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Evidence That an Archaeal α-Like DNA Polymerase Has a Modular Organization of Its Associated Catalytic Activities*

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In this study we report on the evidence that an α-like DNA polymerase purified from the thermoacidophilic archaeon *Sulfolobus solfataricus* has a modular organization of its associated catalytic activities (polymerase and 3′-5′ exonuclease). This enzyme, a monomer of about 100 kDa whose complete primary structure is available, has a protease hypersensitive site that is likely to be cleaved by the action of endogenous proteases during the purification procedure. As a consequence of that, two proteolytic fragments of about 50 and 40 kDa, in addition to the intact 100-kDa molecular species, can be detected upon SDS-PAGE of highly purified *S. solfataricus* DNA polymerase samples. The amino-terminal microsequence analysis by Edman degradation has revealed that the 50- and the 40-kDa polypeptides correspond to the carboxyl- and the amino-terminal portion of the protein molecule, respectively. Using the bidimensional activity gel assay procedure, recently described by Longley and Mosbaugh (Longley, M. J., and Mosbaugh, D. W. (1991) *Biochemistry* 30, 2655–2664), we have demonstrated that the 50-kDa fragment retains a Mg^{2+}-dependent DNA polymerizing activity, whereas the 40-kDa polypeptide is able to catalyze the excision of mispaired nucleotides at the 3′-OH terminus of a primer/template DNA substrate in the presence of Mn^{2+} ions. On the other hand, the 100-kDa protein possess both activities. To date, this is the first report indicating, on the basis of direct functional data, that the polymerization and the 3′-5′ exonuclease activity of a family B DNA polymerase can be ascribed to physically distinct modules of the enzyme molecule.

DNA polymerases are multifunctional enzymes having associated to the synthetic function one (or two) degradative exonucleolytic activities either in the same polypeptide chain or as separate subunits of holoenzymatic complexes (reviewed in Ref. 1). Most of our information regarding the enzymology of template-directed DNA synthesis has derived from a combination of biochemical, genetic, and crystallographic studies on *Escherichia coli* DNA polymerase I (2–5). The three principal catalytic activities carried out by this enzyme have been ascribed to structurally and functionally independent modules of its molecule; the extreme amino-terminal domain retains the 5′-3′ exonuclease activity (6), whereas the middle and the carboxyl-terminal domains are responsible for the proofreading and the polymerization function, respectively (7, 8). This structural organization can be quite likely extrapolated to the other DNA polymerases of family A, whose primary structure is closely related to *E. coli* DNA polymerase I prototype (9, 10). Intriguingly, members of the other major class of DNA polymerases (the family B), that includes eukaryotic cellular and viral DNA polymerases and T4 and φ29 phage replicases, share with the family A enzymes only three segments of sequence similarity referred to as Exo motifs (11–13). They are centered around carboxylate residues reported to be critical for the Klenow fragment proofreading activity (14, 15) and are located in the same relative segmental arrangement within the amino-terminal region of each polypeptide chain (11). Site-specific mutagenesis experiments by several laboratories have demonstrated that the Exo segments are essential also for the editing activity of family B DNA polymerases (11, 16–18). These findings have supported the hypothesis that the proofreading domain could be structurally and functionally conserved between family A and B of DNA polymerases, being both distinct and independent from the polymerase active site (11). Moreover, primary structure alignments among DNA polymerases of family B have revealed the presence of at least five sequence similarity motifs occurring in the same linear order within their carboxyl-terminal half (19, 20). Recently, site-specific mutagenesis studies have supplied evidence for the catalytic significance of such conserved sequence regions for the synthetic function of family B DNA polymerases, thereby suggesting a carboxyl-terminal location of their polymerase active site (21, 22).

On the basis of all these results, it has been proposed that the two different DNA polymerase types, in spite of a noticeable primary structure divergence, could share a common tridimensional fold, similar to that described for the Klenow fragment and predicting a structural and functional separation of the proofreading and polymerization catalytic sites (10, 20, 23). However, certain other lines of evidence seem to conflict with this unifying hypothesis. In fact, it has been pointed out that a more direct interdependence and cooperation could take place during DNA synthesis between the 3′-5′ exonuclease and the polymerase active site of replicases from Herpes simplex (type 1) (24) and Adenovirus (type 2) (25).

In this study we present the first direct evidence that in a family B DNA polymerase the associated synthetic and degradative activities reside on structurally independent protein domains. Our model system is a thermophilic and thermostable DNA polymerase activity purified to homogeneity from cellular extracts of the thermoacidophilic archaeon *Sulfolobus solfataricus* (26, 27). The primary structure of this enzyme, a monomer of about 100 kDa, is available, since the corresponding gene (*polS*) has been cloned and sequenced (28). Computer-assisted similarity searches (9) revealed that *S. solfataricus* DNA polymerase shares with family B DNA polymerases the

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five highly conserved carboxyl-terminal sequence regions above discussed, in addition to the Exo motifs (19, 20). This finding is consistent with the kinetic behavior of the enzyme that was reported to be sensitive to aphidicolin and resistant to the inhibition by dideoxynucleotides (26).

EXPERIMENTAL PROCEDURES

Materials

Chemicals—All chemicals used in this study were reagent grade. [γ-^{32}P]ATP (specific activity: <5000 Ci/mmol, 10 mCi/ml), [α-^{32}P]dCTP (specific activity: <3000 Ci/mmol, 10 mCi/ml), Rainbow™ molecular weight markers, and ^{14}C-methylated proteins were from Amersham International. Sephadex-G25 and molecular weight standard proteins were purchased from Pharmacia. Polyvinylidene difluoride membrane (Problott™) was from Applied Biosystems. Bovine fibrinogen was purchased from Miles Laboratories. M13mp18(+)-strand DNA was from United States Biochemical Corp. Oligodeoxyribonucleotides 24-mer (5′-ACTCTAGAGGATCCCCGGGTACCG-3′) and 25-merT (5′-GCCAAGCTTGCATGCCTGCAGGTCT-3′), synthesized by conventional solid phase methods and purified by gel filtration, were purchased from Primm (Milan, Italy). The 24-mer was designed to be complementary to positions 6241–6264 of M13mp18(+)-strand DNA. On the other hand, the 25-merT was complementary to positions 6264–6289 of the same template, but formed a 3′-terminal (T:C) mispair at position 6265.

Enzymes—T4 DNA polynucleotide kinase was purchased from Promega. DNA polymerase from *S. solfataricus* (strain MT4) was purified as described previously (26, 27).

Methods

Preparation of DNA Substrates—Deblocked/deprotected oligonucleotides were labeled at the 5′-end in reactions (25 μl) containing 50 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, 50 pmol of [γ-^{32}P]ATP, 5 pmol of oligonucleotides, and 10 units of T4 polynucleotide kinase. After incubation at 37 °C for 45 min, reactions were terminated by the addition of EDTA, pH 8.0, to 10 mM. Unincorporated ATP molecules were removed by gel filtration on 1-ml Sephadex G-25 spun column. The resulting oligonucleotide had a specific activity of about 5–10 × 10^6 cpm/pmol of 5′-ends, as estimated by Cerenkov radiation counting on a Beckman β-counter (model LS 6000TA). Annealing of each labeled oligonucleotide to M13mp18 single-stranded DNA (0.25 pmol of 5′-ends/μg of DNA) was obtained by incubation at 65 °C for 5 min and slow cooling to 25 °C.

Protein Concentration Determination—Protein concentration of the enzyme samples was determined by Bio-Rad assay, using bovine serum albumin (Miles Laboratories) as a standard.

DNA Polymerase Standard Activity Assay—DNA polymerase activity was determined by the incorporation of [^{3}H]dTTP into a trichloroacetic acid-precipitable form in a 10-min reaction at 75 °C, as described previously (26). One unit of DNA polymerase is able to incorporate 1 nmol of dTMP into activated calf thymus DNA in 1 min at 75 °C.

Electrophoretic Analysis—Protein gel electrophoreses were performed using the Mini Protean system (Bio-Rad). SDS-PAGE[1] was carried out as described (29). Gel electrophoresis in native conditions at pH 4.5 was according to Reisfeld (30).

Analytical Gel Filtration—Gel filtration of *S. solfataricus* DNA polymerase was carried out on a Protein Pak Glass 300 SW HPLC column (Waters), equilibrated with 50 mM sodium phosphate, pH 7.0, 150 mM NaCl. Fractions containing DNA polymerase were detected by measuring absorbance at 280 nm and by activity assays. The column was calibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), ribonuclease A (13 kDa). Equilibration, loading, and elution of the column were performed at a constant flow rate of 0.5 ml/min at room temperature. Molecular weights were estimated according to Andrews (31).

Elution of Proteins from SDS-Polyacrylamide Gels—The proteolytic fragments of *S. solfataricus* DNA polymerase were purified by elution from a preparative SDS-polyacrylamide gel, essentially as described (32). Protein were visualized in a reference lane by Coomassie staining. The bands of interest were cut out and crushed with a Teflon pestle and protein eluted overnight in the cold room by diffusion in the following buffer: 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol. The mixture was centrifuged to pellet the crumbled gel and the protein present in the supernatant concentrated by diafiltration using the Microcon™ system (Amicon). The purity and the quantity of the eluted proteins were estimated by SDS-PAGE.

Bidimensional Activity Gel Electrophoresis—Denaturing activity gel analyses were performed essentially as described by Longley and Mosbaugh (33). Briefly, *S. solfataricus* DNA polymerase samples (0.5 μg) were denatured by heating at 95 °C for 5 min in a 25 μl of mixture containing 50 mM Tris/HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue and resolved through a 10% polyacrylamide gel (thickness: 0.75 mm), which contained 375 mM Tris/HCl, pH 8.8, O.1% SDS, 50 μg/ml bovine fibrinogen, 1.6 μg/ml ^{32}P-5′-end-labeled 24-mer/M13mp18 single-stranded DNA (for DNA polymerase activity assay) or 1.6 μg/ml ^{32}P-5′-end-labeled 25-merT/M13mp18 single-stranded DNA (for 3′-5′ exonuclease activity assay). In adjacent gel lanes Rainbow™ molecular weight markers were run. The electrophoreses were performed using a Mini Protean gel apparatus in cold room at 100 volts. After the electrophoretic run, the lane containing the prestained markers was cut from the gel and stored in 7% acetic acid. The remaining part of the gel was incubated in buffer 10 mM Tris/HCl, pH 8.0, 25% isopropanol for 1 h at room temperature with gentle agitation to extract the detergent (two changes of 500 ml). *In situ* enzyme renaturation was performed by incubating the gel at 4 °C for 16 h in about 300 volumes of 50 mM Tris/HCl, pH 8.0, 7 mM β-mercaptoethanol, 2.5 mM $MgCl_2$. When the 3′-5′ exonuclease activity was tested, the renaturation was performed in the same buffer as before without $MgCl_2$. Following renaturation, *in situ* enzymatic reactions were initiated by immersing longitudinal gel slices (about 0.2 × 7–8 cm) in screw-cap tubes containing 5 ml of reaction buffer (50 mM Tris/HCl, pH 8.0, 7 mM β-mercaptoethanol, 2.5 mM $MgCl_2$, 0.16 mM dATP and 0.16 mM dGTP, for DNA polymerase assay; 50 mM Tris/HCl, pH 8.0, 7 mM β-mercaptoethanol, and 2.5 mM $MnCl_2$, for 3′-5′ exonuclease activity assay). After incubation at 45 °C for 45 min, reactions were stopped by adding EDTA, pH 8.0, to 10 mM and placed into ice for about 30 min. The products of enzymatic synthesis (or degradation) were analyzed by electrophoresis in a second dimension. The longitudinal gel slices were cast after 90° rotation within 20% polyacrylamide, 8 M urea gels (thickness: 1 mm). Electrophoreses were carried out in TBE buffer (134 mM Tris base, 44 mM boric acid, 2.6 mM EDTA) at 850 volts. The gels were then exposed to Fuji RX autoradiographic films at room temperature for 16 h.

Monodimensional Activity Gel Electrophoresis—Monodimensional activity gel analysis was performed according to Karawya (34) with some modifications. Briefly, *S. solfataricus* DNA polymerase samples were subjected to denaturing gel electrophoresis by the procedure of Laemmli (29) through a 10% polyacrylamide SDS-slab gel containing 0.5 mg/ml activated calf thymus DNA. Immediately after the run, the gel was incubated in buffer 10 mM Tris/HCl, pH 8.0, with gentle agitation for 30 min. This step was repeated twice and then 1 liter of buffer 50 mM Tris/HCl, pH 8.0, 2.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol was added and the incubation continued in cold room overnight to allow *in situ* renaturation of proteins. The following day, the gel was placed into a sealed plastic bag containing the reaction buffer: 50 mM Tris/HCl, pH 8.0, 2.5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 160 μM each dATP, dGTP, dTTP, and 1.6 μM [α-^{32}P]dCTP (50 μCi; specific activity: 3000 Ci/mmol). Incubation was at 65 °C for 6 h. Thereafter, the gel was washed in 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate with gentle agitation for 30 min twice. The gel was then transferred into 1 liter of fresh washing solution and incubated overnight in cold room. The following day, it was dried and autoradiographed.

RESULTS AND DISCUSSION

An α-like DNA polymerase purified from the thermoacidophilic archaeon *S. solfataricus* has a protease hypersensitive site that is partially cleaved during the purification procedure by the action of endogenous proteases (26, 27). This phenomenon could be only partly counteracted by the inclusion of several protease inhibitors in the buffers employed and has been observed also during the purification of the recombinant form of the enzyme, recently overexpressed in *E. coli*.[2] *Panel A* of Fig. 1 shows the results of SDS-PAGE of purified *S. solfataricus* DNA polymerase. In addition to the 100-kDa protein band corresponding to the uncleaved enzyme molecule, additional polypeptides of about 50, 40, and 23 kDa were detected. Western blot analysis using a polyclonal antiserum against the 100-

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

[2] F. M. Pisani and G. Manco, unpublished results.

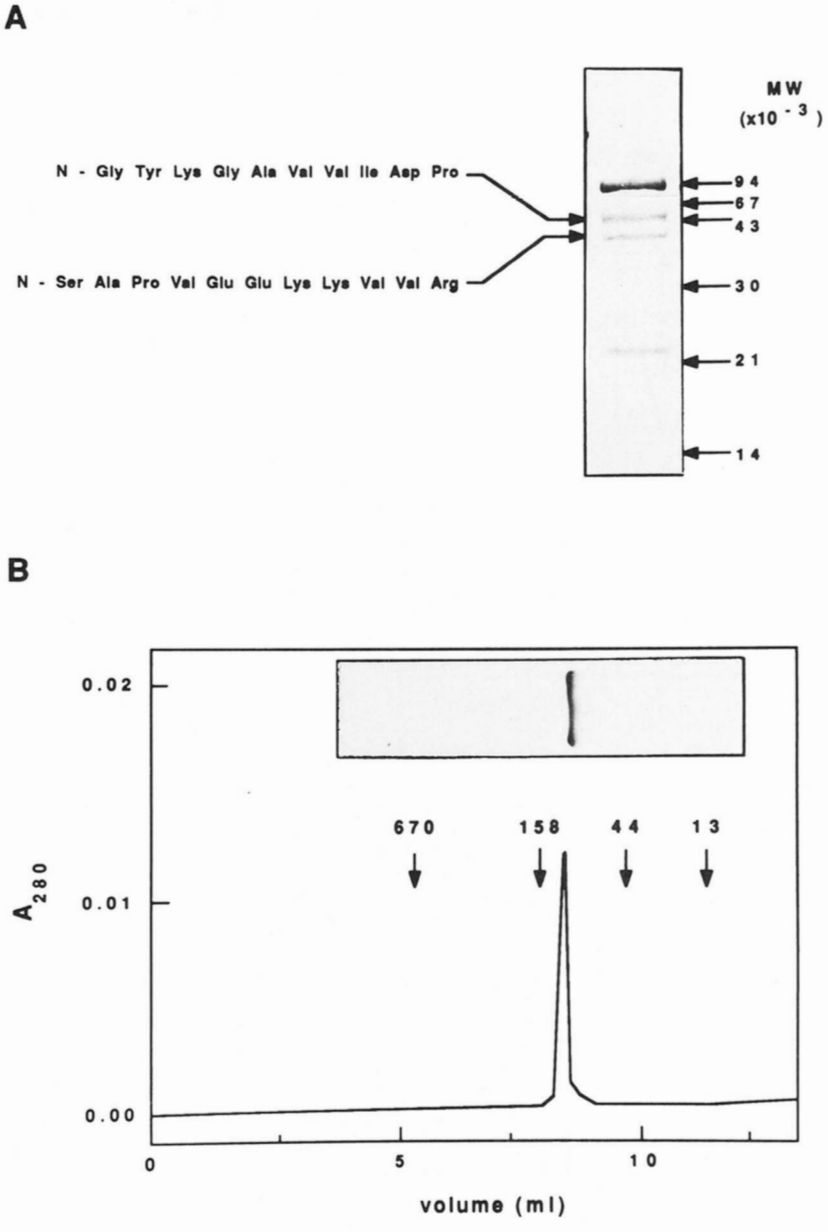

FIG. 1. **Electrophoretic analysis and gel filtration chromatography of *S. solfataricus* DNA polymerase.** *Panel A*, SDS-PAGE of highly purified *S. solfataricus* DNA polymerase (3 μg) was performed on a 10% polyacrylamide slab gel by the method of Laemmli (29). *Right*, the position of the standard proteins resolved in an adjacent lane is indicated. *Left*, the amino-terminal sequence of the 50- and 40 kDa proteolytic fragments is reported (28). *Panel B*, analytical gel filtration of highly purified *S. solfataricus* DNA polymerase (15 μg) was carried out on a Protein Pak Glass 300 SW HPLC column. Protein was detected by absorbance at 280 nm (–). The elution volume of the standard proteins employed to calibrate the column is indicated by *vertical arrows* (see "Experimental Procedures"). Electrophoresis under nondenaturing conditions at pH 4.5 (*inset*) of highly purified enzyme (3 μg) was performed on a 7% polyacrylamide slab gel according to Reisfeld (30). The migration was from *right* to *left*.

kDa molecular species revealed that the 50- and 40-kDa species are immunologically related (27). Amino-terminal sequence analysis of the 40- and 50-kDa polypeptides (see Fig. 1, *panel A*) enabled us to assign them, respectively, to the amino- and carboxyl-terminal portion of *S. solfataricus* DNA polymerase, since the complete enzyme primary structure is available (28). Furthermore, the proteolytic cleavage site was located within a region of the polypeptide chain that is likely to form an unstructured loop on the molecule surface due to its elevated hydrophilicity and high content in glycine residues (data not shown). On the other hand, amino-terminal sequence analysis of the 23-kDa protein species allowed us to rule out the possibility that it could be an additional proteolytic fragment of the enzyme.[3] However, its stable association with the DNA polymerase throughout the entire purification procedure suggests the hypothesis that it could be an accessory subunit more than a contaminant species of the enzyme preparation. Indeed, analytical gel filtration of highly purified enzyme samples on a Protein Pak Glass 300 SW HPLC column yielded a single sharp protein peak at an elution volume corresponding to a 100 ± 15-kDa globular molecule, as shown in Fig. 1 (*panel B*). Furthermore, gel electrophoresis under nondenaturing conditions at pH 4.5 gave only one Coomassie-stained band (Fig. 1, *panel B*). This biochemical analysis suggests that *S. solfataricus* DNA polymerase is folded into two globular domains connected by a flexible protease sensitive loop. Several additional packing interactions are likely to provide a tight linkage between the two domains, since they do not dissociate when the interconnecting loop is nicked, as indicated by the results of native PAGE and gel filtration experiments.

In order to assess whether specific catalytic functions could be assigned to the proteolytic fragments of *S. solfataricus* DNA polymerase, we exploited the novel bidimensional activity gel assay procedure described by Longley and Mosbaugh (33). This technique relied upon the use of a defined ^{32}P-labeled oligonucleotide annealed to M13 single-stranded DNA, which is cast within a SDS-polyacrylamide resolving gel. Following separation of the polypeptides and *in situ* enzyme renaturation and reaction, radiolabeled DNA products of polymerization (or degradation) are separated according to size in a second dimension of electrophoresis through a denaturing gel. The substrate employed to detect the DNA polymerase activity consisted of a

[3] F. M. Pisani, unpublished results.

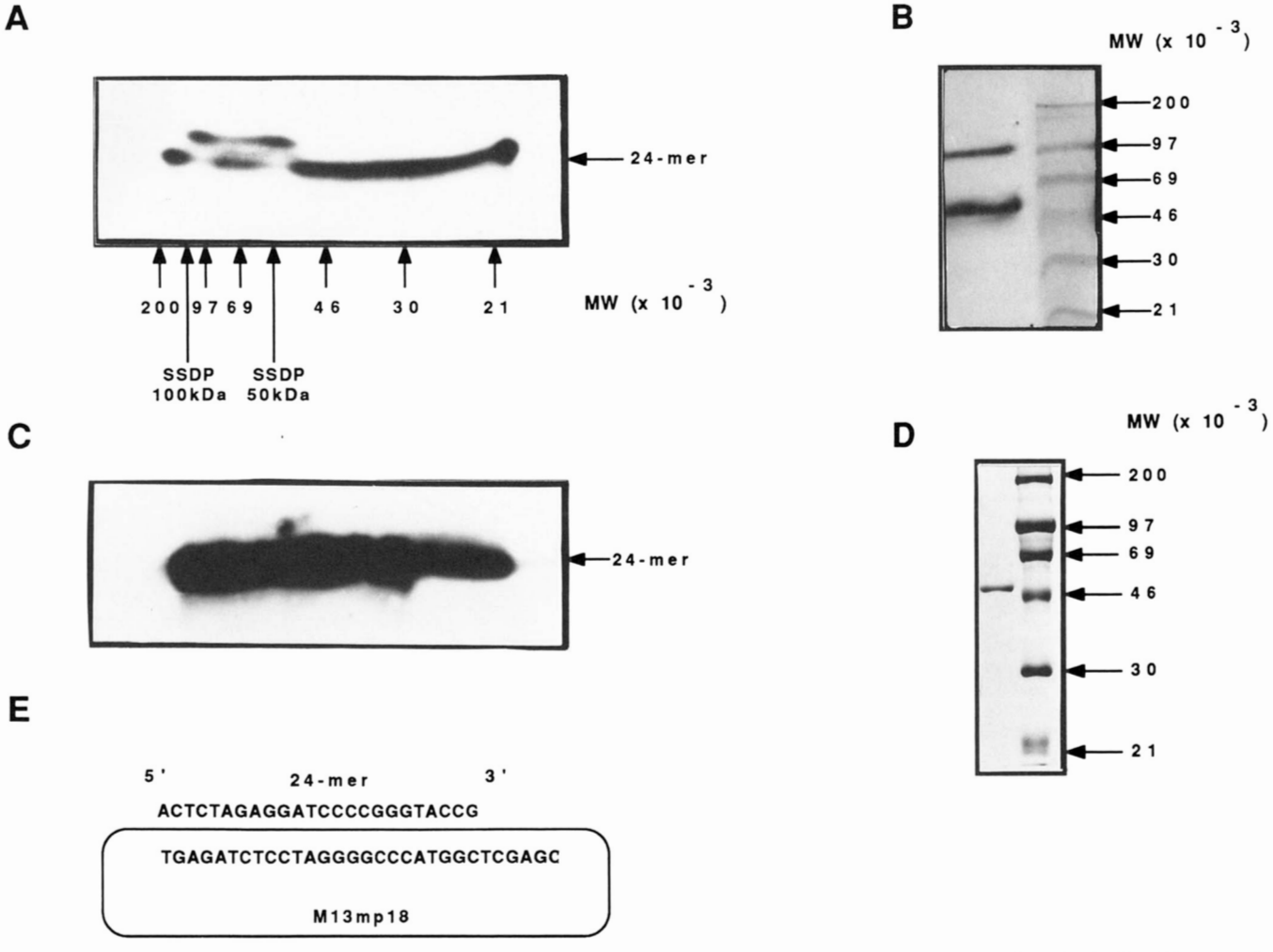

FIG. 2. ***In situ* detection of *S. solfataricus* DNA polymerase active polypeptides by denaturing activity gel analysis.** *Panel A*, a sample of *S. solfataricus* DNA polymerase (0.5 μg) was subjected to the activity gel assay procedure of Longley and Mosbaugh (33), as described under "Experimental Procedures." The autoradiography of the polyacrylamide/urea gel used for the second dimension of electrophoresis is shown. The *horizontal arrow* locates the ^{32}P-labeled 24-mer primer. The active polypeptides molecular weights were determined relative to prestained proteins loaded onto an adjacent lane: myosin (200,000), phosphorylase *b* (97,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), trypsin inhibitor (21, 500). *Vertical arrows* indicate the position of standard proteins and of *S. solfataricus* DNA polymerase active polypeptides of 100 (*SSDP, 100 kDa*) and 50 (*SSDP, 50 kDa*) kDa. *Panel B*, autoradiogram of the activity gel of *S. solfataricus* DNA polymerase according to Karawya (Ref. 34, see "Experimental Procedures") is shown. A sample of the homogeneous enzyme (1 μg) was loaded onto the *left lane*. ^{14}C-Methylated protein markers (*right lane*) were as in *panel A*. *Panel C*, bidimensional activity gel analysis of the *S. solfataricus* DNA polymerase 50-kDa protein fragment purified by elution from a denaturing gel. The autoradiogram of the second dimension of electrophoresis is shown. *Panel D*, a sample (3 μg) of the *S. solfataricus* 50 kDa protein fragment, purified as described in the text, was run through a 10% polyacrylamide gel (*left lane*) by the method of Laemmli (29). The molecular size markers (*right lane*) were as in *Panel A*. Protein was stained with Coomassie Brilliant Blue. *Panel E*, schematic representation of the substrate employed for DNA polymerase *in situ* activity gel assay. The oligonucleotide 24-mer, fully complementary to positions 6241–6264 of M13mp18(+)-strand DNA, was 5′-end-^{32}P-labeled and hybridized to this template, as described under "Experimental Procedures."

^{32}P-5′-end-labeled 24-mer oligonucleotide fully matched to M13mp18(+)-strand DNA; on the other hand, the substrate for the 3′-5′ exonuclease activity assay was a 25-mer oligonucleotide forming with the same DNA template a 3′-terminal (T:C) mispair. In order to prevent complete melting of the primers from the template, both DNA polymerase and exonuclease activity assays were carried out at a reaction temperature of 45 °C, although it was far below the optimal value reported for this thermophilic enzyme (26, 27). Mg^{2+} ions were required in the renaturation and assay buffer to detect the polymerase activity. Since the DNA synthesis reaction was allowed to occur in the presence of dATP and dGTP as the only polymerizable deoxynucleotides, the 24-mer primer could be maximally elongated to a 26-mer oligonucleotide (see Fig. 2). Both the 100- and the 50-kDa polypeptides retain this synthetic capability. The same result was obtained using the monodimensional *in situ* activity gel analysis according to Karawya (34), as shown in Fig. 2 (*panel B*). In addition, the 50-kDa proteolytic fragment, purified by elution from a denaturing polyacrylamide gel and subjected to the bidimensional activity gel assay, was demonstrated to possess polymerase activity (Fig. 2, *panel C* and *D*).

On the other hand, the 3′-5′ exonuclease assay was performed in a reaction buffer containing Mn^{2+} ions at 2.5 mM. In these conditions, degradation of the 25-merT primer was carried out by both the 100- and the 40-kDa polypeptides (see Fig. 3). The 40-kDa polypeptide was shown to retain the degradative activity also after isolation by elution from a SDS-preparative gel, when assayed by the bidimensional activity gel analysis (see Fig. 3, *panels B* and *C*).

It has long been known that divalent metal ions are essential for the enzymatic activities performed by DNA polymerases, although the chemical identity of the cations bound *in vivo* to a catalytically competent enzyme is presently unknown (1, 35, 36). Crystallographic studies revealed the presence of metal

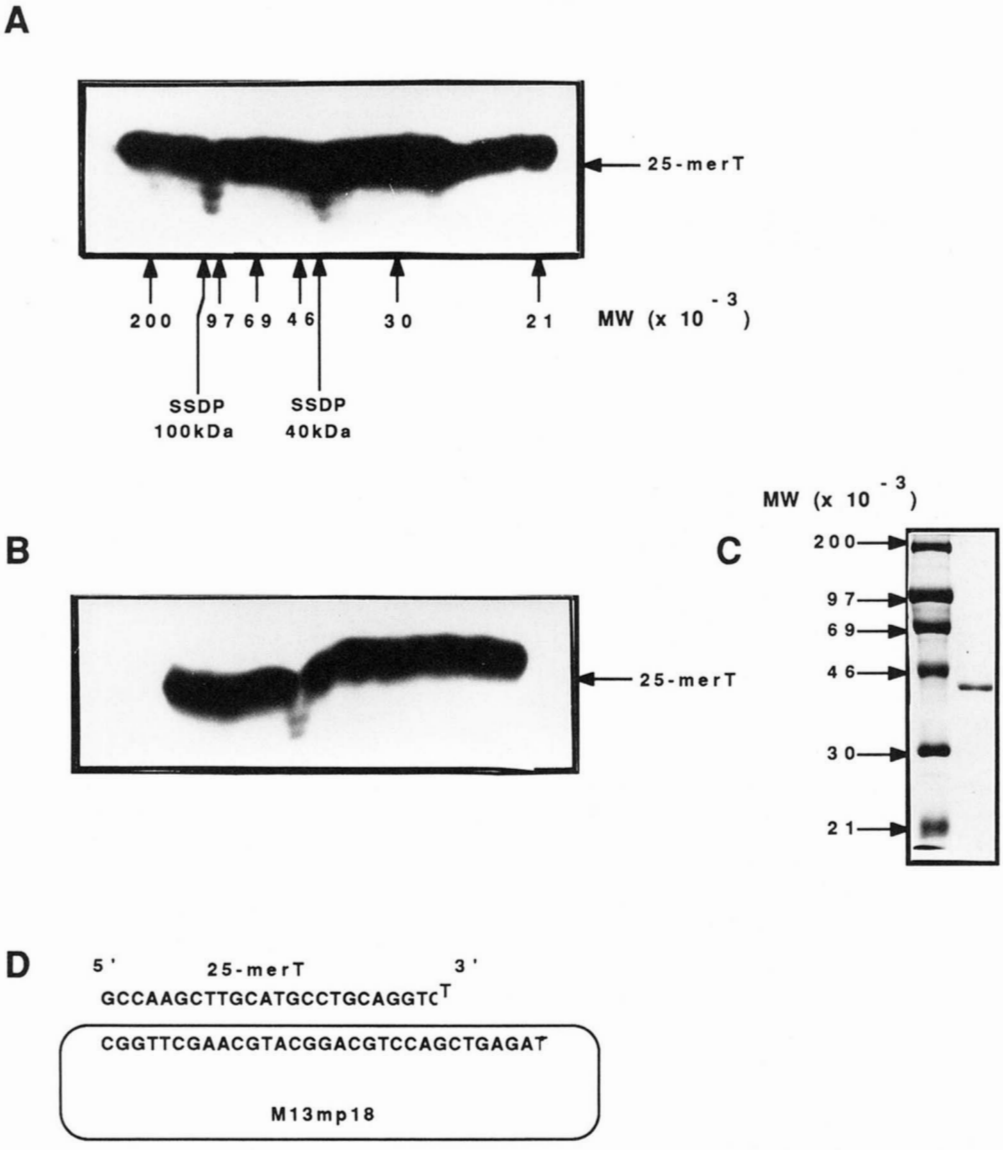

FIG. 3. ***In situ* detection of the 3′-5′ exonuclease activity of *S. solfataricus* DNA polymerase polypeptides by denaturing bidimensional activity gel analysis.** *Panel A*, a sample of *S. solfataricus* DNA polymerase (0.5 μg) was subjected to 3′-5′ exonuclease activity gel assay, as described under "Experimental Procedures." The autoradiographic film of the polyacrylamide/urea gel run in the second dimension to resolve the exonucleolytic degradation products is shown. The *horizontal arrow* locates the ^{32}P-labeled 25-merT primer. Rainbow™ molecular weight markers were the same as in Fig. 2, *panel A*. Their position is indicated by *vertical arrows*, as well as that of *S. solfataricus* DNA polymerase active polypeptides of 100 (SSDP, 100 kDa) and 40 kDa (SSDP, 40 kDa). *Panel B*, a sample (0.5 μg) of *S. solfataricus* 40-kDa protein fragment, purified as described in the text, was subjected to the bidimensional 3′-5′ exonuclease activity gel assay. The autoradiogram of the second dimension of electrophoresis is shown. *Panel C*, a sample (3 μg) of *S. solfataricus* 40-kDa protein fragment, purified as described in the text, was run through a 10% polyacrylamide denaturing gel according to Laemmli (29; *left lane*). The standard proteins loaded onto the *right lane* were as in Fig. 1, *Panel A*. Protein was stained with Coomassie Brilliant Blue. *Panel D*, Schematic representation of the 3′-5′ exonuclease DNA substrate utilized in the gel assay analysis. The oligonucleotide 25-merT was fully complementary to positions 6264–6289 of M13mp18(+)-strand, but formed a 3′-terminal (T:C) mispair at position 6265. The preparation of this primer/template substrate is described under "Experimental Procedures."

ions binding sites in both Klenow fragment active centers that could be filled by Mg^{2+}, Mn^{2+}, or Zn^{2+} (14, 15, 37). The 3′-5′ exonuclease activity of *S. solfataricus* DNA polymerase has a remarkable preference for Mn^{2+} with respect to Mg^{2+} as a metal activator.[4] Accordingly, in other systems, Mn^{2+} ions have been reported to activate the exonucleolytic activity on both single- and double-stranded DNA substrates (38, 39). However, the specific divalent cations requirement for *S. solfataricus* DNA polymerase catalytic functions needs to be further investigated by *in vitro* kinetic characterization of the native enzyme.

The exonuclease activity of the 40-kDa proteolytic fragment could involve the ability to recognize and bind the mispaired 3′ terminus of the primer 25-merT, that is likely to be partially melted from the M13 template at the temperature (45 °C) utilized for the activity assay. Indeed, the structural analysis of Klenow fragment co-crystallized with DNA has supplied evidence that the exonuclease active site could accept only single-stranded DNA molecules (40). On the other hand, Catalano and Benkovic reported that although primer/template DNA molecules having at the 3′-end the nucleotide analogue 2′,3′-epoxy-AMP were able to completely inhibit by irreversible binding the Klenow fragment polymerase active site, in the meantime the exonuclease catalytic site could effect degradation of the 3′ terminus of a second duplex DNA molecule at an undiminished rate (41). All that considered, we cannot rule out the possibility that the 40-kDa polypeptide retains some double-stranded DNA binding capability. Neither we can exclude that it could

[4] F. M. Pisani, unpublished observations.

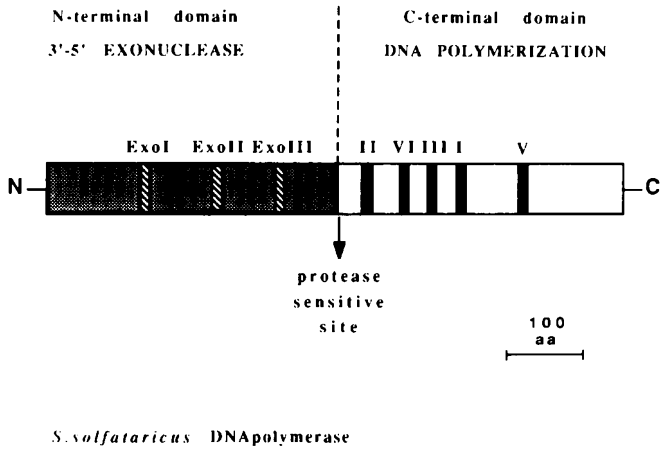

FIG. 4. **Diagram of the domain structure proposed for *S. solfataricus* DNA polymerase.** The molecular mass of the intact enzyme has been estimated to be about 100 kDa (28). Exo motifs are according to Salas (11, 13). The sequence regions I, II, III, V, and VI have been described by Wang (19).

possess an intrinsic editing specificity, as reported for the 32-kDa ϵ subunit of *E. coli* DNA polymerase III, which is able to excise a mispaired 3'-end from a primer/template DNA substrate, even if isolated from the other components of the holoenzymatic complex (42).

Conclusively, on the basis of our biochemical analysis, we propose that *S. solfataricus* DNA polymerase consists of two distinct structural domains with different catalytic activity that are able to function in an independent fashion. In accordance with our findings, Cheng and Kuchta (43) have recently proposed on the basis of the differential inactivating effect of aphidicolin and methylbromoacetate on the polymerase and exonuclease activities of calf thymus DNA polymerase ϵ a physical separation of the respective catalytic sites. Interestingly, as schematically depicted in Fig. 4, the amino-terminal domain responsible for the 3'-5' exonuclease activity includes the three Exo motifs conserved among DNA polymerases of families A and B (11–13); whereas the carboxyl-terminal domain, to which the polymerization function has been assigned, contains the five highly conserved sequence regions described by Wang (19). All that considered, it is tempting to speculate that *S. solfataricus* DNA polymerase could adopt an overall tertiary fold similar to that described for *E. coli* Klenow fragment (2, 4). Indeed, recent amino acid sequence alignments extended to all nucleotides polymerizing enzymes (including reverse transcriptases and RNA polymerases) have indicated that a few key residues are conserved among them, thereby suggesting a common evolutive origin (10, 25, 44). This unifying hypothesis has been recently confirmed by crystallographic studies on human immunodeficiency virus-type 1 (HIV-1) reverse transcriptase, whose polymerase domain, particularly the catalytic site, seems folded in a way globally similar to the Klenow fragment (45).

The construction of deletion mutants of *S. solfataricus* DNA polymerase gene expressing only the 3'-5' exonuclease or the polymerase domain is under way and will be helpful in order to further investigate the modular organization proposed for this enzyme.

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REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) in *DNA Replication*, 2nd Ed., W. H. Freeman and Co., New York
2. Joyce, C. M., and Steitz, T. A. (1987) *Trends Biochem. Sci.* **12,** 288–292
3. Carroll, S. S., and Benkovic, S. J. (1990) *Chem. Rev.* **90,** 1291–1298
4. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., and Steitz, T. A. (1985) *Nature* **313,** 726–766
5. Beese, L. S., Derbyshire, V., and Steitz, T. A. (1993) *Science* **260,** 352–355
6. Klenow, H., and Henningsen, I. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **65,** 168–175
7. Polesky, A. H., Steitz, T. A., Grindley, N. D. F., and Joyce, C. M. (1990) *J. Biol. Chem.* **265,** 14579–14591
8. Freemont, P. S., Ollis, D. L., Steitz, T. A., and Joyce, C. M. (1986) *Proteins* **1,** 66–73
9. Braithwaite, D. K., and Ito, J. (1993) *Nucleic Acids Res.* **21,** 787–802
10. Joyce, C. M. (1991) *Curr. Opin. Struct. Biol.* **1,** 123–129
11. Bernad, A., Blanco, L., Làzaro, J. M., Martin, G., and Salas, M. (1989) *Cell* **59,** 219–228
12. Reha-Krantz, L. J. (1992) *Gene (Amst.)* **112,** 133–137
13. Blanco, L., Bernad, A., and Salas, M. (1992) *Gene (Amst.)* **112,** 139–144
14. Derbyshire, V., Grindley, N. D. F., and Joyce, C. M. (1991) *EMBO J.* **10,** 17–24
15. Beese, L. S., and Steitz, T. A. (1991) *EMBO J.* **10,** 25–33
16. Soengas, M. S., Esteban, J. A., Làzaro, J. M., Bernad, A., Blasco, M. A., Salas, M., and Blanco, L. (1992) *EMBO J.* **11,** 4227–4237
17. Morrison, A., Bell, J. B., Kunkel, T. A., and Sugino, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9473–9477
18. West Frey, M., Nossal, N. G., Lapson, T. L., and Benkovic, S. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2578–2583
19. Wang, T. S. F., Wong, W. W., and Korn, D. (1989) *FASEB J.* **3,** 14–21
20. Blanco, L., Bernad, A., Blasco, M. A., and Salas, M. (1991) *Gene (Amst.)* **100,** 27–38
21. Bernad, A., Làzaro, J. M., Salas, M., and Blanco, L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4610–4614
22. Blasco, M. A., Làzaro, J. M., Bernad, A., Blanco, L., and Salas, M. (1992) *J. Biol. Chem.* **267,** 19427–19434
23. Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990) *Protein Eng.* **3,** 461–467
24. Gibbs, J. S., Weisshart, K., Digard, P., De Bruymkops, A., Knipe, D. M., and, Coen, D. M. (1991) *Mol. Cell. Biol.* **11,** 4786–4795
25. Chen, M., and Horowitz, M. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6116–6120
26. Rossi, M., Rella, R., Pensa, M., Bartolucci, S., De Rosa, M., Gambacorta, A., Raia, C. A., and Dell'Aversano Orabona, N. (1986) *Syst. Appl. Microbiol.* **7,** 337–341
27. Rella, R., Raia, C. A., Pisani, F. M., D'Auria, S., Nucci, R., Gambacorta, A., De Rosa, M., and Rossi, M. (1990) *Ital. J. Biochem. (Engl. Ed.)* **39,** 83–99
28. Pisani, F. M., De Martino, C., and Rossi, M. (1992) *Nucleic Acids Res.* **20,** 2711–2716
29. Laemmli, U. K. (1970) *Nature* **227,** 680–685
30. Reisfeld, R. A., Lewis, V. J., and Williams, D. E. (1962) *Nature* **195,** 281–283
31. Andrews, P. (1964) *Biochem. J.* **91,** 222–233
32. Hager, D. A., and Burgess, R. R. (1980) *Anal. Biochem.* **109,** 76–86
33. Longley, M. J., and Mosbaugh, D. W. (1991) *Biochemistry* **30,** 2655–2664
34. Karawya, E., Swack, J. A., and Nelson, S. H. (1983) *Anal. Biochem.* **135,** 318–325
35. Lehman, I. R., and Richardson, C. C. (1964) *J. Biol. Chem.* **239,** 233–241
36. Mullen, G. P., Sepersu, E. H., Ferrin, L. J., Loeb, L. A., and Mildvan, A. S. (1990) *J. Biol. Chem.* **265,** 14327–14334
37. Steitz, T. A. (1993) *Curr. Opin. Struct. Biol.* **3,** 31–38
38. Kong, H., Kucera, R. B., and Jack, W. E. (1993) *J. Biol. Chem.* **268,** 1965–1975
39. Esteban, J. A., Bernad, A., Salas, M., and Blanco, L. (1992) *Biochemistry* **31,** 350–359
40. Derbyshire, V., Freemont, P. S., Sanderson, M. R., Beese, L., Freedman, J. M., Joyce, C. M., and Steitz, T. A. (1988) *Science* **240,** 199–201
41. Catalano, C. E., and Benkovic, S. J. (1989) *Biochemistry* **28,** 4374–4382
42. Brenowitz, S., Kwack, S., Goodman, M. F., O' Donnell, M., and Echols, H. (1991) *J. Biol. Chem.* **266,** 7888–7892
43. Cheng, C. H., and Kuchta, R. D. (1993) *Biochemistry* **32,** 8568–8574
44. Poch, O., Sauvaget, I., Delarue, M., and Tordo, N. (1989) *EMBO J.* **8,** 3867–3874
45. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) *Science* **256,** 1783–1790