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Properties of DNA ligase from uninfected and virus-infected HeLa cells

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ABSTRACT

HeLa cells contain a high M.W. form of DNA ligase which can be completely converted to a low M.W. form. Stokes radius, frictional ratio, sedimentation coefficient, molecular weight, pH dependence, and heat inactivation rate of the two forms have been studied. The major properties of the two forms of DNA ligase in HeLa cells (in particular molecular weights and pH dependence) resemble those of the "dimer" and "monomer" structures described in cultured human cells (Pedrali, G., Spadari, S., Ciarrocchi, G., Pedrini, M., Falaschi, A. (1973) Eur. J. Biochem., 39 343).

In synchronized HeLa cells, the DNA ligase shows a two fold increase during S phase and parallels the increase in the DNA synthesis rate. DNA ligase increases in parallel with viral DNA synthesis after infection of HeLa cells with vaccinia and Herpes virus but its cofactor requirements and physical properties (including the dimer → monomer conversion) are unchanged, suggesting that the newly formed ligase is not virus-coded.

INTRODUCTION

In previous papers (1, 2) we described the purification and some properties of the DNA ligase from cultured human cells. The enzyme requires ATP and shows unique properties such as a bimodal pH curve, a partial dependence on added heat stable protein fraction and fractionation into two molecular forms, one having a molecular weight of 190,000 and the other 95,000.

In the present paper I have studied some molecular properties of the DNA ligase purified from uninfected HeLa cells and compared it to the DNA ligase induced after infection of HeLa cells with vaccinia or Herpes viruses.

In virus infected HeLa cells the DNA ligase rises in parallel with

viral DNA synthesis. The possible correlation between the level of DNA ligase and DNA synthesis is studied in more detail during the S phase of synchronized HeLa cells.

MATERIALS AND METHODS

Reagents

Diethylaminoethylcellulose (DE-52), phosphocellulose (P11) and cellulose powder (CF11) were purchased from Whatman Co., England. Hydroxylapatite (Bio-Gel HT) was supplied by Bio-Rad Laboratories, California. Terminal transferase was purified from calf thymus according to the procedure of Chang and Bollum (3) and was a gift of Dr. C.Harvey of the Research Division of Hoffmann, La Roche Inc.. NP-40 detergent was obtained from Shell Co..

Growth of cells and viruses and infection procedures.

HeLa cells were grown in suspension cultures in F-13 medium (Gibco) supplemented with 5% fetal calf serum (Gibco). The cells were harvested at a density of $5x10^5$ cells per ml by centrifugation at 1000xg and washed as described by Berkowitz et al. (4).

The WR strain of vaccinia virus and vaccinia infected HeLa cells were grown as described by La Colla and Weissbach (5). HeLa S-3 cells, growing in logarithmic phase, were collected by centrifugation and suspended at a concentration of $5x10^6$ cells/ml in F-13 medium supplemented with 5% heat-inactivated fetal calf-serum. Vaccinia virus was added to the culture (12 PFU/cell) and allowed to adsorb in the presence of 20 mM $MgCl_2$ for one hour at 37°C. The cells were then washed and resuspended at $5x10^5$ cells/ml in F-13 medium supplemented with 5% heat-inactivated fetal calf serum, 1% lactalbumin hydrolyzate (Gibco), 1% non essential aminoacids (Gibco). This time was taken as 0 hour after infection. The infected cells were maintained in spinner culture at 37°C. At intervals after infection samples of the cells were centrifuged and washed (4).

Abbreviations: PFU = plaque-forming unit; MEM= minimal Eagle medium; HSV = herpes simplex I virus; M.W. = molecular weight.

HSV-1 MPdK$^-$ (a deletion mutant) virus was grown in HeLa F cells (Flow Laboratories) as described by Weissbach et al. (6). Cells (seeded at about $3x10^8$ per roller bottle) were infected with 10 PFU/cell. The virus was allowed to adsorb in the presence of 15 ml of MEM (Gibco, F-11). After one hour of adsorption the roll was layered with 250 ml of MEM supplemented with 2% heat-inactivated fetal calf serum. This time was taken as 0 hour. Cells were centrifuged and washed at the indicated intervals after infection.

Synchronization of cells and preparation of subcellular fractions.

HeLa S-3 cells were grown and synchronized as described by Spadari and Weissbach (7). Cytoplasmic fraction and Triton N-101 washed nuclei were prepared by Dounce homogenization according to Spadari and Weissbach (8).

Assay of DNA ligase.

The assay described by Spadari et al. (1) was used. The substrate was prepared according to Bertazzoni et al. (9). Oligo(dT)-cellulose, prepared by the method of Gilham (10) was a gift from Dr. S.Kerwar of the Roche Institute of Molecular Biology. It contained approximately 0.4 nmol of nucleotide residue per milligram of cellulose. The short oligo (dT) chains, covalently joined to cellulose, were extended with terminal transferase and the poly(dT) chains synthesized were of suitable length (40-50 nucleotides) for annealing polydA. Cellulose-^{3}H poly(dT).poly (dA) had 15,000 counts x min^{-1} x $nmol^{-1}$. One unit of enzyme is defined as the amount catalyzing the joining of 1 nmol of nucleotide residues of poly(dT) to cellulose per hour.

Boiled crude extract (5 µg/assay) was present in all assays after chromatography of DNA ligase on DEAE cellulose (2).

Assay of thymidine kinase.

Thymidine kinase was assayed according to Munyon et al. (11).

Purification of DNA ligase from uninfected and virus-infected HeLa cells.

The purification procedure described by Spadari et al. (1) was modified as follows: the dialyzed ammonium sulfate fraction (containing 85% of total activity) was adsorbed on a column of DE-52 at a proportion of 6

mg of protein per ml of packed resin. The column had been previously equilibrated with 10 mM Tris pH 7.5, 0.5 mM dithiothreitol (buffer A). The column was washed with one column volume of this buffer and then was eluted with 8 column volumes of linear gradient from 0 to 0.3 M NaCl in buffer A. The DNA ligase eluted as single peak of activity around 0.16 M NaCl.

Pooled fractions (40% of total activity) were dialyzed against 10 mM KPO_4 (pH 7.5), 0.5 mM dithiothreitol, 20% glycerol, 0.1% NP-40, 0.05 M KCl (buffer B), and adsorbed onto a phosphocellulose column, previously equilibrated with the same buffer, at a ratio of 4 mg of protein per ml of packed resin. The column was washed with one column volume of buffer B and eluted with 8 column volumes of a linear gradient between 0.05 and 0.45 M KCl in buffer B. The activity was eluted as single peak around 0.22 M KCl. Pooled fractions (35% of total activity) were dialyzed against 0.02 M KPO_4 (pH 7.5), 20% glycerol, 0.1% NP-40, 0.5 mM dithiothreitol (buffer C) and adsorbed onto a hydroxylapatite column at a concentration of 1 mg of protein per ml of packed resin previously equilibrated with buffer C. Elution was performed with 8 column volumes of a linear gradient from 0.02 to 0.3 M KPO_4 (pH 7.5) in buffer C. The activity was present in an asymmetric peak eluted around 0.15 M KPO_4. Yield from column was 90% and corresponded to 30% of the activity initially present. Peak fractions were purified 1000-fold over crude extracts.

Sucrose gradient centrifugation.

Five to twenty per cent (w/v) sucrose gradients were prepared in 50 mM Tris-HCl (pH 7.5), 0.15 M KCl, 1 mM dithiothreitol. The gradients were centrifuged for 15 hours at 2°C at 40,000 rpm in a Spinco SW 50 L rotor. Fractions (approximately 40) were collected dropwise from the bottom of each tube and assayed for DNA ligase. Aldolase, bovine serum albumin and myoglobin were used as references.

Gel filtration.

Analytical gel filtration was done on a column of Sephadex G-100, 1.5x23 cm, equilibrated with 50 mM Tris-HCl (pH 7.5), 0.15 M KCl, 1mM dithiothreitol. 0.5 ml fractions were collected at a flow rate of 2 ml per

hour. The column was previously calibrated with blue dextran, catalase, bovine serum albumin, ovalbumin and myoglobin.

Determination of molecular weights and frictional ratios.

The molecular weights and frictional ratios were estimated according to Siegel and Monty (12), using the values of Stokes radius measured by gel filtration and the $\underline{s}^{\circ}_{20,w}$ values determined by sedimentation in sucrose gradient.

Protein determination.

The fluorimetric assay for protein described by Bohlen et al. (13) has been used through all stages of the enzyme purification except the crude extracts where the Lowry method (14) has been used.

RESULTS

Properties of DNA ligase from HeLa cells.

When subcellular fractions are prepared by rupture of cells in aqueous systems as described by Spadari and Weissbach (8), approximately 80% of the DNA ligase activity is found in the cytoplasm compartment. The presence of DNA ligase in the cytoplasmic soluble fraction might of course be due to leakage of the enzyme from nuclei during cell rupture.

The experiments described in this paper have been done with the cytoplasmic fraction, but similar properties (unpublished results) were found for the DNA ligase purified from the nuclei.

When DNA ligase is purified from fresh HeLa cells, as described in Materials and Methods (the purification procedure is completed within 5 days) the enzyme eluted from the hydroxylapatite column is present in a form showing a high molecular weight (approximately 200,000) on Sephadex G-100 (Fig. 1). Its K_{av} value and Stokes radius (12) are 0.08 and 50 Å respectively. The sedimentation coefficient, estimated by sucrose density gradient centrifugation, is 8.6 (Fig. 2). Together with the Stokes radius this $\underline{s}^{\circ}_{20,w}$ value gives the approximate molecular weight of 177,000 and a frictional ratio of 1.35.

Upon freezing in acetone - CO_2 and thawing 4 times all the activity moves to a lower molecular weight on Sephadex G-100 (approximately

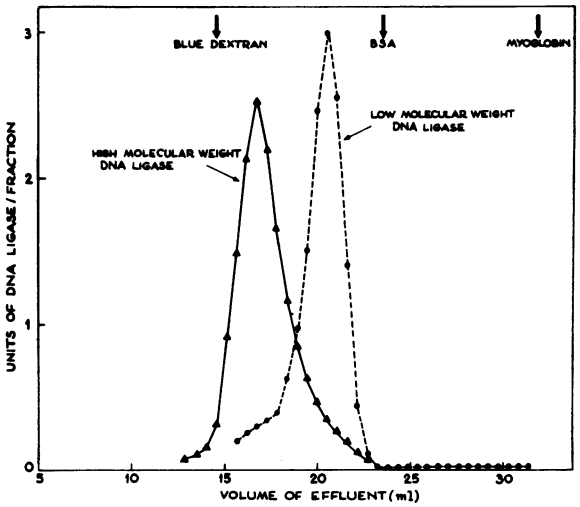

Fig. 1 - Gel filtration of HeLa DNA ligase on Sephadex G-100. 200 µl aliquots containing 100 Units/ml of either high M.W. (unfrozen hydroxylapatite fraction) or low M.W. (the same after freezing and thawing) DNA ligase in 0.15 M KCl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, were chromatographed as described in Methods.

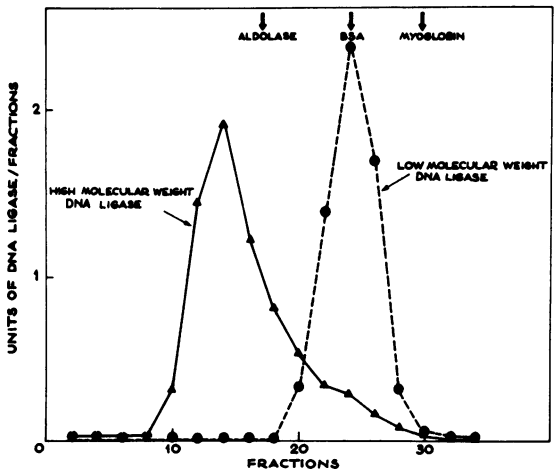

Fig. 2 - Sucrose gradient analysis of HeLa DNA ligase. 200 µl aliquots containing 100 Units/ml of either high or low M.W. DNA ligase (see legend to Fig. 1) in 0.15 M KCl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin were layered on a 5 to 20% (w/v) sucrose gradient and run as described in Methods. Similar results were obtained using 0.3 M KCl.

100,000) (Fig. 1) with a K_{av} value and Stokes radius of 0.237 and 44 Å respectively. The sedimentation coefficient, estimated by sucrose gradient centrifugation, of the low molecular weight activity, is 4.4 (Fig. 2) and the corresponding molecular weight, calculated from the Stokes radius and the $\underline{s}^{\circ}_{20,w}$ value, is 80,000. We have examined for the same properties the DNA ligase purified from vaccinia and HSV infected HeLa cells (see next paragraph). Molecular parameters of the two forms of DNA ligase, whether extracted from uninfected or from vaccinia and HSV infected HeLa cells, are summarized in Table I.

TABLE I

Molecular form	K_{av}	r	f/fo	$\underline{s}^{\circ}_{20,w}$	M.W.
High M.W.	0.08	50 Å	1.35	8.6 S	177,000
Low M.W.	0.237	44 Å	1.52	4.4 S	80,000

r= Stokes radius; f/fo = Frictional ratio; $\underline{s}^{\circ}_{20,w}$ = sedimentation value; M.W. = molecular weight; $K_{av} = \frac{Ve - Vo}{Vt - Vo}$

The two forms of DNA ligase present a first order inactivation kinetics at 45°C with similar inactivation rates; furthermore the heat inactivation curves at different temperatures (Fig. 3)are essentially identical for the two forms.

My conclusion is that the enzyme purified from HeLa cells shows the same dimer→ monomer conversion described previously in other human cells (2, 22) and that the smallest catalytically active unit has a molecular weight of approximately 80,000.

The similarity between the DNA ligase from HeLa and EUE cells (2) is further supported by the fact that the two forms of DNA ligase from HeLa cells show a pH dependence curve similar to that previously reported by Pedrali et al. (2). The results are shown in Fig. 4. The high molecular weight form is active over a broader pH range while the low molecular weight form has its pH optimum at 7.4.

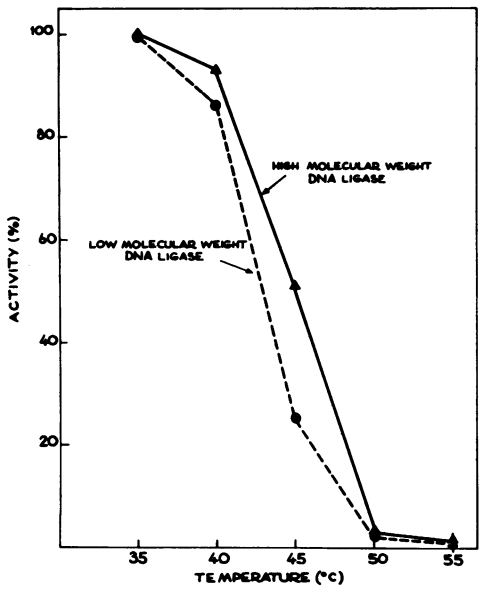

Fig. 3 - Heat inactivation of HeLa DNA ligase. 0.2 Units of high and low molecular weight DNA ligase (hydroxylapatite fractions) in 0.05 M Tris-HCl pH 7.5, 1 mM dithiothreitol, 0.1 M KCl and 0.5 mg/ml bovine serum albumin, were heated separately for 5 minutes at the indicated temperatures and assayed for DNA ligase activity.

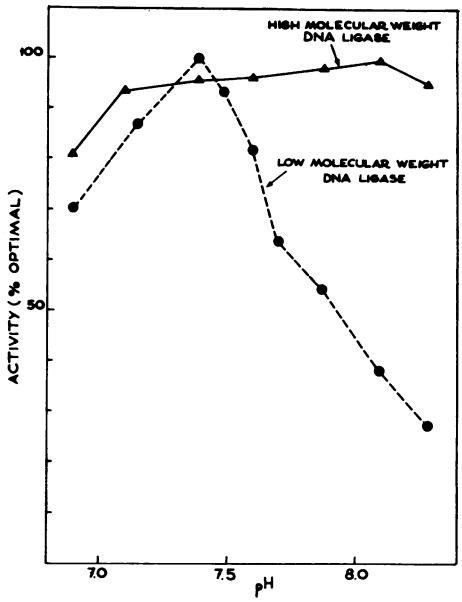

Fig. 4 - pH dependence of HeLa DNA ligase. Enzyme activity was measured in 50 mM Tris-HCl having the indicated pH at 20°C.

Levels of DNA ligase during S phase of synchronized HeLa cells.

The role of the ligase in DNA replication brings to the prediction that its level should be correlated with the rate of cellular DNA replication.

I have accordingly measured the levels of DNA ligase during S phase in synchronized HeLa cells obtained after a double thymidine block (7). The results are summarized in Fig. 5. DNA synthesis, as measured by ^{3}H-thymidine incorporation, continues for 8-10 hours reaching a peak about 5 hours after release of the thymidine block. During this period DNA ligase increases in parallel with DNA synthesis showing a maxi-

mum rise of approximately 2.5 fold about 4 hours after the cells have entered the S-phase, thus supporting the idea that DNA ligase is closely related to DNA replication. The rise of DNA ligase during S phase is similar to the rise of DNA polymerase α(7) which occurs with DNA synthesis (7, 15 - 17).

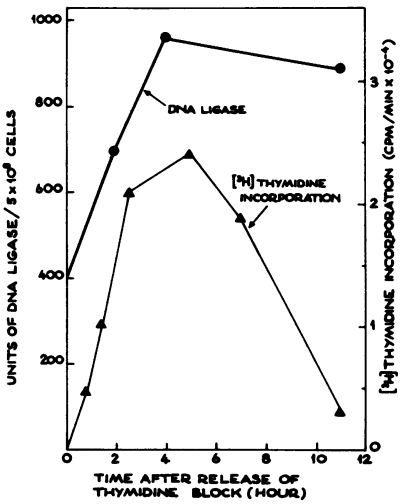

Fig. 5 - Levels of DNA ligase during S phase of synchronized HeLa cells. DNA ligase was assayed in cytoplasmic extracts prepared from 5×10^8 cells removed at the indicated times. A similar curve was obtained when ligase was assayed in nuclear extracts which contained approximately 20% of the total DNA ligase activity. HeLa S-3 cells were synchronized as described by Spadari and Weissbach (7).

Properties of DNA ligase induced in vaccinia- and Herpes-infected HeLa cells.

Sambrook and Shatkin (18) have reported some years ago an increase of DNA ligase activity after infection of HeLa cells with vaccinia virus. The cofactor requirement of the induced DNA ligase in crude extracts was unchanged and they had no evidence that this increase in activity was caused by the appearance of a viral-coded enzyme.

I have purified, by the procedure described in Methods, the DNA ligase induced in cells infected with vaccinia or Herpes Simplex I virus

and examined the purified enzymes for the properties described for the DNA ligase from uninfected HeLa cells.

The increase in DNA ligase activity in HeLa cells infected with vaccinia and Herpes viruses is reported in Fig. 6 A and B respectively. Enzyme activity is expressed as increase in specific activity relative to specific activity of uninfected cells at time zero.

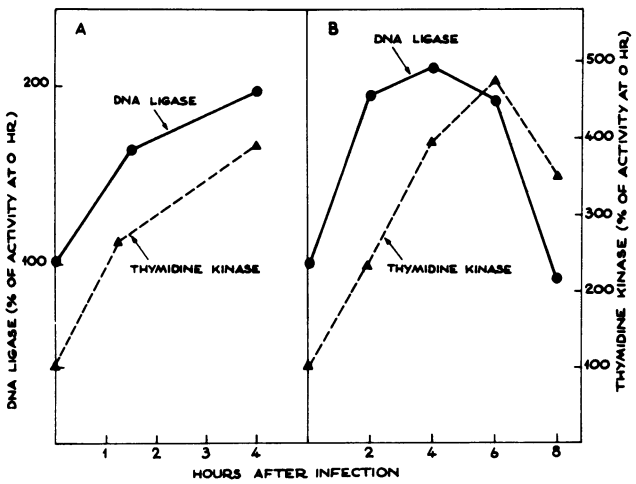

Fig. 6 - Increase of DNA ligase activity in HeLa cells infected with vaccinia (A) and Herpes Simplex I (B) viruses. In both cases the increase in thymidine kinase activity is reported for comparison. Specific activity of DNA ligase in uninfected cells at time zero was 350 Units.

The increase in thymidine kinase activity in the same cells is reported for comparison.

For the large scale purification of DNA ligase approximately 1 g of cells were harvested 3 and 5 hours after infection with vaccinia and Herpes viruses respectively, when the rate of ^{3}H-thymidine incorporation into viral DNA reaches its maximum (5, 6).

In both cases the partially purified enzymes were examined for the properties described for the DNA ligase obtained from uninfected cells.

The chromatographic properties of these ligases on DEAE cellulose, phosphocellulose and hydroxylapatite columns were identical. Mg^{++} and

ATP cofactors could not be replaced by Mn^{++} and NAD^{+} respectively. The purified enzymes present in hydroxylapatite fractions appeared as the high molecular weight form in both cases as measured by gel filtration on G-100 and sucrose sedimentation studies, and could be completely converted into a low molecular weight activity by freezing and thawing.

Molecular parameters of the two forms of DNA ligase from infected cells were identical to those reported in Table I for the HeLa cell DNA ligase. The pH dependence, crude boiled extract dependence and inactivation kinetics at 45°C were also undistinguishable from the values found for the DNA ligase from uninfected cells. I conclude that, based on all physical and biochemical properties examined, the newly formed ligase in virus infected cells is identical to the host ligase.

Ligase activity could not be detected in purified vaccinia or Herpes Simplex particles.

DISCUSSION

The present data and our earlier results (1, 2) indicate that DNA ligase is present in mammalian cells as high molecular weight form which can be completely converted in vitro to a low molecular weight form. The two forms differ in several parameters (molecular weight, K_{av}, Stokes radius, sedimentation coefficient, frictional ratio, pH dependence) but have in common the cofactor requirements (Mg^{++} and ATP), the crude boiled extract-dependence, the heat-inactivation kinetics.

Our data seem consistent with the dissociation of a dimeric protein into active monomers. But since we have never observed a reversion of the phenomenon in vitro, we cannot rule out the possibility of a dissociation of a protein tightly bound to the active ligase. Generation of a smaller active form by proteolysis seems unlikely since freezing and thawing completely dissociate the high molecular weight form.

Because of a tendency for many proteins to give aggregate forms including DNA polymerases α and β from HeLa cells (21) we feel that the lower molecular weight of 80,000 daltons probably corresponds to the catalytically active DNA ligase.

The high M.W. form closely resembles the DNA ligase I, that is the major ligase activity described in calf thymus by Söderhäll and Lindahl (19). These authors have also described a partial conversion of the calf thymus DNA ligase I to a low M.W. form (DNA ligase I "monomers") and, in addition, reported a minor DNA ligase activity in calf thymus, named DNA ligase II (19, 21). The enzyme has a lower M.W. (77,000 daltons), is not antigenically related to DNA ligase I and can be separated by ligase I on hydroxylapatite column. The low M.W. DNA ligase II differs in some properties, such as heat-inactivation and pH dependence, from the DNA ligase I "monomers". While the similar heat stability of the high and low molecular weight forms of the calf thymus DNA ligase I reported by these authors agrees with my present results, my studies show that the pH dependence of the low molecular weight form differs from that of the high molecular weight form and it is similar to that of Lindahl's low molecular weight DNA ligase II. These data taken together with the finding that the high molecular weight form can be completely dissociated to a low M.W. form should stimulate further experiments to obtain final proof that DNA ligase I and II (20, 22) are two separate enzymes.

Another relevant point in this present paper is the correlation between the level of DNA ligase and DNA synthesis. This observation is pertinent to the fact that DNA ligase increases with viral DNA synthesis after infection of HeLa cells with vaccinia and Herpes viruses and that it parallels DNA synthesis during the S-phase of synchronized HeLa cells. That DNA ligase is closely related to DNA replication is confirmed by the 15-fold increase of DNA ligase I activity during rat liver regeneration (22).

In both cases similar increases of DNA polymerase α have been reported (7, 23) and one may postulate that DNA ligase could act together with DNA polymerase α in DNA replication.

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