Thermodynamics of nucleotide binding to NBS-I of the Bacillus subtilis preprotein translocase subunit SecA

Tanneke den Blaauwen¹, Jeroen P.W. van der Wolk, Chris van der Does, Karel H.M. van Wely, Arnold J.M. Driessen*

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

Received 20 July 1999; received in revised form 16 August 1999

Abstract SecA is the dissociatable nucleotide and preprotein binding subunit of the bacterial translocase. The thermodynamics of nucleotide binding to soluble SecA at nucleotide binding site I were determined by isothermal titration calorimetry. Binding of ADP and non-hydrolyzable ATPs is enthalpy-driven (ΔH° of −14.44 and −5.56 kcal/mol, respectively), but is accompanied by opposite entropic contributions (ΔS° of −18.25 and 9.55 cal/mol/K, respectively). ADP binding results in a large change in the heat capacity of SecA (ΔCp = −780 cal/mol/K). It is suggested that ADP binding promotes the interaction between the two thermodynamically discernible domains of SecA which is accompanied by a shielding of hydrophobic surface from solvent.

© 1999 Federation of European Biochemical Societies.

Key words: ATP; Differential scanning calorimetry; Isothermal titration calorimetry; Preprotein translocation; SecA

1. Introduction

SecA catalyzes the ATP-driven translocation of preproteins across the cytoplasmic membrane of bacteria (for reviews, see [1,2]). Although SecA is responsible for the force generation by pushing the preprotein in a stepwise fashion across the membrane [3,4], the mechanism of energy coupling is largely unknown. It is generally assumed that structural changes of the SecA protein are coupled to nucleotide binding and hydrolysis in the catalytic cycle [5,6]. Translocation of preprotein segments may occur by co-insertion with SecA domains into the membrane. SecA has two essential nucleotide binding sites (NBSs) [7-11]: a high affinity binding site (Kd ≈ 0.15 μM) is confined to the amino-terminal domain of the protein (NBS-I) and a low affinity binding site (Kd ≈ 340 μM) is located in the second half of the protein (NBS-II) near the subunit interface [12]. The functional role of the separate NBSs is still largely unresolved, but the binding of ATP to NBS-I suffices to insert SecA domains into the membrane [6]. Biophysical and biochemical studies indicate that SecA adopts a compact conformation in the presence of ADP, whereas in the presence of ATP (non-hydrolyzable ATP analogues), the protein adopts a more open state [13-15]. For the understanding of the mechanism of mechanical force generation, it is, however, essential to determine more precisely the nature and thermodynamics of these conformational changes.

The opportunity to study the interaction of SecA with the other subunits of the translocase in micellar solution [16] provides the components and conditions for a detailed thermodynamic analysis of the conformations of SecA. The first step in such an analysis is presented in this study and concerns the binding of nucleotides to SecA in the absence of the other components of the translocase. We now confirm the proposed thermodynamic structure domain of SecA and further analyze the conformational changes induced by nucleotide occupation of NBS-I. The results are discussed in terms of a model in which SecA acts as a mechanical force generating device.

2. Materials and methods

2.1. Bacterial strains, growth media and biochemicals

Strains were grown in Luria Bertani (LB) broth or on LB agar [17] supplemented with 50 μg/ml ampicillin, 0.5% (w/v) glucose or 1 mM iso-propyl-1-thio-β-galactopyranoside as required. Bacillus subtilis SecA and SecA K106N were overexpressed in Escherichia coli JM109 [18] and NO2947 [18] containing pMKL4 or pMKL21 [8], respectively. Proteins were purified as described [10]. PrePhoB was overexpressed in E. coli SF100 containing pET461, purified and ¹¹C-labelled as described [19]. The protein concentration was determined by the method of Bradford [20] and by total amino acid composition analysis (Eurosequence, Groningen, The Netherlands).

2.2. Construction and expression of the amino-terminal SecA domain

The amino-terminal (1-443) domain of B. subtilis SecA was cloned in two steps by PCR using plasmid pMKL4 [8] as template and the primers N1 forward and N1 reverse (5'-GGGATCCATGGTTGGGAATTITAAATAAA and 5'-CAGCTTACGATTCGATTCG) for nucleotide residues 1-691 and the primers N2 forward and N2 reverse (5'-GCTGCAAAAGTGACTAAGGCTG and 5'-GCCTCTTGAGACTACTATTGAGTTCG) for nucleotide residues 691-1325. This introduced a NcoI site at the start codon (replacing a leucine by a valine) and a SfiI site at nucleotide 691 in the N1 fragment and two stop codons and a Xhol site at the end of the N2 fragment. The two PCR fragments were cloned in pET400, a pBlue-script (SK⁺) derivative with a NcoI site in the multiple cloning site, and completely sequenced on a Vistra DNA sequencer 725 (Amer- sham, Buckinghamshire, UK) using the automated ataq sequencing kit (Amerham). The N-secA gene was subsequently ligated into the NcoI/XhoI sites of pET302 [16,21] to yield the vector pET1105 that harbors the N-secA gene in frame with a hexa histidine-tag under control of the trc promoter. The wild-type length His-tagged SecA in pET1106 was derived from pET1105 by conventional cloning techniques.

Cells overexpressing His-tagged N-secA and SecA were harvested by centrifugation, resuspended in 50 mM HEPES-KOH, pH 7.5, 50 mM KCl, 20% sucrose and lysed by two passages through a French-pressure cell at 8000 psi. Unbroken cells were removed by
centrifugation (15 min, 7600 ×g, 4°C), the supernatant was cleared by ultra centrifugation (Ti 70, 40 min, 120 000 ×g, 4°C) and subsequently 1:1 diluted with 50 mM HEPES-KOH, pH 7.5, 50 mM KCl. The solution was applied to a 25 ml Ni²⁺-NTA agarose beads column (Qiagen, Chatsworth, CA, USA) at 4°C, washed with 12 mM imidazole in the same buffer and the protein was eluted with a 12–100 mM imidazole gradient. The eluted protein was further purified byMonoQ chromatography (Pharmacia, Uppsala, Sweden) using a 0–1 M NaCl gradient in 50 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 10% glycerol. Immunoblots confirmed that the protein samples were free of residual E. coli SecA.

2.3. Differential scanning calorimetry (DSC)

DSC experiments were performed using a microcalorimeter (Microcal MC-2, Microcal, Northampton, MA, USA) at a pressure of two atmosphere. A differential scanning rate of 1°C/min was employed. The DSC experiments were carried out with a B. subtilis SecA concentration of 1–2 mg/ml in TKM buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl and 5 mM MgCl₂). The reversibility of the DSC transitions was checked by re-heating the solution from the first run in the calorimetric cell after cooling (1°C/min). A thermogram corresponding to water was used as instrumental baseline. The dependence of the molar heat capacity on temperature was analyzed using the Origin software (Microcal). Fitting of the data and subtraction of the instrumental baseline data were done as described [22]. The data were normalized, assuming that the SecA dimer dissociated after or during the most stable transition [13].

2.4. Isothermal titration calorimetry

Nucleotide binding to B. subtilis SecA was analyzed in a Microcal Omega titration calorimeter (Microcal). The instrument and the equations used to fit calorimetric data have been described in detail previously [23]. Purified SecA was exchanged in TKM buffer by ultrafiltration (Amicon, W.R. Grace, Beverly, MA, USA). The TKM buffer was adjusted with HCl to correct for temperature effects on the final pH of the buffer. Nucleotide solutions were prepared in the flow through of the last buffer exchange and all solutions were thoroughly degassed by gentle stirring under vacuum. In a typical experiment, the 1.36 ml sample cell was filled with a 3–10 μM (monomer) solution of SecA. Subsequently, 5 μl injections of a 100 μM or 300 μM solution of ADP or ATP were, respectively, added from a 100 μl syringe rotating at 400 rpm. Typically, 20 injections of 10.6 s duration were made at 4 min intervals. Control experiments were performed under identical conditions with a sample cell containing only buffer. After subtraction of the contribution from the heat of dilution of each injection, the sum of the heat evolved was plotted against the total ligand concentration to produce the binding isotherm. The binding constant (Kₐ), enthalpy of binding (∆Hₐ) and stoichiometry (n) were determined by fitting the binding isotherm to the equation described previously [22] for ligand binding to a macromolecule possessing one set of independent ligand binding sites. The data were deconvoluted using a non-linear least squares algorithm incorporated in the Origin software provided with the instrument, assuming the presence of a single high affinity NBS per SecA monomer. ∆G*, ∆H*, and ∆S* values were calculated from Kₐ values (∆G* = -RTlnKₐ) and ∆S* values (∆S* = (∆G*-∆H*)/T).

2.5. Translocation assays

Membrane vesicles were derived from E. coli SF100 cells that overexpress the B. subtilis SecYEG complex (Swanning, van Wely and Driessen, manuscript submitted) as described [16] and treated with a polyclonal antiserum against E. coli SecA to remove the endogenous SecA [3]. Translocation of [¹²⁵]IprePhoB into the E. coli membrane vesicles harboring the overexpressed B. subtilis SecYEG was assayed by its accessibility to proteinase K [19].

3. Results

DSC of the B. subtilis SecA has demonstrated the presence of two endothermic transitions with midpoints (T_m1 and T_m2) of 40 and 49°C [13] (see also Fig. 2). The first transition is completely reversible and the second is irreversible due to aggregation (exothermic) with an onset temperature of 60°C.

Both transitions can be simulated by a two state transition with a van’t Hoff enthalpy (ΔH_vH) approximately identical to the calorimetric enthalpy. This suggests that SecA consists of at least two thermally independent folding domains with approximately similar sizes. Saturation of NBS-I with ADP causes a rather large upshift in transition temperature of T_m1 [13]. Since NBS-I is localized in the amino-terminal halve of SecA, it appears that the more stable domain (T_m2) represents the amino-terminal domain (N-domain), while the lower transition, i.e. the interacting domain that becomes stabilized, may correspond to the carboxyl-terminal domain (C-domain) [13]. To verify the identity of these domains, the thermal unfolding properties of a defined SecA fragment were investigated. Since in the presence of ADP, a site around amino acid 443 of the B. subtilis SecA has been shown to be inaccessible to protease [10], an amino-terminal fragment of SecA containing amino acids 1–443 (His-N-SecA) was cloned and overexpressed in E. coli as a hexa-His-tagged protein and further purified by Ni²⁺-NTA affinity and ion-exchange chromatography. As a control, the secA gene was also fused to a hexa-His-tag, expressed and isolated. The activity of the His-tagged SecA was tested in an in vitro system that consists of E. coli membrane vesicles derived from a strain that overexpresses the B. subtilis SecYEG complex using purified B. subtilis [¹²⁵]I-labelled prePhoB as a substrate. His-tagged SecA and wild-type SecA showed similar ATP-dependent translocation activities (Fig. 1), indicating that the His-tag does not interfere with the SecA activity. The DSC thermogram of His-N-SecA protein shows only one endothermic transition at 50.3°C and subsequently an exothermic transition indicating aggregation similar to the second transition of intact SecA (Fig. 2B, Table 1). As previously shown for the wild-type SecA [13], the transition could be simulated optimally by a two state transition assuming the His-N-SecA protein to be a dimer (Table 1). The transition is about 3°C upshifted compared to T_m2 of the wild type SecA, but only 1.5°C upshift as compared to the His tagged SecA (T_m1 and T_m2 of 39 and 48.8°C, respectively) (data not shown). Therefore, it appears that T_m2 is somewhat affected by the presence of the His-tag at the amino-terminus. The similarity in the thermal unfolding behavior of His-N-
SecA confirms our hypothesis that $T_{m2}$ corresponds to the unfolding of the N-domain and suggests that $T_{m1}$ corresponds to the unfolding of the less stable C-domain.

The observation that the saturation of NBS-I with ADP causes a rather large upshift in $T_{m1}$, i.e. the C-domain of SecA, while the ATP analogue AMP-PNP had no effect on the unfolding pattern of both domains [13] urged us to determine the effect of nucleotides in more detail by isothermal titration calorimetry (ITC). This method determines directly the heat of dilution of the nucleotide in the buffer, but this signal of about 300 W was subtracted from the corresponding data with SecA in the cell. A representative titration experiment for ADP and ATP$S$ is shown in Fig. 3. The association constant ($K_a$), number of binding sites ($n$) and enthalpy and entropy contributions to the Gibbs free energy of association ($\Delta G^0 = \Delta H^0 - T\Delta S^0$) were determined from this binding isotherm (Table 2). The binding of ADP at 25°C is driven by a negative enthalpy change, $\Delta H^0$, of $-14.44$ kcal/mol with a $K_a$ of 270 nM. The latter is similar to the $K_a$ for ADP binding to NBS-I measured previously by tryptophan fluorescence [13]. Since the $\Delta G^0$ corresponds to $-9.0$ kcal/mol, the $\Delta S^0$ is equal to $-18.3$ cal/mol/K. The data demonstrate that binding of ADP to SecA is an entropically unfavorable event. Binding of ATP$S$ at 25°C is also driven by a negative enthalpy, $\Delta H^0$ of $-5.6$ kcal/mol, and occurs with a $K_a$ of 0.74 $\mu$M. The latter dissociation constant is about three times higher than the $K_a$ for ADP binding which either reflects the difference between ATP and ATP$S$ or corresponds indeed to the affinity for ATP. The $\Delta G^0$ is calculated to be $-8.4$ kcal/mol and the favorable entropy $\Delta S^0$ is 9.6 cal/mol/K.

To ascertain that the observed thermodynamics were due to the specific binding of nucleotides at NBS-I, the ITC with nucleotides was repeated with a NBS-I K106N SecA mutant that is unable to bind nucleotides at this site [10]. This mutant is defective in the endogenous and translocation ATPase activity and binds ADP only at NBS-II with a $K_a$ of 365 $\mu$M [10]. Moreover, K106N SecA does not support prePhoB translocation in the in vitro translocation assay (Fig. 1). As compared to wild-type, no major differences in the thermal unfolding transition temperatures were found (Table 1), indicating that the mutation has no major effects on the overall structure and stability of the protein. No thermal effects were observed upon titration of 8.6 $\mu$M K106N SecA at 30°C with an ADP solution up to 800 $\mu$M (Fig. 3A), while titration with a 300 $\mu$M solution of ATP$S$ at 30°C gives only a very weak signal of about $-0.6$ kcal/mol (Fig. 3A). The weakness of the exothermic signal indicates that SecA K106N either has some affinity for ATP$S$ at NBS-I or binds the nucleotide non-specific. It can be concluded that the thermodynamic parameters obtained for binding of nucleotides to SecA represent the occupation of NBS-I and the resulting conformational changes.

The binding energies for ADP and ATP$S$ to NBS-I of SecA do not differ much, the entropies in contrast are very different and even have an opposite sign. Since this is indicative for a change in heat capacity, the heat of nucleotide binding as a function of temperature was determined. The $K_a$ and the enthalpy for the interaction between SecA and the two nucleotides were determined between 10–30°C. Below 10°C, the heat of the reaction was insufficient to be measured and above 30°C, the enthalpy change becomes a mixture of nucleotide binding, C-domain unfolding and stabilization of this domain due to increased interaction with the N-domain (Fig.

---

**Table 1**

Thermodynamics of the unfolding of SecA

<table>
<thead>
<tr>
<th>Buffer, 50 mM</th>
<th>$T_{m1}$, °C</th>
<th>$\Delta H_{m1}$, kcal/mol</th>
<th>$\Delta H_{H1}$, kcal/mol</th>
<th>$\Delta H_{H1}/\Delta H_{m1}$</th>
<th>$T_{m2}$, °C</th>
<th>$\Delta H_{m2}$, kcal/mol</th>
<th>$\Delta H_{H2}$, kcal/mol</th>
<th>$\Delta H_{H2}/\Delta H_{m2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SecA</td>
<td>39.6 ± 0.5</td>
<td>150 ± 23</td>
<td>152 ± 20</td>
<td>1.01</td>
<td>47.3 ± 0.5</td>
<td>160 ± 24</td>
<td>161 ± 24</td>
<td>1.01</td>
</tr>
<tr>
<td>His-N-SecA</td>
<td>39.6 ± 0.5</td>
<td>145 ± 20</td>
<td>144 ± 20</td>
<td>0.85</td>
<td>47.9 ± 0.5</td>
<td>213 ± 43</td>
<td>125 ± 25</td>
<td>1.7</td>
</tr>
<tr>
<td>K106N</td>
<td>37.7 ± 0.5</td>
<td>143 ± 28</td>
<td>121 ± 24</td>
<td>0.85</td>
<td>47.9 ± 0.5</td>
<td>213 ± 43</td>
<td>125 ± 25</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Experiments were performed in TKM buffer.
4). The plot of $v_H$ as a function of temperature for the binding of ADP (Fig. 4A) clearly shows a linear relationship up to 30°C and from the slope, the heat capacity change, $v_C$, of association is obtained as $3780 \pm 160$ cal/mol/K for ADP (Table 2). The $v_C$ for ATP binding is much smaller, i.e. $3190 \pm 60$ cal/mol/K (Fig. 4B and Table 2). As a consequence of the large negative $v_C$ for ADP binding, the enthalpic and entropic contributions are strong functions of the temperature, whereas the Gibbs energy of association is practically independent of the temperature (Fig. 4A). The large negative $v_C$ also indicates a considerable change in the solvent accessible area and a conformational change of the protein upon binding of ADP.

### Table 2

Thermodynamic parameters of the binding of ADP and ATP to *B. subtilis* SecA at 25°C and the entropic contributions where folding is coupled to association

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ADP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>1.08 (0.02)</td>
<td>1.03 (0.03)</td>
</tr>
<tr>
<td>$K_a$ (mol$^{-1}$)</td>
<td>$3.6 (7.1) \times 10^6$</td>
<td>$1.4 (4.3) \times 10^6$</td>
</tr>
<tr>
<td>$\Delta H^\circ$ (kcal/mol)</td>
<td>$-14.44 (1.14)$</td>
<td>$-5.56 (0.88)$</td>
</tr>
<tr>
<td>$\Delta S^\circ$ (cal/mol/K)</td>
<td>$-18.25 (3.69)$</td>
<td>$9.55 (3.08)$</td>
</tr>
<tr>
<td>$\Delta G^\circ$ (kcal/mol)</td>
<td>$-9.00 (0.08)$</td>
<td>$-8.42 (0.22)$</td>
</tr>
<tr>
<td>$\Delta C_T$ (cal/mol/K)</td>
<td>$-780 (160)$</td>
<td>$-186 (60)$</td>
</tr>
</tbody>
</table>

S.D. is shown between parentheses. Stoichiometry, $n$; equilibrium association constant, $K_a$; calorimetric enthalpy change, $\Delta H^\circ$; calorimetric entropy change, $\Delta S^\circ$; change in free energy of the formation of the protein-ligand complex, $\Delta G^\circ$; binding-induced change in heat capacity, $\Delta C_T$.

### 4. Discussion

A main unresolved question in bacterial protein translocation is how conformational changes of SecA lead to force generation and how preprotein translocation is coupled to ATP hydrolysis. In this report, we have used calorimetry to further analyze the thermal domain organization of SecA and its interaction with nucleotides. DSC measurements of the *B. subtilis* SecA confirm the notion that SecA consists of at least two independently unfolding domains [13]. The second transition ($T_m$) in the thermogram can now be assigned to unfolding of the N-domain of SecA, which leaves the first transition ($T_m$) to the unfolding of the C-domain. We have previously shown that saturation of NBS-I with ADP causes a rather large upshift in the transition temperature of $T_m$, i.e. the C-domain of SecA, while the ATP analogue AMP-PNP had no effect on the unfolding pattern of both domains [13]. Since this NBS is located in the N-domain [7–10], it appears that ADP binding to NBS-I stabilizes the C-domain of SecA. Site-directed tryptophan fluorescence studies have previously shown that this binding event elicits a conformational change that propagates to the C-domain [13]. The thermolabile C-domain includes the region of SecA that has been suggested to insert into the membrane [5,6,25]. It is remarkable that the onset of the unfolding reaction of the C-domain is close to the growth temperature of *E. coli*. In this respect, when overexpressed as a fragment (residues 387–850), the C-domain was found to aggregate into a state that could not be used for DSC analysis (T. den Blaauwen, unpublished data).
[Q]

more modest change is evident for ATP appears to be independent of the temperature. A plot of \( v \) at the temperature where slope near unity (\( = 0.974 \)) and an enthalpy intercept (enthalpy indicates a strong enthalpy-entropy compensation with a strong correlation exists between dynamics of ATP

of ADP binding.

Q

modynamics of ATP

versus T

results, a large change in heat capacity for ADP binding (\( 190 \pm 60 \text{ cal/mol/K} \)). With the binding of ADP, the \( \Delta G \) appears to be independent of the temperature. A plot of \( \Delta H \) versus T\&S for the binding of ADP at different temperatures indicates a strong enthalpy-entropy compensation with a slope near unity (\( = 0.974 \)) and an enthalpy intercept (enthalpy at the temperature where \( \Delta S \) equals 0) of \( -9.14 \text{ kcal/mol} \) (\( r^2 = 0.999 \)) (data not shown). This weak temperature-dependence of \( \Delta G \) and the strong enthalpy-entropy compensation are direct consequences of the large \( \Delta C_p \) [26]. Similar observations have been made in other protein-ligand interactions (for other examples, see [27]). The \( \Delta G \) for ATP\&S binding is more temperature-dependent and the \( \Delta H \) is only partially compensated for by the T\&S with a slope of 1.46 and an enthalpy intercept of \( -9.59 \text{ kcal/mol} \). This dependence on temperature of \( \Delta G \) and the favorable \( \Delta S \) of 9.55 cal/mol/K suggest that the thermodynamics of ATP\&S binding are more complex than those of ADP binding.

The heat capacity of interactions that involve proteins, such as protein folding and protein-ligand binding, is determined by hydrophobic and hydrophilic interactions [28-30]. A strong correlation exists between \( \Delta C_p \) and the surface area buried on forming a complex between the ligand and protein [31,32]. Removal of protein surface area from exposure to aqueous solvent has been shown to lead to a large negative \( \Delta C_p \) [32]. The large \( \Delta C_p \) and strong dependence of the enthalpy and entropy on the temperature of the reaction suggest that ADP binding is accompanied by changes in the solvent accessible surface and conformation of SecA. No significant changes in the circular dichroism spectra of SecA in the presence of ADP or ATP\&S were observed (results not shown). This suggests that this large reduction of the solvent accessible surface area is the result from only a small conformational change of SecA upon binding of ADP. This is probably the consequence of the increased interaction between the C- and the N-domain in the presence of ADP. This is also suggested by the unfavorable \( \Delta S \) that accompanies ADP binding. It is important to note that previous DSC data indicate that saturation of NBS-II with ADP drives SecA in an even more compact conformation [13] and that NBS-II is located near the subunit interface [12]. The precise role of NBS-II in protein translocation is, however, still unresolved [6,9,12]. Due to the low nucleotide binding affinity, a thermodynamic analysis of NBS-II by titration calorimetry is technically not feasible.

The calorimetric studies suggest a model in which SecA undergoes two major conformational changes. Upon ADP binding at NBS-I, the protein adopts a more compact state. Most of the enthalpy and entropy changes that accompany this event are due to a conformational change in which the C- and N-domain have been induced to share a considerable part of their surface area. This interaction stabilizes the membrane-inserting C-domain [13]. The ADP-bound state of SecA might be the energy-loaded form of the molecule and this energy may be released to perform mechanical work during the successive ADP-ATP exchange reaction. This event could be coupled to a molecular displacement of the SecYEG-bound C-domain relative to the preprotein binding N-domain. The thermodynamic analysis of the binding of nucleotides to SecA in the absence of the other components of the preprotein translocation pathway provides only a limited picture of the conformational changes in SecA during preprotein translocation. This study provides the basis for an extensive thermodynamic analysis of the effects of the preprotein and other trans-
locase subunits on the nucleotide binding-induced conformations of SecA.

Acknowledgements: The authors thank Drs Wim Meiberg, Erik Manting and Juke Lolkema for stimulating discussions. This work was supported by a PIONIER Grant of the Netherlands Organization for Scientific Research (N.W.O.), the Netherlands Foundation for Chemical Research (C.W.) and the E.C. as part of the Biotech program BI02-CT-930254 and BI04-CT-960097.

References