Purification and Characterization of Poly(ADP-ribose) Synthetase from Calf Thymus*

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Poly(ADP-ribose) synthetase from calf thymus has been purified to apparent homogeneity by a simple and rapid method with a recovery of 10 to 20%. The enzyme activity absolutely requires the presence of DNA. Histone further stimulates the reaction. The $K_m$ for NAD and the maximal velocity at 25°C and pH 8.0 in the presence of both compounds are 58 μM and 1,400 nmol/min/mg, respectively. The sedimentation coefficient ($s_{20,w}$) of the enzyme is 5.80 S. The molecular weight is calculated to be 108,000 by sedimentation equilibrium method using a partial specific volume of 0.736 ml/g. This value is in good agreement with the molecular weight values of 115,000 and 120,000 determined by gel filtration on Sephadex G-200 and gel electrophoresis in the presence of sodium dodecyl sulfate, respectively. The enzyme is colorless and its absorption spectrum shows a maximum at 280 nm. From a CD spectrum, a helical content is estimated to be approximately 30%. The enzyme is a basic protein having a pI value of 9.8 and is rich in lysine rather than arginine. Neutral sugar, phospholipid, and DNA are not detected in the final preparation. These data indicate that the purified enzyme is a simple globular protein composed of a single polypeptide having an approximate molecular weight of 110,000.

Poly(ADP-ribose) synthetase is a ubiquitous nuclear enzyme which catalyzes the synthesis of acceptor-bound poly(ADP-ribose) from the ADP-ribosyl moiety of NAD (1-4). Various nuclear proteins, both histones (5-11) and nonhistone proteins (12-14), are acceptors of this modification reaction.

Poly(ADP-ribose) synthetase has been partially purified from a variety of sources (15-19). Common prominent characteristics of these enzyme preparations are their activation by DNA and histone (16-17). However, Mandel et al. (20) have recently reported that the highly purified calf thymus enzyme migrates as a single band on gel electrophoresis, but it does not require DNA for full activity. On the other hand, Yoshihara et al. (21) have reported that the highly purified enzyme from the same source does require DNA absolutely for the reaction, but it migrates as two bands on gel electrophoresis.

In order to resolve the above discrepancy and obtain further insight into the mechanism of the poly(ADP-ribose) synthetase reaction, we have purified the enzyme to homogeneity with a rapid and simple method and determined the physicochemical properties as well as enzymological characteristics of the protein. In this paper, evidence is presented that poly(ADP-ribose) synthetase is a simple globular protein composed of a single polypeptide of $M_r = 110,000$ that absolutely requires the addition of DNA for the reaction.

EXPERIMENTAL PROCEDURES AND RESULTS

Details of the experimental procedures used and of the results are presented in the miniprint supplement which follows.

DISCUSSION

We have purified calf thymus poly(ADP-ribose) synthetase to apparent homogeneity and characterized the molecular properties of the enzyme. These are summarized in Table I. An agreement of the molecular weight values obtained by the three methods suggests that poly(ADP-ribose) synthetase is composed of a single polypeptide of $M_r = 110,000$. This value is similar to that of $M_r = 120,000$ by Mandel et al. (20) but is different from the value of $M_r = 150,000$ to 160,000 by Yoshihara et al. (21). The frictional coefficient ratio, $f/f_0 = 1.39$, indicates that it is a globular protein with slight asymmetry. Since neutral sugar and phospholipid are not detected in the final enzyme preparation, the enzyme appears to be a simple protein.

The enzyme is a basic protein having a pI value of 9.8. Amino acid composition in Table V shows that the total number of acidic amino acids, glutamate and aspartate, is greater than that of basic amino acids, lysine and arginine. Considering the pI value, this result suggests that the enzyme contains considerable amounts of the amide form of these acidic amino acids. Several attempts were unsuccessful in determining the NH$_2$-terminal amino acid residue of the carboxymethylated enzyme (200 to 500 μg) by the dansyl chloride method (23) in the presence of 0.2 to 0.3% sodium dodecyl sulfate. Therefore, the NH$_2$-terminal amino acid appears to be blocked.

Yoshihara et al. (21) reported that the acrylamide gel electrophoresis (7.5%, pH 4.0, 6 M urea) of the purified enzyme showed two main bands and two minor bands. As mentioned above, our enzyme preparation migrated as a single band on gel electrophoresis.

1 Portions of this paper (including "Experimental Procedures," "Results," additional references, Figs. 1 to 6, and Tables II to V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 950 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-1558, cite author(s), and include a check or money order for $2.40 per set of photocopies. The abbreviation used is SDS, sodium dodecyl sulfate.

The abbreviation used is: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

3 S. Ito, unpublished experiments.

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the gel under the same conditions, indicating that the enzyme consists of a single polypeptide. It is possible, however, that the purified enzyme has different mobilities on the gel under the above conditions when some residues of glutamine and asparagine in the enzyme protein have been converted into glutamic acid and aspartic acid during manipulation. In this connection, Mandel et al. (20) recently reported that their enzyme preparation from calf thymus has a pI value of 6.5. In this case, however, their enzyme preparation appears to be an enzyme DNA complex, thus exhibiting an acidic pI since any procedure to remove DNA from the enzyme fraction is not required for the addition of DNA for full activity. In our purification procedures, separation of DNA from the enzyme fraction was accomplished by protamine sulfate treatment and thereafter, the enzyme always required DNA for the reaction.

The addition of histone stimulated the synthesis of poly(ADP-ribose) approximately 2-fold and resulted in an increase in the average chain length of the polymer without any increase in the number of sites for ADP-ribosylation. These results indicate that, in the purified enzyme system, histone acts as a kind of allosteric activator rather than acceptor for ADP-ribosylation as described by Okayama et al. (24) and Yoshihara et al. (25). Therefore, the acceptor for ADP-ribosylation in the purified system appears to be the enzyme itself or an endogenous molecule attached to or co-purified with the enzyme protein. Then, the initial attachment of poly(ADP-ribose) to the acceptor and subsequent transfer of the polymer to histone may be catalyzed by two different enzymes. These problems, including definite determination of the sites for ADP-ribosylation in the purified system as well as those in the crude system, are now under investigation in our laboratory.

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Additional references will be found on p. 3650.
Supplementary Material

To

Poly(ADP-ribose) Synthetase from Calf Thymus

EXPERIMENTAL PROCEDURES

MATERIALS

Calf thymus was kindly provided by the Department of Bacteriology, University of Wisconsin. Poly(ADP-ribose) synthetase was prepared from the precipitate obtained after dialyzing the enzyme filtrate (20 ml) against 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride and 0.05 M sodium cacodylate (adjusted to pH 7.0 with sodium hydroxide). The precipitate was collected by centrifugation at 10,000 x g for 30 min, washed twice with 0.1 M sodium cacodylate, and finally dissolved in 15 ml of water (filtered). The enzyme solution was stored at -15°C.

Commercially pure protein was used as a standard and a solution of ADP-ribose (0.1 M) was added to the enzyme solution to give a final concentration of 0.05 M. The pH was brought to 7.0 with sodium hydroxide and the solution was kept at 37°C for 1 hr. The enzyme was then precipitated with 60% (v/v) ethanol and 0.2 M sodium acetate buffer, pH 5.0. The precipitate was collected by centrifugation at 10,000 x g for 30 min, washed three times with 0.1 M sodium cacodylate, and finally dissolved in 15 ml of water (filtered). The enzyme solution was stored at -15°C.

RESULTS

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Additional references are included in the supplementary material.
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Ultraviolet Absorption and Circular Dichroism Spectra

The ultraviolet spectrum of poly(ADP-ribose) synthetase at pH 8.0 is given in Fig. 3A. The enzyme was colorless and the absorption maximum at about 250 nm increased slightly at about 350 nm and the absorption was about 1.75 at 275 nm.

The CD spectrum of the purified enzyme is shown in Fig. 3B. The enzyme exhibits a positive CD in the ultraviolet region below 260 nm and a negative CD at 260 nm. The percentage of β-structural elements calculated from the ratio of the mean residue ellipticity at 208 nm (10,000 d and optical rotation) was 96% for the enzyme.

Amino Acid Composition and Other Determinations

The amino acid analysis of the purified enzyme is shown in Table I. The enzyme contained 743 mg of protein and the results were in good agreement. The enzyme contained relatively large amounts of asparagine and glutamine and tyrosine residues were much less than tyrosine residues. Using the data in Table I, a value of 0.73 mg/ml was calculated for the partial specific volume. In the experiments using an isoelectric focusing column, the enzyme was found to be a single peak. The absolute polarimetry was analyzed by the method of Howie (15) using 1.0 mg of the purified enzyme. In accordance with this observation, the staining for carbohydrates with periodic acid-Schiff reagent (27) after gel electrophoresis gave negative results. Also, an emulsion was detected in the enzyme preparation, when 1.0 mg of the purified enzyme was analyzed by the method of Morton (23) using 1 mg of the purified enzyme.

TABLE IV

| Protein concentration (mg/ml) | Molecular weight (kDa) |
|-------------------------------|------------------------|
| 0.10                         | 1.0                    |
| 0.20                         | 2.0                    |
| 0.30                         | 3.0                    |
| 0.40                         | 4.0                    |

TABLE V

| Amino acid composition of poly(ADP-ribose) synthetase Analyzed according to the method of Wood and Moore, 1964 |
|-----------------------------------------------|
| Amino acid hydrolysis (mg/ml) | Amino acid concentration (mg/ml) |
|--------------------------------|---------------------------------|
| Valine                         | 0.03                            |
| Leucine                        | 0.02                            |
| Isoleucine                     | 0.02                            |
| Threonine                      | 0.03                            |
| Aspartic acid                  | 0.03                            |
| Glutamic acid                  | 0.03                            |
| Glycine                        | 0.02                            |
| Alanine                        | 0.02                            |
| Proline                        | 0.02                            |

TABLE II

| Purification of poly(ADP-ribose) synthetase From calf thymus |
|---------------------------------------------------------------|
| Total protein activity | Specific activity (mg/ml) | Yield |
| Crude extract        | 29,000                     | 1.0   | 100 |
| Deoxycholate fraction | 20,000                     | 1.0   | 100 |
| Sepharose 4B fraction | 8,000                      | 1.0   | 100 |
| Deoxycholate fraction | 4,000                      | 1.0   | 100 |
| Deoxycholate fraction | 2,000                      | 1.0   | 100 |
| Deoxycholate fraction | 1,000                      | 1.0   | 100 |

TABLE III

| Requirements for the purified enzyme for poly(ADP-ribose) synthetase |
|---------------------------------------------------------------------|
| Conditions | Enzyme activity (mg/ml) |
|----------------|------------------------|
| Complete | 0.03 | 1.0 |
| DNA       | 0.02 | 0.0 |
| RNA       | 0.02 | 0.0 |
| GS        | 0.02 | 0.0 |
| HEPES    | 0.02 | 0.0 |
| EDTA      | 0.02 | 0.0 |
| Deoxycholate fraction | 0.02 | 0.0 |
| Boiled enzyme in place of active enzyme | 0.0 |

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Fig. 3A. Effects of DNA and histone on poly(ADP-ribose) synthetase. The reaction was carried out under the standard conditions except for the absence (---) or presence of histone: ---, 20 µg/ml; --, 50 µg/ml; —, 100 µg/ml. A, Lineareaver-Diagram of the poly(ADP-ribose) synthetase reaction. The experiments were carried out in the absence (a) and presence (b) of histone (10 µg/ml) as described under "Materials" except that the concentration of NAD was varied as specified and the incubation time of 1 min was employed for the reaction.

Fig. 3B. Absorption spectrum of poly(ADP-ribose) synthetase. A sample solution (1.0 µg/ml) in 50 mM Tris-Cl (pH 8.0) containing 10% glycerol and 0.15 M 2-mercaptoethanol was used for the determination. The absorbance was measured in the CD spectrum of poly(ADP-ribose) synthetase at the wavelength range from 250 to 400 nm. The measurement of CD spectrum was performed with an enzyme solution (0.25 µg/ml) in 1 cm light path quartz cuvet at 25°C. The solvent used was 50 mM Tris-Cl (pH 8.0) containing 10% glycerol and 0.2 M NaCl. Ordinate: A, represents the mean residue ellipticity, taking the mean residue values of 1032.

Fig. 4A. Determination of molecular weight of poly(ADP-ribose) synthetase by sedimentation equilibrium. A, Distance in centimeters from the center of rotation, r = 0. Fringe displacement determined after 24 h at a rotor speed of 10,000 rpm and a temperature of 20°C. B, Sedimentation coefficient, s, of poly(ADP-ribose) synthetase as a function of protein concentration. Values of s were determined at protein concentrations of 0.3, 1.5, 3.0, 5.0 and 6.0 mg/ml. Three runs were performed at 140 or 200°C with a rotor speed of 60,000 rpm. The solvent used was the same as described above.
