Purification and characterization of DNA ligase I from the trypanosomatid *Crithidia fasciculata*

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ABSTRACT

A DNA ligase has been purified approximately 5000-fold, to near homogeneity, from the trypanosomatid *Crithidia fasciculata*. The purified enzyme contains polypeptides with molecular masses of 84 and 80 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both polypeptides formed enzyme-adenylate complexes in the absence of DNA, contained an epitope that is highly conserved between human and bovine DNA ligase I and yeast and vaccinia virus DNA ligases, and were identified in fresh lysates of *C. fasciculata* by antibodies raised against the purified protein. Hydrodynamic measurements indicate that the enzyme is an asymmetric protein of approximately 80 kDa. The purified DNA ligase can join oligo(dT) annealed to poly(dA), but not oligo(dT) annealed to poly(rA), and can ligate blunt-ended DNA fragments. The enzyme has a low K_m for ATP of 0.3 μ M. The DNA ligase absolutely requires ATP and Mg^{2+} , and is inhibited by *N*-ethylmaleimide and by KCl. Substrate specificity, K_m for ATP, and the conserved epitope all suggest that the purified enzyme is the trypanosome homologue of DNA ligase I.

INTRODUCTION

DNA ligases catalyze phosphodiester bond formation between adjacent 5'-phosphoryl and 3'-hydroxyl termini in nicked DNA during replication, repair, and recombination (1–5). Three distinct DNA ligases have been identified in mammalian cells (6). DNA ligase I is the major ligase activity in proliferating mammalian cells (7), and, like DNA polymerase α , its activity is induced in regenerating rat liver (8, 9). It is localized in the nucleus of mammalian cells (10, 11), and the gene encoding human DNA ligase I can complement the DNA replication defect of a *Saccharomyces cerevisiae* DNA ligase (*cdc9*) mutant (12). These findings all suggest that DNA ligase I functions in DNA replication. DNA ligase I has been purified from a number of higher eukaryotes, including mammalian tissues and cells (7, 13–16), *Drosophila melanogaster* (17, 18), and *Xenopus laevis* (19), but not from any protozoan species.

Trypanosomatids are protozoan parasites of considerable economic and medical importance, causing serious disease in humans and livestock. They are among the most ancient of eukaryotes, having diverged from the main eukaryotic lineage very early in evolution (20, 21). Two proteins expected to be involved in chromosomal DNA replication, a type I topoisomerase (22) and replication protein A (23), have been purified to near-homogeneity from the trypanosomatid *Crithidia fasciculata*. These proteins are biochemically similar to the homologous proteins in higher eukaryotes, suggesting that the basic DNA replication machinery found in animals and fungi is also present in trypanosomes. We have purified a DNA ligase from *C. fasciculata* and found it to be similar, in most of the biochemical properties analyzed, to DNA ligase I from higher eukaryotes, suggesting that DNA ligase I function is conserved between evolutionarily distant organisms.

MATERIALS AND METHODS

Cell growth

C. fasciculata Cf-C1 was grown, harvested, and stored frozen at -75°C as described (24).

DNA ligase assays

DNA ligase assay substrate was prepared by labelling 10 μ g of oligonucleotide (either (dT)_{12–18} or (rA)_{12–18}) with [γ -³²P] ATP using T4 polynucleotide kinase. An equal amount (10 μ g) of unlabelled polynucleotide [poly (dA), poly (rA), or poly (dT)] was added, followed by incubation at 70°C for 10 minutes. The substrate was slowly cooled to room temperature and unincorporated ³²P was removed by G25 spun column chromatography.

DNA ligase assays (10 μ l) contained 50 mM Hepes, pH 7.5, 10 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 1 mM ATP, 9.9 μ g/ml polynucleotide substrate (1 μ M 5' ends, 3000 cpm/pmol 5' ends), and were incubated for 15 minutes at 30°C. Alkaline phosphatase solution (10 μ l) containing 100 mM Tris, pH 9.0, 2 mM MgCl₂, 0.2 mM ZnCl₂, 2 mM spermidine, and 0.05 U/ μ l calf intestinal alkaline phosphatase

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was added and incubation was continued for 15 minutes at 56°C. Phosphatase-resistant phosphodiesterases were quantitated by spotting the reactions on DE-81 paper and washing with 0.5 M NaPO₄, pH 7.0, followed by liquid scintillation counting. One unit of DNA ligase activity converts 1 nmol of 5' phosphates to a phosphatase-resistant form in 15 minutes at 30°C. DNA ligase assays for determination of the K_m for ATP contained 5 μ M 5' ends of polynucleotide substrate, and 0.002 U of fraction VIII DNA ligase.

Adenylation reactions (10 μ l) contained 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 μ Ci [α -³²P] ATP (800 Ci/mmol), and DNA ligase, and were incubated for 15 minutes at 30°C. SDS sample buffer was added, and the reactions were boiled for 5 minutes. Proteins were separated by SDS-PAGE and analyzed by autoradiography.

Ligation of blunt-ended DNA fragments was performed in ligase assay buffer, with 200 ng of Hae III-digested pUC 19 DNA, 0 to 15% (w/v) polyethylene glycol 8000, and 0.02 U of fraction VIII DNA ligase I. Reactions were incubated at 30°C for 2 hours and products were analyzed on 1% agarose gels.

Ligase I purification

Fifty grams of frozen cells (approximately 5×10^{11} cells) were thawed in 250 ml of 50 mM Hepes, pH 7.5, 10 mM EDTA, 5 mM β -mercaptoethanol, 5 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl chloride (PMSF), 10 mM benzamidine. After homogenization with a motor-driven teflon pestle, NaCl was added to 0.5 M, and the lysate was stirred for 30 minutes at 0°C. The lysate was clarified by centrifugation in a Beckman Ti45 rotor (40,000 rpm, 60 minutes, 2°C). An equal volume of a saturated solution of (NH₄)₂SO₄ was added to the clear amber supernatant, which was then stirred for 30 minutes. The precipitate was collected, dissolved in 300 ml of 25 mM Hepes, pH 7.5, 0.02% (w/v) Brij 58, 10% (w/v) sucrose, 2.5 mM β -mercaptoethanol, 1 μ g/ml leupeptin, 0.5 μ g/ml pepstatin A, 0.25 mM PMSF (Buffer A) and was loaded onto a 4.8 $\times$ 5.5 cm hydroxyapatite (LKB Ultrogel HA) column equilibrated in Buffer A plus 50 mM (NH₄)₂SO₄. The column was washed with two column volumes of Buffer A, and eluted with three volumes of Buffer A plus 25 mM KPO₄. The eluate was loaded directly onto a 2.5 $\times$ 4.1 cm Heparin-sepharose column equilibrated with Buffer A plus 1 mM EDTA and 10 mM KCl. Ligase activity was eluted with three column volumes of Buffer A plus 1 mM EDTA and 200 mM KCl. An equal volume of glycerol was added, and the heparin column fraction was stored at -20°C. Four heparin column fractions were pooled and dialysed against Buffer B (25 mM MES, pH 6.8, 0.1 mM EDTA, 20% sucrose, 0.02% Brij 58, 1 μ g/ml leupeptin, 2.5 mM β -mercaptoethanol). The dialysate was applied to a 2.5 $\times$ 8.7 cm Bio-Rex 70 column in Buffer B plus 10 mM KCl. The column was washed with Buffer B plus 10 mM KCl, and eluted with a 600 ml 10 mM to 350 mM KCl gradient in Buffer B. Active fractions were pooled, concentrated using a Centrprep 30 (Amicon), and applied to a 1.8 $\times$ 98 cm Sephacryl S200 column equilibrated in Buffer C (25 mM imidazole, pH 7.5, 0.1 mM EDTA, 0.02% Brij 58, 10% sucrose, 1 μ g/ml leupeptin, 2.5 mM β -mercaptoethanol) plus 10 mM KCl. Active fractions were applied to an FPLC mono Q column (HR 5/5, Pharmacia), which was eluted with a 10 mM to 500 mM KCl gradient in Buffer C. Active fractions, eluting at approximately 250 mM KCl, were pooled, diluted five-fold with Buffer C, and applied to an FPLC polyanion SI column (HR 5/5, Pharmacia). The polyanion SI

column was eluted with a 10 mM to 600 mM KCl gradient in Buffer C. Protein concentration was assayed using a modification of the Bradford method (25). Polyacrylamide gel electrophoresis was performed essentially as described (26). Proteins were detected by silver staining (27).

Immunological procedures

Antibodies to purified DNA ligase I were raised in Balb/c mice, using DNA ligase (fraction VIII) that had been transferred to nitrocellulose, excised, dissolved in DMSO, and emulsified with complete Freund's adjuvant (primary injection) or incomplete Freund's adjuvant (subsequent injections). Animals were immunized with approximately 1 μ g of ligase, boosted every 4 weeks, and serum was collected 7 days after each boost. Antiserum was used at a 1:200 dilution for probing immunoblots. Rabbit polyclonal antiserum raised against the 17-mer peptide CGISLRFPFRFTRIREDK that is highly conserved among yeast, human, and vaccinia virus DNA ligases (14) was kindly provided by Dr. Tomas Lindahl, and was used at a 1:100 dilution. Immunoblots were processed as described (23).

Native molecular mass determination

Sedimentations were performed using 4 ml linear glycerol gradients (10–30% (v/v)) in 25 mM HEPES, pH 7.5, 0.1 mM EDTA, 50 mM KCl, 0.02% Brij 58, 2.5 mM β -mercaptoethanol, 2 μ g/ml leupeptin. Purified ligase (0.03 units of fraction VIII) was sedimented with 250 μ g of yeast alcohol dehydrogenase, 50 μ g of bovine carbonic anhydrase, and 500 μ g of bovine catalase as markers. Centrifugation was at 54,000 rpm for 22 h at 2°C in an SW60 rotor (Beckman). Fractions of 0.1 ml were collected from the bottom and assayed for ligase activity using the adenylation assay.

Size exclusion chromatography was performed on a Superose 12 column (Pharmacia) in Buffer C plus 50 mM KCl using 0.07 units of purified DNA ligase (fraction VIII). Marker proteins were yeast alcohol dehydrogenase, bovine serum albumin, and bovine carbonic anhydrase (10 μ g each). The total and excluded volumes were determined using benzamidine and blue dextran. Fractions of 0.5 ml were collected and assayed using the adenylation assay.

The Stokes radius, sedimentation coefficient, molecular mass, and frictional ratio were calculated as described (28, 29).

RESULTS

Purification of *Crithidia fasciculata* DNA ligase I

The purification procedure is summarized in Table 1. The enzyme was purified greater than 5000-fold, with a final yield of approximately 6%. DNA ligase assays of fractions eluting from the polyanion SI column (Figure 1a) show a single peak of ligase activity. Analysis of the same fractions by SDS-polyacrylamide gel electrophoresis (PAGE) (Figure 1b) shows the co-elution of an 84 kDa polypeptide with DNA ligase activity. A lower molecular weight polypeptide (of approximately 80 kDa) co-elutes with the 84 kDa protein, and with DNA ligase activity, and appears to be related to the 84 kDa polypeptide (see below). Both proteins are always present in the polyanion SI fraction, although the relative amounts vary from one purification to the next. To determine the molecular weight of the polypeptide(s) responsible for the DNA ligase activity, fraction VIII was incubated with [α -³²P] ATP in the absence of nicked DNA substrate. Under these conditions, DNA ligases are adenylylated, forming a

Table 1. Purification of DNA ligase from *Crithidia fasciculata*.

Fraction	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (x-fold)
I. Clarified extract	— ^a	8462	—	—	—
II. (NH ₄) ₂ SO ₄ precipitate	202.4	4325	0.047	100	1
III. Hydroxylapatite	170.1	1213	0.140	84	3.0
IV. Heparin-sepharose	146.0	366.7	0.398	72	8.5
V. Bio-Rex 70	57.7	37.8	1.53	28.5	322
VI. Sephacryl S200	35.3	12.1	2.92	17.4	62
VII. Mono Q	18.0	0.659	27.3	8.9	581
VIII. Polyanion SI	12.2	0.052	237	6.0	5040

^aThe activity of DNA ligase I could not be assayed in this fraction.

covalent ligase-AMP adduct. Proteins can then be resolved by SDS-PAGE, and ligase-AMP adducts identified by autoradiography. This analysis is shown in Figure 2, lane 1 and demonstrates that both the 84 kDa and the 80 kDa polypeptides form ligase-AMP adducts in the absence of DNA. In addition, the AMP adducts could be discharged by subsequent incubation with oligo(dT)·poly(dA) ligase substrate (data not shown), suggesting that both polypeptides are DNA ligases.

***Crithidia fasciculata* DNA ligase I contains an epitope conserved in human, bovine, yeast, and vaccinia virus DNA ligases**

The sequences of *Saccharomyces cerevisiae* (30), *Schizosaccharomyces pombe* (31), vaccinia virus (32), and human (12) DNA ligases all contain a highly conserved 16 amino acid sequence near the C-terminus (32). A polyclonal antiserum against a synthetic peptide corresponding to this sequence specifically recognizes bovine and human DNA ligase I enzymes on immunoblots (14). The anti-peptide antiserum was used to probe immunoblots of the purified *C.fasciculata* DNA ligase (Figure 2, lane 2), and identifies the same polypeptides that were labelled in the adenylation experiment in lane 1. Non-immune rabbit serum did not identify any polypeptides in a parallel experiment (data not shown). Therefore, the purified *C.fasciculata* DNA ligase contains this highly conserved C-terminal epitope. The 80 kDa polypeptide is also identified by the anti-peptide antiserum, and so also contains this conserved epitope, further suggesting that it is related to the 84 kDa polypeptide.

Identification of DNA ligase I in fresh *C.fasciculata* lysates

To determine the size of the DNA ligase *in vivo*, antiserum was raised against the purified enzyme following SDS-PAGE and transfer to nitrocellulose to remove minor contaminants. This antiserum was used to probe western blots of the purified DNA ligase (Figure 3, lane 1) and of crude extract of *C.fasciculata* lysed by boiling directly in SDS reducing buffer (Figure 3, lane 2). The antiserum identifies both the 84 kDa and the 80 kDa polypeptides in both the purified and the crude sample. This indicates that both of the polypeptides are present *in vivo*, that the 80 kDa polypeptide is not solely the result of a modification taking place during the purification procedure, and that the purified enzyme reflects the size of the intact protein found *in vivo*. Non-immune mouse serum failed to recognize any polypeptides in an immunoblot of identical samples (Figure 3, lanes 3 and 4).

Figure 1. Purification of *C.fasciculata* DNA ligase I by FPLC polyanion SI chromatography. (a) Activity profile of fractions from the polyanion SI column elution. Ligase assays were performed as described under 'Materials and Methods.' (b) 7.5% SDS-PAGE of fractions 45–58 (10 µl each) from the polyanion SI column. Proteins were detected by silver staining. The sizes of the molecular mass markers (M) are indicated, as is the size of DNA ligase I.

Substrate specificity of *C.fasciculata* DNA ligase I

The substrate specificity of the purified DNA ligase was tested on a series of synthetic polynucleotide substrates (Table 2). The *C.fasciculata* enzyme joins oligo(dT) molecules annealed to a poly(dA) template, but is incapable of joining oligo(dT) molecules annealed to a poly(rA) template. This substrate specificity is characteristic of DNA ligase I (16, 33, 34), but not of DNA ligase II or III, both of which can join oligo(dT) annealed to poly(rA) (6, 16, 18, 33). The *C.fasciculata* DNA ligase joins oligo(rA) annealed to poly(dT), as was found for mammalian DNA ligase I (6), but with 250-fold lower efficiency than it joins oligo(dT) annealed to poly(dA). The *C.fasciculata* DNA ligase also ligates blunt-ended DNA fragments in the presence of 15% polyethylene glycol 8000, conditions under which mammalian DNA ligase I catalyzes blunt end ligation, but mammalian DNA ligase II does not (33) (Figure 4, lane 5). Thus, the substrate specificity of DNA ligase purified from *C.fasciculata* closely resembles that of DNA ligase I enzymes from higher eukaryotes.

Figure 2. Identification of the *C.fasciculata* DNA ligase I polypeptides by adenylation and immunoblot analysis. Lane 1: purified DNA ligase I (fraction VIII; 100 ng) was adenylylated as described under 'Materials and Methods', separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. Lane 2: the nitrocellulose filter from Lane 1 was probed with antibodies against the synthetic peptide described under 'Materials and Methods.'

Figure 3. Immunoblot analysis of *C.fasciculata* DNA ligase I in crude lysates. Immunoblots of purified DNA ligase I (fraction VIII; 10 ng; lanes 1 and 3) and *C.fasciculata* lysed directly in SDS reducing buffer (3.75×10^6 cells; lanes 2 and 4) were probed with a 1:200 dilution of antiserum raised against fraction VIII DNA ligase I (lanes 1 and 2) or with a 1:200 dilution of non-immune mouse serum (lanes 3 and 4). Positions of the molecular weight markers and DNA ligase I are indicated.

Table 2. Substrate specificity of *Crithidia fasciculata* DNA ligase. DNA ligase was assayed under standard conditions, except that the specific activity of the substrate was 30000 cpm/pmol 5' ends.

Substrate	DNA ligase activity (5' ends ligated)
oligo(dT)·poly(dA)	0.754 pmol
oligo(dT)·poly(rA)	<0.001 pmol
oligo(rA)·poly(dT)	0.003 pmol

Table 3. Requirements for DNA ligase activity

Components	Relative activity (%)
Complete ^a	100
-ATP	<0.5
-ATP, +20 μ M dATP	<0.5
-ATP, +20 μ M GTP	<0.5
-ATP, +20 μ M NAD	<0.5
-MgCl ₂	<0.5
-DTT	98.0
-DTT, +0.5 mM NEM	15.5
+50 mM KCl	51.7

^aComplete reaction contains 50 mM HEPES (pH 7.1), 1 mM ATP, 1 mM DTT, 1 μ g/ml BSA, 5 mM MgCl₂, 10 pmol oligo dT·poly dA.

Figure 4. Ligation of blunt-ended DNA fragments by *C.fasciculata* DNA ligase I. Hae III fragments of pUC19 DNA (200 ng) were incubated with DNA ligase I and 0% (lane 2), 5% (lane 3), 10% (lane 4), or 15% (w/v) PEG 8000 (lane 5). Lane 1 contains 15% PEG 8000, but no DNA ligase. Products were resolved on an agarose gel, stained with ethidium bromide, and visualized by UV transillumination.

Native molecular weight of *C.fasciculata* DNA ligase I

Gel filtration of the purified enzyme on a Superose 12 column with several reference proteins yielded a Stokes radius of 36 angstroms (Figure 5a). Sedimentation of the purified enzyme in glycerol density gradients with marker proteins yielded a sedimentation coefficient of 5.4 S (Figure 5b). The 84 kDa DNA ligase polypeptide was assayed in these experiments by adenylation and SDS-PAGE analysis. Assuming a partial specific volume of 0.725 g/ml, the native molecular mass of the DNA ligase is approximately 80 kDa, indicating that the enzyme is a monomer. The frictional ratio of the DNA ligase is 1.3, indicating that the enzyme has an asymmetric shape, although the asymmetry is less than that found for bovine DNA ligase I, which has a frictional ratio of 1.9 (14).

Properties of the purified enzyme

The dependence of DNA ligase activity on the components of the reaction mixture is shown in Table 3. The purified DNA ligase required ATP for activity, with dATP, GTP, and NAD all unable to substitute for ATP. The enzyme also absolutely required the presence of Mg²⁺. Omitting DTT had little effect, but inclusion of 0.5 mM *N*-ethylmaleimide inhibited activity, suggesting that the enzyme contains sulfhydryl groups essential for its activity. Addition of KCl inhibited DNA ligase activity, with 50 mM KCl giving approximately 50% inhibition. The K_m for ATP for the DNA ligase was estimated by the method of Lineweaver and Burk (35) to be 0.34 μ M (Figure 6). A low K_m for ATP is typical of DNA ligase I enzymes. The K_m for ATP for *C.fasciculata* DNA ligase I is similar to the K_m for ATP reported for DNA ligase I from rabbit bone marrow (0.2 μ M (36)), and slightly lower than those reported for bovine DNA ligase I (2 μ M (13)) and *Drosophila melanogaster* DNA ligase I (1.6 μ M (34)).

DISCUSSION

A DNA ligase has been purified greater than 5000-fold from the trypanosomatid *C.fasciculata*. The enzyme is similar to DNA ligase I enzymes from higher eukaryotes in a number of its

Figure 5. Measure of the Stokes radius and sedimentation coefficient of *C.fasciculata* DNA ligase I. (a) DNA ligase I (fraction VIII; 0.07 U) was applied to a Superose 12 gel filtration column as described under 'Materials and Methods.' (b) 0.03 U of fraction VIII were sedimented in a 10–30% glycerol gradient as described under 'Materials and Methods'. The elution and migration positions of the 84 kDa DNA ligase I polypeptide are indicated by the arrows.

properties. It has a low K_m for ATP, ligates blunt-ended DNA fragments, and ligates oligo(dT) annealed to poly(dA) but not oligo(dT) annealed to poly(rA), all of which are characteristics of DNA ligase I. The purified protein also contains an epitope that is highly conserved among other DNA ligase I enzymes, which together with the biochemical data suggests that it is the trypanosome homologue of DNA ligase I.

Purified *C.fasciculata* DNA ligase I consists of two related polypeptides, of 84 kDa and 80 kDa. As shown in Figure 3, both polypeptides are also present in fresh lysates of *C.fasciculata*, suggesting that the 80 kDa form is not simply the result of proteolysis during the purification procedure. Limited proteolysis of the purified DNA ligase *in vitro* using subtilisin produced a 73 kDa DNA ligase fragment at the expense of both the 84 and 80 kDa forms, but did not change the relative amounts of the 84 and 80 kDa polypeptides (not shown). These findings are reminiscent of DNA ligase I from *D. melanogaster*, which was purified as two related polypeptides of 83 and 75 kDa which could be converted by proteolysis *in vitro* to a 64 kDa polypeptide (17). The 84 and 80 kDa polypeptides do not seem to be related by

Figure 6. K_m for ATP of the *C.fasciculata* DNA ligase I. Double-reciprocal plot of the variation of ligation reaction rate with ATP concentration.

phosphorylation, as treatment with calf intestinal alkaline phosphatase, bacterial alkaline phosphatase, or potato acid phosphatase had no effect on the migration of either the 84 or the 80 kDa polypeptide in SDS-polyacrylamide gels (not shown). Since both polypeptides are identified in immunoblots of fresh cell lysates using antibodies raised against the purified DNA ligase I, the size of the intact DNA ligase *in vivo* is not clear, although it is unlikely to be larger than 84 kDa. Further studies are required to determine the relationship between the 84 and 80 kDa DNA ligase I polypeptides.

The similarity of *C.fasciculata* DNA ligase I to DNA ligase I enzymes from higher eukaryotes is particularly striking considering the evolutionary distance between trypanosomatids and the animals and fungi (20, 21). This similarity underscores the importance of the cellular function of DNA ligase I, and is consistent with the finding that DNA ligase I is essential for the joining of Okazaki fragments during DNA replication in yeast (3). Isolation of the gene encoding *C. fasciculata* DNA ligase I will allow evaluation of the conservation of DNA ligase I between evolutionarily distant organisms.

DNA replication represents a good target for rational design of anti-trypanosomal drugs. Little is known, however, about the regulation of DNA replication or of replication proteins in trypanosomes. DNA ligase I activity is known to increase during proliferation of mammalian cells (7), and expression of DNA ligase I is cell-cycle regulated in *S.cerevisiae* (37). Further study of DNA ligase I in *C.fasciculata* may provide insight into the mechanisms of cell-cycle control of DNA replication in trypanosomes. Since the distamycin derivative FCE24517, an anti-tumor drug, has been found to interact specifically with human DNA ligase I, blocking adenylation of the enzyme (38), the DNA ligase I protein itself is a potential target for the development of anti-trypanosomal drugs.

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