Janick, P. A., Rueger, D. C., Krueger, R. J., Barber, M. J., & Siegel, L. M. (1983b) *Biochemistry 22*, 396–408.
Lee, W. A., Calderwood, T. S., & Bruice, T. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4301–4305.
McRee, D. E., Richardson, D. C., Richardson, J. S., & Siegel, L. M. (1986) *J. Biol. Chem. 261*, 10277–10281.
Meckstroth, M. L., Norris, B. J., & Heineman, W. R. (1981) *Bioelectrochem. Bioenerg. 8*, 63–70.
Michaelis, L., & Smythe, C. V. (1931) *J. Biol. Chem. 44*, 329–341.
Morishima, I., Shiro, Y., & Nakajima, K. (1986) *Biochemistry 25*, 3576–3584.
Phillippi, M. A., & Goff, H. M. (1982) *J. Am. Chem. Soc. 104*, 6026–6034.
Richardson, P. F., Chang, C. K., Spaulding, L. D., & Fajer, J. (1979) *J. Am. Chem. Soc. 101*, 7736–7738.
Rutter, R., Hager, L. P., Dhonau, H., Hendrich, M., Valentine, M., & Debrunner, P. G. (1984) *Biochemistry 23*, 6809–6816.
Salerno, J. C., Yoshida, S., & King, T. E. (1986) *J. Biol. Chem. 261*, 5480–5486.
Scheidt, W. R., Geiger, D. K., Lee, Y. J., Reed, C. R., & Lang, G. (1987) *Inorg. Chem. 26*, 1039–1045.
Scholz, W. F., Reed, C. A., Lee, Y. J., & Scheidt, W. R. (1982) *J. Am. Chem. Soc. 104*, 6791–6793.
Schulz, C. E., Rutter, R., Sage, J. T., Debrunner, P. G., & Hager, L. P. (1984) *Biochemistry 23*, 4743–4754.
Siegel, L. M., & Davis, P. S. (1974) *J. Biol. Chem. 249*, 1587–1598.
Siegel, L. M., Murphy, M. J., & Kamin, H. (1973) *J. Biol. Chem. 248*, 251–264.
Siegel, L. M., Rueger, D. C., Barber, M. J., Krueger, R. J., Orme-Johnson, N. R., & Orme-Johnson, W. H. (1982) *J. Biol. Chem. 257*, 6343–6350.
Siegel, L. M., Wilkerson, J. O., & Janick, P. J. (1986) in *Inorganic Nitrogen Metabolism* (Ullrich, W. R., Aparicio, P. J., Syrett, P. J., & Castillo, F., Eds.) pp 118–122, Springer-Verlag, New York.
Stolzenberg, A. M., Spreer, L. O., & Holm, R. J. (1979) *J. Chem. Soc., Chem. Commun.*, 1077–1078.
Stolzenberg, A. M., Spreer, L. O., & Holm, R. J. (1980) *J. Am. Chem. Soc. 102*, 364–370.
Suh, M. P., Sweptson, P. N., & Ibers, J. A. (1984) *J. Am. Chem. Soc. 106*, 5164–5171.
Young, L. J., & Siegel, L. M. (1988) *Biochemistry 27*, 2790–2800.

Polypeptide Domains of ADP-Ribosyltransferase Obtained by Digestion with Plasmin†

Kalman G. Buki‡ and Ernest Kun*,§
Department of Pharmacology and the Cardiovascular Research Institute, School of Medicine, University of California, San Francisco, San Francisco, California 94143-0130

Received September 9, 1987; Revised Manuscript Received January 7, 1988

ABSTRACT: Proteolysis by plasmin inactivates bovine ADP-ribosyltransferase; therefore, enzymatic activity depends exclusively on the intact enzyme molecule. The transferase was hydrolyzed by plasmin to four major polypeptides, which were characterized by affinity chromatography and N-terminal sequencing. Based on the cDNA sequence for human ADP-ribosyltransferase enzyme [Uchida, K., Morita, T., Sato, T., Ogura, T., Yamashita, R., Noguchi, S., Suzuki, H., Nyunoya, H., Miwa, M., & Sugimura, T. (1987) *Biochem. Biophys. Res. Commun. 148*, 617–622], a polypeptide map of the bovine enzyme was constructed by superposing the experimentally determined N-terminal sequences of the isolated polypeptides on the human sequence deduced from its cDNA. Two polypeptides, the N-terminal peptide (M_r 29 000) and the polypeptide adjacent to it (M_r 36 000), exhibited binding affinities toward DNA, whereas the C-terminal peptide (M_r 56 000), which accounts for the rest of the transferase protein, bound to the benzamide–Sepharose affinity matrix, indicating that it contains the NAD^+-binding site. The fourth polypeptide (M_r 42 000) represents the C-terminal end of the larger C-terminal fragment (M_r 56 000) and was formed by a single enzymatic cut by plasmin of the polypeptide of M_r 56 000. The polypeptide of M_r 42 000 still retained the NAD^+-binding site. The plasmin-catalyzed cleavage of the polypeptide of M_r 56 000–42 000 was greatly accelerated by the specific ligand NAD^+. Out of a total of 96 amino acid residues sequenced here, there were only 6 conservative replacements between human and bovine ADP-ribosyltransferase.

ADP-ribosyltransferase [ADPRT,[1] poly(ADP-ribose) polymerase, EC 2.4.2.30] is a DNA-associated nuclear enzyme that synthesizes protein-bound homopolymers of ADP-ribose exhibiting helical conformation (Minaga & Kun 1983a,b) utilizing NAD^+ as a substrate. The enzyme itself is the primary acceptor protein for ADP-ribose (Bauer et al., 1986). The purified enzyme requires double-stranded DNA for activity and consists of a single large polypeptide chain of M_r

† This research was supported by grants from the Air Force Office of Scientific Research (AFO-SR-85-0377 and AFO-SR-86-0064) and the National Institutes of Health (HL-27317).
* Correspondence should be addressed to this author.
‡ Visiting Scientist from the Semmelweis University School of Medicine, Budapest, Hungary.
§ Recipient of the Research Career Award of the U.S. Public Health Service.

[1] Abbreviations: ADPRT, adenosine diphosphoribosyltransferase, poly(ADP-ribose) polymerase (EC 2.4.2.30); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PTH-, phenylthiohydantoinyl-; BSA, bovine serum albumin; bp, base pairs, HEPES, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-5990$01.50/0

116 000–120 000 (cf. reviews: Ueda & Hayaishi, 1985; Gaal & Pearson, 1985; Althaus & Richter, 1987). The enzyme is present in eukaryotes above the genus *Saccharomyces* (Scovassi et al., 1986). The polypeptide structure of this enzyme protein had been first studied by Holtlund et al. (1983) and by Kameshita et al. (1984), and the latter group reported that limited chymotryptic digestion cleaves ADPRT into two polypeptides of M_r 66 000 and 54 000, respectively, and papain at 0 °C generates another pair of fragments (M_r 74 000 and 46 000). Distinct NAD^+- and DNA-binding domains of ADPRT were proposed, and a peptide of M_r 22 000 was suggested as the ADP-ribose acceptor domain (Kameshita et al., 1984). Combination of the polypeptides of M_r 74 000 and 46 000 in the presence of coenzymic DNA recovered about 20% of the original enzymatic activity (Kameshita et al., 1986), suggesting that interaction of both polypeptides is needed for catalysis. The amino terminus of ADPRT is known to be blocked, but the nature of the blocking group has not been identified (Holtlund et al., 1981).

Limited proteolysis by suitable proteases would be expected to yield polypeptides that may provide further information regarding the domain structure of ADPRT. Kameshita et al. (1984) were able to present a gross picture of the primary enzyme structure by splitting the enzyme into two halves either with papain or with α-chymotrypsin. In our experience, time-dependent proteolysis of ADPRT by α-chymotrypsin and papain yielded a progressive breakdown of polypeptides (not shown). In contrast to these, plasmin, a trypsin-like serine protease, produced four relatively stable polypeptides after a digestion for 30 min. Three of these polypeptides accounted for the whole enzyme molecule. We exploit plasmin's fastidiousness (Robbins & Summaria, 1970) here to obtain a more refined picture of the domain structure of the ADPRT enzyme protein.

Sequential assignment of the plasmin-generated polypeptides was based on a recent publication of the cDNA sequence of human ADPRT (Uchida et al., 1987). Shortly after the appearance of this paper, the human cDNA sequence was also reported by Kurosaki et al. (1987). Our matching of sequences is based on the earlier paper (Uchida et al., 1987).

Experimental Procedures

Materials. Reactive Red 120–agarose (R503) and plasmin (P7911) were purchased from Sigma (St. Louis, MO), and 3-methoxybenzamide was purchased from Pierce Chemicals (Rockford, IL). The Spherogel TSK 3000 SW (60-cm-long) molecular size exclusion column was obtained from Beckman-Altex (Berkeley, CA) and Mono-S HR 5/5 cation exchanger from Pharmacia (Piscataway, NJ). SDS–PAGE molecular weight standards were from Bio-Rad (Richmond, CA). DNA–cellulose was prepared according to Alberts and Herrick (1971). Benzamide–Sepharose 4B affinity matrix, bovine ADPRT enzyme protein, and dihydroxy-Reactive Red 120 were prepared as described earlier (Buki et al., 1987). Coenzymic DNA was obtained as a side product from the ADPRT purification (Buki et al., 1987) and was rendered protein-free by phenol extraction. It consisted mainly of double-stranded DNA of 300 bp on average (Yoshihara et al., 1978; Niedergang et al., 1979). Lysine–Sepharose 4B was made according to Chibber et al. (1974), starting with CNBr-activated Sepharose 4B (Pharmacia). All other chemicals were of analytical grade.

Buffers. Buffer I (for digestion with plasmin) contained 50 mM HEPES and 150 mM NaCl, pH 8.0. Buffer II (for affinity chromatographies) contained 50 mM Tris, 200 mM NaCl, 50 mM sodium bisulfite, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 15% ethylene glycol, pH 7.3.

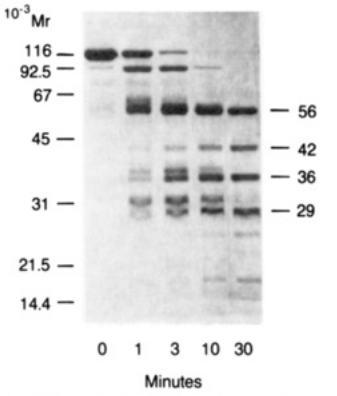

FIGURE 1: SDS–PAGE analysis of digestion products of ADPRT with plasmin. Digestion was carried out as described under Results. At indicated time points, aliquots were withdrawn, mixed with an equal volume of SDS–PAGE sample buffer containing 5 mM PMSF, and heated to 90 °C for 1 min. Each lane represents a total of 4 μg of protein, stained with Coomassie blue. Molecular mass standards (as $M_r \times 10^{-3}$) are shown along the left side of the gel: β-galactosidase, phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. The molecular masses of polypeptides are indicated on the right side. All other figures of gels contain the same notation.

Methods. ADPRT enzyme activity was assayed as described (Buki et al., 1987). Electrophoretic separation of polypeptides was done according to Laemmli (1970) in 0.75-mm-thick slabs of 10% SDS–polyacrylamide gel (SDS–PAGE). High-performance liquid chromatography (HPLC) of polypeptides was carried out with Beckman-Altex 100A pumps, gradient controller 421, and variable wavelength detector. The amino acid sequences of the polypeptides were determined in a Model 470-A gas-phase sequencher with an on-line 120A PTH-analyzer (Applied Biosystems, Foster City, CA) according to Hunkapiller et al. (1983). The polypeptides in SDS–PAGE were stained with Coomassie Brillant Blue R250 (0.1% in 50% aqueous ethanol/10% acetic acid) after brief fixation in 10% trichloroacetic acid. The protein content of electrophoretically separated bands was determined by extraction of the protein-bound Coomassie blue from the gel slices according to Ball (1986).

Results

Digestion of ADPRT with Plasmin. Incubation of ADPRT (2 mg/mL, in buffer I) with 33 μg/mL plasmin at 25 °C either in the absence or presence of coenzymic DNA (Yoshihara et al., 1978; Niedergang et al., 1979) resulted in complete disappearance of ADPRT activity, inactivation following first-order kinetics (not shown). When the digestion of ADPRT by plasmin was arrested at various time intervals with soybean trypsin inhibitor (20 μg/mL of digestion mixture) and the quantity of the remaining unhydrolyzed ADPRT protein determined in SDS–PAGE bands (see Experimental Procedures), enzyme inactivation and the rate of disappearance of the ADPRT molecule (M_r 120 000) ran exactly parallel. It was apparent that the enzymatic activity depended on the intact ADPRT molecule and component peptides had no residual activity.

The progression of the proteolytic degradation of ADPRT by plasmin is illustrated in a typical experiment (Figure 1). The intact ADPRT molecule was hardly visible on the gel after digestion for 10 min. Two groups of polypeptides were formed which could be distinguished by their rates of appearance and decay. There was a transient formation and decay of polypeptides of M_r 90 000 and 65 000 (between 0 and 3 min) and

Table I: Conversion of M_r 56 000 Polypeptide into M_r 42 000 by Plasmin in the Presence of Different Ligands[a]

	protein in polypeptide bands after digestion (μg)	
	M_r 56 000	M_r 42 000
no ligand present	0.8	0.4
0.6 M dihydroxy-Reactive Red 120	0.6	0.4
0.2 mg/mL coenzymic DNA	0.8	0.5
1.7 mM NAD^+	0.0	1.3

[a] Two micrograms of purified polypeptide of M_r 56 000 was digested with 0.2 μg of plasmin for 20 min in the presence or absence of the ligands indicated. At the end of incubation, an equal volume of sample buffer was added and the entire sample was subjected to SDS–PAGE. The quantity of Coomassie-stained peptides was determined according to Ball (1986). Analyses were carried out in triplicates, and they agreed within 10%.

of M_r 31 000 and 39 000 (between 1 and 10 min). The other group consisted of the four more stable polypeptides (M_r 29 000, 36 000, 42 000, and 56 000), which appeared within 1 min after initiation of proteolysis and clearly separated into dominant bands after 30 min. A decay to smaller polypeptides (smaller than M_r 29 000) was also apparent.

The sum of the masses of polypeptides with M_r 29 000, 36 000, and 56 000 accounts for the entire ADPRT molecule, and the progressive relatively slower accumulation of the M_r 42 000 species indicated its generation from polypeptide of M_r 56 000, as demonstrated experimentally. When the isolated and purified polypeptide of M_r 56 000 was incubated with plasmin, polypeptide of M_r 42 000 was produced, which by its migration on SDS–PAGE and binding to benzamide–Sepharose was indistinguishable from the polypeptide of M_r 42 000 isolated from the digest of the intact ADPRT molecule. Concomitant with the conversion of the polypeptide of M_r 56 000 to the M_r 42 000 species, smaller fragments (M_r 15 000 and 16 000) appeared, which can also be seen during the digestion of ADPRT (see Figure 1, 10 and 30 min). Interestingly, the cleavage of the polypeptide of M_r 56 000 was greatly accelerated in the presence of NAD^+ but not by other ligands, such as coenzymic DNA, which does not bind to this polypeptide, or dihydroxy-Reactive Red 120 (Buki et al., 1987), which is a relatively poor NAD^+ analogue. These experiments are shown in Table I.

Binding Properties of Polypeptides to Affinity Columns. The benzamide–Sepharose 4B affinity matrix that recognized NAD^+-binding sites (Burtscher et al., 1986; Buki et al., 1987) strongly bound the polypeptide of M_r 56 000 and only partly bound the polypeptide of M_r 42 000, which also appeared in the flow-through fraction along with the nonadsorbed basic polypeptides (Figure 2). The polypeptides retained on the benzamide affinity column were eluted with 1 mM 3-methoxybenzamide. On the basis of these observations, we conclude that the polypeptide of M_r 56 000 has higher affinity to the benzamide column than the polypeptide of M_r 42 000. On the other hand, the DNA–cellulose column retained the basic polypeptides of M_r 29 000 and 36 000, but not the 56 000 and 42 000 species (Figure 3). Elution of the basic polypeptides from the DNA–cellulose column was carried out with a limited volume (3 mL) of 1 M NaCl, which left traces of the M_r 56 000 and 42 000 polypeptides nonspecifically adsorbed (lane 3, Figure 3). However, more extensive washing with the low-salt buffer prior to the application of 1 M NaCl readily removed these nonspecifically attached polypeptides. Reactive Red 120 is believed to recognize NAD^+- or $NADP^+$-binding sites (Watson et al., 1978). Since we used this dye for purification of ADPRT (Buki et al., 1987), it was of interest to

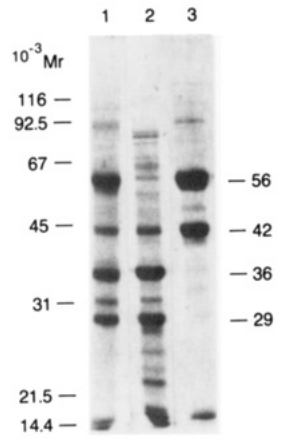

FIGURE 2: SDS–PAGE analysis of polypeptides obtained by benzamide–Sepharose chromatography. After digestion with plasmin as described under Results, 200 μg of ADPRT digest was diluted with an equal volume of buffer II, containing 5 mM PMSF, and the solution was applied to a benzamide–Sepharose column of 0.5 mL volume. The column was washed with 2 mL of buffer II and then eluted with 2 mL of 1 mM 3-methoxybenzamide dissolved in the same buffer. The wash and the eluate were concentrated in Centricon 10 microconcentrators and applied to SDS–PAGE (5 μg of protein per lane). Lane 1, unseparated plasmin digest; lane 2, flow-through fraction; lane 3, polypeptides eluted with 3-methoxybenzamide.

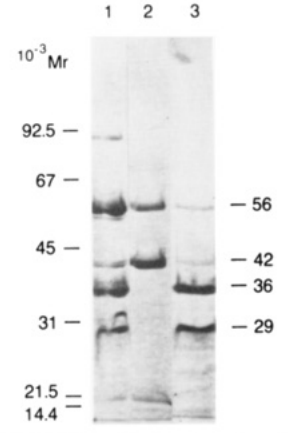

FIGURE 3: SDS–PAGE analysis of polypeptides separated by DNA–cellulose chromatography. As described in the legend to Figure 2, 100 μg of plasmin digest was prepared and applied to a DNA–cellulose column of 1 mL volume. The column was washed with 3 mL of buffer II and then eluted with the same buffer containing 1.0 M NaCl. The wash and the eluate were concentrated in Centricon 10 microconcentrators and applied to SDS–PAGE (3 μg of protein per lane). Lane 1, unseparated plasmin digest; lane 2, flow-through fraction; lane 3, polypeptides eluted with 1.0 M NaCl.

determine which polypeptide moiety of ADPRT would bind to it. The Reactive Red 120–agarose column completely retained from buffer II all the polypeptides except for the one of M_r 42 000, which was bound only partially. The bound polypeptides had differential affinities toward the dye matrix as demonstrated by their stepwise elution with increasing concentrations of salt in the buffer (Figure 4).

Large-Scale Isolation of Polypeptides, Their Amino Acid Analyses, and Partial Sequences. One-half milligram of ADPRT protein was digested with plasmin for 30 min, as described in the legend to Figure 1. The digestion was terminated by filtering the reaction mixture through a 1-mL bed volume of lysine–Sepharose 4B, which specifically binds plasmin (Deutsch & Mertz, 1970), and the resulting polypeptides were applied to a benzamide–Sepharose 4B affinity column (1-mL bed volume). The column was washed with 3 mL of buffer II, the wash containing the nonadsorbed polypeptides (see Figure 2). The bound polypeptides were then

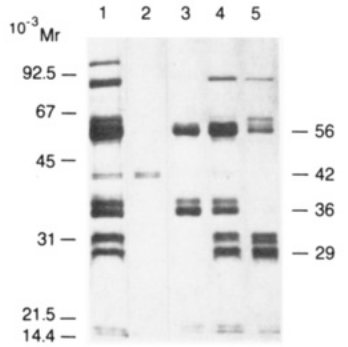

FIGURE 4: SDS–PAGE analysis of polypeptides separated by chromatography on Reactive Red 120–agarose. ADPRT (200 μg) was digested with plasmin, as described under Results, for 10 min. The digestion was stopped as described in the legend to Figure 2. The peptide mixture was applied to a Reactive Red 120–agarose column of 0.5 mL volume and eluted stepwise with 1-mL portions of buffer II containing increasing concentrations of NaCl. Lane 1, unseparated plasmin digest; lane 2, wash fraction with buffer II, lanes 3–5, eluates with 0.4, 0.6, and 0.8 M NaCl in Buffer II, respectively.

eluted with 3 mL of the same buffer containing 1 mM 3-methoxybenzamide. Both the flow-through and eluate fractions were concentrated to ~100 μL in Centricon 10 (Amicon, Danvers, MA) ultrafiltration units. Peptides present in the concentrated flow-through fraction were further separated on a MonoS HR 5/5 cation-exchange column by a salt gradient (legend to Figure 5), and the three major peaks were collected and concentrated. Peaks A and B (in Figure 5) exhibited the same mass (central inset), corresponding to M_r 36 000, but peak C was not homogeneous and had to be repurified. After concentration, this fraction was injected into the same MonoS HR 5/5 cation-exchange column and developed by the same salt gradient but at higher pH (8.1), yielding one main peptide (M_r 29 000) and a second polypeptide (M_r 31 000) amounting to ~10% of the former. The proportion of these two polypeptide bands cannot be visually ascertained on the basis of the inset of Figure 5 because the gel was overloaded. The two polypeptides have the same blocked N-terminus and therefore most probably represent slightly different peptides of the same region, differences being due to an alternate cutting site by plasmin.

Separation of the polypeptides that were eluted with 3-methoxybenzamide from the benzamide–Sepharose 4B column (M_r 42 000 and 56 000) was accomplished on a Spherogel TSK-3000 SW size exclusion column in 0.5 M sodium acetate, pH 5, at a low flow rate of 0.2 mL/min.

N-Terminal Sequences of the Isolated Polypeptides. The polypeptide of M_r 29 000 had a blocked amino terminus; therefore, this polypeptide is the probable N-terminal fragment of ADPRT. The nature of the blocking group is unknown, except its identity with pyroglutamic acid was ruled out by its insensitivity to pyroglutaminase (not shown). The two similar (A and B in Figure 5) polypeptides of M_r 36 000 were sequenced up to 51 cycles, yielding for A the sequence

KSKKEKDKETKLEKALKAQNDLIWNVKDELKKA-
(C)STNDLKE(L)(L)IFNKQEVP

with parentheses indicating uncertainties of amino acids. The single letter codes are used according to IUPAC–IUB (1968). The 36 000 B species proved to be one lysine residue shorter; its sequence started with serine.

The polypeptide of M_r 42 000, though always a doublet on SDS–PAGE (lane 2 in Figure 4), had a uniform amino terminus and was sequenced to 32 cycles, yielding the sequence

(?)LTVNPGTKSKLPKPVQNLIKMIFDVESMK(K)A

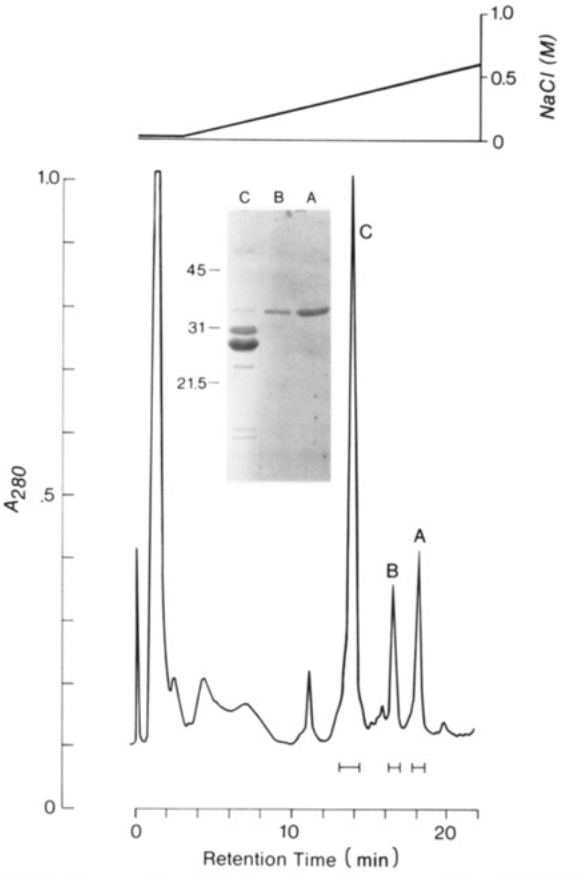

FIGURE 5: Separation of basic polypeptides on a MonoS HR 5/5 cation-exchange column. A MonoS HR 5/5 column was fitted with HPLC components as described under Experimental Procedures. Buffer A was 25 mM HEPES, 50 mM NaCl, and 10 mM 2-mercaptoethanol, pH 7.5; buffer B was the same buffer with 1.0 M NaCl. The mixture of basic peptides (~200 μg) was equilibrated with buffer A in a Centricon 10 microconcentrator and injected into the column. The flow rate was 1 mL/min. After 4 min of isocratic flow with buffer A, a linear gradient from A to B (30 min) was started (indicated at top), and peak fractions were collected by hand (marked as |—|). Aliquots of the collected fractions A–C were analyzed by Coomassie blue stained SDS–PAGE (inset).

Table II: N-Terminal Sequencing Data for the M_r 56 000 Polypeptides[a]

cycle no.	amino acid found	matching sequences in human ADPRT[b]
1	R, M, L	R (522), M (523), L (525)
2	M, K, T	M, K, T
3	K, L	K, L, L
4	L, T, K	L, T, K
5	T, L, G	T, L, G
6	L, K, G	L, K, G (530)
7	K, G, A	K, G, A
8	G, A	G, G (530), A
9	G, A, V	G (530), A, V
10	A, D	A, A, D

[a] Sequencing was performed as described under Experimental Procedures. [b] Uchida et al. (1987).

Even though the polypeptide of M_r 56 000 exhibited a single band on SDS–PAGE, sequencing disclosed that this peptide was a mixture of three nearly identical components. Each sequencing cycle produced a mixture of three or two amino acid signals. The sequencing data along with the corresponding stretches of human ADPRT, which are based on the cDNA sequence of Uchida et al. (1987), are shown in Table II.

DISCUSSION

Although ~50% of the ADPRT protein was completely

Table III: Differences in the Residues of Human and Bovine ADPRT within the Sequence from Positions 223 to 274

human[a]	bovine
aspartic acid (231)	glutamic acid
serine (233)	threonine
isoleucine (248)	valine
valine (255)	alanine
glutamine (271)	glutamic acid

[a] Uchida et al., 1987.

degraded within 30 min, digestion with plasmin revealed the domain structure of the molecule. ADPRT has a high basic amino acid residue content [126 lysine and 34 arginine residues, out of a total of 1014; cf. Uchida et al. (1987)], each basic residue providing a cleavage site to a trypsin-like endopeptidase, e.g. plasmin. However, plasmin apparently cleaves preferentially at only very limited sites, probably at exposed regions between compact structures of ADPRT, and thus large parts of the molecule remain undigested for a long enough time to permit isolation with good yields.

Since the time course of the digestion (Figure 1) shows gradual accumulation of the major, relatively stable polypeptides of M_r 29 000, 36 000, 42 000, and 56 000 from as early as 1 min, plasmin is likely to cut simultaneously at multiple sites, although with different rates; e.g., the formation of M_r 42 000 is the slowest. A distinct protein structure related proteolytic cleavage site was revealed by the NAD^+-promoted hydrolysis of the polypeptide of M_r 56 000–42 000 (Table I). Further analysis of this effect, relevant to both the mechanism of NAD^+ binding and polypeptide structure, is in progress.

From the kinetic behavior of transient polypeptides during digestion and from their tendency to copurify with other polypeptides, we can draw the following conclusion. The polypeptide of M_r 90 000 may represent ADPRT without its N-terminal domain. The M_r 65 000 species, since it has affinity toward DNA, is likely to be composed of the two basic polypeptides (M_r 29 000 and 36 000). The persisting polypeptides of M_r 31 000 and 39 000 (Figure 1; 1–10 min) seem to be larger variants of those of M_r 29 000 and 36 000, respectively, the former eventually being trimmed to the final size. It seems feasible to assume that these alternative cuts might be responsible for the net loss of ADPRT protein.

The amino acid compositions of ADPRTs isolated from different mammals are almost identical (Althaus & Richter, 1987), and the ADPRTs obtained from a wider variety of species are still fairly uniform in mass and in immunoreactivity; therefore, this protein seems to be conserved during evolution (Scovassi et al., 1986). A complete nucleotide sequence of cDNA isolated from the library of a SV 40 transformed human fibroblast was published recently (Uchida et al., 1987). Combining this information with our N-terminal sequencing data, we positioned each of the peptides generated from bovine ADPRT onto the published human sequence as follows.

Since the polypeptide of M_r 29 000 has a blocked amino end, it must be the N-terminal part of the enzyme; therefore, the bovine ADPRT seems to be blocked just like the human enzyme (Suzuki et al., 1987).

The polypeptide of M_r 36 000 starts at position 233 (36 000A) and at 234 (36 000B) of the human sequence. A remarkable homology exists within the analyzed stretch of 51 residues, where only 5 residues are found to be different, and even these substitutions are conservative (see Table III).

It seems appropriate to point out that the cDNA-deduced sequence of Kurosaki et al. (1987) and that of Uchida et al. (1987) differ at six positions and also the former sequence is one residue shorter. Since one of the differing amino acids

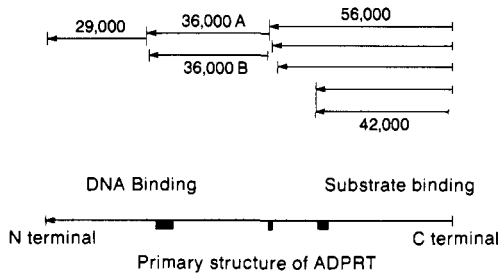

FIGURE 6: Proposed arrangement of isolated polypeptides in the primary structure of ADPRT. The solid boxes refer to the sequenced regions.

(position 237) lies within our directly sequenced stretch of the bovine enzyme, and this residue was alanine, just as found by Uchida et al. (1987), we compared our results to the sequence reported by the latter authors.

Earlier studies suggested that the DNA-binding half of ADPRT was a single domain of M_r 66 000 (Kameshita et al., 1984). Plasmin cuts the DNA-binding moiety of ADPRT into the above two basic polypeptides, and only one of these (M_r 36 000) leaves a footprint on a specific restriction fragment of SV40 viral DNA which is identical with that produced by the whole enzyme itself (S. S. Sastry, K. G., Buki, and E. Kun, unpublished results); therefore, the other basic polypeptides (M_r 29 000) must also have a distinct function.

The next plasmin-generated polypeptide starts at residues 522, 523, and 525 (see Table II). Other possible cleavage sites were excluded since there was no other set of three N-termini, which upon sequencing could yield the experimentally obtained amino acids per cycle. Furthermore, the distance of 130–133 residues between the putative N-termini of M_r 56 000 and the established amino end of M_r 42 000 fits the size of the cleaved-off peptides of M_r 15 000–16 000 that are observed on SDS–PAGE after converting the polypeptide of M_r 56 000 to 42 000. The assignment of N-terminals was supported by the fact that the sequenched 13 residues in this region exactly matched the corresponding human sequence between positions 522 and 534. It is of interest that Kurosaki et al. (1987) also observed multiple cutting sites by papain similar to the multiple cuts by plasmin observed here.

The polypeptide of M_r 42 000 starts at residue 655, and its sequenced 32 residues differ only at one position from the sequence of the human enzyme (human, aspartic acid 671; bovine, asparagine 671). Since this polypeptide is always a closely spaced doublet on SDS–PAGE, its C-terminus is apparently not uniform. This heterogeneity may be already present in the whole enzyme molecule or it may be introduced by cleaving off an oligopeptide by plasmin.

The suggested polypeptide map for ADPRT is shown in Figure 6. Although we cannot rule out minor gaps or overlaps between the polypeptides isolated here, M_r 29 000, 36 000, and 56 000 reasonably add up to the full size of the enzyme. Figure 6 also identifies the apparent functions of the polypeptides, as defined by their binding to different affinity matrices.

ACKNOWLEDGMENTS

We are indebted to Dr. Andras Patthy (Biomolecular Resource Center) for sequencing.

Registry No. ADPRT, 58319-92-9; NAD, 53-84-9.

REFERENCES

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

Althaus, F. R., & Richter, Ch. (1987) *Mol. Biol. Biochem. Biophys. 37*.

Ball, E. H. (1986) *Anal. Biochem. 155*, 23–27.
Bauer, P. I., Hakam, A., & Kun, E. (1986) *FEBS Lett. 195*, 331–338.
Buki, K. G., Kirsten, E., & Kun, E. (1987) *Anal. Biochem. 167*, 160–166.
Burtscher, H. J., Auer, B., Klocker, H., Schweiger, M., & Hirsch-Kauffmann, M. (1986) *Anal. Biochem. 152*, 286–290.
Chibber, B. A. K., Deutsch, D. G., & Mertz, E. T. (1974) *Methods Enzymol. 34*, 424–432.
Deutsch, D. G., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 1095.
Gaal, J. C., & Pearson, C. K. (1985) *Biochem. J. 230*, 1–18.
Holtlund, J., Kristensen, T., Ostvold, A.-C., & Laland, S. G. (1981) *Eur. J. Biochem. 119*, 23–29.
Holtlund, J., Jemtland, R., & Kristensen, T. (1983) *Eur. J. Biochem. 130*, 309–314.
Hunkapiller, M. W., Hewick, K. M., Dreyer, W. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 399–413.
IUPAC–IUB (1968) *J. Biol. Chem. 243*, 3557–3559.
Kameshita, I., Matsuda, Z., Taniguchi, T., & Shizuta, Y. (1984) *J. Biol. Chem. 259*, 4770–4776.
Kameshita, I., Matsuda, M., Nishikimi, M., Ushiro, H., & Shizuta, Y. (1986) *J. Biol. Chem. 261*, 3863–3868.
Kurosaki, T., Ushiro, H., Mitsuuchi, Y., Suzuki, S., Matsuda, M., Matsuda, Y., Katunuma, N., Kangawa, K., Matsuo, H., Hirose, T., Inayama, S., & Shizuta Y. (1987) *J. Biol. Chem. 262*, 15990–15997.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Minaga, T., & Kun, E. (1983a) *J. Biol. Chem. 258*, 725–730.
Minaga, T., & Kun, E. (1983b) *J. Biol. Chem. 258*, 5726–5730.
Niedergang, C., Okazaki, H., & Mandel, P. (1979) *Eur. J. Biochem. 102*, 43–57.
Robbins, K. C., & Summaria, L. (1970) *Methods Enzymol. 19*, 184–199.
Scovassi, A. I., Izzo, R., Franchi, E., & Bertazzoni, U. (1986) *Eur. J. Biochem. 159*, 77–84.
Suzuki, H., Uchida, K., Shima, H., Sato, T., Okamoto, T., Kimura, T., & Miwa, M. (1987) *Biochem. Biophys. Res. Commun. 146*, 403–409.
Uchida, K., Morita, T., Sato, T., Ogura, T., Yamashita, R., Noguchi, S., Suzuki, H., Nyunoya, H., Miwa, M., & Sugimura, T. (1987) *Biochem. Biophys. Res. Commun. 148*, 617–622.
Ueda, K., & Hayaishi, O. (1985) *Annu. Rev. Biochem. 54*, 73–100.
Watson, D. H., Harvey, M. J., & Dean, P. D. G. (1978) *Biochem. J. 173*, 591–596.
Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H., & Kamiya, T. (1978) *J. Biol. Chem. 253*, 6459–6466.

4-Bromo-2-octenoic Acid Specifically Inactivates 3-Ketoacyl-CoA Thiolase and Thereby Fatty Acid Oxidation in Rat Liver Mitochondria†

Jianxun Li and Horst Schulz*
Department of Chemistry, City College of the City University of New York, New York, New York 10031
Received January 22, 1988; Revised Manuscript Received April 12, 1988

ABSTRACT: In an attempt to develop a compound which would specifically inhibit 3-ketoacyl-CoA thiolase (EC 2.3.1.16) in whole mitochondria, 4-bromo-2-octenoic acid was synthesized and studied. After rat liver mitochondria were preincubated with 4-bromo-2-octenoic acid for 3 min, respiration supported by either palmitoylcarnitine or pyruvate was completely abolished, whereas no inhibition was observed with rat heart mitochondria. Addition of carnitine stimulated respiration supported by pyruvate without relieving inhibition of palmitoylcarnitine-dependent respiration. Hence, this compound seems to be a specific inhibitor of β-oxidation. When the enzymes of β-oxidation were assayed in a soluble extract prepared from mitochondria preincubated with 4-bromo-2-octenoic acid, only 3-ketoacyl-CoA thiolase was found to be inactivated. 4-Bromo-2-octenoic acid is metabolized by mitochondrial β-oxidation enzymes to 3-keto-4-bromooctanoyl-CoA which effectively and irreversibly inhibits 3-ketoacyl-CoA thiolase but not acetoacetyl-CoA thiolase (EC 2.3.1.9). Even though 3-keto-4-bromooctanoyl-CoA inhibits the latter enzyme reversibly, 4-bromo-2-octenoic acid does not inhibit ketogenesis in rat liver mitochondria with acetylcarnitine as a substrate. It is concluded that 4-bromo-2-octenoic acid specifically inhibits mitochondrial fatty acid oxidation by inactivating 3-ketoacyl-CoA thiolase in rat liver mitochondria.

Compounds which specifically inhibit key regulatory enzymes are important tools in studying the control of metabolic pathways. Fatty acid oxidation is one of the pathways whose regulation has been probed by use of several inhibitors (Olowe & Schulz, 1982; Declereq et al., 1987). The known inhibitors of this pathway affect one of three reactions [for a recent review, see Schulz (1987)]: those catalyzed by carnitine palmitoyltransferase I, a key regulatory enzyme in liver (McGarry & Foster, 1980); 3-ketoacyl-CoA thiolase, a suggested regulatory enzyme in heart (Olowe & Schulz, 1980); and acyl-CoA dehydrogenase, which catalyzes the first step of β-oxidation.

Although several inhibitors of 3-ketoacyl-CoA thiolase are known, none inactivates only this enzyme. For example, 4-bromocrotonic acid is metabolized intramitochondrially to 3-keto-4-bromobutyryl-CoA which effectively and irreversibly

† This investigation was supported in part by Grants HL 18089 and HL 30847 of the National Heart, Lung, and Blood Institute as well as by a City University Faculty Research Award.

0006-2960/88/0427-5995$01.50/0