Articles

Mono(ADP-ribosylation) in Rat Liver Mitochondria†

Balz Frei‡ and Christoph Richter*

Laboratorium für Biochemie, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland

Received May 6, 1987; Revised Manuscript Received September 22, 1987

ABSTRACT: This paper investigates protein mono(ADP-ribosylation) in rat liver mitochondria. In isolated inner mitochondrial membranes, in the presence of both ADP-ribose and NAD^+, a protein is mono-(ADP-ribosylated) with high specificity. The reaction apparently consists of enzymatic NAD^+ glycohydrolysis and subsequent binding of free ADP-ribose to the acceptor protein. In terms of chemical stability, the resulting bond is unique among the ADP-ribose linkages thus far characterized. Formation of a Schiff base adduct between free ADP-ribose and the acceptor protein is excluded. In intact mitochondria at least three classes of proteins are ADP-ribosylated in vivo. One ADP-ribose–protein linkage is of the carboxylate ester type as indicated by its lability in neutral buffer. Another class of ADP-ribosylated proteins requires hydroxylamine for release of ADP-ribose. The third class is stable in hydroxylamine but labile to alkali, similar to the ADP-ribose–cysteine linkage in transducin formed by pertussis toxin.

Adenosine(5′)diphosphoribosylation (ADP-ribosylation)[1] is a posttranslational modification in which the ADP-ribose moiety of NAD^+ is covalently attached to proteins (Ueda & Hayaishi, 1985; Althaus & Richter, 1987). Mono(ADP-ribosyl) transferases are found in viruses, bacteria, and eukaryotic cells. The roles of mono(ADP-ribosylation) catalyzed by a number of bacterial toxins are well established. For example, cholera toxin and *Escherichia coli* heat-labile enterotoxin irreversibly activate adenylate cyclase by mono-(ADP-ribosylating) the α-component of the stimulatory guanine nucleotide binding protein of the adenylate cyclase system (Moss & Vaughan, 1977; Gill & Richardson, 1980). Pertussis toxin, an ADP-ribosyltransferase produced by *Bordetella pertussis*, attenuates adenylate cyclase inhibition by catalyzing the mono(ADP-ribosylation) of the α-subunit of the inhibitory guanine nucleotide binding protein of the adenylate cyclase system (Katada & Ui, 1982). Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A inhibit protein synthesis by mono(ADP-ribosylating) elongation factor 2 (Honjo et al., 1968; Iglewski et al., 1977). Furthermore, a nitrogenase activity of *Rhodospirillum rubrum* is regulated by reversible mono(ADP-ribosylation) (Lowery et al., 1986). Animal cells themselves possess mono(ADP-ribosyl) transferase activities (Ueda & Hayaishi, 1985). However, the roles of these endogenous mono(ADP-ribosyl) transferases are still unclear. Some of them may catalyze the ADP-ribosylation of the acceptor sites which also serve as targets for the bacterial toxins, but in a physiologically controlled manner.

In rat liver most of the cellular mono(ADP-ribosylated) proteins are associated with the mitochondrial fraction (Adamietz et al., 1981). In mitochondria two mono(ADP-ribosylating) systems have been described, one in the soluble (matrix) fraction (Kun et al., 1975; Hilz et al., 1984), the other in submitochondrial particles (SMP, inverted inner membrane vesicles) (Richter et al., 1983; Hilz et al., 1984). The ADP-ribosylated matrix protein has a molecular mass of 100 kDa and appears to consist of two major subunits of equal mass. The stability of the conjugate between ADP-ribose and the 100-kDa acceptor protein has been investigated (Kun et al., 1975). In SMP of both rat liver (Richter et al., 1983) and beef heart (Hilz et al., 1984), there is one major acceptor protein for mono(ADP-ribose), which migrates with an apparent molecular mass of 30 kDa in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Mono-(ADP-ribosylation) of the acceptor protein of beef heart SMP was suggested to occur nonenzymically. The chemical stability also of this ADP-ribose–protein conjugate has been investigated (Hilz et al., 1984). In rat liver SMP, ADP-ribosylation of the 30-kDa protein was proposed to occur at an arginine residue and is readily reversible in the presence of ATP (Richter et al., 1983). The characteristics of this ADP-ribosylation reaction, i.e., protein specificity and sensitivity to ATP, together with the observation that intramitochondrial hydrolysis of $NAD(P)^+$ is accompanied by release of Ca^{2+} from mitochondria suggest a functional link between mitochondrial protein ADP-ribosylation and Ca^{2+} release (Hofstetter et al., 1981; Richter et al., 1985).

Protein ADP-ribosylation in intact mitochondria has not yet been studied in detail. Labeling of intramitochondrial proteins with radioactive ADP-ribose is hindered by the poor permeability of the inner mitochondrial membrane for NAD^+ in vitro and insufficient radioactive labeling of intramitochondrial $NAD(P)^+$ in vivo. In this study we made use of a newly developed fluorescent technique (Jacobson et al., 1984; Payne et al., 1985). We first investigated the chemical stability of the ADP-ribose–protein conjugate in rat liver SMP and then studied ADP-ribosylation in intact mitochondria. We describe

† This work was supported by Grant 3.503-0.83 from the Schweizerischer Nationalfonds. B.F. was also supported by an EMBO short-term fellowship.

* Author to whom correspondence should be addressed.

‡ Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

[1] Abbreviations: ADP-ribose, adenosine(5′)diphosphoribose; 5′-AMP, adenosine 5′-monophosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; GME buffer, 6 M guanidinium chloride, 50 mM MOPS, and 10 mM EDTA, pH 4.0 (4 °C); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; kDa, kilodaltons; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SMP, submitochondrial particles.

0006-2960/88/0427-0529$01.50/0

the nonenzymic formation of a novel type of ADP-ribose–protein linkage in rat liver SMP and present evidence for at least three classes of ADP-ribosylated proteins in rat liver mitochondria.

Experimental Procedures

Isolation of rat liver mitochondria and SMP was done as reported (Frei et al., 1986).

Standard Incubation Procedure for SMP. SMP, 10 mg of protein/mL, were incubated at 25 °C in 50 mM potassium chloride, 5 mM magnesium chloride, 3.3 mM potassium fluoride, 1 mM mercaptoethanol, and 20 mM Hepes, pH 6.5, in the presence of 1 mM radiolabeled NAD^+ or ADP-ribose under constant stirring. At intervals 25-μL aliquots were withdrawn and proteins precipitated with 0.5 mL of 12% (w/v) perchloric acid. The precipitate was collected on Whatman GF/A filters and rinsed with 10 mL of 12% perchloric acid. The retained radioactivity was determined by liquid scintillation counting.

Analysis of Radiolabeled SMP Proteins by SDS–PAGE and Autoradiography. SMP were incubated under standard conditions in the presence of 1 mM [*adenylate*-^{32}P]NAD^+ in a final volume of 30 μL. After 60 min, the reaction was stopped by addition of 0.5 volume of 3-fold concentrated PAGE sample buffer. Noncovalently bound label was removed by column centrifugation using 0.5-mL Sephadex G-25 desalting columns equilibrated with PAGE sample buffer [cf. Jacobson et al. (1984)].

Slab gel electrophoresis of the desalted samples was done in SDS on acid gels using 10% polyacrylamide (Bame & Scarborough, 1980). Dried gels were exposed to Kodak X-Omat AR films for 5–8 h.

Determination of NAD^+ Glycohydrolase Activity of SMP. SMP were incubated under standard conditions in the presence of 1 mM NAD^+. At intervals 25-μL aliquots were withdrawn, and [NAD^+] was assessed by the yeast alcohol dehydrogenase assay (Williamson & Corkey, 1969). Reduction of NAD^+ was monitored at 340 nm.

Preparation of radiolabeled ADP-ribose was according to the method of Payne et al. (1985). The purity was 90.3 ± 6.1% (n = 5) as judged by high-performance liquid chromatography (HPLC) analysis.

Determination of Covalently Bound Mono(ADP-ribose) Residues by Incubation of the Proteins in the Absence and Presence of Hydroxylamine. This was essentially done according to the method of Jacobson et al. (1984) and Payne et al. (1985). Briefly, suspensions of mitochondria (2 mg of protein/mL) or of SMP (10 mg of protein/mL) incubated under standard conditions were mixed with 0.25 volume of 100% (w/v) trichloroacetic acid. The acid-insoluble material was washed twice with anhydrous diethyl ether, dried under high vacuum, and dissolved completely in 0.5 mL of 6 M guanidinium chloride, 50 mM 4-morpholinepropanesulfonic acid (MOPS), and 10 mM (ethylenedinitrilo)tetraacetic acid (EDTA), pH 4.0 (GME buffer). The proteins were freed of noncovalently bound nucleotides by Sephadex G-25 (superfine) column centrifugation and then incubated at pH 7.0 at 37 °C in 1 M ammonium chloride or in 1 or 3 M hydroxylamine. When SMP were used, 200-μL aliquots of the incubation mixture were withdrawn at time intervals, and protein-bound radioactivity was determined after acid precipitation with 0.5 mL of 12% (w/v) perchloric acid as described above. When mitochondria were used, the mono(ADP-ribose) residues released from the proteins after 12 h of incubation were isolated by batch affinity adsorption on dihydroxyboronyl-Bio-Rex. ADP-ribose was then reacted with chloroacetaldehyde to form the highly fluorescent analogue 1,N^6-etheno(ADP-ribose). 1,N^6-Etheno(ADP-ribose) was purified by affinity chromatography on dihydroxyboronyl-Sepharose and quantitated by fluorescence detection following strong anion-exchange HPLC using 100 mM potassium phosphate, pH 4.7, as elution buffer.

Determination of Covalently Bound Mono(ADP-ribose) Residues by Alkaline Treatment of the Proteins. Samples of mitochondria or SMP were prepared as described above. After the desalting step, the protein fraction (usually 0.75 mL) was mixed with an equal volume of 2 M sodium hydroxide. Alternatively, for SMP only, the pH of the protein fraction was adjusted to pH 9.3 by dropwise addition of 5 M ammonium hydroxide. The radioactivity remaining bound to SMP during alkaline treatment was determined as above. When mitochondria were used, the samples (containing 1 M sodium hydroxide) were incubated for 2 h at 37 °C. This treatment not only releases mono(ADP-ribose) from the protein(s) but also converts ADP-ribose to adenosine 5′-monophosphate (5′-AMP) (Kaplan et al., 1951; Goebel et al., 1977; Alvarez-Gonzalez et al., 1983). For each set of experiments, a "buffer control" (0.75 mL of GME buffer) and, to determine the recovery over the assay, a "label control" [80 000 dpm of [*adenosine*-^{14}C(U)]ADP-ribose added to a desalted protein sample] were treated identically as the samples. After incubation, samples were cooled to room temperature, and an equal volume (usually 1.5 mL) of 1 M hydrochloric acid was added, followed by 2 mL of deionized water and 555 μL of ammonium acetate, pH 4.5. If necessary, the pH of the samples was adjusted to 4.5 with concentrated hydrochloric acid.

Formation of the highly fluorescent 1,N^6-etheno analogues of ADP-ribose and 5′-AMP was accomplished by incubation of the samples with 100 mM chloroacetaldehyde for 4 h at 60 °C. 1,N^6-Etheno(ADP-ribose) and 1,N^6-etheno(5′-AMP) were purified by batch affinity adsorption on Affi-Gel 601 as follows. Samples were cooled to 4 °C and adjusted to pH 9.4 with concentrated ammonium hydroxide. One milliliter of a suspension containing freshly washed Affi-Gel 601 (equilibrated as described below and equivalent to 0.5 mL of packed resin) was added to each sample. Samples were vigorously agitated in a Buchler vortex-evaporator at 4 °C for 30 min and then transferred to Econo columns and washed with 10 mL of 0.25 M ammonium chloride, pH 9.0, and 0.5 mL of 30 mM phosphoric acid (elution buffer). The outlet of the columns was capped, 1.5 mL of elution buffer was added, and the capped columns were vortexed. Caps were removed, and the 1,N^6-etheno compounds were eluted from the columns by a 5 min spin at 1500 g. The pH of the eluate (usually 3.0–3.5) was adjusted to 4.7 with dilute ammonium hydroxide. 1,N^6-Etheno(ADP-ribose) and 1,N^6-etheno(5′-AMP) in 1.0 mL of the eluate were quantitated by fluorescence detection (excitation at 220 nm, emission at >370 nm) following strong anion-exchange HPLC using 50 mM potassium phosphate, pH 4.7, as elution buffer at a flow rate of 1.0 mL/min. A typical fluorescence chromatogram following HPLC analysis of a mitochondrial sample and the distribution of radioactivity originating from radiolabeled ADP-ribose as "label control" are shown in Figure 1A. The recovery of radioactivity over the entire assay was 46.2 ± 6.3% (n = 6); 71.2 ± 4.2% (n = 6) of the recovered radioactivity was associated with the fluorescent peak corresponding to 1,N^6-etheno(5′-AMP), and 3.4 ± 0.5% (n = 6) was detected together with 1,N^6-etheno(ADP-ribose). After each set of sample analysis, the HPLC system was gauged with external standards of 1,N^6-etheno(ADP-ribose) and 1,N^6-etheno(5′-AMP) (Figure 1B). The relationship between fluorescence intensity and the quantity

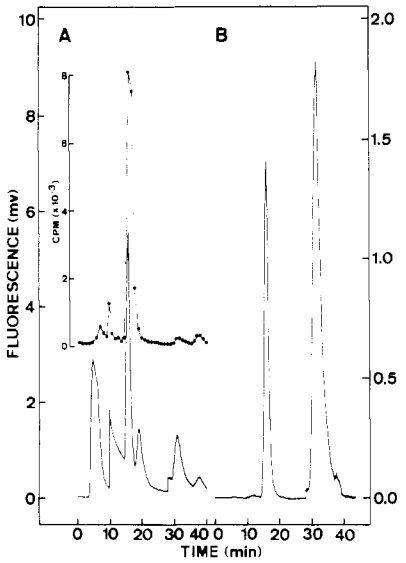

FIGURE 1: Determination of protein-bound mono(ADP-ribose) in rat liver mitochondria following treatment with strong alkali. (A) A mitochondrial sample (0.8 mg of protein) was subjected to G-25 (superfine) column centrifugation, and the protein fraction was analyzed for mono(ADP-ribose) following treatment with 1 M sodium hydroxide at 37 °C for 2 h as described. The fluorescence chromatogram following HPLC is shown. The filled circles represent radioactivity originating from [*adenosine*-^{14}C(U)]ADP-ribose added to a separate mitochondrial sample prior to alkaline treatment. (B) Equimolar amounts (30 pmol) of standards of 1,N^6-etheno(5′-AMP) and 1,N^6-etheno(ADP-ribose) were analyzed by HPLC with fluorescence detection. The peak at 16.5 min corresponds to 1,N^6-etheno(5′-AMP) and the one at 31.5 min to 1,N^6-etheno-(ADP-ribose). For both panels, the sensitivity of the fluorometer was increased 20-fold and 5-fold after 10 and 28 min, respectively (inflections in the chromatograms). Numbers on the left-hand ordinate represent fluorescence after the first change in sensitivity and numbers on the right-hand ordinate fluorescence after the second change.

of both 1,N^6-etheno(5′-AMP) (data not shown) and 1,N^6-etheno(ADP-ribose) [cf. Payne et al. (1985)] is perfectly linear.

The assay was tested with SMP that were incubated for 80 min in the presence of 1 mM NAD^+. The very high ratio of free to protein-bound nucleotides under these conditions (cf. Figure 4A) required rigorous desalting of the SMP extracts by two successive gel filtration steps using the column centrifugation method (Jacobson et al., 1984). Analysis of the modified submitochondrial proteins by the assay described above revealed a clear increase in protein-bound ADP-ribose from 128 pmol/mg of protein at time zero to 311 pmol/mg of protein after 80 min of incubation, indicating incorporation of 183 pmol of ADP-ribose/mg of protein. This result is in excellent agreement with that obtained by determination of acid-precipitable radioactivity of SMP incubated in the presence of 1 mM radiolabeled NAD^+ (see Results).

Equilibration of Affi-Gel 601 for Affinity Chromatography. Affi-Gel 601, stored in the dark at 4 °C in 0.25 M ammonium acetate, pH 4.5 (4 °C) as a 1:4 suspension (volume of packed resin:volume of buffer), was washed and equilibrated immediately before use as follows. The required amount of resin (2.5 mL of stock suspension per sample) was sedimented at 1500*g* for 5 min in a conical polypropylene centrifuge tube and washed with 10 volumes of each of the following solutions: 0.25 M ammonium chloride, pH 9.0 (application buffer); 30 mM phosphoric acid (elution buffer); and application buffer twice. Finally, the resin was resuspended in an equal volume of application buffer.

Materials. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): rotenone, Hepes, MOPS, Sephadex G-25-50 (superfine), poly(L-lysine) hydrochloride, poly(L-arginine) hydrochloride, ADP-ribose, NAD^+, and 5′-AMP. Mannitol, sucrose, hydroxylamine hydrochloride, 70% perchloric acid, EDTA, sodium cyanoborohydride, and guanidine hydrochloride were obtained from Fluka (Buchs, Switzerland). Chloroacetaldehyde (45% in water) was purchased from ICN Pharmaceuticals, Inc. (Plainview, NY). Polypropylene Econo columns (0.8-cm i.d.) and Affi-Gel 601 were obtained from Bio-Rad Laboratories (Richmond, CA). The following radiolabeled compounds were obtained from New England Nuclear (Boston, MA): [*adenosine*-^{14}C(U)]NAD^+, [*adenylate*-^{32}P]NAD^+, [*adenine*-2,8-^{3}H]NAD^+, and [^{14}C(U)]5′-AMP. [*ribose*(NMN)-^{14}C]NAD^+ was kindly provided by Dr. K. Ueda (Kyoto University, Japan). All other chemicals used were of reagent grade or the highest purity commercially available.

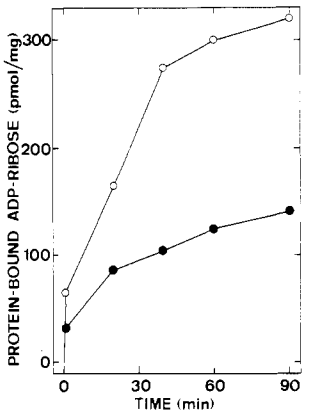

FIGURE 2: ADP-ribosylation of rat liver submitochondrial particles in the presence of [*ribose*(NMN)-^{14}C]NAD^+ and [*adenine*-2,8-^{3}H]NAD^+. SMP were incubated according to the standard incubation procedure in the presence of 1 mM [*ribose*(NMN)-^{14}C]NAD^+ at 26 dpm/pmol (○) or 1 mM [*adenine*-2,8-^{3}H]NAD^+ at 58 dpm/pmol (●).

Results

Incubation of rat liver SMP with [*adenylate*-^{32}P]NAD^+ or with NAD^+ radiolabeled in the adenine ring results in a time-dependent increase of acid-precipitable radioactivity (Hofstetter et al., 1981; Richter et al., 1983). In the presence of [*carbonyl*-^{14}C]NAD^+ no radioactivity is incorporated into SMP (data not shown). Furthermore, treatment of the modified protein with phosphodiesterase liberates mainly 5′-AMP (Richter et al., 1983). These results strongly suggest mono(ADP-ribosylation). However, they do not exclude protein modification by 5′-AMP or 5′-ADP. We therefore used [*ribose*(NMN)-^{14}C]NAD^+, which is labeled at the ribose ring next to the nicotinamide moiety. As shown in Figure 2, this compound, too, labels SMP, unambiguously demonstrating covalent modification with ADP-ribose. The rate and extent of ADP-ribosylation with [*ribose*(NMN)-^{14}C]NAD^+ as substrate are considerably higher than with [*adenine*-2,8-^{3}H]-NAD^+ (Figure 2). This provides circumstantial evidence for the presence in SMP of an enzyme that cleaves protein-bound ADP-ribose between the ribose(NMN) and the adenine moiety.

The chemical stability of the ADP-ribose–protein conjugate was investigated with SMP covalently modified by standard incubation for 90 min. Such incubation yields 179 ± 47 (n = 20) pmol of bound ADP-ribose/mg of protein, as judged from acid-precipitable radioactivity. The modified, acid-precipitated SMP were immediately washed and dried. The resulting powder was completely dissolved in a buffer containing 6 M guanidine. After separation from residual noncovalently bound radioactivity (Jacobson et al., 1984), the

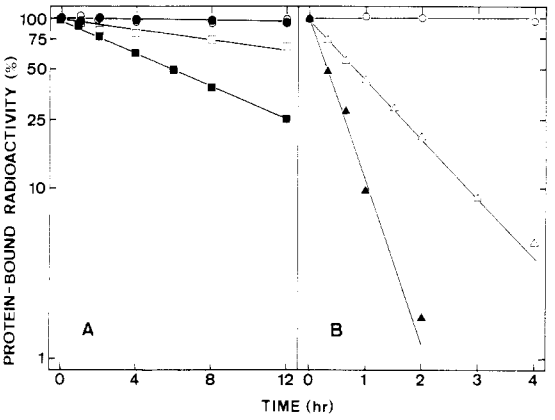

FIGURE 3: Chemical stability of the ADP-ribose–protein conjugate formed in rat liver SMP. SMP were incubated under standard conditions in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 46–53 dpm/pmol. After 90 min, 0.25 volume of 100% (w/v) trichloroacetic acid was added. The acid-insoluble material was washed, dried, dissolved in GME buffer, and subjected to two successive G-25 (superfine) column centrifugation steps. Aliquots of the protein fraction were then incubated at pH 4.0, 4 °C, without further additions (○) or, as depicted in panel A, at pH 7.0, 37 °C, in the absence (●) or presence (□) of 1 M hydroxylamine or in the presence of 3 M neutral hydroxylamine at 37 °C (■). (B) Aliquots of the protein fraction were incubated at pH 9.3, 37 °C (△) or in 1 M sodium hydroxide at 37 °C (▲). At the indicated times, protein-bound radioactivity in 200-μL aliquots was determined after acid-precipitation of the proteins with 500 μL of 12% (w/v) perchloric acid and filtration. 100% = 2120–2600 dpm.

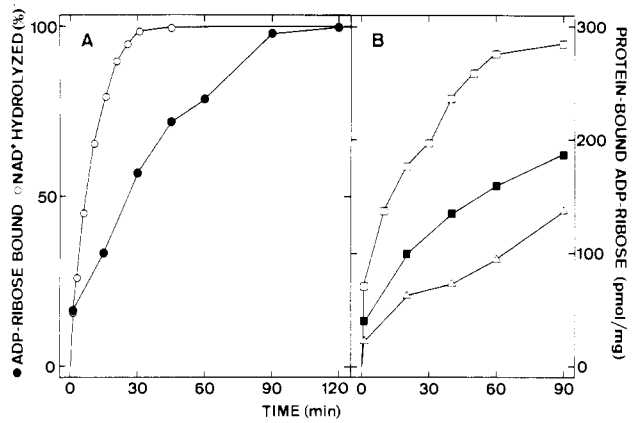

FIGURE 4: Kinetic analysis of NAD$^+$ hydrolysis and ADP-ribosylation in rat liver SMP and incorporation of radiolabel into submitochondrial proteins from [^{3}H]NAD$^+$ and [^{3}H]ADP-ribose. (A) SMP were incubated according to the standard incubation procedure in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 50 dpm/pmol. Hydrolysis of NAD$^+$ (○) and ADP-ribosylation (●) (Experimental Procedures) were assayed in the same experiment. 100% = 1096 pmol of NAD$^+$ hydrolyzed/mg of protein, and 183 pmol of ADP-ribose bound/mg of protein, respectively. (B) SMP were incubated according to the standard incubation procedure with 1 mM [*adenine*-2,8-^{3}H]-ADP-ribose at 50 dpm/pmol (□) or 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 46 dpm/pmol (■), or 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 46 dpm/pmol in the presence of 1 mM unlabeled ADP-ribose (△).

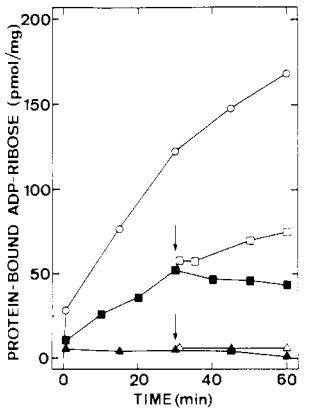

FIGURE 5: Inhibition of ADP-ribosylation in rat liver SMP by carbonyl reagents. SMP were incubated according to the standard incubation procedure in the presence of 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ at 74 dpm/pmol. Conditions were as follows: no inhibitor present (○); in the presence of 100 mM phenylhydrazine (■, □); in the presence of 100 mM hydroxylamine (▲, △). (□, △) Obtained after addition of a second dose of 1 mM [*adenine*-2,8-^{3}H]NAD$^+$ at the *arrows*.

proteins were subjected to various treatments (Figure 3). The ADP-ribose–protein conjugate proved stable for at least 12 h at pH 4.0 and 4 °C, as well as in the presence of 1 M ammonium chloride at neutral pH and 37 °C (Figure 3A). (The latter condition henceforth will be referred to as "in the absence of hydroxylamine".) The linkage is labile to 1 and 3 M neutral hydroxylamine at 37 °C, with a half-life in 3 M hydroxylamine of 6 h (Figure 3A). In 4 M hydroxylamine the half-life of the linkage is 4.0 h (data not shown). At basic pH (Figure 3B), radioactivity is released very fast from the protein, with a half-life at pH 9.3 of 50 min and of 20 min in 1 M sodium hydroxide. This is in agreement with and extends our previous findings (Richter et al., 1983). The release of protein-bound radioactivity follows single first-order kinetics under all conditions (Figure 3). This indicates that only one class of mono(ADP-ribosylated) proteins has been formed by incubation of SMP with NAD$^+$.

When SMP are incubated with 1 mM NAD$^+$, virtually all NAD$^+$ is hydrolyzed within 30 min (Figure 4A). At this time, incorporation of ADP-ribose into the submitochondrial proteins reaches about 100 pmol/mg of protein. ADP-ribosylation continues for another hour (Figure 4A). This clear discrepancy of the kinetics of NAD$^+$ hydrolysis and ADP-ribosylation could be due to nonenzymatic covalent protein modification with free ADP-ribose previously formed by enzymatic hydrolysis of NAD$^+$. A similar reaction sequence has been proposed (Hilz et al., 1984) for the mono(ADP-ribosylation) of a 30-kDa acceptor protein in beef heart SMP. In fact, modification of rat liver SMP is faster and more efficient in the presence of free ADP-ribose instead of NAD$^+$ (Figure 4B). Also, addition of 1 mM unlabeled ADP-ribose to the incubation of SMP with 1 mM radiolabeled NAD$^+$ clearly diminishes incorporation of radioactivity into the protein (Figure 4B). This suggests a competition between the two nucleotides for the same acceptor site. Indeed, the covalent modification with free ADP-ribose has the same specificity with respect to the acceptor protein as with NAD$^+$ (see Figure 6).

Nonenzymatic ADP-ribosylation in rat liver SMP is further indicated by the following observations. Trapping of free ADP-ribose by the carbonyl reagents phenylhydrazine and hydroxylamine strongly inhibits or completely eliminates incorporation of NAD$^+$-derived ADP-ribose into submitochondrial proteins (Figure 5). Neither carbonyl reagent affects the hydrolysis of NAD$^+$ by SMP (data not shown). The moderate covalent modification of SMP in the presence of phenylhydrazine peaks after 30 min of incubation, just when NAD$^+$ hydrolysis is complete (cf. Figure 3A). When more NAD$^+$ is added at this time, again moderate ADP-ribosylation is observed. Still, no ADP-ribosylation is elicited by a second dose of NAD$^+$ to SMP incubated for 30 min in the presence of hydroxylamine (Figure 5).

Mono(ADP-ribosylation) of rat liver SMP is effectively inhibited by the arginine-blocking reagents phenylglyoxal and butanedione (Richter et al., 1983). The half-life in 3 M neutral hydroxylamine of the submitochondrial ADP-ribose–protein conjugate (see Figure 3A) is similar to that of the Schiff base

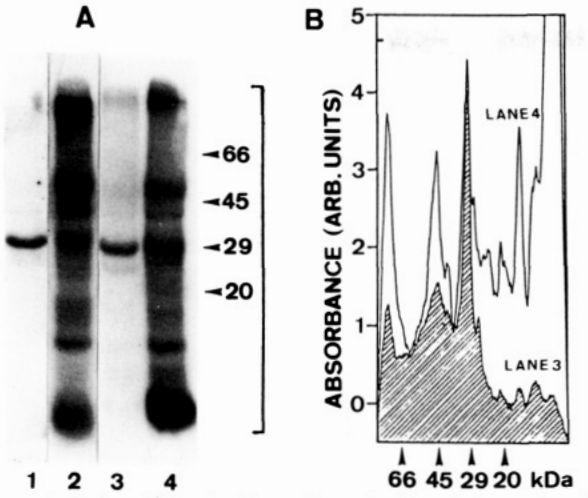

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography of rat liver SMP after incubation with [^{32}P]-ADP-ribose and [^{32}P]NAD$^+$ in the absence and presence of sodium cyanoborohydride. SMP were incubated for 60 min under standard conditions in the presence of either 1 mM [*adenylate*-^{32}P]ADP-ribose at 1100 dpm/pmol (lanes 1 and 2) or 1 mM [*adenylate*-^{32}P]NAD$^+$ at 1250 dpm/pmol (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 mM sodium cyanoborohydride. After incubation, samples were freed of noncovalently bound radioactivity by G-25 (superfine) column centrifugation and subjected to SDS–PAGE (10% polyacrylamide) according to the method of Bame and Scarborough (1980). Gels were immediately dried, and the radiolabeled proteins were visualized by autoradiography. (A) Autoradiograms. The numbers represent apparent molecular masses (in kilodaltons) of protein standards. (B) Lanes 3 and 4 scanned at 540 nm over the stretch indicated in panel A.

adducts of ADP-ribose to polymers of basic amino acids (Hilz et al., 1984; Payne et al., 1985). These results could be taken as evidence that nonenzymatic formation of a Schiff base adduct accounts for mono(ADP-ribosylation) of rat liver SMP. Seemingly consistent with this notion, we found that the covalent modification of rat liver SMP is dramatically enhanced by sodium cyanoborohydride, a substance known to stabilize Schiff base adducts at arginine, lysine, and histidine residues by selective reduction (Acharya et al., 1983). However, analysis of the radiolabeled proteins by SDS–PAGE reveals that the enhanced incorporation of radioactivity from both free ADP-ribose and NAD$^+$ is due to covalent modification of a large number of submitochondrial proteins (Figure 6A). Most of these proteins are also very weakly labeled without sodium cyanoborohydride (Figure 6B). This indicates that some Schiff base adducts between free ADP-ribose and proteins are indeed formed by incubation of SMP with NAD$^+$. However, the 30-kDa protein, which is very specifically labeled in the presence of free ADP-ribose or NAD$^+$ alone, is not modified more efficiently in the presence of sodium cyanoborohydride than in its absence (Figure 6 A,B). The same is true when SMP are incubated with NAD$^+$ for only 30 min (data not shown), i.e., when the 30-kDa acceptor protein is far from being maximally ADP-ribosylated (cf. Figure 5). These findings strongly suggest that the 30-kDa acceptor protein in rat liver SMP is not modified by formation of a Schiff base adduct between free ADP-ribose and an arginine, lysine, or histidine residue. Furthermore, when poly(L-arginine) or poly(L-lysine) is included in the standard incubation of SMP with NAD$^+$, the labeling of the 30-kDa protein is not decreased, although much radioactivity is associated with the poly(L-amino acid)s (not shown). Very much radiolabel is incorporated into poly(L-lysine) and less into poly(L-arginine). With free ADP-ribose the relative labeling efficiency is the same, but the absolute amounts incorporated are higher (not shown). In the absence of submitochondrial proteins, the poly(L-amino acid)s are labeled by free ADP-ribose to about the same extents as in their presence, whereas incorporation from NAD$^+$ is almost eliminated (not shown). Again these results indicate that under the conditions employed formation of Schiff base adducts between free ADP-ribose and basic amino acids can take place and that Schiff base formation does not account for mono(ADP-ribosylation) of the 30-kDa acceptor protein.

Table I: Protein-Bound Mono(ADP-ribose) in Rat Liver Mitochondria[a]

treatment	ADP-ribose released (pmol/mg of protein)
1 M NH_4Cl, pH 7.0, 37 °C, 12 h	6.0 ± 2.1
1 M NH_2OH, pH 7.0, 37 °C, 12 h	23.8 ± 5.7
3 M NH_2OH, pH 7.0, 37 °C, 12 h	54.3 ± 6.9
1 M NaOH, 37 °C, 2 h	205.6 ± 17.7

[a] The acid-insoluble material from rat liver mitochondria was dissolved and subjected to G-25 (superfine) column centrifugation. The samples (containing 0.8–2.0 mg of protein) were incubated under the conditions given in the table and assessed for protein-bound mono(ADP-ribose) fluorometrically as described under Experimental Procedures. Each value represents the average ± standard deviation of three mitochondrial preparations. For each mitochondrial preparation and incubation condition at least three samples were examined.

Although presumably nonenzymatic, the specific mono(ADP-ribosylation) of the 30-kDa protein may fulfill a physiological function in intact mitochondria. When freshly isolated rat liver mitochondria are assessed for endogenous protein-bound mono(ADP-ribose) by the method of Jacobson and co-workers (Jacobson et al., 1984; Payne et al., 1985), 23.8 and 6.0 pmol of ADP-ribose/mg of protein are detected following incubation of the mitochondrial proteins for 12 h in the presence and absence, respectively, of 1 M hydroxylamine (Table I). Incubation with 3 M neutral hydroxylamine for 12 h liberates 54.3 pmol of ADP-ribose/mg of mitochondrial protein. As determined by the newly developed assay described under Experimental Procedures, treatment of the mitochondrial proteins with 1 M sodium hydroxide for 2 h releases approximately 200 pmol of ADP-ribose equivalents/mg of protein (Table I). The portion of covalently bound ADP-ribose residues that is released in the absence of hydroxylamine most probably is linked to the mitochondrial acceptor protein(s) via a carboxylate ester linkage similar to that between ADP-ribose and histones formed in chromatin of rat liver by poly(ADP-ribose) synthetase (Ogata et al., 1980a,b). Since this linkage is extremely labile both in the absence and in the presence of hydroxylamine (Ogata et al., 1980a,b), the portion of ADP-ribose residues released from the mitochondrial proteins in the absence of hydroxylamine is presumably also released in its presence. Therefore, the "hydroxylamine-requiring" ADP-ribose residues in rat liver mitochondria amount to about (23.8 − 6.0) = 17.8 pmol/mg of protein when 1 M neutral hydroxylamine is used for chemical release compared to about (54.3 − 6.0) = 48.3 pmol/mg of protein when 3 M hydroxylamine is used.

Discussion

The ADP-ribose–protein bond formed by incubation of rat liver SMP with NAD$^+$ is stable in the absence of hydroxylamine and has a half-life of 6 h in 3 M neutral hydroxylamine and of 20 min in 1 M sodium hydroxide. It is unique among the linkages so far characterized. For example, the ADP-ribose–cysteine linkage in transducin formed by pertussis toxin (Payne et al., 1985; Hsia et al., 1985) and the ADP-ribose–diphthamide linkage in elongation factor 2 formed by

diphtheria toxin (Nishizuka et al., 1969; Payne et al., 1985) are both stable to 3 M neutral hydroxylamine for 12 h. On the other hand, the linkage formed by a NAD–arginine ADP-ribosyltransferase from turkey erythrocytes between ADP-ribose and arginine residues of histones (Moss et al., 1983; Payne et al., 1985) has a half-life of only 1.5 h in 1 M neutral hydroxylamine. The ADP-ribose–arginine bond formed by cholera toxin (Hsia et al., 1985) and the carboxylate ester linkage between ADP-ribose and histones formed in chromatin of rat liver by poly(ADP-ribose) synthetase (Ogata et al., 1980a,b) are even more labile to hydroxylamine. The stability of the nonenzymatic ADP-ribose–protein adducts described in the literature is also clearly different from that of the ADP-ribose–protein conjugate of rat liver SMP described in this study. In contrast to the latter, the nonenzymic ADP-ribose–protein conjugate of beef heart SMP is stable in 3 M neutral hydroxylamine for 6 h (Hilz et al., 1984). Furthermore, the ADP-ribosylated 100-kDa protein in the soluble fraction of rat liver mitochondria, which is also thought to be formed nonenzymically (Hilz et al., 1984), is labile at pH 6.5 (Kun et al., 1975), whereas the ADP-ribosylated 30-kDa protein of rat liver SMP is stable at pH 4.0 and pH 7.0. The histone H1–mono(ADP-ribose) conjugate synthesized by reaction of free ADP-ribose with H1 is labile in the absence of hydroxylamine, and its half-life in 3 M neutral hydroxylamine is 3 h (Kreimeyer et al., 1985). Finally, although the half-life of the conjugate in rat liver SMP in 3 M neutral hydroxylamine is similar to that of Schiff base adducts between free ADP-ribose and polymers of basic amino acids [4 h (Payne et al., 1985) or 6 h (Hilz et al., 1984)], formation of a Schiff base adduct between ADP-ribose and the 30-kDa acceptor protein is also excluded since sodium cyanoborohydride does not enhance the ADP-ribosylation of this acceptor and poly(L-arginine) and poly(L-lysine) do not competitively inhibit the reaction.

No evidence for significant mono(ADP-ribosyl) transferase activity in rat liver SMP was obtained in the present study. Rather, the ADP-ribosylation reaction appears to consist of enzymic formation of free ADP-ribose from NAD^+ and subsequent specific nonenzymic ADP-ribosylation of the 30-kDa acceptor protein. An identical reaction sequence may account for mono(ADP-ribosylation) of a 30-kDa acceptor protein of beef heart SMP (Hilz et al., 1984). However, the two linkages clearly differ in chemical stability. That covalent modification of rat liver SMP with free ADP-ribose is more efficient than with NAD^+-derived ADP-ribose is in contrast to our previous finding (Richter et al., 1983). This may arise from the different methods of preparing ADP-ribose, i.e., enzymatic versus chemical hydrolysis of NAD^+.

The "hydroxylamine-requiring" residues in intact rat liver mitochondria are very similar in terms of stability toward 1 and 3 M neutral hydroxylamine to the ADP-ribose residues bound to SMP by incubation with NAD^+. This can be inferred from the finding that incubation for 12 h in 3 M neutral hydroxylamine of both the ADP-ribose–protein conjugates formed in vivo and in vitro liberates about 2.5 times the amount of ADP-ribose released from the corresponding conjugate by treatment with only 1 M hydroxylamine (see Figure 3A and Table I). Considering also the unique properties of the ADP-ribose–protein linkage described here (see above), these results suggest that in vivo the 30-kDa protein of the *mitochondrial inner membrane* is ADP-ribosylated and also by the same mechanism as in vitro. However, in mitochondria additional ADP-ribose–protein linkages are formed in vivo. One is the carboxylate ester like linkage. The presence of yet another type of linkage is indicated by the high amount of ADP-ribose residues released by strong alkali. The latter conclusion is based on the notion that the assay determining 5′-AMP following alkaline treatment is specific for protein-bound mono(ADP-ribose). The following observations corroborate this: (i) Only 5′-AMP, ADP-ribose, and NAD^+ give rise to significant amounts of 5′-AMP after alkaline treatment (Goebel et al., 1977). Some few percent of NADH and 5′-ADP are also degraded to 5′-AMP in alkali. No 5′-AMP is liberated by this treatment from any other nucleotide containing the 5′-AMP moiety (Goebel et al., 1977). (ii) G-25 (superfine) column centrifugation very effectively separates noncovalently bound nucleotides from proteins (Payne et al., 1985), eliminating 5′-AMP, NAD^+, NADH, 5′-ADP, and ADP-ribose. (iii) tRNA and DNA, which could conceivably be recovered in the protein fraction, do not yield 5′-AMP by alkaline treatment (Goebel et al., 1977). (iv) Although released from the protein(s), polymeric residues of ADP-ribose are stable in strong alkali (Alvarez-Gonzalez et al., 1983). (v) Determination of ADP-ribosylation in SMP by liberating 5′-AMP through alkaline treatment or by acid precipitation of radioactivity gives very similar results.

Assuming that the in vivo bound hydroxylamine-requiring ADP-ribose residues in mitochondria are identical with those bound in vitro in SMP, incubation of mitochondrial proteins in 3 M neutral hydroxylamine for 12 h can be expected to liberate approximately 75% of the hydroxylamine-requiring ADP-ribose residues (cf. Figure 3A). All these residues should be released by strong alkali. On this basis, the hydroxylamine-requiring residues in mitochondria amount to about 65 pmol/mg of protein (cf. Table I). The ADP-ribose residues linked to the mitochondrial acceptor protein(s) via a carboxylate ester linkage (6 pmol/mg of protein) are also released by strong alkali. This gives a sum of about 70 pmol/mg of protein. In contrast, more than 200 pmol of ADP-ribose/mg of protein is released from mitochondria by incubation in strong alkali. As mentioned above, this strongly points to (an) additional ADP-ribose–protein conjugate(s) formed in vivo being stable in neutral hydroxylamine but labile to alkali. Indeed, the ADP-ribosylated 100-kDa protein of the soluble fraction of rat liver mitochondria is very labile in alkali and stable in hydroxylamine (Kun et al., 1975). However, it should be noted that the stability in hydroxylamine was tested under milder conditions (0.4 M hydroxylamine, pH 7.4, 25 °C) than in this study and after incubation for only 40 min.

A previous report analyzed the mono(ADP-ribose)–protein conjugates formed in vivo by the use of hydroxylamine. It demonstrated the existence of carboxylate ester type and ADP-ribosyl–arginine linkages in rat liver (Payne et al., 1985). Evidence for a third type of ADP-ribose–protein conjugate formed in vivo was recently presented by the groups of Iglewski (Iglewski et al., 1984; Lee & Iglewski, 1984) and Moss (Hsia et al., 1985), who demonstrated mono(ADP-ribosylation) of elongation factor 2 by endogenous activities, most probably at the diphthamide residue. However, a "diphthamide-like" linkage would not have been detected with our treatment, since it is completely stable in both hydroxylamine and strong alkali (Payne et al., 1985). Thus, the hydroxylamine-stable, alkali-labile ADP-ribose–protein linkage in mitochondria described here represents a novel linkage formed in vivo. In terms of chemical stability, it resembles most closely the ADP-ribose–cysteine linkage in transducin formed in vitro by pertussis toxin (Payne et al., 1985).

The importance of mono(ADP-ribosylation) for the functioning of cells is not established. The existence of cellular

mono(ADP-ribosyl) transferases (Ueda & Hayaishi, 1985) and of enzymes that process protein-bound ADP-ribose (Smith et al., 1985; Moss et al., 1985, 1986) suggests a control of cellular metabolism by mono(ADP-ribosylation). We previously proposed regulation by protein ADP-ribosylation of Ca^{2+} release from mitochondria (Hofstetter et al., 1981; Richter et al., 1985). Indeed, we have preliminary evidence that the level of mono(ADP-ribosylated) mitochondrial proteins increases transiently during Ca^{2+} release. It will be of interest to see whether the different mitochondrial acceptor sites described in the present work are differentially susceptible to mono(ADP-ribosylation) during Ca^{2+} release.

ACKNOWLEDGMENTS

We thank Drs. M. K. Jacobson and D. M. Payne for their hospitality and advice. We are grateful to Dr. K. Ueda for the very generous gift of [*ribose*(NMN)-^{14}C]NAD$^+$ and to Dr. K. H. Winterhalter for his interest.

REFERENCES

Acharya, A. S., Sussman, L. G., & Manning, J. M. (1983) *J. Biol. Chem. 258*, 2296–2302.

Adamietz, P., Wielckens, K., Bredehorst, R., Lengyel, H., & Hilz, H. (1981) *Biochem. Biophys. Res. Commun. 101*, 96–1031.

Althaus, F. R., & Richter, C. (1987) *ADP-Ribosylation of Proteins*, Springer-Verlag, Berlin.

Alvarez-Gonzalez, R., Juarez-Salinas, H., Jacobson, E. L., & Jacobson, M. K. (1983) *Anal. Biochem. 135*, 69–77.

Bame, J. B., & Scarborough, G. A. (1980) *Biochemistry 19*, 2931–2937.

Frei, B., Winterhalter, K. H., & Richter, C. (1986) *Biochemistry 25*, 4438–4443.

Gill, D. M., & Richardson, S. H. (1980) *J. Infect. Dis. 141*, 64–70.

Goebel, M., Stone, P. R., Lengyel, H., & Hilz, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 13–21.

Hilz, H., Koch, R., Fanick, W., Klapproth, K., & Adamietz, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3929–3933.

Hofstetter, W., Mühlebach, T., Lötscher, H. R., Winterhalter, K. H., & Richter, C. (1981) *Eur. J. Biochem. 117*, 361–367.

Honjo, T., Nishizuka, Y., Hayaishi, O., & Kato, I. (1968) *J. Biol. Chem. 243*, 3553–3555.

Hsia, J. A., Tsai, S.-C., Adamik, R., Yost, D. A., Hewlett, E. L., & Moss, J. (1985) *J. Biol. Chem. 260*, 16187–16191.

Iglewski, B. H., Liu, P. V., & Kabat, D. (1977) *Infect. Immun. 15*, 138–144.

Iglewski, W. J., Lee, H., & Muller, P. (1984) *FEBS Lett. 173*, 113–118.

Jacobson, M. K., Payne, D. M., Alvarez-Gonzalez, R., Juarez-Salinas, H., Sims, J. L., & Jacobson, E. L. (1984) *Methods Enzymol. 106*, 483–494.

Kaplan, N. O., Colowick, S. P., & Barnes, C. C. (1951) *J. Biol. Chem. 191*, 461–472.

Katada, T., & Ui, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3129–3133.

Kreimeyer, A., Adamietz, P., & Hilz, H. (1985) *Biol. Chem. Hoppe-Seyler 366*, 537–554.

Kun, E., Zimber, P. H., Chang, A. C. Y., Puschendorf, B., & Grunicke, H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1436–1440.

Lee, H., & Iglewski, W. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2703–2707.

Lowery, R. G., Saari, L. L., & Ludden, P. W. (1986) *J. Bacteriol. 166*, 513–518.

Moss, J., & Vaughan, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4396–4400.

Moss, J., Yost, D. A., & Stanley, S. J. (1983) *J. Biol. Chem. 258*, 6466–6470.

Moss, J., Jacobson, M. K., & Stanley, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5603–5607.

Moss, J., Oppenheimer, N. J., West, R. E., Jr., & Stanley, S. J. (1986) *Biochemistry 25*, 5408–5414.

Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M., & Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol. 34*, 781–786.

Ogata, N., Ueda, K., & Hayaishi, O. (1980a) *J. Biol. Chem. 255*, 7610–7615.

Ogata, N., Ueda, K., Kagamiyama, H., & Hayaishi, O. (1980b) *J. Biol. Chem. 255*, 7616–7620.

Payne, D. M., Jacobson, E. L., Moss, J., & Jacobson, M. K. (1985) *Biochemistry 24*, 7540–7549.

Richter, C., Winterhalter, K. H., Baumhüter, S., Lötscher, H. R., & Moser, B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3188–3192.

Richter, C., Schlegel, J., & Frei, B. (1985) in *ADP-Ribosylation of Proteins* (Althaus, F. R., Hilz, H., & Shall, S., Eds.) pp 530–535, Springer-Verlag, Berlin.

Smith, K. P., Benjamin, R. C., Moss, J., & Jacobson, M. K. (1985) *Biochem. Biophys. Res. Commun. 126*, 136–142.

Ueda, K., & Hayaishi, O. (1985) *Annu. Rev. Biochem. 54*, 78–100.

Williamson, J. R., & Corkey, B. E. (1969) *Methods Enzymol. 13*, 434–509.