Mono- and poly-ADP-ribosylation of proteins in mouse kidney after castration and testosterone treatment

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Protein-bound mono(ADP-ribose) and poly(ADP-ribose) residues were determined in mouse kidney after castration and testosterone substitution. After these treatments, the mouse kidney undergoes significant alterations in the extent and pattern of transcription without changes in the amount of DNA and nuclear protein. The amount of mono(ADP-ribose)–protein conjugates (the hydroxylamine-sensitive and -resistant subfractions) decreased by 40% after castration, and returned to normal within 1 week after daily testosterone injections. Polymeric ADP-ribose residues, which amounted to less than 0.3% of the total protein-bound monomeric ADP-ribose, increased after castration and rapidly decreased on testosterone administration. The magnitude of these effects indicates that the decrease in mono(ADP-ribose) was not caused by a shift of monomeric residues into the polymer form. Nuclear ADP-ribosyltransferase activity showed a retarded decrease after castration, reaching 60% of the control value by day 20. After testosterone injections, enzyme activity rose to normal within 3–4 days. The amounts of the substrate NAD^+ as well as of $\text{NAD}^+ + \text{NADH}$ also declined after castration, and rapidly returned to values slightly above normal when the androgen was substituted. The differential response of monomeric and polymeric ADP-ribose residues to castration and testosterone treatment suggests that the two modifications serve different functions.

Modification of nuclear proteins by transfer of ADP-ribose residues from NAD to form mono(ADP-ribose)– and poly(ADP-ribose)–protein conjugates occurs in all eukaryotic cells so far studied. These modification reactions have been implicated in the regulation of DNA synthesis, transcription, DNA repair and cell differentiation, largely based on measurements of ADP-ribosyltransferase activities in isolated nuclear systems (for review see Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell *et al.*, 1980). Since the enzymes involved in formation and degradation of (ADP-ribose)_n–protein conjugates can be profoundly influenced by unphysiological changes in chromatin structure (e.g. DNA breakage; cf. Miller, 1975; Davies *et al.*, 1977; Berger *et al.*, 1978), reliable data as to the function of the ADP-ribosylation reactions are difficult to obtain.

Quantitative analyses in hepatic tissues of endogenous amounts of protein-bound ADP-ribose residues had indicated that most protein acceptor sites are occupied by single ADP-ribose residues, not by polymeric ADP-ribose chains. Recent data suggest that protein modification by poly(ADP-ribose) may

be involved in DNA-repair processes (Berger *et al.*, 1980; Benjamin & Gill, 1979; Juarez-Salinas *et al.*, 1979; Durkacz *et al.*, 1980). On the other hand, changes in the amounts of mono(ADP-ribose)–protein conjugates were observed during tissue differentiation (cf. Stone & Hilz, 1975; Hilz *et al.*, 1979) and during the cell cycle (Wielckens *et al.*, 1979). An extension of these studies to systems undergoing changes in response to steroid hormones appeared to be appropriate, since cortisol induces altered rates of ADP-ribosylation in isolated chick-embryo liver nuclei (Kitamura *et al.*, 1979). We chose as a model system the kidney of male mice, which is a target organ for androgens. After castration the kidney weight decreases by one-third. The original weight can be restored by testosterone administration. These changes represent trophic responses to the hormone of the epithelial cells in the proximal convoluted tubules. The cells do not divide, nor do they synthesize significant amounts of DNA, after hormone treatment (Kochakian & Harrison, 1962; Ohno & Lyon, 1970). These and other reports support the concept

that testosterone regulates transcription in mouse kidney, including the formation of several specific enzymes and their mRNA species (Kochakian, 1962; Ohno *et al.*, 1970; Dofuku *et al.*, 1971; Paigen *et al.*, 1979). In the present study we investigated the amounts of protein-bound mono-(ADP-ribose) residues and of two subfractions in mouse kidney after castration and testosterone administration. These data were compared with the amounts of protein-bound poly(ADP-ribose), the ADP-ribosyltransferase activity and the substrate NAD.

Materials and methods

Testosterone propionate was kindly provided by Dr. F. Neumann (Schering A.G., Berlin). NAD⁺ (99.8% pure), phosphoenolpyruvate, ATP, adenylylase kinase and pyruvate kinase were purchased from Boehringer (Mannheim, Germany). ADP-ribose was obtained from ICN (Cleveland, OH, U.S.A.). 5'-AMP-specific antiserum was drawn from rabbits after the third booster with N⁶-carboxymethyl-ADP-ribose conjugated to bovine serum albumin as described previously (Bredehorst *et al.*, 1978a,b). 5'-[2-³H]AMP (3.55 × 10¹⁰ d.p.m./μmol) was a product of Amersham-Buchler (Braunschweig, Germany). [adenine-2-³H]NAD (5.9 × 10⁷ d.p.m./μmol) was prepared from [2-³H]ATP (Amersham-Buchler) as described previously (Nolde & Hilz, 1972).

Animals and their treatment

Male mice of the NMRI strain, about 12 weeks old and weighing 35–43 g, were castrated by the scrotal route under ether anaesthesia. Testosterone treatment was performed by daily intraperitoneal injections of 10 mg of testosterone propionate (suspended in 0.25 ml of sesame oil) starting on day 20 after castration. The mice were killed by cervical dislocation at the times indicated in the Figure legends, and the kidneys were immediately excised, freeze-clamped, weighed and coarsely ground in a mortar under liquid N₂. The kidney powder was stored under liquid N₂. Control mice received daily injections of 0.25 ml of sesame oil for 7 days before being killed on day 8.

Enzymic determination of NAD⁺

This was done by the method of Klingenberg (1974), and determination of NAD⁺ + NADH was by radioimmunoassay as described previously (Bredehorst *et al.*, 1979a).

Determination of monomeric ADP-ribose residues

This has been described in detail previously (Bredehorst *et al.*, 1978c).

Determination of poly(ADP-ribose)

Trichloroacetic acid-insoluble fractions were prepared as described previously (Bredehorst *et al.*, 1978b); 100 mg of the dried material was dissolved in 6 M-guanidine hydrochloride/50 mM morpholine buffer, pH 8.5. Poly(ADP-ribose) was released from the protein by alkali treatment (0.3 M-NaOH, 1 h at 56°C), neutralized with 6 M-HCl, and freed from DNA by centrifugation (175 000 g for 17 h). The supernatant was chromatographed at pH 8.5 on a Bio-Rex 70-boronate column (prepared from Bio-Rex 70 and *p*-aminophenylboronic acid; see Braeuer *et al.*, 1981). Two samples of the eluted poly(ADP-ribose) fraction were incubated in phosphate buffer, pH 7.5, for 1 h with and without 2.4 munits of purified phosphodiesterase I, followed by heat-inactivation of the enzyme. Contaminating 5'-AMP was converted into ATP as described by Goebel *et al.* (1977), and residual AMP was determined by radioimmunoassay (Bredehorst *et al.*, 1978b). Phosphoribosyl-AMP formed by the phosphodiesterase treatment was then quantified in a radioimmunoassay using an antiserum to 2'-phosphoribosyl-AMP which was coupled to bovine serum albumin by the periodate method (Sakura *et al.*, 1978). [³H]Poly(ADP-ribose) tracer was added before the removal of DNA to correct for losses. The validity of the method was controlled by an independent procedure involving enzymic conversion of poly(ADP-ribose) into phosphoribosyl-AMP before ion-exchange and high-pressure liquid chromatography, yielding identical results. The details of the procedure are described by Wielckens *et al.* (1981).

Enzyme assays

ADP-ribosyltransferase activity was assayed in kidney nuclei under conditions slightly modified from those described by Hilz & Kittler (1971). About 150 mg of kidney powder was homogenized in 3 ml of buffer A (0.9 mM-MgCl₂, 0.9 mM-CaCl₂, 25 mM-KCl, 0.14 mM-spermidine, 1 mM-dithiothreitol, 5 mM-Tris/HCl, pH 7.5; Chevallier & Philippe, 1973) containing 0.5 M-sucrose and 0.2% Triton X-100, by six strokes in a glass/Teflon homogenizer. The homogenate was mixed with 6 ml of buffer A containing 2.2 M-sucrose, and centrifuged for 40 min at 30 000 rev./min and 4°C in a Beckman SW40 rotor through a 3.5 ml cushion of 2.2 M-sucrose in buffer A. The nuclear pellet was resuspended in 0.5 ml of 5 mM-MgCl₂/1 mM-dithiothreitol/5 mM-Tris/HCl, pH 8.0. The recovery of DNA was usually 60% of the cellular DNA. For the assay of ADP-ribosyltransferase activity, 75 μl of the nuclear suspension was incubated for 10 min at 25°C in a total volume of 150 μl containing 10 mM-MgCl₂, 0.1 mM-dithiothreitol, 100 mM-Tris/HCl, pH 8.0, and 1 mM-[³H]NAD⁺. Enzyme activity

was also assayed after addition of 200 μ g of deoxyribonuclease I/ml and Triton X-100 (0.05% final concn.) to the reaction mixture. Enzyme activity is expressed as μ mol of ADP-ribose made acid-insoluble/10 min per mg of DNA.

β -Glucuronidase was determined by the procedure of Talalay *et al.* (1946).

Other analytical procedures

RNA was measured by the method of Schneider (1957) with yeast tRNA as a standard, and DNA by that of Burton (1968), with calf thymus DNA as a standard. The biuret reaction, modified by Kingsley (1939), or the method of Lowry *et al.* (1951), was used for the determination of protein, with bovine serum albumin as a standard. Polyamine-stabilized nuclei (Hewish & Burgoyne, 1973) were used to determine the protein content in kidney nuclei.

Results

Alteration of amounts of protein-bound mono- and poly-(ADP-ribose)

It is the aim of the present paper to study the ADP-ribosylation of proteins in mouse kidney as a

function of castration and testosterone treatment. Therefore a brief illustration of the experimental system is appropriate. After castration of male mice, the kidney wet weight decreased within 10 days by 28% (see Fig. 1). When testosterone was administered to these castrated animals, the kidneys regained their original weight in a short period. Total protein and total RNA decreased after castration by 28% and 39% respectively, and rapidly returned to normal within 5 days of testosterone treatment. β -Glucuronidase activity fell to a low value when testosterone supply was withdrawn, and showed an overshooting reaction after a lag phase after injection of testosterone. In contrast with these parameters, the contents of DNA per kidney and of nuclear protein (see legend to Fig. 1) were not significantly affected by castration and testosterone treatment. These results demonstrate that the observed changes in wet weight and gene expression do not relate to cell and DNA replication, thus confirming previous reports by other investigators (Kochakian & Harrison, 1962; Dofuku *et al.*, 1971).

Kidneys of untreated male mice were found to contain 6.2 nmol of total protein-bound mono-(ADP-ribose) residues/mg of DNA. Mono(ADP-

Fig. 1. Effect of castration and testosterone administration on various kidney parameters

Male mice were castrated and killed 10 or 20 days later. Testosterone administration to castrated males (daily intraperitoneal injections of 10 mg of testosterone propionate) was started on day 20 after castration. The mice were killed at 2, 5 and 24 h and 2, 5 and 7 days after the first injection. The kidneys from each experimental group (nine or ten mice) were pooled before being powdered; ○, wet wt. (mean values for 20 determinations). Standard deviations varied from ± 6 to $\pm 10\%$. Kidney weight (mean of left and right kidneys) is expressed as mg/20 g body wt. ●, DNA content (mean values for four determinations). Standard deviations varied from ± 5 to $\pm 18\%$. Δ , β -Glucuronidase activity. □, RNA content (mean values for two determinations). $\blacktriangle$, Total cellular protein content (mean values for two determinations). The nuclear protein content in kidneys of untreated mice, of mice 20 days after castration and of castrated mice after 7 days of testosterone administration were determined as 2.2 ± 0.1 , 2.3 ± 0.2 and 2.2 ± 0.1 mg of protein/mg of DNA respectively. C, castration; T, onset of daily testosterone injections.

ribose)-protein conjugates can be subdivided into two fractions, one releasing ADP-ribose on treatment with neutral hydroxylamine, the other being hydroxylamine-resistant. Both subfractions, however, are sensitive to cleavage by alkali. The ADP-ribose residues released by NaOH are therefore designated total protein-bound mono(ADP-ribose) residues (Bredehorst *et al.*, 1978c). In untreated animals the hydroxylamine-released and -resistant fractions accounted for 29% and 71% respectively of the total (see Fig. 2). Castration produced a 38% decrease in the total ADP-ribose

Fig. 2. Amounts of protein-bound mono(ADP-ribose) residues after castration and testosterone administration

Mice were castrated and injected with testosterone at the times indicated in the legend of Fig. 1. About 1.2 g of kidney powder from each experimental group was precipitated with trichloroacetic acid and used for determination as described in the Materials and methods section. Δ , Total mono(ADP-ribose) residues (released by NaOH). $\circ$, Hydroxylamine-released subfraction of monomeric ADP-ribose residues. Data points represent mean values $\pm$ S.D. for five determinations (controls) and three determinations (mice after 20 days of castration and castrated mice injected with testosterone for 7 days). All other experimental points are mean values from two separate determinations, involving 5–12 radioimmunoassays from separate suspensions and hydrolysed in duplicates. C, castration; T, onset of daily testosterone injections.

residues within 10 days. After daily testosterone injections to castrated animals, the amount of mono-ADP-ribosylated conjugates increased gradually, returning to the value observed in untreated mice within 7 days. The response of both mono(ADP-ribose)-protein-conjugate subfractions to castration and testosterone substitution was similar to that of total monomeric ADP-ribose residues, no significant changes in the ratio of hydroxylamine-sensitive to hydroxylamine-resistant fractions being observed. Since the target cells of testosterone comprise only about two-thirds of the total kidney cells, the change in mono-ADP-ribosylation in proximal-tubule cells of castrated animals may be even more pronounced.

It might be expected that the decrease in monomeric ADP-ribose residues observed after castration (-2400 pmol; Fig. 2) was caused by an elongation to the polymeric form. If such a mechanism existed, the polymeric fraction should increase correspondingly, i.e. by at least 3×2400 pmol of ADP-ribose residues if the monomers present were elongated to trimers. To test this possibility we determined the amount of ADP-ribose residues present in the polymer form after castration and testosterone treatment. In normal kidney, polymeric ADP-ribose residues were found in very low concentrations, less than 0.3% of that of monomeric residues (Table 1). Castration produced an increase in poly(ADP-ribose) (Table 1), but this increase (16 pmol of ADP-ribose residues/mg of DNA) was more than two orders of magnitude lower than the number of monomers disappearing under these conditions. This excludes the possibility that the observed decrease in monomeric ADP-ribose residues was caused by an elongation to the polymeric form. Such a conclusion is also in line with recent data from our laboratory on the intracellular distribution of ADP-ribosylated proteins. They indicate that a substantial amount, if not the majority, of the mono-ADP-ribosylated proteins is not located in the nucleus, but is found in extra-nuclear compartments (P. Adamietz, K. Wielckens, R. Bredehorst & H. Hilz, unpublished work).

ADP-ribosyltransferase activity

In view of the differential response of mono- and poly-ADP-ribosylated proteins after castration and testosterone treatment, it was decided to monitor the activity of ADP-ribosyltransferase under these conditions. To avoid perturbation of the assay by factors present in crude tissue homogenates (cf. Hilz & Stone, 1976), we determined the transferase activity in purified nuclei. Enzyme activity proceeded linearly for at least 10 min in nuclei from both control and castrated animals (Fig. 3, inset). Furthermore, enzyme activity per unit of DNA was independent of the actual yield in nuclei (results not

Table 1. Protein-bound polymeric and monomeric ADP-ribose residues in kidneys under the influence of castration and testosterone replacement

Results are mean values $\pm$ s.d. performed in quadruplicates [poly(ADP-ribose)] or of eight determinations [mono(ADP-ribose)] from pooled (9–11 animals) acid-insoluble fractions. For further details see the Materials and methods section.

Treatment	ADP-ribose residues (pmol/mg of DNA)		
	Polymeric	Monomeric	Poly/mono
None	19 $\pm$ 3	6760 $\pm$ 220	2.8 $\times 10^{-3}$
Castrated, 20 d	34 $\pm$ 6	3670 $\pm$ 200	9.3 $\times 10^{-3}$
Castrated + testosterone, 2 h	34 $\pm$ 5	4080 $\pm$ 570	8.3 $\times 10^{-3}$
1 day	16 $\pm$ 3	3460 $\pm$ 1760	4.6 $\times 10^{-3}$
5 days	21 $\pm$ 3	5660 $\pm$ 490	3.7 $\times 10^{-3}$

Fig. 3. Changes in nuclear ADP-ribosyltransferase activity after castration and testosterone administration. Kidney nuclei were prepared and incubated with [3 H]NAD $^+$ as described in the Materials and methods section. Enzyme activity was assayed in the absence (O) or presence (Δ) of deoxyribonuclease (DNAase) I and Triton X-100. Data points with bars represent mean activities $\pm$ s.d. for three nuclei preparations; the other points are mean values from two nuclei preparations. C, castration; T, onset of daily testosterone injections. Inset: kinetics of ADP-ribosyltransferase activity without (O) and with (Δ) added DNAase I and Triton X-100 (control animals).

transferase activity in the presence and absence of this nuclease. Addition of deoxyribonuclease I to kidney nuclei stimulated the enzyme activity by 12–30%. These data indicate that endogenous deoxyribonucleases were sufficiently active to elicit nearly maximal ADP-ribosyltransferase activity.

ADP-ribosyltransferase activity in kidney nuclei from untreated mice averaged about 1.2 μ mol of ADP-ribose/min per mg of DNA when the enzyme was assayed after stimulation by deoxyribonuclease I. Fig. 3 shows that castration produced a gradual decrease in enzyme activity. By day 20 after castration, activity had reached a value of about 60% of that in untreated mice. It is noteworthy that the decrease in ADP-ribosyltransferase activity after castration was clearly retarded when compared with the changes in mono(ADP-ribose) residues. On the other hand, when testosterone was injected into castrated mice, a relatively rapid restitution of enzyme activity was observed. Thus the response of total nuclear ADP-ribosyltransferase activity could be correlated neither with the changes in amounts of poly(ADP-ribose) conjugate nor with the alterations in amounts of mono(ADP-ribose)-protein.

From studies with permeabilized cells, it may be concluded that, compared with the enzyme activity in isolated nuclei, ADP-ribosyltransferase activity *in vivo* is much lower (Berger *et al.*, 1978; Hall-dorsson *et al.*, 1978; Bredehorst *et al.*, 1979b). Corresponding analyses in kidney tissue, however, must await the development of reliable procedures to measure the 'intrinsic' activity in whole tissues or tissue slices.

Amounts of NAD $^+$ and NADH

Although deprivation of testosterone resulted in divergent reactions of polymeric and monomeric ADP-ribose residues, the decrease in the bulk material [mono(ADP-ribose)-protein conjugates] could still be caused by a decrease in the substrate NAD $^+$, if the enzymes involved in synthesis of the subfractions had different K_m values. We therefore included an analysis of the amounts of NAD $^+$.

shown). As ADP-ribosyltransferase activity in nuclei or in permeabilized cells of various tissues can be stimulated by the addition of deoxyribonuclease I (Miller, 1975; Berger *et al.*, 1978), we measured the

Castration produced a decrease in NAD^+ . By day 10, it fell to about 72% of the controls (Fig. 4). After daily injections of testosterone NAD^+ returned to control values within a rather short period. Continuing the testosterone treatment produced a further increase of the NAD^+ above normal amounts. In order to exclude the possibility that the changes in NAD^+ concentrations were caused by a shift between the oxidized and the reduced forms of the coenzyme, we also determined the sum of $\text{NAD}^+ + \text{NADH}$ by a newly developed procedure (Brede-

horst *et al.*, 1979a). These measurements showed that NADH accounted for about one-third of the total coenzyme (Fig. 4), which compares favourably with earlier reported values (Jedeikin & Weinhouse, 1955). The changes in $\text{NAD}^+ + \text{NADH}$ seen after castration and testosterone substitution were nearly identical with the changes in NAD^+ . Since the nicotinamide nucleotides and the wet weight responded similarly to castration and testosterone treatment, it is not surprising that the amounts of NAD(H) based on wet weight remained nearly unchanged. This could mean that the concentration of the substrate remained unaltered after castration in spite of an absolute loss per tubular cell.

Discussion

The mouse kidney responds to castration and testosterone treatment with marked changes in a number of basic metabolic and cellular parameters, including transcriptional activity (Kochakian, 1962; Paigen *et al.*, 1979), whereas DNA synthesis and total DNA content are not affected (Kochakian & Harrison, 1962; Ohno & Lyon, 1970). The present results show significant alterations in the extent of protein ADP-ribosylation after castration and testosterone administration, which appear to relate to the trophic changes induced by these treatments. To our knowledge this is the first report of changes in the endogenous amounts of mono- and poly-(ADP-ribose) residues as a result of a hormonal stimulus. Previous analyses (cf. Kitamura *et al.*, 1979) were mostly concerned with the activity of ADP-ribosyltransferase to monitor changes in ADP-ribose metabolism.

The mechanism by which the decrease in mono-(ADP-ribose) occurs appears to be of considerable interest. We first investigated the possibility that monomeric residues were elongated to the polymeric form. Although the amount of poly(ADP-ribose) residues increased after castration, the magnitude of this effect excludes the possibility that the decrease in monomeric residues can be explained by an elongation to the polymeric form. Further, recent studies using cell fractionation have indicated that the majority of mono-ADP-ribosylated proteins in intact cells is not localized in the nucleus (P. Adamietz, K. Wielckens, R. Bredehorst & H. Hilz, unpublished work). Since the polymerizing enzyme appears to be confined to the nucleus (cf. Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell *et al.*, 1980), the majority of the monomeric residues cannot be the substrate of an elongation process. Therefore, to explain the decrease in mono-(ADP-ribose) residues after castration, other mechanisms such as the removal of the ADP-ribose group from the acceptor protein, the

Fig. 4. Amounts of cellular $\text{NAD}^+ + \text{NADH}$ after castration and testosterone administration

NAD^+ and $\text{NAD}^+ + \text{NADH}$ were determined in the acid-soluble fractions after homogenization of the powdered kidneys in trichloroacetic acid and HClO_4 , respectively. NAD^+ (O) was measured enzymically. For three experimental groups (untreated mice, mice 20 days after castration and castrated mice treated for 7 days with testosterone) data points represent mean values $\pm$ s.d. of four determinations, and for the other experimental groups means values of two determinations. The amount of $\text{NAD}^+ + \text{NADH}$ ($\square$) was measured radioimmunochemically (Bredehorst *et al.*, 1979a). For untreated mice, mice after 20 days of castration, and castrated mice treated for 7 days with testosterone, data points represent mean values $\pm$ s.d. for five determinations, and for the other experimental groups mean values of two determinations. C, castration; T, onset of daily testosterone injections.

total degradation of the modified proteins or changes in the rates of conjugate synthesis must be considered. Unfortunately, very little is known about the interrelationship of synthesizing and degrading reactions and the enzymes involved. In the present study, we determined the activity of (total) nuclear ADP-ribosyltransferase. This enzyme activity, presumably responsible for the modification of nuclear proteins, is thought to elongate as well as to initiate protein-linked (ADP-ribosyl)_n chains (Okazaki *et al.*, 1980; Kawaichi *et al.*, 1980). At first sight, the activity appeared to relate to the mono(ADP-ribosyl) fraction rather than to that of the polymer. The castration-induced decrease in enzyme activity, however, was retarded as compared with that of the mono(ADP-ribose) protein conjugates. From these data, it may be concluded that the gross changes in both types of conjugates (mono- and poly-ADP-ribosylated proteins) were independent of total nuclear transferase activity (cf. Wielckens *et al.*, 1980). This is certainly not surprising with respect to that fraction of the mono(ADP-ribose) proteins which is not localized in the nucleus, probably the majority of the mono(ADP-ribose) conjugates (P. Adamietz, K. Wielckens, R. Bredehorst & H. Hilz, unpublished work). They must be synthesized by transferases distinct from the nuclear enzyme. Indeed, recent data from our laboratory show the existence of ADP-ribosyltransferases in the extra-nuclear compartments (R. Bredehorst, P. Adamietz, K. Wielckens & H. Hilz, unpublished work). Previous studies on avian erythrocytes have also demonstrated the occurrence of an ADP-ribosyltransferase in the plasma membrane (Moss & Vaughan, 1978).

In summary, the independent changes of mono- and poly-(ADP-ribose) residues and their localization in various compartments of the cell indicate that covalent modification of proteins by ADP-ribosyl groups is a more general and more versatile means to alter the function of multiple proteins than was previously thought. In this respect it becomes comparable with the modification of proteins by phosphorylation.

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