Enzymic, cysteine-specific ADP-ribosylation in bovine liver mitochondria

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NAD⁺ glycohydrolase (NADase) and non-enzymic ADP-ribosylation have been thought to be involved in the regulation of mitochondrial Ca²⁺ fluxes. In this study it was found that several conditions (5 mM nicotinamide, 5 mM 3-aminobenzamide, 2 mM EDTA, 1 mM ATP, 10 mM dithiothreitol) known to strongly inhibit the NADase decreased ADP-ribosylation in bovine liver mitochondrial membranes with [³²P]NAD⁺ as substrate to only a limited extent, if at all. The reaction led to the specific modification of two proteins with apparent molecular masses of approx. 26 and 53 kDa. An excess of added free ADP-ribose diminished the incorporation of label from [³²P]NAD⁺ only slightly. Dithiothreitol inactivated the NADase, whereas ADP-ribosylation was unaffected. At low concentrations (25 µM) ADP-ribosylation was efficient with NAD⁺, but not ADP-ribose,

as substrate. Under these conditions mitochondrial ADP-ribosylation seems to occur as an enzymic reaction rather than a non-enzymic transfer of ADP-ribose previously liberated from NAD⁺ by NAD⁺ glycohydrolase. The chemical stability of the protein-ADP-ribose bonds in the mitochondrial membranes indicated that cysteine residues are the predominant acceptors. Moreover, yeast aldehyde dehydrogenase, known to be a substrate for thiol-associated ADP-ribosylation, was efficiently ADP-ribosylated by using the mitochondrial activity and NAD⁺ as substrate. The modification of a cysteine residue in the aldehyde dehydrogenase was verified by the observation that pretreatment of this acceptor protein with *N*-ethylmaleimide substantially decreased its modification. It is therefore concluded that bovine liver mitochondria contain a cysteine-specific ADP-ribosyltransferase.

INTRODUCTION

The involvement of NAD⁺ in signalling pathways has gained tremendous interest in recent years. Several reactions have been identified that utilize NAD⁺ as substrate for covalent protein modification (ADP-ribosylation) or formation of a signalling molecule, cyclic ADP-ribose (cADPR). cADPR has been found to exert a Ca²⁺-mobilizing activity comparable with that of inositol 1,4,5-trisphosphate, in a variety of cells and tissues (reviewed in [1,2]).

Enzymic ADP-ribosylation of proteins includes the direct transfer of the ADPR moiety from NAD⁺ to the acceptor protein. A number of eukaryotic mono-ADP-ribosyltransferases have been isolated and characterized [3]. Many of these enzymes attach the ADPR to arginine residues of the acceptor protein [3]. Cysteine residues have also been found to be acceptor sites for ADPR [4,5]. However, it has been suggested that cysteine modification might be non-enzymic, involving the cleavage of NAD⁺ by NAD⁺ glycohydrolase (NADase) followed by a non-enzymic reaction of ADPR with cysteine residues [3,6].

In mitochondria the hydrolysis of pyridine nucleotides has been linked to the efflux of Ca²⁺ [7,8]. It has been proposed that under oxidative conditions the mitochondrial NADase is activated, leading to the accumulation of free ADPR. Subsequent non-enzymic ADP-ribosylation would then cause the activation of a specific Ca²⁺-release pathway [9]. Recent studies have indeed provided evidence that the oxidation of vicinal thiols, presumably the formation of a disulphide bridge, activates the mitochondrial NADase [10,11]. However, this enzyme was demonstrated to possess ADP-ribosyl cyclase activity. It was therefore proposed that the pro-oxidant-induced release of Ca²⁺ from mitochondria might be the result of a signalling pathway involving the formation of the messenger molecule cADPR [10].

These observations indicated that the role of the mitochondrial NADase might be unrelated to ADP-ribosylation. Moreover, the

physiological significance of non-enzymic mitochondrial ADP-ribosylation has been called into question by the finding that in the presence of nicotinamide the NADase was almost inactive, whereas the extent of ADP-ribosylation was only slightly diminished [12,13]. The present study was therefore undertaken to characterize the relationship between mitochondrial NADase activity and ADP-ribosylation. It is demonstrated that ADP-ribosylation in bovine liver mitochondria proceeds enzymically rather than non-enzymically. The predominant acceptor sites of the modified proteins have been identified as cysteine residues. Furthermore yeast aldehyde dehydrogenase (ALDH) served as an acceptor protein of a cysteine-specific ADP-ribosyltransferase present in the mitochondrial preparation.

EXPERIMENTAL

Materials

³²P-labelled NAD⁺ was obtained from ICN; ¹⁴C-labelled NAD⁺ was from Amersham. 1,*N*⁶-Etheno-NAD⁺ (ε-NAD⁺) was synthesized in this laboratory by the method of Barrio et al. [14]. Yeast ALDH was purchased from Boehringer-Mannheim. All other reagents were of analytical grade.

Isolation of mitochondria

Bovine liver mitochondria were isolated as described [15], resuspended in 10 mM Tris/HCl (pH 7.5)/250 mM sucrose/0.5 mM EDTA and stored frozen (−70 °C). For some experiments the postmitochondrial supernatant was recentrifuged at 180000 *g* and the pellet ('microsomes') resuspended in the same buffer. Mitochondria used for ADP-ribosylation assays were washed twice in 50 mM Tris/HCl, pH 7.5, resuspended in the same buffer and stored frozen. The organelles so prepared respired on

Abbreviations used: (c)ADPR, (cyclic) ADP-ribose; ALDH, aldehyde dehydrogenase; DTT, dithiothreitol; ε-NAD⁺, 1,*N*⁶-etheno-NAD⁺; NADase, NAD⁺ glycohydrolase; NEM, *N*-ethylmaleimide.

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the addition of NADH. Therefore the inner side of the inner membrane was accessible to metabolites such as NAD^+ . Consequently this mitochondrial preparation will be more accurately termed 'mitochondrial membranes'. The assignment to membranes (rather than matrix) of the activities reported here was further supported by control experiments with submitochondrial particles (obtained by cycling the preparation through a French press and subsequent sedimentation of the particles): ADP-ribosylation in these particles proceeded similarly to that in the mitochondrial membranes.

ADP-ribosylation assay

The standard incubation mixture contained, in a final volume of 50 μl , 50 mM Tris/HCl, pH 7.5, and 25 μM [^{32}P]NAD $^+$ (1 $\mu\text{Ci}/\text{nmol}$). Some experiments were conducted with [*adenine- ^{14}C*]NAD $^+$ or [*nicotinamide- ^{14}C*]NAD $^+$. Other additions were made as indicated in the Figure legends. The reaction was started by the addition of mitochondrial membranes (0.2 mg). After incubation at 30 $^{\circ}\text{C}$ for the time indicated (usually 15 min) the reaction was stopped by adding 450 μl of ice-cold acetone followed by a 10 min incubation on ice. Protein was then precipitated by centrifugation. The pellets were resuspended in the sample buffer used for gel electrophoresis. The use of trichloroacetic acid instead of acetone led to a decrease in incorporated label and poorer resolution on SDS/PAGE. After electrophoresis the gels were stained with Coomassie Blue, dried and then subjected to autoradiography. Non-enzymic ADP-ribosylation was studied under the same conditions with [^{32}P]ADPR as the substrate instead of [^{32}P]NAD $^+$. [^{32}P]ADPR was synthesized by the treatment of [^{32}P]NAD $^+$ with NADase from *Neurospora crassa* (Sigma). After the reaction had been completed (as checked by TLC) the solution was heated at 95 $^{\circ}\text{C}$ for 5 min to inactivate the enzyme.

Determination of NADase activity

Mitochondrial NADase from bovine liver was partly purified as described [16]. For NADase measurements a fluorimetric assay using the analogue $\epsilon\text{-NAD}^+$ [14] was performed at room temperature in a solution consisting of 50 mM Tris/HCl, pH 7.5, 0.03% lauryl dimethylamine *N*-oxide and 10 μM $\epsilon\text{-NAD}^+$. The final volume was 700 μl . The reaction was started by adding an appropriate amount of enzyme. Cleavage of $\epsilon\text{-NAD}^+$, which caused an approx. 10-fold fluorescence enhancement, was followed at an emission wavelength of 410 nm. The excitation wavelength was 310 nm.

Protein separation by SDS/PAGE

Proteins were separated in minigels (Hoefer Scientific) containing 12% (w/v) polyacrylamide by the method of Laemmli [17].

Protein determination

Protein was determined by a biuret procedure or the bicinchoninic acid assay from Pierce. BSA was used as standard.

All data presented are representative of at least three independent preparations.

RESULTS

ADP-ribosylation of proteins was detected after separation by SDS/PAGE after the incubation of isolated organelles in the presence of ^{32}P -labelled NAD $^+$. Covalent modification of proteins

Figure 1 Influence of various effectors on mitochondrial ADP-ribosylation

Mitochondrial membranes (0.2 mg) were incubated in a final volume of 50 μl for 15 min at 30 $^{\circ}\text{C}$ in the presence of the compounds indicated below. The medium consisted of 50 mM Tris/HCl, pH 7.5, and 25 μM [^{32}P]NAD $^+$. After incubation, protein was precipitated and the pellet was resuspended in sample buffer as described in the Experimental section. Separation was performed by SDS/PAGE (12% gel). The autoradiograph of the gel is shown. The molecular masses of marker proteins (in Da) are shown at the right. Lane 1, no additions (control); lane 2, 5 mM nicotinamide; lane 3, 0.5 mM ADPR; lane 4, 5 mM 3-aminobenzamide; lane 5, 2 mM EDTA; lane 6, 2.5 mM MgCl_2 ; lane 7, 1 mM ATP.

was then visualized by autoradiography. Figure 1 shows that specific incorporation of the label occurred primarily into two proteins of the mitochondrial membrane preparation with molecular masses of approx. 26 and 53 kDa. It has been reported [12,13] and is here confirmed that nicotinamide and 3-aminobenzamide at concentrations sufficient to block NADase activity almost completely, i.e. 5 mM, decreased mitochondrial ADP-ribosylation only slightly (Figure 1, lanes 2 and 4). At 5 mM nicotinamide no NADase activity could be detected with the fluorescence assay [16]. The presence of a 20-fold excess of unlabelled ADPR over [^{32}P]NAD $^+$ had only little effect (Figure 1, lane 3). The addition of 2 mM EDTA seemed to enhance the extent of mitochondrial ADP-ribosylation (Figure 1, lane 5), whereas the NADase was almost inactive under these conditions [16]. ADP-ribosylation was hardly detectable at 2.5 mM Mg^{2+} (Figure 1, lane 6). This effect was due to the stimulation of an enzymic activity catalysing the removal of the label from the modified proteins rather than to the inhibition of ADP-ribosylation, because the addition of Mg^{2+} after ADP-ribosylation had taken place also removed the label within a few minutes (results not shown). Whereas 1 mM ATP decreased NADase activity by more than 50% (results not shown), this nucleotide stimulated ADP-ribosylation in bovine liver mitochondrial membranes (Figure 1, lane 7). Because this stimulation was greater than that observed with EDTA (Figure 1, lane 5), it seems likely to be due to a more specific effect than the chelation of metal ions.

ADP-ribosylation as described for mitochondrial membranes (Figure 1, lane 5) was also tested in the crude tissue homogenate and in the microsomal fraction. Labelling of the two protein bands found in mitochondria (Figure 1) was approx. 4-fold and 10-fold higher in mitochondria (per mg of protein) than in the homogenate and microsomes respectively. Accordingly, succinate dehydrogenase activity was enriched approx. 7-fold in the mitochondria compared with the homogenate (results not shown).

The nature of the incorporated label was identified as ADPR as follows. First, radioactive label was incorporated from ^{14}C -

Figure 2 ADP-ribosylation of mitochondrial proteins with [*nicotinamide-¹⁴C]NAD⁺ or [*adenine-¹⁴C]NAD⁺ as substrate**

The experiment was performed as described in the legend to Figure 1 with ¹⁴C as label instead of ³²P at a specific radioactivity of 10 nCi/nmol. The upper panel shows the gel stained with Coomassie Blue [the molecular masses of marker proteins (in Da) are shown at the left]; the lower panel shows the autoradiograph. Lane M, marker proteins; lane 1, labelling with [*nicotinamide-¹⁴C]NAD⁺; lane 2, labelling with [*adenine-¹⁴C]NAD⁺.**

Table 1 Chemical stability of the protein-ADP-ribosyl linkages in mitochondrial membranes

Mitochondrial membranes were incubated in the presence of 25 μ M [³²P]NAD⁺ for 2 h at 30 °C in a medium consisting of 50 mM Tris/HCl, pH 7.5, 10 mM DTT and 1 mM EDTA. Thereafter, unbound radioactive nucleotide was removed by washing five times with incubation medium (after four washings no further decrease in the protein-associated radioactivity was detected). After the last washing the pellet of the labelled mitochondrial membranes was resuspended in 50 mM Mops, pH 7.0, and the compounds were added to the final concentrations indicated. The pH of 1 M hydroxylamine was adjusted to pH 7.0. After incubation for 2 h at 30 °C, protein was precipitated with 10% (w/v) trichloroacetic acid (final concentration); the radioactivity was determined and related to the amount of precipitated protein. The values given ($\pm$ S.E.M., $n = 3$) are corrected for the release of radioactivity determined in the control sample (incubation without addition for 2 h).

Treatment	Activity released (% of control)
44% (v/v) Formic acid	20 $\pm$ 5
1 M NaOH	90 $\pm$ 7
1 M Hydroxylamine	7 $\pm$ 4
10 mM HgCl ₂	62 $\pm$ 11

labelled NAD⁺ carrying the label in the adenine moiety (Figure 2, lane 2), but not when only the nicotinamide moiety was labelled (Figure 2, lane 1). Secondly, after the labelling with [³²P]NAD⁺ and the removal of unbound label, treatment of mitochondrial membranes with phosphodiesterase from snake venom liberated [³²P]AMP (results not shown).

The chemical stability of the ADP-ribosyl-protein bond(s) is reported in Table 1. Strong alkali (1 M NaOH) removed the incorporated ADPR almost completely. A large fraction of the label was detached from the protein in the presence of 10 mM HgCl₂, suggesting that the predominant acceptors were cysteine

Figure 3 Influence of disulphide reduction on mitochondrial ADP-ribosylation and NADase activity

(A) Autoradiograph of an ADP-ribosylation assay performed as described in Figure 1. Mitochondrial membranes were preincubated for 15 min at room temperature in the absence (lane 1) or presence (lane 2) of 10 mM DTT. The assay was then started by adding mitochondrial membranes to the assay medium lacking (lane 1) or containing (lane 2) 10 mM DTT. (B) The activity of partly purified mitochondrial NADase was measured fluorimetrically as described in the Experimental section, with ϵ -NAD⁺ as substrate. The buffer contained 50 mM Tris/HCl, pH 7.5, 0.03% lauryl dimethylamine *N*-oxide and 10 μ M ϵ -NAD⁺. The final volume was 700 μ l. DTT was added to one sample (trace 2) to a final concentration of 10 mM as indicated, whereas the control sample (trace 1) received an equal volume (7 μ l) of water. The enzyme used for the bottom trace (trace 3) was preincubated for 15 min in the presence of 10 mM DTT and then added to the assay medium also containing 10 mM DTT.

residues. There seemed to be a significant fraction (approx. 20%) of acid-labile linkages, which have been detected in mitochondria [18]. It should be noted that in these experiments the ADP-ribosylation of mitochondrial proteins was conducted under conditions that excluded a significant contribution from non-enzymic labelling [10 mM dithiothreitol (DTT)/1 mM EDTA], because the NADase was strongly inhibited (see below).

The experiments presented in Figure 1 suggested that ADP-ribosylation proceeded primarily via an enzymic pathway. This notion was substantiated by the experiments presented in Figure 3. Whereas the ADP-ribosylation of mitochondrial proteins was unaffected in the presence of 10 mM DTT (Figure 3A), the NADase was virtually inactive under these conditions (Figure 3B). Consequently there was no measurable liberation of free ADPR, which is a prerequisite for non-enzymic ADP-ribosylation with NAD⁺ as substrate. In addition, when mitochondrial membranes were incubated for 15 min in the presence of 25 μ M [³²P]ADPR (results not shown) the incorporation of label was not detected. The incorporation of radioactivity from

Figure 4 Non-enzymic ADP-ribosylation of mitochondrial membranes and yeast ALDH

Mitochondrial membranes (0.2 mg, lane 1) or ALDH (10 μ g, lane 2) were treated as described in the legend to Figure 1, except that [32 P]ADPR (25 μ M) was used instead of [32 P]NAD $^{+}$. The assay medium was supplemented with 10 mM DTT and 1 mM EDTA. The duration of the reaction was 60 min. This autoradiograph was exposed three times longer than that in Figure 1, to reveal the labelling.

Figure 5 Enzymic ADP-ribosylation of ALDH by mitochondrial ADP-ribosyl-transferase

ALDH (20 μ g) was incubated with 25 μ M of [32 P]NAD $^{+}$ or [32 P]ADPR for 15 min in the absence (lanes 1 and 3) or presence (lane 2) of 10 μ g of mitochondrial membranes. The assay medium contained 50 mM Tris/HCl, pH 7.5, 1 mM EDTA and 10 mM DTT. Mitochondrial membranes used for lane 2 were preincubated in the presence of 10 mM DTT for 15 min before their addition to the reaction mixture. The reaction was started by adding the radioactive substrate. After the incubation the samples were treated as described in the legend to Figure 1. The autoradiograph of the gel is shown. Lane 1, [32 P]ADPR; lane 2, [32 P]NAD $^{+}$ (and mitochondrial membranes); lane 3, [32 P]NAD $^{+}$. Lane 4 represents a control sample that did not contain ALDH, containing instead 0.2 mg of mitochondrial membranes and 25 μ M [32 P]NAD $^{+}$ (similar to Figure 4, lane 1).

[32 P]ADPR was detectable only when the incubation time was increased to 60 min (Figure 4, lane 1). Nevertheless the protein-bound label was a fraction of that observed with [32 P]NAD $^{+}$ as substrate and after incubation for only 15 min (Figure 1). (Note that for Figure 4 the exposure time of the gel to the X-ray film had to be increased to reveal the labelling.) A prolonged incubation of mitochondrial membranes at 1 mM [32 P]ADPR led to appreciable, but rather non-specific, labelling of mitochondrial proteins (results not shown). The non-enzymic ADP-ribosylation of ALDH has been described under similar conditions [19].

Figure 6 Modification of thiol groups in the ALDH prevents its ADP-ribosylation by mitochondrial ADP-ribosyl transferase

ALDH (20 μ g) was incubated in the presence (lane 2) of 1 mM NEM in a medium containing 50 mM Tris/HCl, pH 7.5, and 1 mM EDTA. After incubation for 30 min at 30 $^{\circ}$ C, 20 mM DTT was added and the incubation continued for 30 min. For the control sample (lane 1) aldehyde dehydrogenase was added to the medium after 1 mM NEM and 20 mM DTT had been preincubated for 30 min; then 25 μ M [32 P]NAD $^{+}$ and 10 μ g of mitochondrial membranes were added. After incubation for 15 min at 30 $^{\circ}$ C, proteins were precipitated and then separated by SDS/PAGE. The autoradiograph of the gel is shown.

However, as shown in Figure 4 (lane 2), at 25 μ M [32 P]ADPR the modification of ALDH was very weak even after 1 h.

The results presented so far indicated the presence of a cysteine-specific ADP-ribosyl transferase in our mitochondrial preparation. Such an activity was further implicated by the finding that incubating ALDH in the presence of 25 μ M [32 P]NAD $^{+}$ led to significant labelling of the protein, but only if mitochondrial membranes were added (compare lanes 2 and 3 of Figure 5). No labelling was detected when 25 μ M [32 P]ADPR was used (Figure 5, lane 1). It had been shown previously that ALDH contains an active-site cysteine residue that is specifically modified at high concentrations of ADPR and long incubation times [19]. It seems reasonable to expect that this cysteine residue was labelled by using the mitochondrial enzyme activity with NAD $^{+}$ as substrate. The modification of a cysteine residue in the ALDH with the mitochondrial activity was supported by the results shown in Figure 6. Preincubation of the acceptor (ALDH) in the presence of the thiol-specific reagent *N*-ethylmaleimide (NEM) followed by incubation with excess DTT (to inactivate the unreacted NEM) led to a substantial decrease in its modification by using NAD $^{+}$ as substrate with the mitochondrial enzyme activity.

DISCUSSION

This study establishes that bovine liver mitochondria contain an enzymic activity that catalyses the transfer of ADPR from NAD $^{+}$ to specific acceptor proteins. The enzymic nature of this process has been addressed in several ways. Mitochondrial ADP-ribosylation proceeded under a number of different conditions that cause substantial inhibition of the NADase. In addition, ADP-ribosylation was more efficient by far when NAD $^{+}$ was used as substrate rather than free ADPR, the substrate of non-enzymic ADP-ribosylation. Finally, ALDH, a potential physiological target for ADP-ribosylation [19] was found to be modified in the presence of mitochondrial membranes and NAD $^{+}$, but not by NAD $^{+}$ alone or only very weakly by free ADPR. Because

ADP-ribosylation occurred similarly in submitochondrial particles (results not shown), both the enzyme activity and the acceptors seem to reside within the membrane fraction.

The major acceptor sites of enzymic ADP-ribosylation in mitochondrial membranes were identified as cysteine residues, indicating a cysteine-specific ADP-ribosyltransferase. Similar enzyme activities have been identified in human [4] and bovine [5] erythrocytes as well as in platelets [20]. Therefore this protein modification is likely to be of physiological significance. The mitochondrial ALDH from yeast was non-enzymically modified by free ADPR, apparently in an active-site cysteine residue, leading to the inactivation of the enzyme [19]. As shown here, the efficiency of the enzymic ADP-ribosylation of ALDH with the mitochondrial ADP-ribosyltransferase activity greatly exceeds that of the non-enzymic modification. It would be reasonable to suggest that the activity of mitochondrial ALDH might be regulated by enzymic ADP-ribosylation of an active-site cysteine residue under physiological conditions.

The apparent molecular mass (26 kDa) of the smaller labelled protein is close to that of the adenine nucleotide translocase. Interestingly, this carrier contains reactive thiols and has been suggested recently to be a target of thyroid hormone-stimulated ADP-ribosylation [21]. In our experiments the application of a specific inhibitor (atractyloside) or ADP did not influence the extent nor the pattern of ADP-ribosylation (results not shown). In addition, ATP enhanced the modification of both labelled proteins (Figure 1). Therefore at this point our results do not provide further evidence for the ADP-ribosylation of the translocase.

Studies of the relationship between mitochondrial NADase and ADP-ribosylation have led to controversial results. Although non-enzymic ADP-ribosylation has been documented [22–24], the concentration (millimolar) of ADPR required to achieve substantial modification seems to be far beyond a physiologically relevant value. Moreover, even at 1 mM ADPR, it took several hours to modify yeast (mitochondrial) ALDH to a functionally significant extent [19]. It seems unlikely that such a mechanism of ADP-ribosylation might be of relevance to the regulation of ALDH activity.

Similarly to the results reported here for bovine liver mitochondrial membranes, rat liver or brain mitochondrial ADP-ribosylation was decreased only slightly at concentrations of nicotinamide or 3-aminobenzamide that caused virtually complete inhibition of the NADase [12,13]. The occurrence of ADP-ribosylation independently of the NADase activity is also of interest with regard to recent observations that the mitochondrial NADase possesses ADP-ribosyl cyclase activity [10]. It has been

concluded that the physiological role of the NADase might be related to the metabolism of cADPR rather than to the covalent modification of proteins with ADPR. As cADPR is a known Ca^{2+} -mobilizing agent, its intramitochondrial metabolism is likely to be a regulator of Ca^{2+} fluxes. The results of the present study further support the view that pro-oxidant-induced release of Ca^{2+} from the organelles is unrelated to (non-enzymic) ADP-ribosylation, because reducing conditions, which led to virtually complete inhibition of the NADase, did not alter this protein modification.

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REFERENCES

- 1 Lee, H. C. (1996) *Rec. Prog. Horm. Res.* **51**, 355–389
- 2 Ziegler, M., Jorcke, D. and Schweiger, M. (1997) *Rev. Physiol. Biochem. Pharmacol.* **131**, 89–126
- 3 Okazaki, I. J. and Moss, J. (1996) *Rev. Physiol. Biochem. Pharmacol.* **129**, 51–104
- 4 Tanuma, S., Kawashima, K. and Endo, H. (1987) *J. Biochem. (Tokyo)* **101**, 821–824
- 5 Saxty, B. A. and van Heyningen, S. (1995) *Biochem. J.* **310**, 931–937
- 6 McDonald, L. J., Wainschel, L. A., Oppenheimer, N. J. and Moss, J. (1992) *Biochemistry* **31**, 11881–11887
- 7 Lehninger, A. L., Vercesi, A. and Bababunmi, E. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3929–3933
- 8 Lötscher, H.-R., Winterhalter, K. H., Carafoli, E. and Richter, C. (1980) *J. Biol. Chem.* **255**, 9325–9330
- 9 Richter, C. and Kass, G. E. N. (1991) *Chem.-Biol. Interact.* **77**, 1–23
- 10 Ziegler, M., Jorcke, D. and Schweiger, M. (1997) *Biochem. J.* **326**, 401–405
- 11 Schweizer, M. and Richter, C. (1994) *Biochemistry* **33**, 13401–13405
- 12 Masmoudi, A. and Mandel, P. (1987) *Biochemistry* **26**, 1965–1969
- 13 Masmoudi, A., Islam, F. and Mandel, P. (1988) *J. Neurochem.* **51**, 188–193
- 14 Barrio, J. R., Secrist, III, J. A. and Leonard, N. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2039–2042
- 15 Zhang, J., Ziegler, M., Schneider, R., Klocker, H., Auer, B. and Schweiger, M. (1995) *FEBS Lett.* **377**, 530–534
- 16 Ziegler, M., Jorcke, D., Zhang, J., Schneider, R., Klocker, H., Auer, B. and Schweiger, M. (1996) *Biochemistry* **35**, 5207–5212
- 17 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- 18 Cervantes-Laurean, D., Loflin, P. T., Minter, D. E., Jacobson, E. L. and Jacobson, M. K. (1995) *J. Biol. Chem.* **270**, 7929–7936
- 19 McDonald, L. J. and Moss, J. (1993) *J. Biol. Chem.* **268**, 17878–17882
- 20 Tanuma, S., Kawashima, K. and Endo, H. (1988) *J. Biol. Chem.* **263**, 5485–5489
- 21 Mowbray, J. and Hardy, D. L. (1996) *FEBS Lett.* **394**, 61–65
- 22 Richter, C., Winterhalter, K. H., Baumhüter, S., Lötscher, H.-R. and Moser, B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3188–3192
- 23 Hilz, H., Koch, R., Fanick, W., Klapproth, K. and Adamietz, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3929–3933
- 24 Frei, B. and Richter, C. (1988) *Biochemistry* **27**, 529–535