O^6-Methylguanine Methyltransferase in Rat Liver†

James F. Hora, Alan Eastman, and Edward Bresnick*

ABSTRACT: The protein which catalyzes the repair of O^6-methylguanine in DNA has been purified 3800-fold from rat liver. This protein acts as a methyltransferase, with the methyl group transferred to a protein-associated cysteine residue. From kinetic and physical studies, we conclude that the methyl group is transferred to the protein responsible for the activity, resulting in inactivation of the enzyme. The enzyme is asymmetric, with a molecular weight of approximately 18 500. Following methylation, there is an apparent aggregation of methylated proteins which is independent of the concentration of NaCl or nonionic detergent. Upon denaturation and analysis by gel electrophoresis, the aggregated methylated protein migrates as a single peak with a molecular weight of 18 900. The activity does not require any cofactors or divalent cations but is inhibited by NaCl. The activity also shows a preference for double-stranded DNA in terms of kinetics and efficiency of repair.

The carcinogenic and mutagenic actions of simple alkylating agents such as *N*-methyl-*N*-nitrosourea (MNU)[1] are believed to be mediated by conversion within the cell to a highly reactive methyl carbonium ion (Lijinsky et al., 1972). This species is capable of reacting with cellular macromolecules, with DNA considered to be the critical target. MNU reacts with DNA to produce a variety of products, with evidence suggesting that methylation at the 6-position of guanine is the critical lesion in mutagenesis and carcinogenesis (Lawley, 1974; Pegg, 1977). Loveless (1969) suggested that the premutagenic nature of O^6-alkylguanine is due to disruption of normal base pairing with cytosine in DNA. It has subsequently been shown that when O^6-methylguanine was present in DNA, bacterial RNA polymerase (Gerchman & Ludlum, 1973) and DNA polymerase I (Abbott & Saffhill, 1979) catalyzed the incorporation of the inappropriate nucleotide into RNA and DNA, respectively.

In *Escherichia coli*, O^6-methylguanine is repaired by an inducible protein, which is part of the adaptive response of this organism to exposure to simple alkylating agents (Schendel & Robins, 1978; Cairns et al., 1981). This protein acts in a novel fashion, with transfer of the methyl group directly to a cysteine residue on the protein with resultant inactivation of the methyltransferase (Olsson & Lindahl, 1980). The *E. coli* methyltransferase has been purified to apparent homogeneity, and its physical properties have been characterized (Demple et al., 1982).

In mouse (Bogden et al., 1981) and rat (Pegg, 1978) liver, a similar activity has been identified. We have previously demonstrated that the repair activity in mouse liver also acts as a methyltransferase, with the formation of *S*-methylcysteine (Bogden et al., 1981). The rat hepatic methyltransferase appears to be inducible in response to a number of challenges, e.g., administration of carbon tetrachloride (Pegg & Perry, 1981) or dimethylnitrosamine (Montesano et al., 1980). However, this may be a general phenomenon related to cellular proliferation because rats which have been partially hepatectomized also exhibit an increase in repair activity (Pegg et al., 1981).

Our laboratory has been interested in inducible systems in carcinogenesis. Important information regarding the mutuality of the methyltransferase and methyl-accepting protein, substrate specificity, and physicochemical characteristics must first be obtained before questions concerning the mechanism of induction of the O^6-methylguanine repair system can be adequately addressed. In this paper, we present a protocol for the partial purification and physical characterization of the rat liver protein which we have called O^6-methylguanine methyltransferase. We also present data on the methylated protein in an effort to further understand this unique DNA repair system.

Experimental Procedures

Materials. The sources for materials were the following: *N*-[^{3}H]methyl-*N*-nitrosourea (1.6 Ci/mmol), New England Nuclear Corp., Boston, MA; calf thymus DNA, Sigma Chemical Co., St. Louis, MO; Cellex-410 cellulose powder and Cellex-P, Bio-Rad Laboratories, Rockville Center, NY; heparin–agarose, Bethesda Research Laboratories, Gaithesburg, MD; Sephadex G-75, Pharmacia, Piscataway, NJ; poly(ethylene glycol), M_r 6000 (PEG-6000), J.T. Baker Chemical Co., Phillipsburg, NJ. O^6-Methylguanine was a generous gift from Dr. David Ludlum of Albany Medical College.

Preparation of Substrate. Calf thymus DNA at a concentration of 3.5 mg/mL in 0.2 M Tris-HCl, pH 8.0, was alkylated with *N*-[^{3}H]methyl-*N*-nitrosourea by a modification of the method of Lawley & Shah (1973). To 7 mL of the DNA solution was added 1 mL of 1.0 mCi/mL *N*-[^{3}H]-methyl-*N*-nitrosourea, and the solution was incubated for 1 h at 37 °C. The DNA was precipitated by adding 0.1 volume of 2.5 M sodium acetate, followed by 2 volumes of cold 95% ethanol. After 2 h at −20 °C, the DNA was collected by centrifugation, and the pellet was washed 6 times with 95% ethanol. The residual ethanol was removed by lyophilization, and the pellet was resuspended in 8 mL of TEDG and allowed to stand at 4 °C overnight. The alkylated DNA was aliquoted into 500-μg lots, precipitated, and washed as before. Alkylated

† From the Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405. *Received March 14, 1983.* Supported by Grant CA 23514 from the National Institutes of Health.

* Address correspondence to this author at the Eppley Institute for Research in Cancer and Allied Diseases, Omaha, NE 68105.

[1] Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; PEG-6000, poly(ethylene glycol), M_r 6000; DTT, dithiothreitol; Na_2EDTA, disodium ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; TEDG, 50 mM Tris-HCl, pH 7.8, 1 mM Na_2EDTA, 5 mM DTT, 5% glycerol, and 500 μM PMSF.

0006-2960/83/0422-3759$01.50/0

DNA was stored desiccated as lyophilized pellets at −20 °C.

Assay of Methyltransferase Activity. The standard assay was performed in TEDG containing 10 mM Na_2EDTA at 37 °C for 30 min; each assay contained 50 μg of alkylated DNA (1.2–1.3 pmol of O^6-methylguanine) in a total volume of 1 mL. The reactions were stopped by the addition of 40 μL of ice-cold 70% perchloric acid and allowed to stand on ice for 30 min. Pellets were collected by centrifugation in a Beckman microfuge at full speed for 5 min. The supernatants were carefully removed, 300 μL of 0.1 N HCl was added to the pellets, and hydrolysis was performed at 70 °C for 30 min (Lawley & Warren, 1975). The hydrolysates were placed on ice and recentrifuged, and the supernatants were removed. Following neutralization of the hydrolysates, O^6-methylguanine standard was added, and reverse-phase high-performance liquid chromatography was performed for each assay on a Waters C_{18} μBondapak column (0.39 × 20 cm) eluted isocratically as previously described (Bogden et al., 1981).

One unit of methyltransferase activity is defined as the amount that removes 1 pmol of O^6-methylguanine from the alkylated DNA. Protein concentrations were determined by the modification of the Lowry method described by Bensadoun & Weinstein (1976) with bovine serum albumin as the standard.

Purification of O^6-Methylguanine Methyltransferase. All operations were performed at 4 °C. Male Sprague-Dawley rats (125–150 g) were fasted for 16 h prior to sacrifice. The animals were sacrificed by cervical dislocation, and the livers were perfused in situ with 10 mL of ice-cold TEDG buffer. The livers were homogenized in 2 volumes of TEDG and centrifuged for 20 min at 10000*g*. The supernatants were removed and saved. An additional 2 volumes of buffer was added to the pellets. The pellets were homogenized with a Brinkman polytron at setting 6 for two bursts of 15 s. This mixture was added back to the supernatant to give fraction I and centrifuged for 2 h at 150000*g*. The supernatants from this centrifugation were collected (fraction II), made 1.7 M with respect to NaCl, and extracted for 2 h. Solid PEG-6000 was added to the latter to a final concentration of 10% (w/v). After 1 h, the slurry was centrifuged at 25000*g* for 30 min, and the supernatant was collected and dialyzed overnight against 60 volumes of TEDG containing 50 mM NaCl.

Phosphocellulose Chromatography. Cellex-P was prepared according to the manufacturer's specification and equilibrated with TEDG containing 50 mM NaCl. The dialyzed PEG-6000 fraction was centrifuged at 25000*g* for 30 min to remove any insoluble material which formed during dialysis. The resultant supernatant was applied to a Cellex-P column (25 × 2.5 cm) at a flow of 80 mL/h. The Cellex-P column was washed with TEDG containing 50 mM NaCl until the absorbance at 280 nm (A_{280}) was below 0.1. Elution of the methyltransferase was performed at 40 mL/h with TEDG containing 150 mM NaCl. This material was designated fraction III.

DNA–Cellulose Chromatography. Fraction III was diluted with 2 volumes of TEDG buffer and applied to a DNA–cellulose column (12 × 1.6 cm) prepared by the method of Alberts & Herrick (1971) and that had been preequilibrated with TEDG containing 50 mM NaCl. The DNA–cellulose was washed with the latter buffer until the A_{280} was zero, and the methyltransferase activity was eluted with a linear NaCl gradient from 50 to 500 mM NaCl over a total volume of 100 mL at a flow rate of 12 mL/h. Active fractions were pooled and dialyzed against 50 volumes of TEDG containing 50 mM NaCl (fraction IV).

Table I: Purification of O^6-Methylguanine Methyltransferase

no.	fraction	total protein (mg)	sp act. (units/mg)	purification (fold)	yield (%)
I	rat liver homogenate	5450	0.022		100
II	150000*g* supernatant	2180	0.045	2	72
III	phosphocellulose	72	0.93	42	49
IV	DNA-cellulose	2.60	17.54	797	33
V	heparin-agarose	0.90	28.98	1317	21
VI	Sephadex G-75	0.07	82.80	3763	4

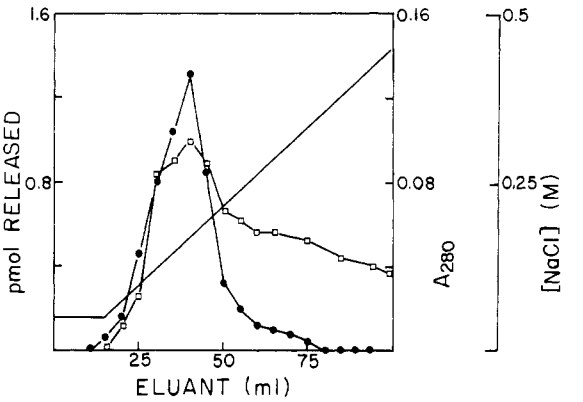

FIGURE 1: DNA–cellulose chromatography. The experimental details are given under Experimental Procedures. The picomoles of methyl groups transferred to protein are given on the left ordinate (●) as a function of milliliters of eluant. The concentration of NaCl (M) is presented on the right ordinate (—); the A_{280} of the eluant is also given on the right ordinate (□). These data are representative of a large number of individual trials.

Heparin–Agarose Chromatography. Fraction IV was applied at a flow rate of 12 mL/h to a heparin–agarose column (6 × 1.6 cm) preequilibrated with TEDG containing 50 mM NaCl. The heparin–agarose was washed until the A_{280} was zero, and the activity was eluted with a 50–500 mM NaCl linear gradient over a total volume of 100 mL. Active fractions were pooled (fraction V).f

Gel Filtration. Fraction V was adjusted to 500 mM NaCl by using solid NaCl, and gel filtration was performed on a Sephadex G-75 column (75 × 2.5 cm). The column was preequilibrated with TEDG containing 500 mM NaCl and calibrated with standards of known Stokes radius; elution was at 10 mL/h. The partition coefficients, K_{av} for each standard and the transferase, were determined as described by Laurent & Killander (1964). The Stokes radius of the transferase was determined from a plot of Stokes radius of standards vs. K_{av}. The active fractions were pooled and desalted by dialysis.

Glycerol Gradients. The sedimentation coefficient of the methyltransferase was determined by using 5–20% (v/v) glycerol gradients of 4.5 mL volume. Centrifugation was performed at 4 °C in a Beckman SW 50.1 rotor for 32 h at 45 000 rpm. 1 milligram each of cytochrome *c*, horse heart myoglobin, chymotrypsinogen A, and ovalbumin was run separately in parallel gradients. Fractions were collected by piercing the bottom of the tubes.

Results

Purification. The protein which catalyzes the transfer of methyl groups from O^6-methylguanine has been purified approximately 3800-fold relative to the initial homogenate (Table I). All of the methyltransferase activity was bound to phosphocellulose at 50 mM NaCl and was completely eluted

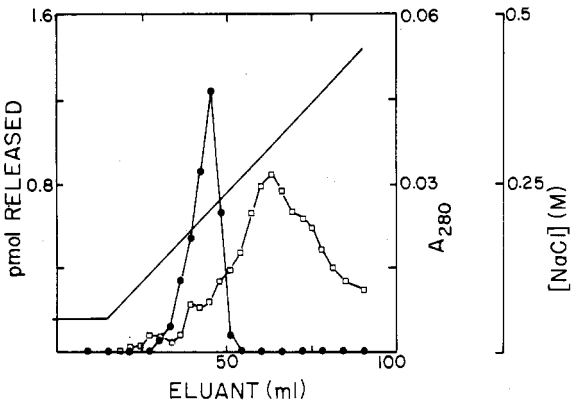

FIGURE 2: Heparin–agarose chromatography. The experimental details are presented under Experimental Procedures. On the left ordinate (●) are given the picomoles of transferred methyl groups while on the right ordinate both the NaCl concentration (M) (—) and the A_{280} (□) are presented.

Table II: Physical Properties of the Methyltransferase

molecular weight (gel filtration)	28 500 ± 1000
molecular weight (glycerol gradient)	18 500 ± 500[a]
molecular weight ($NaDodSO_4$-PAGE of methylated protein)[b]	18 900 ± 500
Stokes radius (Å)	23.8
$s_{20,w}$ ($\times 10^{13}$) (S)	1.9
frictional coefficient, f/f_0	1.4[a]

[a] Calculated from Stokes radius and sedimentation coefficient as described by Siegel & Monty (1966). [b] $NaDodSO_4$-PAGE, $NaDodSO_4$-polyacrylamide gel electrophoresis.

with 150 mM NaCl. Figures 1 and 2 show the elution profiles of the methyltransferase from DNA–cellulose and heparin–agarose. The purification is summarized in Table I. A major loss in activity occurred during Sephadex G-75 chromatography, and further attempts at purification were unsuccessful.

Physical Properties. The molecular weight of the methyltransferase was determined by gel filtration to be 28 500 ± 1000. From these data, the Stokes radius of the methyltransferase was determined to be approximately 24 Å. In our calculations, we assumed the partial specific volume of the protein to be 0.725. The activity sedimented in 5–20% of glycerol gradients with a sedimentation coefficient of 1.9 S. From the Stokes radius and sedimentation coefficient, a molecular weight of 18 500 and a frictional coefficient, f/f_0, of 1.4 were calculated (Siegel & Monty, 1966).

The question of mutuality of methyltransferase and acceptor protein was examined by gel filtration of the methylated protein. A reaction mixture was scaled up, and the methylated protein was purified from the DNA by centrifugation through CsCl as described by Mehta et al. (1981). The methylated protein was chromatographed by gel filtration by using the same Sephadex G-75 as employed above. The methylated protein appeared in the void volume of the column, which was characteristic of proteins with a Stokes radius in excess of 32 Å.

The void volume which contained the methylated protein was collected and analyzed under denaturing conditions in 10% polyacrylamide gels by using the buffer system of Laemmli (1970). The methylated protein migrated as a single peak with molecular weight of 18 900 ± 500. A comparison of the physical parameters for both the activity and the methylated protein is shown in Table II.

Substrate Specificity of Transferase. The transfer of methyl group as a function of time and substrate is shown in Figure 3. The methyltransferase reaction is rapid with the double-stranded DNA as substrate, reaching 90% of maximum by 2 min. With the single-stranded DNA, maximum release of methyl groups was not observed until 20 min and was much less efficient than with the double-stranded DNA, reaching only 26% of the release observed with double-stranded DNA.

Effects of NaCl. The methyltransferase does not require any divalent metal ion for activity so high (10 mM) levels of Na_2EDTA were used in all reactions to keep nonspecific nuclease activity at a minimum. The activity, however, is affected by NaCl. As the NaCl concentration is increased, the removal of methyl groups decreases with a 50% decrease in activity seen at 200 mM NaCl. This is probably due to the inability of the methyltransferase to bind to DNA since the decrease in activity parallels the elution of the methyltransferase from DNA–cellulose.

Discussion

In our studies, we have found that the methyltransferase activity is very labile, and accordingly, the protocol presented in this paper was designed to minimize the time required to obtain partially purified protein. A high concentration of dithiothreitol aids in the stabilization during the purification, although it does not prevent the gradual loss of activity. Nonionic detergents such as Lubrol PX or Emulgen 911 at a concentration of 0.1% were able to stabilize the activity to some degree, with 30% of the fraction VI activity remaining 1 month after storage at −70 °C as compared to total loss of activity in the absence of detergent.

Renard & Verly (1980) were able to demonstrate an activity in chromatin which repaired O^6-methylguanine lesions. This activity also acted as a methyltransferase, with the ethyl group transferred to a cysteine residue (Renard & Verly, 1980). We have not been able to obtain significant methyltransferase activity from purified nuclei (data not shown), and therefore we have utilized a total rat liver homogenate as our initial fraction.

The molecular weight of the activity calculated from gel filtration and sedimentation data is approximately 18 500. This is comparable to the molecular weight determined for the *E. coli* transferase as reported by Demple et al. (1982). However, in contrast to the *E. coli* protein, the rat liver enyzme is asymmetric with a frictional coefficient of 1.4.

In addressing the question of mutuality of methyltransferase and methyl-accepting activities, we performed a gel filtration experiment with the methylated protein under the same conditions used to analyze the activity. We found that the methylated protein was contained entirely in the void volume of the Sephadex G-75 column. This result proved independent of NaCl or nonionic detergent concentration. When the methylated protein fraction from this experiment was denatured and analyzed by $NaDodSO_4$ gel electrophoresis, it was found to have a molecular weight of 18 900 ± 500. This value is in good agreement with the molecular weight of 18 500 calculated for the activity and is consistent with a mechanism in which the methyltransferase inactivates itself during the course of methylation of a cysteine residue. Demple et al. (1982) concluded that the *E. coli* transferase and methyl acceptor were the same protein by gel filtration of the methylated protein in 6 M guanidine hydrochloride. Under these denaturing conditions, they would not have observed any aggregation of the methylated proteins.

Methylation of the methyltransferase apparently induces some structural changes within the protein which result in the facile aggregation with other methylated proteins. It is not clear from our studies if this aggregation is only a test-tube phenomenon or does take place in situ. Demonstration of this

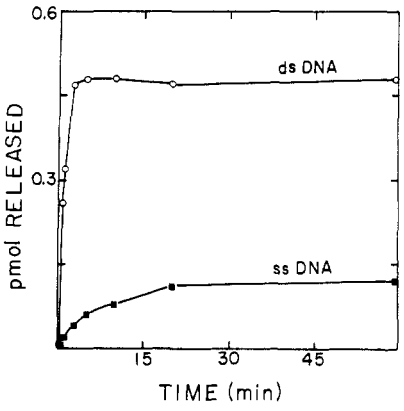

FIGURE 3: Kinetics of methyltransferase with single-stranded (ss) and double-stranded (ds) DNA. Single-stranded calf thymus DNA was produced by heating a solution at 100 °C for 5 min and then plunging the tube in an ice bath. Alkylation of this single-stranded DNA was performed as described under Experimental Procedures. Six micrograms of fraction V was preincubated for 1 min at 37 °C in the standard reaction buffer. Reactions were initiated by the addition of 50 μg of the appropriate substrate and were run for the times indicated, followed by precipitation and analysis as described under Experimental Procedures.

aggregation in vivo could have important physiological significance for the regulation of this repair system. In our studies (unpublished), we have been unable to reverse the methylation process by the addition of SH-containing materials, e.g., glutathione. The methyltransferase activity appears irreversibly inactivated during the methyl transfer. Hence, new methyltransferase synthesis would be required during times of high levels of alkylation in order to protect the cell from mutation or transformation. The aggregated methylated protein may be a signal which initiates the synthesis of new methyltransferase molecules. Alternatively, a breakdown product of the methylated protein may provide such a signal. In this case, the aggregated form of the protein would serve as a preferred substrate for proteolytic enzymes.

Under the assumption that the rat liver transferase has a molecular weight of 18 500 and that one methyl group is transferred per methyltransferase protein molecule, a theoretical specific activity of 54 000 units/mg would be necessary in order to obtain a homogeneous preparation. This would require a purification of 2.4×10^6-fold relative to the initial homogenate. This indicates that there are very low levels of the transferase in the normal rat liver. This is not surprising since at any given time, the normal liver would not be exposed to high levels of DNA alkylation. However, the lability of the protein, particularly during the latter phases of purification, leads us to believe that the specific activity of our final preparation is really understated. Pegg et al. (1983) have recently published a partial purification of the O^6-methylguanine methyltransferase from the livers of partially hepatectomized rats. They have determined the molecular weight to be over 20 000 by gel filtration but did not consider its sedimentation coefficient in their calculations.

From the observations that nonmethylating hepatotoxins can lead to aberrant methylation of DNA (Barrows & Shank, 1980, 1981) and that increases in cellular proliferation result in higher levels of methyltransferase activity (Pegg & Perry, 1981; Pegg et al., 1981), it appears that the methyltransferase performs a general protective function. The enzyme is required for recognition and repair of O^6-methylguanosine lesions which may result during abnormal nuclear metabolism.

The methyltransferase has a requirement for double-stranded DNA for efficient repair of O^6-methylguanine lesions although single-stranded DNA is more susceptible to alkylation by MNU [for review, Singer (1975)]. Cordeiro-Stone et al. (1982) have shown a preferential alkylation by MNU of DNA near the sites of replication during the S phase of synchronized C3H/10T1/2 fibroblasts. These results indicate that the time during the cell cycle when there is aberrant methylation of DNA may be important in determining whether or not the O^6-methylguanine lesions will be repaired.

Despite differences in constitutive levels of the methyltransferase between *E. coli* and rat liver relative to total cell protein and differences in molecular shape, there appears to be conservation of the repair mechanism between prokaryotic and eukaryotic organisms. Questions regarding the inducibility of the transferase and its controls are important in understanding the protective function of this DNA repair system. The data presented in this paper should allow for further inquiries into these mechanisms.

Registry No. DNA O^6-methylguanine methyltransferase, 77271-19-3; O^6-methylguanine, 20535-83-5.

References

Abbott, P. J., & Saffhill, R. (1979) *Biochim. Biophys. Acta 562*, 51–56.

Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.

Barrows, L. R., & Shank, R. C. (1980) *Proc. Am. Assoc. Cancer Res. 21*, 110.

Barrows, L. R., & Shank, R. C. (1981) *Toxicol. Appl. Pharmacol. 60*, 334–345.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–256.

Bogden, J. M., Eastman, A., & Bresnick, E. (1981) *Nucleic Acids Res. 9*, 3089–3103.

Cairns, J., Robins, P., Sedgwick, B., & Talmud, P. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 26*, 237–244.

Cordeiro-Stone, M., Topal, M. D., & Kaufman, D. G. (1982) *Carcinogenesis (N.Y.) 3*, 1119–1127.

Demple, B., Jacobsson, A., Olsson, W., Robins, P., & Lindahl, T. (1982) *J. Biol. Chem. 257*, 13776–13780.

Gerchman, L. L., & Ludlum, D. B. (1973) *Biochim. Biophys. Acta 308*, 310–316.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Laurent, T. C., & Killander, J. (1964) *J. Chromatogr. 14*, 317–330.

Lawley, P. D. (1974) *Mutat. Res. 23*, 283–295.

Lawley, P. D., & Shah, S. A. (1973) *Chem.-Biol. Interact. 7*, 115–120.

Lawley, P. D., & Warren, E. (1975) *Chem.-Biol. Interact. 11*, 55–57.

Lijinsky, W., Garcia, H., Keefer, L., Loo, J., & Ross, A. E. (1972) *Cancer Res. 32*, 893–897.

Loveless, A. (1969) *Nature (London) 223*, 206–207.

Mehta, J. R., Ludlum, D. B., Renard, A., & Verly, W. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6766–6770.

Montesano, R., Bresil, J., Planche-Martel, G., Margison, G. P., & Pegg, A. E. (1980) *Cancer Res. 40*, 452–458.

Olsson, M., & Lindahl, T. (1980) *J. Biol. Chem. 255*, 10569–10571.

Pegg, A. E. (1977) *Adv. Cancer Res. 25*, 195–269.

Pegg, A. E. (1978) *Biochem. Biophys. Res. Commun. 84*, 166–173.

Pegg, A. E., & Perry, W. (1981) *Carcinogenesis (N.Y.) 2*, 1195–1200.

Pegg, A. E., Perry, W., & Bennett, R. A. (1981) *Biochem. J. 197*, 305–310.

Pegg, A. E., Wiest, L., Foote, R. S., Mitra, S., & Perry, W.

(1983) J. Biol. Chem. 258, 2327–2333.
Renard, A., & Verly, W. G. (1980) FEBS Lett. 114, 98–102.
Schendel, P. F., & Robins, P. E. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 6017–6020.
Siegel, L. M., & Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362.
Singer, B. (1975) Prog. Nucleic Acid Res. Mol. Biol. 15, 219–284.

Isolation and Characterization of Rat Pancreatic Elastase†

Corey Largman

ABSTRACT: Proelastase has been purified to homogeneity from rat pancreatic tissue by a combination of CM-Sephadex and immobilized protease inhibitor affinity resins. Trypsin activation yields an elastolytic enzyme that possesses a specificity toward small hydrophobic residues in synthetic amide substrates, similar to those of porcine elastase 1 and canine elastase. However, the rat enzyme also rapidly hydrolyzes a substrate containing tyrosine in the P_1 position. N-Terminal sequence analysis reveals that rat proelastase has an identical activation peptide with that of porcine proelastase 1 and has two conservative amino acid sequence differences from the activation peptide of canine proelastase. The sequence data established that rat proelastase corresponds to the elastase 1 mRNA clone isolated by MacDonald et al. [MacDonald, R. J., Swift, G. H., Quinto, C., Swain, W., Pictet, R. L., Nikovits, W., & Rutter, W. J. (1982) *Biochemistry 21*, 1453]. The sequence and substrate data obtained for rat and canine elastases suggest that there is a family of pancreatic elastases with properties similar to those of the classically described porcine elastase 1.

The pancreatic serine proteases are related by extensive sequence homology, maintenance of crucial active site catalytic residues, and highly conserved three-dimensional structure. X-ray crystal structure studies have provided rational explanations for the substrate specificities of trypsin (Stroud et al., 1971) and chymotrypsin (Mathews et al., 1967). In contrast, there is no general agreement concerning the peptide-bond specificity of pancreatic elastases. Much of this ambiguity results from the fact that elastases are operationally defined on the basis of their capacity to hydrolyze elastin (Shotten, 1970). In recent years, a number of pancreatic and nonpancreatic serine proteases with elastolytic activity have been reported that preferentially hydrolyze low molecular weight substrates containing different P_1[1] residues (Shotton, 1970; Gertler et al., 1977; Largman et al., 1976; Zimmerman & Ashe, 1977).

Essentially all of the early studies on elastases were focused on porcine pancreatic elastase 1 (Shotton, 1970). The few studies on the frequency of peptide bonds cleaved by porcine elastase 1 indicate a broad specificity for isoleucine, valine, and alanine (Naughton & Sanger, 1961, Powers et al., 1977). Nevertheless, the desire for a rapid and simple chemical assay based on synthetic substrates (Gertler & Hofman, 1969; Atlas & Berger, 1972; Bieth & Wermuth, 1973; Kasafirek et al., 1976) has resulted in the development of a literature that has emphasized the specificity of porcine pancreatic elastase 1 for hydrolysis of substrates with alanine in the P_1 position. The X-ray crystal structure of porcine elastase 1 has been interpreted in support of this specificity by suggesting that a partially obstructed binding pocket exists in this enzyme compared to chymotrypsin (Shotton & Watson, 1970). However, Mallory & Travis (1975) isolated an alanine-specific protease from human pancreas tissue that lacks elastolytic activity. Furthermore, a second elastase isolated from porcine pancreatic tissue and a similar enzyme isolated from human pancreas (Gertler et al., 1977; Largman et al., 1976) degrade elastin but possess specificities for more bulky amino acids in the P_1 position of synthetic substrates (Gertler et al., 1977; Del Mar et al., 1980). These elastases have chymotrypsin-like activation peptides (Lamy et al., 1977; Largman et al., 1980a) but, unlike chymotrypsin, are capable of rapid elastolysis. Finally, the finding that a serine protease from leukocytes rapidly hydrolyzes elastin but possesses a specificity for valine in the P_1 position (Zimmerman & Ashe, 1977) further obscures the relationship between the apparent configuration of the substrate binding site of an enzyme and its capacity to effect elastolysis.

MacDonald et al. (1982) have recently reported the nucleotide sequences of two messenger RNAs from rat pancreas, which the authors suggest correspond to the two types of pancreatic elastase described in other species. These authors have relied on comparisons of the inferred amino acid sequences with those of known pancreatic proteases and the existing substrate and X-ray structure data to make assignments for the proteins corresponding to these two messenger RNAs as elastolytic enzymes. At present, however, neither protein has been expressed from the cloned messenger RNA or compared with proteins isolated from rat pancreas tissue. The authors note that the isolation of the cDNA clones corresponding to the nucleotide sequence for pancreatic elastase(s) presents opportunities for the engineering and production of altered enzymes by specific mutagenesis, which would facilitate further understanding of the catalytic mechanism and determinants required for serine protease activity. It appears that such experiments would be especially useful in gaining insight

† From the Biochemical Research Laboratory, Martinez Veterans Administration Medical Center, Martinez, California 94553, and the Departments of Internal Medicine and Biological Chemistry, University of California, Davis, California 95616. *Received March 14, 1983.* This work was supported by the Research Service of the Veterans Administration and a grant from the National Institutes of Health (HL25408).

[1] The nomenclature introduced by Schechter & Berger (1967) is used to describe the positions of amino acids in a substrate. Amino acid residues and terminal acyl substituents are numbered P_1, P_2, P_3, etc. in the N-terminal direction from the scissle bond. The corresponding subsites of the enzyme's active site are numbered S_1, S_2, S_3, etc. in an analogous fashion.