Volume 212, number 2, 259–262 FEB 04449 February 1987

Probing DNA polymerase α with monoclonal antibodies

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Received 8 December 1986

DNA polymerase α was purified from human KB cells by immunoaffinity chromatography. Enzyme activity was inhibited by three different monoclonal antibodies (SJK-132, SJK-211, SJK-287). Kinetic analysis showed that each antibody neutralized polymerase activity by a different mechanism. SJK-132 was competitive with DNA indicating it interacts with the DNA binding domain of the polymerase. SJK-287 showed a biphasic response to dCTP suggesting two dCTP binding sites exist on polymerase α. SJK-211 was non-competitive with DNA, dCTP and dATP.

DNA polymerase α; Monoclonal antibody; Enzyme kinetics

1. INTRODUCTION

The availability of monoclonal antibodies to DNA polymerase α [1] has permitted detailed analysis of the properties and distribution of this enzyme. These antibodies have been used to localize DNA polymerase α to the cell nucleus [2] and assisted in defining the separation of the polymerase α and DNA primase active sites [3,4]. Furthermore, a rapid immunoaffinity purification of this enzyme was developed by employing the monoclonal antibodies [3].

Inhibition kinetics have proven to be a powerful tool in elucidating the reaction mechanism of DNA polymerase α [5]. The coupling of this information with details of the structural organization of this enzyme may eventually lead to a comprehensive understanding of how polymerase α functions.

In this communication the use of $MgCl_2$ to immunopurify KB cell DNA polymerase α is presented and the application of neutralizing monoclonal antibodies as kinetic probes of polymerase α is described.

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2. MATERIALS AND METHODS

2.1. *Assay of DNA polymerase α*

The standard assay has been described in detail [6]. Kinetic analyses were performed using these assay conditions with variables indicated in the figures. Prior to initiating the reaction, enzyme was added to a mixture of antibody and substrate, and incubated for 10 min. Activated salmon sperm DNA served as template/primer in these experiments after extraction with phenol and dialysis into 10 mM Tris-HCl, pH 8.1, 1 mM EDTA.

2.2. *DNA polymerase α immunopurification*

Polymerase α was extracted from human KB cells (9 l, 3.5×10^5 cells/ml) and purified by phosphocellulose chromatography [3]. The phosphocellulose pool was rotated 12 h at 4°C with SJK-237 antibody [1] covalently coupled to Sepharose 4B (Pharmacia). The resin (1.5 ml) was pelleted by centrifugation, washed twice with 20 ml phosphate-buffered saline (PBS), transferred to a column and washed with another 20 ml PBS. The resin was then washed with 20 ml solution A (50 mM Tris-HCl, pH 8.1, 1 mM 2-mercaptoethanol) containing 1.0 M NaCl and eluted with 2.0 M $MgCl_2$ in solution A. Fractions

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00145793/87/$3.50

(1.0 ml) were assayed, pooled and desalted by Sephadex G-50 (Pharmacia) chromatography into solution B (solution A containing 20 mM NaCl, 20% ethylene glycol). The Sephadex G-50 pool was concentrated by dialysis against 30% sucrose, 0.05% Triton X-100 in solution B.

3. RESULTS AND DISCUSSION

3.1. *Purification of DNA polymerase α*

The availability of monoclonal antibodies to DNA polymerase α has made it possible to prepare highly purified enzyme. Immunoaffinity purification of polymerase α from mouse, human and bovine tissues has been described [3,7–9]. To overcome the difficulty of dissociating the polymerase from the antibody, DNA polymerase α from KB cells was purified in conjunction with the antibody [3]. Although there was no apparent influence of the antibody on the catalytic properties of the enzyme [10], it was unsuitable for studying the effects of antibody binding.

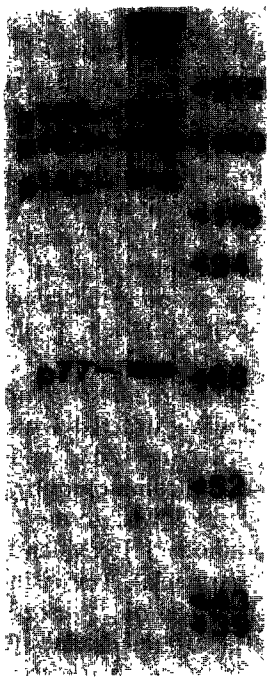

Fig.1. Subunit composition of immunoaffinity purified DNA polymerase α. Enzyme was precipitated with 10% trichloroacetic acid, subjected to SDS-gel electrophoresis in 8% polyacrylamide [21] and protein visualized by silver staining [22].

To obtain KB cell polymerase α without an associated antibody molecule, a procedure developed for the purification of calf thymus polymerase was used [7]. KB cell polymerase α was bound to antibody covalently linked to Sepharose and eluted with 2.0 M $MgCl_2$. Lower concentrations of $MgCl_2$ did not efficiently elute polymerase α while higher concentrations irreversibly inhibited activity. Analysis of the protein components of DNA polymerase α by SDS-polyacrylamide gel electrophoresis (fig.1) showed that the core polymerase polypeptides of 180, 165, 140 and 77 kDa were the major species present. The levels of 55 and 49 kDa proteins, usually considered part of the polymerase α complex [3], were greatly reduced. This polypeptide pattern resembled the subunit structure of calf thymus polymerase α [7] indicating a comparable efficiency of purification. Although the overall yield of polymerase obtained with this procedure was lower than the yield obtained from the protocol originally developed for the KB enzyme (25%), no immunoglobin subunits (52 and 23 kDa) were evident, thus ensuring this enzyme preparation was suitable for the kinetic experiments performed.

3.2. *Kinetic characterization of antibody inhibition*

Monoclonal antibodies are among the most specific binding molecules known [11] and this specificity, when combined with neutralizing capabilities, makes them excellent reagents for kinetic studies. However, both the high dissociation constant of the antibody-antigen complex and the size of the immunoglobin protein pose difficulties for their use in kinetic analysis. Polyclonal sera may inhibit enzyme activity through a specific interaction with the enzyme or by steric hindrance. Affinity purification of an antibody from sera greatly reduces inhibition caused by steric hindrance [12]. Monoclonal antibodies are highly purified so it can be assumed that their neutralizing ability is due to a specific interaction with DNA polymerase. Although several kinetic studies of antibody inhibition have been described using Michaelis-Menten graphics [12,13], they are generally unsuited to studying tight-binding reactions. Equations derived for interactions involving high dissociation constants are available and have been applied to antibody inhibition [14].

The inhibition of DNA polymerase α by the monoclonal antibodies SJK-132, SJK-211 and SJK-287 [1] may result from an interaction at one of the substrate binding sites. The tight-binding equations of Henderson [15] were used to examine the relationship of antibody inhibition and DNA. The data are summarized in fig.2. In the primary plot, a set of straight lines was obtained with SJK-132 antibody which increased in slope as higher DNA concentrations were added (fig.2). A secondary plot of slope versus DNA concentration (fig.2, inset) gave a straight line with a slope >0 which is typical of competitive inhibition [15]. A K_i of 0.06 μg was calculated from the y-intercept of the secondary plot (fig.2, inset). A similar study with SJK-211 showed that this antibody was non-competitive with DNA (fig.2, inset). Data obtained with SJK-287 were inconclusive since curved lines were obtained in the primary plot (not shown).

In addition to binding DNA, polymerase α has a binding site for deoxynucleotide 5'-triphosphates. Kinetic experiments designed to determine

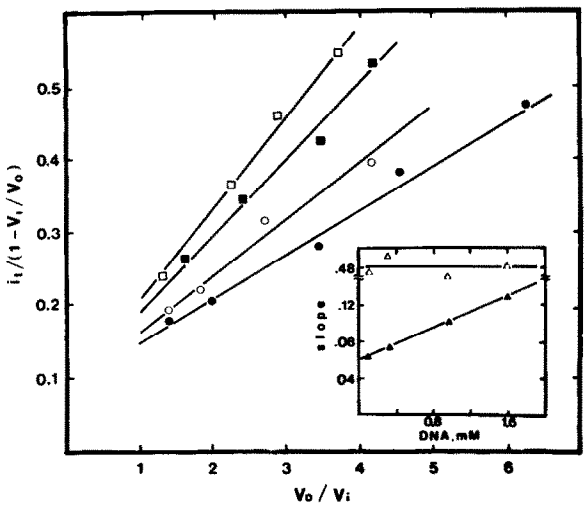

Fig.2. Graphical representation of kinetic data obtained by inhibition of DNA polymerase α by monoclonal antibodies under conditions of varied DNA concentration. Primary plot of data given by SJK-132. Each data point represents the average of duplicate assays and lines were determined by least squares analysis. Symbols are for 0.08 mM (●), 0.32 mM (○), 0.96 mM (■) and 1.6 mM (□) DNA. (Inset) Secondary plot of data presented for SJK-132 (▲) including data for SJK-211 (△).

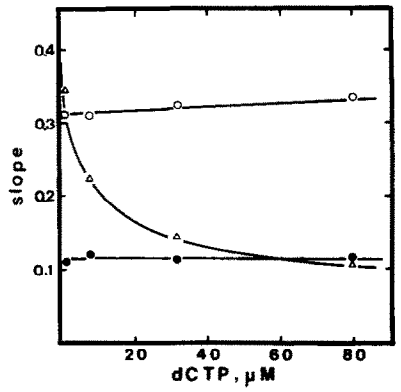

Fig.3. Secondary plot of kinetic data obtained with varied dCTP concentrations. Evaluation of the data was performed as described in fig.2. Results for SJK-132 (●), SJK-211 (○) and SJK-287 (△) are shown.

the effects of the antibodies on the interaction of polymerase α with dATP showed they were all non-competitive (not shown). The secondary plot of a similar study with dCTP is shown in fig.3. Both SJK-132 and SJK-211 were non-competitive with dCTP, producing lines with a slope = 0. In contrast, SJK-287 gave a curved line representing a series of lines in the primary plot with decreasing slope at higher dCTP concentration. This pattern is consistent with uncompetitive inhibition [15]. This indicated that there is an interaction of the antibody with a polymerase-dCTP complex. Although this conclusion is clear from fig.3, the primary plot (not shown) showed a break in the lines at $i_t/(1 - \nu_i/\nu_0) = 0.3$. At this point the slope of each line became 1.2, thus indicating non-competitive inhibition. The biphasic nature of the SJK-287 kinetics possibly reflects two separate events related to dCTP binding. These events may involve, among other possibilities, two dCTP binding sites on a single enzyme or different affinities of dCTP for the various forms of the catalytic subunit (p180, p165, p140). Structural analysis of both KB and calf thymus DNA polymerase has proven that the high molecular mass subunits show extensive homology and are probably derived from a common precursor [10,16]. Since SJK-287 interacts to the same extent with p180, p165 and p140 [10] it is unlikely that sufficient differences exist between these polypeptides to alter their interaction with dCTP. Indications that two dCTP binding sites are present on

polymerase α, however, have been made. There is evidence for the presence of an allosteric site which binds dCTP [17] and it has been suggested that the enzyme is composed of two catalytic subunits [7,10]. Further investigations are necessary to clarify the nature of this observation since insufficient information is available to interpret these data.

Previous studies have shown the presence of two binding domains on DNA polymerase α for DNA, one for the template and another for the primer terminus [5,18]. Interaction of polymerase α with DNA occurs in two stages [5], binding of the template and subsequent binding of primer terminus. The small amounts of available protein made testing for inhibition of DNA binding unsuccessful by filter binding [19] or gel electrophoresis [20] so that the effect of SJK-132 antibody on template binding could not be determined. It must be noted, however, that an analogous situation exists for poly(ADP-ribose) synthetase where the inhibitor of a specific interaction between the synthetase and DNA was competitive with DNA yet DNA binding was unaffected [19] and inhibitors of DNA binding were non-competitive (Zahradka and Ebisuzaki, unpublished).

In this report a procedure for the immunoaffinity purification of KB DNA polymerase α is presented which circumvents the use of less purified enzyme fractions for immunokinetic studies. The results obtained with this enzyme preparation show that the neutralizing antibodies developed against DNA polymerase α interact with unique substrate binding domains present on the enzyme. These data further indicate that each antibody inhibits enzyme activity by affecting a different catalytic process of the polymerization reaction.

ACKNOWLEDGEMENTS

These studies were supported by Research Grant CA-14835 from the National Cancer Institute and by a gift from the George D. Smith Fund. I would like to thank Stan Dunn for the helpful comments.

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