Domain Organization and DNA-Induced Conformational Changes of an Archaeal Family B DNA Polymerase†

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ABSTRACT: Family B DNA polymerase from the thermoacidophilic archaeon *Sulfolobus solfataricus* (Sso DNA pol) is a monomer of about 100 kDa with two associated catalytic functions: 3′-5′ exonuclease and DNA polymerase activities. The structure of this enzyme in the free and DNA-bound states was probed by limited proteolysis and fluorescence spectroscopy measurements. The results of partial trypsin proteolysis experiments on the recombinant Sso DNA pol pinpointed three major sites of protease sensitivity: near the N-terminus, within the center, and near the C-terminal end of the polypeptide chain. When partial trypsin digestion was carried out in the presence of either activated calf thymus DNA or primed M13mp18 single-stranded DNA, changes in cleavage pattern and in susceptibility to protease were detected. This phenomenon was dependent on the nucleic acid concentration and suggested the occurrence of DNA-induced conformational changes. These were also probed by steady-state fluorescence spectroscopy measurements using acrylamide as a quencher. Fine mapping of the DNA-specific cleavage sites allowed us to precisely locate the protein subdomains which were affected by these structural changes. Importantly, a specific proteolytic fragment of about 8 kDa was recovered after partial digestion of Sso DNA pol only in the presence of nucleic acid ligands. It was found to start at residues 392−394 and to span the protease-hypersensitive central region of the polypeptide chain. Its involvement in critical polymerase functions, such as substrate binding and/or enzyme processivity, was discussed. In addition, we found that controlled trypsin digestion of Sso DNA pol did not inactivate either polymerase or 3′-5′ exonuclease activity concomitantly with the disappearance of full-sized enzyme. Activity gel analysis revealed that proteolytic products corresponding to the amino- and carboxyl-terminal halves of the enzyme retained 3′-5′ exonuclease and DNA polymerase activity, respectively. These results are in line with the model of modular organization proposed for Sso DNA pol in a previous report [Pisani & Rossi (1994) *J. Biol. Chem. 269*, 7887−7892].

DNA polymerases have a key role in the maintenance of genetic information during an organisms lifetime and in its faithful hereditary transmission, being at the heart of fundamental biological processes, such as replication and repair of the genetic material [for a review, see Kornberg and Baker (1992)]. To accomplish all these critical tasks, DNA polymerases interact with accessory subunits responsible for specific enzymatic functions, or, alternatively, they possess some of the auxiliary catalytic functions associated in a single (usually multimodular) polypeptide chain. The prototype of this kind of structural organization is *Escherichia coli* DNA pol I,[1] whose three principal catalytic activities (5′-3′ and 3′-5′ exonucleases and polymerase activities) have been ascribed to independent modules of the protein molecule (Joyce & Steitz, 1994). On the basis of sequence similarities, DNA polymerases have been classified into families A, B, C, and X, which include enzymes similar to *Escherichia coli* DNA pol I, II, and III, and eukaryotic DNA pol β, respectively (Braithwaite & Ito, 1993). DNA polymerases of families A and B with 3′-5′ exonuclease activity possess three short sequence motifs (Exo I, II, and III) located in the same relative order within their N-terminal halves (Bernad et al., 1989). The Exo motifs contain invariant amino acid residues demonstrated to be catalytically critical for the 3′-5′ exonuclease activity (Bernad et al., 1989; Derbyshire et al., 1991; Beese et al., 1991; Blanco et al., 1992; Soengas et al., 1992; Morrison et al., 1991; Simon et al., 1991; Reha-Krantz & Nonay, 1993). As for the polymerase function, primary structure alignments, extended to both RNA- and DNA-dependent polymerases, have pinpointed a small number of highly conserved amino acid residues (Delarue et al., 1990; Joyce, 1991; Blanco et al., 1991). The catalytic significance of these residues for the polymerase activity has been demonstrated by site-specific mutagenesis studies in a certain number of enzymes (Méndez et al., 1994; Polesky et al., 1992; Copeland & Wang, 1993; Blasco et al., 1995). These findings have led to the hypothesis that the polymerase active site could be structurally and functionally conserved among all kinds of nucleotide polymerizing enzymes. This notion has recently received great support from the comparison of the tertiary structure of Klenow fragment, HIV-1 reverse transcriptase, and

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[1] Abbreviations: DNA pol, DNA polymerase; Sso, *Sulfolobus solfataricus*; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); OD, optical density; RF, replicative form; HIV, human immunodeficiency virus.

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bacteriophage T7 RNA polymerase (Joyce & Steitz, 1994; Steitz, 1993; Arnold et al., 1995). A remarkable similarity has been found in the overall structural organization of these enzymes, in spite of a very limited sequence similarity. In particular, their polymerase domain has been described as a right hand holding the DNA substrate in a deep cleft, defined by three protein subdomains referred to as palm, fingers, and thumb. The palm subdomain is at the bottom of the cleft and contains invariant active site carboxylate residues. Its topology, which consists of two α-helices and two antiparallel β-strands forming an $\alpha\beta\beta\alpha$ fold, is highly conserved among these structures, suggesting the existence of some conformational constraints dictated by a critical catalytic function. The fingers interact with the single-stranded region of DNA substrate near the primer terminus and contribute also to dNTP binding within the polymerase active site. On the other hand, the thumb subdomain forms a sort of α-helical flexible protrusion which contacts the minor groove of the product duplex.

More recently, the X-ray structure of the 31 kDa C-terminal catalytic domain of rat DNA polymerase β has been reported independently by two groups (Davies et al., 1994; Sawaya et al., 1994). Although it contains a big U-shaped cleft with subdomains analogous to fingers, thumb, and palm and its catalytically critical carboxylate residues are located at the bottom of this cleft within the palm region, nevertheless no structural homology has been found with any of the other known polymerase structures.

Unfortunately, much less is known regarding the structural organization of family B DNA polymerases, since there are no crystallographic data available so far about any representative of this group. Some clues have been derived from sequence alignments and site-specific mutagenesis studies, which have predicted for the family B enzymes a Klenow-like modular organization with a functional and structural separation of the 3′-5′ exonuclease and polymerase active sites (Bernad et al., 1989). However, it is not clear whether this unifying model could be extended to all family B DNA polymerases. In fact, it has been reported that a more direct interplay could take place during DNA synthesis between the exonuclease and polymerase functions of certain family B replicases, such as that from *Herpes simplex virus* (Weisshart et al., 1994), and bacteriophage T4 (Lin et al., 1994).

In this study, we have used as a model system a thermophilic and thermostable family B DNA polymerase from the thermoacidophilic archaeon *Sulfolobus solfataricus* (Sso DNA pol). This enzyme is a monomer of about 100 kDa, whose 3′-5′ exonuclease and polymerase activities reside on structurally independent protein domains, as previously described (Pisani et al., 1992; Pisani & Rossi, 1994). Herein, we have investigated the structure of the recombinant Sso DNA pol in the free and DNA-bound states by limited proteolysis experiments and fluorescence quenching measurements. This analysis has allowed us (i) to further investigate the model of modular organization previously proposed for this enzyme (Pisani & Rossi, 1994), (ii) to map precisely the sites of protease sensitivity, and (iii) to identify regions within the polypeptide chain affected by the DNA-induced conformational changes. Their involvement in DNA binding and enzyme processivity is discussed.

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were reagent grade. [α-^{32}P]dCTP (specific activity <3000 Ci/mmol, 10 mCi/mL) and [*methyl*-1′,2′-^{3}H]dTTP (specific activity <90 Ci/mmol) and Rainbow molecular size markers were from Amersham International. Oligodeoxyribonucleotide 24-mer (5′-ACTCTAGAGGATCCCCGGGTACCG-3′), synthesized by conventional solid phase methods and purified by gel filtration, was designed to be complementary to positions 6241−6264 of M13mp18(+)-strand DNA.

Enzymes. Overexpression of Sso DNA pol was obtained by subcloning its encoding gene into a pT7 vector derivative under the control of T7 RNA polymerase Φ10 promoter (Studier & Moffatt, 1986). The construction of this plasmid, as well as the purification of the recombinant enzyme, will be described elsewhere (unpublished results).

Preparation of DNA Templates. Activated calf thymus DNA was prepared according to Grippo et al. (1975). For the fluorescence measurements it was phenol-extracted to remove extraneous fluorescent proteins. 3′-End, ^{32}P-labeled activated calf thymus DNA (specific activity 150 000 cpm/μg) and 3′-end, ^{3}H-labeled activated calf thymus DNA (specific activity 3500 cpm/μg) were prepared essentially as described by Spanos et al. (1981). Annealing of 24-mer oligonucleotide to M13mp18 single-stranded DNA (0.25 pmol of 5′-ends/μg of DNA) was obtained by incubation at 75 °C for 5 min and slow-cooling to 25 °C.

Enzymatic Assays. Calf thymus activated DNA was used as substrate for polymerase activity assays, performed as previously described (Pisani & Rossi, 1994). Reaction mixture for quantitative 3′-5′ exonuclease assays (final volume 50 μL) contained 50 mM Tris/HCl, pH 8.0, 2.5 mM $MnCl_2$, 3 mM 2-mercaptoethanol, 200 μg/mL bovine serum albumin, and 500 μg/mL 3′-end, ^{3}H-labeled activated calf thymus DNA (specific activity 3500 cpm/μg). Reaction was started by addition of Sso DNA pol (about 0.3 μg). After incubation for 5 min at 75 °C (a layer of mineral oil was stratified over the top of the reaction mixture to prevent evaporation), 75 μL of trichloroacetic acid 10% (w/v) was added and the acid-soluble radioactivity measured by using a Packard Tri-Carb 300 liquid scintillation counter.

Limited Proteolysis by Trypsin. All protease digestions were carried out at 37 °C in the following buffer: 10 mM Tris/HCl, pH 8.0, 15 mM NaCl, 190 μM 2-mercaptoethanol, 190 μM $MgCl_2$, 153 μM $CaCl_2$, 3.8% glycerol. For the digestion time-course experiment described in Figure 1, the incubation mixture contained 70 μg of Sso DNA pol and 90 ng of trypsin in a final volume of 350 μL. Aliquots (40 μL) were withdrawn at the indicated time points and immediately pipetted into 1.5-mL Eppendorf tubes in a dry ice/ethanol bath. For the electrophoretic analysis (see below), each protein sample was denatured by heating at 95 °C for 5 min after addition of 3× loading buffer (20 μL). When the effect of trypsin digestion on Sso DNA pol catalytic activities was studied, the proteolysis experiment was carried out exactly as previously described, except that half of each aliquot was used for the denaturing gel electrophoresis and half for enzymatic assays (each assay was performed in duplicate immediately after withdrawal of the aliquot from the reaction mixture). When the effect of nucleic acid ligand on the tryptic digestion pattern of Sso DNA pol was analyzed, the protein (6 μg in a final reaction volume of 25

μL) was preincubated with the indicated amount of DNA at 37 °C for 10 min in the reaction buffer previously described. Then, proteolysis was initiated by addition of protease (6 ng) to each reaction mixture and incubation at 37 °C continued, typically, for an additional 30 min. Trypsin digestion was terminated by putting samples into a dry ice/ethanol bath. Subsequently, proteolytic products were subjected to SDS−PAGE, as described below.

Electrophoretic Analysis. Protein gel electrophoreses were performed using the Mini-Protean system (Bio-Rad). SDS−PAGE was carried out using the Tris/glycine buffer system, as described by Laemmli (1970). To identify low molecular size proteolytic fragments, the Tris/Tricine denaturing system was employed (Schagger & von Jagow, 1987). When indicated, densitometric scanning of Coomassie-stained gels was performed using a Sharp Scanner JX-325 apparatus (Pharmacia).

Activity Gel Analyses. Monodimensional polymerase activity gel of Sso DNA pol partially digested with trypsin was performed according to Karawya et al. (1983) with the modifications previously described (Pisani & Rossi, 1994).

3′-5′ exonuclease gel assay was carried out as described by Spanos et al. (1981) except that the renaturation buffer was 50 mM Tris/HCl, pH 8.0, 2 mM 2-mercaptoethanol, and after renaturation the gel was incubated at 65 °C for about 6 h in a reaction buffer of the same composition as the previous one with the addition of 2.5 mM $MnCl_2$.

N-Terminal Amino Acid Sequence Analysis. N-terminal amino acid sequence analysis of proteolytic products was performed after their separation by SDS−PAGE and electrotransfer onto PVDF membrane ProBlott (Applied Biosystems), as described by Matsudaira (1987) using an Applied Biosystems gas-phase sequencer (Model 477A) equipped with an on-line 120A PTH-analyzer. Tryptic fragments to be subjected to Edman degradation were prepared by digesting Sso DNA pol (60 μg) with trypsin (125 ng) at 37 °C for 30 min (reaction volume 250 μL), as previously described. Limited proteolysis of Sso DNA pol upon calf thymus DNA binding was carried out by incubating the protein (100 μg) with trypsin (260 ng) for 30 min at 37 °C in the presence of 160 μg of DNA (reaction volume 350 μL), as previously described. Half of this reaction mixture was subjected to denaturing electrophoresis on a 10% polyacrylamide gel in order to separate proteolytic products ranging from 48 to 27 kDa. The remaining portion of the sample was electrophoresed through a 16.5% polyacrylamide resolving gel overlaid with a 10% polyacrylamide spacer gel using a Tricine buffer system (Schagger & von Jagow, 1987) in order to recover the 8 kDa tryptic fragment.

Fluorescence Measurements. Fluorescence was measured on a Jasco FP-77 spectrofluorometer (Japan Spectroscopic Co., Ltd). This instrument employs the dinode feedback system to automatically correct the intensity variation and the spectral characteristics of the light source and of the excitation monochromator. Excitation was at 295 nm (to avoid the contribution of tyrosine residues), and emission was recorded either between 300 and 400 nm or at a fixed emission wavelength (347.5 nm, λ_{max} of Sso DNA pol). Fluorescence experiments were done using 1 mL quartz semimicrocuvettes in 500 μL solutions containing 80 nM Sso DNA pol in 20 mM sodium phosphate buffer, pH 7.2. Correction for the inner filter effect was not applied since low concentrations of acrylamide were used and the absorbance at 285 nm was much lower than 0.1 OD (Eftink & Ghiron, 1975; Schmid, 1989). Intensities from buffer and quencher were measured in the same manner and subtracted from sample intensities. Data were acquired with Sso DNA pol alone and in the presence of either activated calf thymus DNA (final concentration 42 μg/mL) or primed M13mp18 single-stranded DNA (final concentration 4.2 μg/mL).

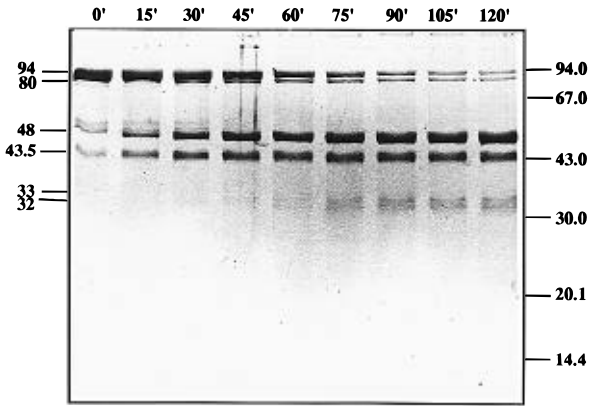

FIGURE 1: Limited proteolysis of Sso DNA pol by trypsin. Sso DNA pol (70 μg; specific activity 6624 units/mg) was incubated at 37 °C with 90 ng of trypsin (reaction volume 360 μL). At the indicated times, aliquots (4 μg of polymerase) were withdrawn and immediately put into a dry ice/ethanol bath to stop the reaction. Proteolytic digests were run through a 10% polyacrylamide denaturing gel. The position and the molecular size (in kDa) of the protein fragments are indicated on the left margin. The position of markers run on the same gel is indicated on the right margin.

RESULTS

Limited Tryptic Digestion of Sso DNA pol. Limited proteolysis under nondenaturing conditions is widely utilized to define the domain organization of proteins. This method is based on the observation that endoproteases prefer unstructured substrates that are flexible and able to adopt a conformation that fits their catalytic site. Thus, under mild conditions, preferential sites of proteolytic attack within a folded protein are segments with the highest conformational mobility, hydrophilicity, and exposure to the solvent. Since interdomain linkers usually possess the above features, controlled proteolysis experiments allow us to precisely locate domain boundaries of multimodular proteins (Price & Johnson, 1989).

The availability of highly purified recombinant Sso DNA pol in large amounts made it feasible to utilize partial trypsin digestion under native conditions to further characterize the domain structure of this protein and to evidentiate segments of high conformational flexibility within its polypeptide chain. We first analyzed the proteolytic products obtained by varying either the trypsin/Sso DNA pol ratio or the digestion times at a given fixed protease/protein ratio. This analysis allowed us to select the best conditions in order to identify stable and unstable proteolytic fragments. A typical example of a limited digestion time course experiment is given in Figure 1. Highly purified Sso DNA polymerase was partially digested with trypsin for 120 min at a protease/polymerase ratio of 1:800 (w/w). The intensity of the two major protein bands of about 48 and 43.5 kDa rapidly increased within the first 45 min of incubation and then became stable. Simultaneously, a minor, less stable fragment of about 80 kDa was detected. Minor proteolytic products

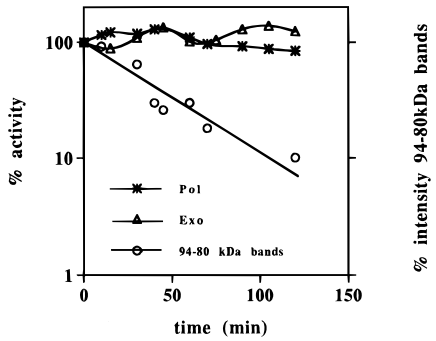

FIGURE 2: Effects of trypsin limited proteolysis on enzymatic activities of Sso DNA pol. A sample of Sso DNA pol (70 μg) was digested with trypsin as described in Figure 1. Aliquots of the reaction mixture were removed at the indicated time points and used to measure the enzymatic activities, as described under Materials and Methods. The percentage of polymerase (*) and 3′-5′ exonuclease (△) activity is plotted on a semi-logarithmic scale (left axis) versus the digestion time. Additionally, a portion of each removed aliquot was analyzed by SDS−PAGE in order to monitor the extent of trypsin digestion. The percentage of 94 plus 80 kDa bands, determined by densitometric scanning of Coomassie-stained gels, is plotted on a semi-logarithmic scale (right axis) versus the digestion time (○). The reported values are the average of three independent experiments.

of about 33 and 32 kDa appeared with slower kinetics. Densitometric analysis of Coomassie-stained gels revealed that the half-life of the 94/80 kDa protein species was about 30 min (see Figure 2).

Effect of Limited Tryptic Proteolysis on Sso DNA pol Enzymatic Activities. Effects of partial proteolysis with trypsin on the associated catalytic activities of Sso DNA pol were investigated by digestion time course experiments similar to that described in Figure 1. A sample of Sso DNA pol was digested with trypsin at a protease/polymerase ratio of 1:800 (by weight) for 120 min. At the indicated time points (from 0 to 120 min), aliquots were withdrawn and assayed for DNA polymerase and 3′-5′ exonuclease activities by standard methods (see Materials and Methods). At the same time, the residual fraction of high molecular size Sso DNA pol (94 plus 80 kDa protein bands) was quantitated by SDS−PAGE analysis and densitometric scanning of the Coomassie-stained gels (Figure 2). We found that trypsin digestion did not inactivate either the polymerase or the 3′-5′ exonuclease activities of Sso DNA pol: after 120 min incubation, both enzymatic functions remained substantially unchanged, whereas the amount of high molecular size Sso DNA pol was greatly reduced. Effects of partial tryptic proteolysis on Sso DNA pol catalytic activities were also monitored by monodimensional activity gels. This analysis allowed us to assess whether any of the proteolytic fragments retained a specific enzymatic function. Figure 3 (panel A) shows the result of a polymerase gel assay on the protein digested with trypsin. It can be noticed that, as the proteolytic digestion proceeded, a gradual reduction of the full-sized polymerase active band(s) took place and, in parallel, an additional activity band, corresponding to the 43.5 kDa tryptic fragment, could be detected. Similarly, gel analysis for 3′-5′ exonuclease activity shows a gradual reduction of the radioactive signal at the level of high molecular size protein band(s), which was accompanied by a concomitant increase of an active band of 48 kDa. Besides, a light *in situ* DNA degradation by the 43.5 kDa tryptic fragment can also be observed (see Figure 3, panel B). The

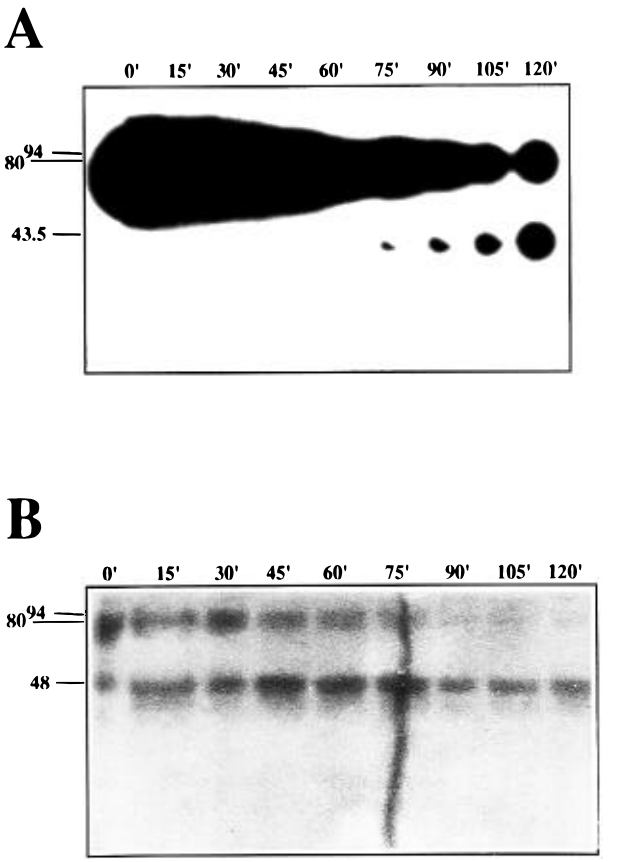

FIGURE 3: Activity gel analysis of partially proteolyzed Sso DNA pol. Sso DNA pol (140 μg) was subjected to limited proteolysis by trypsin, as described for the digestion time course experiment of Figure 1. At each of the indicated time points aliquots (12 μg of polymerase) were withdrawn from the reaction mixture. Half of each aliquot was run through a 10% polyacrylamide denaturing gel. The remaining portion of each aliquot was subjected to activity gel analysis. The autoradiogram of the polymerase activity gel is reported in panel A; the negative autoradiographic image of the 3′-5′ exonuclease activity gel is shown in panel B. The times (min) when each sample was removed are shown along the top of each panel. The molecular sizes (in kDa) of the putative active proteolytic fragments are indicated on the left margin of each panel.

limited resolution of the activity gel technique did not allow us to assess whether the 80 kDa tryptic fragment retained 3′-5′ exonuclease and/or polymerase activity.

Effect of DNA Ligands on the Trypsin Cleavage Pattern of Sso DNA pol. In a subsequent set of experiments, Sso DNA pol was subjected to limited proteolysis with trypsin upon binding of various DNA molecules (see Figure 4). In a typical experiment, Sso DNA pol was preincubated with the indicated amounts of ligand for 10 min at 37 °C. After the addition of trypsin (a protease/polymerase ratio of 1:1000 was employed), the incubation was continued for an additional 30 min at the same temperature. Using either calf thymus activated DNA or primed M13mp18 single-stranded DNA, changes in the cleavage pattern and in susceptibility to protease could be observed (compare lanes 2 and 4 of panel A and lanes 2 and 6 of panel B in Figure 4). This phenomenon was dramatically dependent on the nucleic acid concentration. When calf thymus activated DNA was present at a concentration higher than 90 μg/mL (see lanes 4−7 in Figure 4, panel A) or when primed M13 DNA was present at a concentration higher than 18 μg/mL (see lanes 6 and 7 in Figure 4, panel B), an almost complete disappearance of high molecular size (94 and 80 kDa) polypeptides of Sso

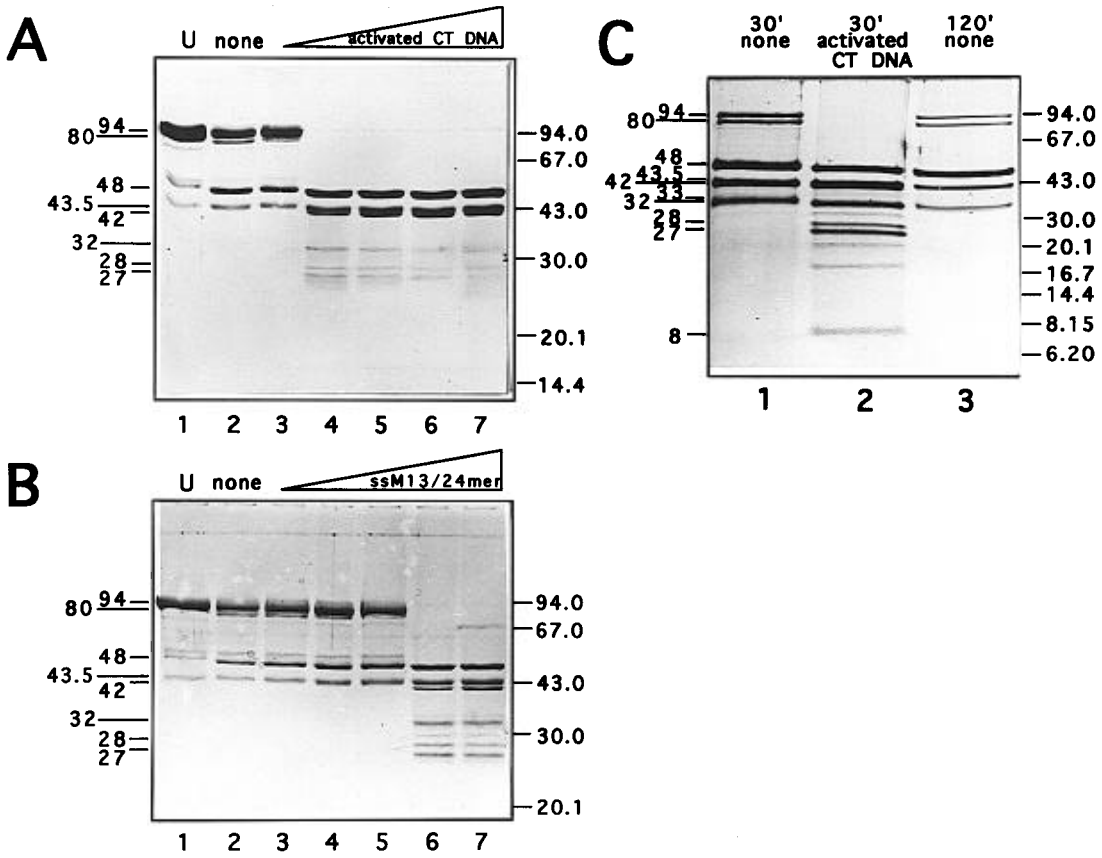

FIGURE 4: Trypsin cleavage sensitivity of Sso DNA pol upon DNA binding. (Panel A) 6 μg of Sso DNA pol was digested with trypsin (reaction volume 25 μL) at 37 °C for 30 min at a protease/polymerase ratio (w/w) of 1:1000 in the absence (lane 2) or presence of 225 ng (lane 3), 2.25 μg (lane 4), 6 μg (lane 5), 12 μg (lane 6), or 21 μg (lane 7) of activated calf thymus DNA. Sso DNA pol was preincubated for 10 min at 37 °C with nucleic acid before addition of protease. (Panel B) 6 μg of Sso DNA pol was digested with trypsin, as described for the experiment of panel A, in the absence (lane 2) or presence of 50 ng (lane 3), 100 ng (lane 4), 150 ng (lane 5), 450 ng (lane 6), or 900 ng (lane 7) of primed single-stranded M13mp18 DNA. For both panels A and B, the reaction products were analyzed by denaturing electrophoresis through a 10% polyacrylamide gel and Coomassie-staining; Sso DNA pol untreated (U) with protease was run as a control in parallel (lane 1). (Panel C) 9 μg of Sso DNA pol was digested at 37 °C with trypsin at a protease/polymerase ratio by weight of 1:250 (reaction volume 35 μL) for 30 min in the absence (lane 1) or in the presence of 16 μg of activated calf thymus DNA (lane 2); or for 120 min in the absence of DNA (lane 3). When DNA was present, the protease was added after preincubation of polymerase with nucleic acid at 37 °C for 10 min. Proteolytic products were subjected to Tricine−SDS electrophoresis through a 16.5% polyacrylamide resolving gel overlaid with a 10% polyacrylamide spacer gel, as described (Schagger & von Jagow, 1987). In panels A, B, and C, protein markers are indicated on the right margin, whereas the position and the size (in kDa) of the proteolytic fragments are shown on the left margin.

DNA pol was obtained, whereas at least three new tryptic fragments of 42, 28, and 27 kDa appeared, in addition to those of 48 and 43.5 kDa observed also in the absence of DNA. To assess whether any smaller proteolytic product was present, an aliquot of the Sso DNA pol digest was subjected to denaturing gel electrophoresis using the Tricine buffer system (Schagger & von Jagow, 1987), and a protein band of about 8 kDa could be detected in a Coomassie-stained gel (Figure 4, lane 2 of panel C). This seemed to be a DNA-specific proteolytic product, since it was observed also when the digestion was carried out in the presence of M13 DNA (data not shown), but not in the absence of any nucleic acid ligand, even when the incubation was prolonged up to 120 min (see Figure 4, lanes 1 and 3 of panel C). The minor proteolytic products of about 20 and 17 kDa, visible in Figure 4 (lane 2 of panel C), were not taken into account since they were not reproducibly detected.

Results similar to those described here were obtained when partial trypsin proteolysis was performed in the presence of various other nucleic acid molecules, such as *Xho*I-linearized pGEM7-zf(+) plasmid or RF M13mp18 DNA (data not shown).

Mapping of Trypsin Cleavage Sites within Sso DNA pol. The tryptic fragments of Sso DNA pol were identified by sequential Edman degradation analysis after separation on SDS−polyacrylamide gels and electrotransfer to PVDF membranes (Matsudaira, 1987). Their partial amino-terminal sequence is reported in Table 1, and a diagrammatic tryptic cleavage map is depicted in Figure 5 (panel A). It should be pointed out that the recombinant Sso DNA pol polypeptide chain starts at Ser^{28}, very likely because of a proteolytic degradation during the purification procedure or some posttranslational modification inside *E. coli* transformed cells. Both the 80 and 48 kDa protein fragments were found to have the same N-terminal end originated by trypsin cleavage between Lys^{34} and Lys^{35} (site a). Sequence analysis revealed that the 43.5 kDa protein band contained two polypeptide species in an almost equimolar amount: the fragment referred to as 43.5 kDa (N) starts at residue 35, while the fragment named 43.5 kDa (C) begins with Gly^{493}. The proteolytic products of 33 and 32 kDa were both found to start with Gly^{493}. Therefore, their N-terminus, as well as that of the 43.5 kDa (C) fragment, was produced by trypsin cleavage after Lys^{492} (site f). The carboxyl end of the 80 kDa

Table 1: N-Terminal Sequence Analysis of Tryptic Peptide Fragments of Sso DNA pol[a]

sample	content (%)	N-terminal sequence
94 kDa	100	^{28}SAPVEEKKV
80 kDa	100	^{35}KVVRREWLE
48 kDa	70	^{35}KVVRREWLE
	30	^{36}VVRREWLEE
43.5 kDa	57	^{35}KVVRREWLEEA
	43	^{493}GYKGAVVIDPP
33 kDa	100	^{493}GYKGAVVI
32 kDa	100	^{493}GYKGAVVI
48 kDa^{DNA}	70	^{35}KVVRREWLE
	30	^{36}VVRREWLEE
43.5 kDa^{DNA}	50	^{491}GKGYKGAVVI
	50	^{493}GYKGAVVIDP
42 kDa^{DNA}	40	^{491}GKGYKGAVVID
	20	^{493}GYKGAVVIDPP
	40	^{35}KVVRREWLEEA
32 kDa^{DNA}	30	^{491}GKGYKGAVVID
	50	^{493}GYKGAVVIDPP
	20	^{496}GAVVIDPPAGIF
28 kDa^{DNA}	100	^{240}GRIPDSQKAEF
27 kDa^{DNA}	100	^{35}KVVRREWLEEAQ
8 kDa^{DNA}	40	^{392}VKVDTLISFLDV
	60	^{394}VDTLISFLDVEK

[a] For a description of the isolation of the peptides, see Materials and Methods. The single-letter amino acid code is used. Fragments are identified by their apparent molecular size, as shown in Figure 5. The number at the left side of each sequence indicates the amino acid residue in the intact Sso DNA pol polypepetide chain. When the analyzed sample contains more than one polypeptide species, the percentage of each sequence in the total mixture is indicated. The superscript *DNA* indicates the tryptic fragment obtained with digestion carried out in the presence of activated calf thymus DNA.

proteolytic product can be only tentatively located near sites g or h, as estimated from its molecular mass. Besides, the C-terminus of the 48 kDa fragment is likely to correspond to trypsin cleavage site f on the basis of its apparent molecular size. On the other hand, the size difference between the 48 and 43.5 kDa (N) fragments (which have the same N-terminus) predicts that cleavage sites at their C-terminal ends are about 40 residues distant from each other (sites f and d, respectively). The apparent molecular size of polypeptide 43.5 kDa (C) makes it unlikely that it includes the C-terminus of Sso DNA pol (Ser^{882}). Therefore, we have placed its end about 60−65 amino acids upstream of residue 882, likely at Lys^{819}, or Arg^{821}, or Lys^{823} (site l). In fact, all these residues lie in a region of the polypeptide chain predicted to possess high surface probability according to Emini et al. (1985; unpublished results). However, we cannot exclude that cleavage at the nearby site l took place during the purification procedure or as a result of some processing of the recombinant protein within *Escherichia coli*, as observed for the N-terminal end of the polypeptide chain. This could account for the molecular size of recombinant Sso DNA pol estimated to be 94 kDa, a value which is smaller than that predicted on the basis of the corresponding gene sequence (103 kDa; Pisani et al., 1992). As for the kinetics of trypsin cleavage, we propose, from the time-dependent change in the staining intensity of the various proteolytic products, the following order of cleavage site accessibility to protease: site a = site l > site f ≫ site d > site g = site h.

The fragments obtained by digestion with trypsin in the presence of activated calf thymus DNA are schematically depicted in Figure 5 (panel B), and their N-terminal sequence is reported in Table 1. As previously pointed out, the 42,

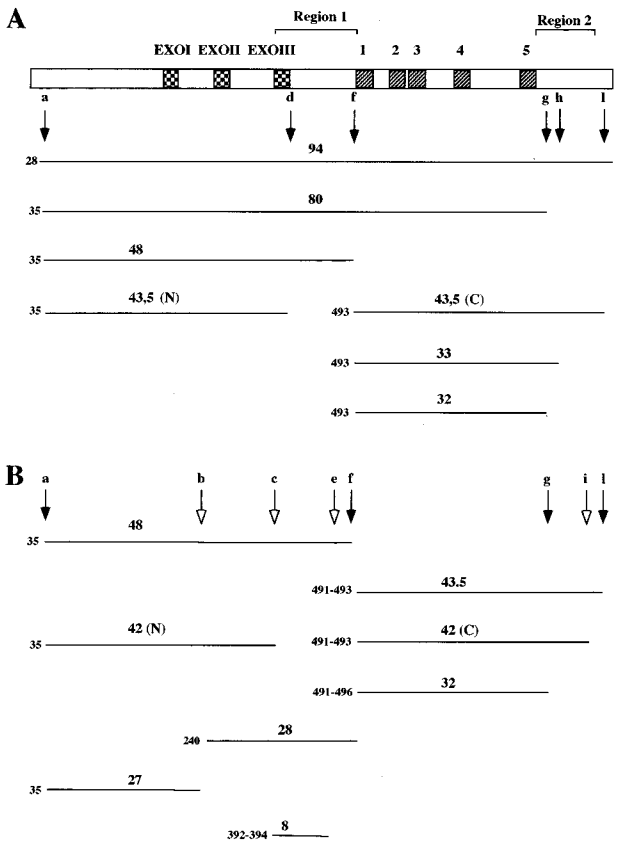

FIGURE 5: Cleavage map of trypsin on Sso DNA pol. The top diagram indicates the position of the sequence similarity motifs of family B DNA polymerases (Blanco et al., 1991) within the Sso DNA pol primary structure. Lines represent the proteolytic fragments obtained by trypsin digestion in the absence (part A) or in the presence of activated calf thymus DNA (part B). The apparent molecular size of each fragment is indicated in kDa. The cleavage sites at the N-terminal end were determined by protein sequencing, as described in detail under Materials and Methods. The location of C-terminal ends is approximate. Relative distance of cleavage sites from protein N-terminus is indicated by the alphabetical order of the arrows. Cleavage sites, which are present only when trypsin digestion was performed in the presence of activated calf thymus DNA, are represented by open arrows. Hot spots of accessibility to trypsin within the center and the C-terminal end of Sso DNA pol are indicated above the top diagram as region 1 and region 2, respectively. The sum of the sizes of fragments is not necessarily equal to full-length Sso DNA pol, since estimation of the proteolytic products molecular mass by SDS−PAGE is approximate.

28, 27 and 8 kDa protein bands were specific of trypsin cleavage pattern observed in the presence of DNA. The amino-terminal sequence of the 42 kDa protein band revealed the presence of two polypeptide species: the 42 kDa (N) fragment which starts at Gly^{35} and the 42 kDa (C) fragment, which is an almost equimolar mixture of two sequences produced by cleavage at Lys^{490} and at Lys^{492}. The C-terminus of the 42 kDa (C) fragment is likely to be located at an amino acid residue (site i) just upstream of site l, which corresponds to the carboxyl end of the 43.5 kDa (C) polypeptide. The 27 and 28 kDa proteolytic fragments, which start at residues 35 and 240, respectively, could have originated from cleavage of the 48 kDa polypeptide at site b. Sequence determination revealed that the 8 kDa species possessed a heterogeneous N-terminal end with two components. The major component begins with Val^{394}, while the minor component starts at Val^{392}. Judging from its apparent molecular size, this fragment is likely to be about 70 residues long. This places its C-terminus upstream of

cleavage site f, around position 462 (cleavage site e). Besides, in the diagram reported in Figure 5, we have also hypothesized that the C-terminus of the 42 kDa (N) fragment and the N-terminus of the 8 kDa peptide were both generated by cleavage at site c (positions 392−394). In fact, the apparent molecular size of 42 kDa closely matches the estimated molecular mass of a polypeptide spanning residues 35 to 392−394.

In summary, these proteolytic digestion experiments indicate that trypsin cleaves Sso DNA pol at three major locations: near the N-terminal end (cleavage site a, residue 35); within the center of the polypeptide chain (region 1, from Val^{392} to Gly^{491}); and near the C-terminus (region 2, from site g to site l). On the other hand, in the presence of nucleic acid ligands, the spectrum of Sso DNA pol proteolytic fragments was altered, so that new trypsin cleavage sites were detected (sites b, c, e, and i), and other sites became less susceptible to proteolytic attack (sites d and h). In addition, a fragment of about 8 kDa starting at positions 392−394 was fully protected from protease digestion.

Fluorescence Measurements on Sso DNA pol. We used the intrinsic tryptophan fluorescence as a sensitive marker to further investigate nucleic acid-induced structural changes of Sso DNA pol. The spectroscopic approach relied upon the endogenous fluorescence of the protein, which is due to the presence of nine tryptophan residues (Pisani et al., 1992). The background fluorescence emission spectrum of Sso DNA pol excited at 295 nm showed a λ_{max} of about 347.5 nm, which was indicative of tryptophan residues largely exposed to the solvent. The intrinsic fluorescence of Sso DNA pol was substantially unchanged (±5% of the maximal emission intensity value) following addition of activated calf thymus DNA or primed M13mp18 DNA, and no change in λ_{max} was observed. Nevertheless, we attempted to probe conformational changes induced upon DNA binding by using acrylamide as an efficient fluorescence quencher (Eftink & Ghiron, 1975). Acrylamide quenched the tryptophan fluorescence of Sso DNA pol by a collisional process. In fact, we found a proportionality of quenching rate constant (K_{SVeff}) against T/η, where T is the absolute temperature and η is the medium viscosity (Lehrer, 1971; data not shown). All that considered, differences in the quenching parameters (f_{aeff} and K_{SVeff}) of enzyme−ligand complexes compared to enzyme alone could be ascribed to changes in the accessibility of tryptophan residues to acrylamide. Fluorescence intensity data, acquired at 37 °C with Sso DNA pol alone and in the presence of either activated calf thymus DNA or primed M13mp18 DNA, were fitted to the modified Stern−Volmer equation (Eftink & Ghiron, 1975; Lehrer, 1971; see Figure 6). The plots obtained in the range 0−500 mM acrylamide concentration were downwardly curving as not unexpected for a complex system of nine fluorophores (data not shown). In Figure 6, only the points obtained at the lower quencher concentrations (range 0−140 mM) are reported since derivation of K_{SVeff} and f_{aeff} requires extrapolation of the plotted values to $1/Q = 0$ after fitting data points to a linear equation. The resulting K_{SVeff} and f_{aeff} values (means of two independent experiments) were 48 ± 2.5 M^{-1} and 55 ± 2% for Sso DNA pol alone; 21.5 ± 2.6 M^{-1} and 65.5 ± 7% for calf thymus DNA addition; and 11 ± 2 M^{-1} and 81.5 ± 10.5% for primed M13 DNA addition, respectively.

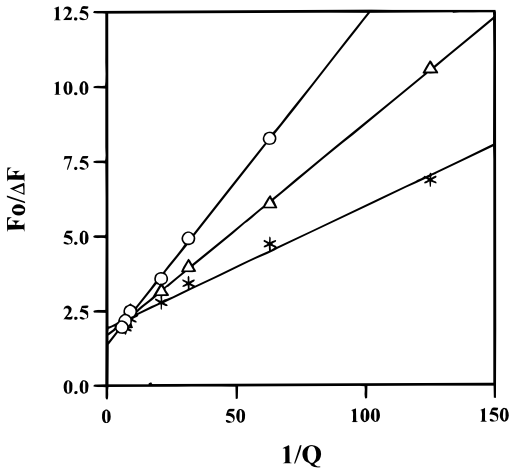

FIGURE 6: Determination of fluorescence quenching parameters. The figure reports modified Stern−Volmer plots according to the equation $F_0/\Delta F = 1/f_a + 1/f_a K_{SV}[Q]$, where F_0 is the fluorescence in the absence of quencher, $\Delta F = F_0 - F$, [Q] is the concentration of quencher, f_a is the fraction of the total emission accessible to the quencher, and K_{SV} is the Stern−Volmer constant (Eftink & Ghiron, 1975; Lehrer, 1971). Quenching of Sso DNA pol fluorescence was obtained by small additions of a 4 M acrylamide solution in 20 mM sodium phosphate buffer, pH 7.2. Measurements were acquired on the protein alone (*), in the presence of activated calf thymus DNA (△) or primed single-stranded M13mp18 DNA (○), as described under Materials and Methods. K_{SVeff} and f_{aeff} were derived from the plots shown, which represent the best fit to the above equation of data acquired in a representative experiment. The correlation coefficient values for the plots shown were 0.9938 (*), 0.9996 (△) and 0.9994 (○).

DISCUSSION

Domain Structure of Sso DNA pol. In a previous report, we demonstrated by analyzing two naturally occurring proteolytic fragments that Sso DNA pol is composed of two functional and structural domains separated by a protease hypersensitive site. We showed by bidimensional gel assays that the isolated amino- and the carboxy-terminal fragments retain 3′-5′ exonuclease and DNA polymerase activity, respectively. Additional and important evidence emerges from the limited proteolysis analysis herein reported. The partial trypsin digestion of the enzyme under native conditions (Figure 1) produced relatively stable tryptic fragments corresponding to the N- and C-terminal halves of the protein molecule, which were found to retain 3′-5′ exonuclease and polymerase activity, respectively, by means of activity gel analysis. However, it should be pointed out that the proteolytic fragments of the nicked DNA pol purified from *S. solfataricus* had different molecular sizes in comparison with those derived from the trypsinized recombinant enzymes. This finding could be accounted for by a different cleavage specificity of endogenous *S. solfataricus* proteolytic activities in comparison with trypsin. Moreover, the recombinant Sso DNA pol polypeptide chain starts at Ser^{28} whereas we were unable to determine the N-terminal residue of native Sso DNA pol because it was blocked (Pisani & Rossi, 1994). Finally, this study revealed new hypersensitive sites, besides the ones previously identified: near the N-terminal end (cleavage site a, residue 35) and the C-terminus (region 2, from site g to site l; see Table 1 and Figure 5).

By visual inspection of the autoradiograms in Figure 3, we noticed that the apparent specific activity of the tryptic fragments was lower if compared to the full-length protein

(this was especially evident for the polymerase active bands). This could be due to a less complete renaturation and/or an intrinsic lower catalytic efficiency and/or a reduced thermal stability of the separated proteolytic fragments. Attempts at isolating the proteolytic products either under native conditions or in the presence of dissociating agents (such as urea and Tween-20) by various chromatographic techniques (such as gel filtration and ion exchange) were unsuccessful, suggesting that they were physically associated by means of hydrophobic interactions and/or hydrogen bonds (data not shown). These data are consistent with the finding that trypsin time course digestion of SsoDNA pol did not affect either the 3′-5′ exonuclease or the polymerase activities (see Figure 2). In fact, we could imagine that nicked enzyme molecules retain activity and thermal stability features comparable to the uncleaved full-length protein.

These data are not completely in agreement with recent reports on the structural and functional organization of the DNA polymerase from *Herpes simplex* virus (Weisshart et al., 1994) and bacteriophage T4 (Lin et al., 1994). For both these family B DNA polymerases, attempts at identifying a protein domain responsible for the synthetic function failed, whereas their 3′-5′ exonuclease activity seemed to reside on a separable independent N-terminal protein fragment. Therefore, it is likely that 3′-5′ exonuclease and polymerase functions of these enzymes are interdependent and the respective active sites partially overlap. In contrast, the DNA polymerase from the archaeon *S. solfataricus* could be reminiscent of an ancestral prototype of DNA polymerizing enzyme whose proofreading and synthesizing functions very likely resided on independent protein domains. This would be consistent with the hypothesis that genes encoding multifunctional DNA polymerases originated by means of an exon-shuffling event (Bernad et al., 1989; Doolittle, 1995).

DNA-Induced Conformational Changes. The limited proteolysis technique can also be employed to detect subtle conformational changes of a protein upon binding to a specific ligand (Price & Johnson, 1989). Our analysis indicated that Sso DNA pol following DNA binding did not undergo a global conformational change, since we did not observe a striking modification of the trypsin cleavage pattern. In fact, as was true in the absence of DNA, we found hot spots of protease accessibility at the N-terminus, within the center, and at the C-terminus of the polypeptide chain (site a, regions 1 and 2 of Figure 5, respectively). However, a more fine mapping indicates that in the DNA-bound state within region 1 cleavage site d (approximately located at residue 450) was less solvent-exposed, while the accessibility of sites c (Lys^{391} or Lys^{393}) and e (around position 461) was noticeably increased. Similarly, we observed that solvent exposure of sites h and i within region 2 was reduced and increased, respectively, as compared to unbound Sso DNA pol.

Furthermore, it should be noticed that globally the susceptibility of the above locations to the proteolytic attack was noticeably increased in the presence of defined amounts of nucleic acid ligands. The minimal DNA concentration required to obtain the complete disappearance of full-length Sso DNA pol depended on the kind of nucleic acid utilized. This behavior is likely to correlate with the different binding affinity of the enzymes for the various substrates. Interestingly, in line with our results, limited proteolysis studies on *Herpes simplex virus* DNA pol revealed that binding to various nucleic acid molecules increased the cleavage sensitivity by trypsin near the center of the protein, between residues 597 and 693 (Weisshart et al., 1993). Similarly, partial proteolysis experiments on bacteriophage T4 DNA pol using chymotrypsin indicated a site of high protease accessibility around residue 392, in the middle of the polypeptide chain (Lin et al., 1994), but in this latter case no data are available on the cleavage susceptibility of the polymerase in the DNA-bound state.

The sensitivity of tryptophanyl residue exposure to acrylamide quenching was exploited to confirm protein conformational changes upon DNA binding. In preliminary measurements, we found that the Sso DNA pol intrinsic fluorescence did not change in the presence of nucleic acids, suggesting that the emission available for quenching was the same in both the free and the DNA-bound state. The decrease of K_{SVeff} values measured in the presence of these ligands (2−4-fold) implicated that the environments of previously accessible tryptophans were less readily penetrated by quencher in the enzyme−DNA complex. On the other hand, the fractional accessibility (f_{aeff}) increased by about 20% and 50% for calf thymus and M13 DNA addition, respectively, indicating that the previously-buried tryptophan emission had become accessible to the quencher in the enzyme−DNA complex. This finding, together with the difference in size and structure of the two kinds of DNA ligands utilized, allowed us to rule out steric effects as a possible explanation of the observed difference in the K_{SVeff} values (Eftink & Ghiron, 1975). A similar approach was utilized by Philips et al. (1987) to demonstrate ligand-induced conformational changes of human adenosine deaminase.

The biochemical analysis herein reported allowed us to map precisely the sites where structural changes occurred within the Sso DNA pol polypeptide chain upon nucleic acid binding. It is interesting to observe that limited trypsin digestion in the presence of DNA produced a proteolytic fragment of about 8 kDa, which was not found in the absence of nucleic acid ligands (as shown in Figure 4, panel C). This polypeptide is likely to extend from residue 392 or 394 (site c) to residue 461 (site e) and would then include the majority of region 1 (as depicted in Figure 5). The appearance of this fragment only after partial tryptic digestion in the presence of DNA suggests its involvement in the DNA binding function so that site occupancy by ligand would exclude access by proteases. Sequence alignments among family B DNA polymerases (Braithwaite & Ito, 1993) show that region 1 of Sso DNA pol as well as the previously discussed segment of high protease accessibility of the DNA pol from *Herpes simplex* (Weisshart et al., 1993, 1994) and bacteriophage T4 (Lin et al., 1994) all lie within a not conserved region located between the Exo III motif and the similarity motif 1 (which contains the signature sequence "Asp - - Ser Leu Tyr Pro") according to Blanco et al. (1991). In attempts to model the three-dimensional structure of family B DNA polymerases onto that of Klenow fragment, the above regions have been located nearby the tip of the thumb subdomain (Blanco et al., 1991). Therefore, assuming that these structural predictions are true, our results could raise the intriguing possibility that region 1 of Sso DNA pol (see Figure 5) corresponds to a putative highly-flexible thumb subdomain and that the conformational changes it undergoes upon DNA binding might be critical for substrate interaction and enzyme processivity, as reported for *E. coli* Klenow

fragment (Joyce & Steitz, 1994; Steitz, 1993) and HIV-1 reverse transcriptase (Kohlstaetd et al., 1992; Jacobo-Molina et al., 1993).

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