was maintained at constant saturation level (200 $\mu$M) in the forward reaction. (B) NADPH as variable substrate (5, 10, 20, 30, 50, 100, and 200 $\mu$M) while 3-dehydroshikimate was maintained at constant saturation level (200 $\mu$M) in the forward reaction. (C) Shikimate as variable substrate (5, 10, 20, 30, 50, 100, and 200 $\mu$M) while NADP$^+$ was maintained at constant saturation level (200 $\mu$M) in the forward reaction. (D) NADP$^+$ as variable substrate (5, 10, 20, 30, 50, 100, and 200 $\mu$M) while shikimate was maintained at constant near-saturation level (100 $\mu$M) in the forward reaction.
obtained in the reverse reaction in 100 mM Tris–HCl, pH 7.0, containing M. tuberculosis SD enzyme by the Arrhenius equation. All data were carried out in 100 mM Tris–HCl, pH 7.0, assay mixture containing 200 μM SHK, 100 μM NADP+, and 0.24 nM of homogenous M. tuberculosis SD enzyme and the reaction was measured for 1 min at 25 °C.

From the linear plots of logk versus 1/T (K⁻¹), a value of 35.2 kJ mol⁻¹ for $E_a$ was obtained for mtSD (Fig. 5). It should be pointed out that the $E_a$ value calculated from the Arrhenius plot is an apparent or “average value,” and that the pre-exponential factor (A) was considered as temperature-independent in the temperature range used in our experiments. Keeping that in mind, 35.2 kJ mol⁻¹ can be considered as the minimal amount of energy required to initiate the mtSD-catalyzed chemical reaction, since we measured the enzyme activity at saturating concentrations of substrates (200 μM SHK and 100 μM NADP+). Interestingly, the Arrhenius plot is linear indicating no change in the rate-limiting step of the mtSD-catalyzed chemical reaction at different temperatures. In addition, there was no sudden drop in the Arrhenius plot at low 1/T (high T) values that could indicate protein denaturation, which is consistent with mtSD thermal stability up to 55 °C.

The three-dimensional structures of shikimate dehydrogenase from E. coli [29,31], H. influenzae [23,32], and M. jannaschii [22] have been determined. Sequence alignment of shikimate dehydrogenase from M. tuberculosis H37Rv strain, E. coli, H. influenzae, M. jannaschii was carried out using ClustalW [33,34]. The identity between the SD sequences from M. tuberculosis and E. coli is 24%. The substrate-binding site in the E. coli 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 [29]. This binding site is in a pocket where most of the residues absolutely conserved in the shikimate dehydrogenase (SDH) family are located, i.e., Ser14, Ser16, Lys65, Asn86, Thr101, Asp102, and Gln244 (E. coli SD numbering). These residues are conserved in the mtSD polypeptide sequence (Fig. 6) corresponding to the following residues: Ser18, Ser20, Lys69, Asn90, Thr104, Asp105, and Gln243 (M. tuberculosis SD numbering). However, the 3-dehydroshikimate-binding site has not unambiguously been shown for E. coli shikimate dehydrogenase since it was inferred from DTT and sulfate ions bound to the crystal and position of the nicotinamide ring of NADP+ [29]. The sequence identity (24%) between mtSD and M. jannaschii SD is similar to the identity between the sequences of M. tuberculosis and E. coli SDs. The N-terminal domain (domain I) of M. jannaschii SD is responsible for 3-dehydroshikimate substrate binding [22]. The putative active site residues within domain I that are likely to be involved with substrate binding are invariant polar residues include Lys70, Asn91, and Asp106 (M. jannaschii SD numbering). Further examination of the active site cleft of M. jannaschii SD revealed another strictly conserved residue, Gln254. These residues correspond to Lys69, Asn90, Asp105, and Gln244 in M. tuberculosis SD.

It has been proposed that these residues are likely to be involved in catalytic reduction of DHS to SHK catalyzed by M. jannaschii SD [22]. The amino acids Ser14, Ser16, Lys65, Asn85, Asp102, and Gln245 (H. influenzae SD numbering) have been suggested as some of the potential residues involved in 3-dehydroshikimate-binding site in H. influenzae SD [23]. All these residues are conserved in the mtSD polypeptide sequence (Fig. 6), and correspond to the same residues observed in the E. coli SD sequence. More recently, the crystal structure of a newly characterized shikimate dehydrogenase-like protein (H10607) from H. influenzae has been determined [32], and the conserved residues Lys67, Asn88, Asp103, and Gln242 have been proposed to be involved in either catalysis or substrate binding. It should be pointed out that the three-dimensional structures of shikimate dehydrogenases determined were in complex with the cofactor and no three-dimensional structure in complex with 3-dehydroshikimate/shikimate molecules have been reported to date.
The work presented here describes, to the best of our knowledge, the first purification protocol of recombinant M. tuberculosis SD to homogeneity and determination of its oligomeric state. In addition, here we present a detailed characterization of the homogeneous mtSD. It should be pointed out that expression of mtSD in soluble and active form proved to be laborious to achieve [15]. Protein production and crystallization must be optimized if structural genomics will ever reach its goal of solving the three-dimensional structure of the whole proteome encoded by a given genome [35]. 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 [36]. 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 [37].

The protocol for mtSD purification presented here should provide protein in quantities necessary for further enzymological studies and three-dimensional structure determination efforts. Determination of mtSD kinetic mechanism by steady-state and pre-steady-state kinetics, and isotope effects, site-directed mutagenesis, and chemical rescue will allow elucidation of its chemical and catalytic mechanism. The three-dimensional structures of shikimate dehydrogenases from E. coli [29,31], H. influenzae [23,32], and M. jannaschii [22] should facilitate screening of experimental conditions to obtain crystals of mtSD protein and may provide templates for mtSD structure determination by molecular replacement. All of the enzymes that make up the shikimate pathway are potential targets for the design of novel drugs directed against pathogenic bacteria. The structure of M. tuberculosis shikimate dehydrogenase and the understanding of the enzyme mode of action will be used as a platform for the design of effective inhibitors of this pathway aiming at the development of antitubercular agents.

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References


