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ALTERATIONS OF SURFACE MORPHOLOGY CAUSED BY THE METABOLISM OF MENADIONE IN MAMMALIAN CELLS ARE ASSOCIATED WITH THE OXIDATION OF CRITICAL SULFHYDRYL GROUPS IN CYTOSKELETAL PROTEINS

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Abstract—Incubation of freshly-isolated (rat hepatocytes) or cultured (HeLa, GH_3, and McCoy) mammalian cells with menadione (2-methyl-1,4-naphthoquinone) resulted in the appearance of numerous cell surface protrusions. The perturbation of surface structure was associated with an increase in the amount of cytoskeletal protein and the oxidation of sulfhydryl groups in actin, leading to the formation of high-molecular weight aggregates sensitive to treatment with thiol reductants. Our findings indicate that the oxidation of thiol groups in cytoskeletal proteins may be responsible for menadione-induced cell surface abnormalities in mammalian cells.

The metabolism of menadione (2-methyl-1,4-naphthoquinone) has been widely employed as a model to investigate the mechanisms and consequences of oxidative stress in mammalian cells [1–3]. In hepatocytes, menadione can undergo either one- or two-electron reduction reactions. One-electron reduction of the quinone results in the formation of the semiquinone free radical which, in the presence of molecular oxygen, is readily reoxidized to the parent quinone with the concomitant production of superoxide anion. At toxic levels of menadione, redox cycling of the quinone results in extensive O_2^- and H_2O_2 formation followed by glutathione oxidation and depletion [2, 4], NAD(P)H oxidation [1], protein thiol modification [2, 3], disruption of intracellular Ca^{2+} homeostasis [1, 3, 5, 6] and, finally, cell death [1, 3].

In addition to the biochemical alterations, morphological changes, characterized by the appearance of numerous surface blebs, are also observed in cells exposed to toxic levels of menadione [7]. We have recently demonstrated that menadione-induced bleb formation in hepatocytes is associated with cytoskeletal abnormalities, of which the oxidation of sulfhydryl groups in actin seems to play a crucial role [8]. We can now report that similar alterations occur during the metabolism of toxic doses of menadione in various cultured cells, suggesting that cytoskeletal thiol oxidation may represent the general mechanism of menadione-induced cell surface abnormalities in mammalian cells.

MATERIALS AND METHODS

Collagenase (grade II) was obtained from Boehringer Mannheim (Mannheim, F.R.G.) and menadione was from Sigma (St. Louis, MO). All tissue culture media and sera were from Flow Laboratories (Herts, U.K.). Other reagents were of the highest grade of purity commercially available and were obtained from local suppliers.

Hepatocytes were isolated from male Sprague–Dawley rats by collagenase perfusion of the liver as described in [9]. Incubation conditions were similar to those reported by Mirabelli *et al.* [8]. GH_3 cells were cultured in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum, HeLa cells in Eagle's minimum essential medium supplemented with 10% fetal calf serum, and McCoy cells in RPMI 1640 medium supplemented with 10% fetal calf serum. All cultures were performed in 90 mm plastic Petri dishes. The cultures were washed twice with phosphate-buffered saline (PBS) prior to the addition of the indicated concentrations of menadione in PBS.

Hepatocyte cytoskeleton was prepared as described in [8]. The cytoskeletal fraction from cultured cells was obtained by extracting the cell layer (previously washed twice with PBS) with 5 ml of a solution containing 40 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5, and 1% Triton X-100 for 30 min on ice. The layer remaining adherent to the plastic surface was then scraped with a rubber policeman and centrifuged at 4000 *g* for 20 min. The pellet obtained was washed twice in the same buffer, dissolved in 8 M urea/1% SDS and used for protein and thiol measurements and for polyacrylamide gel electrophoresis (PAGE).

PAGE was performed on 10–15% gradient slab gels by the procedure of Laemmli [10]. Immunoblotting of actin was performed essentially as described by Mirabelli *et al.* [8]. Intracellular GSH

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was measured by the HPLC technique described by Reed *et al.* [11]. Protein sulfhydryl groups were assayed using the procedure described in [2], and protein concentration was determined by the method of Lowry *et al.* [12].

RESULTS AND DISCUSSION

As shown in Fig. 1, incubation with menadione resulted in the appearance of cell surface abnormalities characterized by the protrusion of large (hepatocytes), intermediate (HeLa and McCoy cells), or small (GH_3) blebs. In all instances, the formation of surface blebs preceded cell killing, as measured by the uptake of Trypan blue. The appearance of the morphological changes were, in turn, preceded by biochemical alterations, including glutathione and protein thiol depletion (Table 1). These occurred, although at different rates and to various extents, in all four cell types investigated,

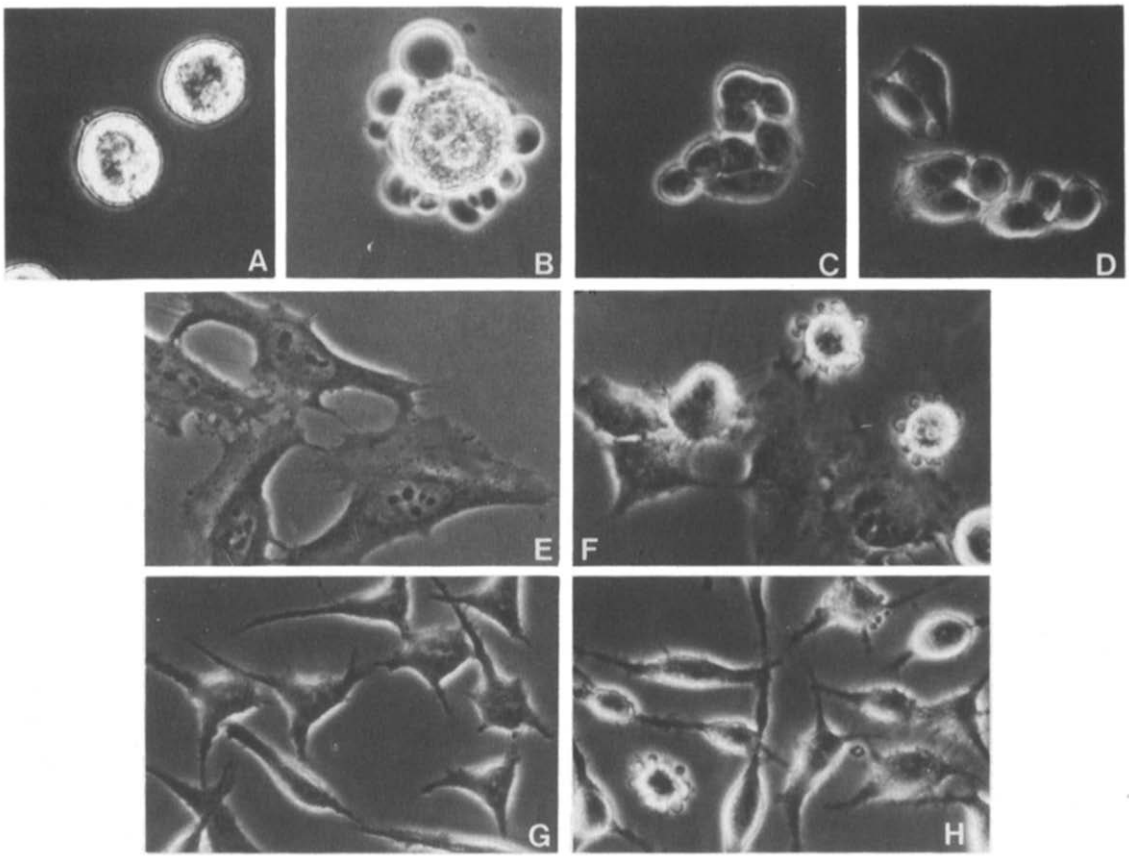

Fig. 1. Alterations in surface morphology following the metabolism of menadione in mammalian cells. Hepatocytes (A, B), GH_3 cells (C, D), HeLa cells (E, F), and McCoy cells (G, H) were incubated for 60 min in the absence (A, C, E, G) or presence (B, D, F, H) of 200 μM menadione. The cells were then harvested and submitted to light microscopy under phase contrast (magnification: 400×).

Table 1. The metabolism of menadione causes glutathione and protein thiol depletion and alterations of cell surface morphology in mammalian cells

	Cell types			
	Hepatocytes	GH_3	HeLa	McCoy
Cells exhibiting surface protrusions (%)	100	64	75	68
Intracellular GSH (% of control)	5	14	12	10
Protein thiols (% of control)	52	54	56	49

Hepatocytes, GH_3, HeLa, and McCoy cells were incubated with 200 μM menadione as described in Methods. After 60 min, samples were taken for evaluation of surface abnormalities and for GSH and protein thiol measurements. The levels of GSH and protein thiols (nmol/mg protein) in untreated hepatocytes, GH_3 cells, HeLa cells, and McCoy cells were 22 ± 5, 8 ± 2, 14 ± 2, 26 ± 6 and 96 ± 22, 88 ± 14, 90 ± 16, 98 ± 18, respectively (mean ± SD of three experiments).

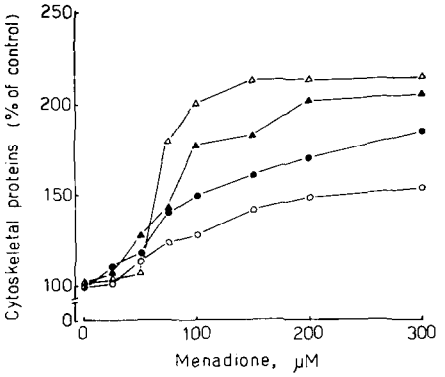

Fig. 2. Menadione-induced increase in the amount of cytoskeleton-associated protein in mammalian cells. Hepatocytes (●), GH_3 cells (△), HeLa cells (▲), and McCoy cells (○) were incubated for 60 min with the indicated concentrations of menadione. The cytoskeleton was then extracted and the amount of protein measured as described in Methods. The amounts of cytoskeleton-associated protein (μg/mg of cellular protein) in untreated cells were (mean ± SD of three experiments): Hepatocytes, 66 ± 8; GH_3 cells, 78 ± 12; HeLa cells, 69 ± 13; and McCoy cells, 52 ± 11.

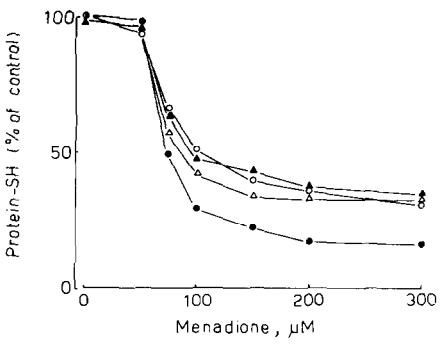

Fig. 3. Menadione-induced loss of cytoskeletal protein thiols in mammalian cells. Hepatocytes (●), GH_3 cells (△), HeLa cells (▲), and McCoy cells (○) were incubated for 60 min with the indicated concentrations of menadione. The cytoskeleton was then extracted and protein thiols measured as described in Methods. The amounts of cytoskeletal protein thiols (nmol/mg protein) in untreated cells were (mean ± SD of four experiments): Hepatocytes, 55 ± 15; GH_3 cells, 44 ± 8; HeLa cells, 61 ± 6; McCoy cells, 58 ± 12.

suggesting that common pathways may be involved in the metabolism of menadione and in menadione-induced cell damage. This assumption received further support from the finding that GH_3, HeLa, and McCoy cells, like hepatocytes, can catalyze redox cycling of menadione [1, 13, 14], leading to intracellular thiol depletion [2, 4, 15, 16] (F. Mirabelli and G. Bellomo, unpublished observation).

Previous studies with hepatocytes have suggested that plasma membrane bleb formation following toxic cell injury is associated with a perturbation of normal cytoskeletal organization [17, 18]. A relationship between the two events is also indicated by the finding that two cytoskeletal toxins, cytochalasin B and phalloidin, cause bleb formation in hepatocytes [19, 20].

To investigate the effects of menadione exposure on cytoskeletal organization in the various cell types, the cytoskeleton was prepared by the conventional Triton X-100 extraction method [21–23]. As illustrated in Fig. 2, menadione caused a dose-dependent increase in Triton X-100 non-extractable protein, suggestive of an enhanced association of non-cytoskeletal proteins with the cytoskeleton. This effect

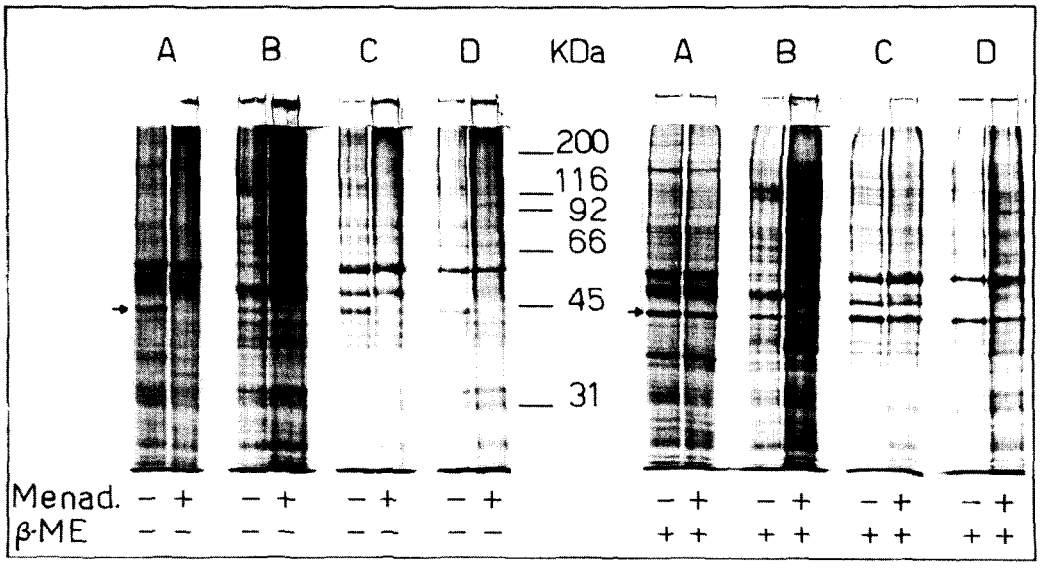

Fig. 4. Alterations in cytoskeletal polypeptide composition induced by the metabolism of menadione in mammalian cells. Hepatocytes (A), GH_3 cells (B), HeLa cells (C), and McCoy cells (D) were incubated for 60 min in the absence or presence of 200 μM menadione. The cytoskeletal fraction was then extracted and analyzed by PAGE (80–100 μg protein) under non-reducing (left panel) or reducing (right panel) conditions. Numbers between the two panels refer to the molecular mass (kDa) of protein standards. Arrows indicate a protein band corresponding to actin. β-ME: β-mercaptoethanol; Menad.: menadione.

was most pronounced in GH_3 and McCoy cells but was also apparent in hepatocytes and HeLa cells. Concomitant with the increase in cytoskeleton-associated protein, there was a marked decrease in free sulfhydryl groups in this fraction (Fig. 3). Both the increase in cytoskeleton-associated protein and the decrease in thiol groups occurred well before cell killing.

When the polypeptide composition of the cytoskeleton from control and menadione-treated cells was analyzed by means of PAGE, several major changes appeared, depending on the cell type (Fig. 4, left panel). However, two of these alterations were consistently present in all four cell types, namely, a decrease or disappearance, of a protein migrating like actin (44 kDa) and the concomitant appearance of high-molecular weight aggregates which did not enter the gel. Pretreatment of the same samples with the thiol reductant β-mercaptoethanol prevented both the disappearance of the 44 kDa polypeptide and the formation of protein aggregates (Fig. 4, right panel), suggesting that the latter contained actin molecules cross-linked by disulfide bonds. The identification of the 44 kDa polypeptide was achieved by immunoblotting with monoclonal anti-actin antibodies. As shown in Fig. 5, the cytoskeletal fraction extracted from menadione-treated cells did not react with anti-actin antibodies when analyzed under non-reducing conditions. However, prior reduction of the cytoskeletal fraction from menadione-treated cells with β-mercaptoethanol, led to the recovery of a protein reacting with the anti-actin antibodies.

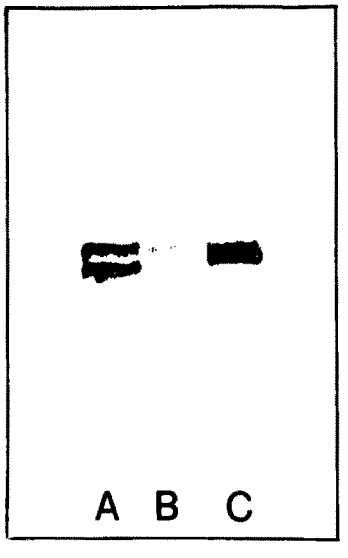

Fig. 5. Immunological identification of actin in the cytoskeletal fraction isolated from control and menadione-treated hepatocytes. Hepatocytes (10^6 cells/ml) were incubated without (lane A) or with (lanes B and C) 200 μM menadione for 60 min. The cytoskeleton was then extracted and analyzed by PAGE under non-reducing (lanes A and B) or reducing (lane C) conditions. Proteins were then transferred to a nitrocellulose sheet and actin identified using monoclonal anti-actin antibodies, as described in Methods. The presence of two different bands of 44 and 42 kDa molecular mass, reacting with the anti-actin antibodies, in lane A (control cells, non-reduced sample) was due to spontaneous, partial autooxidation of actin. Reduction of the sample with β-mercaptoethanol led to the recovery of a single band migrating in the 44 kDa region (not shown).

The data reported in this study demonstrate that the metabolism of menadione in cultured mammalian cells can induce morphological and cytoskeletal changes comparable to those observed in isolated hepatocytes [8]. Our findings suggest that a common mechanism may be responsible for the morphological and cytoskeletal alterations seen in mammalian cells exposed to oxidative stress. A direct relationship between the cytoskeletal alterations and surface blebbing in menadione-treated cells is also strongly indicated by the protective effects of thiol reductants against both surface blebbing and cytoskeletal abnormalities [8]. Thus, it appears that the oxidation of thiol groups in actin is the predominant mechanism underlying surface blebbing in mammalian cells exposed to oxidative stress. Although other mechanisms, e.g. ATP depletion [25] and the activation of Ca^{2+}-dependent enzymes acting on the cytoskeleton [26, 27], may contribute to cytoskeletal alterations during toxic cell injury, they probably play a less important role in oxidative cell damage.

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REFERENCES

1. Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA and Orrenius S, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated rat hepatocytes. *J Biol Chem* **257**: 12419–12425, 1982.
2. DiMonte D, Ross D, Bellomo G, Eklöw L and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235**: 334, 1984.
3. DiMonte D, Bellomo G, Thor H, Nicotera P and Orrenius S, Menadione-induced cytotoxicity is associated with protein thiol oxidation and alterations in intracellular Ca^{2+} homeostasis. *Arch Biochem Biophys* **235**: 342–350, 1984.
4. Bellomo G, Mirabelli F, DiMonte D, Richelmi P, Thor H, Orrenius C and Orrenius S, Formation and reduction of glutathione-protein mixed disulfides during oxidative stress. *Biochem Pharmacol* **36**: 1313–1320, 1987.
5. Bellomo G, Jewell SA and Orrenius S, The metabolism of menadione impairs the ability of rat liver mitochondria to take up and retain Ca^{2+}. *J Biol Chem* **257**: 11558–11562, 1982.
6. Thor H, Hartzell P, Svensson S-Å, Orrenius S, Mirrabelli F, Marinoni V and Bellomo G, On the role of thiol groups in the inhibition of liver microsomal Ca^{2+} sequestration by toxic agents. *Biochem Pharmacol* **34**: 3717–3723, 1985.
7. Jewell SA, Bellomo G, Thor H, Orrenius S and Smith MT, Bleb formation in hepatocytes during drug metabolism is caused by disturbances in intracellular thiol and calcium ion homeostasis. *Science* **217**: 1257–1259, 1982.
8. Mirabelli F, Salis A, Marinoni V, Finardi G, Bellomo G, Thor H and Orrenius S, Menadione-induced bleb formation in hepatocytes is associated with the oxi-

dation of thiol groups in actin. *Arch Biochem Biophys*, accepted for publication.
9. Moldéus P, Högberg J and Orrenius S, Isolation and use of liver cells. *Meth Enzymol* **51**, 60–71, 1978.
10. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond)* **277**: 680–685, 1970.
11. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW, High-performance liquid-chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.
12. Lowry OH, Rosebrough MJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
13. Powis G, Metabolism and reactions of quinoid anti-cancer agents. *Pharmacol Ther* **35**: 57–62, 1987.
14. Lind C, Hochstein P and Ernster L, DT-diaphorase as a quinone reductase: a cellular control device against semi-quinone and superoxide radical formation. *Arch Biochem Biophys* **216**: 178–185, 1982.
15. Wendel A. Glutathione peroxidase. *Meth Enzymol* **77**: 325–333, 1981.
16. Babson JR, Abell NS and Reed DJ, Protective role of the glutathione redox cycle against adriamycin-mediated toxicity in isolated hepatocytes. *Biochem Pharmacol* **30**: 2299–2304, 1981.
17. Orrenius S, Jewell SA, Thor H, Bellomo G, Eklöw L and Smith MT, Drug-induced alterations in the surface morphology of isolated hepatocytes. In: *Isolation, Characterization, and Use of Hepatocytes* (Eds. Harris RA and Cornell NW) pp. 333–340, Elsevier Science Publishing Co. Inc., New York, 1983.
18. Smith MT, Thor H, Jewell SA, Bellomo G, Sandy MS and Orrenius S, Free radical-induced changes in the surface morphology of isolated hepatocytes. In: *Free Radicals in Molecular Biology, Aging and Disease* (Ed. Armstrong G) pp. 103–118, Raven Press, New York, 1984.
19. Mesland DM, Los G and Spiele H. Cytochalasin B disrupts the association of filamentous web and plasma membrane in hepatocytes. *Exp Cell Res* **135**: 431–435, 1981.
20. Prentki M, Chaponnier C, Janrenaud B and Gabbiani G, Actin microfilaments, cell shape and secretory processes in isolated rat hepatocytes. *J Cell Biol* **81**: 592–607, 1979.
21. Hinshaw DB, Sklar RA, Bohe B, Schraufstatter I, Hyslop PA, Rossi MW, Spragg RG and Cochrane CG, Cytoskeletal and morphological impact of cellular oxidant injury. *Am J Pathol* **123**: 454–464, 1986.
22. Davies GE, Association of actin with the platelets membrane. *Biochim Biophys Acta* **772**: 149, 1984.
23. Birchmeyer W, Cytoskeleton structure and function. *Trends Biochem Sci* **9**: 192–195, 1984.
24. Sahyoun N, Stenbuck P, Le Vine III H, Bronson D, Moncharmont B, Henderson C and Cuatrecasas P, Formation and identification of cytoskeletal components from liver cytosolic precursors. *Proc Natl Acad Sci USA* **79**, 7341–7345, 1982.
25. Lemasters JJ, DiGuiseppi J, Nieminen AL and Herman B, Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. *Nature (Lond)* **325**: 78–81, 1987.
26. Fox JEB, Goll DE, Reynolds CC and Phillips DR, Identification of two proteins (actin binding protein and P235) that are hydrolyzed by endogenous Ca^{2+}-dependent protease during platelet activation. *J Biol Chem* **260**: 1060–1066, 1985.
27. Puszkin EG and Raghuraman V, Catalytic properties of a calmodulin-regulated transglutaminase from human platelets and chicken gizzard. *J Biol Chem* **260**, 16012–16020, 1985.