Poly(ADP-Ribose) Synthetase

SEPARATION AND IDENTIFICATION OF THREE PROTEOLYTIC FRAGMENTS AS THE SUBSTRATE-BINDING DOMAIN, THE DNA-BINDING DOMAIN, AND THE AUTOMODIFICATION DOMAIN*

Isamu Kameshita, Zen‘e Matsuda, Taketoshi Taniguchi, and Yutaka Shizuta
From the Department of Medical Chemistry, Kochi Medical School, Nankoku, Kochi 781-51, Japan

Poly(ADP-ribose) synthetase of \( M_r = 120,000 \) is cleaved by limited proteolysis with \( \alpha \)-chymotrypsin into two fragments of \( M_r = 54,000 \) (54K) and \( M_r = 66,000 \) (66K). When the native enzyme is modified with 3-(bromoacetylp)pyridine, both portions of the enzyme are alkylated; however, alklylation of the 54K portions of the enzyme is protected by the addition of the substrate, NAD, or its analog, nicotinamide, suggesting that the substrate-binding site is localized in the 54K fragment. When the enzyme previously auto- modified with a low concentration of \([adenine-U-^{14}C]\)NAD is digested with \( \alpha \)-chymotrypsin, the radioactivity is detected exclusively in the 66K fragment. The 66K fragment labeled is further cleaved with papain into two fragments of \( M_r = 46,000 \) and \( M_r = 22,000 \). With these two fragments, the label is detected only in the 22K fragment, but not in the 46K fragment. The 46K fragment binds to a DNA-cellulose column with the same affinity as that of the native enzyme, while the 22K fragment and the 54K fragment have little affinity for the DNA ligand. These results indicate that poly(ADP-ribose) synthetase contains three separable domains, the first possessing the site for binding of the substrate, NAD, the second containing the site for binding of DNA, and the third acting as the site(s) for accepting poly(ADP-ribose).

Poly(ADP-ribose) synthetase, an enzyme localized in the nucleus of eukaryotic cells, catalyzes the polymerization of the ADP-ribose moiety of NAD to form poly(ADP-ribose) which is covalently bound to various nuclear proteins (1, 2). Although the physiological function of this enzyme reaction is not yet fully understood, it has been suggested that the reaction participates in DNA repair (3, 4), DNA synthesis (5), and histone H2B are known to serve as the acceptors of the ADP-ribose moiety of NAD to form poly(ADP-ribose) synthetase. The costs of publication in this journal are defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication, December 12, 1983
EXPERIMENTAL PROCEDURES

Materials—Commercial sources of materials used were as follows: [adenine-U-\( ^{14}C \)]NAD (286 mCi/mmol) from the Radiochemical Centre, Amersham; sodium borohydride (347 mCi/mmol) from New England Nuclear; NAD, phenylmethylsulfonyl fluoride, calf thymus DNA, calf thymus histone H1, \( \alpha \)-chymotrypsin (Type II), bovine serum albumin, ovalbumin, and lysozyme from Sigma; \( \beta \)-galactosidase and phosphorylase a from Boehringer Mannheim; papain and snake venom phosphodiesterase from Worthington Biochemical Corp.; polyethyleneimine cellulose plates from Macherey-Nagel; Tonein TF from Otsuka Assay Laboratories. All other chemicals were from Nakarai Chemicals and were of analytical grade. Ep-galactosidase was prepared by bromination of 3-acetylpyridine as described by Albert and Herrick (26). The DNA-cellulose thus prepared contained approximately 3 mg of DNA/ml of packed volume. Fragmented DNA with 20–30 base pairs was prepared by incubating calf thymus DNA (0.5 mg) at 27 °C for 1 min with 20 μg of DNase I in a buffer containing 0.1 M Tris-HCl (pH 8.0) and 10 mM 2-mercaptoethanol.

DNA-cellulose was prepared according to the method of Albert and Herrick (26). The DNA-cellulose thus prepared contained approximately 3 mg of DNA/ml of packed volume. Fragmented DNA with 20–30 base pairs was prepared by incubating calf thymus DNA (0.5 mg) at 27 °C for 1 min with 20 μg of DNase I in a buffer containing 0.1 M Tris-HCl (pH 8.0) and 10 mM MgCl₂ in a total volume of 1 ml.

Preparation of 3-(Bromomethyl)pyridine—3-(Bromomethyl)pyridine was prepared by bromination of 3-ethylpyridine as described by Woenckhaus et al. (27), and recrystallized three times from glacial acetic acid. The sample thus obtained was subjected to thin layer chromatography on polyethyleneimine cellulose and found to migrate as a single spot using both solvent systems of 0.25 M LiCl (\( R_f 0.64 \)) and 1-butanol/pyridine/H₂O (1:1:1, v/v) (\( R_f 0.67 \)) when detected by 5,5'-dithiobis(2-nitrobenzoic acid) spray for reactive halogen com-

4770
Three Functional Domains of Poly(ADP-Ribose) Synthetase

Pounds (28) and by visualization with ultraviolet irradiation. Quantitative determination of 3-bromomethylpyridine was performed by its reaction with glutathione as described by Hartman (29). Aqueous solutions of 3-bromomethylpyridine were capable of storage frozen at -70°C for several months without noticeable change.

Assay of Poly(ADP-Ribose) Synthetase—The standard reaction mixture contained Tris-HCl buffer (pH 7.5), 10 mM dithiothreitol, 100 μM [adenine-U-14C]NAD (5000 dpm/nmol), 10 μg of calf thymus DNA and calf thymus histone H1, and varying amounts of enzyme in a total volume of 0.1 ml. The reaction was carried out at 25°C for 3 min unless otherwise specified and terminated by the addition of 5 ml of 10% trichloroacetic acid containing 1% diethylpyrocarbonate. The resulting solution was heated at 100°C for 10 min to denature enzyme-bound acetylpyridine. When 10 mM nicotinamide was employed in place of 5 mM NAD, the modification was almost completely removed. The labeled enzyme thus prepared was then diluted with Buffer B to a concentration of 100 μg of protein/ml and subjected to limited proteolysis with α-chymotrypsin or with papain.

Proteolytic Digestion with α-Chymotrypsin or with Papain—The purified preparation of poly(ADP-ribose) synthetase (100–200 μg/ml) in Buffer B was digested at 25°C with α-chymotrypsin in a protein ratio of 50:1. After an appropriate time of digestion, the reaction was terminated by adding either trichloroacetic acid at a final concentration of 20% or phenylmethylsulfonyl fluoride at a final concentration of 1 mM. Digestion of the enzyme with papain was performed under the same conditions as those with α-chymotrypsin except that the incubation was performed at 0°C and that the reaction was terminated by the addition of trichloroacetic acid at a final concentration of 20% or Ep-475 at a final concentration of 2.5 μg/ml.

Polyacrylamide Gel Electrophoresis—The enzyme sample digested with α-chymotrypsin or with papain was diluted with SDS-sample buffer and subjected to electrophoresis in the presence of SDS-PAGE. The gels, which were run in the absence of any sodium dodecyl sulfate, were visualized by staining with Coomassie Blue R-250. The molecular weights of the enzyme was estimated to be 66,000 and 54,000, respectively, by comparison with reference proteins. Since the molecular weight of the native enzyme was estimated to be 120,000, it was concluded that α-chymotrypsin cleaved the enzyme into two fragments (Fig. 1B). Upon prolonged incubation, the 54K fragment was further cleaved into two fragments of Mr = 41,000 and 15,000, respectively. The 66K fragment, however, was resistant to further digestion with α-chymotrypsin. In parallel with the disappearance of the intact enzyme, poly(ADP-ribose) synthetase activity was lost progressively (Fig. 1B). The addition of calf thymus with highly polymerized or fragmented DNA to the reaction mixture affected neither the rate nor the pattern of digestion as judged by SDS-polyacrylamide gel electrophoresis.

Identification of the Fragment Containing the Substrate-binding Site—When poly(ADP-ribose) synthetase was incubated at 25°C with α-chymotrypsin in a protein ratio of 50:1, the enzyme was cleaved into a number of fragments which were separated by SDS-polyacrylamide gel electrophoresis (Fig. 1A). In the early stage of the digestion, there appeared two major fragments with the simultaneous disappearance of the native enzyme. The molecular weights of these fragments were estimated to be 66,000 and 54,000, respectively, by comparison with reference proteins. Since the molecular weight of the native enzyme was estimated to be 120,000, it was concluded that α-chymotrypsin cleaved the enzyme into two major fragments. Upon prolonged incubation, the 54K fragment was further cleaved into two fragments of Mr = 41,000 and 15,000, respectively. The 66K fragment, however, was resistant to further digestion with α-chymotrypsin. In parallel with the disappearance of the intact enzyme, poly(ADP-ribose) synthetase activity was lost progressively (Fig. 1B). The addition of calf thymus with highly polymerized or fragmented DNA to the reaction mixture affected neither the rate nor the pattern of digestion as judged by SDS-polyacrylamide gel electrophoresis.

Identification of the Fragment Containing the Substrate-binding Site—When poly(ADP-ribose) synthetase was incubated at 25°C with α-chymotrypsin in a protein ratio of 50:1, the enzyme was rapidly inactivated (Fig. 2). However, inactivation by this reagent was significantly prevented by the presence of 5 mM nicotinamide, a potent competitive inhibitor with respect to the binding of the substrate, NAD (38, 39). The addition of fragmented DNA had no effect on the rate of inactivation both in the absence and presence of nicotinamide. These results suggest that nicotinamide protects the enzyme from inactivation by 3-bromomethylpyridine by binding to the catalytic site of poly(ADP-ribose) synthetase.

To locate the substrate-binding site of poly(ADP-ribose) synthetase, the enzyme modified with 3-(bromomethyl)pyridine and then labeled with sodium boro[H]hydride as described under "Experimental Procedures," was digested with a-chymotrypsin and subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, left, the radioactivity was retained on the four main protein bands which corresponded to the native enzyme (120K) and the 66K, 54K, and 41K fragments, respectively. When the modification was carried out in the presence of nicotinamide, incorporation of tritium into the 54K protein band significantly decreased in comparison with that of the 66K fragment. Under these conditions,
Three Functional Domains of Poly(ADP-Ribose) Synthetase

Inactivation of poly(ADP-ribose) synthetase by 3-(bromoacetyl)pyridine. The enzyme (16 μg) was incubated at 25°C with 0.2 mM 3-(bromoacetyl)pyridine in 50 μl of Buffer A in the presence (open symbols) or absence (closed symbols) of 5 mM nicotinamide. The chemical modification was also carried out in the presence (○, □) or absence (●, ■) of 0.25 μg of fragmented DNA. At the indicated times, aliquots (10 μl) were removed from the incubation mixture and assayed for the residual enzyme activity as described under “Experimental Procedures.” As a control, the enzyme sample was incubated without 3-(bromoacetyl)pyridine (△).

Identification of the Fragment Containing the DNA-binding Site—In order to determine which fragments possess the DNA-binding site, column chromatography on DNA-cellulose was carried out. The enzyme fragments obtained by digestion with a-chymotrypsin were applied to a column of DNA-cellulose equilibrated with Buffer B, and eluted with a linear concentration gradient of NaCl from 0.1 to 1.2 M. As shown in Fig. 5A, two protein peaks were detected; one passed through the column when washed with Buffer B (peak 1), and the other eluted at approximately 0.6 M NaCl (peak 2). Aliquots of these two peaks were subjected to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5B, peak 1 contained the 54K and 41K fragments, and peak 2 contained the 66K fragment. When the native enzyme was applied onto the column, the enzyme was eluted at the same salt concentration as that of the 66K fragment. These results indicate that the DNA-binding site was located on the 66K fragment. The 66K fragment was resistant to further digestion with α-chymotrypsin, but it was cleaved by papain into two fragments of M_e = 46,000 and 22,000 (Fig. 6B). To determine which of these two fragments, like the parent intact enzyme, has affinity for DNA, chromatography on DNA-cellulose was again carried out. When the two fragments were passed over the column equilibrated with Buffer B, the 22 K fragment was slightly retarded relative to the breakthrough fractions, indicating that this fragment has weak affinity for DNA. On the other hand, the 46K fragment was bound tightly to the column under the above conditions. When elution was performed using a linear salt gradient between 0.1 and 1.2 M, the protein peak of the 46K fragment appeared at 0.6 M NaCl (Fig. 6). These findings indicate that the 46K fragment contains the

The labeled enzyme was also digested with papain to further elucidate the localization of the substrate-binding site (Fig. 3, right). After digestion, there appeared two predominant fragments of M_e = 74,000 and 46,000 as shown in Fig. 3C. In this case, incorporation of tritium into the 74K portion of the enzyme was markedly protected by nicotinamide (Fig. 3D), indicating that the substrate-binding site is located in the 74K fragment. Weak protection was also observed in a minor 56K band, which was derived from the 74K fragment (24).

To establish the above lines of evidence, the substrate, NAD, was employed for protecting the active site of the

the native enzyme and the 41K portion were also protected by nicotinamide (Fig. 4), indicating that the substrate-binding site is located in the 41K fragment. The 66K fragment was slightly retarded relative to the breakthrough fractions, indicating that this fragment has weak affinity for DNA. On the other hand, the 46K fragment was bound tightly to the column under the above conditions. When elution was performed using a linear salt gradient between 0.1 and 1.2 M, the protein peak of the 46K fragment appeared at 0.6 M NaCl (Fig. 6). These findings indicate that the 46K fragment contains the
DNA-binding site of the enzyme.

Identification of the Fragment Containing the Site(s) for Accepting Poly(ADP-Ribose)—Poly(ADP-ribose) synthetase itself is known to be automodified with poly(ADP-ribose) (21-23). In order to locate the sites for poly(ADP-ribose)ylation in the enzyme molecule, the enzyme previously automodified with [3H]NAD was digested with α-chymotrypsin or with papain, and analyzed by SDS-polyacrylamide gel electrophoresis. In this experiment, the poly(ADP-ribose)ylation reaction was controlled by lowering the concentration of [3H]NAD (1.4 μM, 423 dpm/pmol) so that the increase in molecular weight due to the attachment of the polymer might not affect the electrophoretic mobility of the fragments. Under the conditions employed, 1.6 ADP-ribose units were incorporated per molecule of enzyme, and an average chain length of the polymer formed was 3.2 ADP-ribose units. The results of fluorography of an electrophoresed gel is shown in Fig. 7. When the enzyme previously automodified with [3H]NAD was digested with α-chymotrypsin, the radioactivity was detected only on the 66K fragment (Fig. 7b). To elucidate the localization of the site(s) for poly(ADP-ribose)ylation, the 66K fragment was further digested by papain and again analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 7c, the radioactivity due to poly(ADP-ribose) was detected exclusively in the protein band corresponding to the Mr = 22,000. Upon prolonged digestion with papain, no additional band containing radioactivity appeared up to 120 min. These results indicate that the site(s) for poly(ADP-ribose)ylation is localized in the 22K fragment.

DISCUSSION

In the present study, three functional domains of poly(ADP-ribose) synthetase have been separated from each other by limited proteolysis with α-chymotrypsin and papain. The first domain of Mr = 54,000 possesses the site for the substrate binding, the second one of Mr = 46,000 retains the site for DNA binding, and the third one of Mr = 22,000 contains the site(s) for accepting poly(ADP-ribose) (Fig. 8). It may be pointed out, however, that small fragments other than these three fragments are released in the process of limited proteolysis and not detected on SDS-polyacrylamide gel electrophoresis under the present conditions, because the primary structure of each fragment is not yet fully determined. Nevertheless, total amino acid analyses of these fragments, in addition to our present data on the molecular size of each fragment, support the above conclusion that the enzyme essentially consists of these three fragments.

The substrate-binding domain of poly(ADP-ribose) synthetase was deduced by chemical modification with 3-(bromoacetyl)pyridine in the presence and absence of NAD or its analog, nicotinamide, followed by labeling with sodium [3H]hydroxoxine. When the labeled enzyme was subjected to SDS-polyacrylamide gel electrophoresis after partial proteolytic digestion, the radioactivity was found in all of the chymotryptic fragments corresponding to the site(s) for accepting poly(ADP-ribose) (Fig. 8). These three fragments are easily separable from each other by chromatography and summation of amino acid compositions of these fragments coincides well with those of the intact poly(ADP-ribose) synthetase (M. Agemori, I. Kameshita, and Y. Shizuta, manuscript in preparation).
Three Functional Domains of Poly(ADP-Ribose) Synthetase

FIG. 4. Digestion of poly(ADP-ribose) synthetase with α-chymotrypsin or papain after modification with 3-(bromoacety1)pyridine in the presence and absence of NAD. Modification of the enzyme in the presence of 5 mM NAD was carried out under the same conditions as described in Fig. 3, except that 80 µl of 20 mM sodium borohydride (347 mCi/mmol) was used for the labeling of the modified enzyme. The enzyme sample (25 µg) thus labeled was digested with 0.5 µg of α-chymotrypsin or 0.5 µg of papain and subjected to SDS-polyacrylamide gel electrophoresis; left, the enzyme digested with α-chymotrypsin for 2 min; right, the enzyme digested with papain for 5 min. A and C, quantification of the radioactivity in SDS-polyacrylamide gels as described under “Experimental Procedures.” The enzyme was modified with 3-(bromoacety1)pyridine in the absence (O) or presence (●) of nicotinamide. B and D, the difference between the values denoted by O and ● in A and C was replotted.

FIG. 5. DNA-cellulose column chromatography of the enzyme fragments obtained by digestion with α-chymotrypsin. The enzyme (400 µg) in Buffer B was incubated at 25 °C with 8 µg of α-chymotrypsin in a total volume of 4 ml. After 20 min, phenylmethylsulfonyl fluoride at a final concentration of 0.1 M was added to the reaction mixture. The mixture was then applied onto a column (0.7 × 2.6 cm) of DNA-cellulose which was equilibrated with Buffer B. After washing the column with 3 ml of Buffer B, elution was carried out with a linear concentration gradient of NaCl, from 0.1 to 1.2 M, 10 ml each in Buffer B solution. Protein in each fraction (580 µl) was determined using an aliquot (50 µl) as described under “Experimental Procedures.” The concentration of NaCl was estimated by measuring the value of conductivity of each fraction. A, elution profile of DNA-cellulose column chromatography. B, SDS-polyacrylamide gel electrophoretic patterns showing the enzyme fragments obtained by digestion with α-chymotrypsin (O), components of peak 1 (1), and of peak 2 (2), respectively.

FIG. 6. DNA-cellulose column chromatography of the fragments obtained by digestion of the 66K fragment with papain. The 66K fragment prepared by DNA-cellulose column chromatography as shown in Fig. 5 was dialyzed at 4 °C for 3 h against 500 ml of Buffer B. The 66K fragment (140 µg) was incubated at 0 °C for 10 min with 2.8 µg of papain in a total volume of 1.8 ml. To the mixture was then added Ep-475 at a final concentration of 2.5 µg/ml to terminate further digestion. Column chromatography on DNA-cellulose and SDS-polyacrylamide gel electrophoresis were carried out in the same manner as described in Fig. 5. A, elution profile of DNA-cellulose column chromatography. B, SDS-polyacrylamide gel electrophoretic patterns showing the fragments obtained by digestion of the 66K fragment with papain (0), components of peak 1 (1), and of peak 2 (2), respectively.

NAD or its analog was included in the reaction mixture. The covalent and nonspecific incorporation of tritium observed in the 66K and 46K fragments appears to be derived from acetylpyridine attached to some cysteinyl residues which are not required for the binding of the substrate, NAD.³ There-

³ I. Kameshita and Y. Shizuta, unpublished results.
The enzyme activity was lost progressively in parallel with the disappearance of the native enzyme (Fig. 1). The addition of DNA to the reaction mixture affected neither the rate nor the extent of inactivation of the enzyme by this protease. In contrast, the digestion with papain was somewhat retarded by the presence of DNA and nearly 20% of the initial activity was detected even after the enzyme was completely split into the 74K and 46K fragments (24). These differences might be derived from the difference in the sites of the primary sequence of poly(ADP-ribose) synthetase which are attacked by these two different proteases. Both the 46K fragment and the 74K fragment as obtained by digestion with a-chymotrypsin appear to lose the original conformation almost completely because the 54K fragment is not able to bind to the DNA ligand.

REFERENCES

Three Functional Domains of Poly(ADP-Ribose) Synthetase 4775

FIG. 7. Digestion of automodified poly(ADP-ribose) synthetase with a-chymotrypsin and with papain. Poly(ADP-ribose) synthetase (40 &g;g) was incubated at 25 °C for 1 min with 1.4 &mu;M [adenine-U-14C]NAD (423 dpm/pmole) in 100 &mu;l Tris-HCl (pH 8.0) containing 1 &mu;M dithiothreitol, 10 &mu;M MgCl2, 10% glycerol, and 100 &mu;g/ml of calf thymus DNA in a final volume of 800 &mu;l. To the mixture was then added nicotinamide at a final concentration of 10 mM. The enzyme (37.5 &mu;g) thus automodified was digested with a-chymotrypsin (0.75 &mu;g) at 25 °C for 20 min in a total volume of 750 &mu;l. The digestion was terminated by the addition of phenylmethylsulfonyl fluoride at a final concentration of 1 mM. The enzyme (35 &mu;g) treated with a-chymotrypsin was further digested at 0 °C for 5 min with papain (0.7 &mu;g). In each step of digestion, aliquots (50 &mu;l) were removed and subjected to SDS-polyacrylamide gel electrophoresis and the radioactivity was detected by fluorography as described under "Experimental Procedures." a, the automodified enzyme, b, the enzyme fragment after digestion with a-chymotrypsin, and c, the fragment obtained by digestion with papain after a-chymotryptic digestion.

A  
\[ \begin{array}{c}
\text{Substrate Binding} \\
\text{DNA Binding} \\
\text{Autodomination}
\end{array} \]

54K  
66K

B  
\[ \begin{array}{c}
\text{Substrate Binding} \\
\text{DNA Binding} \\
\text{Autodomination}
\end{array} \]

74K  
46K

C  
\[ \begin{array}{c}
\text{Substrate Binding} \\
\text{DNA Binding} \\
\text{Autodomination}
\end{array} \]

54K  
22K  
46K

FIG. 8. Schematic representation for the domain structure of poly(ADP-ribose) synthetase. The chymotryptic and papain cleavage sites on the enzyme molecule are indicated by arrows. The left end may not represent the NH2 terminus of the native enzyme as the group analysis of each fragment was not performed.
Three Functional Domains of Poly(ADP-Ribose) Synthetase

Poly (ADP-Ribose) synthetase. Separation and identification of three proteolytic fragments as the substrate-binding domain, the DNA-binding domain, and the automodification domain.

I Kameshita, Z Matsuda, T Taniguchi and Y Shizuta


Access the most updated version of this article at http://www.jbc.org/content/259/8/4770

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/8/4770.full.html#ref-list-1